

# **ICCVAM Test Method Evaluation Report on Using the Murine Local Lymph Node Assay for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products**

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)



National Institute of Environmental Health Sciences  
National Institutes of Health  
U.S. Public Health Service  
Department of Health and Human Services

# About the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

In 1997, the National Institute of Environmental Health Sciences (NIEHS), one of the National Institutes of Health, established ICCVAM to:

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- Coordinate cross-agency issues relating to validation, acceptance, and national and international harmonization of new, modified, and alternative toxicological test methods

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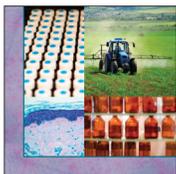
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The NICEATM-ICCVAM earth-and-sun graphic symbolizes the important role of new and alternative toxicological methods in protecting and advancing the health of people, animals, and our environment.



On the cover: This collage of pictures representing the murine local lymph node assay (LLNA) test method includes (clockwise from top left): scintillation vials used to measure the quantity of radioactive tracer chemical incorporated into dividing lymph node cells; a tractor with a spray attachment spraying a field of crops to represent the use of pesticides in agriculture; brown bottles used for storage of light-sensitive chemicals or chemical mixtures; a photomicrograph of a cross-section of human epidermis. The lavender border below and to the left of the collage is a color-adjusted picture of a human skin rash.

**ICCVAM Test Method Evaluation Report  
on Using the Murine Local Lymph Node Assay for Testing  
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and Other Products**

**Interagency Coordinating Committee on the  
Validation of Alternative Methods**

**National Toxicology Program Interagency Center for the  
Evaluation of Alternative Toxicological Methods**

**National Institute of Environmental Health Sciences  
National Institutes of Health  
U.S. Public Health Service  
Department of Health and Human Services**

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## Table of Contents

<b>List of Tables</b> .....	<b>v</b>
<b>List of Abbreviations and Acronyms</b> .....	<b>vi</b>
<b>Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives</b> .....	<b>vii</b>
<b>Acknowledgements</b> .....	<b>viii</b>
<b>Preface</b> .....	<b>xiii</b>
<b>Executive Summary</b> .....	<b>xv</b>
<b>1.0 Introduction</b> .....	<b>1</b>
<b>2.0 ICCVAM Recommendations for the Updated Assessment of the Validity of the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products</b> .....	<b>4</b>
2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations.....	4
2.2 ICCVAM Recommendations: Test Method Protocol.....	6
2.3 ICCVAM Recommendations: Future Studies.....	7
2.4 ICCVAM Recommendations: Performance Standards.....	7
<b>3.0 Evaluation of the LLNA Applicability Domain</b> .....	<b>8</b>
3.1 Test Method Description.....	8
3.2 LLNA Applicability Domain Database.....	8
3.3 Reference Test Method Data.....	10
3.4 Test Method Accuracy.....	10
3.5 Animal Welfare Considerations: Reduction, Refinement, and Replacement.....	13
<b>4.0 ICCVAM Consideration of Public and SACATM Comments</b> .....	<b>14</b>
4.1 Public Comments in Response to 72 FR 27815 (May 17, 2007).....	14
4.2 Public Comments in Response to 72 FR 52130 (September 12, 2007).....	16
4.3 Public Comments in Response to 73 FR 1360 (January 8, 2008).....	17
4.4 Public Comments in Response to 73 FR 25754 (May 7, 2008).....	18
4.5 Public Comments in Response to 73 FR 29136 (May 20, 2008).....	19
4.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008.....	19
4.7 Public Comments in Response to 74 FR 8974 (February 27, 2009).....	19
4.8 Public Comments in Response to 74 FR 19562 (April 29, 2009).....	20
4.9 Public Comments in Response to 74 FR 26242 (June 1, 2009).....	20
4.10 Public and SACATM Comments: SACATM Meeting on June 25-26, 2009.....	20
<b>5.0 References</b> .....	<b>22</b>
<b>Appendix A ICCVAM Evaluation Timeline</b> .....	<b>A-1</b>

<b>Appendix B</b>	<b>ICCVAM-Recommended Protocol: The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products .....</b>	<b>B-1</b>
<b>Appendix C</b>	<b>Comparison of LLNA Responses for Substances Tested in CBA and BALB/c Mice .....</b>	<b>C-1</b>
<b>Appendix D</b>	<b>Final Assessment of the Validity of the LLNA for Pesticide Formulations and Other Products, Metals, and Aqueous Solutions: 2010 Addendum to NIH Pub. No. 99-4494 .....</b>	<b>D-1</b>
<b>Appendix E</b>	<b>Independent Scientific Peer Review Panel Assessments .....</b>	<b>E-1</b>
	E1 Summary Minutes from the Independent Scientific Peer Review Panel Meeting on March 4-6, 2008 .....	E-3
	E2 Peer Review Panel Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.....	E-33
	E3 Summary Minutes from the Independent Scientific Peer Review Panel Meeting on April 28-29, 2009 .....	E-73
	E4 Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products .....	E-91
<b>Appendix F</b>	<b><i>Federal Register</i> Notices and Public Comments.....</b>	<b>F-1</b>
	F1 <i>Federal Register</i> Notices .....	F-3
	F2 Public Comments Received in Response to <i>Federal Register</i> Notices .....	F-23
	F3 Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments: SACATM Meeting on June 18-19, 2008 .....	F-107
	F4 Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments: SACATM Meeting on June 25-26, 2009 .....	F-121
<b>Appendix G</b>	<b>Relevant Skin Sensitization Regulations and Testing Guidelines .....</b>	<b>G-1</b>
	G1 Table of Relevant Skin Sensitization Test Regulations.....	G-3
	G2 EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization (March 2003).....	G-7
	G3 ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Delayed-type Hypersensitivity (2002).....	G-25
	G4 OECD Test Guideline 429: Skin Sensitisation – Local Lymph Node Assay (Adopted April 2002).....	G-27
	G5 OECD Test Guideline 406: Skin Sensitisation (Adopted July 1992).....	G-37

## List of Tables

<b>Table 1</b>	Summary of LLNA Performance for Testing Pesticide Formulations and Other Products, Metal Compounds, and Substances in Aqueous Solutions .....	xix
<b>Table 3-1</b>	Summary of Data Sources and Rationale for Substance Selection .....	12
<b>Table 3-2</b>	Summary of LLNA Performance for Testing Pesticide Formulations and Other Products, Metal Compounds, and Substances in Aqueous Solutions .....	14
<b>Table 4-1</b>	Opportunities for Public Comment .....	20

## List of Abbreviations and Acronyms

AOO	Acetone: olive oil (4:1 by volume)
BAuA	Federal Institute for Occupational Safety and Health (Germany)
BRD	Background review document
BT	Buehler test
CPSC	U.S. Consumer Product Safety Commission
DMSO	Dimethyl sulfoxide
DNCB	Dinitrochlorobenzene
EC3	Estimated concentration needed to produce a stimulation index of 3
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FR	<i>Federal Register</i>
GP	Guinea pig
GPMT	Guinea pig maximization test
HCA	Hexyl cinnamic aldehyde
HSUS	Humane Society of the United States
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
LLNA	Murine local lymph node assay
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organisation for Economic Co-operation and Development
P.L.	Public Law
rLLNA	Reduced murine local lymph node assay
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
U.S.C.	United States Code

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- <sup>2</sup> Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products*.
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## Preface

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin-sensitizing chemicals and products. ACD results in lost workdays<sup>1</sup> and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). To minimize the occurrence of ACD, regulatory authorities require testing to identify substances that may cause skin sensitization. Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary to avoid development of ACD.

Skin-sensitization testing has typically required the use of guinea pigs (Buehler 1965; Magnusson and Kligman 1970). However, in 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated and recommended an alternative known as the murine (mouse) local lymph node assay (“traditional LLNA”).<sup>2</sup> The traditional LLNA provides several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time to perform, and availability of dose-response information. Based on the validation database and performance, ICCVAM recommended the LLNA as an alternative test method for assessing the skin sensitization potential of most types of substances (ICCVAM 1999). United States and international regulatory agencies subsequently accepted the traditional LLNA as a valid alternative test method for ACD testing.

In 2007, the U.S. Consumer Product Safety Commission requested that ICCVAM evaluate the usefulness and limitations of the LLNA for testing mixtures, metals, and substances in aqueous solutions (i.e., an evaluation of the current applicability domain of the LLNA), among other activities related to the LLNA. ICCVAM assigned this activity a high priority after considering comments from the public and ICCVAM’s Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). As part of their ongoing collaboration with ICCVAM, scientists from the European Centre for Validation of Alternative Methods (ECVAM) and the Japanese Center for Validation of Alternative Methods (JaCVAM) served as liaisons to the ICCVAM Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA applicability domain evaluation is included with this report.

This test method evaluation report provides ICCVAM’s recommendations regarding the usefulness and limitations of the LLNA for assessing the ACD potential of pesticide formulations, metals, substances tested in aqueous solutions, and other products. The report also provides the updated ICCVAM-recommended LLNA test method protocol. The database of substances used to evaluate the current applicability domain of the LLNA is discussed and summarized.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the evaluation process. ICCVAM considered the SACATM comments, the Independent Scientific Peer Review Panel’s report, and all public comments before finalizing this ICCVAM Test Method Evaluation Report. The ICCVAM Test Method Evaluation Report will be provided to U.S. Federal regulatory agencies for consideration and be made available to the public. The ICCVAM Authorization Act requires that Federal agencies respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. Agency responses will be posted on the NICEATM-ICCVAM website<sup>3</sup> as they become available.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for

---

<sup>1</sup> <http://www.blf.gov/IIF>

<sup>2</sup> The “traditional LLNA” refers to the validated ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of <sup>3</sup>H-methyl thymidine or <sup>125</sup>I-iododeoxy-uridine into the cells of the draining auricular lymph nodes (ICCVAM 1999, Dean et al. 2000).

<sup>3</sup> <http://iccvam.niehs.nih.gov>

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This comprehensive ICCVAM evaluation of the LLNA applicability domain should facilitate regulatory agency decisions on the acceptability of the LLNA for evaluating the allergic contact dermatitis potential of pesticide formulations, metals, substances tested in aqueous solutions, and other products. Use of the method by industry can be expected to significantly reduce and refine animal use for ACD testing while continuing to support the protection of human health.

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Chair, ICCVAM

## Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the applicability domain of the murine local lymph node assay (LLNA). Applicability domain refers to defined chemicals and products for which a test method can be used to obtain accurate and reliable results. The LLNA assesses the potential of substances to cause allergic contact dermatitis (ACD). ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. This Test Method Evaluation Report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA for testing pesticide formulations, metals, substances in aqueous solutions, and other products (i.e., the current applicability domain of the LLNA). This report includes the updated ICCVAM-recommended LLNA test method protocol, the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999), and recommendations for future studies and performance standards.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and the ICCVAM Immunotoxicity Working Group prepared an initial draft Addendum and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (Panel) and the public for comment. The initial draft Addendum reviewed LLNA data from a database of more than 500 test substances. It built on the original ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). The Panel met twice in public session to review the initial and updated draft Addendums and draft ICCVAM recommendations. A detailed timeline of the evaluation of the LLNA applicability domain is included with this report.

The Panel initially met in public session on March 4–6, 2008, to discuss its peer review of the ICCVAM initial draft Addendum and to provide conclusions and recommendations regarding the LLNA applicability domain. The Panel also reviewed how well the information contained in the initial draft Addendum supported ICCVAM's draft test method recommendations. The Panel agreed with ICCVAM that the LLNA appeared useful for the testing of metal compounds, with the exception of nickel. The Panel agreed with the ICCVAM recommendations, which stated that more data were necessary before a recommendation could be made on the usefulness and limitations of the LLNA for testing mixtures and substances in aqueous solutions.

NICEATM obtained the additional data and updated the initial draft Addendum. The updated draft Addendum evaluated data derived from a database of more than 600 substances tested in the LLNA (including pesticide formulations and other products). The Panel reconvened in public session on April 28–29, 2009, to review the ICCVAM updated draft Addendum and to finalize its conclusions and recommendations on the current LLNA applicability domain. In finalizing this Test Method Evaluation Report and the Addendum, which is included as an appendix, ICCVAM considered (1) the conclusions and recommendations of the Panel, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and (3) public comments.

### **ICCVAM Recommendations: Test Method Usefulness and Limitations**

ICCVAM concludes that the accuracy performance of the LLNA supports its use for testing (1) pesticide formulations and other products; (2) metals, with the exception of nickel; (3) substances tested in aqueous solutions; and (4) other products and substances, unless these materials have unique physiochemical properties associated with them that might interfere with the LLNA's ability to detect sensitizing substances. To achieve adequate exposure, substances in aqueous solutions must be tested in an appropriate vehicle (e.g., 1% Pluronic L92 [Boverhoff et al. 2008]) that will maintain adequate contact of the test substance with the skin. The determination that a specific modification of the LLNA test method protocol is valid for evaluating new chemical classes should be relevant to other valid versions of the LLNA test method protocol (e.g., LLNA: DA and LLNA: BrdU-ELISA).

As shown in **Table 1**, the LLNA is more likely than the guinea pig test to yield a positive result for many substances. Therefore, the potential for overclassification may be a limitation of the LLNA. Federal agencies should assess how well the test materials and findings in the updated draft Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

**ICCVAM Recommendations: Test Method Protocol**

ICCVAM recently updated the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a). ICCVAM recommends this revised protocol for all future LLNA studies.

Additionally, in testing situations that do not require dose-response information, the LLNA should be considered as a reduced LLNA test method protocol. The reduced LLNA tests only the high dose, further reducing animal use.

**ICCVAM Recommendations: Future Studies**

ICCVAM recommends several future studies to further characterize the usefulness and limitations of the LLNA. However, ICCVAM discourages formal validation of the LLNA for new classes/types of test substances unless there is a biologically-based rationale. An integrated assessment of available information, including computer-assisted structure–activity relationships, prediction/measurement of biotransformation to potential reactive species, and possibly peptide, protein, or lipid binding should be conducted for new classes of test materials. Before any animal testing is conducted, the need to test a substance for skin sensitization potential should be considered.

**Table 1 Summary of LLNA Performance for Testing Pesticide Formulations and Other Products, Metal Compounds, and Substances in Aqueous Solutions**

Comparison	n	Accuracy		False Positive Rate		False Negative Rate	
		%	No.	%	No.	%	No.
<b><i>Pesticide Formulations</i></b>							
LLNA vs. GP <sup>1</sup>	23	57	13/23	50	10/20	0	0/3
<b><i>Dyes</i></b>							
LLNA vs. GP <sup>1</sup>	6	33	2/6	100	1/1	60	3/5
<b><i>Natural Complex Substances</i></b>							
LLNA vs. Human <sup>2</sup>	12	42	5/12	75	6/8	25	1/4
<b><i>Metal Compounds</i></b>							
LLNA vs. GP <sup>1</sup>	6	83	5/6	100	1/1	0	0/5
LLNA vs. Human <sup>2</sup>	14	86	12/14	40	2/5	0	0/9
<b><i>Substances Tested in Aqueous Solutions</i></b>							
LLNA vs. GP <sup>1</sup>	25	56	14/25	48	10/21	25	1/4

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; n = number of substances included in this analysis; No. = number (data on which the percentage calculation is based).

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method; false positive rate = the proportion of all negative substances that are falsely identified as positive; false negative rate = the proportion of all positive substances that are falsely identified as negative.

<sup>1</sup> GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

<sup>2</sup> Human refers to outcomes obtained by studies conducted using the human maximization test or a human patch test allergen kit.

## ICCVAM Recommendations: Performance Standards

ICCVAM, the European Centre for the Validation of Alternative Methods, and the Japanese Center for the Validation of Alternative Methods have developed internationally harmonized test method performance standards for the LLNA (ICCVAM 2009a).<sup>4</sup> These performance standards can be used to evaluate the validity of LLNA test methods that incorporate specific modifications of the traditional LLNA test method.

### Validation Status of the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

The Addendum summarizes information from a review of LLNA data derived from a database of more than 600 substances (including pesticide formulations and other products). It builds on the 1998-99 ICCVAM evaluation of the LLNA (ICCVAM 1999) that considered a database of 209 substances. To minimize duplication, metal formulations were not analyzed, and metal compounds were restricted to those testing single substances. The updated reference database includes (1) data for metal compounds from the original ICCVAM evaluation, (2) data published since that evaluation, and (3) data submitted in response to a *Federal Register* notice (72 FR 27815)<sup>5</sup> requesting LLNA, guinea pig, and/or human skin sensitization data and experience.

**Pesticide Formulations:** The updated LLNA database contains data for 104 pesticide formulations. Fifty-four percent of these formulations were LLNA positive, and 46% were LLNA negative.

Twenty-three pesticide formulations had associated guinea pig data for the complete formulation. An additional 46 formulations had guinea pig data for one or more of the active ingredients included in the formulation tested in the LLNA. Fourteen formulations had guinea pig data for a substance related to an active ingredient or for a related formulation.

Among the 23 formulations that had both LLNA and guinea pig data, the LLNA classified 52% (12 of 23) as sensitizers while the guinea pig tests classified 13% (3 of 23) as sensitizers. All three pesticide formulations identified as sensitizers in the guinea pig test were also identified as sensitizers in the LLNA. Overall, the LLNA and the guinea pig results had 57% agreement (accuracy) in 13 of 23 tests (**Table 1**). The LLNA identified as sensitizers an additional seven formulations that the guinea pig test classified as nonsensitizers, a possible overprediction (false positive) rate of 50% (10 of 20) (**Table 1**). However, human data were not available for these pesticide formulations to confirm their sensitization potential in humans.

**Dyes:** The current LLNA database contains data for six dyes that have comparative LLNA and guinea pig data. The LLNA classified 50% of the dyes as sensitizers and 50% as nonsensitizers. By comparison, the guinea pig maximization test (GPMT) classified 83% as sensitizers and 17% as nonsensitizers. Overall, the LLNA and GPMT results had 33% accuracy (**Table 1**). The overprediction (false positive) rate for the LLNA was 100% (1 of 1), and the underprediction (false negative) rate was 60% (3 of 5) (**Table 1**).

**Natural Complex Substances:** The current LLNA database contains data for 12 natural complex substances (essential oils and absolutes) with comparative LLNA and human data. The LLNA classified 75% (9 of 12) of these substances as sensitizers and 25% (3 of 12) as nonsensitizers. However, human clinical studies identified only 33% (4 of 12) as sensitizers. The LLNA identified three of these four as sensitizers (75%), but six more tested positive that did not produce positive results in the human testing. Compared to human outcomes, the LLNA had an accuracy of 42% (5 of 12), a false positive rate of 75% (6 of 8), and a false negative rate of 25% (1 of 4) (**Table 1**).

<sup>4</sup> Available at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PerfStds.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm).

<sup>5</sup> Available at [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_9544.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf).

**Metal Compounds:** The current LLNA database contains test results from 48 studies of 16 metal compounds. The compounds represent 13 different metals. (Formulations containing metals were excluded from this analysis.) All 16 metal compounds had comparative human data, and eight had comparative guinea pig data. Because nickel was classified as a sensitizer in three of seven studies and as a nonsensitizer in four of seven studies, nickel compounds were excluded from the LLNA metals performance analysis.

For the remaining 14 metal compounds (13 metals), the LLNA had an accuracy of 86% (12 of 14), a false positive rate of 40% (2 of 5), and a false negative rate of 0% (0 of 9) when compared to human results (**Table 1**). The two false positive compounds were copper chloride and zinc sulfate.

The LLNA classified as sensitizers all six of the metal compounds with comparative guinea pig test results (six different metals with nickel compounds excluded). For these metal compounds, the LLNA had an accuracy of 83%, a false positive rate of 100%, and a false negative rate of 0% (**Table 1**) when compared to guinea pig test results.

The performance of the LLNA and the guinea pig tests was compared to human results for the six metal compounds tested in all three species. The LLNA had accuracy of 83%, a false positive rate of 100%, and a false negative rate of 0%. By comparison, the guinea pig tests had an accuracy of 100%, a false positive rate of 0%, and a false negative rate of 0% relative to the human outcomes.

**Substances Tested in Aqueous Solutions:** The current LLNA database of substances tested in aqueous solutions includes results from 171 studies representing 139 substances. Ninety-one percent of these substances (123 LLNA studies) are pesticide formulations and pure compounds. Forty-eight percent (48 LLNA studies) are aqueous eluates of medical devices. The two groups were analyzed separately because of differences in the protocols for sample preparation. Of the 91 pesticide formulations and pure compounds, 63% (57 of 91) were LLNA positive, and 37% (34 of 91) were LLNA negative. The substances included in this evaluation were tested at a final concentration of at least 20% water.

Guinea pig data were available for 25 substances tested in aqueous solutions. The LLNA and the guinea pig test results disagreed for 11 (44%) of the substances. Ten of the 11 discordant substances (91%) were pesticide formulations tested in aqueous 1% Pluronic L92. These were the same 10 substances previously discussed for the pesticide formulations analysis. The LLNA overpredicted all 10 with respect to the guinea pig results (48% [10 of 21] false positive rate) (**Table 1**). The LLNA underpredicted one additional substance, neomycin sulfate, which was tested in 25% EtOH (25% [1 of 4] false negative rate) (**Table 1**). The LLNA and guinea pig results had overall agreement (accuracy) of 56% (14/25) (**Table 1**).

All 48 of the medical device eluates were negative in the LLNA. These eluates were not analyzed to determine their constituents or to determine whether any compound(s) were in fact eluted from the medical device tested.

### **ICCVAM Consideration of Public and SACATM Comments**

The ICCVAM evaluation process provides numerous opportunities for stakeholder involvement. The public may submit written comments and provide oral comments at ICCVAM independent scientific peer review panel meetings and SACATM meetings. From May 2007 to June 2009, there were a total of 12 opportunities for public comment on the ICCVAM evaluation of the LLNA applicability domain. During this time, ICCVAM received 46 public comments, nine of which pertained directly to the LLNA applicability domain. In addition, SACATM reviewed and commented on the draft ICCVAM recommendations and associated conclusions of the Panel during their annual meetings in June 2008 and June 2009. ICCVAM considered both public and SACATM comments in finalizing the test method recommendations provided in this report.

## 1.0 Introduction

The murine local lymph node assay (traditional LLNA)<sup>1</sup> is an alternative skin sensitization test method that requires fewer animals and less time than currently accepted guinea pig (GP) tests (e.g., the guinea pig maximization test and the Buehler test). It also avoids animal discomfort that can occur in the GP tests when substances cause allergic contact dermatitis (ACD). The LLNA measures cell proliferation in the draining auricular lymph nodes of the mouse by analyzing incorporation of a radioactive marker into newly synthesized DNA. The LLNA was the first alternative test method evaluated and recommended by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). International regulatory authorities have now recognized the traditional LLNA as an acceptable alternative to GP tests for most testing situations.

The current LLNA applicability domain was one of several LLNA-related topics nominated by the U.S. Consumer Product Safety Commission (CPSC) for evaluation by ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).<sup>2</sup> For this evaluation, the LLNA was assessed for its ability to correctly identify the sensitization potential of pesticide formulations and other products, metals, and substances tested in aqueous solutions.

The ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285I-3) charged ICCVAM with coordinating the technical evaluations of new, revised, and alternative test methods with regulatory applicability. After considering comments from the public and ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), ICCVAM members unanimously agreed that an evaluation of the LLNA applicability domain should have a high priority for evaluation. A detailed timeline of this evaluation is provided in **Appendix A**. The updated ICCVAM-recommended LLNA test method protocol, a comparison of LLNA results for substances tested in two different mouse strains, and the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999, hereafter Addendum) are provided in **Appendices B, C, and D**, respectively.

The ICCVAM Immunotoxicity Working Group (IWG) was formed to work with NICEATM in evaluating the test methods. Dr. Silvia Casati was the European Centre for the Validation of Alternative Methods (ECVAM) liaison, and Dr. Hajime Kojima was the Japanese Center for the Validation of Alternative Methods (JaCVAM) liaison to the IWG.

To facilitate peer review of the LLNA applicability domain evaluation, the IWG and NICEATM, which administers ICCVAM and provides scientific and operational support for ICCVAM activities, prepared a comprehensive initial draft Addendum that provided information and data from validation studies and the scientific literature. A May 17, 2007, *Federal Register* (FR) notice (72 FR 27815)<sup>3</sup> requested data and information on these test methods and nominations of individuals to serve on an international independent scientific peer review panel (Panel). The request was also disseminated via the ICCVAM electronic mailing list and through direct requests to over 100 stakeholders. In response to this request, three individuals or organizations nominated members to the Panel (see **Section 4.0**).

In the initial draft Addendum, ICCVAM examined data derived from a database of over 500 substances (including pesticide formulations and other products) tested in the LLNA. In the original ICCVAM evaluation of the LLNA (ICCVAM 1999), the performance of the LLNA was compared to (1) results from GP tests and (2) information about sensitizers in humans (e.g., human

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<sup>1</sup> The "traditional LLNA" refers to the validated ICCVAM-recommended LLNA protocol, which measures lymphocyte proliferation based on incorporation of <sup>3</sup>H-methyl thymidine or <sup>125</sup>I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 2009a).

<sup>2</sup> Available at [http://iccvam.niehs.nih.gov/methods/immunotox/llnadoocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadoocs/CPSC_LLNA_nom.pdf)

<sup>3</sup> Available at [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_9544.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf)

maximization test results, substances used in human repeat insult patch test, clinical case reports), where available. The initial draft Addendum updated the LLNA performance analyses for pesticide formulations and other products, metals, and substances tested in aqueous solutions when compared to human and GP results. On January 8, 2008, ICCVAM announced the availability of the initial draft Addendum to the public and a public Panel meeting to review the validation status of the LLNA applicability domain (and other LLNA-related activities) (73 FR 1360).<sup>4</sup> All of the information provided to the Panel, including the ICCVAM initial draft Addendum, draft test method recommendations, and all public comments received prior to the Panel meeting, were made publicly available via the NICEATM-ICCVAM website.<sup>5</sup>

The first Panel meeting was a public session held on March 4-6, 2008, to review the ICCVAM evaluation of the LLNA for testing pesticide formulations and other products, metals, and substances in aqueous solutions and the completeness of the ICCVAM initial draft Addendum. The Panel evaluated (1) the extent to which the initial draft Addendum addressed established validation and acceptance criteria and (2) the extent to which the initial draft Addendum supported ICCVAM's draft proposed test method uses, recommended protocol, draft test method performance standards, and proposed future studies. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. The Panel considered these comments as well as those submitted prior to the meeting before concluding their deliberations. The Panel recommended that NICEATM and ICCVAM solicit more data on pesticide formulations and other products and substances tested in aqueous solutions, before making recommendations about the usefulness of the LLNA for testing such substances. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations<sup>6</sup> (see **Appendix E**) on the NICEATM-ICCVAM website for public review and comment (announced in 73 FR 29136).<sup>7</sup>

ICCVAM provided SACATM with the updated draft Addendum and initial draft test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 18-19, 2008, where public stakeholders were given another opportunity to comment.

NICEATM subsequently obtained a detailed test method protocol and data from an additional 140 substances and updated the initial draft Addendum to include this new information. The updated draft Addendum included an accuracy evaluation for the expanded database of over 600 substances (as compared with over 500 substances included in the January 2008 draft). Based on the analyses included in the updated draft Addendum, ICCVAM prepared updated draft test method recommendations for proposed test method uses and limitations, recommended protocol, test method performance standards, and future studies for the LLNA. ICCVAM released the updated draft documents to the public for comment on February 27, 2009, and announced a second meeting of the Panel (74 FR 8974).<sup>8</sup> The Panel reconvened on April 27-28, 2009, to again evaluate the LLNA applicability domain. The Panel also reviewed the completeness of the ICCVAM updated draft Addendum and the extent to which the information therein supported the ICCVAM updated draft test method recommendations. On June 1, 2009, ICCVAM posted the second report of the Panel's recommendations<sup>9</sup> (see **Appendix E**) on the NICEATM-ICCVAM website for public review and comment (announced in 74 FR 26242).<sup>10</sup>

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<sup>4</sup> Available at [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_25553.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf)

<sup>5</sup> Available at <http://iccvam.niehs.nih.gov>

<sup>6</sup> Available at [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)

<sup>7</sup> Available at <http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf>

<sup>8</sup> Available at <http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-4280.pdf>

<sup>9</sup> Available at [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2009.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2009.pdf)

<sup>10</sup> Announced in 74 FR 26242 <http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-12360.pdf>

ICCVAM provided SACATM with the revised draft Addendum, the second Panel report, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment.

After SACATM's meeting, ICCVAM and the IWG considered the SACATM comments, the Panel report, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and the Addendum provided in this report. As required by the ICCVAM Authorization Act, ICCVAM will make this test method evaluation report and the accompanying final addendum available to the public and to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. Agency responses will be made available to the public on the NICEATM-ICCVAM website as they are received.

## 2.0 ICCVAM Recommendations for the Updated Assessment of the Validity of the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

ICCVAM has updated the original validation report of the LLNA (ICCVAM 1999) based on a comprehensive review of available data and information regarding the current validity of the LLNA for assessing the skin-sensitizing potential of pesticide formulations and other products, metal compounds, and substances in aqueous solutions. The information is based on a retrospective review of data derived from over 600 substances, including 104 pesticide formulations, tested in the LLNA. The current evaluation builds on the previous ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). The Addendum updates the LLNA performance analyses for pesticide formulations and other products, metal compounds, and substances in aqueous solutions when compared to (1) the results from GP tests and (2) information about sensitizers in humans (e.g., human maximization test results, substances used in human repeat insult patch test, clinical case reports), where available (see **Section 3.0** and **Appendix D**).

### 2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations

**Pesticide Formulations:** The current LLNA database contains test results on 104 pesticide formulations, 23 of which have comparative GP data. None have comparative human data. Ten out of the approximately 450 active ingredients registered with EPA were represented among these 23 formulations. Furthermore, approximately 40 different classes of pesticides are registered with EPA, of which these 10 active ingredients represent a small proportion (i.e., one insecticide, one microbioocide, six herbicides and two fungicides). Based on these 23 pesticide formulations, the concordance (accuracy) of the LLNA results compared to GP data is 57% (13/23), with an overprediction (“false positive”) rate of 50% (10/20) and underprediction (“false negative”) rate of 0% (0/3). Thus, there is a greater likelihood of obtaining a positive result in the LLNA (13/23; 57%) than in a GP test (3/23; 13%). All three formulations that were identified as positive in the GP tests were also identified as positive in the LLNA. Although human data are not available for these pesticide formulations to confirm their human sensitization potential, these data indicate that the LLNA is more likely to classify a pesticide formulation as a sensitizer than the GP tests. It should be noted that all 23 formulations were tested in the LLNA in the aqueous vehicle 1% Pluronic L92. Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects. If there is any primary testing or postmarketing reports of skin sensitization, they should be used for comparison with LLNA results.

The LLNA can be used for testing pesticide formulations unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of pesticide formulations may be a limitation of the LLNA.

**Natural Complex Substances:** The current LLNA database also contains data for 12 natural complex substances for which there are comparative LLNA and human data. Based on LLNA results for these natural complex substances, 75% (9/12) were sensitizers and 25% (3/12) were nonsensitizers. However, based on human clinical studies, only 33% (4/12) of these substances tested as sensitizers. Based on this limited database, the concordance (accuracy) of the LLNA results compared to human sensitization data is 42% (5/12), with an overprediction (“false positive”) rate of 75% (6/8) and underprediction (“false negative”) rate of 25% (1/4). There are no comparative data from GP tests with these natural complex substances. Therefore, a comparison of the performance of the LLNA and the GP tests relative to the human outcome is not possible. Federal agencies should

assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing natural complex substances unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of natural complex substances may be a limitation of the LLNA.

**Dyes:** The current LLNA database contains data for six dyes, for which there are LLNA and GP data. Compared to GPMT outcomes, the LLNA concordance (accuracy) is 33% (2/6), the overprediction (“false positive”) rate is 100% (1/1) and the underprediction (“false negative”) rate is 60% (3/5). Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing dyes unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of dyes may be a limitation of the LLNA.

**Metal Compounds:** The current LLNA database contains test results on 48 studies involving 16 metal compounds representing 13 different metals (formulations containing metals are excluded from this analysis). All 16 metal compounds had comparative human data and eight had comparative GP data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, and three times as nickel chloride. Because nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in the other four, nickel compounds were excluded from the LLNA metals performance analysis.

For these remaining 14 metal compounds (13 metals), the LLNA concordance (accuracy) is 86% (12/14), the overprediction (“false positive”) rate is 40% (2/5) and the underprediction (“false negative”) rate is 0% (0/9), when compared to human results. The two false positive compounds were copper chloride and zinc sulfate. All six of the metal compounds (six different metals with nickel compounds excluded) with comparative GP test results were predicted as sensitizers by the LLNA. For these metal compounds, the LLNA concordance (accuracy) is 83% (5/6), the overprediction (“false positive”) rate is 100% (1/1), and the underprediction (“false negative”) rate is 0% (0/5), when compared to GP test results. When comparing the performance of the LLNA and the GP tests for the six metal compounds tested in all three species (i.e., mice, GPs, and humans) to human results, the LLNA concordance (accuracy) is 83% (5/6), the overprediction (“false positive”) rate is 100% (1/1) and the underprediction (“false negative”) rate is 0% (0/5). By comparison, the GP test concordance (accuracy) is 100% (6/6), the overprediction (“false positive”) rate is 0% (0/1) and the underprediction (“false negative”) rate is 0% (0/5) against the human. Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing metal compounds, with the exception of nickel, unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. Inconsistent results for nickel compounds obtained with the traditional LLNA suggest that the LLNA may not be suitable for testing substances containing nickel. Until the LLNA has been found to accurately identify ACD potential in substances containing nickel, further testing using a different test system is recommended when negative results are obtained for such substances.

**Substances Tested in Aqueous Solutions:** The current LLNA database contains test data on 44 studies that involved testing 25 substances in an aqueous solution. Pesticide formulations that were considered in the analysis discussed previously were also included in this evaluation, so this database

has the same limitations as discussed previously. The substances included in this evaluation contain at least 20% water. Most (23/25) of these substances were tested in the vehicle 1% Pluronic L92. Based on LLNA results for these substances 48% (12/25) were sensitizers and 52% (13/25) were nonsensitizers. However, based on GP results, only 20% (5/25) tested as sensitizers. Based on this limited database, the concordance (accuracy) of the LLNA compared to GP sensitization data is 56% (14/25), the overprediction (“false positive”) rate is 48% (10/21) and the underprediction (“false negative”) rate is 25% (1/4). Among the 11 substances for which LLNA and GP results were discordant, only one (i.e., neomycin sulfate) is negative in the LLNA and positive in the GP. These data suggest that the LLNA is more likely than the GP to classify a substance tested in an aqueous solution as a sensitizer. Human data are available for one substance that is discordant between the LLNA and the GP (i.e., neomycin sulfate). This substance is also discordant between the LLNA (i.e., negative) and the human (i.e., positive). Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle, to maintain the test substance in contact with the skin (e.g. 1% Pluronic L92 [Boverhoff et al. 2008]) so an adequate exposure is achieved, as demonstrated by positive control results. It should be recognized that the potential for possible overclassification of aqueous substances may be a limitation of the LLNA.

### **Independent Peer Review Panel Conclusions and Recommendations**

The Panel concurred that the available data supported the ICCVAM updated draft test method recommendations for the LLNA with regard to testing pesticide formulations, dyes, natural complex substances, metal compounds and substances tested in aqueous solutions, in terms of the proposed test method usefulness and limitations.

On the basis of the available information, unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances, the Panel considered all of these test materials as candidates for testing in the LLNA, subject to the limitations outlined in the ICCVAM Test Method Recommendations.

## **2.2 ICCVAM Recommendations: Test Method Protocol**

An updated version of the validated ICCVAM-recommended LLNA test method protocol has recently been developed (Appendix A of ICCVAM 2009a). This revised protocol is recommended for all future LLNA studies and includes the following key aspects:

- The high dose should be the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation. The measurement of ear swelling is a potentially valuable adjunct for identifying local irritation.
- A minimum of four animals per dose group is recommended.
- Collection of individual animal data is recommended.
- Inclusion of a concurrent vehicle control and positive control in each study is recommended.

Additionally, ICCVAM recommends that there should be a measure of variability of the positive control response over time. Laboratories should maintain a historical database of positive control SI values such that results can be compared to the mean historical SI. There could be cause for concern when a negative test substance result is accompanied by a concurrent positive control SI value significantly lower than the mean historical SI.

In testing situations where dose-response information is not required, the LLNA should be considered for use as a reduced LLNA test method protocol in which only the high dose is tested, thus further reducing animal use.

### **Independent Peer Review Panel Conclusions and Recommendations**

The Panel concluded that updated information on various elements in the Addendum did not suggest the need for changes to recommendations for the development of a revised standard method. Whenever discretion is permitted, the Panel recommended the inclusion of a suitable (representative) positive control from the same category of materials to be tested (e.g., for testing pesticides, select one representative positive control pesticide).

## **2.3 ICCVAM Recommendations: Future Studies**

ICCVAM recommends the following future studies to further characterize the usefulness and limitations of the LLNA:

- To more comprehensively evaluate the ability of the LLNA to be used for testing nickel compounds, additional data from LLNA studies on such compounds with comparative human and/or GP data are needed.
- Where available, solubility data should be provided in future studies so that thermodynamic activity can be computed and compared to maximum theoretical percutaneous penetration. This information should be considered when comparing the data from LLNA studies in lipophilic delivery systems compared to that in aqueous systems. Studies done in aqueous systems should use 1% Pluronic L92 as the vehicle in order to expand the existing database for that vehicle, unless adequate scientific rationale is provided for using another aqueous vehicle.
- Revalidation of the LLNA for new classes/types of test substances should be avoided unless there is a biologically based rationale. For new classes of test materials, an integrated assessment of available information should be conducted. This should include computer-assisted structure-activity relationships, prediction/measurement of biotransformation to potential reactive species, and possibly peptide, protein, or lipid binding. Before any animal testing is conducted, consideration should be given to the necessity for a substance to be tested for skin sensitization potential.
- If any variant of the LLNA is validated for use to test novel classes, then the findings should be relevant to the family of validated LLNA tests.

### **Independent Peer Review Panel Conclusions and Recommendations**

The Panel concurred with ICCVAM's recommendations for future studies. The Panel also suggested that, before additional animal testing is conducted, consideration should be given to the necessity for the substance to be tested for skin sensitization potential.

## **2.4 ICCVAM Recommendations: Performance Standards**

In conjunction with ECVAM and JaCVAM, ICCVAM has developed internationally harmonized test method performance standards for the LLNA (ICCVAM 2009a)<sup>11</sup> to evaluate the performance of LLNA test methods that incorporate specific protocol modifications (e.g., procedures to measure lymphocyte proliferation) compared to the traditional LLNA.

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<sup>11</sup> Available at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PerfStds.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm)

### 3.0 Evaluation of the LLNA Applicability Domain

The following is a synopsis of the information in the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999) (**Appendix D**, hereafter, Addendum), which reviews the available data and information for the LLNA applicability domain. The Addendum describes the current validation status of the LLNA for testing pesticide formulations and other products, metals, and substances in aqueous solutions, the scope of the substances tested, and standardized protocols used.

#### 3.1 Test Method Description

The purpose of the LLNA test method is to identify potential skin sensitizers by quantifying lymphocyte proliferation in the draining auricular lymph nodes. The magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops after a topical induction exposure to a potential skin-sensitizing substance.

##### 3.1.1 General Test Method Procedures

The LLNA measures lymphocyte proliferation after topical exposure to a potential skin-sensitizing substance. The test substance is administered topically on three consecutive days to the ears of mice at a concentration that provides maximum solubility of the test substance without causing systemic toxicity and/or excessive local irritation. Two days after the final application of the test substance, <sup>3</sup>H-methyl thymidine or <sup>125</sup>I-iododeoxyuridine (in phosphate-buffered saline; 250 µL/mouse) is administered via the tail vein. Five hours later the draining auricular lymph nodes are excised, and a single-cell suspension from the lymph nodes of each animal is prepared for quantifying the incorporation of radioactivity, which correlates with lymph node cell proliferation.

The incorporation of <sup>3</sup>H-methyl thymidine or <sup>125</sup>I-iododeoxyuridine for each mouse is expressed in disintegrations per minute (dpm). The stimulation index (SI) is calculated as the ratio of the mean dpm/mouse for each treatment group against the mean dpm/mouse for the vehicle control group. The threshold for a positive response is an SI ≥ 3.

#### 3.2 LLNA Applicability Domain Database

The information summarized in the Addendum is based on a retrospective review of LLNA data derived from a database of over 600 substances (including pesticide formulations and other products) tested in the LLNA and builds on the previous ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). To minimize duplication in this evaluation, metal formulations were not included in the analysis of pesticide formulations and other products, and metal compounds were restricted to those testing single substances. The reference database includes data for metal compounds from the original ICCVAM evaluation (**Appendix D**, Annex I), data published since that evaluation, and data submitted in response to a request in a FR notice (72 FR 27815)<sup>12</sup> requesting LLNA, GP, and/or human skin sensitization data and experience. An evaluation of the usefulness and limitations of the LLNA for testing pesticide formulations and other products, and substances tested in aqueous solutions was not included in the original ICCVAM validation (**Appendix D**, Annex I) because no data on these substances were available at that time. The reference database for these substances in the Addendum consists of data published since the original ICCVAM evaluation or submitted in response to the FR notice. **Table 3-1** provides information on the sources of the data and the rationale for the substances tested.

Among the LLNA studies for the pesticide formulations, 32% (29/89) used the BALB/c mouse strain rather than the CBA/J or CBA/Ca strains of mice, which are recommended in standardized LLNA

<sup>12</sup> Available at [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_9544.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf)

protocols (ICCVAM 2009a; EPA 2003; OECD 2002). One additional submitted LLNA study (from Dr. Dori Germolec at the National Institute of Environmental Health Sciences [NIEHS]) also used the BALB/c strain. The comparative performance of the LLNA using these different mouse strains relative to the GP is detailed in **Appendix C**.

**Table 3-1 Summary of Data Sources and Rationale for Substance Selection**

<b>Data Source</b>	<b>N</b>	<b>Substance Selection Rationale</b>
AppTec Laboratory Services	48	Aqueous eluates from medical devices.
Dow AgroSciences	52	Pesticide formulations analyzed in the LLNA with associated GP data of various kinds.
Dupont	28	Pesticide formulations analyzed in the LLNA.
ECPA	39	Plant protection products (i.e., pesticides) were evaluated in the LLNA with a novel vehicle to assess its usefulness.
Basketter et al. (1994; 1996; 1999a; 2005)	16	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Lalko and Api (2006)	12	Original research that evaluated essential oils in the LLNA. RIFM and the authors submitted additional data.
Ryan et al. (2000)	2	Interlaboratory study to evaluate the accuracy of the LLNA to identify human sensitizers.
Ryan et al. (2002)	11	Original research with known water soluble haptens and known skin sensitizers to assess the usefulness of a novel vehicle in the LLNA.
E. Debruyne (Bayer Crop Science SA)	10	Original research on different pesticide types and formulations in the LLNA.
Kimber et al. (1991; 1995; 2003)	9	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Gerberick et al. (2005) <sup>1</sup>	6	Compiled from previously conducted LLNA studies (from published literature and unpublished sources) on substances of varying skin sensitization potential.
Bundesanstalt für Arbeitsschutz und Arbeitsmedizin	6	Original LLNA research on dye formulations.
H.W. Vohr (BGIA)	4	Original LLNA research with epoxy resin components as part of a validation effort for nonradioactive versions of the LLNA.
Basketter and Scholes (1992) <sup>2</sup>	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Gerberick et al. (1992)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
D. Germolec (NIEHS)	2	Substances were evaluated by NTP for skin sensitization potential in the LLNA.
Lea et al. (1999)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
M.J. Olson (GlaxoSmithKline)	2	Pharmaceutical substances tested in the LLNA.
Unilever (unpublished data)	2	Metal substances evaluated for skin sensitization potential in the LLNA.
Basketter and Kimber (2006)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Goodwin et al. (1981)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.

*Continued*

**Table 3-1 Summary of Data Sources and Rationale for Substance Selection (Continued)**

Data Source	N	Substance Selection Rationale
Griem et al. (2003)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
Kligman (1966)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential.
J. Matheson (CPSC)	1	Published LLNA data submitted electronically to NICEATM, as a reference.
K. Skirda (CESIO - TNO Report V7217)	1	Data were provided by CESIO member companies for use in paper titled "Limitations of the LLNA as preferred test for skin sensitization: concerns about false positive and false negative test result".
<b>Total</b>	<b>262</b>	

Abbreviations:

BGIA = Berufsgenossenschaftliches Institut für Arbeitsschutz; CESIO = Comité Européen des Agents de Surface et de leurs Intermédiaires Organiques; CPSC = U.S. Consumer Product Safety Commission; ECPA = European Crop Protection Association; ECVAM = European Centre for the Validation of Alternative Methods; GP = guinea pig; LLNA = murine local lymph node assay; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; NIEHS = National Institute of Environmental Health Sciences; NTP = National Toxicology Program; RIFM = Research Institute for Fragrance Materials; TNO = Netherlands Organization for Applied Scientific Research.

<sup>1</sup> These data were evaluated by the ECVAM Scientific Advisory Committee in its evaluation of the LLNA limit dose procedure and were previously submitted to ICCVAM in 1998 for the original evaluation of the validation status of the LLNA (ICCVAM 1999; Gerberick et al. 2005).

<sup>2</sup> These LLNA studies used both male and female mice, but single experiments were limited to one sex.

### 3.3 Reference Test Method Data

The traditional LLNA data used for evaluation of the LLNA applicability domain include the results for all tested doses of each substance. In addition to calculated SI values for each of the tested doses, the vehicles tested and EC3 values (estimated concentration needed to produce an SI value of 3) for substances classified as sensitizers were provided in Gerberick et al. (2005). If EC3 values were not included in the data source, they were calculated, where possible, using either interpolation or extrapolation (Dearman et al. 2007).

The reference data for the GP tests (guinea pig maximization test [GPMT] or Buehler test) and human data (human maximization test, human patch test allergen, or other human data) were obtained from the scientific literature or from the data submitters. The complete database (by each source) is provided in Annex II, III, and IV of the Addendum (**Appendix D**).

### 3.4 Test Method Accuracy

**Table 3-2** presents a summary of performance statistics for the LLNA for testing pesticide formulations, dyes, natural complex substances, metal compounds, and substances tested in aqueous solutions.

**Table 3-2 Evaluation of LLNA Performance for Testing Pesticide Formulations and Other Products, Metal Compounds, and Substances in Aqueous Solutions**

Comparison	n <sup>1</sup>	Accuracy		False Positive Rate		False Negative Rate	
		%	No. <sup>2</sup>	%	No. <sup>2</sup>	%	No. <sup>2</sup>
<b><i>Pesticide Formulations</i></b>							
LLNA vs. GP <sup>3</sup>	23	57	13/23	50	10/20	0	0/3
<b><i>Dyes</i></b>							
LLNA vs. GP <sup>3</sup>	6	33	2/6	100	1/1	60	3/5
<b><i>Natural Complex Substances</i></b>							
LLNA vs. Human <sup>4</sup>	12	42	5/12	75	6/8	25	1/4
<b><i>Metal Compounds</i></b>							
LLNA vs. GP <sup>4</sup>	6	83	5/6	100	1/1	0	0/5
LLNA vs. Human <sup>4</sup>	14	86	12/14	40	2/5	0	0/9
<b><i>Substances Tested in Aqueous Solutions</i></b>							
LLNA vs. GP <sup>3</sup>	25	56	14/25	48	10/21	25	1/4

Abbreviations:

GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

False positive rate = the proportion of all negative substances that are falsely identified as positive

False negative rate = the proportion of all positive substances that are falsely identified as negative

<sup>1</sup> n = number of substances included in this analysis.

<sup>2</sup> The data on which the percentage calculation is based.

<sup>3</sup> GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

<sup>4</sup> Human refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

**Pesticide Formulations:** The current LLNA database contains data for 104 pesticide formulations. Among these formulations, 54% (56/104) were LLNA positive and 46% (48/104) were LLNA negative.

Seventy of the 104 pesticide formulations have LLNA and some type of associated GP reference data. A total of 89 LLNA studies were performed using these 70 formulations. LLNA studies were conducted with either CBA/Ca or CBA/J (61/89) and/or BALB/c (28/89) mouse strains. Six pesticide formulations were tested in multiple LLNA studies (25 studies total); 5/6 multiply tested pesticide formulations had LLNA results in agreement, and 1/6 pesticide formulations produced discordant results (i.e., three positive, two negative). The discordant data were for the pesticide formulation Oxyflourfen EC and were submitted to NICEATM by the European Crop Protection Association. In a five-laboratory study, SI values for the highest concentration tested (33%) ranged from 2.3 to 5.4. All lower concentrations tested showed no SI values  $\geq 3$ .

All 70 pesticide formulations (89/89 studies) were tested in the LLNA in aqueous 1% Pluronic L92, a surfactant and wetting agent that has been evaluated as an alternative aqueous-based vehicle for use in the LLNA (Boverhof et al. 2008; Ryan et al. 2002).

Twenty-three pesticide formulations had associated GP data for the complete formulation, 46 pesticide formulations had GP data for one or more of the active ingredients included in the

complete formulation, and 14 pesticide formulations had GP data for a substance related to an active ingredient or for a related formulation.

For the 23 formulations for which there were GP data, the LLNA classified 52% (12/23) of the formulations as sensitizers while the GP tests classified only 13% (3/23) of the formulations as sensitizers. All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA. Overall, the LLNA and the GP results were in agreement (accuracy) 57% (13/23) of the time (**Table 3-2**). The LLNA also identified an additional seven substances as sensitizers that were classified as nonsensitizers in the GP test, an overprediction (false positive) rate of 50% (10/20) (**Table 3-2**). Three of the LLNA studies for the 23 pesticide formulations were done with BALB/c mice. If these three studies are removed from the analysis, the LLNA and the GP results were in agreement 60% (12/20) of the time, and the overprediction was 47% (8/17). There were no instances of underprediction by the LLNA for these 23 pesticide formulations. Human data were not available for these pesticide formulations to confirm their sensitization potential in humans.

**Dyes:** The current LLNA database contains data for six dyes for which there are LLNA and GP data. Based on LLNA results for these six dyes, 50% (3/6) were sensitizers and 50% (3/6) were nonsensitizers. By comparison, based on GP results, 83% (5/6) were sensitizers and 17% (1/6) were nonsensitizers. The LLNA and the GP results were in agreement (accuracy) 33% of the time (**Table 3-2**). The overprediction (false positive rate) for the LLNA was 100% (1/1) and the underprediction (false negative rate) was 60% (3/5) (**Table 3-2**).

**Natural Complex Substances:** The current LLNA database also contains data for 12 natural complex substances (essential oils and absolutes) for which there are comparative LLNA and human data. Based on LLNA results for these substances, 75% (9/12) were sensitizers and 25% (3/12) nonsensitizers. However, based on human clinical studies, only 33% (4/12) of these substances tested as sensitizers. Therefore, compared to human outcomes for these 12 substances, the LLNA was able to identify three out of four of the substances that were positive in human testing. However, an additional six substances that did not produce positive results in the human testing were positive in the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5/12), a false positive rate of 75% (6/8) and a false negative rate of 25% (1/4) (**Table 3-2**). There were no comparative data from GP tests with these substances. Therefore, a comparison of the performance of the LLNA and the GP tests relative to the human outcome was not possible.

**Metal Compounds:** The current LLNA database contains test results on 48 studies involving 16 metal compounds representing 13 different metals (formulations containing metals were excluded from this analysis). All 16 metal compounds had comparative human data and eight had comparative GP data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, and three times as nickel chloride. Nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in the other four. Two positive results occurred in aqueous vehicles, one positive result occurred in a nonaqueous vehicle, and the four negative results all occurred in nonaqueous vehicles. Because of these discordant results, a performance analysis for metals was also conducted with nickel compounds excluded.

For the remaining 14 metal compounds (13 metals), the LLNA had an accuracy of 86% (12/14), a false positive rate of 40% (2/5) and a false negative rate of 0% (0/9), when compared to human results (**Table 3-2**). The two false positive compounds were copper chloride and zinc sulfate. All six of the metal compounds (six different metals with nickel compounds excluded) with comparative GP test results were predicted as sensitizers by the LLNA. For these metal compounds, the LLNA had an accuracy of 83% (5/6), a false positive rate of 100% (1/1), and a false negative rate of 0% (0/5) (**Table 3-2**), when compared to GP test results. When comparing the performance of the LLNA and the GP tests for the six metal compounds tested in all three species to human results, the LLNA had

an accuracy of 83% (5/6), a false positive rate of 100% (1/1) and a false negative rate of 0% (0/5). By comparison, the GP tests had an accuracy of 100% (6/6), a false positive rate of 0% (0/1) and a false negative rate of 0% (0/5) relative to the human.

**Substances Tested in Aqueous Solutions:** The current LLNA database of substances tested in aqueous solutions includes results from 171 studies representing 139 substances; 91 (123 LLNA studies) of these substances are pesticide formulations and pure compounds, and 48 of these substances (48 LLNA studies) are aqueous eluates of medical devices. Because of differences in the protocols for sample preparation between the 91 pesticide formulations and pure compounds and the 48 medical device eluates, these groups were analyzed separately. Of the 91 pesticide formulations and pure compounds, 63% (57/91) are LLNA positive and 37% (34/91) are LLNA negative. LLNA studies were done with either CBA (66 studies) and/or BALB/c (28 studies) mouse strains. The mouse strain was unspecified for 29 studies. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water.

GP data were available for 25 (four sensitizers/21 nonsensitizers in the GP) substances tested in aqueous solutions. The outcomes of 11 substances were discordant between the LLNA and the GP tests. Ten of the 11 discordant substances were pesticide formulations tested in aqueous 1% Pluronic L92; these were the same 10 substances previously discussed for the pesticide formulations analysis, and all were overpredicted by the LLNA with respect to the GP results (48% [10/21] false positive rate) (**Table 3-2**). One additional substance, neomycin sulfate, which was tested in 25% EtOH, was underpredicted by the LLNA with respect to the GP results (25% [1/4] false negative rate) (**Table 3-2**). Overall, the LLNA and the GP results were in agreement (accuracy) 56% (13/25) of the time (**Table 3-2**).

Human data were available for only four substances (three sensitizers/one nonsensitizer in humans) tested in aqueous solutions, while there were only two substances tested in aqueous solutions in the LLNA for which there was comparative GP and human data. Therefore, the database of substances tested in multiple test methods (i.e., LLNA, GP, and/or human) is too few to allow for a meaningful assessment of performance.

All 48 of the medical device eluates were negative in the LLNA. None of these eluates had associated GP or human data. These eluates were not analyzed to determine their constituents, or whether in fact any compound(s) were eluted from the medical device tested. Since the LLNA results were uniformly negative and no sample preparation control was included in the studies, the effectiveness of the sample preparation could not be determined. Therefore, the results from these eluates were not included with those from the pesticide formulations and pure substances tested in aqueous solutions.

### **3.5 Animal Welfare Considerations: Reduction, Refinement, and Replacement**

This comprehensive evaluation of the LLNA applicability domain should facilitate regulatory agency decisions on the acceptability of submitted LLNA studies for pesticide formulations and other products, metals, and substances tested in aqueous solutions. Following regulatory acceptance, use of the method by industry may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress in the LLNA procedure. This can be expected to significantly reduce the number of animals required for ACD testing while continuing to support the protection of human health.

## 4.0 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process incorporates a high level of transparency. This process is designed to provide numerous opportunities for stakeholder involvement, including submitting written public comments and providing oral comments at ICCVAM independent peer review panel meetings and SACATM meetings. **Table 4-1** lists the 12 different opportunities for public comment that were provided during the ICCVAM evaluation of the validation status of new versions and applications of the LLNA. The number of public comments received in response to each of the opportunities is also indicated. A total of 49 comments were submitted. Comments received in response to or related to the *Federal Register* notices are available on the NICEATM-ICCVAM website.<sup>13</sup> The following sections, delineated by *Federal Register* notice, briefly discuss the public comments received.

### 4.1 Public Comments in Response to 72 FR 27815 (May 17, 2007): The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

NICEATM requested the following:

1. Public comments on the appropriateness and relative priority of evaluation of the validation status of
  - a. The LLNA as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification
  - b. The reduced LLNA approach (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b)
  - c. Nonradioactive LLNA methods
  - d. The use of the LLNA for testing mixtures, aqueous solutions, and metals
  - e. The current applicability domain
2. Nominations of expert scientists to consider as members of a possible peer review panel
3. Submission of data for the LLNA and/or modified versions of the LLNA

In response to this FR notice, NICEATM received 17 comments. Six comments included additional data and information, while two others offered data and information upon request. Three commenters nominated four potential panelists for consideration. Three commenters suggested reference publications for consideration during the Panel evaluation. The nominees were included in the database of experts from which the Panel was selected. The data and suggested references were included in the initial draft ICCVAM review documents that were provided to the Panel at the March 2008 meeting.

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<sup>13</sup> Available at <http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm>

**Table 4-1 Opportunities for Public Comment**

<b>Opportunities for Public Comments</b>	<b>Date</b>	<b># of Public Comments Received</b>
72 FR 27815: The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data	May 17, 2007	17
72 FR 52130: Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments	September 12, 2007	4
73 FR 1360: Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments	January 8, 2008	7
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	March 4-6, 2008	16
73 FR 25754: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	May 7, 2008	1
73 FR 29136: Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	May 20, 2008	0
SACATM Meeting, Radisson Hotel, RTP, NC	June 18-19, 2008	0
74 FR 8974: Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments	February 27, 2009	1
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	April 28-29, 2009	2
74 FR 19562: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 29, 2009	0
74 FR 26242: Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	June 1, 2009	1
SACATM Meeting, Hilton Arlington Hotel, Arlington, VA	June 25-26, 2009	0

1. A commenter suggested rearranging the priority sequence of test method evaluation from most to least pressing: a, e, d, b, and c (see list above).

ICCVAM did not establish a relative priority for these activities because they were all considered to be high-priority activities. Accordingly, all LLNA-related activities described above were discussed at the March 2008 Panel meeting.

Two comments pertained to the LLNA applicability domain.

1. One commenter noted that the LLNA is the only method that can be used in the United Kingdom for assessment of skin sensitization potential for regulatory purposes and highlighted that in some areas of the chemical industry there is concern regarding the applicability of the LLNA for testing of preparations, mixtures and irritant substances. The commenter also noted that there is concern with regard to the view that the LLNA has not always provided results consistent with existing knowledge of the test substance or related test substances. The commenter indicated that since the LLNA offers significant scientific and animal welfare advantages over GP models for many product types, and, in the U.K., the LLNA is effectively the only available method for evaluation of skin sensitization potential for regulatory purposes, an assessment of the LLNA is welcomed.

ICCVAM initiated an assessment of the peer-reviewed literature and available data, and prepared a comprehensive background review document, to assess the LLNA applicability domain.

2. Another commenter indicated that available information should allow ICCVAM to make a rapid determination of the applicability and limitations of the LLNA for testing aqueous mixtures and metals, and, if not, then further validation efforts in this regard, should instead focus on *in vitro* methods.

In addition to *in vivo* refinement (less pain and distress) alternatives (such as the LLNA), ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

#### **4.2 Public Comments in Response to 72 FR 52130 (September 12, 2007): Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments**

NICEATM requested public comments on the September 2007 draft ICCVAM-recommended LLNA performance standards developed to facilitate evaluation of modified LLNA test method protocols with regard to the traditional LLNA. In response to this FR notice, NICEATM received four comments, two of which suggested clarifications to the text. Another comment recommended that test substances chosen for testing in the various LLNA methods should be pure, with conclusive structures, and should not be mixtures. Most comments specifically addressed the LLNA performance standards, although one comment pertained to the LLNA in general.

1. One commenter supported the development of performance standards that expedite the validation of new protocols similar to previously validated methods but was disappointed that NICEATM-ICCVAM had chosen to develop performance standards for such a narrow scope of applicability (i.e., modifications of the standard LLNA that involve incorporation of nonradioactive methods of detecting lymphocyte proliferation). The commenter suggested that limited resources available to NICEATM-ICCVAM would be better spent on activities that would have greater impact on the reduction, refinement, or replacement of animal use, such as evaluating the use of human cell lines or *in vitro* skin models as a replacement for the LLNA.

ICCVAM considered the comment and concludes that the proposed modifications to the LLNA test method protocol and expanded applications have the potential to further reduce and refine animal use. ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

There were no comments that specifically addressed the LLNA applicability domain.

#### **4.3 Public Comments in Response to 73 FR 1360 (January 8, 2008): Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments**

NICEATM requested public comments on the drafts for the January 2008 BRDs, ICCVAM test recommendations, test method protocols, and LLNA performance standards for an international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received 23 comments in response to this FR notice; seven written comments were received in advance of the meeting, and 16 oral comments were offered at the Panel meeting.

Two written comments were relevant to the LLNA applicability domain.

1. One commenter indicated that the limited data prevented a conclusive recommendation for the use of the LLNA to predict the skin sensitization potential of mixtures, metals, and aqueous solutions. Thus, the commenter viewed that the approach to expand the applicability domain of the LLNA had not been successful, and recommended that further resources be directed towards the pursuit of *in vitro* methods.

ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

2. Another commenter indicated that the dataset used to evaluate mixtures was limited due to the lack of human data for comparison (i.e., only comparative GP data were available). The commenter questioned the likelihood that GP data is representative of the human response. Thus, they did not consider using GP data as reference data to be appropriate. In addition, the usefulness of the data was limited further by the fact that information on the ingredients was known for only one of the 15 mixtures and 11 were tested in the LLNA in an aqueous vehicle (noting that the usefulness and limitations of the LLNA for testing substances in aqueous solutions was also being evaluated).
  - As indicated in the January 2008 ICCVAM draft recommendations the limitations with the database indicated that more data were needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures could be made.

The commenter further noted that Lalko and Api (2006) evaluated essential oils and included analytical data on the composition of the oils as well as LLNA data on the identified major constituents and that these data should have been included in the evaluation and not just mentioned as other available scientific reports.

- These data are included in the ICCVAM final Addendum for the LLNA applicability domain (see **Appendix D**).

The same commenter also agreed with the January 2008 ICCVAM draft recommendation that the LLNA is useful for the testing of metal compounds but questioned the importance or need to assess the LLNA's ability to detect metal allergens since the allergenic potential in humans of most known metals has already been established. Further, whether or not the LLNA is useful for testing nickel

compounds is of limited importance as nickel is a known human contact allergen. In addition, since only one of the 14 metal compounds with LLNA and human data was tested in an aqueous vehicle, the comparison did not add much value to the assessment, especially in light of the fact that the performance of the LLNA using aqueous vehicles was being assessed in this same report.

- ICCVAM considers it important to characterize the ability of the LLNA to appropriately detect the sensitization status of metals because metals may be components of formulated products that require testing to determine their skin sensitization potential.

The commenter also agreed with the January 2008 ICCVAM draft recommendation that an assessment of the suitability of the LLNA for testing substances in aqueous solutions should not be conducted until a sufficient quantity of quality data become available.

Two oral comments were relevant to the LLNA applicability domain.

1. One commenter noted that the LLNA could be used to test pesticide formulations and supported the efforts of the EPA and ICCVAM to confirm the validity of the LLNA for testing mixtures/formulations. If the LLNA is not accepted for testing formulations in the United States, international companies will be required to conduct both the LLNA and GP tests to satisfy the differing regulatory requirements for each formulation developed for global distribution. Such additional animal would be counter to the ICCVAM goal of reducing, refining, and replacing animal use in regulatory safety testing.

As outlined in the test method recommendations (see **Section 2.0**), ICCVAM recommends that the LLNA can be used for testing pesticide formulations, complex natural substances, dyes, metal compounds (except nickel), and substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle to maintain the test substance in contact with the skin (e.g., 1% Pluronic L92 [Boverhoff et al, 2008]) so an adequate exposure is achieved, as demonstrated by a positive control response.

2. Another commenter expressed reservations about using the LLNA to test complex mixtures and formulations because it was developed to test single substances. The commenter also stated that, since most metals have already been tested (and their sensitization potential characterized), it does not seem worthwhile to try to optimize the LLNA for hazard and potency categorization for testing metals.
  - As outlined in the test method recommendations (see **Section 2.0**), the LLNA can be used for testing pesticide formulations, complex natural substances, dyes, metal compounds (except nickel), and substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle, to maintain the test substance in contact with the skin (e.g. 1% Pluronic L92 [Boverhoff et al. 2008]) so an adequate exposure is achieved, as demonstrated by positive control results.
  - ICCVAM considers it important to characterize the ability of the LLNA to appropriately detect the sensitization status of metals because metals may be components of formulated products that require testing to determine their skin sensitization potential.

#### **4.4 Public Comments in Response to 73 FR 25754 (May 7, 2008): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)**

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. One public comment was received in response to this FR notice. The commenter made

a general comment that the members of SACATM do not represent a cross-section of the American public.

The SACATM charter indicates that the Committee shall consist of 15 members, including the Chair. Voting members shall be appointed by the Director, NIEHS, and include representatives from an academic institution, a State government agency, an international regulatory body, or any corporation developing or marketing new or revised or alternative test methodologies, including contract laboratories. Knowledgeable representatives from public health, environmental communities, or organizations using new or alternative test methodologies may be included as appropriate. There shall be at least one knowledgeable representative having a history of expertise, development, or evaluation of new or revised or alternative test methods from each of the following categories: (1) personal care, pharmaceutical, industrial chemicals, or agricultural industry; (2) any other industry that is regulated by one of the Federal agencies on ICCVAM; and (3) a national animal protection organization established under section 501(c)(3) of the Internal Revenue Code of 1986. The Director, NIEHS, shall select the Chair from among the appointed members of SACATM.

#### **4.5 Public Comments in Response to 73 FR 29136 (May 20, 2008): Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments**

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. No public comments were received in response to this FR notice.

#### **4.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008**

The June 18-19, 2008, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method.

There were no public comments specific to the LLNA applicability domain.

Regarding the LLNA applicability domain, one SACATM member indicated that there was not enough data and information to offer an informed opinion.

As indicated in the January 2008 ICCVAM draft recommendations, more data and information were needed to make final recommendations for the LLNA applicability domain. NICEATM subsequently obtained additional data for pesticide formulations, dyes, and natural complex substances for inclusion in the updated draft Addendum that was evaluated by the Panel in April 2009.

#### **4.7 Public Comments in Response to 74 FR 8974 (February 27, 2009): Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments**

NICEATM requested public comments on the updated drafts for the BRDs, Addendum, ICCVAM test method recommendations, and test method protocols for the second international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received three comments in response to this FR notice; one written comment, and two oral comments offered at the Panel meeting.

1. This was a general comment expressing concern that the extensive time and resources that ICCVAM has devoted to this evaluation has detracted from focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

ICCVAM considers the evaluations conducted to date have significant potential to further reduce and refine animal use, particularly where the use of the LLNA is precluded due to restrictions associated with the use of radioactivity. ICCVAM is also committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

The commenter further made one comment relevant to the LLNA applicability domain.

1. The commenter stated that the limited availability of data or the lack of clear definition of the test substance prevented a conclusive recommendation from the previous ICCVAM review for the use of the LLNA. The commenter noted that the updated recommendations from the current review of formulation and aqueous solutions offered a potential for expanded use, if overclassification was accepted (presumably by both the manufacturer and the regulatory agency). The commenter further noted that, in the interim, little had changed in the availability of comparative human data and they supported the ICCVAM recommendation that there is a need to identify relevant human data and human experience in order to continue to evaluate the applicability of LLNA to mixtures and aqueous solutions. The commenter indicated that this approach would provide the most valuable information and would not involve further animal testing, and therefore should be a priority.

- ICCVAM will consider this comment when prioritizing future activities.

#### **4.8 Public Comments in Response to 74 FR 19562 (April 29, 2009): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)**

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. No public comments were received in response to this FR notice.

#### **4.9 Public Comments in Response to 74 FR 26242 (June 1, 2009): Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments**

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. One comment was received in response to this FR notice.

The commenter did not make a comment relevant to the LLNA applicability domain.

#### **4.10 Public and SACATM Comments: SACATM Meeting on June 25-26, 2009**

The June 25-26, 2009, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method.

There were no public comments specific to the LLNA applicability domain.

In general, SACATM was supportive of the Panel report. However, there was general concern regarding the potential for over-labeling substances that may occur by using LLNA test results. They emphasized the need for developing non-animal test methods for identifying potential skin sensitizers.

Regarding the LLNA applicability domain, one SACATM member expressed concern about the limited additional data for the pesticide formulations. Compared to the original work on single substances, these data show that the pesticide formulations appear to produce false positives in the LLNA. The difference in sensitivity between the Buehler test and the GPMT was clarified. For the 22 substances for which there were comparative tests, 18 of the GPMTs were actually Buehler tests, so

there is a question as to whether they could have been concordant if they had been GPMTs. Strictly comparing the performance of the LLNA and the GPMT for those 22 substances, the accuracy is not great because the trend was to get a positive result more often in the LLNA.

As indicated in the ICCVAM final test method recommendations (**Section 2.1**), the potential for possible overclassification of pesticide formulations may be a limitation of the LLNA.

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**Appendix A**  
**ICCVAM Evaluation Timeline**

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### ICCVAM Evaluation Timeline

<b>January 10, 2007</b>	ICCVAM receives a letter from the Consumer Product Safety Commission (CPSC) nominating six murine local lymph node assay (LLNA) review activities for evaluation, including the LLNA applicability domain.
<b>January 2007</b>	The ICCVAM Immunotoxicity Working Group (IWG) is re-established to work with NICEATM to carry out LLNA evaluations.
<b>January 24, 2007</b>	ICCVAM endorses the six CPSC-nominated LLNA review activities, including evaluation of the LLNA applicability domain.
<b>May 17, 2007</b>	<i>Federal Register</i> notice (72 FR 27815) – The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data.
<b>June 12, 2007</b>	SACATM endorses with high priority the six CPSC-nominated LLNA review activities, including evaluation of the LLNA applicability domain.
<b>November 12–13, 2007</b>	ECVAM Workshop on Alternative Methods (Reduction, Refinement, Replacement).
<b>January 8, 2008</b>	<i>Federal Register</i> notice (73 FR 1360) – Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments.
<b>March 4–6, 2008</b>	Independent Scientific Peer Review Panel holds a public meeting, with opportunity for oral public comments, at CPSC Headquarters in Bethesda, MD, to discuss LLNA review activities, including the LLNA applicability domain. The Panel is charged with reviewing the current status of the LLNA applicability domain and commenting on the extent to which the information in the draft LLNA Addendum on the validity of the LLNA for mixtures, metals, and aqueous solutions supported the draft ICCVAM recommendations.
<b>May 20, 2008</b>	<i>Federal Register</i> notice (73 FR 29136) – Announcement of the Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments.
<b>June 18–19, 2008</b>	SACATM public meeting for comments on the 2008 Panel report.

- February 27, 2009** *Federal Register* notice (74 FR 8974) – Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments.
- April 28–29, 2009** Independent Scientific Peer Review Panel holds a public meeting with opportunity for oral public comments, at NIH Natcher Conference Center in Bethesda, MD, to discuss LLNA review activities, including the updated LLNA applicability domain. The Panel is charged with reviewing the current status of the LLNA applicability domain and commenting on the extent to which the information in the revised draft LLNA Addendum on the validity of the LLNA for mixtures, metals, and aqueous solutions supported the revised draft ICCVAM recommendations.
- June 1, 2009** *Federal Register* notice (74 FR 26242) – Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments.
- June 25–26, 2009** SACATM public meeting for comments on the 2009 Panel report.
- October 28, 2009** ICCVAM endorses the TMER for the LLNA applicability domain, which includes the final LLNA Addendum on the validity of the LLNA for mixtures, metals, and aqueous solutions.

## **Appendix B**

### **ICCVAM-Recommended Protocol**

#### **The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products**

Annex I

An Approach to Dissection and Identification of the Draining (“Auricular”) Lymph Nodes B-12

Annex II

An Example of How to Reduce the Number of Animals in the Concurrent Positive Control Group of the Local Lymph Node Assay.....B-15

Annex III

Evaluating Local Irritation and Systemic Toxicity in the Local Lymph Node Assay .....B-17

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## Preface

The murine local lymph node assay (LLNA) is a test method developed to assess whether a chemical has the potential to induce allergic contact dermatitis (ACD) in humans. In 1998, the LLNA was submitted to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for evaluation as an alternative (i.e., stand-alone) test method to the guinea pig (GP) sensitization tests accepted by U.S. regulatory agencies. In 1999, based on a comprehensive evaluation of the LLNA by an independent scientific peer review panel (Panel),<sup>1</sup> ICCVAM concluded that the LLNA is an acceptable alternative to the GP test methods to assess the ACD hazard potential of most substances (Dean et al. 2001). The Panel also concluded that the LLNA offers animal welfare advantages compared to use of the traditional GP methods, in that it provides for animal use refinement (i.e., elimination of distress and pain) and reduces the total number of animals required. An ICCVAM Immunotoxicity Working Group (IWG) reviewed the 1999 Panel report and developed recommendations applicable to the regulatory use of the LLNA. The IWG then worked with the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to produce a recommended test method protocol (ICCVAM 2001)<sup>2</sup> that would accurately reflect the ICCVAM and Panel recommendations (ICCVAM 1999).

In March 2008, ICCVAM and NICEATM convened an independent scientific peer review panel (Panel) to evaluate new versions and applications of the LLNA. The Panel provided conclusions and recommendations in their report, many of which were applicable to the traditional LLNA test method protocol.<sup>3</sup> ICCVAM subsequently considered the Panel's conclusions and recommendations, as well as comments from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and public, and updated the 2001 ICCVAM-recommended LLNA test method protocol. The updated ICCVAM-recommended LLNA test method protocol will be forwarded with the Panel's report to agencies for their consideration.

The updated ICCVAM-recommended test method protocol for the LLNA is based on evaluation of previous experience and scientific data. It is provided to Federal agencies for their consideration as a standardized test method protocol recommended for generation of data for regulatory purposes. Prior to conducting an LLNA test to meet a regulatory requirement, the appropriate regulatory agency should be contacted for their current guidance on the conduct and interpretation of this assay. Additional information on the ICCVAM LLNA review process and deliberations of the Panel can be found at the ICCVAM website (<http://iccvam.niehs.nih.gov>) or in the Panel report (ICCVAM 2008a).

We want to express our sincere appreciation to the ICCVAM IWG for their careful deliberations and efforts in updating the LLNA test method protocol, and especially appreciate the efforts of the Working Group Co-Chairs, Abigail Jacobs, Ph.D., from the U.S. Food and Drug Administration and Joanna Matheson, Ph.D., from the U.S. Consumer Products Safety Commission. We also want to acknowledge the outstanding support provided by NICEATM and the Integrated Laboratory Systems, Inc., support staff. Lastly, we appreciate the efforts of the Panel members for their diligent review, and the comments provided by SACATM and numerous stakeholders, including the public.

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<sup>1</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/llnarep.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf)

<sup>2</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/LLNAProt.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf)

<sup>3</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

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## 1.0 General Principle of Detection of Skin Sensitization Using the Local Lymph Node Assay

The basic principle underlying the murine local lymph node assay (LLNA) is that sensitizers induce proliferation of lymphocytes in the lymph node draining the site of substance application. Under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cellular proliferation as a function of *in vivo* radioisotope incorporation into the DNA of dividing lymphocytes. The LLNA assesses this proliferation in the draining lymph nodes proximal to the application site (see **Annex I**). This effect occurs as a dose response in which the proliferation in test groups is compared to that in the concurrent vehicle-treated control group. A concurrent positive control is added to each assay to provide an indication of appropriate assay performance.

## 2.0 Description of the Local Lymph Node Assay

### 2.1 Sex and strain of animals

Young adult female mice (nulliparous and nonpregnant) of the CBA/Ca or CBA/J strain are recommended.<sup>4</sup> Females are used because most data in the existing database were generated using mice of this gender. At the start of the study, mice should be age 8–12 weeks. All mice should be age matched (preferably within a one-week time frame). Weight variations between the mice should not exceed 20% of the mean weight.

### 2.2 Preparation of animals

The temperature of the experimental animal room should be 21°C (±3°C) and the relative humidity 30%–70%. When artificial lighting is used, the light cycle should be 12 hours light: 12 hours dark. For feeding, an unlimited supply of standard laboratory mouse diets and drinking water should be used. The mice should be acclimatized for at least five days prior to the start of the test (ILAR 1996). Mice should be housed in small groups unless adequate scientific rationale for housing mice individually is provided (ILAR 1996). Healthy mice are randomly assigned to the control and treatment groups. The mice are uniquely identified prior to being placed in the study. The method used to mark the mice should not involve identification via the ear (e.g., marking, clipping, or punching of the ear). All mice should be examined prior to the initiation of the test to ensure that there are no skin lesions present.

### 2.3 Preparation of doses

Solid test substances should be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the mice. Liquid test substances may be dosed directly (i.e., applied neat) or diluted prior to dosing. Fresh preparations of the test substance should be prepared daily unless stability data demonstrate the acceptability of storage.

### 2.4 Test Conditions

#### 2.4.1 Solvent/vehicle

The selected solvent/vehicle must not interfere with or bias the test result and should be selected on the basis of maximizing the test concentrations while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone: olive oil (4:1 v/v), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide, but others may be used (Kimber and Basketter 1992). Particular care should be taken to

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<sup>4</sup> Male mice or other strains of mice may be used if it is sufficiently demonstrated that these animals perform as well as female CBA mice in the LLNA.

ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles may need to be avoided. It may be necessary for regulatory purposes to test the substance in the clinically relevant solvent or product formulation.

### 2.4.2 Controls

Concurrent negative (solvent/vehicle) controls should be included in each test to ensure that the test system is functioning properly and that the specific test is valid. In some circumstances (e.g., when using a solvent/vehicle not recommended in **Section 2.4.1**), it may be useful to include a naïve control. Except for treatment with the test substance, the mice in the negative control groups should be handled in an identical manner to the mice of the treatment groups.

Concurrent positive controls are used to ensure the appropriate performance of the assay by demonstrating that the test method is responding with adequate and reproducible sensitivity to a sensitizing substance for which the magnitude of the response is well characterized. Inclusion of a concurrent positive control is also important since it can confirm technical competence in performing the test and can demonstrate intra- and interlaboratory reproducibility and comparability. The positive control should produce a positive LLNA response (i.e., a stimulation index [SI]  $\geq 3$  over the negative control group). In particular, for negative LLNA studies, the concurrent positive control must induce a SI  $\geq 3$  relative to its vehicle-treated control. The positive control dose should be chosen such that the induction is reproducible but not excessive (i.e., SI  $> 20$ ). Preferred positive control substances are hexyl cinnamic aldehyde or mercaptobenzothiazole. There may be circumstances where, given adequate justification, other positive control substances may be used.

Although the positive control substance should be tested in the same vehicle as the test substance, there may be certain regulatory situations where it is necessary to test the positive control substance in both a standard and a non-standard vehicle (e.g., a clinically/chemically relevant formulation) to test for possible interactions.

Inclusion of a positive control with each test is recommended to ensure that all test method protocol procedures are being conducted properly and that all aspects of the test system are working properly such that they are capable of producing a positive response. However, periodic testing (i.e., at intervals  $\leq 6$  months) of the positive control substance may be considered in laboratories that conduct the LLNA regularly (i.e., conduct the LLNA at a frequency of no less than once per month) and that have a history and a documented proficiency for obtaining consistent results with positive controls. Adequate proficiency with the LLNA can be successfully demonstrated by generating consistent results with the positive control in at least 10 independent tests conducted within a reasonable period of time (i.e., less than one year). A positive control group should always be included when there is a procedural change to the LLNA (i.e., change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals, etc.), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the positive control results. Users should be aware that the decision to only include a positive control on a periodic basis instead of concurrently will have ramifications on the adequacy and acceptability of negative study results generated without a concurrent positive control during the interval between each periodic positive control study. For example, if a false negative result is obtained in the periodic positive control study, all negative test substance results obtained in the interval between the last acceptable periodic positive control study and the unacceptable periodic positive control study will be questioned. In order to demonstrate that the prior negative test substance study results are acceptable, a laboratory would be expected to repeat all negative studies, which would require additional expense and increased animal use. These implications should be carefully considered when determining whether to include concurrent positive controls or to only conduct periodic positive controls.

Consideration should also be given to using fewer animals in the concurrent positive control group when this is scientifically justified, as discussed below and in **Annex II**.

Benchmark controls may be useful to demonstrate that the test method is functioning properly for detecting the skin sensitization potential of substances of a specific chemical class or a specific range of responses, or for evaluating the relative skin sensitization potential of a test substance. Appropriate benchmark controls should have the following properties:

- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data on known effects in animal models
- Known potency for sensitization response

## 2.5 Methodology

A minimum of four animals per dose group is recommended. The collection of lymph nodes from individual mice is necessary in order to identify if any of the individual animal responses are outliers (e.g., in accordance with statistical tests such as Dixon's test). This will aid in avoiding false negative results for weaker sensitizers (i.e., substances that normally would induce an SI just above 3 might be incorrectly classified as negative due to a low outlier value, because the resulting mean SI may be less than 3 if an outlier is not identified and excluded). Individual animal measurements allow for the assessment of interanimal variability, a statistical comparison of the difference between test substance and vehicle control group measurements, and the evaluation of statistical power for different group sizes. Finally, evaluating the possibility of reducing the number of mice in the positive control group is only feasible when individual animal data are collected.

As noted above, concurrent negative and positive control groups should be included, unless a laboratory can demonstrate adequate proficiency that would support the use of a periodic positive control study. The number of mice in the concurrent positive control group might be reduced compared to the vehicle and test substance groups, if the laboratory demonstrates, based on laboratory-specific historical data,<sup>5</sup> that fewer mice can be used without substantially increasing the frequency with which studies will need to be repeated. An example of how to reduce the number of mice in the concurrent positive control group is provided in **Annex II**.

Test substance treatment dose levels should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Panel Report (ICCVAM 1999). Dose levels are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. The maximum concentration tested should be the highest achievable level while avoiding excessive local irritation and overt systemic toxicity (**Annex III**). Efforts should be made to identify existing information that may aid in selecting the appropriate maximum test substance dose level. In the absence of such information, an initial prescreen test, conducted under identical experimental conditions except for omission of an assessment of lymph node proliferative activity, may be necessary. In order to have adequate information from which to select a maximum dose level to use in the definitive test and to identify a dose-response relationship, data should be collected on at least three test substance dose levels with two mice per dose group, in addition to the concurrent solvent/vehicle control group.

The LLNA experimental procedure is performed as follows:

- Day 1.** Identify and record the weight of each mouse before applying the test substance. Apply 25  $\mu$ L/ear of the appropriate dilution of the test substance, or the positive control, or the solvent/vehicle only, to the dorsum of both ears of each mouse.

<sup>5</sup> A robust historical dataset should include at least 10 independent tests, conducted within a reasonable period of time (i.e., less than one year), with a minimum of four mice per negative and positive control groups.

**Days 2 and 3.** Repeat the application procedure as carried out on Day 1.

**Days 4 and 5.** No treatment.

**Day 6.** Record the weight of each mouse. Inject 250  $\mu\text{L}$  of sterile phosphate-buffered saline (PBS) containing 20  $\mu\text{Ci}$  of tritiated ( $^3\text{H}$ )-methyl thymidine or 250  $\mu\text{L}$  PBS containing 2  $\mu\text{Ci}$  of  $^{125}\text{I}$ -iododeoxyuridine ( $^{125}\text{IU}$ ) and  $10^{-5}$  M fluorodeoxyuridine into each mouse via the tail vein (Kimber et al. 1995; Loveless et al. 1996). Five hours later, euthanize each mouse and collect the draining (“auricular”) lymph nodes of both ears and place in PBS (one container per mouse). Both bilateral draining lymph nodes must be collected (see diagram and description of dissection in **Annex I**). Prepare a single-cell suspension of lymph node cells (LNC) for each individual mouse. The single-cell suspension is prepared in PBS by either gentle mechanical separation through 200-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension. Wash LNC twice with an excess of PBS and precipitate the DNA with 5% trichloroacetic acid (TCA) at  $4^\circ\text{C}$  for approximately 18 hours.

For the  $^3\text{H}$ -methyl thymidine method, resuspend pellets 1 mL TCA and transfer to 10 mL of scintillation fluid. Incorporation of  $^3\text{H}$ -methyl thymidine is measured by  $\beta$ -scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the  $^{125}\text{IU}$  method, transfer the 1 mL TCA pellet directly into gamma-counting tubes. Incorporation of  $^{125}\text{IU}$  is determined by gamma counting and also expressed as dpm/mouse.

## 2.6 Observations

Mice should be carefully observed for any clinical signs, either of local irritation at the application site or of systemic toxicity (**Annex III**). Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded and records maintained for each individual mouse. Animal monitoring plans must include criteria to promptly identify mice exhibiting systemic toxicity or excessive irritation or corrosion of skin for euthanasia.

## 3.0 Calculation of Results

Results for each treatment group are expressed as the mean SI. Each SI is the ratio of the mean dpm/mouse within each test-substance treatment group or the positive control treated group against the mean dpm/mouse for the solvent/vehicle treated control group. The investigator should be alert to possible outlier responses for individual mice within a group that may necessitate analysis both with and without the outlier.

In addition to a formal assessment of the magnitude of the SI, a statistical analysis for presence and degree of dose response may be conducted, which is possible only with the use of individual animals. Any statistical assessment should include an assessment of the dose-response relationship as well as suitably adjusted comparisons of test groups (e.g., pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Analyses may include, for instance, linear regression, William’s test to assess dose-response trends, or Dunnett’s test for pairwise comparisons. In choosing an appropriate method of statistical analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis.

## 4.0 Evaluation and Interpretation of Results

In general, when the SI for any single treatment dose group is  $\geq 3$ , the test substance is regarded as a skin sensitizer (Kimber et al. 1994; Basketter et al. 1996; ICCVAM 1999) and a test substance not meeting this criterion is considered a non-sensitizer in this test. However, the magnitude of the observed SI should not be the sole factor used in determining the biological significance of a skin

sensitization response. Additional factors that could be considered include the outcomes of statistical analyses, the strength of the dose-response relationship, chemical toxicity, and solubility. For instance, a quantitative assessment may be performed by statistical analysis of individual mouse data and may provide a more complete evaluation of the test substance's ability to act as a sensitizer (see **Section 3.0**). Equivocal results (e.g., the SI does not reach 3, but it is near 3 and there is a positive dose-response relationship) should be clarified by performing statistical analysis, and by considering structural relationships, available toxicity information, and dose selection.

## 5.0 Data and Reporting

### 5.1 Data

Individual animal dpm data should be presented in tabular form, along with the group mean dpm/mouse, its associated error term, and the mean SI (and associated error term) for each dose group compared against the concurrent solvent/vehicle control group.

### 5.2 Test Report

The test report should contain the following information:

#### *Test Substances and Control Substances*

- Identification data and Chemical Abstracts Service Registry Number, if known
- Physical nature and purity
- Physiochemical properties relevant to the conduct of the study
- Stability of the test substance, if known
- Lot number of the test substance

#### *Solvent/Vehicle:*

- Justification for choice of solvent/vehicle
- Solubility and stability of the test substance in the solvent/vehicle

#### *Test Animals:*

- Strain of mice used
- Number, age, and sex of mice
- Source, housing conditions, diet, etc.
- Individual weight of the mice at the start and end of the test, including body weight range, as well as mean and associated error term for each group
- Microbiological status of the mice

#### *Test Conditions:*

- Concurrent and historical positive and negative (solvent/vehicle) control data
- Data from range-finding study, if conducted
- Rationale for dose-level selection
- Details of test substance preparation
- Details of the administration of the test substance
- Details of food and water quality
- Detailed description of treatment and sampling schedules
- Methods for measurement of toxicity
- Criteria for considering studies as positive, negative, or equivocal

#### *Results:*

- Signs of systemic toxicity and/or local irritation
- Values for dpm/mouse for each mouse within each treatment group

- Mean and associated error term for dpm/mouse for each treatment group and the results of outlier analysis for each dose group should be provided
- Calculated SI and an appropriate measure of variability that takes into account the interanimal variability in both the test substance dosed and control groups
- Dose-response relationship
- Statistical analyses and method applied
- Concurrent and historical positive and negative (solvent/vehicle) control data as established in the test laboratory
- Concurrent positive control data or, if not done, the date and laboratory report for the most recent periodic positive control and a report detailing the historical positive control data for the laboratory justifying the basis for not conducting a concurrent positive control.

#### *Discussion of the Results*

#### *Conclusion*

#### *A Quality Assurance Statement for GLP-compliant Studies*

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

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## **Annex I: An Approach to Dissection and Identification of the Draining ("Auricular") Lymph Nodes**

### **1.0 Background**

Although minimal technical training of the murine local lymph node assay (LLNA) is required, extreme care must be taken to ensure appropriate and consistent dissection of the lymph nodes. It is recommended that technical proficiency in the dissection and identification of the lymph nodes draining the ear be achieved by practice on mice that have been (a) injected with a colored agent (dye) and/or (b) sensitized with a strong positive sensitizer. Brief descriptions of these practice dissections are provided below. Recognizing that nodes from vehicle-treated and naïve mice are smaller, laboratories performing the LLNA must also gain proficiency in the dissection of these nodes. It may be helpful for laboratories inexperienced in this procedure to request guidance from laboratories that have successfully performed the LLNA.

### **2.0 Training and Preparation for Node Identification**

#### **2.1 Identification of the Draining Node – Dye Treatment**

There are several methods that can be used to provide color identification of the draining nodes. These techniques may be helpful for initial identification and should be performed to ensure proper isolation of the appropriate node. Examples of such treatments are listed below. It should be noted that other such protocols might be used effectively.

##### **2.1.1 Evan's Blue Dye treatment:**

Inject approximately 0.1 mL of 2% Evan's Blue Dye (prepared in sterile saline) intradermally into the pinnae of an ear. Euthanize the mouse after several minutes and continue with the dissection as noted below.

##### **2.1.2 Colloidal carbon and other dye treatments:**

Colloidal carbon and India ink are examples of other dye treatments that may be used (Tilney 1971).

#### **2.2 Identification of the Draining Node – Application of Strong Sensitizers**

For the purpose of node identification and training, a strong sensitizer is recommended. This agent should be applied in the standard acetone: olive oil vehicle (4:1). Suggested sensitizers for this training exercise include 0.1% oxazolone, 0.1% (w/v) 2,4-dinitrochlorobenzene, and 0.1% (v/v) dinitrofluorobenzene. After treating the ear with a strong sensitizer, the draining node will dramatically increase in size, thus aiding in identification and location of the node.

Using a procedure similar to that described in the test method protocol, apply the agent to the dorsum of both ears (25 µL/ear) for 3 consecutive days. On the fourth day, euthanize the mouse.

Identification and dissection (listed below) of the node should be performed in these animals prior to practice in non-sensitized or vehicle-treated mice, where the node is significantly smaller.

Please note: Due to the exacerbated response, the suggested sensitizers are not recommended as controls for assay performance. They should only be used for training and node identification purposes.

### **3.0 Dissection Approach**

#### **3.1 Lateral Dissection (Figure B-1)**

Although lateral dissection is not the conventional approach used to obtain the nodes draining the ear, it may be helpful as a training procedure when used in combination with the ventral dissection. Perform this approach bilaterally (on both sides of the mouse). After euthanizing the mouse, place it in a lateral position. Wet the face and neck with 70% ethanol. Use scissors and forceps to make an initial cut from the neck area slightly below the ear. Carefully extend the incision toward the mouth and nose. Angle the tip of the scissors slightly upward during this procedure to prevent the damage of deeper tissue. Gently retract the glandular tissue in the area using the forceps. Using the masseter muscle, facial nerves, blood vessels, and the bifurcation of the jugular vein as landmarks, isolate and remove the draining node (**Figure B-1**). The draining node (“auricular”) will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

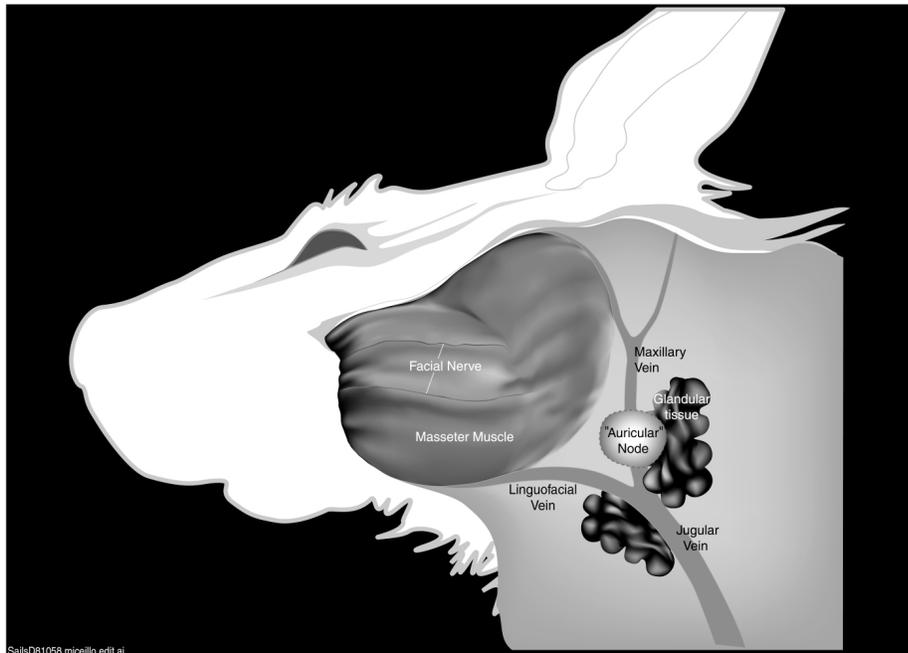
#### **3.2 Ventral Dissection (Figure B-2)**

The most commonly used dissection approach is from the ventral surface of the mouse. This approach allows both right and left draining nodes to be obtained without repositioning the mouse. With the mouse ventrally exposed, wet the neck and abdomen with 70% ethanol. Use scissors and forceps to carefully make the first incision across the chest and between the arms. Make a second incision up the midline perpendicular to the initial cut, and then cut up to the chin area. Reflect the skin to expose the external jugular veins in the neck area. Take care to avoid salivary tissue at the midline and nodes associated with this tissue. The nodes draining the ear (“auricular”) are located distal to the masseter muscle, away from the midline, and near the bifurcation of the jugular veins.

### **4.0 Accuracy in Identification**

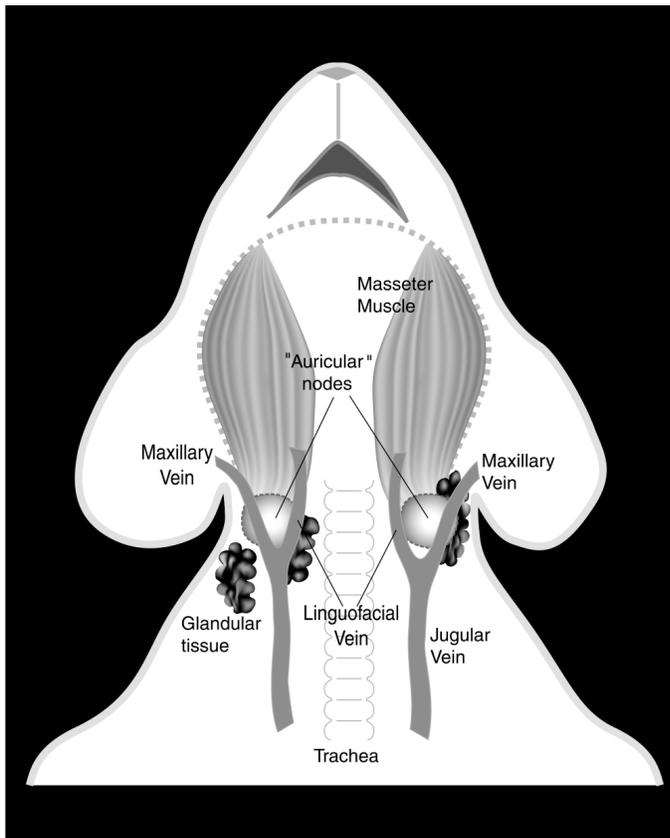
The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the node size. If a dye is injected for training purposes, the node will take on the tint of the dye.

**Figure B-1 Lateral Dissection**



Credit: Dee Sailstad, U.S. EPA

**Figure B-2 Ventral Dissection**



Credit: Dee Sailstad, U.S. EPA

## Annex II: An Example of How to Reduce the Number of Animals in the Concurrent Positive Control Group of the Local Lymph Node Assay

As stated in the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Murine Local Lymph Node Assay (LLNA) test method protocol (**Section 2.4.2 of Appendix B**), a concurrent positive control is recommended to ensure the appropriate performance of the assay. Appropriate performance is demonstrated when the test method responds with adequate and reproducible sensitivity to a sensitizing substance for which the magnitude of the response is well characterized. The number of mice in the concurrent positive control group may possibly be reduced if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used without compromising the integrity of the study (i.e., positive control results should always be positive compared to the vehicle control results). As illustrated in the example and accompanying explanation below, reducing the number of animals in the positive control group is only feasible when individual animal data are collected.

The stimulation index (SI) results for each positive control test can be used to generate mean SI values for every possible combination of SI values for as few as two animals. The mean SI values for every combination of numbers for each group size can then be used to calculate the failure rate of the positive control for each group size (i.e., the percentage of the combinations for which the mean  $SI < 3$ ). **Table B-1** provides an example of positive control results from four tests in one laboratory of 30% hexyl cinnamic aldehyde (HCA) using six CBA/J mice per group. In these tests, with six animals, HCA produced “borderline” positive results (i.e., the mean SI values were marginally greater than 3). To determine whether the number of animals can be reduced, sample size reductions (i.e.,  $N = 5, 4, 3,$  or  $2$ ) can be evaluated by taking all possible samples from the six values for each test given in **Table B-1**, which can occur in the following ways:  $N = 2$  (15 samples),  $N = 3$  (20 samples),  $N = 4$  (15 samples), and  $N = 5$  (6 samples).

**Table B-1 Example of SI Results from Four Local Lymph Node Assay Positive Control Studies with 30% HCA**

Test	1	2	3	4
Animal 1	2.13	3.56	4.68	0.78
Animal 2	4.55	1.54	4.44	9.16
Animal 3	3.64	3.00	5.41	6.66
Animal 4	1.98	3.87	3.32	3.02
Animal 5	3.09	3.79	2.89	2.32
Animal 6	3.77	3.96	1.81	2.91
<b>Mean SI</b>	<b>3.19</b>	<b>3.29</b>	<b>3.76</b>	<b>4.14</b>

Abbreviations: HCA = hexyl cinnamic aldehyde; SI = stimulation index

The failure rate of the positive control was then calculated using the SI results for each group of two, three, four, or five values to determine the likelihood of obtaining a mean  $SI < 3$ . The results for these four “borderline” HCA tests were then added to the results from an additional 12 robust positive control tests included in this laboratory’s historical database to determine the overall likelihood of obtaining a mean  $SI < 3$  for the positive control substance (**Table B-2**). The failure rate reflects the frequency with which a positive control test will fail, which would result in retesting the positive control and any concurrent test substances. Each laboratory is encouraged to determine the lowest number of animals to use in the positive control group based on the highest failure rate considered acceptable by the laboratory.

**Table B-2 Example of Positive Control Failure Rate for 30% HCA Based on Data Collected in Single Laboratory**

<b>Number of Animals</b>	<b>HCA Test 1</b>	<b>HCA Test 2</b>	<b>HCA Test 3</b>	<b>HCA Test 4</b>	<b>Results from Other Tests<sup>1</sup></b>	<b>Overall Likelihood of a Mean SI &lt; 3</b>
5	17% (1/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/72)	1% (1/96)
4	27% (4/15)	13% (2/15)	0% (0/15)	7% (1/15)	0% (0/180)	3% (7/240)
3	40% (8/20)	30% (6/20)	5% (1/20)	20% (4/20)	0% (0/240)	6% (19/320)
2	47% (7/15)	33% (5/15)	13% (2/15)	40% (6/15)	1% (1/180)	9% (21/240)

Abbreviations: HCA = hexyl cinnamic aldehyde; SI = stimulation index

<sup>1</sup> These represent 12 positive control studies in the same laboratory where all mice in the positive control groups treated with 30% HCA produced an SI  $\geq$  3.

### Annex III: Evaluating Local Irritation and Systemic Toxicity in the Local Lymph Node Assay

As noted in the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Murine Local Lymph Node Assay (LLNA) protocol, at least three dose levels of a test substance should be evaluated. The highest dose level tested should be a concentration of 100% (i.e., neat substance for liquid substances) or the maximum soluble concentration (for solids), unless available information suggests that this concentration induces systemic toxicity or excessive local irritation after topical application.

In the absence of such information, a prescreen test should be performed using three dose levels of the test substance, in order to define the appropriate dose level to test in the LLNA. Six mice (two per concentration) are used, and the prescreen is conducted under identical conditions as the main LLNA study, except there is no assessment of lymph node proliferation. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. For example, observations might occur before and after treatment on Days 1, 2, and 3. Body weights are recorded pre-test and prior to termination (Day 6). Both ears of each mouse are observed for erythema (and scored using **Table B-3**). Ear thickness measurements are taken using a thickness gauge (e.g., digital micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose), and Day 6.

Excessive local irritation is indicated by an erythema score  $\geq 3$  and/or ear swelling of  $\geq 25\%$ .

**Table B-3 Erythema Scores**

Observation	Value
No visual effect	0
Slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema (beet redness)	3
Eschar (i.e., piece of dead tissue that is cast off from the surface of the skin)	4

A 25% increase in ear swelling has been used as an initial step to identify substances that cause a skin reaction due to an irritant response rather than sensitization (Reeder et al. 2007; ICCVAM 2008b). A statistically significant difference from control animals has also been used to delineate irritants from non-irritants in the LLNA (Hayes et al. 1998; Homey et al. 1998; Woolhiser et al. 1998; Hayes and Meade 1999; Ehling et al. 2005; Vohr and Jürgen 2005; Patterson et al. 2007). While these statistical differences often occur when ear swelling is less than 25%, they have not been associated specifically with excessive irritation (Woolhiser et al. 1998; Ehling et al. 2005; Vohr and Jürgen 2005; Patterson et al. 2007). Additionally, an adequately robust statistical comparison would require that a vehicle control group be included and that more than two animals per group be tested. Both of these requirements would substantially increase the number of animals used for this prescreen test. For this reason, a threshold increase in ear swelling above pre-dosing levels is recommended for this prescreen test.

Test guidelines for assessing acute systemic toxicity recommend a number of clinical observations for assessing systemic toxicity (OECD 1987; EPA 1998). The following observations, which are based on test guidelines and current practices (ICCVAM 2009), may indicate systemic toxicity when used

as part of an integrated assessment and therefore may indicate that the maximum dose recommended for the LLNA has been exceeded:

- Clinical signs:
  - Changes in nervous system function (e.g., piloerection, ataxia, tremors, and convulsions)
  - Changes in behavior (e.g., aggressiveness, change in grooming activity, marked change in activity level)
  - Changes in respiratory patterns (i.e., changes in frequency and intensity of breathing such as dyspnea, gasping, and rales)
  - Changes in food and water consumption
  - Lethargy and/or unresponsiveness
  - Any clinical signs of more than slight or momentary pain and distress
- Reduction in body weight >10% from Day 1 to Day 6
- Mortality

## **Appendix C**

### **Comparison of LLNA Responses for Substances Tested in CBA and BALB/C Mice**

Comparison of LLNA Responses for Substances Tested in CBA and BALB/c Mice.....	C-1
Annex I:	
Data for Substances Tested in the LLNA in CBA and BALB/c Mice .....	C-15

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## 1.0 Introduction

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the LLNA is a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many types of substances (Haneke, et al., 2001). The LLNA provides several advantages compared to guinea pig methods, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information (Dean, et al. 2001; Sailstad et al., 2001). The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel assessment of LLNA validation status (ICCVAM 1999).

The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (OECD 2002; ISO 2002; EPA 2003) and is now commonly used worldwide. The recently updated ICCVAM-recommended LLNA protocol states that mouse strains other than CBA may be used in the LLNA if it is sufficiently demonstrated that these animals perform as well as CBA mice in the LLNA (ICCVAM 2009).

Although CBA/J and CBA/Ca mice are currently recommended as the preferred mouse strains in national and international LLNA test guidelines (OECD 2002; EPA 2003), the LLNA was originally developed using BALB/c mice (Kimber et al. 1986). Kimber and Weisenberger (1989) observed that *in vitro* proliferation of lymph node cells in response to exposure to 2,4-dinitrochlorobenzene was stronger in CBA/Ca mice than in BALB/c, and chose to focus on using CBA/Ca mice in further development efforts for the LLNA.

Woolhiser and co-workers assessed LLNA responses in various mouse strains including CBA and BALB/c. They found essentially equal levels of lymph node proliferation (as measured by incorporation of 3H-thymidine into the draining auricular lymph nodes) in both strains following exposure to the sensitizers  $\alpha$ -hexylcinnamaldehyde (HCA), 2,4-dinitrofluorobenzene (DNFB) and toluene diisocyanate (Woolhiser et al., 2000). Other U.S. groups have also published LLNA studies using BALB/c mice, including the National Institute for Occupational Safety and Health, the Dow Chemical Corporation, and the National Toxicology Program (Anderson et al. 2009; Boverhof et al. 2009; NTP 2005) and continue to use them today.

In order to further evaluate the impact of using different strains and substrains of mice in the LLNA, the study reported here is a retrospective evaluation of the performance of the LLNA in studies using CBA mice with studies using BALB/c mice. LLNA results are compared from studies done with CBA and BALB/c mice using the same test substances in the same vehicles.

## 2.0 Methodology

The information summarized here is based on LLNA data derived from a database of over 600 substances tested in the LLNA. Data were extracted from published reports or submissions in response to a *Federal Register (FR)* notice requesting LLNA, guinea pig, and/or human skin sensitization data and experience (Vol. 72, No. 95, pp. 27815-27817<sup>1</sup>). Key words used in the online searches for this evaluation were "LLNA" OR "Local Lymph Node" OR "Local lymph node" OR "local lymph node". Papers that contained studies on BALB/c were identified by appending AND "balb/c" to this search string. Forty-one such papers identified by the AND "balb/c" search were examined for BALB/c data appropriate for inclusion in this study.

The primary consideration for inclusion of data from published studies was the identification of test substances for which LLNA studies in the same vehicle existed. In general, published studies that were included in this evaluation followed the LLNA protocol in the Organisation for Economic Co-

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<sup>1</sup> Available at [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_9544.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf)

operation and Development (OECD) Test Guideline 429 (OECD 2002). However, some exceptions were made since many of the published BALB/c studies were done prior to the formal adoption of TG 429. Exceptions to the OECD protocol include studies in which lymph nodes were harvested on days 3, 4, 5, and 6 after study initiation, as well as studies that used 2 or 3 mice per treatment group. Studies that included other modifications (e.g., pretreatment of mice with sodium lauryl sulfate before application of the test substance) were excluded. The complete database is in **Annex I**.

An LLNA result was identified as positive if an SI value  $\geq 3.0$  occurred at any concentration tested. Overall LLNA outcomes for individual substances were made according to the most prevalent outcome, or on a most conservative basis if an equal number of positive and negative studies were found (i.e., considered positive). Since this was a retrospective study, there were substances with multiple studies using the same strain. For each such substance, LLNA outcome was based on the most prevalent study result (positive vs. negative), or considered positive if an equal number of positive and negative studies were found. EC3 values (the concentration of a test substance necessary to cause an SI value of 3) were calculated according to the methods used by Ryan and co-workers (Ryan et al., 2007). In the event that an EC3 value could not be calculated using these methods due to an inadequate dose response, the study was still designated as either positive or negative for the purpose of calculating agreement between strains, based on the decision criterion of  $SI > 3$  as the basis for a positive.

### 3.0 Results

#### 3.1 Characteristics of the Database

A summary of the responses in LLNA studies conducted with CBA and BALB/c mice is shown in **Table C-1**.

**Table C-1 Summary of LLNA Responses from CBA and BALB/c**

Test Substance	Vehicle	No. of Studies								
		All Strains	CBA			BALBc			Avg EC3 (%)	
		Total	Total	Pos	Neg	Total	Pos	Neg	CBA	BALBc
3-Amino-5-mercapto-1,2,4-triazole	DMSO	2	1	1	0	1	1	0	11.6	5.2
Benzocaine	AOO	5	4	1	3	1	0	1	NC	NC
Cobalt chloride	DMSO	3	2	2	0	1	0	1	0.6	NC
2,4-DNCB	AOO	14	10	10	0	4	4	0	0.052	0.116
2,4-DNFB	AOO	3	1	1	0	2	2	0	0.016	0.024
Eugenol	AOO	9	8	8	0	1	1	0	14.3	13.8
Eugenol	ACE	2	1	1	0	1	0	1	18.2	NC
Formaldehyde	DMF	2	1	1	0	1	1	0	0.27	0.11
Glutaraldehyde	DMF	2	1	1	0	1	1	0	0.07	0.09
HCA	ACE	5	4	4	0	1	1	0	5.8	12.9
Isoeugenol	AOO	33	32	32	0	1	1	0	1.4	0.8

*continued*

**Table C-1 Summary of LLNA Responses from CBA and BALB/c (continued)**

Test Substance	Vehicle	No. of Studies								
		All Strains	CBA			BALBc			Avg EC3 (%)	
			Total	Total	Pos	Neg	Total	Pos	Neg	CBA
Methyl salicylate	AOO	7	6	0	6	1	0	1	NC	NC
Nickel sulfate	DMSO	2	1	1	0	1	0	1	1.5	NC
Oxazolone	AOO	6	5	5	0	1	1	0	0.0018	IDR
Potassium dichromate	DMSO	10	8	8	0	2	1	1	0.09	0.2
Trimellitic anhydride	AOO	3	1	1	0	2	2	0	9.2	0.15
Total No. Studies		108	86	77	9	22	16	6		

Abbreviations: ACE = acetone; AOO = acetone/olive oil; DMF = dimethylformamide;

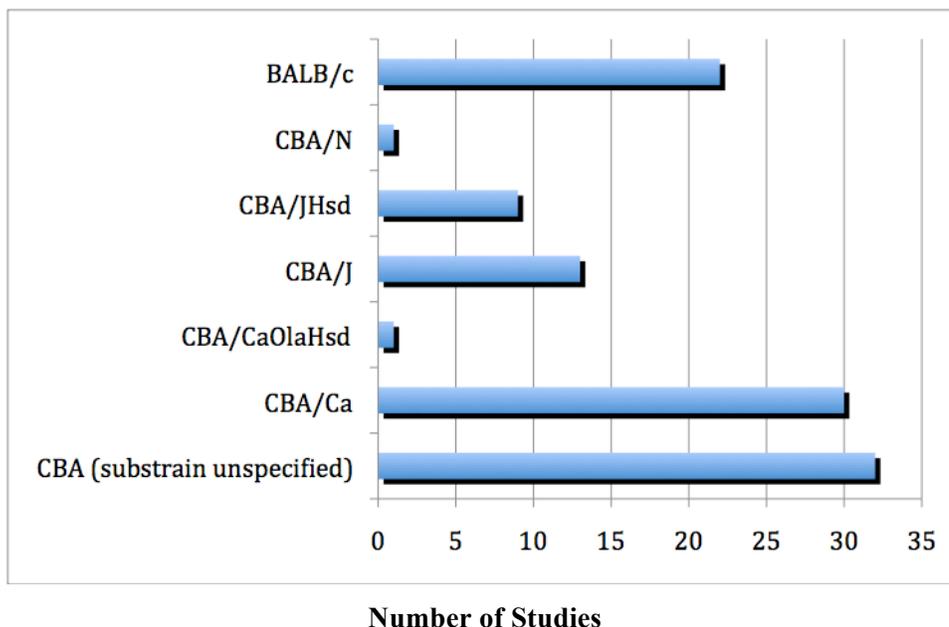
DMSO = dimethylsulfoxide; DNCB = dinitrochlorobenzene; DNFB = dinitrofluorobenzene; EC3 = estimated concentration needed to produce a stimulation index of 3; HCA =  $\alpha$ -hexylcinnamic aldehyde;

IDR = Inadequate dose response to calculate an EC3 value; LLNA = local lymph node assay; N = No;

NC = not calculated; Neg = negative; Pos = positive.

The database evaluated contains results from a total of 108 independent LLNA studies, representing 16 different test substances; 86 of the studies were done with CBA and 22 with BALB/c. Substrains of CBA mice used in the studies were not always specified; specified CBA substrains included CBA/Ca, CBA/CaHsd, CBA/J, CBA/JHsd and CBA/N. None of the studies using BALB/c mice specified a substrain. **Figure C-1** shows a frequency distribution of the substrains used in the studies analyzed. The substrain used in a particular study and the supplier (if known) is indicated for each study in **Annex 1**.

**Figure C-1 Substrain Frequency Distribution**



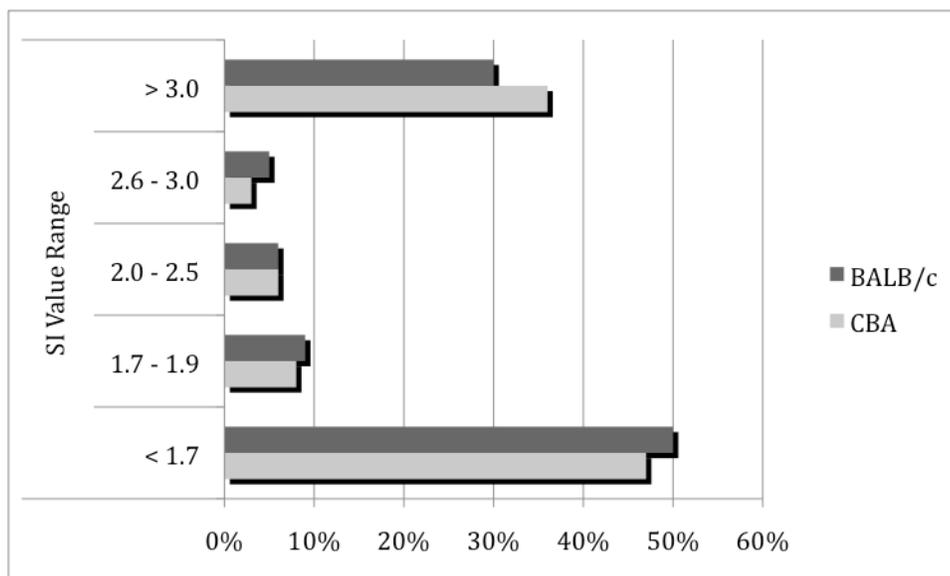
Four different vehicles were represented, with acetone-olive oil (AOO, 80 studies) being the most prevalent, followed by dimethylsulfoxide (DMSO, 17 studies), acetone (ACE, 5 studies) and dimethylformamide (DMF, 4 studies). Only one nonsensitizer (as classified by results in guinea pigs and humans), methyl salicylate, was included. The EC3 values for the 15 sensitizers (as determined from CBA LLNA data) included in the database ranged from 0.0018% (for oxazolone in AOO) to 18.2% (for eugenol in ACE) (**Table C-1**).

Current ICCVAM-recommended LLNA performance standards (ICCVAM 2009) recommend that EC3 values for HCA and DNCB determined in different laboratories should fall into a range of 0.5-2.0x of a reference value; in this study, 29% of the EC3 values for all sensitizers determined in BALB/c fall within this range, if the EC3 value determined in CBA is used as the reference. Neither the EC3 value determined in BALBc for DNCB, or for HCA, falls within this range (**Table C-1**). However, it should be noted that most of the EC3 values determined in both strains were based on a very limited number of studies; for CBA, 8/16 EC3 values were based on one or two LLNA studies, for BALB/c, 13/16 EC3 values were based on one or two LLNA studies. No EC3 value for oxazolone was determined in BALB/c because the dose response data were inadequate to do so.

### 3.2 Comparison of Responses in the LLNA from CBA and BALB/c Databases

Initially, results from LLNA studies using CBA mice (75 substances, 83 LLNA studies) were compared to results from LLNA studies using BALB/c mice (39 substances, 41 LLNA studies) (ICCVAM 2009). The percentage of positive LLNA studies (i.e.,  $SI \geq 3.0$ ) using either CBA (59% [49/83]) or BALB/c (63% [26/41]) mice were similar. **Figure C-2** shows the frequency distribution of LLNA responses from 277 test substance doses that fall into the indicated ranges of SI values. However, this does not include a comparison of results from the same substances tested in the same vehicles. The study described in this report was done to compare results of substances tested in the same vehicle in both CBA and BALB/c.

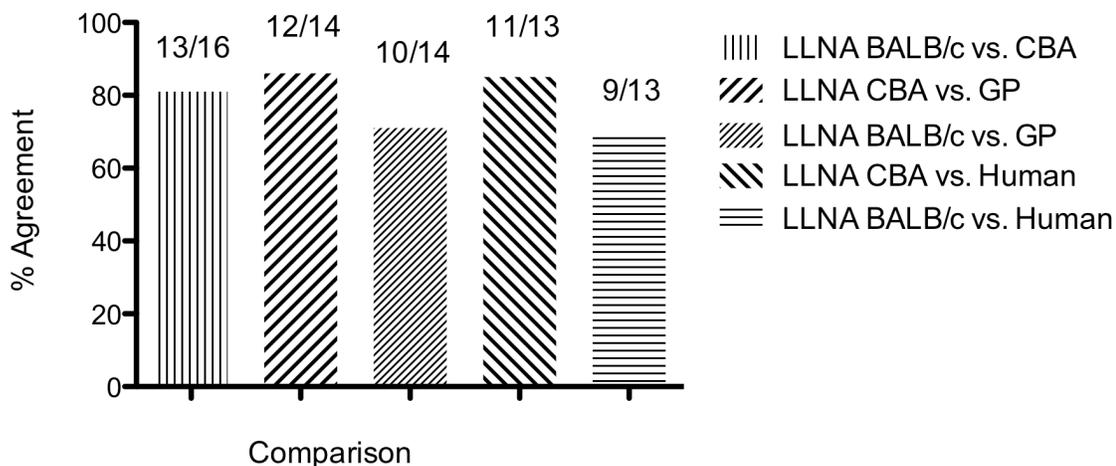
**Figure C-2 Comparison of LLNA Responses from CBA and BALB/c Databases (ICCVAM 2009)**



Abbreviation: No. = number; SI = stimulation index

The database analyzed here contains data for 16 substances for which there is LLNA data for both CBA and BALB/c in the same vehicle. Thirteen of these substances had GP reference data and 12 had human reference data. Two substances, 3-Amino-5-mercapto-1,2,4-triazole and 2,4-dinitrofluorobenzene, had neither GP nor human reference data; and one substance, trimellitic anhydride, had GP reference data but no human reference data. For this database, 92% (12/13) of the substances were classified as sensitizers in the GP, 92% (11/12) of the substances were classified as sensitizers in humans, 8% (1/13) were classified as nonsensitizers in the GP and 8% (1/12) were classified as nonsensitizers in humans. **Figure C-3** provides a comparison of the performance of the LLNA when the two strains are compared to each other, and to GP and human outcomes.

**Figure C-3 Comparison of the Performance of the LLNA using CBA or BALB/c Mice**



Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = local lymph node assay; No. = number.

GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test. Human refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

LLNA outcomes using BALB/c are in agreement with LLNA outcomes obtained with CBA for 81% (13/16) of the test substances. LLNA outcomes with CBA agree with GP outcomes for 86% (12/14) of the test substances and with human outcomes for 85% (11/13) of the test substances; in contrast, LLNA outcomes with BALB/c agree with GP outcomes for 71% (10/14) of the test substances and with human outcomes for 69% (9/13) of the test substances.

**Table C-2** contains LLNA data for three substances (cobalt chloride, nickel sulfate, and eugenol) for which the overall LLNA results were different between CBA and BALB/c, or between one of the mouse strains and guinea pig or human reference data. In the LLNA studies for cobalt chloride and nickel sulfate considered in this investigation, the LLNA results using CBA were concordant with guinea pig and human reference tests, while those using BALB/c were discordant. However, the discordant results obtained in BALB/c were based on a single study for each metal compound. The negative study for nickel sulfate using BALB/c was a 4-day study, while the positive study in CBA was a 6-day study. Furthermore, the LLNA response was a borderline positive in CBA (maximum SI=3.1), and the maximum SI for BALB/c mice was SI=2.46; **Table C-2**). For these reasons there is insufficient information to draw conclusions about the LLNA response to metals in BALB/c. It should also be noted that metal compounds (ICCVAM 1999) are known to produce variable LLNA responses in CBA.

Table C-2 Substances Discordant Between the LLNA, GP, and Human

Chemical Name	LLNA Vehicle	Conc. (%)	SI	EC3 (%)	Mouse Strain	LLNA Call	LLNA Study Length (Days)	Overall LLNA Call <sup>2</sup> (CBA)	Overall LLNA Call <sup>2</sup> (BALB/c)	Overall GP <sup>1</sup> Call <sup>2</sup>	Overall Human <sup>3</sup> Call <sup>2</sup>	LLNA Ref	GP Ref	Human Ref
Eugenol	ACE	25, 50, 75	5.4, 10.6, 10.5	18.5	CBA/J	+	5	+	-	+	+	Gerberick et al. (1992)	Basketter et al. (1999)	Basketter et al. (1999)
		10, 20	1.07, 1.89	NC	BALB/c	-	4					Sailstad et al., (1995)		
Cobalt chloride	DMSO	0.5, 1.0, 2.5	3.2, 3.7, 2.8	0.4	CBA/Ca	+	5					Basketter and Scholes (1992)	Basketter et al. (1999)	Kligman (1966)
		0.5, 1.0, 2.5, 5.0	2.1, 3.5, 3.8, 7.2	0.8	CBA/N	+	4	+	-	+	+	Ikarashi (1992b)		
		1.0, 2.5, 5.0	1.5, 1.6, 2.7	NC	BALB/c	-	4						Manderville et al. (1997)	
Nickel sulfate	DMSO	0.25, 0.5, 1, 2.5, 5	1.3, 1.4, 1.4, 1.8, 3.1	4.8	CBA/J	+	6					Ryan et al. (2002)	Basketter and Scholes (1992)	Kligman (1966)
		2.5, 5,	2.19, 2.46	NC	BALB/c	-	4	+	-	+	+	Ikarashi et al. (1992a)		

Abbreviations:

AOO = acetone/olive oil; Conc. = concentration; DMSO=dimethylsulfoxide; EC3 = estimated concentration needed to produce a stimulation index of 3; GP = guinea pig; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NC = not calculated since SI<3.0; SI = stimulation index; Veh. = vehicle

<sup>1</sup> GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

<sup>2</sup> Human refers to outcomes obtained by studies conducted using either the human repeat insult patch test or the human maximization test, or inclusion in a human patch test allergen kit.

<sup>3</sup> (-) = nonsensitizer, (+) = sensitizer

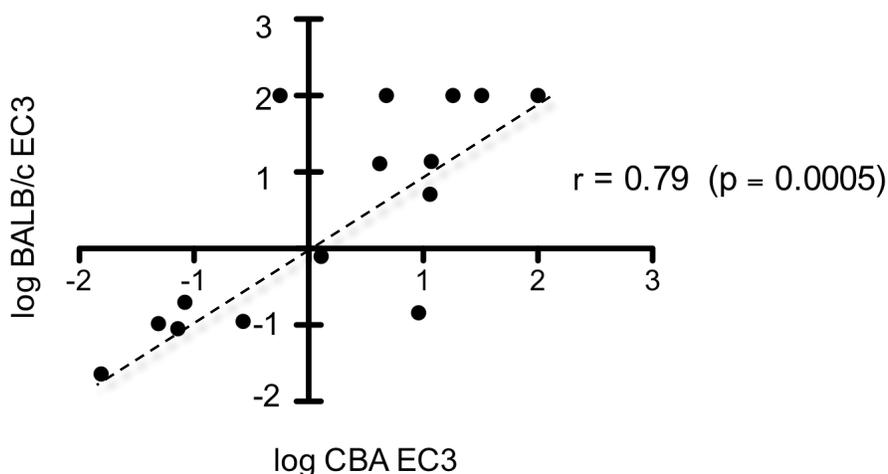
In the LLNA studies for eugenol with acetone as the vehicle, the LLNA results using CBA were concordant with guinea pig and human reference tests, while those using BALB/c were discordant. The differences between CBA and BALB/c studies may be due the large differences in the concentration ranges used, where the maximum concentration used in the CBA study was almost 4-fold higher than that used in the BALB/c study. It should also be noted that BALB/c and CBA studies for eugenol in which AOO was used as the vehicle were both positive. (Annex 1).

### 3.3 Correlation of EC3 Values Obtained with CBA and BALB/c Mice

A correlation analysis between EC3 values calculated using LLNA data from each of the two strains was done. If there were multiple LLNA studies for a strain, a geometric mean EC3 value was used in the correlation analysis. Since the EC3 values for the test substances in this analysis spanned six orders of magnitude (range = 0.0018% to 100%), the mean EC3 values were log transformed prior to analysis. Oxazolone was not included in this analysis because the dose response obtained with BALB/c mice was inadequate to allow calculation of an EC3 value (Table C-1).

Spearman’s rank correlation is used for rating the extent of agreement with the ‘true’ ranking of a set of observations (Steel and Torrie, 1980). In this analysis, the CBA EC3 results were considered the “true” ranking. A highly significant ( $p \leq 0.0005$ ) positive correlation ( $r = 0.79$ ) was obtained between EC3 values calculated from LLNA studies in both strains (Figure C-4).

Figure C-4 Correlation of EC3 Values Obtained with CBA and BALB/c Mice



Log-transformed geometric mean EC3 values for 15 of the 16 substance-vehicle groups shown in Table 2.  $r$  = Spearman’s Rank correlation coefficient.

NOTE: An EC3 value of 100% was assigned to negative LLNA results in order to exceed all positive values, so that they could be included in the correlation analysis.

Among the 10 substances for which an EC3 was calculated in both CBA and BALB/c studies, 5/10 were lower CBA and 5/10 were lower in BALB/c. (Table C-1).

As stated previously, it should be noted that most of the EC3 values determined in both strains were based on a very limited number of studies; for CBA, 50% (8/16) EC3 values were based on one or two LLNA studies, and for BALB/c, 81% (13/16) EC3 values were based on one or two LLNA studies (Table C-1).

### 3.4 Conclusions

This study complements a previous study (ICCVAM 2009), which concluded that the percentage of positive LLNA responses study were the same between studies with CBA or BALB/c mice. However, there was no substance-by-substance comparison (i.e., the respective databases were compared *in toto*, regardless of test substance or vehicle). Therefore, the present study compares results from LLNA studies with CBA and BALB/c mice using the same test substances in the same vehicles.

Current testing guidelines (OECD 2002; EPA 2003) recommend using CBA mice unless it is sufficiently demonstrated that significant strain-specific differences in the LLNA response do not exist. When compared to LLNA studies using CBA mice (the strain specified in the ICCVAM-recommended LLNA protocol [ICCVAM 2009]), results of studies done on the same substances in BALB/c were in agreement most of the time (81% [13/16]) (Figure C-3). Also, there was a positive rank correlation ( $r = 0.79$ ) between EC3 values ( $p \leq 0.0005$ ) (Figure C-4). Where there were different outcomes ( $n=3$ ) between the two mouse strains, the CBA studies were positive (which was also concordant with the human and GP outcomes) while the BALB/c studies were negative (and thereby discordant with the human and GP outcomes) (Table C-2).

These results suggest that further characterization of strain and substrain differences is needed. Until such additional information becomes available, caution should be used prior to selecting a mouse strain other than CBA for use in the LLNA for regulatory testing.

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## **Annex I**

### **Data for Substances Tested in the LLNA in s CBA and BALB/c Mice**

#### List of Abbreviations and Acronyms

ACE	acetone
AOO	acetone: olive oil (4:1)
CASRN	Chemical Abstract Services Registry Number
Conc.	concentration
DMF	N, N-dimethyl formamide
DMSO	dimethyl sulfoxide
EC3	estimated concentration needed to produce a stimulation index of 3
GP	guinea pig
LLNA	murine local lymph node assay
MEK	methyl ethyl ketone
NA	not available
Veh.	Vehicle
SI	Stimulation index
+	Sensitizer
-	Non-sensitizer

ICCVAM LLNA Applicability Domain Evaluation Report

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain
3-Amino-5-mercapto-1,2,4-triazole	16691-43-3	DMSO	5, 15, 25	2.95, 6.2, 8.66	5.2	BALB/c
3-Amino-5-mercapto-1,2,4-triazole	16691-43-3	DMSO	1, 5, 15, 25	1.23, 2.13, 3.45, 4.08	11.6	CBA
Benzocaine	94-09-7	AOO	2.5, 5, 10, 25, 50	2.1, 1.8, 2.7, 1.8, 1.2	NC	CBA
Benzocaine	94-09-7	AOO	1, 5, 25	1.3, 1.8, 2.9	NC	CBA/Ca
Benzocaine	94-09-7	AOO	10, 25, 50	1.7, 2.0, 0.9	NC	CBA/Ca
Benzocaine	94-09-7	AOO	5, 10, 20	4.5, 7.2, 7.6	3.4	CBA/Ca
Benzocaine	94-09-7	AOO	10, 25	0.95, 1.05	NC	BALB/c
Cobalt chloride	1332-82-7	DMSO	0.5, 1, 2.5	3.2, 3.7, 2.8	0.4	CBA/Ca
Cobalt chloride	1332-82-7	DMSO	0.5, 1, 2.5, 5	2.1, 3.5, 3.8, 7.2	0.8	CBA/N
Cobalt chloride	1332-82-7	DMSO	1, 2.5, 5	1.5, 1.6, 2.7	NC	BALB/c
2,4-Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.5, 1.8, 2.4, 8.9, 38.0	0.055	CBA/JHsd
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.4, 2.2, 4.0, 9.8, 16.2	0.036	CBA/Ca
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	2.0, 2.3, 5.3, 10.5, 35.5	0.027	CBA/Ca
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	0.8, 1.8, 3.3, 8.7, 40.9	0.046	CBA/Ca
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.1, 1.4, 2.5, 4.6, 11.5	0.062	CBA/J

Appendix C – Comparison of LLNA in CBA and BALB/c Mice

Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Taconic Laboratories (Germantown, NY)	+	Klink & Meade (2003)	NA	NA	
Taconic Laboratories (Germantown, NY)	+	Klink & Meade (2003)			
Harlan Olac, Bicester, Oxfordshire, UK	-	Gerberick et al. (2005)	Basketter and Scholes (1992)	Kligman (1966c)	
B&K Universal AB, Sollentuna, Sweden	-	Montelius et al. (1994)			
Harlan Olac, Bicester, Oxfordshire, UK	-	Basketter et al. (1995)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al (1989b)			
Japan SLC Inc, Shizuoka, Japan	-	Ikarashi et al, (1993a)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter and Scholes (1992)	Basketter et al. (1999b)	Kligman (1966c)	
Japan SLC Inc, Shizuoka, Japan	+	Ikarashi et al. (1992b)			
Charles River, Germany	-	Mandervelt et al. (1997)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Gerberick et al. (2005)	Basketter et al. (1999b)	Kligman (1996b)	
Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al. (1995)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al. (1995)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al. (1995)			
Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			

ICCVAM LLNA Applicability Domain Evaluation Report

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	0.8, 1.2, 1.7, 3.1, 22.5	0.094	CBA/J
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.3, 1.5, 2.1, 7.7, 43.9	0.057	CBA/J
2,4- Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.5, 1.9, 3.1, 6.5, 25.0	0.05	CBA/Ca
2,4- Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.2, 0.9, 2.9, 4.5, 13.0	0.06	CBA/Ca
2,4- Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	2.5, 2.9, 3.2, 7.1, 25.0	0.033	CBA/JHsd
2,4- Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.2, 1.1, 1.9, 2.0, 7.1	0.13	BALB/c
2,4- Dinitrochloro benzene	97-00-7	AOO	0.03, 0.1, 0.3, 1.0	1.6, 5.0, 15.8, 24.6	0.06	BALB/c
2,4- Dinitrochloro benzene	97-00-7	AOO	0.5, 1.0	8.7, 12.9	0.19	BALB/c
2,4- Dinitrochloro benzene	97-00-7	AOO	0.1, 0.5, 1.0	3.5, 7.4, 12.3	0.083	BALB/c
2,4- Dinitrochloro benzene	70-34-8	AOO	0.02, 0.1, 0.5	6.4, 28.0, 39.9	0.016	CBA/Ca
2,4- Dinitrochloro benzene	70-34-8	AOO	NA	NA	0.032	BALB/c
2,4- Dinitrochloro benzene	70-34-8	AOO	0.01, 0.025, 0.05	2, 4.5, 6.5	0.016	BALB/c
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	1.6, 1.5, 2.4, 5.5, 16.1	11.9	CBA/Ca

Appendix C – Comparison of LLNA in CBA and BALB/c Mice

Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Charles River Laboratories (location unspecified)	+	NTP Study Submitted by: Dori Germolec			
Charles River Japan Laboratories, Atugi, Kanagawa, Japan	+	Fukuyama et al. (2008b)			
Japan SLC Inc, Shizuoka, Japan	+	Ikarashi et al, (1993a)			
Japan SLC Inc, Shizuoka, Japan	+	Ikarashi et al, (1993a)			
B&K Universal AB, Sollentuna, Sweden	+	Montelius et al. (1994)	NA	NA	
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter et al. (1997a)			
Taconic Laboratories, Rockville, MD	+	Patterson et al. (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter and Scholes (1992)	Basketter et al. (1999d)	Basketter et al. (1999d)	SI values were estimated from a graph of dpm vs conc in LLNA Ref

ICCVAM LLNA Applicability Domain Evaluation Report

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain
Eugenol	97-53-0	AOO	25, 50	1.2, 4.0	40.9	CBA/Ca
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	2.0, 2.8, 3.2, 13.0, 17.0	5.8	CBA/Ca
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	1.6, 1.5, 2.4, 5.5, 16.0	14.5	CBA/Ca
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	1.1, 1.7, 1.8, 9.1, 12.4	8.9	CBA/JHsd
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	2.4, 2.1, 1.2, 5.3, 9.6	13.8	CBA/JHsd
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	1.5, 4.3, 4.6, 14.0, 6.1	6	CBA/JHsd
Eugenol	97-53-0	AOO	10, 25, 50	2.4, 5.5, 16.1	12.9	CBA/Ca
Eugenol	97-53-0	ACE	25, 50, 75	5.4, 10.6, 10.5	18.2	CBA/J
Eugenol	97-53-0	AOO	5, 10, 25	1, 2, 6	13.8	BALB/c
Eugenol	97-53-0	ACE	10, 20	1.1, 1.9	NC	BALB/c
Formaldehyde	50-00-0	DMF	1, 10, 20	6.7, 13.2, 17.7	0.27	CBA/J
Formaldehyde	50-00-0	DMF	10, 25, 50	8.6, 9.7, 9.0	0.11	BALB/c
Glutaraldehyde	111-30-8	DMF	0.1, 0.75, 2.5	4.9, 16.4, 31.5	0.07	CBA
Glutaraldehyde	111-30-8	DMF	0.1, 0.75, 2.5	3.5, 12.7, 25.5	0.09	BALB/c

Appendix C – Comparison of LLNA in CBA and BALB/c Mice

Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Barrièred Animal Breeding Unit, Adderly Park, UK	+	Kimber & Weisenberger (1991)			Mice were exposed to AOO under an occluded patch 5 days before exposure to eugenol in AOO on the ears.
Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Bertrand et al. (1997)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Gerberick et al. (1992)			Mice were treated with the test substance for 4 consecutive days instead of 3 days as per the ICCVAM protocol
Harlan Olac, Bicester, Oxfordshire, UK	+	Hilton et al. (1996a)			SI values were estimated from a graph of dpm x 10 <sup>3</sup> vs conc in LLNA Ref
Charles River, Raleigh, NC	-	Sailstad et al. (1995)			
Jackson Laboratories, Bar Harbor, ME	+	Ryan et al. (2002)	Basketter et al. (1999b)	Kligman (1966c)	
Harlan Olac, Bicester, Oxfordshire, UK	+	Hilton et al. (1996b)			
Taconic Laboratories, Germantown, NY	+	Azadi et al. (2004)	Gad et al. (1986)	Marzulli & Maibach (1974)	
Taconic Laboratories, Germantown, NY	+	Azadi et al. (2004)			

ICCVAM LLNA Applicability Domain Evaluation Report

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain
Hexyl cinnamic aldehyde	101-86-0	ACE	3, 10, 30	4.6, 6.6, 9.9	1.2	CBA/CaOla Hsd
Hexyl cinnamic aldehyde	101-86-0	ACE	1, 3, 10	1.8, 3.2, 3.7	2.7	CBA/J
Hexyl cinnamic aldehyde	101-86-0	ACE	1, 3, 10	1.8, 2.4, 3.3	8	CBA/J
Hexyl cinnamic aldehyde	101-86-0	ACE	5, 25, 50	2.5, 4.1, 9.4	11.3	CBA
Hexyl cinnamic aldehyde	101-86-0	ACE	5, 25, 50	1.7, 5, 10.9	12.9	BALB/c
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.7, 2.3, 13.8	1	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.8, 1.6, 14.1	1.1	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.8, 2.8, 5.6	2.1	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.9, 6.3, 31	0.5	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.9, 1, 7.2	1.9	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1, 1.1, 12.4	1.2	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1, 1.3, 7.5	1.8	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.1, 1.8, 23.2	0.8	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.1, 1.9, 15.3	1.3	CBA

Appendix C – Comparison of LLNA in CBA and BALB/c Mice

Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Charles River Laboratories, Inc., Kingston, NY	+	Report; Project No.: BGIA Project FP251, submitted by Bayer	Basketter et al. (1999b)	Basketter et al. (1999b)	
Charles River, Germany	+	BASF, submitted by C. Hastings			
Charles River, Germany	+	BASF, submitted by C. Hastings			
Jackson Laboratories, Bar Harbor, ME	+	Woolhiser et al. (2000)			
Jackson Laboratories, Bar Harbor, ME	+	Woolhiser et al. (2000)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)	Wahlberg & Boman (1985)	Basketter et al. (1999b)	
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Gerberick et al. (2005)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			

ICCVAM LLNA Applicability Domain Evaluation Report

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.2, 4.2, 18.4	0.7	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.2, 1.4, 19.3	1.8	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.2, 3.2, 8.7	1.3	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.3, 2.2, 13.1	1	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.3, 3.3, 14.7	1.5	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.4, 1.5, 4.9	2.6	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.4, 1.2, 6.7	2	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.5, 2.6, 19.2	0.8	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.5, 2.5, 29.8	0.6	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.6, 1.6, 14.7	1.4	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.6, 2.2, 7.5	1.6	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.6, 2.2, 19	0.8	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.6, 4.3, 24.4	0.6	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.7, 1.2, 5	2.6	CBA

Appendix C – Comparison of LLNA in CBA and BALB/c Mice

Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			

ICCVAM LLNA Applicability Domain Evaluation Report

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.8, 2.9, 23.2	0.6	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	2, 1.4, 7.6	1.6	CBA
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	2.3, 1.6, 23.6	0.6	CBA
Isoeugenol	97-54-1	AOO	NA	NA	1.3	CBA/Ca
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	1.5, 2.2, 2.5, 4.9, 10	1.3	CBA/Ca
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	1, 1.3, 2.1, 2.3, 4.1	3.3	CBA/Ca
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	2.9, 1.7, 2.3, 3.8, 6.8	1.8	CBA/Ca
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	0.7, 0.7, 0.9, 2.1, 7.2	3.1	CBA/Ca
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	1.2, 1.7, 2.6, 4.3, 11	1.6	CBA/Ca
Isoeugenol	97-54-1	AOO	5, 10, 25	7, 8.5, 26	0.8	BALB/c
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	1.1, 1, 1.1, 1.6, 1.9	NC	CBA/J
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	1.2, 1.5, 1.2, 1.8, 2.9	NC	CBA/Ca
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	2.1, 1.4, 1.5, 1.9, 2.1	NC	CBA/J

Appendix C – Comparison of LLNA in CBA and BALB/c Mice

Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Hilton et al. (1996a)			SI values were estimated from a graph of dpm x 10 <sup>3</sup> vs conc in Ref1
Harlan Sprague Dawley Inc, Indianapolis, IN	-	Kimber et al. (1995)	Basketter et al. (1999b)	Basketter et al. (1999b)	
Harlan Olac, Bicester, Oxfordshire, UK	-	Kimber et al. (1995)			
Harlan Sprague Dawley Inc, Indianapolis, IN	-	Kimber et al. (1995)			

ICCVAM LLNA Applicability Domain Evaluation Report

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	0.7, 0.9, 0.8, 0.5, 1.1	NC	CBA/J
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	0.9, 1.2, 1.8, 1.6, 2.3	NC	CBA/Ca
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	1, 1.1, 1.6, 1.4, 0.9	NC	CBA/JHsd
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	0.9, 1.2, 1.2, 1.4, 1.7	NC	BALB/c
Nickel sulfate	7786-81-4	DMSO	0.25, 0.5, 1.0, 2.5, 5.0	1.3, 1.4, 1.4, 1.8, 3.1	1.5	CBA/J
Nickel sulfate	7786-81-4	DMSO	2.5, 5.0	2.2, 2.5	NC	BALBc
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	2.9, 4.9, 12, 22, 33	0.0026	CBA/JHsd
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	3.4, 4.4, 4, 5.9, 8.9	0.002	CBA/Ca
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	3.9, 4.8, 6, 12, 13	0.0014	CBACa
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	4, 6.9, 16, 40, 59	0.0025	CBA/JHsd
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	3.8, 6.2, 7.7, 15, 23	0.0007	CBA/JHsd
Oxazolone	15646-46-5	AOO	1, 2, 4	25.2, 25.5, 19	IDR	BALB/c
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.6, 1.4, 3.8, 5.3, 16.1	0.08	CBA/J
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.4, 2.5, 9.5, 25.9, 10.1	0.05	CBA/J

Appendix C – Comparison of LLNA in CBA and BALB/c Mice

Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Harlan Sprague Dawley Inc, Indianapolis, IN	-	Kimber et al. (1995)			
Harlan Olac, Bicester, Oxfordshire, UK	-	Kimber et al. (1995)			
Harlan Sprague Dawley, Inc., Frederick, MD	-	Gerberick et al. (2005)			
Charles River Laboratories (location unspecified)	-	NTP Study Submitted by: Dori Germolec			
Jackson Laboratories, Bar Harbor, ME	+	Ryan et al. (2002)	Basketter and Scholes (1992)	Kligman (1966c)	
Japan SLC Inc, Shizuoka, Japan	-	Ikarashi et al, (1993a)			
Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)	Basketter et al. (1999b)	Basketter et al. (1999b)	
Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
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Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Charles River, Germany	+	Mandervelt et al. (1997)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Gerberick et al. (2005)	Basketter et al. (1999b)	Kligman (1966c)	
Jackson Laboratories, Bar Harbor, ME	+	Ryan et al. (2002)			

ICCVAM LLNA Applicability Domain Evaluation Report

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.1, 1.3, 2.3, 5.1, 13.1	0.15	CBA/Ca
Potassium dichromate	7778-50-9	DMSO	0.1, 0.25, 0.5	3.5, 10.2, 10.4	0.03	CBA/Ca
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.7, 2.9, 4.5, 10.4, 19.1	0.058	CBA/Ca
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.2, 2.1, 3.4, 4.5, 11.2	0.132	CBA/J
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.9, 1.7, 2.2, 5.9, 13	0.122	CBA/J
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.6, 1.4, 3.8, 5.3, 16.1	0.126	CBA/J
Potassium dichromate	7778-50-9	DMSO	0.5, 1, 2	1.8, 1.4, 1.5	NC	BALB/c
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25	1.2, 1.8, 2.2, 3.4	0.2	BALB/c
Trimellitic anhydride	552-30-7	AOO	1, 2.5, 5, 10, 25	1.1, 2.0, 2.0, 3.2, 4.6	9.2	CBA
Trimellitic anhydride	552-30-7	AOO	0.5, 1.0, 2.5, 5.0, 10	2.6, 2.7, 3.7, 7.5, 11.6	0.11	BALB/c
Trimellitic anhydride	552-30-7	AOO	5, 10, 25	7, 8.5, 26	0.19	BALB/c

Appendix C – Comparison of LLNA in CBA and BALB/c Mice

Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter et al. (1999a)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter and Scholes (1992)			
Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al. (1995)			
Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
Charles River, Germany	-	Mandervelt et al. (1997)			
Charles River Laboratories (location unspecified)	+	NTP Study Submitted by: Dori Germolec			
Harlan Olac, Bicester, Oxfordshire, UK	+	Gerberick et al. (2005)	Basketter and Scholes (1992)	NA	
Charles River Laboratories, Inc., Kingston, NY	+	Boverhof et al. (2009)			
Charles River Japan Laboratories, Atugi, Kanagawa, Japan	+	Fukuyama et al. (2008b)			

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## **Appendix D**

**Assessment of the Validity of the LLNA for Pesticide Formulations, Metals,  
Substances in Aqueous Solutions, and Other Products**

**2010 Addendum to NIH Publication Number 99-4494:**

**The Murine Local Lymph Node Assay (LLNA):**

**A Test Method for Assessing the Allergic Contact Dermatitis Potential of  
Chemicals/Compounds**

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**Final Assessment of the Validity of the LLNA for Pesticide Formulations,  
Metals, Substances in Aqueous Solutions, and Other Products**

**2010 Addendum to NIH Publication Number 99-4494:  
The Murine Local Lymph Node Assay (LLNA):  
A Test Method for Assessing the Allergic Contact Dermatitis Potential of  
Chemicals/Compounds**

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## Table of Contents

<b>List of Tables</b> .....	<b>D-7</b>
<b>List of Figures</b> .....	<b>D-8</b>
<b>List of Abbreviations and Acronyms</b> .....	<b>D-9</b>
<b>Interagency Coordinating Committee on the Validation of Alternative Methods: Agency Representatives</b> .....	<b>D-11</b>
<b>Acknowledgements</b> .....	<b>D-12</b>
<b>Preface</b> .....	<b>D-15</b>
<b>Executive Summary</b> .....	<b>D-17</b>
<b>1.0 Introduction</b> .....	<b>D-21</b>
<b>2.0 Substances Used for the Revised Evaluation of the Applicability Domain for the LLNA</b> .....	<b>D-24</b>
<b>3.0 Comparative <i>In Vivo</i> Reference Data</b> .....	<b>D-28</b>
<b>4.0 LLNA Data and Results</b> .....	<b>D-29</b>
<b>5.0 Accuracy of the LLNA: Revised Applicability Domain</b> .....	<b>D-30</b>
5.1 Testing of Pesticide Formulations and Other Products .....	D-30
5.2 Testing of Metal Compounds.....	D-42
5.3 Testing of Substances in Aqueous Solutions .....	D-45
<b>6.0 LLNA Data Quality</b> .....	<b>D-52</b>
<b>7.0 Other Scientific Reports and Reviews</b> .....	<b>D-53</b>
7.1 Maibach (1986) .....	D-53
7.2 Sharma and Kaur (1990) .....	D-53
7.3 Lisi (1992).....	D-53
7.4 Basketter et al. (1999a).....	D-53
7.5 Wright et al. (2001) .....	D-53
7.6 Ikarashi et al. (2002).....	D-54
7.7 Griem et al. (2003) .....	D-54
7.8 Hostynek and Maibach (2003 and 2004) .....	D-54
7.9 Penagos et al. (2004).....	D-55
7.10 Tinkle et al. (2004).....	D-55
7.11 Lalko and Api (2006).....	D-55
7.12 Shelnutt et al. (2007).....	D-55
7.13 Chipinda et al. (2008).....	D-56
7.14 Fukuyama et al. (2008).....	D-56
7.15 Horiuchi et al. (2008).....	D-56

7-16 Jowsey et al. (2008) .....	D-56
<b>8.0 References.....</b>	<b>D-58</b>
<b>9.0 Glossary .....</b>	<b>D-63</b>
<b>Annex I      The Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds (NIH Pub. No. 99-4494).....</b>	<b>D-67</b>
<b>Annex II      Available Data and Information for Pesticide Formulations and Other Products Tested in the LLNA .....</b>	<b>D-69</b>
<b>Annex III     Available Data and Information for Metals Tested in the LLNA .....</b>	<b>D-155</b>
<b>Annex IV     Available Data and Information for Substances in Aqueous Solutions Tested in the LLNA .....</b>	<b>D-171</b>
<b>Annex V      Supplementary Analysis of Pesticide Formulations in the LLNA .....</b>	<b>D-215</b>

## List of Tables

<b>Table D-1</b>	Summary of Data Sources and Rationale for Substance Selection .....	D-24
<b>Table D-2</b>	Pesticide Formulations with Multiple LLNA Studies .....	D-31
<b>Table D-3</b>	LLNA Data for Pesticide Formulation with Discordant Results .....	D-31
<b>Table D-4</b>	Evaluation of the Performance of the LLNA for Testing Pesticide Formulations.....	D-34
<b>Table D-5</b>	Pesticide Formulations that are Classified as Sensitizers in the LLNA but Classified as Nonsensitizers in the Guinea Pig .....	D-35
<b>Table D-6</b>	Evaluation of the Performance of the LLNA for Testing Dyes .....	D-38
<b>Table D-7</b>	Dyes Discordant Between the LLNA and GPMT .....	D-39
<b>Table D-8</b>	Evaluation of the Performance of the LLNA for Testing Natural Complex Substances .....	D-40
<b>Table D-9</b>	Natural Complex Substances: Discordant Results Between the LLNA and Human .....	D-41
<b>Table D-10</b>	Behavior of Nickel-containing Compounds in the LLNA .....	D-42
<b>Table D-11</b>	Evaluation of the Performance of the LLNA for Testing Metal Compounds.....	D-43
<b>Table D-12</b>	Substances Tested in Aqueous Solutions in Multiple LLNA Studies.....	D-46
<b>Table D-13</b>	Substances Tested in Multiple LLNA Studies in Aqueous Solutions with Discordant Results.....	D-46
<b>Table D-14</b>	Evaluation of the Performance of the LLNA for Testing Aqueous Solutions .....	D-47
<b>Table D-15</b>	Substances Tested in Aqueous Solution: Discordant Results Between the LLNA and GP .....	D-48
<b>Table D-16</b>	Substances with Human Data Tested in Aqueous Solution .....	D-50

## List of Figures

<b>Figure D-1</b>	Numbers of Positive and Negative LLNA and GP Calls for Pesticide Formulations .....	D-33
<b>Figure D-2</b>	Percentage of Formulations Classified as Sensitizers or Nonsensitizers in Two Mouse Strains.....	D-37

## List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
AOO	Acetone: olive oil
BGIA	Berufsgenossenschaftliches Institut für Arbeitsschutz (German Institute for Occupational Safety and Health)
BRD	Background review document
BT	Buehler Test
CASRN	Chemical Abstracts Service Registry Number
CCA	Chromated copper arsenate
CESIO	Comité Européen des Agents de Surface et de leurs Intermédiaires Organiques (European Committee of Surfactants and their Organic Intermediates)
CoDEC	Cobalt diethyldithiocarbamate
Conc.	Concentration tested
CPSC	U.S. Consumer Product Safety Commission
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EC3	Estimated concentration needed to produce a stimulation index of 3
ECPA	European Crop Protection Association
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
EtOH	Ethanol
FDA	U.S. Food and Drug Administration
FR	<i>Federal Register</i>
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
g/L	Grams per liter
GP	Guinea pig
GPMT	Guinea pig maximization test
GSK	GlaxoSmithKline
GST	Gold sodium thiosulfate
HMT	Human Maximization Test
HRIPT	Human Repeat Insult Patch Test
H <sub>2</sub> O	Water
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
IUD	Intrauterine device
IWG	Immunotoxicity Working Group
K <sub>ow</sub>	Octanol-water partition coefficient
LLNA	Local lymph node assay

MeSH	Medical subject headings
MEST	Mouse ear swelling test
n	Number
No.	Number
NA	Not available
NC	Not calculated
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIOSH	National Institute of Occupational Safety and Health
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
QRA	Quantitative Risk Assessment
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index
TEDCD	Tetraethyldicarbamoyl disulfide
TETD	Tetraethylthiuram disulfide
TG	Test Guideline
TNO	TNO Nutrition and Food Research (Dutch - No English translation)
U.K.	United Kingdom
U.S.	United States
vs.	Versus
w/v	Weight to volume ratio
Veh.	Vehicle
ZDEC	Zinc diethyldithiocarbamate

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## Preface

In 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999). ICCVAM concluded that the LLNA (referred to herein as the “traditional LLNA”) provided several advantages compared to the guinea pig method, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for allergic contact dermatitis testing. It is now commonly used around the world.

However, as described in the ICCVAM evaluation report<sup>1</sup>, based on the lack of available data for aqueous solutions and mixtures and on discordant results for a limited number of studies with metals, ICCVAM recommended that these substances not be tested for skin sensitization potential using the LLNA.

Based on the ICCVAM recommendations, the ICCVAM member agencies that require the regulatory submission of skin sensitization data accepted the LLNA, with the identified limitations, as an alternative to the traditional guinea pig tests (Guinea Pig Maximization Test, Buehler Test).

In 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to reevaluate the usefulness and limitations of the LLNA for testing mixtures, metals, and substances in aqueous solutions, among other activities related to the LLNA. ICCVAM assigned the activity a high priority, and established the ICCVAM Immunotoxicity Working Group (IWG) to work with NICEATM to review the current literature and evaluate available data to assess the status of the LLNA applicability domain. A comprehensive draft Addendum to the 1999 ICCVAM evaluation report provided the information, data and analyses supporting the validation status of the LLNA applicability domain. ICCVAM also developed draft test method recommendations for the LLNA applicability domain regarding usefulness and limitations, test method protocol, performance standards and future studies.

NICEATM and ICCVAM provided the draft Addendum and draft recommendations to an international independent scientific peer review panel for their consideration at a public meeting on March 4-6, 2008. Both the Panel and ICCVAM concluded that, due to the limitations associated with the available database for mixtures (i.e., unknown formulae, lack of human data), more data were needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures could be made. The Panel also stated that the term “mixtures” was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations that were being examined. Public comments at the meeting revealed that additional relevant data from LLNA studies with pesticide formulations and other products were available, which had not previously been provided in response to earlier requests for data. The Panel recommended that NICEATM obtain additional existing data that were not available to the Panel, and reanalyze the performance of the LLNA for testing pesticide formulations and other products. NICEATM subsequently obtained additional data and prepared this revised Addendum. ICCVAM also prepared revised draft test method recommendations based on the revised Addendum. This revised draft Addendum addresses the validation database for the LLNA applicability domain.

The Panel reconvened on April 27-28, 2009 to assess the current validation status of the LLNA applicability domain. The Panel also reviewed the completeness and accuracy of the draft Addendum and the extent to which the information therein supported the ICCVAM draft test method

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<sup>1</sup> ICCVAM (1999), available at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel98.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel98.htm)

recommendations for usefulness and limitations, test method protocol, performance standards and future studies. ICCVAM considered the conclusions and recommendations of the Panel, along with comments received from the public and the Scientific Advisory Committee for Alternative Toxicological Methods, when finalizing this Addendum and test method recommendations on the LLNA applicability domain.

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## Executive Summary

### **Background**

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine local lymph node assay (LLNA) to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods. These test methods assess the potential of many types of substances to cause allergic contact dermatitis, a skin reaction characterized by redness, swelling, and itching. Allergic contact dermatitis can result from contact with a sensitizing chemical or product.

ICCVAM based its recommendation on a comprehensive evaluation that included an assessment of the LLNA's validation status by an independent international scientific peer review panel. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)–ICCVAM website (<http://iccvam.niehs.nih.gov>).

The LLNA was subsequently incorporated into the following national and international test guidelines for assessing skin sensitization:

- U.S. Environmental Protection Agency Health Effect Testing Guidelines on Skin Sensitization (EPA 2003)
- Organisation for Economic Co-operation and Development Test Guideline 429 (OECD 2002)
- International Organization for Standardization 10993-10: Tests for Irritation and Delayed-type Hypersensitivity (ISO 2002)

In 2007, the U.S. Consumer Product Safety Commission formally nominated several LLNA-related activities for evaluation by NICEATM and ICCVAM. The U.S. Consumer Product Safety Commission asked for an assessment of the validation status of the LLNA applicability domain. In response, NICEATM and ICCVAM compiled the information in this Addendum.

This Addendum provides a comprehensive review of available data and information about the usefulness and limitations of the LLNA for assessing the skin-sensitizing potential of pesticide formulations and other products, metals, and substances tested in aqueous solutions (i.e., its current applicability domain). The information is based on a review of traditional LLNA data that were either (1) submitted as part of the original LLNA evaluation (ICCVAM 1999), (2) extracted from peer-reviewed publications, or (3) submitted to NICEATM in response to a May 2007 *Federal Register* notice (72 FR 27815).<sup>2</sup>

### **Revisions to the NICEATM-ICCVAM Evaluation of the LLNA Applicability Domain**

NICEATM and ICCVAM convened a Panel meeting on March 4–6, 2008. The Panel members reviewed the draft Addendum and commented on the extent to which it supported the draft ICCVAM test method recommendations on the usefulness and limitations of the LLNA regarding the applicability domain. Both ICCVAM and the Panel concluded that, because of insufficient information about mixtures (e.g., unknown formulas, lack of human data), more data were needed before a recommendation could be made on the usefulness and limitations of the LLNA for testing mixtures.<sup>3</sup> The Panel also stated that the term “mixtures” was used too broadly (i.e., it can represent an infinite number of materials). The Panel stated that it would be more beneficial to specify types or formulations that are being examined (ICCVAM 2008).

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<sup>2</sup> Available at [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_9544.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf)

<sup>3</sup> Available at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel08.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel08.htm)

Public comments at the meeting revealed additional relevant data from LLNA studies with pesticide formulations and other products. These data had not been provided in response to earlier requests. The Panel recommended that NICEATM obtain and analyze additional data on the performance of the LLNA for testing pesticide formulations and other products. In response, NICEATM obtained additional data and, in some cases, corresponding reference test method data (i.e., guinea pig test and/or human data) (ICCVAM 2008). NICEATM revised the evaluation of the LLNA for testing pesticide formulations and other products<sup>4</sup> (**Section 5.1**) and for testing substances in aqueous solutions (**Section 5.3**). No new LLNA data were received for LLNA tests with metals; therefore, this part of the evaluation remained unchanged (**Section 5.2**).

### ***Validation Database***

The information in this Addendum is based on a review of LLNA data derived from a database of more than 600 substances (including pesticide formulations and other products). In the original ICCVAM evaluation of the LLNA (ICCVAM 1999), the performance of the LLNA was compared to (1) the results from guinea pig tests and (2) information about sensitizers in humans (e.g., human maximization test results, substances used in a human repeat insult patch test, and clinical data), where available. This Addendum updates the LLNA performance analyses for (1) pesticide formulations and other products, (2) metals, and (3) substances tested in aqueous solutions when compared to human and guinea pig test results.

### ***Use of the LLNA for Testing Formulations and Other Products***

**Pesticide Formulations:** The revised LLNA database contains data for 104 pesticide formulations. Among these formulations, 54% (56 of 104) were LLNA positive, and 46% (48 of 104) were LLNA negative.

Seventy of the 104 pesticide formulations have LLNA data and some type of associated guinea pig reference data. Eighty-nine LLNA studies were performed using these 70 formulations. Sixty-one of the 89 LLNA studies used CBA/Ca or CBA/J strains; 28 used BALBc mice. Six pesticide formulations were tested in multiple LLNA studies (25 studies total). Five of the six had LLNA results in agreement, and one of the six produced discordant results (three positive, two negative).

All 70 pesticide formulations (89 of 89 studies) were tested in the LLNA in aqueous 1% Pluronic L92, a surfactant and wetting agent that has been evaluated as an alternative aqueous-based vehicle for use in the LLNA (Boverhof et al. 2008; Ryan et al. 2002).

Twenty-three pesticide formulations had associated guinea pig data for the complete formulation. Forty-six had guinea pig data for one or more of the active ingredients in the complete formulation. Fourteen pesticide formulations had guinea pig data for a substance related to an active ingredient or for a related formulation.

Among the 23 formulations that had guinea pig data, the LLNA classified 52% (12 of 23 formulations) as sensitizers, while the guinea pig tests classified only 13% (3 of 23 formulations) as sensitizers. All three of the pesticide formulations identified as sensitizers in the guinea pig test were also identified as sensitizers in the LLNA. Overall, the LLNA and the guinea pig results were in agreement 57% of the time. The LLNA identified as sensitizers an additional seven substances that the guinea pig test classified as nonsensitizers, an overprediction rate of 50% (10 of 20).

Three of the LLNA studies for these 23 pesticide formulations were done in BALB/c mice. The OECD Test Guideline and ICCVAM protocol use CBA/CA and CBA/J strains. If the three BALB/c studies are therefore excluded from the analysis, the LLNA and guinea pig results were in agreement 60% of the time (12 of 20), and the overprediction rate was 47% (8 of 17). There were no instances of

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<sup>4</sup> Based on the Panel's recommendation, this Addendum does not refer to "mixtures" as a type of substance tested but rather specifies, where possible, the types of products that were tested.

underprediction for the 23 pesticide formulations. Human data were not available for these pesticide formulations to confirm their sensitization potential in humans.

**Dyes:** The current LLNA database contains data for six dyes that have associated LLNA and guinea pig data. The LLNA classified 50% (3 of 6) as sensitizers and 50% (3 of 6) as nonsensitizers. By comparison, the guinea pig maximization test (GPMT) identified 83% (5 of 6) as sensitizers and 17% (1 of 6) as nonsensitizers (when there were multiple calls in the GPMT, the most conservative call was used). The LLNA and the guinea pig results were in agreement 33% of the time. The overprediction rate for the LLNA was 100% (1 of 1), and the underprediction rate was 60% (3 of 5).

**Natural Complex Substances:** The current LLNA database contains data for 12 natural complex substances (essential oils and absolutes) with comparative LLNA and human data. Essential oils are derived from a natural source using steam or pressure. Absolutes are purified extracts from natural products. Both essential oils and absolutes are composed of more than one component.

Of the 12 natural complex substances, the LLNA classified 75% (9 of 12) as sensitizers and 25% (3 of 12) as nonsensitizers. However, human clinical studies identified only 33% (4 of 12) of these substances as sensitizers. Therefore, among these 12 substances, the LLNA was able to identify three out of four of the substances that tested positive in human testing.

Six substances that did not produce positive results in human testing were positive in the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5 of 12), a sensitivity of 75% (3 of 4), a specificity of 25% (2 of 8), a false positive rate of 75% (6 of 8), and a false negative rate of 25% (1 of 4). There are no data from guinea pig tests for these natural complex substances; therefore, the performance of the LLNA and the guinea pig tests could not be compared to the human outcome.

#### ***Use of the LLNA for Testing Metal Compounds***

The NICEATM LLNA database includes test results from 48 studies involving 16 metal compounds. The compounds in turn represent 13 different metals (mixtures containing metals are excluded from this analysis). All 16 metal compounds had comparative human data, and 8 had comparative guinea pig data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, and three times as nickel chloride. Because nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in four, a decision was made to exclude nickel compounds from the LLNA metals performance analysis.

For the remaining 14 metal compounds (13 metals), the LLNA had an accuracy of 86% (12 of 14), a sensitivity of 100% (9 of 9), a specificity of 60% (3 of 5), a false positive rate of 40% (2 of 5), and a false negative rate of 0% (0 of 9) when compared to human results. The two false positive compounds were copper chloride and zinc sulfate.

The LLNA identified as sensitizers all six of the metal compounds (six different metals with nickel compounds excluded) with comparative guinea pig test results. The LLNA results had an accuracy of 83% (5 of 6), a false positive rate of 100% (1 of 1), and a false negative rate of 0% (0 of 5) when compared to guinea pig test results.

NICEATM compared the performance of the LLNA and the guinea pig tests to that of human tests for the six metal compounds tested in all three species. The LLNA had an accuracy of 83% (5 of 6), a false positive rate of 100% (1 of 1), and a false negative rate of 0% (0 of 5). By comparison, the guinea pig test had an accuracy of 100% (6 of 6), a false positive rate of 0% (0 of 1), and a false negative rate of 0% (0 of 5) against the human test.

#### ***Use of the LLNA for Substances Tested in Aqueous Solutions***

The NICEATM LLNA database for aqueous solutions includes data from 171 studies that involved 139 substances. Ninety-one of these substances (123 LLNA studies) are pesticide formulations and pure compounds. Forty-eight substances (48 LLNA studies) are aqueous eluates of medical devices.

Because of differences in the protocols for sample preparation, NICEATM analyzed the two groups separately. Of the 91 pesticide formulations and pure compounds, 63% (57 of 91) were LLNA positive, and 37% (34 of 91) were LLNA negative. Of these 91 LLNA studies, 66 used CBA mice, and 28 used BALBc. The mouse strain was not specified for 29 studies. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water.

Guinea pig data were available for 25 substances tested in aqueous solutions (4 sensitizers/21 nonsensitizers in the guinea pig). Eleven substances had LLNA test results that differed from the guinea pig results. Ten of the 11 discordant substances were pesticide formulations tested in aqueous 1% Pluronic L92. These were the same 10 substances discussed for the pesticide formulations analysis. All were overpredicted by the LLNA with respect to the guinea pig results (48% overprediction [10 of 21 tests]). One additional substance, neomycin sulfate, which was tested in 25% EtOH, was underpredicted by the LLNA (25% underprediction [1 of 4]). Overall, the LLNA and the guinea pig results were in agreement 56% of the time (14 of 25).

Human data were available for only four substances tested in aqueous solutions. Three were classified as sensitizers, and one was classified as a nonsensitizer in humans. Only two substances tested in aqueous solutions in the LLNA had comparative guinea pig and human data. Thus, not enough substances were tested in multiple test methods (e.g., LLNA, guinea pig, and human) to allow for a meaningful calculation.

All 48 of the medical device eluates were negative in the LLNA. None of the eluates had associated guinea pig or human data. They were not analyzed to determine their constituents or whether any compound(s) were in fact eluted from the medical device tested. Because the LLNA results were uniformly negative and no sample preparation control was included in the studies, the effectiveness of the sample preparation could not be determined. Therefore, the results from these eluates were not included in the final analysis with those from the pesticide formulations and pure substances tested in aqueous solutions.

## 1.0 Introduction

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin-sensitizing chemicals and products. ACD results in lost workdays and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). To minimize the occurrence of ACD, regulatory authorities require testing to identify substances that may cause ACD. Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary to avoid development of ACD.

Skin sensitization testing has typically required the use of guinea pigs (Buehler 1965; Magnusson and Kligman 1970). However, in 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999). ICCVAM concluded that the LLNA (referred to herein as the “traditional LLNA”) provided several advantages compared to the guinea pig method, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for ACD testing. It is now commonly used around the world.

In February 1998, ICCVAM received a submission from Drs. G. Frank Gerberick (Procter and Gamble, Cincinnati, United States [U.S.]), David Basketter (Unilever Safety and Environmental Assurance Centre, United Kingdom [U.K.]), and Ian Kimber (Syngenta Central Toxicology Laboratory, U.K.) requesting an evaluation of the validation status of the LLNA as an alternative to the guinea pig maximization test (GPMT) and the Buehler test (BT) for assessing skin sensitization potential. The submission summarized the performance (relevance and reliability) of the LLNA as compared to the GPMT and BT methods. An additional analysis was conducted by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to evaluate, where comparable data existed, the comparative performance of the LLNA and the guinea pig (GP) tests against sensitization results obtained in humans. An independent expert peer review panel (Panel) meeting was convened on September 17, 1998, to review the completeness of the submission, to determine whether the usefulness and limitations of the LLNA had been adequately described, and to decide whether its demonstrated performance supported recommending the LLNA as a stand-alone alternative to the GPMT and BT. The Panel also was asked to evaluate whether the LLNA offered advantages with regard to animal welfare considerations (i.e., refinement, reduction, or replacement<sup>5</sup>).

The Panel considered the performance of the LLNA to be similar to that of the GPMT and BT for identifying moderate to strong sensitizers. The Panel concluded that the LLNA did not accurately predict all weak sensitizers, nor did it adequately discriminate between strong skin irritants and skin sensitizers. The LLNA also produced false negative results with some metals. It was recommended that these issues be evaluated in future studies and workshops. Furthermore, data to support using the LLNA to test mixtures and substances tested in aqueous solutions were not provided and the evaluation of pharmaceuticals was limited. Still, the Panel noted that when compared with the GPMT and BT methods, the LLNA appeared to provide equivalent prediction of risk for human ACD, based on comparisons to available human data.

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<sup>5</sup> *Refinement alternative* is defined as a new or revised test method that refines procedures to lessen or eliminate pain or distress to animals, or enhances animal well-being. *Reduction alternative* is defined as a new or revised test method that reduces the number of animals required. *Replacement alternative* is defined as a new or revised test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate) (ICCVAM 1997).

In addition, the Panel concluded that the LLNA could be considered a refinement alternative to the GPMT and BT, because the pain and distress due to sensitization associated with the guinea pig methods could be virtually eliminated by using the LLNA. ICCVAM agreed that the LLNA test method, when modified and used in accordance with the Panel report, can be used effectively for assessment of skin sensitization potential (ICCVAM 1999 [available in **Annex I**]).

The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline 429 [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and Sensitization [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effect Testing Guidelines on Skin Sensitization [EPA 2003]).

NICEATM conducted this revised evaluation of the LLNA applicability domain in response to a nomination<sup>6</sup> submitted to ICCVAM in January 2007 by the U.S. Consumer Product Safety Commission. This Addendum to the ICCVAM (1999) report contains an evaluation of the current database for the LLNA when used to test pesticide formulations and other products, metals, and substances in aqueous solutions in order to fill some of the data gaps identified in the original evaluation (see **Annex I**).

An independent peer review panel (Panel) reviewed this Addendum in March 2008 to evaluate the extent to which the information contained in this Addendum supported the draft recommendations. The draft recommendations stated that more data would be needed before a recommendation on the usefulness and limitations of the traditional LLNA for testing mixtures could be made, due to the limitations associated with the available mixtures database (i.e., unknown formulae, lack of human data). The Panel agreed that the draft recommendation with respect to the traditional LLNA testing of mixtures appeared valid based on the limitations inherent in the available data set. Still, the Panel urged that the ICCVAM recommendations indicate that the approach may be viable. The Panel further recommended that the test method recommendations summary should indicate that the limitations include relatively poor concordance of traditional LLNA outcomes for mixtures with those obtained in GP tests. Routine comparisons of accuracy according to classification criteria may not be sufficient to evaluate the concordance for mixtures, and furthermore, the GP tests are not necessarily valid for mixtures. The Panel also indicated that the term *mixtures* was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations of mixtures that are being examined. The analyses in this Addendum have been done separately on pesticide formulations, dyes, and natural complex substances in response to the Panel's comment.

The draft recommendations also stated that, based on the available data for metals, the traditional LLNA was useful for the testing of metal compounds, with the exception of nickel. Based on the available information, the Panel agreed that the draft recommendations with regard to testing metals appeared to be valid. A minority Panel opinion stated that it should not be concluded that the traditional LLNA was not suitable for testing nickel compounds, because the different vehicles used may have had a significant impact on the ability of nickel to penetrate the skin and be bioavailable.

The draft recommendations also stated that, due to the limited number of substances tested in aqueous solutions, more data would be needed before a recommendation on the usefulness and limitations of the traditional LLNA for testing substances in aqueous solutions could be made. The Panel agreed that the draft ICCVAM recommendation was appropriate and that more data were required before an adequate evaluation of the use of the traditional LLNA with aqueous solutions could be conducted.<sup>7</sup>

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<sup>6</sup> Available at [http://iccvam.niehs.nih.gov/methods/immunotox/llnadoocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadoocs/CPSC_LLNA_nom.pdf)

<sup>7</sup> Available at [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

The data summarized in this Addendum are based on information obtained from the peer-reviewed scientific literature identified through online searches via PubMed and SCOPUS, through citations in publications, and in response to a *Federal Register (FR)* notice requesting LLNA, guinea pig, and/or human skin sensitization data and experience (Vol. 72, No. 95, pp. 27815-27817<sup>8</sup>). Key words used in the online searches for this evaluation were "LLNA" OR "Local Lymph Node" OR "Local lymph node" OR "local lymph node" AND (mixture\* OR formula\*) OR ("metal\* OR aqueous\*"). Additionally, a weekly search on SCOPUS that uses the key words (TITLE-ABS-KEY(**sensi\***) AND TITLE-ABS-KEY(**skin** OR **dermal**)) is done. Since March 2008, six relevant papers were added to the database.

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<sup>8</sup> Available at [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_9544.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf)

## 2.0 Substances Used for the Revised Evaluation of the Applicability Domain for the LLNA

The information summarized in this Addendum is based on a retrospective review of LLNA data derived from a database of over 600 substances (including pesticide formulations and other products) tested in the LLNA and builds on the previous ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). For this evaluation, to minimize the complexity of the analysis, metal formulations are not included in the analysis of pesticide formulations and other products, and metal compounds were restricted to those testing single substances. The reference database includes data for metal compounds from the original ICCVAM evaluation (**Annex I**), data published since that evaluation, and data submitted in response to a request in the previously cited *FR* notice. Since an evaluation of the usefulness and limitations of pesticide formulations and other products, and substances tested in aqueous solutions were not included in original ICCVAM validation (**Annex I**), because no data on these substances were available, the reference database for these substances consists of data published since the original ICCVAM evaluation or submitted in response to the *FR* notice. **Table D-1** provides information on the sources of the data and the rationale for the substances tested.

**Table D-1 Summary of Data Sources and Rationale for Substance Selection**

Data Source	N	Substance Selection Rationale
AppTec Laboratory Services	48	Aqueous eluates from medical devices
Dow AgroSciences	52	Pesticide formulations analyzed in the LLNA with associated GP data of various kinds
Dupont	28	Pesticide formulations analyzed in the LLNA
ECPA	39	Plant protection products (i.e., pesticides) were evaluated in the LLNA with a novel vehicle to assess its usefulness
Basketter et al. (1994, 1996, 1999a, 2005)	16	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Lalko and Api (2006)	12	Original research that evaluated natural complex substances in the LLNA. Additional data were submitted by the authors and RIFM.
Ryan et al. (2000)	2	Interlaboratory study to evaluate the accuracy of the LLNA to identify human sensitizers.
Ryan et al. (2002)	11	Original research with known water soluble haptens and known skin sensitizers to assess the usefulness of a novel vehicle in the LLNA
E. Debruyne (Bayer Crop Science SA)	10	Original research on different pesticide types and formulations in the LLNA
Kimber et al. (1991, 1995, 2003)	9	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Gerberick et al. (2005) <sup>1</sup>	6	Compiled from previously conducted LLNA studies (from published literature and unpublished sources) on substances of varying skin sensitization potential

*Continued*

**Table D-1 Summary of Data Sources and Rationale for Substance Selection (Continued)**

<b>Data Source</b>	<b>N</b>	<b>Substance Selection Rationale</b>
Bundesanstalt für Arbeitsschutz und Arbeitsmedizin	6	Original LLNA research on dye formulations
H.W. Vohr (BGIA)	4	Original LLNA research with epoxy resin components as part of a validation effort for non-radioactive versions of the LLNA
Basketter and Scholes (1992) <sup>2</sup>	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Gerberick et al. (1992)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
D. Germolec (NIEHS)	2	Substances were evaluated by NTP for skin sensitization potential in the LLNA.
Lea et al. (1999)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
M.J. Olson (GlaxoSmithKline)	2	Pharmaceutical substances tested in the LLNA
Unilever (unpublished data)	2	Metal substances evaluated for skin sensitization potential in the LLNA
Basketter and Kimber (2006)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Goodwin et al. (1981)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Griem et al. (2003)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Kligman (1966)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
J. Matheson (CPSC)	1	Published LLNA data submitted to NICEATM, as a reference
K. Skirda (CESIO - TNO Report V7217)	1	Data were provided by CESIO member companies for use in paper titled “Limitations of the LLNA as preferred test for skin sensitization: concerns about false positive and false negative test result.”
Total	262	

Abbreviations: BGIA = Berufsgenossenschaftliches Institut für Arbeitsschutz; CESIO = Comité Européen des Agents de Surface et de leurs Intermédiaires Organiques; CPSC = U.S. Consumer Product Safety Commission; ECPA = European Crop Protection Association; GP = guinea pig; LLNA=local lymph node assay; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; NIEHS = National Institute of Environmental Health Sciences; NTP = National Toxicology Program; RIFM = Research Institute for Fragrance Materials; TNO = TNO Nutrition and Food Research.

<sup>1</sup> These data were evaluated by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee in its evaluation of the LLNA limit dose procedure and were previously submitted to ICCVAM in 1998 for the original evaluation of the validation status of the LLNA (ICCVAM 1999, Gerberick et al. 2005).

<sup>2</sup> These LLNA studies used both male and female mice, but single experiments were limited to one sex.

LLNA studies for 29/89 of the pesticide formulations (tested in aqueous solutions) used the BALB/c mouse strain rather than the CBA/J and CBA/Ca strains of mice, which are recommended for the LLNA by ICCVAM (ICCVAM 1999, Dean et al. 2001, EPA 2003), and the OECD (OECD 2002). The comparative performance of the LLNA using these different strains relative to the guinea pig is detailed in **Section 5.0**. Two additional submitted LLNA studies (from Dr. Dori Germolec at the National Institute of Environmental Health Sciences [NIEHS]) also used the BALB/c strain. One of these, sodium metasilicate (an aqueous solution), did not have comparative GP or human data and thus was not included in the performance analysis. The other study was for potassium dichromate (a metal), which was positive in the LLNA, GP, and human. As there are 22 LLNA studies for potassium dichromate included in **Annex III-2**, all of which are positive, excluding this study would have no impact on the performance analysis for metals. Two other studies cited in Griem et al. (2003) used both male and female mice, but single experiments were limited to one sex. These data were included in the evaluation.

To the extent possible, **Annexes II-1, II-4, II-6, III-1, and IV-1** provide information on the physicochemical properties (e.g., physical form), Chemical Abstracts Service Registry Number (CASRN), and chemical class for each pesticide formulation, dye, fragrance ingredient, metal compound, and substance tested in an aqueous solution, respectively. This information was obtained from published reports, submitted data, or through literature searches.

When available, chemical classes for the test substances were retrieved from the National Library of Medicine's ChemID Plus database. If chemical classes were not located, where possible, they were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system<sup>9</sup>. Some substances were assigned to more than one chemical class; however, no substance was assigned to more than three classes. One complex pharmaceutical intermediate was simply identified as a pharmaceutical substance. Material families for the active ingredients in the formulations submitted by Dow AgroSciences were provided by Dow AgroSciences.

The generic composition of some of the formulated products evaluated by the European Crop Protection Association (ECPA) (Dinocap EC, Oxyfluorfen EC, Quinoxifen/cyproconazole, and Trifluralin EC) and the formulations submitted by Dow AgroSciences, using the LLNA, is included in **Annex II-3**. For the formulations provided by ECPA, none of the active ingredients have been tested using the LLNA but the active ingredients have been tested previously in a guinea pig test (personal communication by Dr Eric Debruyne, Bayer CropScience in France). Likewise, none of the inerts (e.g., surfactants, solvents, etc.) have been tested independently for these formulations. Dow AgroSciences provided information about LLNA and guinea pig tests on active ingredients and inerts for the formulations they submitted. The component information for the remaining pesticide formulations have been requested by NICEATM, but since some of the data is proprietary, it is not available at this time.

One hundred and four pesticide formulations (i.e., herbicides, fungicides, insecticides) were evaluated for this Addendum. All of these were liquids, though some were in the form of suspensions or emulsions, and were tested in an aqueous vehicle. Six dyes (all solids), and 12 natural complex substances (all liquids), which are a combination of essential oils and absolutes, were also evaluated. Essential oils are oils derived from a natural source using steam or pressure. Absolutes are purified extracts from natural products. Both essential oils and absolutes are substances comprised of more than one component.

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<sup>9</sup> Available at <http://www.nlm.nih.gov/mesh/meshhome.html>

Of the 13 metal compounds evaluated, one (potassium dichromate) is used in leather tanning and as an oxidizer in organic synthesis. Most of the remaining 12 metals in the analysis are used as catalysts, conductors of electricity, or for coating and plating. All of the metal compounds for which information on physical form is identified are solids.

Of the 21 substances tested in aqueous solutions included in this evaluation, six are pesticides (i.e., herbicides, fungicides, and insecticides); this is the only product class represented by more than one substance tested in an aqueous solution.

### 3.0 Comparative *In Vivo* Reference Data

The reference database for this evaluation includes results using currently accepted guinea pig test methods for skin sensitization (i.e., the GPMT and the BT) and human clinical studies and experience (e.g., human repeat insult patch test [HRIPT], human maximization test [HMT], case reports). In the absence of HRIPT or HMT data, the classification of a substance as a human sensitizer was based on the classification of the authors of the report. National and international test guidelines are available for each of these standardized tests and are thus described in detail elsewhere (EPA 2003; OECD 1992).

Ongoing efforts are being made by NICEATM to obtain the original records for all of the reference data used in this evaluation. Ideally, all data supporting the validity of a test method should be obtained and reported from animal studies conducted in accordance with Good Laboratory Practice (GLP) guidelines (EPA 2006a, 2006b; FDA 2007; OECD 1998). Equally, data based on human studies should be conducted in compliance with Good Clinical Practices (GCP) guidelines (ICH 1996). Both sets of guidelines provide an internationally standardized procedure for the conduct of studies, reporting requirements, archival of study data and records, and information about the test protocol, in order to ensure the integrity, reliability, and accountability of a study.

The extent to which the human or guinea pig studies were compliant with GCP or GLP guidelines, respectively, is based on the information provided in published and submitted reports. The GP data obtained from E. Debruyne (Bayer CropScience SA) and P. Botham (ECPA), and Dow AgroSciences, were reportedly conducted according to GLP guidelines. None of the published references from which GP or human data were obtained include specifics on GCP or GLP compliance.

## 4.0 LLNA Data and Results

The data used for this evaluation were obtained from 25 sources (**Table D-1**). No new LLNA studies were conducted to generate data for this evaluation (see **Section 2.0**). Where available, specific information including name, CASRN, physicochemical properties (e.g., molecular weight, Log  $K_{ow}$ ), chemical class<sup>10</sup> and data source are indicated for each pesticide formulation, dye, fragrance ingredient, metal compound, and substance tested in an aqueous solution (**Annexes II-1, II-4, II-6, III-1, and IV-1**, respectively). The concentrations tested, along with calculated stimulation index (SI) and/or EC3 (the concentration that induces an SI of 3) values, are provided in **Annexes II-2, II-5, B7, III-2, and IV-2** for pesticide formulations, dyes, natural complex substances, metal compounds, and substances tested in an aqueous solution, respectively. Individual components and concentrations of the pesticide formulations and substances tested in an aqueous solution submitted by Bayer have been requested, but due to confidential and proprietary issues, Bayer has only been able to provide the generic composition for four formulated products (see **Section 2.0**). Furthermore, provided in the submitted data or study reports, the source or purity of the test substance was not known.

LLNA classification as to whether a substance was a sensitizer or a nonsensitizer was based on study data extracted from the sources listed in **Table D-1** and **Annexes II-1, II-4, II-6, III-1, and IV-1**, with two exceptions. Classification of ammonium tetrachloroplatinate and gold (III) chloride (both of which are metal compounds) as sensitizers by the LLNA was based on published reference classifications (Basketter and Scholes 1992, Basketter et al. 1999a) and not on actual LLNA data.

The LLNA data included in the ICCVAM (1999) database (**Annex I**) were reviewed during the original evaluation. However, the availability of the original data for the other studies included in this evaluation has not yet been established for all data sources. Additionally, coding of substances to avoid potential scoring bias was not described in the previous evaluation of 209 substances (ICCVAM 1999; **Annex I**) or for any of the newly obtained studies used in this evaluation.

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<sup>10</sup> Chemical classes were assigned by NICEATM based on the classification of the National Library of Medicine's Medical Subject Heading (available at <http://www.nlm.nih.gov/mesh/meshhome.html>).

## 5.0 Accuracy of the LLNA: Revised Applicability Domain

The ability of the LLNA to correctly identify pesticide formulations and other products, metal compounds, and substances tested in aqueous solutions as potential skin sensitizers was evaluated when compared to human and guinea pig data. The classification of pesticide formulations, dyes, fragrance ingredients, metal compounds, and substances tested in aqueous solutions and the relevant data for each substance is located in **Annexes II-2, II-5, II-7, III-2, and IV-2**, respectively. For comparison purposes, the performance of the LLNA database reported in the ICCVAM evaluation report (ICCVAM 1999; **Annex I**) is included in **Tables D-4, D-6, D-8, D-11, and D-14**. For this addendum, substances containing multiple components were analyzed separately as pesticide formulations, dyes, and fragrance ingredients.

### 5.1 Testing of Pesticide Formulations and Other Products

The original ICCVAM LLNA report (ICCVAM 1999) (**Annex I**) did not include an analysis on the ability of the LLNA to predict the skin sensitizing potential of pesticide formulations and other products, because data were not available for that evaluation. Thus, all of the analyses below for pesticide formulations, dyes and fragrance ingredients are new material in this addendum.

#### 5.1.1 Testing of Pesticide Formulations

The current LLNA database contains data for 104 pesticide formulations for which LLNA data exists. The physicochemical properties of these formulations are in **Annex II-1**, and the data analyzed here are in **Annex II-2**.

For these formulations, 54% (56/104) were classified as sensitizers in the LLNA, and 46% (48/104) were classified as nonsensitizers. For substances that were tested multiple times in the LLNA, classification as a sensitizer or nonsensitizer was made by a majority call (i.e., the most prevalent call that occurred among the studies). For example, five independent studies were considered for the formulation Oxyfluorfen EC. The highest SI values observed for the various studies were 5.4, 4.9, 3.1, 2.8, and 2.3, respectively (all of these SI values occurred with a test concentration of 33%). Since an SI value  $\geq 3$  occurred in three of the five studies, Oxyfluorfen EC was classified as a sensitizer in the LLNA, even though two studies (SIs = 2.8 and 2.1, respectively) would have resulted in classification as a nonsensitizer if considered alone.

Seventy of the 104 pesticide formulations have LLNA and some type of guinea pig reference data. A total of 89 LLNA studies were performed using these 70 formulations. LLNA studies were conducted with either CBA/Ca or CBA/J (61/89) and/or BALB/c (28/89) mouse strains.

Six formulations were tested in multiple LLNA studies (25 studies total [**Table D-2**]). LLNA results for 5/6 formulations were in agreement across multiple studies, and LLNA results for 1/6 formulations were discordant across multiple studies (3 positive, 2 negative [**Table D-3**]).

Twenty-three formulations had associated GP data for the formulation itself, 46 formulations had GP data for one or more of the active ingredients in the formulation, and 14 formulations had GP data for a substance related to an active ingredient, or for a related formulation. The performance of the LLNA against GP tests for pesticide formulations with GP data for the entire formulation is discussed in **Section 5.1.1.1**. The performance of the LLNA against GP tests for pesticide formulations with GP data for active ingredients or related substances and formulations is discussed in **Annex V**.

All formulations (89/89 studies) were tested in the LLNA in 1% Pluronic L92. Pluronic L92 block copolymer is a surfactant and wetting agent that has been evaluated as an alternative aqueous-based vehicle for use in the LLNA. Pluronic L92 was chosen for evaluation because it promotes test material retention on the ear by preventing run-off, and exhibits low acute toxicity and irritation potential (Boverhof et al. 2008; Ryan et al. 2002). Ryan et al. (2002) assessed the performance of Pluronic L92 relative to other solvents in the LLNA using aqueous soluble haptens. Based on their

results, they determined that, for identification of sensitization hazard of aqueous soluble materials using the LLNA, dimethylformamide (DMF), and dimethylsulfoxide (DMSO) were the preferred vehicles. However, if a test material is not soluble in DMF or DMSO, or if higher test concentrations could be achieved in an aqueous vehicle, then 1% Pluronic L92 might improve assay performance over the use of water as a vehicle.

In an interlaboratory study (n=5 laboratories), Boverhof et al. (2008) conducted LLNA tests on three substances with known sensitization potential (hexylcinnamaldehyde, formaldehyde, and potassium dichromate), and four pesticide formulations for which the sensitization potential in guinea pigs and/or humans had previously been determined, using Pluronic L92 as the vehicle. They concluded that the LLNA results for all of these substances when tested in Pluronic L92 were consistent with previous GP or human results, and that Pluronic L92 was a suitable vehicle to use when testing aqueous solutions in the LLNA.

For the 52 formulations submitted by Dow AgroSciences, a list of all of the components in the formulation (albeit some were listed generically [e.g., emulsifier, biocide, etc.]) was also provided, along with information as to whether each component was a sensitizer. For these components, the criteria for classification as a sensitizer were not specified. **Annex II-3** contains the information on components provided by Dow AgroSciences.

**Table D-2 Pesticide Formulations with Multiple LLNA Studies**

Formulation	Source	No. Studies	Mouse Strain	No. Positive Studies	No. Negative Studies	No. Labs
Atrazine SC	ECPA	2	CBA	2	0	2
Dinocap EC	ECPA	5	CBA	5	0	5
Formulation 7	Dow AgroSciences	2	BALB/c	2	0	1
Oxyfluorfen EC	ECPA	5	CBA	3	2	5
Quinoxifen / cyproconazole	ECPA	6	CBA	6	0	6
Trifluralin EC	ECPA	5	CBA	5	0	5

Abbreviations:

EC = emulsion concentrate; ECPA= European Crop Protection Association; No. = number; SC = suspension concentrate.

**Table D-3 LLNA Data for Pesticide Formulation with Discordant Results**

Formulation	Vehicle	Conc. (%)	SIs	Strain	EC3 (%)	Lab
Oxyfluorfen EC	L92	1, 7, 33	0.8, 1.4, 4.9	CBA/Ca	30.8	1
		1, 7, 33	0.9, 1.4, 2.8	CBA/J	NC	2
		1, 7, 33	0.3, 0.9, 2.3	CBA/J	NC	3
		1, 7, 33	1.1, 1.5, 3.1	CBA/JHsd	30.8	4
		1, 7, 33	1.2, 1.2, 5.4	CBA/CaOlaHsd	18.1	5

Abbreviations:

Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce an SI of 3; L92 = 1% aqueous pluronic L92; NC = not calculated since SI<3.0; SIs = stimulation indices.

#### 5.1.1.1 Testing of Pesticide Formulations: LLNA vs. GP with Available Reference Data for the Entire Formulation

For the 23 formulations that had associated GP data for the formulation itself, 13% (3/23) were classified as sensitizers and 87% (20/23) as nonsensitizers according to the GP results (**Figure D-1**). Twenty-one of these GP tests were BT and 2 were GPMT. These results are based on a positive overall GP call for formulation EXP 10810.<sup>11</sup> Ten out of the approximately 450 active ingredients registered with EPA were represented among these 23 formulations. Furthermore, approximately 40 different classes of pesticides are registered with EPA, of which these nine active ingredients represent a small proportion (i.e., one insecticide, one microbiocide, six herbicides and two fungicides).

Twenty of the LLNA studies were conducted in CBA mice (i.e., the preferred strain for use in the LLNA according to the ICCVAM recommended LLNA protocol and OECD TG 429) and three studies were conducted in BALB/c mice. The LLNA classified 57% (13/23) of the formulations as sensitizers and 43% (10/23) as nonsensitizers (**Figure D-1**). All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA. The LLNA also identified an additional seven substances as sensitizers that were classified as nonsensitizers in the GP test (**Table D-4**).

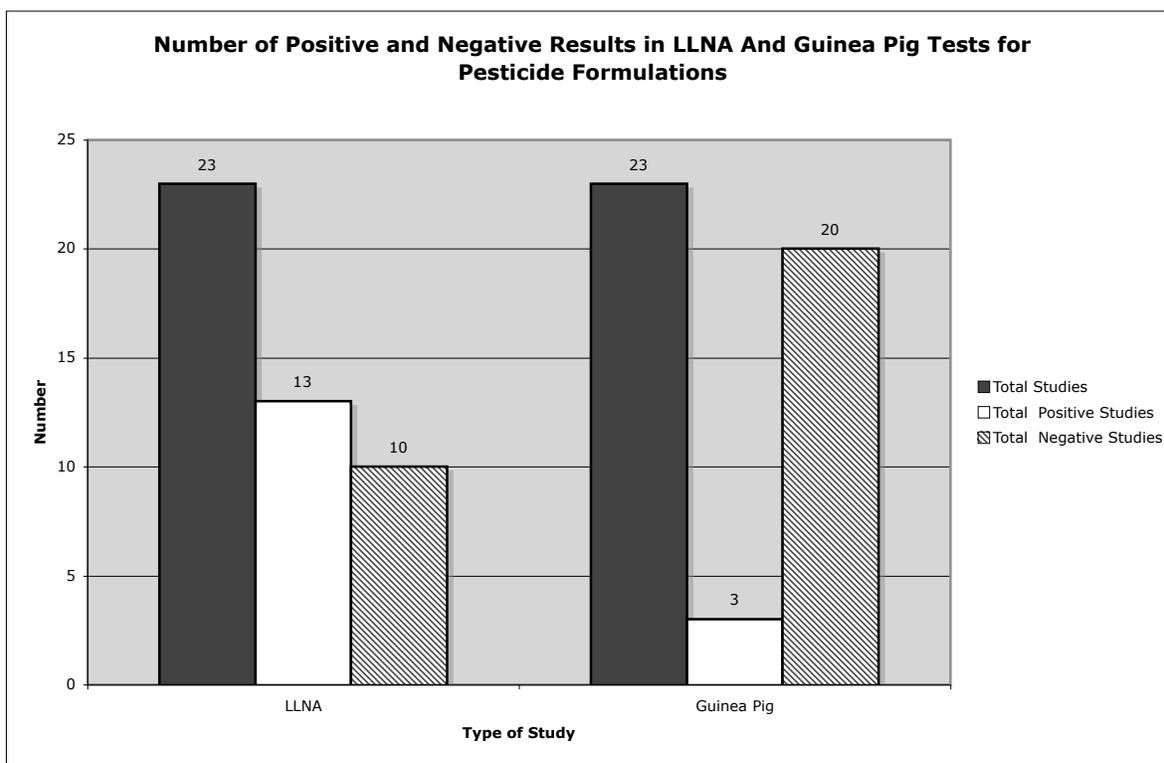
If only LLNA studies using CBA mice are considered, three LLNA studies conducted with BALB/c mice are removed from the database, which eliminates two LLNA positive studies, and one LLNA negative study. Based on the remaining 20 LLNA studies, the LLNA classified 55% (11/20) of the formulations as sensitizers and 45% (9/20) as nonsensitizers (**Figure D-1**). This does not change the fact that all three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA, and that seven substances identified as sensitizers in the LLNA are classified as nonsensitizers in the GP test (**Table D-4**).

There were no comparative human data with which to determine the actual human sensitization potential.

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<sup>11</sup> Formulation EXP 10810 A (submitted by E. Debruyne, Bayer Crop Science), the only formulation for which there was data in both the GPMT and the BT, showed equivocal results in the guinea pig. This formulation tested positive in the GPMT (sensitization incidence 100%), and negative in the BT (sensitization incidence 10%). The patch concentration in the GPMT was the same as the induction concentration in the BT (50%).

**Figure D-1 Numbers of Positive and Negative LLNA and GP Calls for Pesticide Formulations**



Abbreviations: LLNA = local lymph node assay.

Based on the 23 pesticide formulations tested in CBA (n=20) and BALB/c (n=3) strains, the accuracy of the LLNA compared to guinea pig data was 57% (13/23), the sensitivity was 100% (3/3), the specificity was 50% (10/20), the false positive rate was 50% (10/20) and false negative rate was 0% (0/3). If the three studies using BALB/c mice are not considered, the accuracy of the LLNA compared to guinea pig data was 60% (12/20), the sensitivity was 100% (3/3), the specificity was 53% (9/17), the false positive rate was 47% (8/17), and the false negative rate was 0% (0/3) (**Table D-4**).

**Table D-4 Evaluation of the Performance of the LLNA for Testing Pesticide Formulations**

Comparison <sup>1</sup>	n <sup>2</sup>	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>
LLNA <sup>4</sup> vs. GP <sup>5</sup>	23	57	13/23	100	3/3	50	10/20	50	10/20	0	0/3
LLNA <sup>6</sup> vs. GP <sup>5</sup>	20	60	12/20	100	3/3	53	9/17	47	8/17	0	0/3
<i>ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data<sup>7</sup></i>											
LLNA <sup>6</sup> vs. GP <sup>5</sup>	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA <sup>6</sup> vs. Human <sup>9</sup>	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP <sup>5</sup> vs. Human <sup>8</sup>	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:

GP = guinea pig skin sensitization outcomes; LLNA = local Lymph Node Assay; No. = number.

*Accuracy* (concordance) = the proportion of correct outcomes (positive and negative) of a test method

*Sensitivity* = the proportion of all positive substances that are classified as positive

*Specificity* = the proportion of all negative substances that are classified as negative

*False negative rate* = the proportion of all positive substances that are falsely identified as negative

*False positive rate* = the proportion of all negative substances that are falsely identified as positive

<sup>1</sup> This accuracy analysis is only for formulations that have LLNA data and some type of associated GP data; none of the pesticide formulations analyzed had human data, so a comparison between LLNA vs. human and LLNA vs. GP is not included.

<sup>2</sup> n = number of substances included in this analysis

<sup>3</sup> The data on which the percentage calculation is based

<sup>4</sup> LLNA studies conducted with CBA (n=20) and BALB/c (n=3) mice

<sup>5</sup> P refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

<sup>6</sup> LLNA studies conducted with CBA mice

<sup>7</sup> For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; Annex I) showing the overall performance of the LLNA vs. GP and human, and GP vs. human is included here.

<sup>8</sup> *Human* refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

Among the 10 of 23 formulations classified as sensitizers by the LLNA that were classified as nonsensitizers in the GP (**Table D-5**), eight were classified as nonsensitizers based on BT results and two were classified as nonsensitizers based on GPMT results.

**Table D-5 Pesticide Formulations that are Classified as Sensitizers in the LLNA, but Classified as Nonsensitizers in the Guinea Pig**

Substance Name	LLNA Results				GP Results			Skin Irritant?
	Conc. (%) <sup>1</sup>	SI <sup>2</sup>	EC3 (%)	Result <sup>3</sup>	Ind. Conc. (%)	Sens. Incid. (%)	Result <sup>3</sup>	
Atrazine SC	100	7.3	36.4 <sup>4</sup>	+	30	0	- <sup>5</sup>	Nonirritant at ≤ 25% <sup>6</sup>
BASF SE-1	70	22.7	5.5	+	100	0	- <sup>7</sup>	Nonirritant at ≤ 50% <sup>6</sup>
EXP 11120 A	100	5.3	64.9	+	100	0	- <sup>7</sup>	Nonirritant at 100% <sup>6</sup>
F & Fo WG 50 + 25	25	15.2	0.003	+	30	0	- <sup>7</sup>	Nonirritant at ≤ 10% <sup>6</sup>
FAR01060-00	100	3.6	88.5	+	100	0	- <sup>7</sup>	Nonirritant at 100% <sup>6</sup>
Formulation 2 <sup>8</sup>	80	15.8	15.7	+	NA	NA	- <sup>7</sup>	Nonirritant at 80% <sup>9</sup>
Formulation 7 <sup>8</sup>	100	3.2	85	+	100	0	- <sup>7</sup>	Nonirritant at 80% <sup>9</sup>
Fx + Me EW 69	50	8.6	25.2	+	100	0	- <sup>7</sup>	Nonirritant at 100% <sup>6</sup>
Oxyfluorfen EC	33	5.4	30.8 <sup>10</sup>	+	10	26	- <sup>5</sup>	Nonirritant at ≤ 25% <sup>6</sup>
Trifluralin EC	100	75.2	10.3 <sup>11</sup>	+	50	10	- <sup>7</sup>	Nonirritant at ≤ 25% <sup>6</sup>

## Abbreviations:

Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of 3; EW = emulsion, oil in water; GP = guinea pig; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; SC = suspension concentrate; Sens. Incid. = sensitization incidence; SI = stimulation index; WG = water-dispersible granules

<sup>1</sup> Maximum concentration tested in the LLNA

<sup>2</sup> Maximum SI obtained in the LLNA

<sup>3</sup> (-) = nonsensitizer, (+) = sensitizer

<sup>4</sup> Mean value from two studies

<sup>5</sup> Guinea pig maximization test (GPMT) result

<sup>6</sup> Based on challenge concentration from a GPMT or Buehler test (BT)

<sup>7</sup> BT result

<sup>8</sup> LLNA conducted in BALB/c mice

<sup>9</sup> Based on irritation prescreen in mice

<sup>10</sup> Mean from three positive studies

<sup>11</sup> Mean of five studies

The constituents of most of the formulations are unknown (**Annex II-3**). Formulation 2 contains a biocide (at a concentration of 0.54 g/L) that is a sensitizer according to constituent information provided by Dow AgroSciences (**Annex II-3**). Dow Agrosciences categorizes all other constituents of Formulation 2 as nonsensitizers, including the active ingredients fluroxypyr-meptyl and florasulam (**Annex II-3**). Formulation 7 contains the sensitizers quinoxifen (active ingredient at a concentration of 45 g/L) and a biocide (at a concentration of 0.37 g/L); it is unknown whether this is the same biocide that is a constituent of Formulation 2. Formulation 7 also contains the active ingredient myclobutanil, which, when tested by Dow AgroSciences in GP sensitization tests, gave equivocal results (**Annex II-3**).

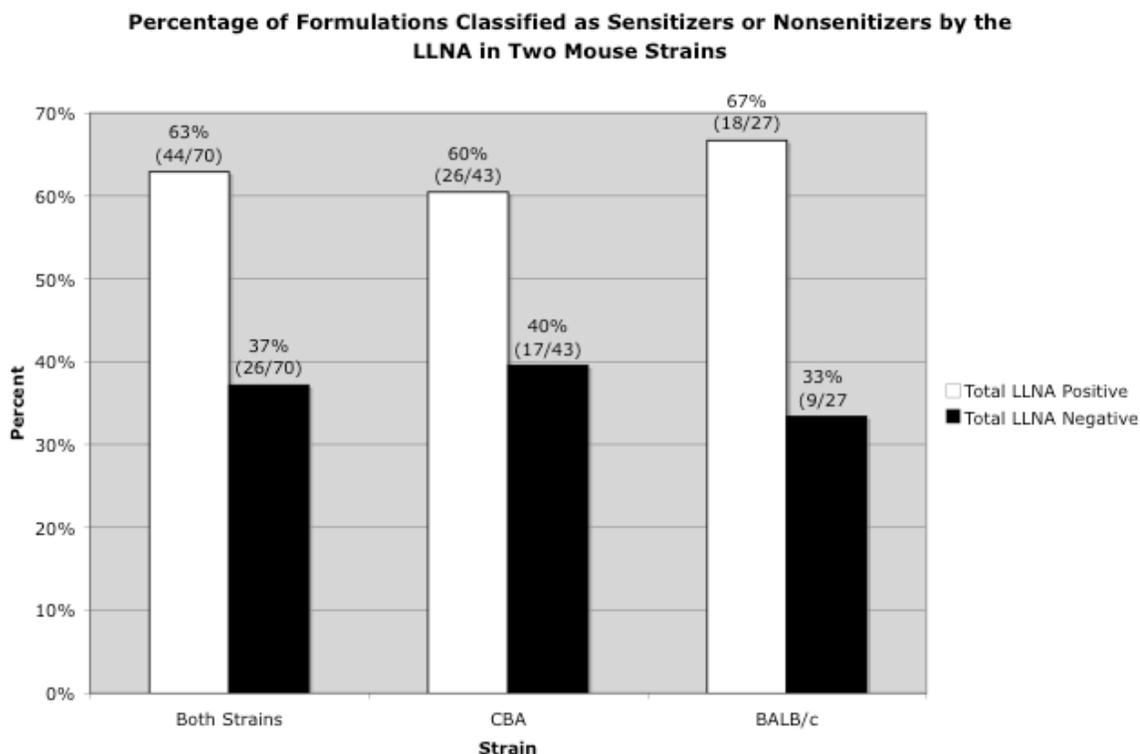
Six of the overpredicted formulations based on LLNA results compared to GP results (BASF SE-1, EXP 11120 A, F & Fo WG 50 + 25, FAR01060-00, Formulation 7, and Fx + Me EW 69; see **Table D-5**) were tested in the GP at induction concentrations equal to or greater than the highest concentration tested in the LLNA. However, atrazine tested as a sensitizer at 100% in the LLNA, but tested as a nonsensitizer at 30% induction concentration in the GPMT; oxyfluorfen tested as a sensitizer at 33% in the LLNA but tested as a nonsensitizer at 10% induction concentration in the GPMT; and trifluralin tested as a sensitizer at 100% in the LLNA, but tested as a nonsensitizer at 50% induction concentration in the BT (**Table D-5**).

The EC3 values for most (9/10) of the formulations indicated that they produced weak to moderate responses in the LLNA (EC3 range of 5.5% to 88.5%) (**Table D-5**). However, the EC3 value for the formulation F & Fo WG 50 + 25 (EC3 = 0.003%) is a very strong LLNA response. This could be because the LLNA dose-response curve approached saturation (i.e., SI = 11.7 at 2.5%, SI = 15.2 at 25%) and the calculation of the EC3 was performed by extrapolation because no responses were below SI = 3 (**Annex II-2**). This EC3 value is likely a poor estimate of the actual value. However, based on the concentrations test, and the resulting SI values, the LLNA data do indicate that the EC3 for formulation F & Fo WG 50 + 25 is less than 2.5% (i.e., SI = 11.7 at 2.5%, the lowest concentration tested).

Five of the overpredicted formulations (Atrazine SC, BASF SE-1, F & Fo WG 50 + 25, Oxyfluorfen EC, and Trifluralin EC) were tested in the LLNA at potentially irritating concentrations. This is based on the concentration tested in the LLNA exceeding the reported challenge concentrations used in the BT or GPMT. According to the respective protocols for these guinea pig tests, the challenge concentration should be the maximum nonirritating concentration of a test substance (**Table D-5**).

#### *5.1.1.2 Testing of Pesticide Formulations: Comparison Between Mouse Strains CBA and BALB/c*

For the 70 pesticide formulations that had associated GP data, 43 were tested in the LLNA in CBA mice and 27 were tested in BALB/c mice. No formulation was tested in the LLNA in both strains. **Figure D-2** shows that the percentage of formulations that were classified as sensitizers was slightly higher in BALB/c mice (67% [18/27]) than in CBA mice (60% [26/43]).



**Figure D-2 Percentage of Formulations Classified as Sensitizers or Nonsensitizers in Two Mouse Strains**

For the 23 pesticide formulations that were tested in both the GP and the LLNA, 20/23 were conducted using CBA mice and 3/22 were conducted using BALB/c mice. As noted in **Section 5.1.1.1**, when data for all 23 formulations is considered (i.e., using both CBA and BALB/c data), the overall accuracy is 57% (13/23), with false positive and false negative rates of 50% (10/20) and 0% (0/3), respectively. If only LLNA studies using CBA mice are considered, removing the three LLNA studies conducted with BALB/c mice from the database eliminates two LLNA positive studies, and one LLNA negative study, which only marginally impacts the overall accuracy (accuracy = 60% [12/20], false positive rate = 47% [8/17], and false negative rate = 0% [0/3]).

As mentioned previously, since comparative human data are not available for any of the formulations analyzed, an evaluation of these formulations in the LLNA compared to human performance could not be assessed. For the same reason, an evaluation of GP versus human outcomes is also not possible. Also, no formulations were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Annex I**), so these data and analyses cannot be compared to previously considered data.

### 5.1.2 Testing of Dyes

The current LLNA database contains data for six dyes, for which there is LLNA and GP data. The physicochemical properties of these dyes are in **Annex II-4**, and the data analyzed here are in **Annex II-5**. For these dyes, 50% (3/6) were classified as sensitizers in the LLNA, and 50% (3/6) were classified as nonsensitizers in the LLNA. In the GPMT, 83% (5/6) dyes tested as sensitizers. **Table D-6** provides the performance statistics for the LLNA when compared to GPMT outcomes for this limited dataset.

**Table D-6 Evaluation of the Performance of the LLNA for Testing Dyes**

Comparison <sup>1</sup>	n <sup>2</sup>	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>
<b>LLNA vs. GPMT</b>	6	33	2/6	40	2/5	0	0/1	100	1/1	60	3/5
<b>ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data<sup>4</sup></b>											
<b>LLNA vs. GP<sup>5</sup></b>	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
<b>LLNA vs. Human<sup>6</sup></b>	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
<b>GP<sup>5</sup> vs. Human<sup>6</sup></b>	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:

GP = guinea pig; GPMT = guinea pig maximization test; LLNA = local lymph node assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

Sensitivity = the proportion of all positive substances that are classified as positive

Specificity = the proportion of all negative substances that are classified as negative

False negative rate: the proportion of all positive substances that are falsely identified as negative

False positive rate = the proportion of all negative substances that are falsely identified as positive

<sup>1</sup> This accuracy analysis is only for dyes that have LLNA data and some type of associated GP data; none of the dyes analyzed had human data, so a comparison between LLNA vs. human and LLNA vs. GP is not included.

<sup>2</sup> n = number of substances included in this analysis

<sup>3</sup> The data on which the percentage calculation is based

<sup>4</sup> For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; Annex I) showing the overall performance of the LLNA vs. GP and human, and GP versus human is included here.

<sup>5</sup> GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test, the Buehler test, or the McGuire test.

<sup>6</sup> Human refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

Four of the six dyes showed discordant results between the LLNA and the GPMT. These substances are shown in **Table 5-6**, including the maximum concentration tested in the LLNA and the maximum SI value attained, as well as the induction concentration and sensitization incidence in the GPMT. These results indicate that the discordant outcomes between the LLNA and the GPMT cannot be explained based on the concentrations tested (i.e., the maximum concentration tested in the LLNA was higher than the GPMT induction concentration in all four cases).

**Table D-7 Dyes Discordant Between the LLNA and GPMT**

Substance Name	LLNA Results					GPMT Results			Skin Irritant?
	Veh.	Conc. (%) <sup>1</sup>	SI <sup>2</sup>	EC3 (%)	Result <sup>3</sup>	Ind. Conc. (%)	Sens. Incid. (%)	Result <sup>3</sup>	
C.I. Reactive Yellow 174	AOO	15	7.8	7.8	+	5	11	-	NA
Dispersionsrot 2754	AOO	9	1	NC	-	5	100	+	NA
Produkt P-4G	AOO	15	2.5	NC	-	5	90	+	NA
Yellow E-JD 3442	AOO	15	0.9	NC	-	5	90	+	NA

## Abbreviations:

AOO = acetone/olive oil; Conc. = concentration; EC3 = estimated concentration needed to produce a stimulation index of three; GPMT = guinea pig maximization test; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI<3.0; ND = not done; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle.

<sup>1</sup> Maximum concentration tested in the LLNA

<sup>2</sup> Maximum SI obtained in the LLNA

<sup>3</sup> (-) = nonsensitizer, (+) = sensitizer

As mentioned previously, since comparative human data are not available for any of the dyes analyzed, an evaluation of these substances in the LLNA or the GP compared to human performance could not be assessed. Also, no dyes were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Annex I**), so these data and analyses cannot be compared to previously considered data.

### 5.1.3 Testing of Natural Complex Substances

The current LLNA database contains data for 12 natural complex substances, for which there are LLNA and human data. The physicochemical properties of these substances are in **Annex II-6**, and the data analyzed here are in **Annex II-7**. For these substances, 75% (9/12) were classified as sensitizers in the LLNA, and 25% (3/12) were classified as nonsensitizers in the LLNA. In the human, 33% (4/12) of these substances tested as sensitizers. One of these human sensitizers (treemoss) was underpredicted by the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5/12), a sensitivity of 75% (3/4), a specificity of 25% (2/8), a false positive rate of 75% (6/8), and a false negative rate of 25% (1/4) (**Table D-8**).

**Table D-8 Evaluation of the Performance of the LLNA for Testing Natural Complex Substances**

Comparison <sup>1</sup>	n <sup>2</sup>	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>
<b>LLNA vs. Human<sup>4</sup></b>	12	42	5/12	75	3/4	25	2/8	75	6/8	25	1/4
<b>ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data<sup>6</sup></b>											
<b>LLNA vs. GP<sup>5</sup></b>	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
<b>LLNA vs. Human<sup>4</sup></b>	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
<b>GP<sup>3</sup> vs. Human<sup>4</sup></b>	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:

GP = guinea pig; LLNA = local lymph node assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

Sensitivity = the proportion of all positive substances that are classified as positive

Specificity = the proportion of all negative substances that are classified as negative

False negative rate: the proportion of all positive substances that are falsely identified as negative

False positive rate = the proportion of all negative substances that are falsely identified as positive

<sup>1</sup> This accuracy analysis is only for substances that have LLNA data and associated human data; none of the natural complex substances analyzed had GP data, so a comparison between LLNA vs. human and LLNA vs. GP is not included.

<sup>2</sup> n = Number of substances included in this analysis

<sup>3</sup> The data on which the percentage calculation is based

<sup>4</sup> Human refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

<sup>5</sup> GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test, the Buehler test, or the McGuire test.

Seven of 12 natural complex substances showed discordant results between the LLNA and the HMT. These substances are shown in **Table D-9**, along with the maximum concentration tested in the LLNA and the maximum SI value attained, and the test concentration and sensitization incidence from the HMT. Most (6/7) of the discordant substances were LLNA positive/human negative. All substances for which concentration information was available for both the LLNA and HMT (5/7) were tested at higher concentrations in the LLNA than the induction concentration in the HMT. All false positives in the LLNA produced maximum SI values greater than 6.0, with the exception of spearmint oil, which produced an SI of 3.6 at a test concentration of 10%. All of the discordant LLNA positive fragrance ingredients had EC3 values in a narrow range (3.6% to 9.6%). All false positives were clearly nonsensitizers in the HMT with a sensitization index of 0%. The one human sensitizer underpredicted by the LLNA (treemoss) is classified as a sensitizer based on a sensitization incidence of 2% (3/145) in humans. The concentrations tested in the LLNA and the human were not available.

**Table D-9 Natural Complex Substances: Discordant Results Between the LLNA and Human**

Substance Name	LLNA Results					HMT Results			Skin Irritant?
	Veh.	Conc. (%) <sup>1</sup>	SI <sup>2</sup>	EC3 (%)	Result <sup>3</sup>	Test Conc. (%)	Sens. Incid. (%)	Result <sup>3</sup>	
Basil oil	EtOH/DEP (1:3)	50	25.2	6.2	+	4	0	-	Mild irritant at 100% <sup>4</sup>
Clove oil	EtOH/DEP (1:3)	50	11.4	7.1	+	5 <sup>5</sup>	0 <sup>5</sup>	-	Severe irritant at 100% <sup>8</sup>
						5 <sup>6</sup>	0 <sup>6</sup>		
						10 <sup>7</sup>	0 <sup>7</sup>		
Lemongrass oil	EtOH/DEP (1:3)	50	13.1	6.5	+	4 <sup>9</sup>	0 <sup>9</sup>	-	Mild irritant at 100% <sup>4</sup>
						4 <sup>10</sup>	0 <sup>10</sup>		
						5 <sup>10</sup>	0 <sup>10</sup>		
Litsea cubeb oil	EtOH/DEP (1:3)	50	16.0	8.4	+	8	0	-	Strong irritant at 100% <sup>4</sup>
Palmarosa oil	EtOH/DEP (1:3)	50	5.0	9.6	+	NA	0	-	NA
Spearmint oil	EtOH/DEP (1:3)	10	3.6	3.6	+	4	0	-	Nonirritant at 100% <sup>4</sup>
Treemoss	EtOH/DEP (1:3)	NA	NA	NC	-	NA	2 <sup>11</sup>	+	Nonirritant at 100% <sup>4</sup>

## Abbreviations:

Conc. = concentration; DEP = diethyl phthalate; EtOH = ethanol; HMT = human maximization test; LLNA = local lymph node assay; NA = Not available; NC = not calculated since SI < 3.0; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle.

<sup>1</sup> Maximum concentration tested in the LLNA

<sup>2</sup> Maximum SI obtained in the LLNA

<sup>3</sup> (-) = nonsensitizer, (+) = sensitizer

<sup>4</sup> Test in mice

<sup>5</sup> Test substance was clove bud oil (Opdyke 1975a)

<sup>6</sup> Test substance was clove stem oil (Opdyke 1975b)

<sup>7</sup> Test substance was clove leaf oil Madagascar (Opdyke 1978)

<sup>8</sup> Test in mice with clove stem oil (Opdyke 1976a)

<sup>9</sup> Test substance was lemongrass oil, East Indian (Opdyke 1976a)

<sup>10</sup> Test substance was lemongrass oil, East Indian (Opdyke 1976b)

<sup>11</sup> HMT or human repeat insult patch test data, submitted by the Research Institute for Fragrance Materials

As mentioned previously, since comparative GP data are not available for any of the natural complex substances analyzed, an evaluation of these substances in the LLNA compared to GP performance could not be assessed. For the same reason, an evaluation of GP versus human outcomes is also not

possible. Also, no natural complex substances were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Annex I**), so these data and analyses cannot be compared to previously considered data.

## 5.2 Testing of Metal Compounds

The ICCVAM LLNA report (ICCVAM 1999) includes a summary on the ability of the LLNA to predict the skin-sensitizing potential of 11 metal compounds, representing 10 different metals (**Annex I**). In this addendum, the original ICCVAM analysis has been revised to include a total number of 16 metal compounds, representing 13 different metals, with corresponding human and/or GP data. The physicochemical properties of these metal compounds are in **Annex III-1**, and the data analyzed here are in **Annex III-2**. To reduce the complexity of the analysis, pesticide formulations and other products containing metals were not classified as metal compounds in this evaluation. Among these 16 metal compounds, 14 were tested in an aqueous vehicle, a nonaqueous vehicle, or both. The vehicle in which the two remaining metal compounds (i.e. cobalt chloride and cobalt sulfate) were tested in was not specified (**Annex III-2**). Similar to pesticide formulations and other products (**Section 5.1**), aqueous vehicles contained at least 20% water, while a nonaqueous vehicle contains no water.

All 16 metal compounds had comparative human data and eight had comparative GP data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, and three times as nickel chloride. The LLNA results for these studies with nickel-containing compounds are shown in **Table D-10**.

**Table D-10 Behavior of Nickel-containing Compounds in the LLNA**

Substance	LLNA Vehicle	LLNA Call	Max. SI (Conc. [%])	Max. Conc. Tested (%)	Mouse Strain	Reference
Nickel chloride	30% ETOH	+	6.6 (10)	10	CBA/J	Gerberick et al. (1992)
Nickel chloride	DMSO	-	2.2 (2.5)	2.5	CBA/Ca	Basketter et al. (1999d)
Nickel chloride	DMSO	-	2.4 (5)	5	CBA/Ca	Basketter and Scholes (1992)
Nickel sulfate	DMSO	+	3.1 (5)	5	CBA/J	Ryan et al. (2002)
Nickel sulfate	DMSO	-	1.5 (2.5)	2.5	CBA/Ca	Basketter and Scholes (1992)
Nickel sulfate	DMF	-	2.2 (5)	5	CBA/J	Ryan et al. (2002)
Nickel sulfate	Pluronic L92 (1%)	+	3 (2,5)	5	CBA/J	Ryan et al. (2002)

Nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in the other four. Two of the three positive results occurred in aqueous vehicles (30% ethanol and 1% Pluronic L92), one of the positive results occurred in a nonaqueous vehicle (DMSO), and all four of the negative results occurred in a nonaqueous vehicle (three in DMSO and one in DMF). Because of these discordant results, a decision was made to exclude nickel compounds from the LLNA metals performance analysis.

Of the 14 remaining metal compounds (13 metals) tested in the LLNA and with human data, nine are sensitizers and five are nonsensitizers in humans. For these 14 metal compounds, the LLNA has an accuracy of 86% (12/14), a sensitivity of 100% (9/9), a specificity of 60% (3/5), a false positive rate

of 40% (2/5), and a false negative rate of 0% (0/9), when compared to human results (**Table D-11**). For the six metal compounds (after excluding nickel compounds) with GP data (five sensitizers and one nonsensitizer in the GP), the LLNA has an accuracy of 83% (5/6), a sensitivity of 100% (5/5), a specificity of 0% (0/1), a false positive rate of 100% (1/1), and a false negative rate of 0% (0/5), when compared to GP test results (**Table D-11**) (**Annex III-2**).

Furthermore, all six of the 14 metal compounds with GP data have human data for comparison and there is a chemical-by-chemical match in classification between the GP and human outcomes (**Table D-11**). In contrast, the LLNA incorrectly identified the one human nonsensitizing metal compound as a sensitizer. For comparative purposes, the corresponding performance of the LLNA in predicting the human response for these same six metal compounds is also provided in **Table D-11**.

**Table D-11 Evaluation of the Performance of the LLNA for Testing Metal Compounds<sup>1</sup>**

Comparison	n <sup>2</sup>	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>
<i>All Metal Compounds (Aqueous and Nonaqueous Vehicles)</i>											
LLNA vs. GP <sup>4</sup>	6	83	5/6	100	5/5	0	0/1	100	1/1	0	0/5
LLNA vs. Human <sup>5</sup>	14	86	12/14	100	9/9	60	3/5	40	2/5	0	0/9
GP <sup>3</sup> vs. Human <sup>5</sup>	6	100	6/6	100	5/5	100	1/1	0	0/1	0	0/5
LLNA vs. Human <sup>5</sup> for the same GP metal compounds	6	83	5/6	100	5/5	0	0/1	100	1/1	0	0/5
<i>Metal Compounds Tested in Aqueous Vehicles<sup>6</sup></i>											
LLNA vs. GP <sup>4</sup>	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
LLNA vs. Human <sup>5</sup>	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
GP <sup>3</sup> vs. Human <sup>5</sup>	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
<i>Metal Compounds Tested in Nonaqueous Vehicles</i>											
LLNA vs. GP <sup>4</sup>	5	80	4/5	100	4/4	0	0/1	100	1/1	0	0/4
LLNA vs. Human <sup>5</sup>	12	92	11/12	100	7/7	80	4/5	20	1/5	0	0/7
GP <sup>3</sup> vs. Human <sup>5</sup>	5	100	5/5	100	4/4	100	1/1	0	0/1	0	0/4
<i>ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data<sup>7</sup></i>											
LLNA vs. GP <sup>4</sup>	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human <sup>5</sup>	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP <sup>3</sup> vs. Human <sup>5</sup>	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:

GP = Guinea pig skin sensitization outcomes; LLNA = local lymph node assay; No. = number.

*Accuracy* (concordance) = the proportion of correct outcomes (positive and negative) of a test method

*Sensitivity* = the proportion of all positive substances that are classified as positive

*Specificity* = the proportion of all negative substances that are classified as negative

*False negative rate* = the proportion of all positive substances that are falsely identified as negative

*continued*

*False positive rate* = the proportion of all negative substances that are falsely identified as positive

- <sup>1</sup> Because of discordant results obtained with nickel-containing compound in multiple studies, nickel-containing compounds were omitted from this analysis.
- <sup>2</sup> n = Number of substances included in this analysis
- <sup>3</sup> The data on which the percentage calculation is based
- <sup>4</sup> *GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
- <sup>5</sup> *Human* refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.
- <sup>6</sup> All the metal compounds tested in an aqueous vehicle were also tested in a nonaqueous vehicle.
- <sup>7</sup> For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; Annex I

Of the six metal compounds with GP data, the vehicle is known for five of the six compounds. Four of these metal compounds were tested only in a nonaqueous vehicle, while one was tested in both an aqueous and nonaqueous vehicle. Thus, when considering only the metal compound with GP data that was tested in an aqueous vehicle, it was a sensitizer in the LLNA and the LLNA correctly classified it compared to the GP data (**Table D-11**). All five of the metal compounds with comparative GP data tested in a nonaqueous vehicle are also classified as sensitizing in the LLNA. Compared to GP data, the LLNA correctly classifies four of the five nonaqueous metal compounds. The accuracy statistics based on this limited dataset are also presented in **Table D-11**.

Of the 14 metal compounds with human data, the vehicle is known for 12 of the 14 compounds. Eleven of these metal compounds were tested only in a nonaqueous vehicle, while one was tested in both an aqueous and nonaqueous vehicle. Thus, when considering only the metal compound with human data that was tested in an aqueous vehicle, the LLNA correctly classified it as a sensitizer compared to the human data (**Table D-11**). In contrast, of the 12 metal compounds with comparative human data tested in a nonaqueous vehicle, eight are classified as sensitizers and the remaining four are nonsensitizers in the LLNA. Compared to human data, the LLNA correctly classifies 11 of the 12 nonaqueous metal compounds. This results in an accuracy of 92% (11/12), a sensitivity of 100% (7/7), a specificity of 80% (4/5), a false positive rate of 20% (1/5) and a false negative rate of 0% (0/7) (**Table D-11**).

Potassium dichromate was the one metal compound with comparative GP and human data that was tested in both an aqueous and nonaqueous vehicle. Vehicle information was available for 20 of the 22 LLNA studies included in this analysis on potassium dichromate, indicating that it was tested six times in an aqueous vehicle (i.e., 1% Pluronic L92) and 14 times in a nonaqueous vehicle (DMF or DMSO). In all cases, it was found to be sensitizing by the LLNA regardless of the vehicle used.

For the purpose of this addendum, a case-by-case analysis was carried out to determine whether the overall LLNA classification for each metal compound is as a sensitizer or a nonsensitizer. In most cases, the majority result determined the overall LLNA skin sensitizing classification for each metal compound. In instances where there were an equal number of reports classifying the metal compound as sensitizing or nonsensitizing, the most severe classification was used. For instance, for zinc sulfate, LLNA data from two studies are considered in this evaluation report (ICCVAM 1999 [**Annex I**] and Basketter et al. 1999a). Zinc sulfate is classified as a sensitizer in ICCVAM 1999 (neither the vehicle nor the raw data were included) whereas Basketter et al. (1999a) classified zinc sulfate as a nonsensitizer when using DMSO as the vehicle (SI = 2.3 at 25%). For the purposes of this evaluation, to be conservative, zinc sulfate is classified as a sensitizer (**Annex III-2**).

Based on the data compiled for this evaluation, the LLNA classification for nine of the 11 metal compounds evaluated in the 1999 ICCVAM report remained the same in this evaluation because either no new data were available or classifications based on new data were consistent with the original classification (**Annex I**). For the remaining two metal compounds (nickel chloride and nickel sulfate), additional LLNA data were available, but as described above, discordant results with nickel compounds in eight different LLNA studies precluded a definitive classification and it was therefore excluded from this analysis.

### 5.3 Testing of Substances in Aqueous Solutions

The ICCVAM report (ICCVAM 1999) did not include an analysis of the ability of the LLNA to predict the skin sensitizing potential of substances tested in aqueous solutions, because data were not available for that evaluation (**Annex I**). The current database contains LLNA data for 139 substances tested in aqueous solutions, representing 171 LLNA studies; 91 (123 LLNA studies) of these substances are pesticide formulations and pure compounds and 48 of these substances (48 LLNA studies) are aqueous eluates of medical devices. As mentioned previously in **Section 5.1.1**, all pesticide formulations were tested in the LLNA in 1% Pluronic L92. Because of differences in the protocols for sample preparation between the 91 pesticide formulations and pure compounds and the 48 medical device eluates, these groups were analyzed separately.

In this addendum, the ICCVAM 1999 report has been revised to include a total of 25 unique substances tested in aqueous solutions from 47 LLNA studies with corresponding human and/or GP data. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water. The group of substances analyzed for this section of the addendum does not include metal compounds tested in aqueous vehicles, which have instead been included in the analyses discussed in **Section 5.2**.

#### 5.3.1 Pesticide Formulations and Pure Compounds Tested in Aqueous Solutions

Of the 91 pesticide formulations and pure compounds considered in this analysis, 63% (57/91) are LLNA positive and 37% (34/91) are LLNA negative. Where available, the physicochemical properties of these substances are in **Annex IV-1**, and the data analyzed here are in **Annex IV-2**. If there were multiple LLNA studies for a substance, a majority call was used, so there was one LLNA call for each substance. Eleven substances were tested in multiple LLNA studies (43 total studies); 9/11 of these substances had concordant LLNA results among all studies, and 2/11 substances had discordant results among two or more studies (**Table D-12**).

LLNA data for the two substances for which discordant LLNA study results occurred are shown in **Table D-13**. The discordance for 1,4 dihydroquinone is likely due to differing concentration ranges between the two LLNA studies (i.e., only one study tested up to at least 5%, where a positive result was first noted). For Oxyfluorfen EC, the range of EC3 values for the positive LLNA studies (> 20%) is associated with a weak response in the LLNA, where the greatest variability would be expected. Similarly, the SI values for the negative LLNA studies (2.3 and 2.8) are near the threshold for a positive response (i.e., SI=3), again where the greatest variability would be expected (**Table D-13**).

**Table D-12 Substances Tested in Aqueous Solutions in Multiple LLNA Studies**

Formulation	Reference	No. Studies	Mouse Strain	Vehicle	No. Positive Studies	No. Negative Studies	No. Labs
Atrazine SC	ECPA	2	CBA	L92	2	0	2
1,4 Dihydroquinone	Lea et al. (1999)	2	NA	ACE/saline (1:1)	1	1	2
2,4 Dinitrobenzene sulfonic acid	Ryan et al. (2002)	2	NA	L92	2	0	1
				H <sub>2</sub> O			
Dinocap EC	ECPA	5	CBA	L92	5	0	5
Formaldehyde	ECPA	7	NA	L92	7	0	6
Formulation 7	Dow AgroSciences	2	BALB/c	L92	2	0	1
Hexyl cinnamic aldehyde	ECPA	5	NA	L92	5	0	5
Methyl 2-nonyanoate	Ryan et al. (2000)	2	NA	80% EtOH	2	0	NA
Oxyfluorfen EC	ECPA	5	CBA	L92	3	2	2
Quinoxifen / cyproconazole	ECPA	6	CBA	L92	6	0	6
Trifluralin EC	ECPA	5	CBA	L92	5	0	6

Abbreviations:

ACE = acetone; EC = emulsion concentrate; ECPA= European Crop Protection Association; EtOH = ethanol (diluent not specified); L92 = 1% aqueous Pluronic L92; NA = not available; No. = number; SC = suspension concentrate.

**Table D-13 Substances Tested in Multiple LLNA Studies in Aqueous Solutions with Discordant Results**

Substance	Vehicle	Conc. (%)	SIs	Strain	EC3	Lab
1,4 Dihydroquinone	ACE/saline (1:1)	0.05, 0.1, 0.25, 0.5, 1.0	0.7, 1.0, 0.9, 1.9, 1.9	NA	NC	1
	ACE/saline (1:1)	0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10	1.4, 0.8, 1.2, 1.3, 1.9, 6.8, 10.9	NA	1.3	2
Oxyfluorfen EC	L92	1, 7, 33	0.81, 1.4, 4.9	CBA/Ca	30.8	1
	L92	1, 7, 33	0.9, 1.4, 2.8	CBA/J	NC	2
	L92	1, 7, 33	0.3, 0.9, 2.3	CBA/J	NC	3
	L92	1, 7, 33	1.1, 1.5, 3.1	CBA/JHsd	30.8	4
	L92	1, 7, 33	1.2, 1.2, 5.4	CBA/CaOlaHsd	18.1	5

Abbreviations:

ACE = acetone; Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of 3; L92 = 1% aqueous Pluronic L92; LLNA = local lymph node assay; NA = Not available; NC = not calculated since SI<3.0; SIs = stimulation indices.

GP data were available for 25 substances (4 sensitizers/21 nonsensitizers in the GP) tested in aqueous solutions. These substances represented a total of 44 LLNA studies. Based on these comparative data, the LLNA has an accuracy of 56% (14/25), a sensitivity of 75% (3/4), a specificity of 52% (11/21), a false positive rate of 48% (10/21), and a false negative rate of 25% (1/4) (Table D-14).

**Table D-14 Evaluation of the Performance of the LLNA for Testing Aqueous Solutions**

Comparison	n <sup>1</sup>	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No. <sup>2</sup>	%	No. <sup>2</sup>	%	No. <sup>2</sup>	%	No. <sup>2</sup>	%	No. <sup>2</sup>
<i>Pesticide Formulations and Pure Compounds Tested in Aqueous Solutions</i>											
LLNA (CBA & BALB/c) vs. GP <sup>3</sup>	25	56	14/25	75	3/4	52	11/21	48	10/21	25	1/4
LLNA (CBA only) vs. GP <sup>3</sup>	22	57	13/22	75	3/4	56	10/18	44	8/18	25	1/4
LLNA (CBA only) vs. Human <sup>4</sup>	4	50	2/4	33	1/3	100	1/1	0	0/1	67	2/3
GP <sup>3</sup> vs. Human <sup>4</sup>	2	100	2/2	100	1/1	100	1/1	0	0/1	0	0/1
<i>ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data<sup>5</sup></i>											
LLNA vs. GP <sup>3</sup>	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human <sup>4</sup>	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP <sup>3</sup> vs. Human <sup>4</sup>	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59
<i>ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data<sup>5</sup></i>											
LLNA vs. GP <sup>3</sup>	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human <sup>4</sup>	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP <sup>3</sup> vs. Human <sup>4</sup>	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:

GP = guinea pig skin sensitization outcomes; LLNA = local lymph node assay; No. = number.

*Accuracy* (concordance) = the proportion of correct outcomes (positive and negative) of a test method

*Sensitivity* = the proportion of all positive substances that are classified as positive

*Specificity* = the proportion of all negative substances that are classified as negative

*False negative rate* = the proportion of all positive substances that are falsely identified as negative

*False positive rate* = the proportion of all negative substances that are falsely identified as positive

<sup>1</sup> n = number of substances included in this analysis.

<sup>2</sup> The data on which the percentage calculation is based.

<sup>3</sup> GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

<sup>4</sup> Human refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

<sup>5</sup> For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; Annex I) showing the overall performance of the LLNA vs. GP and human, and GP vs. human is included here.

Eleven substances were discordant between the LLNA and the GP tests (**Table D-15**). Ten of the 11 discordant substances (all overpredicted by the LLNA) were pesticide formulations tested in aqueous 1% Pluronic L92. These were the same 10 formulations noted in **Section 5.1.1.1**, where a detailed discussion of the discordant results is also detailed. The other discordant substance was neomycin sulfate, which was tested in 25% EtOH. Among the 11 of 25 substances classified as sensitizers by the LLNA that were classified as nonsensitizers in the GP (**Table D-15**), 9/11 were based on BT results and 2/11 were based on GPMT results.

The one false negative substance based on LLNA results as compared to GP results, neomycin sulfate, was tested in the LLNA at a maximum concentration 12.5-fold lower than the induction concentration used in the guinea pig (**Table D-15**). However, it should also be noted that neomycin sulfate also gave a negative result in the LLNA when tested at 25% in DMSO, a nonaqueous vehicle (Basketter et al. 1994).

**Table D-15 Substances Tested in Aqueous Solution: Discordant Results Between the LLNA and GP**

Substance Name	LLNA Results					GP Results			Skin Irritant?
	Veh.	Conc. (%) <sup>1</sup>	SI <sup>2</sup>	EC3 (%)	Result <sup>3</sup>	Ind. Conc. (%)	Sens. Incid. (%)	Result <sup>3</sup>	
Atrazine SC	L92	100	7.3	36.4 <sup>4</sup>	+	30	0	- <sup>5</sup>	Nonirritant at ≤ 25% <sup>6</sup>
BASF SE-1	L92	70	22.7	5.5	+	100	0	- <sup>7</sup>	Nonirritant at ≤ 50% <sup>6</sup>
EXP 11120 A	L92	100	5.3	64.9	+	100	0	- <sup>7</sup>	Nonirritant at 100% <sup>6</sup>
F & Fo WG 50 + 25	L92	25	15.2	0.003	+	30	0	- <sup>7</sup>	Nonirritant at ≤ 10% <sup>6</sup>
FAR01060-00	L92	100	3.6	88.5	+	100	0	- <sup>7</sup>	Nonirritant at 100% <sup>6</sup>
Formulation 2 <sup>8</sup>	L92	80	15.8	15.7	+	NA	NA	- <sup>7</sup>	Nonirritant at 80% <sup>9</sup>
Formulation 7 <sup>8</sup>	L92	100	3.2	85	+	100	0	- <sup>7</sup>	Nonirritant at 80% <sup>9</sup>
Fx + Me EW 69	L92	50	8.6	25.2	+	100	0	- <sup>7</sup>	Nonirritant at 100% <sup>6</sup>
Neomycin sulfate	25% EtOH	2	0.9	NC	-	25	76	+	Nonirritant at ≤ 25% <sup>6</sup>
Oxyfluorfen EC	L92	33	5.4	30.8 <sup>7</sup>	+	10	26	- <sup>5</sup>	Nonirritant at ≤ 25% <sup>6</sup>
Trifluralin EC	L92	100	75.2	10.3 <sup>8</sup>	+	50	10	- <sup>7</sup>	Nonirritant at ≤ 25% <sup>6</sup>

Abbreviations:

Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of 3; EW = emulsion, oil in water; GP = guinea pig test; Ind. Conc. = induction concentration; L92 = 1% aqueous Pluronic L92; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI < 3.0; SC = suspension concentrate; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle; WG = water-dispersible granules.

<sup>1</sup> Maximum concentration tested in the LLNA

- <sup>2</sup> Maximum SI obtained in the LLNA
- <sup>3</sup> (-) = nonsensitizer, (+) = sensitizer
- <sup>4</sup> Mean value from 2 studies
- <sup>5</sup> Guinea pig maximization test (GPMT) result
- <sup>6</sup> Based on challenge concentration from a GPMT or Buehler test (BT)
- <sup>7</sup> BT result
- <sup>8</sup> LLNA conducted in BALB/c mice
- <sup>9</sup> Based on irritation prescreen in mice

Among the substances tested in aqueous solutions, human data were available for only four (3 sensitizers/1 nonsensitizer in humans). Of these four, two were correctly identified by the LLNA when compared to human data. The accuracy statistics for the LLNA for this limited database are presented in **Table D-14**.

Two substances, which had comparative human and GP data, were tested in aqueous solutions. Of these, one (neomycin sulfate) was correctly identified in the GP as a sensitizer, compared to human results (Magnusson and Kligman 1969) (**Table D-16**). Neomycin sulfate, when tested in aqueous solution (25% EtOH) in the LLNA (Gerberick et al. 1992) is false negative in the LLNA when compared to human results. As noted above, the maximum concentration of neomycin sulfate tested in the LLNA in aqueous solution (2%), is 12.5-fold less than the induction concentration (25%) used in both the GPMT and the HMT tests that gave positive results (Kligman 1966), but again, neomycin sulfate was also negative in the LLNA when tested at 25% in DMSO, a nonaqueous vehicle (Basketter et al. 1994). The other substance for which there was both GP and human data, propylene glycol, was false negative in both the LLNA and the GPMT. It was classified as a sensitizer for this study based on its inclusion in a human patch test allergen test kit (ICCVAM 1999), along with the fact that Guillot et al. (1983) note anecdotal evidence of sensitization reactions in humans. However, there is published HMT data for propylene glycol that indicates it is a nonsensitizer (Kligman 1966; Guillot et al. 1983) and a weak human irritant (Basketter et al. 1997). The maximum concentration of propylene glycol that has been tested in humans is 25% (Kligman 1966). Given these uncertainties, this false negative result could be considered equivocal.

**Table D-16 Substances with Human Data Tested in Aqueous Solution**

Substance Name	LLNA Results				GP Results				Human Results				Skin Irritant?	
	Veh.	Conc. (%) <sup>1</sup>	SI <sup>2</sup>	EC3 (%)	Result <sup>3</sup>	Test	Ind. Conc. (%)	Sens. Incid. (%)	Result <sub>3</sub>	Test	Ind. Conc. (%)	Sens. Incid. (%)		Result <sub>3</sub>
Butanol	H <sub>2</sub> O	20	1.6 <sup>4</sup>	NC	-	NA	NA	NA	NA	NA	NA	NA	-	NA
Methyl 2-nonynoate	80% EtOH	20	24.4	2.5	+	NA	NA	NA	NA	HRIPT	0.2	0	+	NA
Neomycin sulfate	25% EtOH	2	0.9	NC	-	GPMT	25	76	+	HMT	25	28	+	NA
Propylene glycol	H <sub>2</sub> O	100	1.6	NC	-	GPMT <sup>5</sup>	1	0	-	--	--	--	+ <sup>6</sup>	Non-irritant at 25% <sup>7</sup>

Abbreviations:

Conc. = concentration; EC3 = estimated concentration needed to produce a stimulation index of 3; EtOH = ethanol; GP = guinea pig; GPMT = guinea pig maximization test; HMT = human maximization test; HRIPT = human repeat insult patch test; Ind. = incidence; Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI < 3.0; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle.

<sup>1</sup> Maximum concentration tested in the LLNA

<sup>2</sup> Maximum SI obtained in the LLNA

<sup>3</sup> (-) = nonsensitizer, (+) = sensitizer

<sup>4</sup> Test concentration that produced this SI was 5%.

<sup>5</sup> Also tested in Buehler test. Ind. Conc. = 0.2, Sens. Ind. = 0%

<sup>6</sup> Positive call on the basis that propylene glycol is included as a human patch test allergen (ICCVAM 1999)

<sup>7</sup> Test in humans

### **5.3.2 Medical Device Eluates Tested in Aqueous Solutions**

Of the 48 medical device eluates considered in this analysis, 100% (48/48) are LLNA negative. The constituents of these eluates were not provided by the submitter, so physicochemical properties of any substances they contained are unknown. The submitted data are provided in **Annex IV-3**.

None of these eluates had associated GP data or human data. All of the LLNA studies were reportedly done according to the ICCVAM-recommended protocol (ICCVAM 1999). The LLNA data provided by the submitter were average dpm for each treatment group (n = 5 animals); the individual animal data were not submitted (although the study report indicates that individual animal data were collected). SI values were calculated by NICEATM based on the submitted average values (**Annex IV-3**).

The sample preparation for these samples was different from that for the pesticide formulations and pure substances discussed in **Section 5.3.1**. The test substances for the LLNA were eluates of medical devices prepared according to standard procedures (ASTM 2008, ISO 2002), rather than dilutions of specific substances. A concurrent positive control was included in each LLNA study. Another treatment group treated with an eluate sample spiked with a known sensitizer, 2,4-dinitrobenzenesulfonic acid, was also included in each LLNA study. The purpose of the spiked samples was reportedly to demonstrate that there was nothing present in the eluate that would attenuate a positive LLNA response.

These eluates were not analyzed to determine their constituents, or whether in fact any compound(s) were eluted from the medical device tested. Since the LLNA results were uniformly negative and no sample preparation control was included in the studies, the effectiveness of the sample preparation could not be determined, so the results from these eluates were not included with those from the pesticide formulations and pure substances discussed in **Section 5.3.1**.

## 6.0 LLNA Data Quality

Based on the available information, the published papers, and data submissions, information on compliance with GLP guidelines was available for data obtained from Dow AgroSciences, Dupont, Gerberick et al. (2005), H.W. Vohr (BGIA), E. Debruyne (Bayer CropScience SA), P. Botham (ECPA), Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, and D. Germolec (NIEHS).

A formal assessment of the quality of the remainder of the LLNA data considered here was not feasible. The published data on the LLNA were limited to tested concentrations and calculated SI and EC3 values. Auditing the reported values would require obtaining the original individual animal data for each LLNA experiment, which have been requested, but not yet obtained. However, many of the studies were conducted according to GLP guidelines, which implies that an independent quality assurance audit was conducted. The impact of any deviations from GLP guidelines cannot be evaluated for the data reviewed here, since no data quality audits were obtained.

As noted in **Section 5.0**, the original records were not obtained for all of the studies included in this evaluation. Data were available for several of the substances included in the ICCVAM (1999) evaluation and thus some of the raw data for these substances were available for review.

## 7.0 Other Scientific Reports and Reviews

A search of Medline, PubMed, and Toxline resulted in 46 published reports relevant to the applicability domain of the LLNA and the use of the LLNA for testing pesticide formulations and other products, metals and aqueous solutions for skin sensitizing potential. Of these reports, 26 have been published since the 1999 ICCVAM report on the LLNA. Included below are the reports most relevant to the evaluation included in this Addendum, with the most salient points summarized for each.

### 7.1 Maibach (1986)

The author evaluated the herbicide glyphosate, an active ingredient of a formulation considered in this Addendum (see **Annex II-3**), for acute and cumulative irritation, photoirritation, and allergic and photoallergic contact sensitization potential in 346 volunteers. The skin sensitization study used a modified Draize protocol in 204 adults with 0.2 mg of a commercial glyphosate formulation applied on patches. It was concluded that glyphosate is a nonsensitizer. A 10% concentration was suggested for a diagnostic patch test series.

### 7.2 Sharma and Kaur (1990)

The authors prepared a patch test series of 37 most prevalent pesticides used in the Chandigarh, India region, including insecticides, fungicides and herbicides. They tested 30 farmers with dermatoses and 20 controls. The only pesticide with active ingredients considered in this Addendum (see **Annex II-3**) that showed a positive patch test reaction was 1% 2,4-D (3/20, incidence = 15%). The only pesticide with active ingredients considered in this Addendum (see **Annex II-3**) that showed a negative patch test reaction was 1% atrazine.

### 7.3 Lisi (1992)

This is a review article that is primarily focused on pesticides sold and used in Italy at the time it was published. It covers both irritants and allergens and a broad array of pesticides (fungicides, herbicides, insecticides, soil fumigants, and contaminants in formulations). It contains a list of pesticides and active ingredients that caused positive reactions, with concentrations tested, for patch tests done by the International Contact Dermatitis Group and the Italian Group for the Study of Contact and Environmental Dermatitis. Pesticides with active ingredients considered in this Addendum (see **Annex II-3**) included in patch test series of 10% glyphosate and 1% dinocap.

### 7.4 Basketter et al. (1999a)

Basketter et al. (1999a) used the LLNA to evaluate the skin sensitization potential of 13 metal salts. For the purposes of their evaluation, eight of the 13 metals were considered to be human sensitizers. Their results show that the LLNA had an accuracy of 85% (11/13), sensitivity 88% (7/8), specificity of 80% (4/5), false negative rate of 12% (1/8), and false positive rate of 20% (1/5). Nickel chloride (tested up to 5% in DMSO) was false negative in the LLNA based on an  $SI \leq 2.4$ . Copper chloride (tested up to 5% in DMSO) was false positive in the LLNA based on an  $SI \geq 8.1$ . The authors concluded that these data support the potential utility of the LLNA for testing metal contact allergens.

### 7.5 Wright et al. (2001)

The authors investigate the influence of application vehicle on sensitizing potency, using the LLNA to examine the activity of four recognized human contact allergens: isoeugenol and cinnamic aldehyde and two fragrance chemicals; 3-dimethylaminopropylamine (a sensitizing impurity of cocamidopropyl betaine, a surfactant used in shower gel) and dibromodicyanobutane (the sensitizing component of Euxyl K 400, a preservative used in cosmetics). The four chemicals were applied in each of seven different vehicles (acetone: olive oil [4:1; AOO]; DMSO: methyl ethyl ketone; dimethylformamide; propylene glycol; and both 50:50 and 90:10 mixtures of ethanol and water). It was found that the vehicle in which a chemical is presented to the epidermis can have a marked effect

on sensitizing activity. EC<sub>3</sub> values ranged from 0.9 to 4.9% for isoeugenol, from 0.5 to 1.7% for cinnamic aldehyde, from 1.7 to > 10% for dimethylaminopropylamine and from 0.4 to 6.4% for dibromodicyanobutane. These authors confirm that the vehicle in which a chemical is encountered on the skin has an important influence on the relative skin sensitizing potency of chemicals and may have a significant impact on the acquisition of allergic contact dermatitis. The data also demonstrate the utility of the LLNA as a method for the prediction of these effects and thus for the development of more accurate risk assessments.

#### **7.6 Ikarashi et al. (2002)**

The authors examined the sensitization potential of gold sodium thiosulfate (GST) in the GP and the mouse. GST has been included in a standard human patch test series, and the incidence of patients showing positive reactions to gold is increasing (contact allergy rates to gold were reported to be in the range 1–23% from various countries). GST was tested in the GPMT and in several *in vivo* assays in the mouse, including the mouse ear swelling test (MEST) (Gad et al. 1986), an ex-vivo variant of the LLNA, the sensitive LLNA (Ikarashi et al. 1993), and the mouse IgE test (Hilton et al. 1995, Dearman et al. 1992). GST was identified as a sensitizer in the GPMT (GST intradermal induction concentration, 1%; sensitization index 60% [6/10]. However, only 2/6 mice showed a positive response (ear swelling  $\geq 20\%$ ) in the MEST, and GST did not induce an SI  $\geq 3$  in either variant of the LLNA. There was a significant difference in total serum IgE concentrations between vehicle- and GST-treated groups ( $p < 0.05$ ). The authors concluded that GST was a weak sensitizer.

#### **7.7 Griem et al. (2003)**

The authors propose a quantitative risk assessment methodology for skin sensitization aimed at deriving "safe" exposure levels for sensitizing substances. In their analysis they used cinnamic aldehyde and nickel as examples of how they apply their risk assessment proposal to sensitizing substances. In their discussion of nickel, they reference data supporting that nickel is an allergen with a relatively low sensitizing potency but a high prevalence in the general population (Kligman 1966; Vandenberg and Epstein 1963). Consequently, as in humans, nickel salts (i.e. nickel chloride and nickel sulfate) are weak sensitizers in animals and often give negative results in standardized tests (e.g., LLNA). Clinical experience in humans indicates that nickel allergy preferentially develops after nickel exposure on irritated or inflamed, but not on healthy skin (Kligman 1966; Vandenberg and Epstein 1963). Similarly, previously false negative results with nickel salts in the mouse LLNA could recently be overcome by the addition of a detergent (1% surfactant in water) to the nickel test solution (Ryan et al. 2002).

#### **7.8 Hostynek and Maibach (2003 and 2004)**

In these two review papers, the authors consider reports of immediate and delayed type immune reactions to cutaneous or systemic exposure to copper in humans. They mention that the electropositive copper ion is potentially immunogenic due to its ability to diffuse through biological membranes to form complexes in contact with tissue protein. Reports of immune reactions to copper include ACD, immunologic contact urticaria, systemic allergic reactions and contact stomatitis. They state that considering the widespread use of copper intrauterine devices (IUDs) and the importance of copper in coinage, items of personal adornment and industry, unambiguous reports of sensitization to the metal are extremely rare, and even fewer are the cases, which appear clinically relevant. Reports of immune reactions to copper mainly describe systemic exposure from IUDs and prosthetic materials in dentistry, implicitly excluding induction of the hypersensitivity from contact with the skin as a risk factor. Based on predictive GP testing and the LLNA, copper has a low sensitization potential. The authors then provide a diagnostic algorithm that might clarify the frequency of copper hypersensitivity.

### 7.9 Penagos et al. (2004)

The authors prepared a pesticide patch test series specific to the most prevalent pesticides used on banana plantations in Panama. They examined 366 plantation workers from four different plantations for dermatoses, and tested 37 workers with dermatoses that they judged most likely to be pesticide-related. Twenty-three control workers, without dermatoses, were also patch-tested. Twenty-four workers showed a positive reaction to one or more of the pesticides tested; these positive reactions included 15 ACD cases (20 positive reactions) in 37 workers diagnosed with dermatoses and three control workers who had allergic reactions to pesticides (4 positive reactions). Pesticides with active ingredients considered in this Addendum (see **Annex II-3**) that showed positive patch test reactions were 10% glyphosate (2/60, incidence = 3.3%), 0.02% oxyfluorfen (1/60, incidence = 1.6%), 1% chlorpyrifos (1/60, incidence = 1.6%), and 0.44% propiconazole (1/60, incidence = 1.6%).

### 7.10 Tinkle et al. (2004)

The authors investigated the skin sensitization potential of beryllium, the cause of chronic beryllium disease, an incurable occupational lung disease that begins as a cell-mediated immune response to beryllium. Since occupational respiratory beryllium exposures have been decreasing and the rate of beryllium sensitization has not declined, the authors hypothesized that skin exposure to beryllium particles might be an alternative route for sensitization. Optical scanning laser confocal microscopy and size-selected fluorospheres were used to demonstrate that ultrafine beryllium particles penetrate the stratum corneum of human skin, reaching the epidermis and, occasionally, the dermis. Skin sensitization in mice was suggested by peripheral blood and LN beryllium lymphocyte proliferation tests (BeLPT), and by changes in LN T-cell activation markers, increased expression of CD44, and decreased CD62L following topical application of beryllium. Topically applied beryllium also increased ear thickness in mice following challenge. The authors believe that these observations are consistent with development of a cell-mediated immune response following topical application of beryllium, and hypothesize a link between the persistent rate of occupational beryllium sensitization and skin exposure to ultrafine particles.

### 7.11 Lalko and Api (2006)

The authors tested seven essential oils (basil, citronella, clove leaf, geranium, litsea cubeba, lemongrass, and palmarosa oils) as well as three of the major components (citral, eugenol, and geraniol) in the LLNA. Each of these essential oils contains one or more known sensitizers. If the concentration of a major component that was a sensitizer was approximately 70% or more, the potency of an essential oil (as indicated by an EC<sub>3</sub> value adjusted for the concentration of the major component as measured by GC/MS or HPLC) showed less than a 2-fold difference from the EC<sub>3</sub> value calculated for that individual component. *Quenching*, a phenomenon that occurs when some component in a mixture inhibits the sensitization potential of a known sensitizer that is present in the mixture at a sensitizing concentration, was not observed for any of the essential oils tested in this study.

### 7.12 Shelnutt et al. (2007)

This is a review of the literature on the skin sensitization potential of hexavalent chromium. Hexavalent chromium is both a dermal irritant and a dermal sensitizer, causing ulceration of the skin and ACD. While the trivalent form of chromium is the naturally occurring valence, hexavalent chromium is one of the more prevalent sensitizers in the environment, present in detergents, cement, cosmetics, and foods. Research indicates that the hexavalent form exhibits greater skin-penetration properties than the trivalent form, although it is hypothesized that hexavalent chromium is transformed to trivalent chromium in the body and it is the trivalent form that induces sensitization. Repeated exposure to 4–25 ppm of hexavalent chromium can both cause sensitization and elicit ACD. Exposure to 20 ppm hexavalent chromium can cause skin ulcers in nonsensitized people. Chromium

ACD can be persistent and debilitating, perhaps because of the high prevalence and ubiquity of hexavalent chromium.

### 7.13 Chipinda et al. (2008)

Zinc diethyldithiocarbamate (ZDEC) and its disulfide, tetraethylthiuram disulfide (TETD) occur in rubber products, and are well-documented contact sensitizers in animals and humans. They are cross-reactive, as sensitization to one often confers sensitization to the other. This paper explored haptenation mechanisms of ZDEC by using high-performance liquid chromatography and mass spectrometry to identify ZDEC oxidation/reduction products and sites of protein binding. The LLNA was employed to test ZDEC and its oxidation products for sensitization potential and to examine possible mechanisms of hapten formation via elimination of oxidation and chelation mechanisms by substituting cobalt for zinc in ZDEC, to produce CoDEC. Oxidation of ZDEC produced TETD, tetraethylthiocarbamoyl disulfide, and tetraethyldicarbamoyl disulfide (TEDCD). The LLNA identified ZDEC, sodium diethyldithiocarbamate, TEDCD, and TETD as sensitizers, and CoDEC, as a nonsensitizer. While ZDEC bound to the copper-containing active site of superoxide dismutase, CoDec did not, suggesting chelation of metal-containing proteins as a possible mechanism of hapten formation.

### 7.14 Fukuyama et al. (2008)

The authors used the LLNA to test the sensitization potential of chromated copper arsenate (CCA), a commonly used wood preservative, and its components, for sensitization potential. LLNA studies were done using both AOO and DMSO as vehicles. CCA components tested included  $As_2O_5$ ,  $CrO_3$ , and  $CuO_2$ . Trimellitic anhydride in AOO was used as a positive control. All metal compounds were detected as sensitizers by the LLNA. EC3 values for metal compounds tested in AOO and DMSO were different (CCA: EC3 in AOO = 1.86%, EC3 in DMSO < 0.3%;  $As_2O_5$ : EC3 in AOO = 0.8%, EC3 in DMSO < 0.3%).  $CuO_2$  (EC3 = 1.69%) and  $CrO_3$  (EC3 < 0.3%) were tested in DMSO only. ATP was also measured in an aliquot of the lymph node suspension via a luciferin-luciferase assay and found to increase with increasing dose of the metal compounds.

### 7.15 Horiuchi et al. (2008)

This paper describes case reports tabulated by the Division of Dermatology, Sake Central Hospital, Saku, Japan from 1975 to 2000. Of pesticides with active ingredients considered in this Addendum (see **Annex II-3**), three cases in which trifluralin was implicated as the causative agent, and two cases in which glyphosate was implicated as the causative agent were documented. These causative agents were identified by either anecdotal evidence related to exposure or by patch testing.

### 7.16 Jowsey et al. (2008)

The authors conducted a retrospective examination of LLNA data in AOO for 18 substances that had been tested multiple times in AOO (2 - 15 studies per substance) to determine the inherent variability in the calculated EC3 values. The highest observed variability was for isoeugenol (31 studies) at 4.1-fold. A second retrospective analysis of data from the literature and previously unpublished studies for 18 substances that had been tested in the LLNA using at least two of 15 different vehicles was conducted. For 6/18 substances (ethylene glycol dimethacrylate, eugenol, geraniol, imidazolidinyl urea, hydroxycitronellal, and nickel sulfate), the variability was less than 5-fold. For 6/18 chemicals (3-dimethylaminopropylamine, cinnamic aldehyde, isoeugenol, p-tert-butyl-a-ethyl hydrocinnamal, methylchloroisothiazolinone/methylisothiazolinone, and potassium dichromate), the variability was greater than 5-fold but less than 10-fold. For 6/18 chemicals (dinitrobenzene sulfonate, 1,4-hydroquinone, 1,4-phenylenediamine, methyl dibromoglutaronitrile, formaldehyde, and glutaraldehyde), the observed range was greater than 10-fold. Further examination of the data for the substances in the highest-variability group suggested that the high variability might be due to an underestimation of potency in the LLNA associated with the use of predominantly aqueous vehicles

or propylene glycol. In contrast, use of AOO, DMF, methyl ethyl ketone, DMSO, and 9:1 ethanol:water resulted in less variable potency estimates for most substances.

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## 9.0 Glossary

**Absolute:** A natural complex substance prepared from plant material by chemical extraction.

**Accuracy<sup>12</sup>:** (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *concordance* (see also *two-by-two table*). Accuracy is highly dependent on the prevalence of positives in the population being examined.

**Allergic contact dermatitis (ACD):** A Type IV allergic reaction of the skin that results from repeated skin contact with a skin sensitizer. Clinical signs of ACD include the development of erythema (redness) and edema (swelling), blistering, and itching. Also referred to as skin sensitization.

**Assay<sup>12</sup>:** The experimental system used. Often used interchangeably with *test* and *test method*.

**Buehler test (BT):** An *in vivo* test method used to assess the skin sensitization potential of a substance. A sensitization phase uses topical application of the test substance using an occluded patch. The sensitization phase is followed by a challenge with the test substance, also with an occluded patch, to elicit an ACD reaction, which occurs if the animal has become sensitized (Buehler 1965).

**Coded substances:** Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

**Concordance<sup>12</sup>:** The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *accuracy* (see also *two-by-two table*). Concordance is highly dependent on the prevalence of positives in the population being examined.

**Dye:** A chemical compound that can impart color when applied to a substance. Various dyes are used as tissue stains, test reagents, therapeutic agents, and coloring agents.

**EC3:** The estimated concentration needed to produce a stimulation index of 3, as compared to the concurrent vehicle control.

**Essential oil:** A natural complex substance, in the form of a concentrated hydrophobic liquid, which contains volatile compounds. Prepared commercially from plants by distillation.

**False negative<sup>12</sup>:** A substance incorrectly identified as negative by a test method.

**False negative rate<sup>12</sup>:** The proportion of all positive substances falsely identified by a test method as negative (see *two-by-two table*). It is one indicator of test method accuracy.

**False positive<sup>12</sup>:** A substance incorrectly identified as positive by a test method.

**False positive rate<sup>12</sup>:** The proportion of all negative substances that are falsely identified by a test method as positive (see *two-by-two table*). It is one indicator of test method accuracy.

**Formulation:** A particular mixture of base chemicals and additives required for a product. Formulations typically contain one or more active ingredients and inert ingredients to facilitate mixing, application, penetration, etc.

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<sup>12</sup> Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003).

**Good Laboratory Practices (GLP)<sup>12</sup>:** Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organization for Economic Cooperation and Development and Japanese authorities, that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

**Guinea pig maximization test (GPMT):** An *in vivo* test method used to assess the skin sensitization potential of a substance. A sensitization phase combines intradermal induction using the test substance and Freund's complete adjuvant, followed by topical application using an occluded patch. The sensitization phase is followed by a challenge with the test substance, also with an occluded patch, to elicit an ACD reaction, which occurs if the animal has become sensitized (Magnusson and Kligman 1969).

**Hazard<sup>12</sup>:** The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

**Human maximization test (HMT):** An *in vivo* test method used to assess the skin sensitization potential of a substance. Skin is pretreated with sodium lauryl sulfate, an anionic surfactant, to cause irritation and facilitate dermal penetration of the test substance. A sensitization phase via topical application of the test substance using an occluded patch follows. The sensitization phase is followed by a challenge with the test substance, also with an occluded patch, to elicit an ACD reaction, which occurs if the person has become sensitized (Kligman 1966c).

**Human repeat insult patch test (HRIPT):** An *in vivo* test method used to assess the skin sensitization potential of a substance. A number of 24-hour or 48-hour exposures to test substances are delivered by occluded patch over a 3-week period to 100–200 volunteers. Two weeks later, a challenge exposure is made at the induction site and a unexposed site, again using a 24-/48-hour patch to elicit an ACD reaction, which occurs if the person has become sensitized (Stots 1980).

**Interlaboratory reproducibility<sup>12</sup>:** A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes and indicates the extent to which a test method can be transferred successfully among laboratories.

**Intralaboratory repeatability<sup>12</sup>:** The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

**Intralaboratory reproducibility<sup>12</sup>:** The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

**Immunological:** Relating to the immune system and immune responses.

***In vivo*:** In the living organism. Refers to assays performed in multicellular organisms.

**Lymphocyte:** A white blood cell found in the blood, lymph, and lymphoid tissues, which regulates and plays a role in acquired immunity.

**Murine local lymph node assay (LLNA):** An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure of the ear to the substance. The traditional LLNA measures lymphocyte proliferation by quantifying the amount of <sup>3</sup>H-thymidine or <sup>125</sup>I-iododeoxyuridine incorporated into the cells of the draining lymph nodes.

**Natural complex substance:** A substance that occurs in nature that is a mixture of several individual chemical constituents. Examples are essential oils and absolutes.

**Negative predictivity**<sup>12</sup>: The proportion of correct negative responses among substances testing negative in a test method (see *two-by-two table*). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

**Nonsensitizer:** A substance that does not cause skin sensitization following repeated skin contact.

**Performance**<sup>12</sup>: The accuracy and reliability characteristics of a test method (see *accuracy, reliability*).

**Positive control:** A substance known to induce a positive response, which is used to demonstrate the sensitivity of the test method and to allow for an assessment of variability in the conduct of the assay over time. For most test methods, the positive control substance is tested concurrently with the test substance and the vehicle/solvent control. However, for some *in vivo* test methods, periodic studies using a positive control substance are considered adequate by the OECD.

**Positive predictivity**<sup>12</sup>: The proportion of correct positive responses among substances testing positive by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

**Prevalence**<sup>12</sup>: The proportion of positives in the population of substances tested (see *two-by-two table*).

**Protocol**<sup>12</sup>: The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria, and procedures for the evaluation of the test data.

**Quality assurance**<sup>12</sup>: A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures is assessed independently by individuals other than those performing the testing.

**Reduction alternative**<sup>12</sup>: A new or modified test method that reduces the number of animals required.

**Reference test method**<sup>12</sup>: The accepted *in vivo* test method used for regulatory purposes to evaluate the potential of a test substance to be hazardous to the species of interest.

**Refinement alternative**<sup>12</sup>: A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhance animal wellbeing.

**Relevance**<sup>12</sup>: The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the *accuracy* or *concordance* of a test method.

**Reliability**<sup>12</sup>: A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reproducibility and intralaboratory repeatability.

**Replacement alternative**<sup>12</sup>: A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

**Reproducibility**<sup>12</sup>: The consistency of individual test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol and test substances (see *intra- and interlaboratory reproducibility*).

**rLLNA:** A variant of the LLNA that employs a single high dose of the test substance rather than multiple doses to determine its skin sensitization potential, thus using fewer animals.

**Sensitivity<sup>12</sup>:** The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy (see *two-by-two table*).

**Skin sensitizer:** A substance that induces an allergic response following skin contact (UN 2005).

**Specificity<sup>12</sup>:** The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy (see *two-by-two table*).

**Stimulation index (SI):** A value calculated for the LLNA to assess the skin sensitization potential of a test substance. The value is calculated as the ratio of radioactivity incorporated into the auricular lymph nodes of a group of treated mice to the radioactivity incorporated into the corresponding lymph nodes of a group of vehicle control mice. For the traditional LLNA and the rLLNA, an  $SI \geq 3.0$  classifies a substance as a skin sensitizer.

**Test<sup>12</sup>:** The experimental system used; used interchangeably with *test method* and *assay*.

**Test method<sup>12</sup>:** A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with *test* and *assay*. See also *validated test method* and *reference test*.

**Transferability<sup>12</sup>:** The ability of a test method or procedure to be accurately and reliably performed in different competent laboratories.

**Two-by-two table<sup>12</sup>:** The two-by-two table can be used for calculating accuracy (concordance) ( $(a+d)/(a+b+c+d)$ ), negative predictivity ( $d/(c+d)$ ), positive predictivity ( $a/(a+b)$ ), prevalence ( $(a+c)/(a+b+c+d)$ ), sensitivity ( $a/(a+c)$ ), specificity ( $d/(b+d)$ ), false positive rate ( $b/(b+d)$ ), and false negative rate ( $c/(a+c)$ ).

		New Test Outcome		
		Positive	Negative	Total
Reference Test Outcome	Positive	a	c	a + c
	Negative	b	d	b + d
	Total	a + b	c + d	a + b + c + d

**Validated test method<sup>12</sup>:** An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

**Validation<sup>12</sup>:** The process by which the reliability and relevance of a procedure are established for a specific purpose.

**Vehicle control:** An untreated sample containing all components of a test system, including the vehicle that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same vehicle.

**Weight-of-evidence (process):** In the weight-of-evidence process, the strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

## **Annex I**

### **The Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds (NIH Publication No. 99-4494)**

**This document is available electronically at:  
[http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/llnarep.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf)**

**This document is also available on request from NICEATM:**

**NICEATM  
National Institute of Environmental Health Sciences  
P.O. Box 1223, MD K2-16  
Research Triangle Park, NC 27709 USA  
Telephone: 919-541-2384 Fax: 919-541-0947  
E-mail: niceatm@niehs.nih.gov**

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## **Annex II**

### **Available Data and Information for Pesticide Formulations and Other Products Tested in the LLNA**

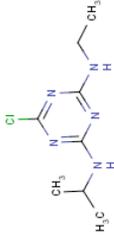
II-1	Physicochemical Properties and Chemical Classes of Pesticide Formulations Tested in the LLNA.....	D-71
II-2	Pesticide Formulations Tested in the LLNA – Comparative Data.....	D-101
II-3	Composition of Pesticide Formulations Tested in the LLNA .....	D-117
II-4	Physicochemical Properties and Chemical Classes of Dye Formulations Tested in the LLNA.....	D-137
II-5	Dye Formulations Tested in the LLNA – Comparative Data.....	D-141
II-6	Physicochemical Properties and Chemical Classes of Natural Complex Substances Tested in the LLNA.....	D-145
II-7	Natural complex substances Tested in the LLNA – Comparative Data.....	D-149

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## **Annex II-1**

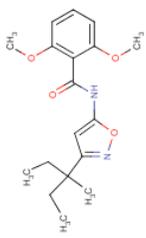
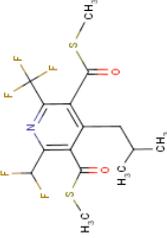
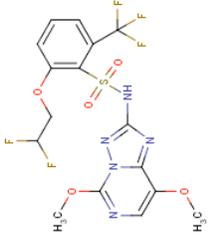
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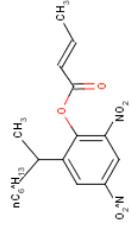
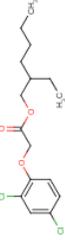
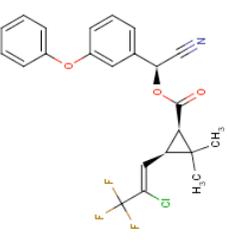
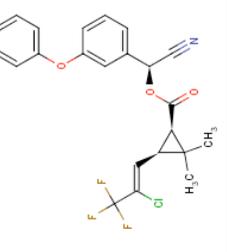
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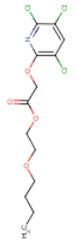
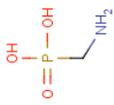
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
AE F016382 00 TK71 A101	NA	NA	NA	NA	NA	Formulation	NA
A SC600	NA	NA	NA	NA	NA	Formulation	NA
Atrazine	Atrazine SC 1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine	1912-24-9	215.68	2.82	Solid	Heterocyclic Compounds	
BASF #1	NA	NA	NA	NA	Emulsion	NA	NA
BASF #2	NA	NA	NA	NA	Emulsion	NA	NA
BASF #3	NA	NA	NA	NA	Liquid	NA	NA
BASF #4	NA	NA	NA	NA	Emulsion	NA	NA
BASF #5	NA	NA	NA	NA	Suspension	NA	NA
BASF #6	BAS 493 05 F	NA	NA	NA	Dispersion	NA	NA
BASF SC-1	NA	NA	NA	NA	Emulsion	NA	NA
BASF SE-1	NA	NA	NA	NA	Emulsion	NA	NA
D EC25	NA	NA	NA	NA	NA	Formulation	NA
D EW 15	NA	NA	NA	NA	NA	Formulation	NA

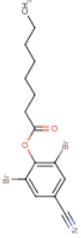
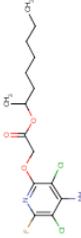
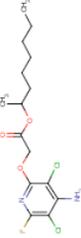
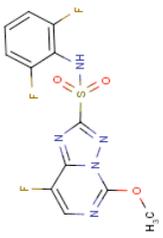
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Dinocap	Butenoic acid, 2-(or 4)-isooctyl-4,6(or 2,6)-dinitrophenyl ester (9CI); Crotonic acid, 2(or 4)-(1-methylheptyl)-4,6(or 2,6)-dinitrophenylester	39300-45-3	364.39	5.76	Liquid	Nitro Compounds; Hydrocarbons, Cyclic	
DU-10	NA	NA	NA	NA	NA	Formulation	NA
DU-11A	NA	NA	NA	NA	NA	Formulation	NA
DU-11B	NA	NA	NA	NA	NA	Formulation	NA
DU-11C	NA	NA	NA	NA	NA	Formulation	NA
DU-12	NA	NA	NA	NA	NA	Formulation	NA
DU-13A	NA	NA	NA	NA	NA	Formulation	NA
DU-13B	NA	NA	NA	NA	NA	Formulation	NA
DU-1A	NA	NA	NA	NA	NA	Formulation	NA
DU-1B	NA	NA	NA	NA	NA	Formulation	NA
DU-1C	NA	NA	NA	NA	NA	Formulation	NA
DU-1D	NA	NA	NA	NA	NA	Formulation	NA
DU-2A	NA	NA	NA	NA	NA	Formulation	NA
DU-2B	NA	NA	NA	NA	NA	Formulation	NA
DU-2C	NA	NA	NA	NA	NA	Formulation	NA
DU-2D	NA	NA	NA	NA	NA	Formulation	NA

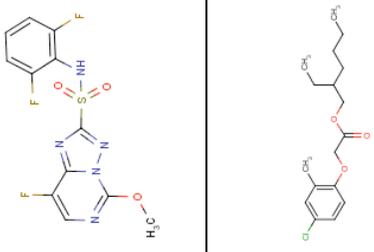
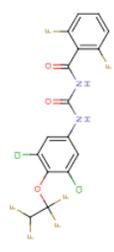
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
DU-2E	NA	NA	NA	NA	NA	Formulation	NA
DU-2F	NA	NA	NA	NA	NA	Formulation	NA
DU-3	NA	NA	NA	NA	NA	Formulation	NA
DU-4	NA	NA	NA	NA	NA	Formulation	NA
DU-5A	NA	NA	NA	NA	NA	Formulation	NA
DU-5B	NA	NA	NA	NA	NA	Formulation	NA
DU-5C	NA	NA	NA	NA	NA	Formulation	NA
DU-6	NA	NA	NA	NA	NA	Formulation	NA
DU-7	NA	NA	NA	NA	NA	Formulation	NA
DU-8A	NA	NA	NA	NA	NA	Formulation	NA
DU-8B	NA	NA	NA	NA	NA	Formulation	NA
DU-9A	NA	NA	NA	NA	NA	Formulation	NA
DU-9B	NA	NA	NA	NA	NA	Formulation	NA
EXP 10810 A	NA	NA	NA	NA	NA	Formulation	NA
EXP 11120 A	NA	NA	NA	NA	NA	Formulation	NA
FAR01042-00	NA	NA	NA	NA	NA	Formulation	NA
FAR01060-00	NA	NA	NA	NA	NA	Formulation	NA

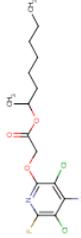
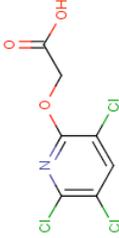
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 1	Isoxaben	82558-50-7	332.40	NA	Liquid	Formulation	
Formulation 10	22.9% w/w dithiopyr	97886-45-8	401.42	NA	Liquid	Formulation	
Formulation 11	0.31 wt % penoxsulam 84.2 wt % acetochlor	219714-96-2 34256-82-1	483.37 269.77	NA	Liquid	Formulation	

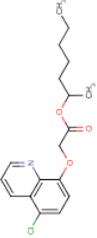
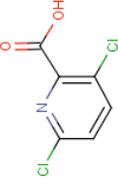
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 12	34.7% w/w 2,4-dinitro-6-(1-methylheptyl)phenyl crotonate DE-126	6119-92-2	364.40	NA	Liquid	Formulation	
Formulation 13	87.6% w/w 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester	1928-43-4	333.25	NA	Liquid	Formulation	
Formulation 14	1.5 wt. % gamma-cyhalothrin Nexide Fentrol	76703-62-3	449.85	NA	Liquid	Formulation	
Formulation 15	5.8 wt. % gamma-cyhalothrin Nexide Fentrol	76703-62-3	449.85	NA	Liquid	Formulation	

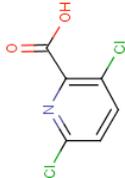
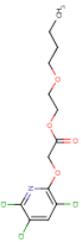
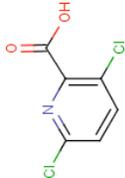
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>10</sub> K <sub>ow</sub> <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 16	85.3% w/w triclopyr butoxyethyl ester	64470-88-8	356.63	NA	Liquid	Formulation	
Formulation 17	50.8% wt/wt glyphosate dimethylammonium salt (active ingredient) 40.1% wt/wt glyphosate (acid equivalent) 8.3% w/w Geronol CF/AS 30 (ammonium adjuvant)	1066-51-9 1071-83-6	111.04 169.02	NA	Liquid	Formulation	

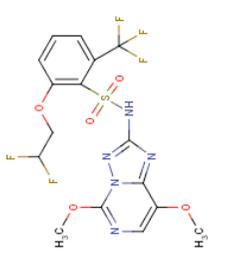
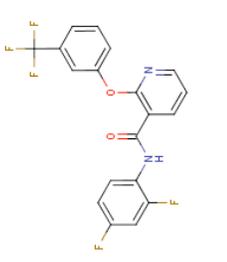
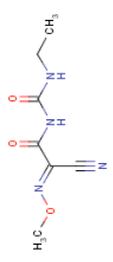
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 19	37.1 wt% bromoxynil octanoate 9.23 wt% fluroxypyr-1-methylheptyl	1689-99-2 81406-37-3	403.11 367.25	NA	Liquid	Formulation	
							
Formulation 2	14.2% w/w fluroxypyr -methyl 0.22% w/w florasulam	81406-37-3 145701-23-1	367.25 359.29	NA	Liquid	Formulation	
							

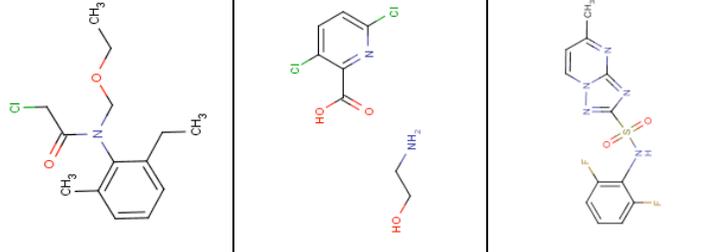
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>10</sub> K <sub>ow</sub> <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 20	0.39 wt% Florasulam 41.9 wt% 2-methyl-4-chlorophenoxyacetic acid 2-ethylhexyl ester (MCPA, 2-ethyl hexyl ester)	145701-23-1 29450-45-1	359.29 312.84	NA	Liquid	Formulation	 <p>The image shows two chemical structures. The top structure is Florasulam, a pyrazolopyrimidinone derivative with a sulfonamide group and a 2-fluorophenyl substituent. The bottom structure is MCPA 2-ethyl hexyl ester, an ester of 2-methyl-4-chlorophenoxyacetic acid and 2-ethylhexanol.</p>
Formulation 21	50.4% Hexaflumuron N-(((3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)phenyl)amino)carbonyl)-2,6-difluoro benzamide	86479-06-3	461.14	NA	Liquid	Formulation	 <p>The image shows the chemical structure of Hexaflumuron, a hexafluoroisobenzamide derivative with a 2,6-difluoro-4-((3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)phenyl)amino)phenyl substituent.</p>

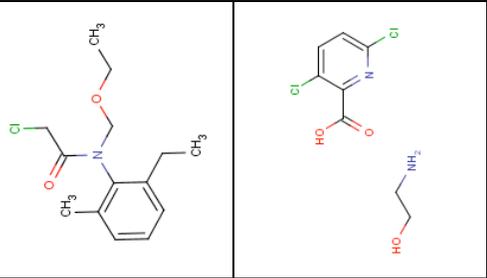
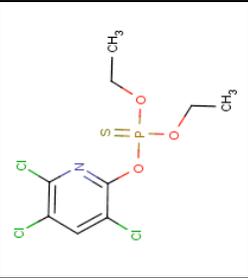
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 22	8.3 wt. % Triclopyr triethylammonium 2.8 wt. % fluoxy-pyr-methyl heptyl ester	57213-69-1 81406-37-3	357.66 367.25	NA	Liquid	Formulation	
							
Formulation 23	16.1 wt% Triclopyr - triethylammonium 11.6 wt% triclopyr acid	57213-69-1 55335-06-3	357.66	NA	Liquid	Formulation	
							

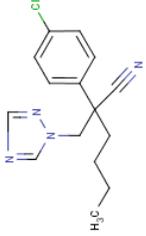
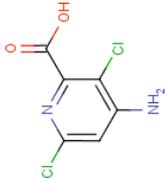
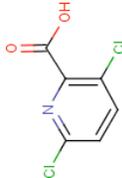
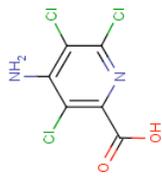
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log K <sub>ow</sub> <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 24	8.8 wt% Cloquintocet-mexyl	99607-70-2	335.83	NA	Liquid	Formulation	
Formulation 25	2.2 wt.% Clopyralid 37.7 wt.% MCPA-2-ethylhexyl ester 8.2 wt.% fluroxypyr -meptyl	1702-17-6 26544-20-7 81406-37-3	192.00 312.84/ 367.25	NA	Liquid	Formulation	

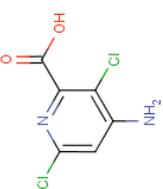
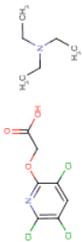
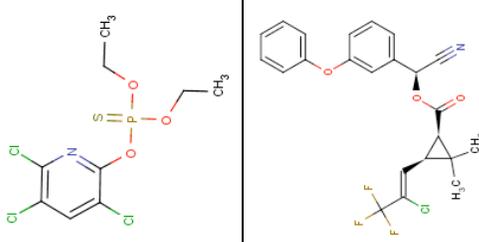
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>10</sub> K <sub>ow</sub> <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 26	5.9 wt. % Clopyralid 32.9 wt. % Triclopyr-butotyl	1702-17-6 64700-56-7	192.00 356.63	NA	Liquid	Formulation	
							
Formulation 27	45.2 wt. % Fluroxypyr-meptyl	81406-37-3	192.00	NA	Liquid	Formulation	

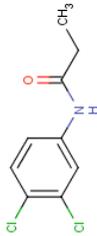
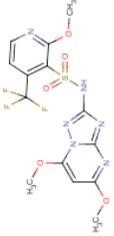
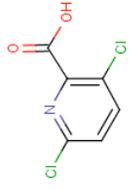
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>10</sub> K <sub>ow</sub> <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 28	1.4 wt. % Penoxsulam 9.37 wt. % Diflufenican	219714-96-2 83164-33-4	483.37 394.30	NA	Liquid	Formulation	
							
Formulation 29	35.6% Mancozeb 4.92% Cymoxanil	8018-01-7 57966-95-7	541.1 198.18	NA	Liquid	Formulation	
							

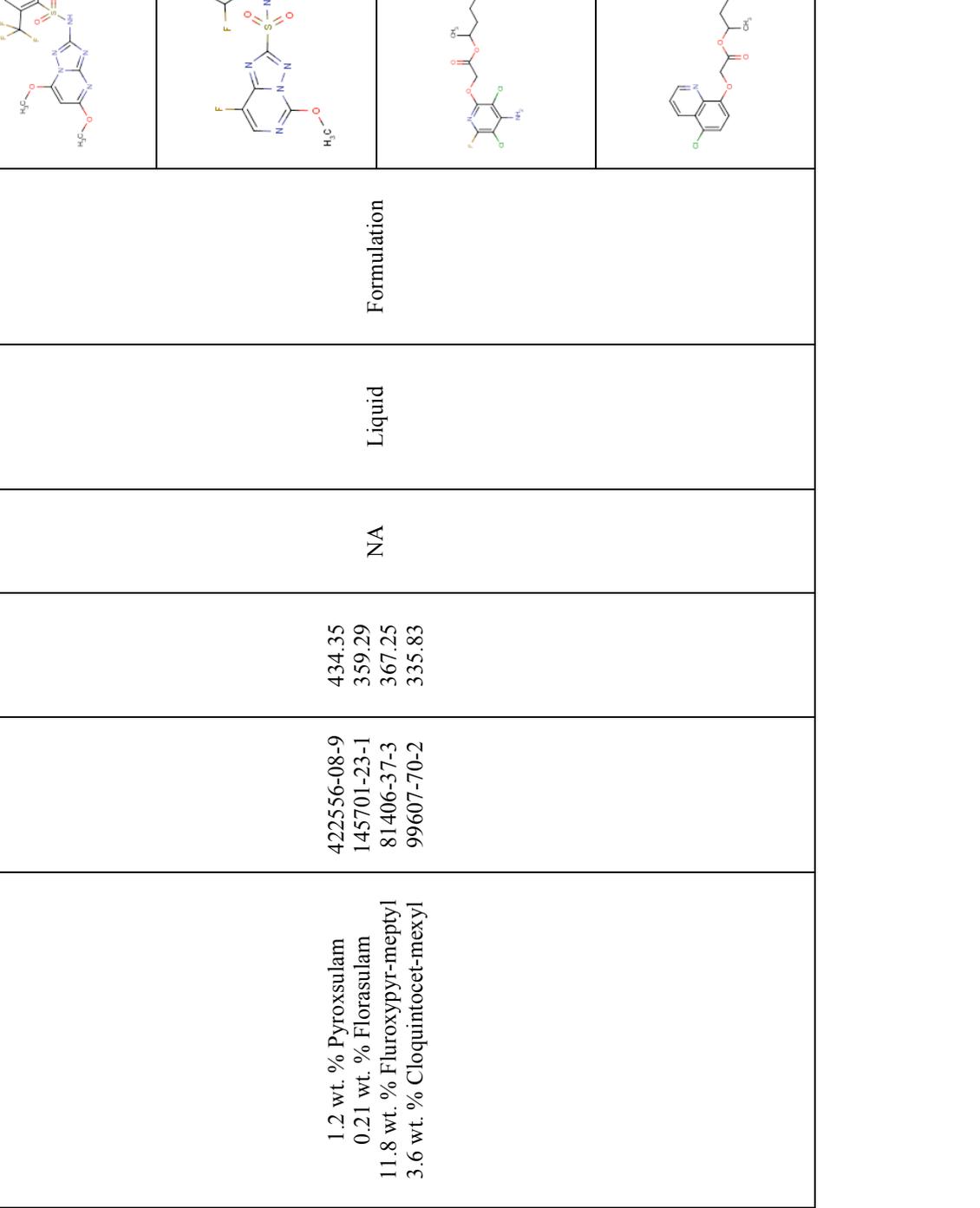
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 3	455 g/L Acetochlor 47 g/L Clopyralid-olamine 14 g/L Flumetsulam	34256-82-1 57754-85-5 98967-40-9	269.77 253.08 325.30	NA	Liquid	Formulation	 <p>The image displays three chemical structures. The top structure is Acetochlor, a benzamide derivative with a methyl group, a chloromethyl group, and a 2-ethoxyethyl group. The middle structure is Clopyralid, a pyridine ring with a chlorine atom and a carboxylic acid group. The bottom structure is Flumetsulam, a pyrimidopyrimidine derivative with a methyl group, a fluorine atom, and a bromine atom.</p>

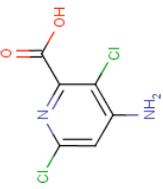
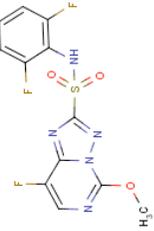
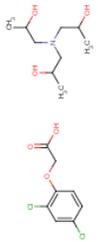
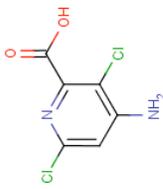
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 30	455 g/L Acetochlor 47 g/L Clopyralid-olamine 14 g/L Flumetsulam	34256-82-1 57754-85-5 98967-40-9	269.77 253.08 325.30	NA	Liquid	Formulation	 <p>The image shows three chemical structures. The top structure is Acetochlor, a benzamide derivative with a methyl group and a chloromethyl group. The middle structure is Clopyralid-olamine, a pyridine ring with a chlorine atom and a carboxylic acid group, and a separate ethanolamine molecule. The bottom structure is Flumetsulam, a pyrimidopyrimidine derivative with a methyl group and a sulfonamide group.</p>
Formulation 31	18.7 wt. % Chlorpyrifos	2921-88-2	350.59	NA	Liquid	Formulation	 <p>The image shows the chemical structure of Chlorpyrifos, which consists of a pyrimidopyrimidine ring system with two chlorine atoms and a phosphorus atom bonded to a sulfur atom and two ethoxy groups.</p>

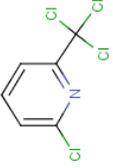
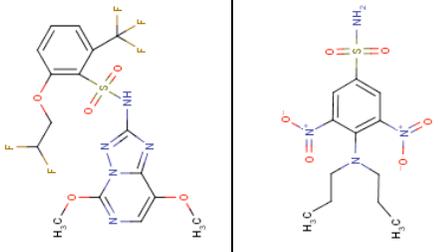
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 32	11.2 wt. % ((E)-2-(1-methylheptyl)-4,6-dinitrophenyl ester-2-butenic acid 4.68% wt/wt Myclobutanil	88671-89-0	288.78	NA	Liquid/ Solid	Formulation	
Formulation 33	4.5 wt. % Aminopyralid-olamine 27.1 wt. % Clopyralid-olamine 8.7 wt. % Picloram-olamine 3.5 wt. % Aminopyralid 20.6 wt. % Clopyralid 7.0 wt. % Picloram	150114-71-9 1702-17-6 1918-02-1	207.02 192.00 241.46	NA	Liquid	Formulation	  

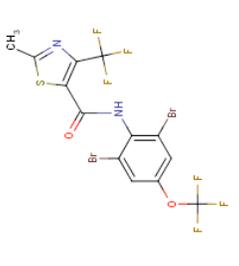
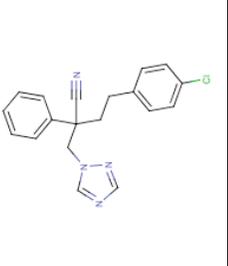
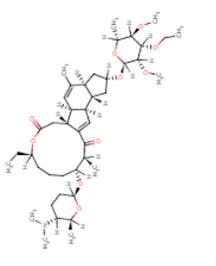
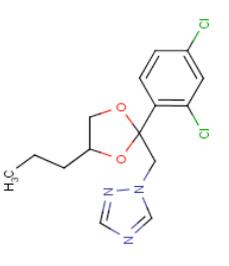
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 34	3.0 wt. % Aminopyralid	150114-71-9		NA	Liquid	Formulation	
Formulation 35	2.15 wt. % Aminopyralid-trisopropanolammonium 16.0 wt. % triclopyr-triethylammonium	566191-89-7 57213-69-1	NA 357.66	NA	Liquid	Formulation	
Formulation 37	30.6 wt. % Chlorpyrifos 0.54 wt. % Gamma-cyhalothrin	2921-88-2 76703-62-3	350.60 449.85	NA	Liquid	Formulation	

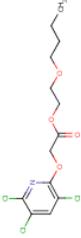
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 38	44.4 wt. % Propanil	709-98-8	218.08	NA	Liquid	Formulation	
Formulation 39	4.2 wt. % Pyroxsulam 8.7 wt. % Cloquintocet mexyl	422556-08-9 99607-70-2	434.35 335.83	NA	Liquid	Formulation	
Formulation 4	100 g/L Clopyralid mono-ethanolamine salt	1702-17-6	192.00	NA	Liquid	Formulation	

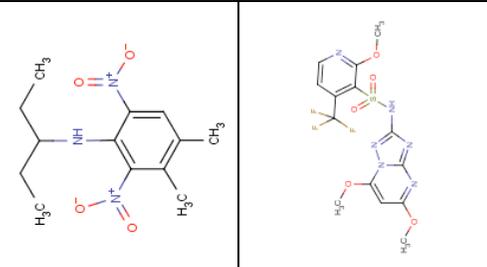
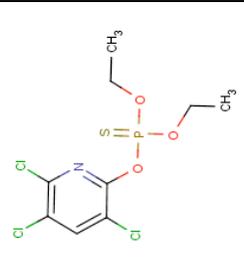
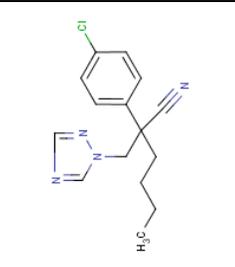
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 40	1.2 wt. % Pyroxsulam 0.21 wt. % Florasulam 11.8 wt. % Fluroxypyr-meptyl 3.6 wt. % Cloquintocet-mexyl	422556-08-9 145701-23-1 81406-37-3 99607-70-2	434.35 359.29 367.25 335.83	NA	Liquid	Formulation	

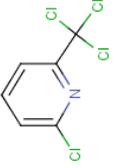
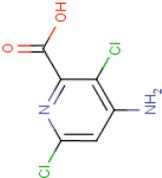
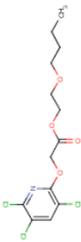
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>1</sub> Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 41	1.10 wt. % Aminopyralid potassium salt 0.47 wt. % Florasulam	150114-71-9 145701-23-1	207.02 359.29	NA	Liquid	Formulation	
							
Formulation 42	31 wt. % 2,4-D-triisopropanolamine 1.52 wt. % Aminopyralid triisopropanolammonium	18584-79-7 150114-71-9	412.31 207.2	NA	NA	Formulation	
							

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>10</sub> K <sub>ow</sub> <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 43	17.9 wt. % Nitrapyrin	1929-82-4	230.91	NA	NA	Formulation	
Formulation 44	0.12 wt. % Penoxsulam 40.38 wt. % Oryzalin	219714-96-2 19044-88-3	483.37 346.36	NA	NA	Formulation	

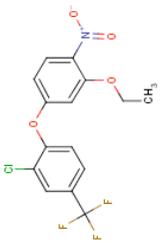
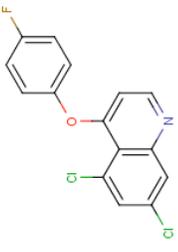
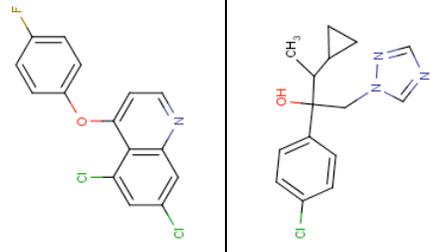
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 45	7.53 wt. % Thifluzamide	130000-40-7	528.06	NA	NA	Formulation	
	9.42 wt. % Fenbuconazole	114369-43-6	336.82				
Formulation 46	5.87 wt. % Spinetoram	187166-15-0	760.02	NA	NA	Formulation	
Formulation 47	14.56 wt. % Propiconazole	60207-90-1	342.22	NA	NA	Formulation	

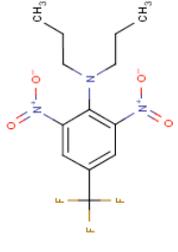
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>1</sub> Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 49	23.7 wt. % Triclopyr BEE	64700-56-7	356.63	NA	Liquid	Formulation	
Formulation 5	3,5,6-trichloro-2-pyridyloxyacetic acid, butoxy ethyl ester Triclopyr-butyl triclopyr BEE	64700-56-7	356.63	NA	Liquid	Formulation	
Formulation 50	Glyphosate dimethylamine salt Glyphosate dimethylammonium salt	34494-04-7 NA	NA	NA	Liquid	Formulation	NA

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 51	29.6 wt. % Pendimethalin 0.51 wt. % Pyroxsulam	40487-42-1 422556-08-9	281.31 434.35	NA	Liquid	Formulation	
Formulation 53	41.1 wt. % Chlorpyrifos	2921-88-2	350.60	NA	Liquid	Formulation	
Formulation 54	49.9 wt. % Glyphosate dimethylammonium salt	NA	NA	NA	Liquid	Formulation	NA
Formulation 55	4.6 wt. % Myclobutanil	88671-89-0	288.78	NA	Liquid	Formulation	

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>1</sub> Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 56	20.5 wt. % Nitrapyrin	1929-82-4	230.91	NA	Liquid	Formulation	
Formulation 6	Aminopyralid potassium + Triclopyr-butotyl form Aminopyralid herbicide	150114-71-9 64700-56-7	207.02	NA	Liquid	Formulation	
							

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log <sub>10</sub> Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 7	45 g/L Myclobutanil + 45 g/L quinoxifen	88671-89-0 124495-18-7	288.78 308.14	NA	Liquid	Formulation	
							
Formulation 8	81.8% w/w 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester 2,4-D EHE	1928-43-4	333.25	NA	Liquid	Formulation	
Formulation 9	NA	NA	NA	NA	Liquid	Formulation	NA
F & Fo WG 50 + 25	NA	NA	NA	NA	NA	Formulation	NA
Fx + Me EW 69	NA	NA	NA	NA	NA	Formulation	NA

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Oxyfluorfen	Oxirane, mono; ((C12-14-alkyloxy) methyl) derivatives	42874-03-3	361.70	5.21	Solid	Ethers	
Quinoxifen	5,7-dichloro-4-(4-fluorophenoxy)quinoline	124495-18-7	308.14	5.69	Liquid	Heterocyclic Compounds	
Quinoxifen / Cyproconazole	5,7-dichloro-4-(4-fluorophenoxy)quinoline/ H-1,2,4-triazole-1-ethanol, alpha-(4-chlorophenyl)-alpha-(1-cyclopropylethyl)-	124495-18-7 113096-99-4	308.14 291.78	5.69 3.25	Liquid	Heterocyclic Compounds	

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Trifluralin	2,6-dinitro-4-trifluoromethyl-N,N-dipropylanilin	1582-09-8	335.28	5.31	NA	Hydrocarbons, Cyclic; Amine	

Abbreviations: CASRN = Chemical Abstract Services Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

<sup>1</sup> Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: [http://www.syrres.com/esc/est\\_kowdemo.htm](http://www.syrres.com/esc/est_kowdemo.htm).

<sup>2</sup> Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: <http://www.nlm.nih.gov/mesh/meshhome.html>.

<sup>3</sup> Chemical structures of active ingredients, based on CASRN, were obtained from ChemID available at: <http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp>.

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## **Annex II-2**

### **Pesticide Formulations Tested in the LLNA – Comparative Data**

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Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference
										GP (F)	GP (any) <sup>3</sup>	GP (AD) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RE) <sup>5</sup>	
A SC600	NA	10, 25, 50, 100	1.4, 1.8, 2.3, 1.6	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	NA	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
AE F016382.00 TK71 A101	NA	3.6, 7.1, 17.9, 35.7	1.0, 0.8, 1.0, 1.1	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	NA	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
Atrazine	SC	12.5, 25, 50, 75, 100	1.8, 2.8, 3.6, 7.1, 7.3	31.3	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical								
		7, 33, 100	0.8, 2.9, 3.7	41.4	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical	+	-	NA	NA	NA	NA	+	NA
BASF #1	NA	10, 30, 70	2.0, 2.9, 4.9	31.2	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF #2	NA	3, 10, 30	0.8, 1.0, 3.0	29.7	1% L92	CBA/J	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF #3	NA	3, 10, 30	6.9, 14.6, 16.1	1.6	ACE	CBA/J	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF #4	NA	3, 10, 50	2.4, 2.7, 5.4	14.1	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF #5	NA	3, 10, 50	1.6, 1.2, 3.9	36.9	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference		
										GP (F)	GP (any) <sup>3</sup>	GP (AD) <sup>4</sup>	BT (AD) <sup>4</sup>	GPMT (AD) <sup>4</sup>	GP (RC/RE) <sup>5</sup>			
BASF #6	NA	3, 10, 30	2.7, 9.9, 23.1	0.3	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA	NA	
BASF SC-1	SC	3, 10, 30	0.8, 1.3, 1.9	NC	1% L92	CBA/Ca	-	BASF, submitted by C. Hastings	-	-	NA	NA	NA	NA	NA	NA	NA	
BASF SE-1	SE	10, 30, 70	8.0, 17.3, 22.7	5.5	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	-	NA	NA	NA	NA	NA	NA	NA	
DEC25®	EC	0.5, 1.0, 2.5	0.6, 0.6, 0.6	NC	1% L92	CBA/Ca	-	Bayer Crop Science, submitted by E. Debruyne	-	-	NA	NA	NA	NA	NA	NA	NA	
DEW 15	EW	2.5, 5.0, 10.0, 25.0	1.9, 1.5, 2.5, 2.5	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	NA	NA	NA	NA	NA	NA	NA	
Dinocap	EC	0.8, 4, 21	2.2, 25.8, 14.4	0.9	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+									
		0.8, 4, 20	1.3, 11.5, 15.6	1.3	1% L92	CBA/J	+											
		0.8, 4, 21	2.0, 4.0, 26.7	1.1	1% L92	CBA/J	+											
		0.8, 4, 10	1.3, 4.1, 10.9	2.8	1% L92	CBA/JHsd	+											
		0.8, 4, 10	2.7, 22.9, 40.5	0.8	1% L92	CBA/Ca OIaHsd	+											
DU-10	NA	0.5, 1, 2.5, 5	1.0, 1.3, 1.5, 1.6	NC	PG	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
DU-11A	NA	5, 25, 50, 100	3.2, 1.6, 0.7, 0.5	NC	AOO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	NA

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference	
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>		
DU-11B	NA	5, 25, 50, 100	1.4, 0.7, 0.7, 1.0	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-11C	NA	5, 25, 50, 100	1.5, 1.1, 0.9, 1.5	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-12	NA	1, 5, 25, 50	0.8, 1.2, 0.8, 1.4	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-13A	NA	5, 25, 50, 100	0.5, 0.4, 0.5, 0.6	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-13B	NA	1, 10, 50, 100	1.2, 1.0, 0.7, 0.6	NC	AOO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-1A	NA	5, 25, 50, 100	0.6, 1.2, 0.7, 1.0	NC	PG	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-1B	NA	1, 5, 10, 25	0.6, 1.1, 1.3, 1.1	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-1C	NA	5, 25, 50, 100	0.7, 1.4, 1.7, 1.3	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-1D	NA	5, 10, 25, 50	0.7, 1.0, 1.3, 1.0	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA
DU-2A	NA	5, 25, 50, 100	4.1, 5.4, 6.7, 6.5	1.2	AOO	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA	NA
DU-2B	NA	5, 25, 50, 100	2.1, 4.5, 7.3, 9.3	12.4	DMF	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA	NA
DU-2C	NA	10, 50, 100	2.1, 2.7, 3.7	62.9	DMF	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA	NA
DU-2D	NA	5, 25, 50, 100	4.5, 8.1, 14.8, 14.5	2.5	DMF	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA	NA
DU-2E	NA	5, 25, 50, 100	1.0, 0.8, 1.1, 1.4	NC	PG	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference		
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>			
DU-2F	NA	5, 25, 50, 100	2.0, 3.8, 7.5, 5.8	15.6	DMF	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA	NA	
DU-3	NA	5, 10, 25, 50	0.6, 0.8, 0.8, 0.6	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-4	NA	5, 25, 50, 100	0.9, 1.0, 1.0, 0.9	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-5A	NA	5, 25, 50, 100	2.7, 1.5, 1.6, 0.9	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-5B	NA	5, 25, 50, 100	0.8, 1.1, 1.0, 1.1	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-5C	NA	1, 5, 25, 100	1.4, 2.0, 1.2, 0.9	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-6	NA	5, 25, 50, 80	1.1, 0.8, 0.9, 0.9	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-7	NA	5, 25, 50, 80	1.9, 1.2, 1.1, 1.3	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-8A	NA	1, 10, 50, 100	1.4, 1.4, 0.8, 1.0	NC	AOO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-8B	NA	5, 25, 50, 100	1.2, 1.9, 1.4, 1.8	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
DU-9A	NA	5, 25, 50, 100	3.6, 5.0, 8.8, 13.5	2.7	AOO	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA	NA	
DU-9B	NA	5, 25, 50, 100	0.8, 0.8, 0.6, 0.5	NC	AOO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA	NA	
EXP 10810 A	NA	10, 25, 50	6.4, 8.4, 9.2	2.1	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	+	+	NA	NA	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>	
EXP 11120 A	NA	10, 25, 50, 100	1.0, 0.7, 1.6, 6.3	64.9	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	NA	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
F & Fo WG 50 + 25	WG	2.5, 5.0, 10.0, 25.0	11.7, 12.6, 14.4, 15.2	0.003	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	NA	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
FAR01042-00	NA	10, 25, 50, 100	1.4, 2.1, 1.4, 2.5	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	NA	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
FAR01060-00	NA	10, 25, 50, 100	0.4, 0.8, 1.0, 3.6	88.5	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	NA	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
Formulation 1	SC	5, 20, 80	1.1, 1.3, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	-	+	+	NA	Submitted by Dow AgroSciences
Formulation 10	EW	2, 10, 50	1, 1, 5.2	29.0	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	-	-	-	NA	NA	Submitted by Dow AgroSciences
Formulation 11	OD	0.4, 2, 10	1.2, 1.2, 3.2	9.2	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	+	NA	Submitted by Dow AgroSciences
Formulation 12	EC	0.2, 1, 5	1.2, 3, 11.6	1.00	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	NA	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 13	EC	1, 5, 25	1.2, 1.3, 10.4	8.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	+	+	Submitted by Dow AgroSciences

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference		
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>			
Formulation 14	CS	0.1, 1, 10	0.7, 0.7, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	+	NA	+	NA	+	NA	Submitted by Dow AgroSciences
Formulation 15	CS	0.2, 1, 5	0.8, 1.4, 3.2	4.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	+	NA	+	NA	Submitted by Dow AgroSciences
Formulation 16	EC	1, 5, 25	1.3, 2.2, 12.3	6.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	+	NA	+	NA	Submitted by Dow AgroSciences
Formulation 17	SL	5, 25, 75	1.7, 9.3, 18.5	8.4	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	NA	NA	+	NA	-	-	Submitted by Dow AgroSciences
Formulation 19	EC	1, 10, 25, 50	4.9, 7.9, 20, 50.5	0.23	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	+	NA	-	-	Submitted by Dow AgroSciences
Formulation 2	SE	5, 20, 80	2, 3.4, 15.8	15.7	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	-	+	-	-	-	-	-	NA	Submitted by Dow AgroSciences
Formulation 20	SE	2, 10, 50	1.1, 1.4, 3.3	43.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	+	NA	+	NA	Submitted by Dow AgroSciences
Formulation 21	TK	5, 25, 100	1.3, 1.2, 1.9	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	-	-	-	NA	-	NA	Submitted by Dow AgroSciences
Formulation 22	ME	5, 25, 100	1.2, 1.4, 5.8	52.3	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	-	-	NA	+	NA	Submitted by Dow AgroSciences
Formulation 23	SL	5, 25, 100	0.8, 1, 1	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	+	+	+	NA	+	NA	Submitted by Dow AgroSciences

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>	
Formulation 24	OD	2, 10, 50	1, 4, 4, 1, 11.7	6.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	NA	NA	NA	+	Submitted by Dow AgroSciences
Formulation 25	EC	1, 5, 25	1, 8, 2, 6, 14.7	5.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	-	+	NA	+	Submitted by Dow AgroSciences
Formulation 26	EC	1, 5, 25	1, 1, 4	18	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 27	EC	1, 5, 25	2, 3, 2, 5, 11.2	6.1	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	-	-	-	NA	-	Submitted by Dow AgroSciences
Formulation 28	SC	5, 25, 100	1, 1, 1, 1	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	-	NA	NA	+	Submitted by Dow AgroSciences
Formulation 29	SC	5, 25, 100	1, 8, 1, 6, 1.5	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	NA	+	+	Submitted by Dow AgroSciences
Formulation 3	SC	5, 20, 80	1, 1, 2, 1, 7	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	-	+	-	-	NA	-	Submitted by Dow AgroSciences
Formulation 30	EW	5, 25, 100	1, 8, 7, 2, 13.6	9.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	+	Submitted by Dow AgroSciences
Formulation 31	CS	5, 25, 100	1, 1, 9, 1, 8	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	+	NA	+	Submitted by Dow AgroSciences
Formulation 32	EC	5, 25, 100	6, 5, 4, 4, 7, 69.3	4.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	NA	NA	+	+	Submitted by Dow AgroSciences

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>	
Formulation 33	SL	5, 25, 100	0.7, 1.4, 1.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	NA	NA	NA	+	Submitted by Dow AgroSciences
Formulation 34	SL	5, 25, 100	1.9, 1.4, 1.5	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	-	NA	-	-	-	Submitted by Dow AgroSciences
Formulation 35	SL	5, 25, 100	1.1, 1.2, 1.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 37	EC	1, 5, 15	1.4, 2.7, 7.5	5.6	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 38	EC	5, 25, 100	1.1, 4.6, 12.7	15.9	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	-	-	-	NA	NA	Submitted by Dow AgroSciences
Formulation 39	OD	1, 5, 25	1.7, 2.5, 3.3	17.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 4	SL	5, 20, 80	1.4, 1.1, 1.2	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	-	-	-	NA	+	Submitted by Dow AgroSciences
Formulation 40	OD	1, 5, 25	1.8, 2.8, 5.7	6.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 41	SE	5, 25, 100	1.9, 1.9, 4.7	54.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 42	SL	10, 50, 100	1.2, 2.0, 3.1	95.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	-	-	NA	NA	NA	Submitted by Dow AgroSciences

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference	
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>		
Formulation 43	CS	5, 25, 75	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	+	NA	+	NA	Submitted by Dow AgroSciences
Formulation 44	SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	-	NA	NA	+	NA	Submitted by Dow AgroSciences
Formulation 45	SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	-	NA	NA	+	NA	Submitted by Dow AgroSciences
Formulation 46	SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	NA	NA	+	NA	Submitted by Dow AgroSciences
Formulation 47	EW	5, 25, 100	2.1, 2.1, 6.0	42.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	NA	Submitted by Dow AgroSciences
Formulation 49	AL	5, 25, 100	0.7, 1.4, 4.7	61.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	NA	Submitted by Dow AgroSciences
Formulation 5	EC	3, 10, 30	1.4, 4, 11.5	7.3	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	NA	Submitted by Dow AgroSciences
Formulation 50	SL	5, 25, 100	1.2, 1.2, 14.7	35	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	-	-	NA	NA	-	NA	Submitted by Dow AgroSciences
Formulation 51	OD	5, 25, 100	1.6, 4.5, 2.9	14.7	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	+	+	+	Submitted by Dow AgroSciences
Formulation 53	EW	2.5, 7.5, 15	1.5, 3.2, 6.7	6.9	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	-	+	NA	Submitted by Dow AgroSciences

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>	
Formulation 54	SL	5, 25, 100	1.3, 1.2, 2.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	-	NA	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 55	EW	5, 25, 100	1.5, 2.5, 3.7	56.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	-	NA	NA	Submitted by Dow AgroSciences
Formulation 56	SL	5, 25, 100	3.3, 6.1, 3.9	4.2	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 6	EW	5, 20, 80	1.3, 2.7, 11.6	23.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 7	SC	20, 80, 100	1, 1.9, 3.2	96.9	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	-	NA	+	Submitted by Dow AgroSciences
		5, 20, 80	2.6, 1.4, 3.2	73.3	1% L92	BALB/c	+									
Formulation 8	EC	1, 5, 25	0.9, 1.1, 7.3	11.1	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 9	SC	4, 20, 80	1.1, 1.7, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	NA	NA	NA	NA	Submitted by Dow AgroSciences
Fx + Me EW 69	EW	5, 10, 25, 50, 100	0.8, 1.6, 3.0, 8.6	25.2	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	-	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference													
										GP (F)	GP (any) <sup>3</sup>	GP (AI) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RF) <sup>5</sup>														
Oxyfluorfen	EC	1, 7, 33	0.81, 1.4, 4.9	18.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+																				
								ECPA LLNA Project Report submitted by Bayer											-										
								ECPA LLNA Project Report submitted by Dow Chemical																					
		1, 7, 33	1.1, 1.5, 3.1	30.8	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Dupont																					
								ECPA LLNA Project Report submitted by Syngenta/RCC																					
								ECPA LLNA Project Report submitted by Dow Chemical																					
Quinoxifen	SC	7, 33, 100	1.1, 0.7, 0.8	NC	1% L92	CBA/J	-	ECPA LLNA Project Report submitted by Dow Chemical	-																				
								ECPA LLNA Project Report submitted by Dow Chemical																					

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result	LLNA Reference	Overall LLNA Result (Majority)	Overall Call						GP Reference				
										GP (F)	GP (any) <sup>3</sup>	GP (AD) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RE) <sup>5</sup>					
Quinoxifen / cyproconazole	NA	7, 33, 100	2.1, 10.7, 20.3	9.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF										ECPA LLNA Project Report submitted by Dow Chemical		
		7, 33, 100	1.2, 7.2, 12.4	14.8	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Bayer												
		7, 33, 100	0.4, 3.8, 2.0	26.9	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical												
		7, 33, 100	1.4, 2.0, 6.2	49.8	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Dow Chemical												
		7, 33, 100	1.3, 6.5, 13.6	15.5	1% L92	CBA/CaO1 aHsd	+	ECPA LLNA Project Report submitted by Dupont												
		12.5, 25, 50, 75, 100	2, 2.3, 8.6, 15.8, 30.1	27.8	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Syngenta/RCC												

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	Overall Call						GP Reference									
										GP (F)	GP (any) <sup>3</sup>	GP (AD) <sup>4</sup>	BT (AI) <sup>4</sup>	GPMT (AI) <sup>4</sup>	GP (RC/RE) <sup>5</sup>										
Trifluralin	EC	7, 33, 100	6.0, 30.0, 75.2	5.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+									ECPA LLNA Project Report submitted by Dow Chemical							
								ECPA LLNA Project Report submitted by Bayer											-	NA	NA	NA	NA		
								ECPA LLNA Project Report submitted by Dow Chemical																	
		7, 33, 100	1.9, 8.7, 25.7	11.2	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Bayer	+																
								ECPA LLNA Project Report submitted by Bayer													-	NA	NA	NA	NA
								ECPA LLNA Project Report submitted by Dow Chemical																	
7, 33, 100	3.1, 26.3, 61.5	7.0	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dupont	+																		
						ECPA LLNA Project Report submitted by Dupont														-	NA	NA	NA	NA	
						ECPA LLNA Project Report submitted by Dupont																			
7, 33, 100	1.0, 7.0, 16.1	15.6	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Syngenta/RCC	+																		
						ECPA LLNA Project Report submitted by Syngenta/RCC														-	NA	NA	NA	NA	
						ECPA LLNA Project Report submitted by Syngenta/RCC																			
7, 33, 100	1.8, 8.2, 20.5	11.9	1% L92	CBA/CaOlaHsd	+	ECPA LLNA Project Report submitted by Syngenta/RCC	+																		
						ECPA LLNA Project Report submitted by Syngenta/RCC														-	NA	NA	NA	NA	
						ECPA LLNA Project Report submitted by Syngenta/RCC																			

Abbreviations: AL = any other liquid; AOO = acetone olive-oil (4:1); ACE = acetone; BT = Buehler Test; Conc. = concentration; CS = capsule suspension; DMF = dimethyl formamide; DMSO = dimethyl sulfoxide; EC = emulsion concentrate; ECPA = European Crop Protection Association; EW = emulsion, oil in water; GPMT = Guinea Pig Maximization Test; LLNA = Local Lymph Node Assay; OD = oil dispersion; ME = micro-emulsion; NA = not available; NC = not calculated since SI>3; PG = propylene glycol; SC = suspension concentrate; SE = suspo-emulsion; SI = stimulation index; SL = soluble concentrate; TK = technical concentrate.

<sup>1</sup> "+" = sensitizer; "-" = nonsensitizer

<sup>2</sup> Overall GP call made on the basis of a test on the entire formulation

<sup>3</sup> Overall GP call made with priority entire formulation > active ingredient > related compound or formulation

<sup>4</sup> Overall GP call made on the basis of a test on an active ingredient

<sup>5</sup> Overall GP call made on the basis of a test on a related compound or formulation

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## **Annex II-3**

### **Composition of Pesticide Formulations Tested in the LLNA**

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Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Dinocap	EC	ECPA	NA	Dinocap	350	NA	NA
			NA	Solvent	542	NA	NA
			NA	Surfactant	78	NA	NA
Formulation 1	SC	Dow AgroSciences	Benzamide	Isoxaben	125	12.14%	- (Dow Data)
			NA	Water	735.2	NA	-
			NA	Thickener	4	NA	- (MSDS)
			NA	Antifoam	2	NA	- (MSDS)
			NA	Surfactant	30	NA	- (MSDS)
			NA	Surfactant	20	NA	- (MSDS)
			NA	Performance Aid	8.5	NA	- (MSDS)
			NA	pH Buffer	1.3	NA	- (MSDS)
			NA	Surfactant	100	NA	- (MSDS)
			NA	Biocide	4	< 0.1%	+ (MSDS)
Formulation 2	SE	Dow AgroSciences	Pyridinyloxy acetic acid	Fluroxypyr-meptyl	144.09	14.53%	- (Dow Data)
			Sulfonamides	Florasulam	2.5	0.25%	- (Dow Data)
			NA	Emulsifier	58.92	NA	- (MSDS)
			NA	Emulsifier	31.84	NA	- (MSDS)
			NA	Solvent	326.8	NA	- (MSDS)
			NA	Suspending Aid	3.24	NA	- (MSDS)
			NA	Suspending Aid	0.91	NA	- (MSDS)
			NA	Emulsifier	1.81	NA	- (MSDS)
			NA	Emulsifier	1.81	NA	- (MSDS)
			NA	Biocide	0.54	0.05%	+ (MSDS)
			NA	Antifoam	1.06	NA	- (MSDS)
			NA	Antifreeze	34.62	NA	- (MSDS)
			NA	Suspending Aid	0.05	NA	- (MSDS)
			NA	Dispersant	0.1	NA	- (MSDS)
			NA	pH Buffer	0.003	NA	- (MSDS)
			NA	Dispersant	0.2	NA	- (MSDS)
NA	Water	383.66	NA	-			

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 3	SC	Dow AgroSciences	Sulfonamides	Florasulam	50	4.84%	- (Dow Data)
			NA	Water	869.12	NA	-
			NA	Biocide	0.93	0.09%	+ (MSDS)
			NA	Dispersant	10.03	NA	- (MSDS)
			NA	Thickener	10.03	NA	- (MSDS)
			NA	Dispersant	1.96	NA	- (MSDS)
			NA	Antifoam	0.21	NA	- (MSDS)
			NA	Thickener	1.76	NA	- (MSDS)
			NA	Antifreeze	89.96	NA	- (MSDS)
			NA	pH Buffer	0.1	NA	- (MSDS)
Formulation 4	SL	Dow AgroSciences	Pyridine carboxylic acids	Clopyralid-olamine (MEA salt)	131.75	12.52%	- (Dow Data) (Clopyralid)
			NA	Water	920.25	NA	-
Formulation 5	EC	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-butotyl	670.39	60.45%	+ (Dow Data)
			NA	Emulsifier	55.45	NA	- (MSDS)
			NA	Solvent	383.16	NA	- (MSDS)
Formulation 6	EW	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-butotyl	333.567	29.44%	+ (Dow Data)
			Pyridine carboxylic acids	Aminopyralid potassium	35.507	3.13%	- (Dow Data) (Aminopyralid)
			NA	Antifreeze	50	NA	- (MSDS)
			NA	Emulsifier	32.5	NA	- (MSDS)
			NA	Emulsifier	32.5	NA	- (MSDS)
			NA	Biocide	1	0.09%	+ (MSDS)
			NA	Thickener	7.5	NA	- (MSDS)
			NA	Thickener	1.875	NA	- (MSDS)
			NA	pH Buffer	27.33	NA	- (MSDS)
			NA	pH Buffer	2.67	NA	- (MSDS)
			NA	Antifoam	2	NA	- (MSDS)
			NA	Water	606.831	NA	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 7	SC	Dow AgroSciences	Triazole	Myclobutanil	45	4.12%	Equivocal (Dow Data)
			Phenoxyquinoline	Quinoxifen	45	4.12%	+ (Dow Data)
			NA	Antifreeze	74.89	NA	- (MSDS)
			NA	Dispersant	31.81	NA	- (MSDS)
			NA	Wetter	14.96	NA	- (MSDS)
			NA	Suspending Aid	7.45	NA	- (MSDS)
			NA	Carrier	57.12	NA	-
			NA	Antifoam	1.09	NA	- (MSDS)
			NA	Biocide	0.37	0.03%	+ (MSDS)
			NA	Water	785.84	NA	-
			NA	Filler	26.5	NA	- (MSDS)
			NA	Thickener	1.97	NA	- (WHO)
Formulation 8	EC	Dow AgroSciences	Phenoxyacetic acids	2,4-D-ethylhexyl	905	81.68%	+ (Dow Data)
			NA	Emulsifier	37	3.34%	- (MSDS)
			NA	Emulsifier	43	3.88%	- (MSDS)
			NA	Solvent	123	NA	- (MSDS)
Formulation 9	SC	Dow AgroSciences	Spinosoids	DE-175	120	11.71%	Equivocal (+/- LLNA)
			Nicotinoates	Wetter	20.5	NA	- (MSDS)
			NA	Antifreeze	61.5	NA	- (MSDS)
			NA	Biocide	2	0.20%	+ (MSDS)
			NA	Thickener	1.8	NA	- (WHO)
			NA	Thickener	4.1	NA	- (MSDS)
			NA	Antifoam	3.6	NA	- (MSDS)
			NA	Dispersant	46.1	NA	- (MSDS)
Formulation 10	EW	Dow AgroSciences	NA	Dithiopyr	240	24%	- (Dow Data)
			NA	Solvent	130	13%	- (MSDS)
			NA	Emulsifier	470	47%	- (MSDS)
			NA	Water	160	16%	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 11	OD	Dow AgroSciences	Chloroacetamides	Acetochlor	950	84.15%	+ (Dow Data)
			Sulfonamides	Penoxsulam	3.5	0.31%	- (Dow Data)
			NA	Suspending Aid	28.5	NA	- (MSDS)
			NA	Antifoam	0.035	NA	- (MSDS)
			NA	Thickener	0.035	NA	- (MSDS)
			NA	pH Buffer	0.014	NA	- (MSDS)
			NA	Dispersant	0.28	NA	- (MSDS)
			NA	Wetter	0.07	NA	- (MSDS)
			NA	Antifreeze	0.21	NA	- (MSDS)
			NA	Water, Deionized	2.84	NA	-
			NA	Nutrient	4.75	0.42%	- (Human Data from IUCLID)
			NA	Related Process Inert Impurities	45.98	NA	- (MSDS)
			NA	Anticaking Agent	0.007	NA	- (MSDS)
			NA	Biocide	0.007	0% (0.007)	+ (MSDS)
NA	Emulsifier	92.94	NA	- (MSDS)			
Formulation 12	EC	Dow AgroSciences	Dinitrophenol	Meptyldinocap	350	35.71%	+ (Dow Data)
			NA	Emulsifier	41.7	NA	- (MSDS)
			NA	Emulsifier	25.76	NA	- (MSDS)
			NA	Solvent	562.54	NA	- (MSDS)
Formulation 13	EC	Dow AgroSciences	Phenoxyacetic acids	2,4-D-ethylhexyl	995.5	87.17%	+ (Dow Data)
			NA	Emulsifier	48	NA	- (MSDS)
			NA	Emulsifier	48	NA	-
			NA	Unspecified Inert	50.5	NA	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 14	CS	Dow AgroSciences	Pyrethroids	Gamma-cyhalothrin	15	1.5%	+ (Dow Data)
			NA	Solvent	10.02	NA	- (MSDS)
			NA	Emulsifier	1.25	NA	- (MSDS)
			NA	Emulsifier	1.25	NA	- (MSDS)
			NA	Encapsulating Agent	1.63	NA	-
			NA	pH Buffer	1	NA	- (MSDS)
			NA	Thickener	0.02	NA	- (MSDS)
			NA	Biocide	1.5	0.15%	+ (MSDS)
			NA	Thickener	1.5	NA	- (MSDS)
			NA	Thickener	0.02	NA	- (MSDS)
			NA	Thickener	15.03	NA	-
			NA	Water	953.8	NA	-
Formulation 15	CS	Dow AgroSciences	Pyrethroids	Gamma-cyhalothrin	60	5.9%	+ (Dow Data)
			NA	Solvent	48.82	NA	- (MSDS)
			NA	Emulsifier	5.09	NA	- (MSDS)
			NA	Emulsifier	5.09	NA	- (MSDS)
			NA	Encapsulating Agent	6.81	NA	-
			NA	Thickener	0.09	NA	- (MSDS)
			NA	Biocide	1.53	0.15%	+ (MSDS)
			NA	Thickener	1.53	NA	- (MSDS)
			NA	Thickener	0.09	NA	- (MSDS)
			NA	pH Buffer	4.07	NA	- (MSDS)
			NA	Thickener	10.68	NA	-
			NA	Water	873.4	NA	-
Formulation 16	EC	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-butotyl	1050.07	83.94%	+ (Dow Data)
			NA	Emulsifier	200.93	NA	- (MSDS)
Formulation 17	SL	Dow AgroSciences	Glycines	Glyphosate dimethyl-ammonium salt	608	50.21%	- (EPA Tolerance)
			NA	Adjuvant	50	4.13%	No Data
			NA	Adjuvant	100	NA	- (MSDS)
			NA	Water	453	NA	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 19	EC	Dow AgroSciences	Pyridinyloxy acetic acid	Fluroxypyr-meptyl	100.865	9.23%	- (Dow Data)
			Benzonitriles	Bromoxynil-octanoate	407.569	37.29%	+ (Dow Data)
			NA	Emulsifier	44	4.03%	- (MSDS)
			NA	Emulsifier	44	NA	- (MSDS)
			NA	Solvent	496.566	45.43%	- (IUCOLID Datasheet)
Formulation 20	SE	Dow AgroSciences	Sulfonamides	Florasulam	4	0.39%	- (Dow Data)
			NA	MCPA-2-ethylhexyl	436.817	42.25%	- (Dow Data); + (EPA RED)
			NA	Emulsifier	12	NA	- (MSDS)
			NA	Thickener	4.34	NA	- (MSDS)
			NA	Dispersant	0.17	NA	- (MSDS)
			NA	Antifoam	1	NA	- (MSDS)
			NA	Stabilizer	1.5	NA	- (MSDS)
			NA	Thickener	0.54	NA	- (MSDS)
			NA	Stabilizer	45.14	NA	- (MSDS)
			NA	pH Buffer	0.01	NA	- (MSDS)
			NA	Stabilizer	0.34	NA	- (MSDS)
			NA	Antifreeze	49.75	NA	- (MSDS)
			NA	Biocide	0.93	0.09%	+ (MSDS)
			NA	pH Buffer	1.03	NA	- (MSDS)
			NA	Water	476.443	NA	-
Formulation 21	TK	Dow AgroSciences	Acyl Ureas	Hexaflumuron	645	50%	- (Dow Data)
			NA	Water	497.42	NA	-
			NA	Biocide	9.68	0.75%	+ (MSDS)
			NA	Surfactant	64.5	NA	- (MSDS)
			NA	Antifoam	3.48	NA	- (MSDS)
			NA	Surfactant	69.92	5.42%	- (MSDS)

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 22	ME	Dow AgroSciences	Pyridinyloxy acetic acid	Fluroxypyr-meptyl	28.8	2.83%	- (Dow Data)
			NA	Triclopyr-triethyl-ammonium	83.67	8.23%	+ (EPA RED)
			NA	Surfactant	29.59	NA	- (MSDS)
			NA	Carrier	29.59	NA	- (MSDS)
			NA	Surfactant	84	NA	- (MSDS)
			NA	Emulsifier	48	NA	- (MSDS)
			NA	Solvent	86.34	NA	- (MSDS)
			NA	Unspecified Inert	104.98	NA	-
Formulation 23	SL	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-triethyl-ammonium	167.36	16%	+ (EPA RED)
			NA	Water	837	NA	-
			NA	Antifoam	0.02	NA	- (MSDS)
			NA	Wetter	3.77	NA	- (MSDS)
			NA	Chelating Agent	8.68	NA	- (MSDS)
			NA	Surfactant	10.04	NA	- (MSDS)
			NA	Neutralizer	11.3	NA	- (67/548/EEC)
			NA	Carrier	7.85	NA	- (67/548/EEC)
Formulation 24	OD	Dow AgroSciences	Sulfonamides	Pyroxsulam	30	2.87%	+ (Dow Data)
			NA	Safener	90	8.6%	+ (EPA Tolerance Petition)
			NA	Emulsifier	40	NA	- (MSDS)
			NA	Emulsifier	50	NA	- (MSDS)
			NA	Emulsifier	20	NA	- (MSDS)
			NA	Stabilizer	10	NA	-
			NA	Suspending Aid	40	NA	- (MSDS)
			NA	Diluent	767	NA	- (MSDS)

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 25	EC	Dow AgroSciences	Pyridine carboxylic acids	Clopyralid	23.34	2.21%	- (Dow Data)
			Pyridinyloxy acetic acid	Fluroxypyr-meptyl	86.455	8.19%	- (Dow Data)
			NA	MCPA-2-ethylhexyl	416.1	39.4%	- (Dow Data); + (EPA RED)
			NA	Solvent	38.54	NA	- (MSDS)
			NA	Emulsifier	52.27	NA	- (MSDS)
			NA	Emulsifier	428.205	NA	- (MSDS)
			NA	Solvent	11.09	NA	- (MSDS)
Formulation 26	EC	Dow AgroSciences	Pyridine carboxylic acids	Clopyralid	60	5.83%	- (Dow Data)
			Pyridinyloxy acetic acid	Triclopyr-butotyl	333.797	32.41%	+ (Dow Data)
			NA	Emulsifier	43.7	NA	- (MSDS)
			NA	Emulsifier	29.2	NA	- (MSDS)
			NA	Solvent	88.9	NA	- (MSDS)
			NA	Solvent	474.403	NA	- (IUCLID Datasheet)
Formulation 27	EC	Dow AgroSciences	Pyridinyloxy acetic acid	Fluroxypyr-meptyl	479.827	45.52%	- (Dow Data)
			NA	Emulsifier	78.46	NA	- (MSDS)
			NA	Solvent	417.253	NA	- (MSDS)
			NA	Emulsifier	78.46	NA	-(MSDS)
Formulation 28	SC	Dow AgroSciences	Unclassified Herbicide	Diflufenican	100	9.48%	- (MSDS)
			Sulfonamides	Penoxsulam	15	1.42%	- (Dow Data)
			NA	Wetter	15	NA	- (MSDS)
			NA	Dispersant	10	NA	- (MSDS)
			NA	Thickener	10	NA	- (MSDS)
			NA	Thickener	2	NA	- (MSDS)
			NA	Biocide	1.5	0.14%	+ (MSDS)
			NA	Antifreeze	50	NA	- (MSDS)
			NA	pH Buffer	0.462	NA	- (MSDS)
			NA	Antifoam	5	NA	- (MSDS)
			NA	Water	846.038	NA	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 29	SC	Dow AgroSciences	Dithiocarbamate	Mancozeb	462	35.95%	Equivocal (EPA RED)
			Unspecified	Cymoxanil	70.03	5.45%	- (EPA Fact Sheet)
			NA	Anticaking Agent	29.81	NA	- (MSDS)
			NA	Stabilizer	25.7	NA	-
			NA	Stabilizer	12.85	NA	-
			NA	Emulsifier	12.85	NA	- (MSDS)
			NA	Dispersant	2.57	NA	- (MSDS)
			NA	Thickener	1.29	NA	- (MSDS)
			NA	Adjuvant	131.58	NA	- (MSDS)
			NA	Water	536.32	NA	-
Formulation 30	EW	Dow AgroSciences	Chloroacetamides	Acetochlor	450	41.82%	+(Dow Data)
			Pyridine carboxylic acids	Clopyralid-olamine	46.11	4.29%	- (Dow Data)
			Sulfonamides	Flumetsulam	14.0	1.3%	- (MSDS)
			NA	pH Buffer	2.37	0.22%	- (67/548/EEC)
			NA	Emulsifier	21.52	2%	- (IUCLID Datasheet)
			NA	Solvent	10.76	1%	- (IUCLID Datasheet)
			NA	Biocide	1.076	0.10%	+ (MSDS)
			NA	Thickener	1.076	0.10%	- (WHO)
			NA	Antifoam	1.61	NA	- (MSDS)
			NA	Dispersant	5.38	NA	- (MSDS)
			NA	Wetter	2.69	NA	- (MSDS)
			NA	Water	519.408	NA	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 31	CS	Dow AgroSciences	Organophosphates	Chlorpyrifos	200	18.96%	Equivocal (Dow Data)
			NA	Encapsulating Agent	6.49	NA	-
			NA	Dispersant	29.59	NA	-(MSDS)
			NA	Biocide	1.055	0.10%	+(MSDS)
			NA	Thickener	5.92	NA	-(MSDS)
			NA	Thickener	0.738	NA	-(MSDS)
			NA	Dispersant	16.47	NA	-(MSDS)
			NA	Solvent	120	NA	-(IUCLID Datasheet)
NA	Water	674.737	NA	-			
Formulation 32	EC	Dow AgroSciences	Dinitrophenol	Meptyldinocap	105	11.27%	+(Dow Data)
			Triazole	Myclobutanil	45	4.83%	Equivocal (Dow Data)
			NA	pH Buffer	15	NA	-(67/548/EEC)
			NA	Emulsifier	23	NA	-(MSDS)
			NA	Emulsifier	68	NA	-(MSDS)
			NA	Solvent	676	NA	-(MSDS)
Formulation 33	SL	Dow AgroSciences	Pyridine carboxylic acids	Clopyralid-olamine	316.206	26.66%	-(Dow Data)
			Pyridine carboxylic acids	Picloram-olamine	100.251	8.45%	-(EPA RED)
			Pyridine carboxylic acids	Aminopyralid-olamine	51.8	4.37%	-(Dow Data)
			NA	Neutralizer	22	NA	-(67/548/EEC)
			NA	Water, Deionized	695.743	NA	-
Formulation 34	SL	Dow AgroSciences	Pyridine carboxylic acids	Aminopyralid	30	2.95%	-(Dow Data)
			NA	Neutralizer	8.1	NA	-(67/548/EEC)
			NA	Water	978.9	NA	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 35	SL	Dow AgroSciences	Pyridine carboxylic acids	Aminopyralid triisopropanol-ammonium	23.08	2.22 %	- (Dow Data) (Aminopyralid)
			Pyridinyloxy acetic acid	Triclopyr-triethyl-ammonium	167.36	16.09 %	+ (EPA RED)
			NA	Neutralizer	1.14	NA	-
			NA	Wetter	38	NA	- (MSDS)
			NA	Antifoam	0.19	NA	- (MSDS)
			NA	Neutralizer	14.82	NA	- (67/548/EEC)
			NA	Sequestrant	8.74	NA	- (MSDS)
			NA	Water	786.67	NA	-
Formulation 37	EC	Dow AgroSciences	Organophosphates	Chlorpyrifos	300	30%	Equivocal (Dow Data)
			Pyrethroids	Gamma-cyhalothrin	5.4	0.54%	+ (DOW Data)
			NA	Emulsifier	55	5.50%	- (MSDS)
			NA	Emulsifier	4.4	0.44%	- (MSDS)
			NA	Solvent	635.2	63.52%	- (IUCLID Datasheet)
Formulation 38	EC	Dow AgroSciences	Acetamides	Propanil	479.81	44.80%	- (EPA RED)
			NA	Solvent	362	NA	- (MSDS)
			NA	Solvent	122.09	NA	- (IUCLID Datasheet)
			NA	Emulsifier	107.1	10%	- (IUCLID Datasheet)
Formulation 39	OD	Dow AgroSciences	Sulfonamides	Pyroxsulam	45	4.31%	+ (DOW Data)
			NA	Safener	90	8.61%	+ (EPA Tolerance Petition)
			NA	Dispersant	6	0.57%	- (MSDS)
			NA	Dispersant	10	NA	- (MSDS)
			NA	Emulsifier	80	NA	- (MSDS)
			NA	Stabilizer	10	0.96%	- (MSDS)
			NA	Suspending Aid	27	NA	- (MSDS)
			NA	Solvent	777	NA	- (MSDS)

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 40	OD	Dow AgroSciences	Sulfonamides	Pyroxsulam	12.8	1.20%	+ (Dow Data)
			NA	Safener	38.5	3.62%	+ (EPA Tolerance Petition)
			NA	Active Ingredient	2.14	0.20%	- (EPA Fact Sheet)
			NA	Active Ingredient	123.199	11.57%	- (Dow Data)
			NA	Dispersant	4	0.38%	- (MSDS)
			NA	Dispersant	10	NA	- (MSDS)
			NA	Emulsifier	80	NA	- (MSDS)
			NA	Stabilizer	10	NA	- (MSDS)
			NA	Thickener	30	NA	- (MSDS)
			NA	Solvent	754.361	NA	- (MSDS)
Formulation 41	SE	Dow AgroSciences	Phenoxyacetic acids	2,4-D-ethylhexyl	271.493	25.61%	+ (Dow Data)
			Pyridine carboxylic acids	Aminopyralid	11.834	1.12%	- (Dow Data)
			Sulfonamides	Florasulam	5	0.47%	- (Dow Data)
			NA	Solvent	73.2	NA	- (MSDS)
			NA	Emulsifier	60.4	NA	- (MSDS)
			NA	Thickener	0.1	NA	- (MSDS)
			NA	Biocide	0.9	0.08%	+ (MSDS)
			NA	Antifoam	2	NA	- (MSDS)
			NA	Dispersant	0.2	NA	- (MSDS)
			NA	Antifoam	0.02	NA	- (MSDS)
			NA	Antifreeze	50.5	NA	- (MSDS)
			NA	Suspending Aid	1.6	NA	- (MSDS)
			NA	pH Buffer	0.1	NA	- (MSDS)
			NA	Water	582.873	NA	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 42	SL	Dow AgroSciences	Phenoxyacetic acids	2,4-D-triisopropanolamine	339	31.00%	- (EPA RED)
			Pyridine carboxylic acids	Aminopyralid triisopropanol-ammonium	17	1.52%	- (Dow Data) (Aminopyralid)
			NA	Neutralizer	4.962	NA	- (MSDS)
			NA	Sequestrant	2.19	NA	- (MSDS)
			NA	Antifreeze	38.26	NA	- (MSDS)
			NA	Water	694.48	NA	-
Formulation 43	CS	Dow AgroSciences	Unspecified nitrification inhibitor	Nitrapyrin	200	17.90%	+ (Dow Data)
			NA	Solvent	234.79	0.12%	+ (R43)
			NA	Solvent	99.65	NA	- (MSDS)
			NA	Thickener	22.31	NA	- (MSDS)
			NA	Dispersant	13.36	NA	+ (MSDS)
			NA	Emulsifier	13.36	0.24%	- (MSDS)
			NA	Dispersant	2.67	1.19%	- (MSDS)
			NA	Thickener	2.14	8.87%	+ (DOW Data)
			NA	Biocide	1.34	NA	- (MSDS)
			NA	Water	534.38	NA	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 44	SC	Dow AgroSciences	Sulfonamides	Penoxsulam	1.4	0.12%	- (Dow Data)
			Dinitroanilines	Oryzalin	478.9	40.38%	Equivocal (Dow Data)
			NA	Antifoam	5.92	NA	- (MSDS)
			NA	Dispersant	71	5.99%	- (MSDS)
			NA	Antifreeze	47.3	NA	- (MSDS)
			NA	Dispersant	17.7	1.49%	- (MSDS)
			NA	Antifreeze	71.1	5.99%	- (MSDS)
			NA	Biocide	0.59	0.05%	+ (MSDS)
			NA	Suspending Aid	1.78	0.15%	- (WHO)
			NA	Carrier	8.88	NA	- (MSDS)
			NA	Antifoam	0.01	NA	- (MSDS)
			NA	Suspending Aid	0.01	0%	- (MSDS)
			NA	pH Buffer	0.01	NA	- (MSDS)
			NA	Dispersant	0.11	0.01%	- (MSDS)
			NA	Wetter	0.03	0%	- (MSDS)
			NA	Water	481.32	40.58%	-
Formulation 45	SC	Dow AgroSciences	Carboxanilide	Thifluzamide	80	7.53%	- (Dow Data) (25%)
			Triazole	Fenbuconazole	100	9.42%	- (Dow Data)
			NA	Adjuvant	51.4008	NA	- (MSDS)
			NA	Wetter	12.8502	NA	- (MSDS)
			NA	Biocide	1.062	0.10%	+ (MSDS)
			NA	Suspending Aid	4.248	NA	- (MSDS)
			NA	Antifoam	5.32	NA	- (MSDS)
			NA	Emulsifier	11.682	NA	- (MSDS)
			NA	Dispersant	40.887	NA	- (MSDS)
			NA	Water	754.55	71.05%	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 46	SC	Dow AgroSciences	Spinosoids	Spinetoram	60	5.87%	Equivocal (+/- LLNA)
			NA	Dispersant	30.75	NA	- (MSDS)
			NA	Wetter	20.5	2%	- (MSDS)
			NA	Antifreeze	61.4	NA	- (MSDS)
			NA	Biocide	2	0.20%	+ (MSDS)
			NA	Thickener	2	0.20%	- (WHO)
			NA	Thickener	4.1	NA	- (MSDS)
			NA	Antifoam	10	0.98%	- (MSDS)
			NA	Water	832.25	81.35%	-
Formulation 47	EW	Dow AgroSciences	Triazole	Propiconazole	150	14.56%	+ (EPA RED)
			NA	Solvent	5.15	NA	- (MSDS)
			NA	Emulsifier	20.6	2.00%	- (MSDS)
			NA	Emulsifier	15.45	0.50%	- (MSDS)
			NA	Antifreeze	51.5	5.00%	- (MSDS)
			NA	Emulsifier	51.5	1.50%	- (MSDS)
			NA	Water		66.44%	-
			NA	Solvent	735.8	5.00%	- (IUCLID Datasheet)
Formulation 49	AL	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-butotyl	200.3	23.16%	+ (Dow Data)
			NA	Diluent	664.7	76.84%	- (IUCLID Datasheet)
Formulation 50	SL	Dow AgroSciences	Glycines	Glyphosate dimethyl-ammonium salt	608	50.54%	- (EPA Tolerance)
			NA	Adjuvant	90	7.48%	- (MSDS for Similar)
			NA	Water	505	41.98%	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 51	OD	Dow AgroSciences	Dinitroanilines	Pendimethalin	314	29.76%	- (EPA RED)
			Sulfonamides	Pyroxulam	5.4	0.51%	+ (Dow Data)
			NA	Safener	5.4	0.51%	+ (EPA Tolerance Petition)
			NA	Stabilizer	5	NA	- (MSDS)
			NA	Suspending Aid	20	NA	- (MSDS)
			NA	Emulsifier	60	NA	- (MSDS)
			NA	Emulsifier	10	0.95%	- (MSDS)
			NA	Emulsifier	30	NA	- (MSDS)
			NA	Antifoam	1	0.09%	- (MSDS)
NA	Solvent	604.2	NA	- (MSDS)			
Formulation 53	EW	Dow AgroSciences	Organophosphates	Chlorpyrifos	450	40.18%	Equivocal (Dow Data)
			NA	Emulsifier	56	5%	No Data
			NA	Antifreeze	28	NA	- (MSDS)
			NA	Dispersant	134.5	12.01%	- (MSDS)
			NA	Biocide	1.12	0.10%	+ (MSDS)
			NA	Antifoam	4.5	NA	- (MSDS)
			NA	Solvent	224	20%	- (IUCALID Datasheet)
			NA	Water	221.88	19.81%	-
Formulation 54	SL	Dow AgroSciences	Glycines	Glyphosate dimethyl-ammonium salt	608	49.88%	- (EPA Tolerance)
			NA	Adjuvant	100	NA	- (MSDS)
			NA	Adjuvant	50	NA	- (MSDS)
			NA	Water	461	37.82%	-

Substance Name	Formulation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information <sup>1</sup>
Formulation 55	EW	Dow AgroSciences	Triazole	Myclobutanil	45	4.5%	Equivocal (Dow Data)
			NA	Emulsifier	26.5	2.65%	- (MSDS)
			NA	Emulsifier	18.5	1.85%	6.0 - (MSDS)
			NA	Antifreeze	100	NA	- (MSDS)
			NA	Solvent	200	20%	- (IUCLID Datasheet)
			NA	Diluent	40.5	NA	- (MSDS)
			NA	Emulsifier	5	0.50%	No Data
			NA	Water	561.5	56.15%	-
			NA	Biocide	3	0.30%	+ (MSDS)
Formulation 56	SL	Dow AgroSciences	Unspecified nitrification inhibitor	Nitrapyrin	216	19.89%	+ (Dow Data)
			NA	Impurities	24	2.21%	No Data
			NA	Stabilizer	14.4	1.33%	- (MSDS)
			NA	Solvent	831.6	76.57%	- (IUCLID Datasheet)
Oxyfluorfen	EC	ECPA	NA	Oxyfluorfen	240	NA	NA
			NA	Solvent	732	NA	NA
			NA	Surfactant	108	NA	NA
Quinoxyfen / Cyproconazole	NA	ECPA	NA	Cyproconazole	80	NA	NA
			NA	Quinoxyfen	75	NA	NA
			NA	Antifreeze	75	NA	NA
			NA	Thickener	10	NA	NA
			NA	Water/Other Components	842	NA	NA
Trifluralin	EC	ECPA	NA	Trifluralin	480	NA	NA
			NA	Solvent	500	NA	NA
			NA	Surfactant	60	NA	NA

Abbreviations: AL = any other liquid; AOO = acetone olive oil (4:1); ACE = acetone; Conc. = concentration; CS = capsule suspension; EC = emulsion concentrate; ECPA = European Crop Protection Association; EEC = European Economic Community; EPA = U.S. Environmental Protection Agency; EW = emulsion, oil in water; IUCLID = International Uniform Chemical Information Database; LLNA = Local Lymph Node Assay; OD = oil dispersion; ME = micro-emulsion; MSDS = Material Safety Data Sheet; NA = not available; RED = reregistration eligibility decision; SC = suspension concentrate; SE = suspo-emulsion; SI = stimulation index; SL = soluble concentrate; TK = technical concentrate; WHO = World Health Organization.

<sup>1</sup> (+) = sensitizer, (-) = nonsensitizer

**Annex II-4**  
**Physicochemical Properties and Chemical Classes**  
**of Dye Formulations Tested in the LLNA**

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Substance Name	Synonyms	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Phys. Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
C.I. Reactive Red 231	NA	NA	NA	NA	Solid	Formulation	NA
C.I. Reactive Yellow 174	1,3,6-Naphthalene-trisulfonic acid, 7-(2-(2-(aminocarbonyl)amino)-4-((4-((2-(2-(ethenylsulfonyl)ethoxy)ethyl)amino)-6-fluoro-1,3,5-triazin-2-yl)amino)phenyl) diazenyl)-, sodium salt (1:3)	106359-91-5	885.72	NA	Solid	Formulation	
Dispersionsrot 2754	NA	NA	NA	NA	Solid	Formulation	NA
Navy 14 08 723	NA	NA	NA	NA	Solid	Formulation	NA
Produkt P-4G	NA	185461-17-0	NA	NA	Solid	Formulation	NA
Yellow E-JD 3442	Benzenesulfonic acid, 3-(2-(2-(acetylamino)-4-(2-(4-(2-hydroxybutoxy)phenyl)diazenyl)phenyl) diazenyl)-, sodium salt (1:1)	147703-65-9	533.54	NA	Solid	Formulation	

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

<sup>1</sup>Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: [http://www.syrres.com/esc/est\\_kowdemo.htm](http://www.syrres.com/esc/est_kowdemo.htm).

<sup>2</sup>Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: <http://www.nlm.nih.gov/mesh/meshhome.html>.

<sup>3</sup>Chemical structures, based on CASRN, were obtained from ChemID available at: <http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp>.

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## **Annex II-5**

### **Dye Formulations Tested in the LLNA - Comparative Data**

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Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result	GPMT i.d. Induction Conc. (%)	GPMT Patch Conc. (%)	GPMT Challenge Conc. (%)	GPMT No. Animals with + rxn After Challenge & Rechallenge	GPMT % Sens. Incidence	GPMT Result <sup>1</sup>	Reference
C.I. Reactive Red 231	Dye	1, 3, 9, 15	4.8, 3.4, 4.4, 4.6	0.6	AOO	CBA/Ca	+	1	75	75	NA	~50	+	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin
C.I. Reactive Yellow 174	Dye	1, 3, 9, 15	4.2, 5.3, 5.5, 7.8	0.3	AOO	CBA/Ca	+	5	25	25	2	11	-	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin
Dispersionsrot 2754	Dye	1, 3, 9	1.0, 0.9, 1.0	NC	AOO	CBA/Ca	-	5	25	25	8	100	+	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin
Navy 14 08 723	Dye	1, 3, 9, 15	5.1, 4.8, 5.7, 5.2	IDR	AOO	CBA/Ca	+	5	25	10	20	100	+	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin
Produkt P-4G	Dye	1, 3, 9, 15	2.4, 2.5, 1.9, 2.5	NC	AOO	CBA/Ca	-	5	25	25	9	90	+	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC <sub>3</sub> (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	GPMT i.d. Induction Conc. (%)	GPMT Patch Conc. (%)	GPMT Challenge Conc. (%)	GPMT No. Animals with + rxn After Challenge & Rechallenge	GPMT % Sens. Incidence	GPMT Result <sup>1</sup>	Reference
Yellow E-JD 3442	Dye	1, 3, 9, 15	1.0, 0.8, 0.9, 0.9	NC	AOO	CBA/Ca	-	5	50	50	2	10	-	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

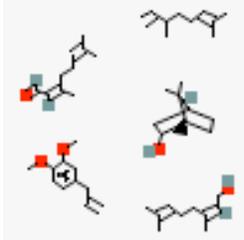
Abbreviations: AOO = acetone olive-oil (4:1); Conc. = concentration; GPMT = Guinea Pig Maximization Test; i.d. = intradermal; IDR = inadequate dose response; LLNA = Local Lymph Node Assay; NA = not available; NC = not calculated since SI<3; rxn = reaction; sens. = sensitization; SI = stimulation index.

<sup>1</sup> "+" = sensitizer; "-" = nonsensitizer

## **Annex II-6**

### **Physicochemical Properties and Chemical Classes of Natural Complex Substances Tested in the LLNA**

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Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Basil oil	Ocimum basilicum oil	8015-73-4	NA	NA	Liquid	Lipids	NA
Citronella oil	Cymbopogon nardus oil	8000-29-1	NA	3.53	Liquid	Lipids	NA
Clove Oil	Clove leaf oil Clove stem oil	8000-34-8	NA	NA	Liquid	Lipids	NA
Geranium oil	Geranium maculatum oil	8000-46-2	NA	NA	Liquid	NA	NA
Jasmine absolute	Gardenia jasminoides, ext.	92457-01-7	NA	NA	NA	NA	NA
Lemongrass oil	Citral terpenes; 1,2-dimethoxy-4-prop-2-enylbenzene	8007-02-1	777.21	NA	Liquid	NA	
Litsea cubeb oil	Litsea cubeba	68855-99-2	NA	NA	Liquid	NA	NA
Oakmoss	Oak moss extract, absolute	68917-10-2	NA	NA	NA	NA	NA
Palmarosa oil	Cymbopogon martini oil	8014-19-5	NA	NA	NA	NA	NA
Spearmint oil	Mentha spicata oil	8008-79-5	NA	NA	Liquid	NA	NA
Treemoss	Cedar moss extract	68648-41-9	NA	NA	NA	NA	NA
Ylang Ylang oil	Cananga oil	68606-83-7 8006-81-3	NA	NA	NA	NA	NA

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

<sup>1</sup>Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: [http://www.syrres.com/esc/est\\_kowdemo.htm](http://www.syrres.com/esc/est_kowdemo.htm).

<sup>2</sup>Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: <http://www.nlm.nih.gov/mesh/meshhome.html>

<sup>3</sup>Chemical structures, based on CASRN, were obtained from ChemID available at: <http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp>.

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**Annex II-7**

**Natural Complex Substances Tested in the LLNA – Comparative Data**

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Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	Overall LLNA Result <sup>1</sup>	LLNA Reference	Test Conc. (%)	% Sens. Incidence	Result <sup>1</sup>	Overall Human Result <sup>1</sup>	Human Reference			
Basil Oil	Fragrance Ingredient	2.5,	3.0,	6.2	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	4	0	-	-	Opdyke (1973a)			
		5,	3.0,														
Citronella Oil	Fragrance Ingredient	10,	8.0,	NC	1:3 EtOH/DEP	CBA/Ca	-	-	Lalko & Api (2006) submitted by RIFM	8	0	-	-	Opdyke (1973b)			
		25,	17.6,							8	0	-					
		50	25.2							8	0	-					
Clove Oil	Fragrance Ingredient	2.5,	1.4,	7.1	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	5	0	-	-	Opdyke (1975a)			
		5,	0.9,												5	0	-
		10,	1.2,														
		25,	1.2,														
		50	2.7														
Clove Oil	Fragrance Ingredient	1.0,	1.1,	7.1	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	5	0	-	-	Opdyke (1978a)			
		2.5,	1.8,												5	0	-
		5,	2.5,														
		10,	3.7,														
		25,	5.9														
		50															
Clove Oil	Fragrance Ingredient	2.5,	1.6,	7.0	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	10	0	-	-	Opdyke (1975b)			
		5,	1.5,												10	0	-
		10,	4.0,														
		25,	9.5,														
		50	11.4														
Geranium Oil	Fragrance Ingredient	1.0,	1.6,	NC	1:3 EtOH/DEP	CBA/Ca	-	-	Lalko & Api (2006) submitted by RIFM	10	0	-	-	Opdyke (1975c)			
		2.5,	1.7,												10	0	-
		5,	2.2,														
		10,	4.2,														
		25,	8.9														
		50															
Geranium Oil	Fragrance Ingredient	2.5,	1.2,	NC	1:3 EtOH/DEP	CBA/Ca	-	-	Lalko & Api (2006) submitted by RIFM	10	0	-	-	Opdyke (1975c)			
		5,	0.7,												10	0	-
		10,	1.7,														
		25,	1.8,														
		50	2.8														

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	Overall LLNA Result <sup>1</sup>	LLNA Reference	Test Conc. (%)	% Sens. Incidence	Result <sup>1</sup>	Overall Human Result <sup>1</sup>	Human Reference
Jasmine Absolute	Fragrance Ingredient	1.0, 2.5, 5, 10, 25	1.2, 1.8, 2.0, 7.4, 11.8	5.9	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	3	8	+ <sup>2</sup>	+	Opdyke (1976c)
		10, 25, 50, 75, 100	1.7, 2.5, 3.6, 1.8, 16.2	36.4	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	3	0	-	-	
Lemongrass Oil	Fragrance Ingredient	2.5, 5, 10, 25, 50	0.9, 2.1, 5.1, 10.3, 13.1	6.5	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	4	0	-	-	Opdyke (1976e)
Litsea cubeb Oil	Fragrance Ingredient	2.5, 5, 10, 25, 50	2.0, 2.3, 3.3, 7.9, 16.0	8.4	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	8	0	-	-	Opdyke (1976d)
Oakmoss	Fragrance Ingredient	NA	NA	3.9	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	10	0	-	+	Opdyke (1976a)
Palmarosa Oil	Fragrance Ingredient	2.5, 5, 10, 25, 50	1.1, 2.1, 3.1, 3.6, 5.0	9.6	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	NA	NA	NA	-	Lalko & Api (2006) submitted by RIFM
Spearmint Oil	Fragrance Ingredient	0.5, 1.0, 2.5, 5, 10	1.2, 1.1, 1.2, 1.9, 3.6	8.2	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	4	0	-	-	Opdyke (1978b)

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result <sup>1</sup>	Overall LLNA Result <sup>1</sup>	LLNA Reference	Test Conc. (%)	% Sens. Incidence	Result <sup>1</sup>	Overall Human Result <sup>1</sup>	Human Reference	
Treemoss	Fragrance Ingredient	NA	NA	NC	1:3 EtOH/DEP	CBA/Ca	-	-	Lalko & Api (2006) submitted by RIFM	NA	NA	NA	+	RIFM, submitted by AM Api	
Ylang Ylang Oil	Fragrance Ingredient	0.5,	1.5,	NC	1:3 EtOH/DEP	CBA/Ca	-	+	Lalko & Api (2006) submitted by RIFM	10	0	-	+	Opdyke (1974)	
		1.0,	1.7,							10	0	-			
		2.5,	2.1,							10	0	-			
		5,	2.6, 2.6												
		10													
		0.5,	1.5,	6.8	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	10	5	+	+	Opdyke (1974)	
	1.4,	10	0							-					
	2.1,	10	0							-					
		5,	2.5,												
		10	3.9												

Abbreviations: Conc. = concentration; DEP = diethyl phthalate; EtOH = ethanol; HMT = Human Maximization Test; HRIPT = Human Repeat Insult Patch Test; LLNA = Local Lymph Node Assay; NA = not available; NC = not calculated since SI < 3; RIFM = Research Institute for Fragrance Materials; Sens. = sensitization; SI = stimulation index.

<sup>1</sup> "+" = sensitizer; "-" = nonsensitizer

<sup>2</sup> Positive result possibly due to "Spillover effect." "In maximization testing, four unrelated materials are tested on each of 25 human subjects. In the event that one of the four test materials turns out to be a potent sensitizer (in this case it was Costus oil, which sensitized 25/25 subjects), false weak positive results may occur with the other three materials. When these three materials are subsequently retested out of the context of the serious allergen, and in the same or different groups of subjects, they prove to be negative. We refer to this as the 'spillover effect'" (Opdyke 1976c).

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### **Annex III**

#### **Available Data and Information for Metals Tested in the LLNA**

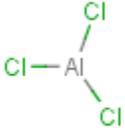
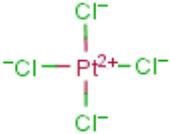
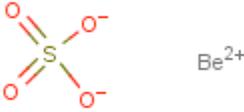
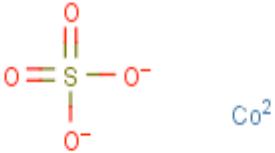
III-1 Physicochemical Properties and Chemical Classes of Metals Tested in the LLNA.....	D-157
III-2 Metals Tested in the LLNA – Comparative Data.....	D-163

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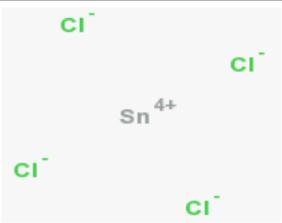
**Annex III-1**

**Physicochemical Properties and Chemical Classes of Metals Tested in the LLNA**

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Substance Name	Synonyms	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Aluminum chloride	Aluminum chloride, anhydrous	7446-70-0	NA	NA	Solid	Inorganic Chemicals, Aluminum Compounds, Inorganic Chemicals, Chlorine Compounds	
<i>Ammonium tetrachloroplatinate</i>	<i>Ammonium platinum chloride; Ammonium chloroplatinate</i>	13820-41-2	372.97	0.47	<i>Solid</i>	<i>Inorganic Chemicals, Platinum Compounds</i>	 6.1
<i>Beryllium sulfate</i>	<i>Beryllium sulfate tetrahydrate</i>	7787-56-6	177.14	NA	<i>Solid</i>	<i>Inorganic Chemicals, Metals, Salts</i>	
<i>Cobalt chloride</i>	<i>Cobaltous chloride</i>	7646-79-9	129.84	0.85	<i>Solid</i>	<i>Inorganic Chemicals, Metals, Salts</i>	$[Cl^-]_{2nt}$ $[Co^{2+}]$
Cobalt (II) salts	NA	NA	NA	NA	Solid	Inorganic Chemicals, Metals, Salts	NA
Cobalt sulfate	Cobaltous sulfate	10124-43-3	154.99	0.63	Solid	Inorganic Chemicals, Metals, Salts	
<i>Copper chloride</i>	<i>Cuprous chloride</i>	7758-89-6	98.99	-0.26	NA	<i>Inorganic Chemicals, Metals, Salts</i>	$Cu-Cl$

Substance Name	Synonyms	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
<i>Gold chloride</i>	<i>Gold tetrachloride</i>	16903-35-8	339.79	0.16	<i>Solid</i>	<i>Inorganic Chemicals, Gold Compounds, Salts</i>	
<i>Lead acetate</i>	<i>Acetic acid, lead salt</i>	15347-57-6	325.29	-0.08	<i>Solid</i>	<i>Inorganic Chemicals, 6.2 Metals, Salts</i>	
Manganese chloride	Manganese chloride, anhydrous	7773-01-5	125.84	0.85	Solid	Inorganic Chemicals, Manganese Compounds, Salts	
<i>Mercuric chloride</i>	<i>Mercuric (II) chloride</i>	7487-94-7	271.5	0.15	<i>Solid</i>	<i>Inorganic Chemicals, Mercury Compounds, Salts</i>	
<i>Nickel chloride</i>	<i>Nickelous chloride</i>	7718-54-9	129.6	0.05	<i>Solid</i>	<i>Inorganic Chemicals, Metals, Salts</i>	
<i>Nickel sulfate</i>	<i>Nickel (II) sulfate</i>	7786-81-4	154.76	-0.17	<i>Solid</i>	<i>Inorganic Chemicals, Metals, Salts</i>	
<i>Potassium dichromate</i>	<i>PDC</i>	7778-50-9	294.18	-2.24	<i>Solid</i>	<i>Inorganic Chemicals, Chromium Compounds, Inorganic Chemicals Potassium Compounds</i>	

Substance Name	Synonyms	CASRN	Mol. Wt. (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Tin chloride	NA	1344-13-14	260.52	NA	Solid	Inorganic Chemicals, Tin Compounds, Salts	
<b>Zinc sulfate</b>	<b><i>Sulfuric acid, zinc salt; Zinc sulphate</i></b>	<b>7733-02-0</b>	<b>NA</b>	<b>NA</b>	<b><i>Solid</i></b>	Inorganic Chemicals, Zinc Compounds, Salts	

Bold, italicized text represents the 11 metals reported in the original LLNA Evaluation Report (ICCVAM 1999).

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

<sup>1</sup> Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: [http://www.syrres.com/esc/est\\_kowdemo.htm](http://www.syrres.com/esc/est_kowdemo.htm).

<sup>2</sup> Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: <http://www.nlm.nih.gov/mesh/meshhome.html>.

<sup>3</sup> Chemical structures, based on CASRN, were obtained from ChemID available at: <http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp>.

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**Annex III-2**

**Metals Tested in the LLNA – Comparative Data**

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## Abbreviations

AOO	Acetone olive-oil (4:1)
BT	Beuhler Test
CASRN	Chemical Abstracts Service Registry Number
Conc.	Concentration
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
ETOH	Ethanol
GPMT	Guinea Pig Maximization Test
IDR	Insufficient data results
LLNA	Local Lymph Node Assay
NA	Not available
NC	Not calculated
SI	Stimulation index

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	CASRN	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	Vehicle	LLNA <sup>1</sup> Result	Overall LLNA Result <sup>1,2</sup>	Overall LLNA Result <sup>1,2,3</sup> (Aqueous Metals)	Overall LLNA Result <sup>1,2,3</sup> (Nonaqueous Metals)
Aluminum chloride	7446-70-0	5, 10, 25	0.8, 0.8, 0.7	NC	Petrolatum	-	-	NA	-
<i>Ammonium tetrachloroplatinate</i> <sup>d</sup>	13820-41-2	2.5, 5, 10	16, 15.4, 18.1	IDR	DMSO	+	+	NA	+
<i>Beryllium sulfate</i>	7787-56-6	NA	NA	0.03	NA	+	+	NA	+
		2.5, 5, 10	8.4, 7.1, 9.4	IDR	DMF	+			
<i>Cobalt chloride</i>	7646-79-9	0.5, 1.0, 2.5	3.2, 2.7, 2.8	0.4	NA	+	+	NA	NA
Cobalt (II) salts	7440-48-4	NA	NA	NA	DMSO	+	+	NA	+
Cobalt sulfate	10124-43-3	NA	NA	NA	NA	+	+	NA	NA
<i>Copper chloride</i>	7758-89-6	1, 2.5, 5	8.1, 13.8, 13.6	0.4	DMSO	+	+	NA	+
		NA	NA	NA	DMSO	+			
<i>Gold chloride</i>	16903-35-8	NA	NA	0.31	DMSO	+	+	NA	+
		5, 10, 25	21.8, 10.9, 17.9	IDR	DMSO	+			
<i>Lead acetate</i>	15347-57-6	2.5, 5, 10	0.7, 0.8, 1	NC	DMSO	-	-	NA	-
		NA	NA	NA	NA	-			
Manganese chloride	1/5/73	5, 10, 25	1.10, 0.60, 1.00	NC	Petrolatum	-	-	NA	-
<i>Mercuric (II) chloride</i>	7484-94-7	5, 10	19.9, 11.8	0.39	AOO	+	+	NA	+
<i>Nickel chloride</i>	7718-54-9	2.5, 5, 10	1.3, 2.6, 6.6	5.5	30% ETOH	+	+	+	-
		0.5, 1.0, 2.5	1, 1.7, 2.2	NC	DMSO	-			
		1, 2.5, 5	1.5, 2.2, 2.4	NC	DMSO	-			
<i>Nickel sulfate</i>	7786-81-4	0.25, 0.5, 1, 2.5	2, 2.4, 2.8, 3	2.5	1% Pluronic L92	+	+	+	-
		0.25, 0.5, 1, 2.5	0.9, 1.1, 1.6, 1.6	NC	DMF	-			
		0.25, 0.5, 1, 2.5	1.3, 1.4, 1.4, 1.8	4.8	DMSO	+			
		0.5, 1.0, 2.5	1.1, 1.5, 1.5	NC	DMSO	-			
<i>Potassium dichromate</i>	7778-50-9	0.025, 0.05, 0.1, 0.25, 0.5	1.6, 1.4, 3.8, 5.3, 16.1	0.08	DMSO	+	+	+	+
		0.025, 0.05, 0.1, 0.25, 0.5	1.4, 2.5, 9.5, 25.9, 10.1	0.05	DMSO	+			
		0.025, 0.05, 0.1, 0.25	1.21, 1.84, 2.22, 3.39	0.2	DMSO	+			
		0.025, 0.05, 0.1, 0.25, 0.5	1.1, 1.1, 1.4, 4.9, 5.4	0.17	1% Pluronic L92	+			
		0.025, 0.05, 0.1, 0.25, 0.5	2.9, 4.3, 9.1, 15.1, 22.6	0.33	DMF	+			
		0.02, 0.1, 0.5	1.5, 4.5, 15.2	0.06	1% Pluronic L92	+			

LLNA References	Guinea Pig Studies Outcome <sup>1</sup> (GPMT/ BT)	Guinea Pig References	Human Outcome <sup>1</sup>	Human References
Basketter et al. (1999a)	NA	NT	-	Basketter et al. (1999a)
Basketter and Scholes (1992); Basketter et al. (1999a,b)	+	Basketter and Scholes (1992); Basketter et al. (1999a)	+ <sup>7</sup>	Basketter et al. (1999a,b)
Basketter et al. (1994); Mandervelt et al. (1997); Basketter et al. (1999a); Schneider and Akkan (2004)	+	Basketter et al. (1999a)	+ <sup>8,9</sup>	Basketter et al. (1994); Kligman (1966); Basketter et al. (1999b)
Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1999b)	+	Basketter and Scholes (1992)	+ <sup>7,8</sup>	Basketter et al. (1999a,b)
Ikarashi et al. (1992); Griem et al. (2003); Mandervelt et al. (1997); Schneider and Akkan (2004)	NA	NT	+ <sup>8</sup>	Kligman (1966); Griem et al. (2003); Schneider and Akkan (2004)
NP	NA	NT	+ <sup>9</sup>	Kligman (1966)
Basketter and Scholes (1992); Basketter et al. (1999a); ICCVAM (1999)	-	Basketter and Scholes (1992); ICCVAM (1999)	-	Basketter et al. (1999a,b)
Basketter et al. (1999a); Schneider and Akkan (2004)	NA	NT	+ <sup>8,9</sup>	Kligman (1966); Basketter et al. (1999a,b); Schneider and Akkan (2004)
Basketter et al. (1999b); ICCVAM (1999)	NA	NT	-	Basketter et al. (1999a,b)
Basketter et al. (1999a)	NA	NT	-	Basketter et al. (1999a,b)
Basketter et al. (1994); Basketter et al. (1999a); Schneider and Akkan (2004)	+	Magnusson and Kligman (1969); Basketter et al. (1999a)	+ <sup>7,8,9</sup>	Kligman (1966); Marzulli and Maibach (1974); Magnusson and Kligman (1969); Basketter et al. (1994); Basketter et al. (1999a,b)
Basketter and Scholes (1992); Gerberick et al. (1992); Basketter et al. (1999a,b); ICCVAM (1999); Griem et al. (2003)	+	Hicks et al. (1979); Goodwin et al. (1981); Möller (1984); Wahlberg and Boman (1985); Basketter and Scholes (1992); Basketter et al. (1999b); ICCVAM (1999)	+	Vandenberg and Epstein (1963); Goodwin et al. (1981); Menne (1994); Basketter et al. (1999a,b); Griem et al. (2003)
Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1999a); Ryan et al. (2000, 2002); Griem et al. (2003)	+	Magnusson and Kligman (1969); Bourrinet et al. (1979); Maurer et al. (1979); Wahlberg and Boman (1985); Gad et al. (1986); Basketter and Scholes (1992)	+ <sup>7,8</sup>	Magnusson and Kligman (1969); Marzulli and Maibach (1976); Bourrinet et al. (1979); Gad et al. (1986); Basketter et al. (1994); Uter et al. (1995); Basketter et al. (1999a,b); Griem et al. (2003)
ECPA LLNA Project Report <sup>5</sup> ; NTP Study <sup>6</sup> ; Kimber et al. (1991); Basketter and Scholes (1992); Basketter et al. (1994); Kimber et al. (1995); Basketter et al. (1999a,b); Ryan et al. (2002); Schneider and Akkan (2004); Basketter and Kimber (2006)	+	Magnusson and Kligman (1969); Goodwin et al. (1981); Gad et al. (1986); Kimber et al. (1991); Basketter and Scholes (1992); Kimber et al. (2003)	+ <sup>7,8,9</sup>	Kligman (1966); Magnusson and Kligman (1969); Marzulli and Maibach (1976); Goodwin et al. (1981); Basketter et al. (1994); Basketter et al. (1999a,b); Schneider and Akkan (2004); Basketter and Kimber (2006)

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	CASRN	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	Vehicle	LLNA <sup>1</sup> Result	Overall LLNA Result <sup>1,2</sup>	Overall LLNA Result <sup>1,2,3</sup> (Aqueous Metals)	Overall LLNA Result <sup>1,2,3</sup> (Nonaqueous Metals)
<i>Potassium dichromate (continued)</i>	7778-50-9	0.02, 0.1, 0.5	1.06, 1.04, 5.55	0.3	1% Pluronic L92	+	+	+	+
		0.02, 0.1, 0.5	2.4, 2.9, 7.9	0.11	1% Pluronic L92	+			
		0.02, 0.1, 0.5	1.4, 1.8, 7.8	0.18	1% Pluronic L92	+			
		0.02, 0.1, 0.5	1.7, 1.5, 4.1	0.33	1% Pluronic L92	+			
		0.025, 0.05, 0.1, 0.25, 0.5	1.1, 1.3, 2.3, 5.1, 13.1	0.15	DMSO	+			
		0.1, 0.25, 0.5	3.5, 10.2, 10.4	0.03	DMSO	+			
		NA	NA	0.46	NA	+			
		0.1, 0.25, 0.5	7.9, 22.6, 33.6	0.07	DMSO	+			
		0.1, 0.25, 0.5	1.8, 5.1, 6.9	0.15	DMSO	+			
		0.1, 0.25, 0.5	NA, 8.8, 10.1	0.01	DMSO	+			
		0.1, 0.25, 0.5	2.0, 4.4, 5.4	0.17	DMSO	+			
		0.025, 0.05, 0.1, 0.25, 0.5	1.7, 2.9, 4.5, 10.4, 19.1	0.058	DMSO	+			
		0.025, 0.05, 0.1, 0.25, 0.5	1.2, 2.1, 3.4, 4.5, 11.2	0.132	DMSO	+			
		0.025, 0.05, 0.1, 0.25, 0.5	1.9, 1.7, 2.2, 5.9, 13.0	0.122	DMSO	+			
0.025, 0.05, 0.1, 0.25, 0.5	1.6, 1.4, 3.8, 5.3, 16.1	0.126	DMSO	+					
0.025, 0.05, 0.1, 0.25, 0.5	NA	0.08	NA	+					
Tin chloride	NA	5, 10, 25	4.1, 6.5, 6.3	3.6	AOO	+	+	NA	+
<i>Zinc sulfate</i>	7730-02-0	5, 10, 25	1.3, 2, 2.3	NC	DMSO	-	+	NA	-
		NA	NA	NA	NA	+			

<sup>1</sup> (+) = sensitizer; (-) = nonsensitizer

<sup>2</sup> Overall LLNA result based on "weight-of-evidence" with the majority and/or most severe result applicable to all chemicals except for nickel chloride.

<sup>3</sup> An aqueous vehicle is any vehicle containing at least 20% water. Conversely, a nonaqueous vehicle is any vehicle containing less than 20% water.

<sup>4</sup> Bold and italicized text represents the 11 metals that were recorded in the ICCVAM LLNA Evaluation Report (ICCVAM 1999).

<sup>5</sup> LLNA Project Report was provided by the European Crop Protection Association (ECPA).

<sup>6</sup> National Toxicology Program (NTP) data were provided by D. Germolec.

<sup>7</sup> Data obtained from the Human Patch Test Allergen

<sup>8</sup> Data obtained from the Human Maximization Test

<sup>9</sup> Data obtained from the Human Repeat Insult Patch Test

LLNA References	Guinea Pig Studies Outcome <sup>1</sup> (GPMT/BT)	Guinea Pig References	Human Outcome <sup>1</sup>	Human References
<p>ECPA LLNA Project Report<sup>5</sup>; NTP Study<sup>6</sup>; Kimber et al. (1991); Basketter and Scholes (1992); Basketter et al. (1994); Kimber et al. (1995); Basketter et al. (1999a,b); Ryan et al. (2002); Schneider and Akkan (2004); Basketter and Kimber (2006)</p>	<p>+</p>	<p>Magnusson and Kligman (1969); Goodwin et al. (1981); Gad et al. (1986); Kimber et al. (1991); Basketter and Scholes 1992); Kimber et al. (2003)</p>	<p>+<sup>7,8,9</sup></p>	<p>Kligman (1966); Magnusson and Kligman (1969); Marzulli and Maibach (1976); Goodwin et al. (1981); Basketter et al. (1994); Basketter et al. (1999a,b); Schneider and Akkan (2004); Basketter and Kimber (2006)</p>
<p>Basketter et al. (1999b)</p>	<p>NA</p>	<p>NT</p>	<p>+</p>	<p>Basketter et al. (1999a,b)</p>
<p>Basketter et al. (1999a); ICCVAM (1999)</p>	<p>NA</p>	<p>NT</p>	<p>-</p>	<p>Basketter et al. (1999a,b)</p>

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## **Annex IV**

### **Available Data and Information for Substances in Aqueous Solutions Tested in the LLNA**

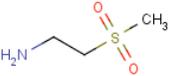
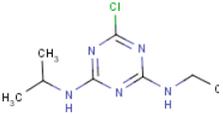
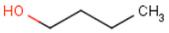
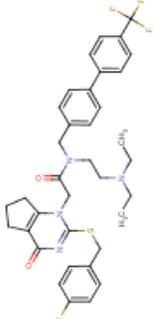
IV-1 Physicochemical Properties and Chemical Classes of Substances Tested in Aqueous Solutions in the LLNA .....	D-173
IV-2 Substances Tested in Aqueous Solutions in the LLNA – Comparative Data.....	D-195
IV-3 Medical Device Eluates Tested in Aqueous Solutions in the LLNA – Comparative Data ..	D-211

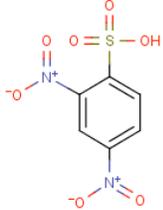
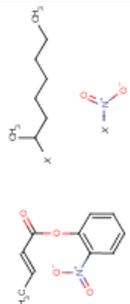
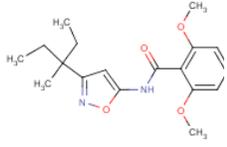
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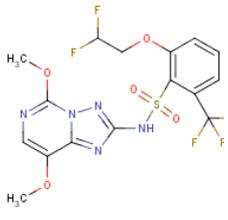
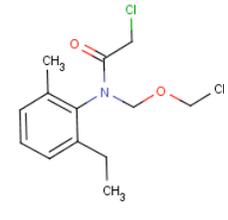
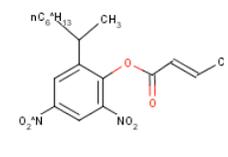
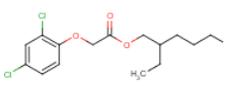
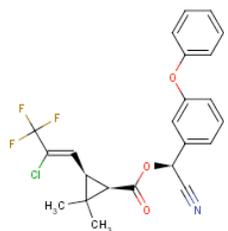
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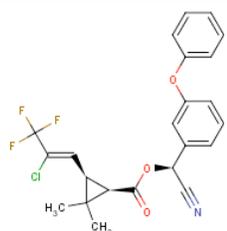
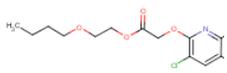
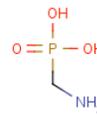
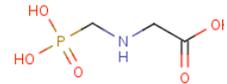
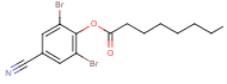
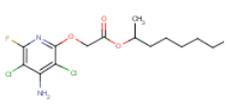
### **Physicochemical Properties and Chemical Classes of Substances Tested in Aqueous Solutions in the LLNA**

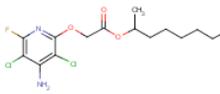
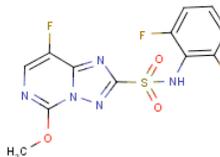
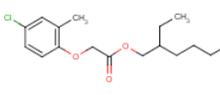
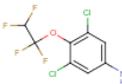
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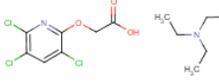
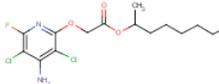
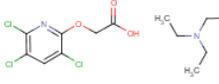
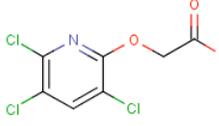
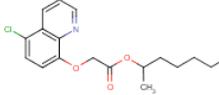
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
AE F016382 00 TK71 A101	NA	NA	NA	NA	NA	Formulation	NA
A SC600	NA	NA	NA	NA	NA	Formulation	NA
2-Aminoethyl-methylsulfone	Ethanamine, 2-(methylsulfonyl)-	49773-20-8	159.63	NA	Solid	Sulfur Compounds	
Atrazine	Atrazine SC 1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine	1912-24-9	215.68	2.82	Solid	Heterocyclic Compounds	
BASF #1	NA	NA	NA	NA	Emulsion	NA	NA
BASF #2	NA	NA	NA	NA	Emulsion	NA	NA
BASF #4	NA	NA	NA	NA	Emulsion	NA	NA
BASF #5	NA	NA	NA	NA	Suspension	NA	NA
BASF #6	BAS 493 05 F	NA	NA	NA	Dispersion	NA	NA
BASF SC-1	Suspension concentrate 1	NA	NA	NA	Emulsion	NA	NA
BASF SE-1	Suspo-emulsion 1	NA	NA	NA	Emulsion	NA	NA
1-Butanol	n-Butyl alcohol	71-36-3	74.12	1.06	Liquid	Alcohols; Lipids	
D EC25	NA	NA	NA	NA	NA	Formulation	NA
D EW 15	NA	NA	NA	NA	NA	Formulation	NA
n-[2-(diethylamino)ethyl]-2-[[[4-fluorophenyl)methyl]thio]-4,5,6,7-tetrahydro-4-oxo-n-[[4-(trifluoromethyl)-[1,1'-biphenyl]-4-yl]methyl]-1h-cyclopentapyrimidine-1-acetamide	Darapladib	356057-34-6	666.78	NA	Solid	Pharmaceutical Intermediate	

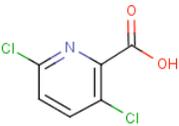
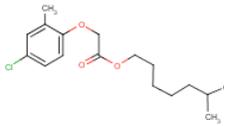
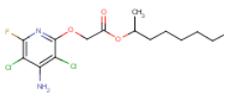
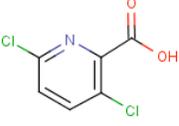
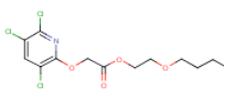
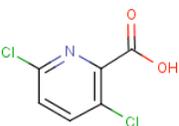
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
1,4-Dihydroquinone	Hydroquinone p-hydroquinone	123-31-9	110.11	1.17	Solid	Phenols	
2,4-Dinitrobenzene sulfonic acid	2,4-Dinitrophenyl-sulfonic acid	89-02-1	248.17	-1.53	Solid	Hydrocarbons, Cyclic	
Dinocap	Butenoic acid, 2-(or 4)-isooctyl-4,6(or 2,6)-dinitrophenyl ester(9CI) Crotonic acid, 2(or 4)-(1-methylheptyl)-4,6(or 2,6)-dinitrophenylester	39300-45-3	364.39	5.76	Liquid	Nitro Compounds; Hydrocarbons, Cyclic	
EXP 10810 A	NA	NA	NA	NA	NA	Formulation	NA
EXP 11120 A	NA	NA	NA	NA	NA	Formulation	NA
FAR01042-00	NA	NA	NA	NA	NA	Formulation	NA
FAR01060-00	NA	NA	NA	NA	NA	Formulation	NA
F & Fo WG 50 + 25	NA	NA	NA	NA	NA	Formulation	NA
Formaldehyde	Formalin	50-00-0	30.03	0.33	Liquid	Aldehydes	
Formulation 1	Isoxaben	82558-50-7	332.40	NA	Liquid	Formulation	

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 10	22.9% w/w dithiopyr	97886-45-8	401.42	NA	Liquid	Formulation	
Formulation 11	0.31 wt.% penoxsulam, 84.2 wt.% acetochlor	219714-96-2 34256-82-1	483.37 269.77	NA	Liquid	Formulation	
							
Formulation 12	34.7% w/w 2,4-dinitro-6-(1-methylheptyl)-phenyl crotonate DE-126	6119-92-2	364.40	NA	Liquid	Formulation	
Formulation 13	87.6% w/w 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester 2,4-D-2-ethylhexyl	1928-43-4	333.25	NA	Liquid	Formulation	
Formulation 14	1.5 wt.% gamma-haloethrin Nexide Fentrol	76703-62-3	449.85	NA	Liquid	Formulation	

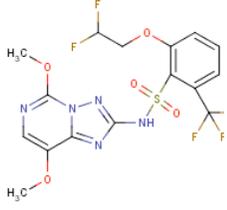
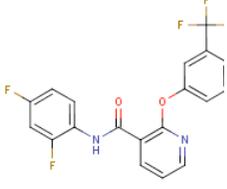
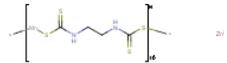
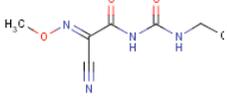
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 15	5.8 wt.% gamma-cyhalothrin Nexide Fentrol	76703-62-3	449.85	NA	Liquid	Formulation	
Formulation 16	85.3% w/w triclopyr butoxyethyl ester	64470-88-8	356.63	NA	Liquid	Formulation	
Formulation 17	50.8% wt/wt glyphosate dimethyl- ammonium salt (active ingredient) 40.1% wt/wt glyphosate (acid equivalent) 8.3% w/w Geronol CF/AS 30 (ammonium adjuvant)	1066-51-9 1071-83-6	111.04 169.02	NA	Liquid	Formulation	
							
Formulation 19	37.1 wt.% bromoxynil octanoate 9.23 wt.% fluroxypyr-1- methylheptyl	1689-99-2 81406-37-3	403.11 367.25	NA	Liquid	Formulation	
							

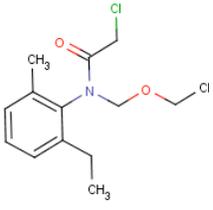
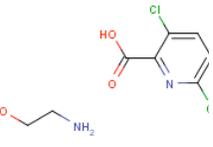
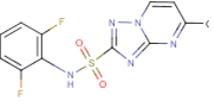
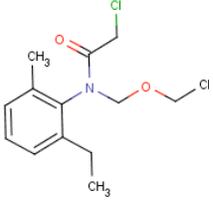
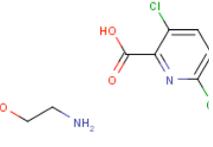
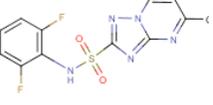
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 2	14.2% w/w fluroxypyr - meptyl 0.22% w/w florasulam	81406-37-3 145701-23-1	367.25 359.29	NA	Liquid	Formulation	
							
Formulation 20	0.39 wt.% Florasulam 41.9 wt.% 2-methyl-4-chlorophenoxyacetic acid 2-ethylhexyl ester (MCPA, 2-ethyl hexyl ester)	145701-23-1 29450-45-1	359.29 312.84	NA	Liquid	Formulation	
							
Formulation 21	50.4% hexaflumuron N-(((3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)-phenyl)amino)-carbonyl)-2,6-difluoro benzamide	86479-06-3	461.14	NA	Liquid	Formulation	 6.3

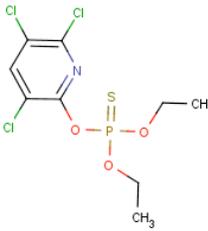
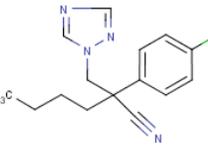
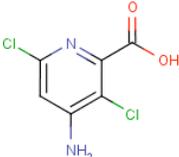
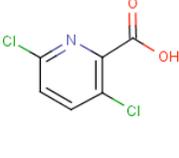
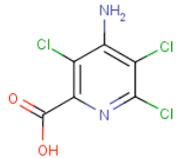
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 22	8.3 wt.% triclopyr triethylammonium 2.8 wt.% fluroxypyr-methyl heptyl ester	57213-69-1 81406-37-3	357.66 367.25	NA	Liquid	Formulation	
							
Formulation 23	16.1 wt.% triclopyr - triethylammonium 11.6 wt.% triclopyr acid	57213-69-1 55335-06-3	357.66	NA	Liquid	Formulation	
							
Formulation 24	8.8 wt.% cloquintocet-mexyl	99607-70-2	335.83	NA	Liquid	Formulation	

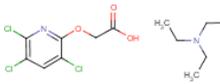
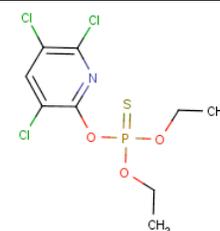
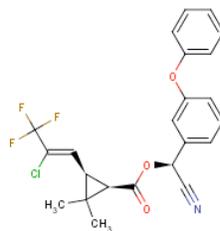
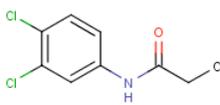
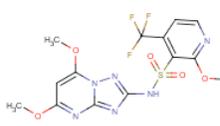
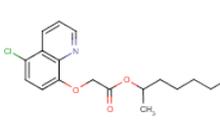
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 25	2.2 wt.% clopyralid 37.7 wt.% MCPA-2-ethyl-hexyl ester 8.2 wt.% fluroxypyr-meptyl	1702-17-62 6544-20-7 81406-37-3	192.00 312.84 367.25	NA	Liquid	Formulation	
							
							
Formulation 26	5.9 wt.% clopyralid 32.9 wt.% triclopyr-butotyl	1702-17-6 64700-56-7	192.00 356.63	NA	Liquid	Formulation	
							
Formulation 27	45.2 wt.% fluroxypyr-meptyl	81406-37-3	192.00	NA	Liquid	Formulation	

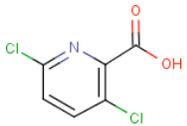
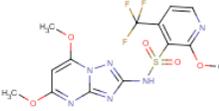
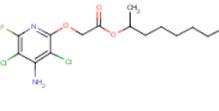
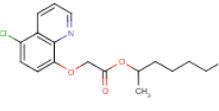
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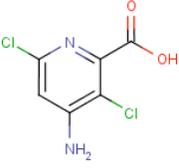
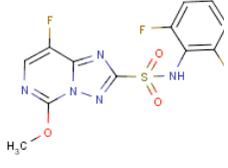
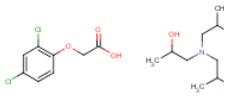
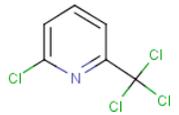
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 28	1.4 wt.% penoxsulam 9.37 wt.% diflufenican	219714-96-2 83164-33-4	483.37 394.30	NA	Liquid	Formulation	
							
Formulation 29	35.6% mancozeb, 4.92% cymoxanil	8018-01-7 57966-95-7	541.1 198.18	NA	Liquid	Formulation	
							

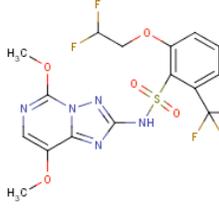
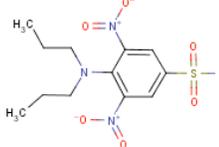
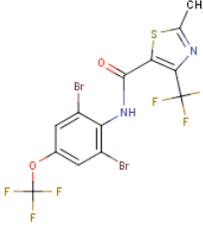
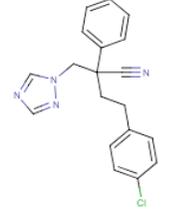
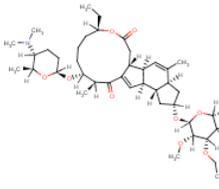
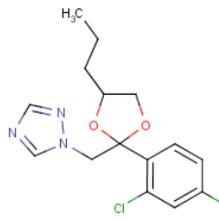
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 3	455 g/L acetochlor 47 g/L clopyralid- olamine 14 g/L flumetsulam	34256-82-1 57754-85-5 98967-40-9	269.77 253.08 325.30	NA	Liquid	Formulation	
							
							
Formulation 30	455 g/L acetochlor 47 g/L clopyralid- olamine 14 g/L flumetsulam	34256-82-1 57754-85-5 98967-40-9	269.77 253.08 325.30	NA	Liquid	Formulation	
							
							

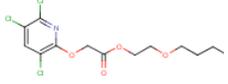
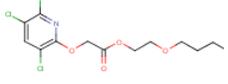
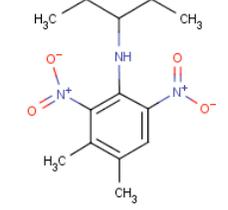
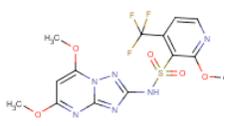
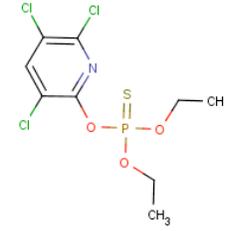
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 31	18.7 wt.% chlorpyrifos	2921-88-2	350.59	NA	Liquid	Formulation	
Formulation 32	11.2 wt.% ((E)-2-(1-methylheptyl)-4,6-dinitrophenyl ester-2-butenic acid 4.68% wt/wt myclobutanil	88671-89-0	288.78	NA	Liquid/Solid	Formulation	
Formulation 33	4.5 wt.% aminopyralid-olamine 27.1 wt.% clopyralid-olamine 8.7 wt.% picloram-olamine 3.5 wt.% aminopyralid 20.6 wt.% clopyralid 7.0 wt.% picloram	150114-71-9 1702-17-6 1918-02-1	207.02 192.00 241.46	NA	Liquid	Formulation	
							
							
Formulation 34	3.0 wt.% aminopyralid	150114-71-9		NA	Liquid	Formulation	

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 35	2.15 wt.% aminopyralid-triisopropanol-ammonium 16.0 wt.% triclopyr-triethylammonium	566191-89-7 57213-69-1	NA 357.66	NA	Liquid	Formulation	NA
							
Formulation 37	30.6 wt.% chlorpyrifos 0.54 wt.% gamma-cyhalothrin	2921-88-2 76703-62-3	350.60 449.85	NA	Liquid	Formulation	
							
Formulation 38	44.4 wt.% propanil	709-98-8	218.08	NA	Liquid	Formulation	
Formulation 39	4.2 wt.% pyroxsulam 8.7 wt.% cloquintocetmexyl	422556-08-9 99607-70-2	434.35 335.83	NA	Liquid	Formulation	
							

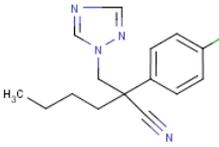
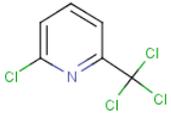
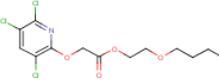
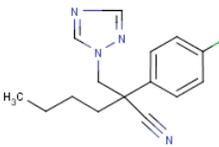
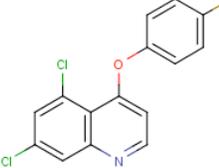
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 4	100 g/L clopyralid mono-ethanolamine salt)	1702-17-6	192.00	NA	Liquid	Formulation	
Formulation 40	1.2 wt.% pyroxsulam 0.21 wt.% florasulam 11.8 wt.% fluroxypyr-meptyl 3.6 wt.% cloquintocetmexyl	422556-08-9 145701-23-1 81406-37-3 99607-70-2	434.35 359.29 367.25 335.83	NA	Liquid	Formulation	
							
							
							

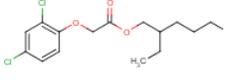
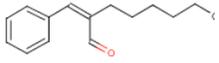
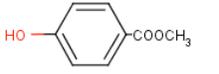
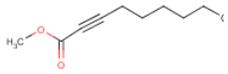
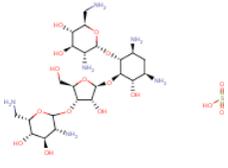
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 41	1.10 wt.% aminopyralid potassium salt 0.47 wt.% florasulam	150114-71-9 145701-23-1	207.02 359.29	NA	Liquid	Formulation	
							
Formulation 42	31 wt.% 2,4-D-triisopropanolamine 1.52 wt.% aminopyralid triisopropanol-ammonium	18584-79-7 150114-71-9	412.31 207.2	NA	NA	Formulation	
							
Formulation 43	17.9 wt.% nitrapyrin	1929-82-4	230.91	NA	NA	Formulation	

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 44	0.12 wt.% penoxsulam 40.38 wt.% oryzalin	219714-96-2 19044-88-3	483.37 346.36	NA	NA	Formulation	
							
Formulation 45	7.53 wt.% thifluzamide 9.42 wt.% fenbuconazole	130000-40-7 114369-43-6	528.06 336.82	NA	NA	Formulation	
							
Formulation 46	5.87 wt.% spinetoram	187166-15-0	760.02	NA	NA	Formulation	
Formulation 47	14.56 wt.% propiconazole	60207-90-1	342.22	NA	NA	Formulation	

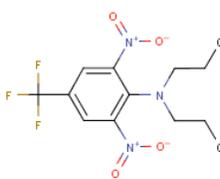
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 49	23.7 wt.% triclopyr BEE	64700-56-7	356.63		Liquid	Formulation	
Formulation 5	3,5,6-trichloro-2-pyridyloxyacetic acid, butoxy ethyl ester Triclopyr-butotyl triclopyr BEE	64700-56-7	356.63		Liquid	Formulation	
Formulation 50	Glyphosate dimethylamine salt Glyphosate dimethyl-ammonium salt	34494-04-7 NA	NA	NA	Liquid	Formulation	NA
Formulation 51	29.6 wt.% pendimethalin 0.51 wt.% pyroxsulam	40487-42-1 422556-08-9	281.31 434.35		Liquid	Formulation	
							
Formulation 53	41.1 wt.% chlorpyrifos	2921-88-2	350.60	NA	Liquid	Formulation	

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 54	t.% glyphosate l-ammonium salt	NA	NA	NA	Liquid	Formulation	NA
Formulation 55	4.6 wt.% myclobutanil	88671-89-0	288.78	NA	Liquid	Formulation	
Formulation 56	20.5 wt.% nitrapyrin	1929-82-4	230.91	NA	Liquid	Formulation	
Formulation 6	Aminopyralid potassium + triclopyr-butotyl form Aminopyralid herbicide	150114-71-9 64700-56-7	207.02	NA	Liquid	Formulation	
							
Formulation 7	45 g/L myclobutanil + 45 g/L quinoxifen)	88671-89-0 124495-18-7	288.78 308.14	NA	Liquid	Formulation	
							

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Formulation 8	81.8% w/w 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester 2,4-D EHE	1928-43-4	333.25	NA	Liquid	Formulation	
Formulation 9	NA	NA	NA	NA	Liquid	Formulation	NA
Fx + Me EW 69	NA	NA	NA	NA	NA	Formulation	NA
Glutaraldehyde	Glutaral	111-30-8	100.12	NA		Aldehydes	
Hexyl cinnamic aldehyde	HCA, alpha-hexylcinnamic aldehyde, alpha-hexyl cinnamaldehyde	101-86-0	216.32	3.77	Liquid	Aldehydes	
Methyl 4-hydroxybenzoate	Methylparaben	99-76-3	152.15	1.28	Solid	Carboxylic Acids	
Methyl 2-nonynoate	Methyl octine carbonate	111-80-8	168.24	2.15	Liquid	Lipids	
Neomycin sulfate	Neomycin, sulfate (salt)	1405-10-3	908.88	NA	Solid	Carbohydrates	

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Oxyfluorfen	Oxirane, mono; ((C12-14-alkyloxy) methyl) derivatives	42874-03-3	361.70	5.21	Solid	Ethers	
Pluronic L92®	NA	NA	NA	NA	NA	NA	NA
Propylene glycol	1,2-dihydroxypropane 1,2-propanediol	57-55-6	76.10	0.43	Liquid	Alcohols	
Quinoxifen	5,7-dichloro-4-(4-fluorophenoxy)-quinoline	124495-18-7	308.14	5.69	Liquid	Heterocyclic Compounds	
Quinoxifen/ Cyproconazole	5,7-dichloro-4-(4-fluorophenoxy) quinoline/ H-1,2,4-triazole-1-ethanol, alpha-(4-chlorophenyl)-alpha-(1-cyclopropylethyl)-	124495-18-7 113096-99-4	308.14 291.78	5.69 3.25	Liquid	Heterocyclic Compounds	
Saturated diglycerin	NA	NA	NA	NA	NA	NA	NA
Sodium lauryl sulfate	Sodium dodecyl sulfate, SLS, SDS, irium	151-21-3	288.38	1.87	Solid	Alcohols, Sulfur Compounds, Lipids	

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow <sup>1</sup>	Physical Form	Chemical Class <sup>2</sup>	Structure <sup>3</sup>
Sodium metasilicate	Silicic acid, disodium salt	6834-92-0	122.063	NA	Solid	Inorganic Chemical, Sodium Compounds, Inorganic Chemical, Silicon Compounds	
Trifluralin	2,6-dinitro-4-trifluoromethyl-N,N-dipropylanilin	1582-09-8	335.28	5.31	NA	Hydrocarbons, Cyclic, Amine	

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

<sup>1</sup> Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: [http://www.syrres.com/esc/est\\_kowdemo.htm](http://www.syrres.com/esc/est_kowdemo.htm).

<sup>2</sup> Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: <http://www.nlm.nih.gov/mesh/meshhome.html>.

<sup>3</sup> Chemical structures, based on CASRN, were obtained from ChemID, available at: <http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp>.

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**Annex IV-2**

**Substances in Aqueous Solutions Tested in the LLNA – Comparative Data**

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## Abbreviations

ACE	Acetone
AL	Any other liquid
AOO	Acetone olive-oil (4:1)
BT	Buehler Test
Conc.	Concentration
CS	Capsule suspension
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
EC	Emulsion concentrate
ECPA	European Crop Protection Association
EW	Emulsion, oil in water
GPMT	Guinea Pig Maximization Test
LLNA	Local Lymph Node Assay
ME	Micro-emulsion
NA	Not available
NC	Not calculated
NT	Not tested
OD	Oil dispersion
PG	Propylene glycol
SC	Suspension concentrate
SE	Suspo-emulsion
SI	Stimulation index
SL	Soluble concentrate
TK	Technical concentrate
WG	Water dispersible granules

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	CASRN	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain
A SC600		NA	10, 25, 50, 100	1.4, 1.8, 2.3, 1.6	NC	1% L92	CBA/J
AE F016382 00 TK71 A101		NA	3.6, 7.1, 17.9, 35.7	1.0, 0.8, 1.0, 1.1	NC	1% L92	CBA/J
2-aminoethyl-methylsulfone	49773-20-8		10, 25, 50	0.4, 0.3, 0.3	NC	0.5% Tween 80/H2O	
Atrazine	1912-24-9	SC	12.5, 25, 50, 75, 100	1.8, 2.8, 3.6, 7.1, 7.3	31.3	1% L92	CBA/J
			7, 33, 100	0.8, 2.9, 3.7	41.4	1% L92	CBA/J
BASF #1		NA	10, 30, 70	2.0, 2.9, 4.9	31.2	1% L92	CBA/Ca
BASF #2		NA	3, 10, 30	0.8, 1.0, 3.0	29.7	1% L92	CBA/J
BASF #4		NA	3, 10, 50	2.4, 2.7, 5.4	14.1	1% L92	CBA/Ca
BASF #5		NA	3, 10, 50	1.6, 1.2, 3.9	36.9	1% L92	CBA/Ca
BASF #6		NA	3, 10, 30	2.7, 9.9, 23.1	0.3	1% L92	CBA/Ca
BASF SC-1		SC	3, 10, 30	0.8, 1.3, 1.9	NC	1% L92	CBA/Ca
BASF SE-1		SE	10, 30, 70	8.0, 17.3, 22.7	5.5	1% L92	CBA/Ca
1-butanol	71-36-3		5, 10, 20	1.6, 1.2, 1.4	NC	H2O	
D EC25®		EC	0.5, 1.0, 2.5	0.6, 0.6, 0.6	NC	1% L92	CBA/Ca
D EW 15		EW	2.5, 5.0, 10.0, 25.0	1.9, 1.5, 2.5, 2.5	NC	1% L92	CBA/J
n-[2-(diethylamino)ethyl]-2-[[[4-(4-fluorophenyl)-methyl]thio]-4,5,6,7-tetrahydro-4-oxo-n-[[4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl]methyl]-1h-cyclopentapyrim-idine-1-acetamide	356057-34-6		5, 10, 25	1.1, 2.4, 12.7	10.8	80% ETOH	
1,4-dihydroquinone	123-31-9		0.05, 0.1, 0.25, 0.5, 1.0	0.7, 1.0, 0.9, 1.9, 1.9	NC	ACE/saline (1:1)	
			0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10	1.4, 0.8, 1.2, 1.3, 1.9, 6.8, 10.9, 11.1	1.3	ACE/saline (1:1)	
2,4-dinitrobenzene sulfonic acid	89-02-1		1, 10, 20	1.7, 1.5, 4.4	15.2	H2O	
			1, 10, 20	0.9, 4.4, 11.6	6.4	1% Pluronic L92/H2O	

LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	GP Call <sup>2</sup>	GP Test	GP Reference	Human Call	Human References
-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
-	GSK <sup>3</sup>	-	NA		NT	NA	NT
+	ECPA LLNA Project Report submitted by Dow Chemical	+	-	GPMT	NA	NA	NT
+	ECPA LLNA Project Report submitted by Dow Chemical						
+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
-	BASF, submitted by C. Hastings	-	-	BT	NA	NA	NT
+	BASF, submitted by C. Hastings	+	-	BT	NA	NA	NT
-	Ryan et al. (2000); Gerberick et al. (2005)	-	NA	NA	NT	-	Ryan et al. (2000)
-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	NA	NA	NT
-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	NA	NA	NT
+	GSK	+	NA	NA	NT	NA	NT
-	Lea et al. (1999)	+	NA	NA	NT	NA	NT
+							
+	Ryan et al. (2002)	+	NA	NA	NT	NA	NT
+							

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	CASRN	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain
Dinocap	39300-45-3	EC	0.8, 4, 21	2.2, 25.8, 14.4	0.9	1% L92	CBA/Ca
			0.8, 4, 20	1.3, 11.5, 15.6	1.3	1% L92	CBA/J
			0.8, 4, 21	2.0, 4.0, 26.7	1.1	1% L92	CBA/J
			0.8, 4, 10	1.3, 4.1, 10.9	2.8	1% L92	CBA/JHsd
			0.8, 4, 10	2.7, 22.9, 40.5	0.8	1% L92	CBA/ CaOlaHsd
EXP 10810 A		NA	10, 25, 50	6.4, 8.4, 9.2	2.1	1% L92	CBA/J
EXP 11120 A		NA	10, 25, 50, 100	1.0, 0.7, 1.6, 6.3	64.9	1% L92	CBA/J
F & Fo WG 50 + 25		WG	2.5, 5.0, 10.0, 25.0	11.7, 12.6, 14.4, 15.2	0.003	1% L92	CBA/J
FAR01042-00		NA	10, 25, 50, 100	1.4, 2.1, 1.4, 2.5	NC	1% L92	CBA/J
FAR01060-00		NA	10, 25, 50, 100	0.4, 0.8, 1.0, 3.6	88.5	1% L92	CBA/J
Formaldehyde	50-00-0		1, 10, 20	1.2, 2.5, 3.6	14.5	H2O	
			1, 10, 20	2, 4.8, 8.8	4.2	1% Pluronic L92/H2O	
			1, 5, 20	1.1, 3.8, 10.6	3.8	1% Pluronic L92/H2O	
			1, 5, 20	1, 2.2, 6.2	8.2	1% Pluronic L92/H2O	
			1, 5, 20	1.6, 2.6, 12	5.6	1% Pluronic L92/H2O	
			1, 5, 20	1.1, 2.5, 4.8	8.3	1% Pluronic L92/H2O	
			1, 5, 20	0.8, 1.3, 4.8	12.3	1% Pluronic L92/H2O	
Formulation 1		SC	5, 20, 80	1.1, 1.3, 1.3	NC	1% L92	BALB/c
Formulation 10		EW	2, 10, 50	1, 1, 5.2	29	1% L92	BALB/c

LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	GP Call <sup>2</sup>	GP Test	GP Reference	Human Call	Human References						
+	ECPA LLNA Project Report submitted by BASF	+	+	BT	NA	NA	NT						
+	ECPA LLNA Project Report submitted by Bayer												
+	ECPA LLNA Project Report submitted by Dow Chemical												
+	ECPA LLNA Project Report submitted by Dupont												
+	ECPA LLNA Project Report submitted by Syngenta/RCC												
+	Bayer Crop Science, submitted by E. Debruyne	+	+	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT						
+	Bayer Crop Science, submitted by E. Debruyne	+	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT						
+	Bayer Crop Science, submitted by E. Debruyne	+	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT						
-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT						
+	Bayer Crop Science, submitted by E. Debruyne	+	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT						
+	Ryan et al. (2002)	+	+	GPMT	ECPA LLNA Project Report; Andersen et al. (1984); Wahlberg and Boman (1985)	+	Kligman (1966); Marzulli and Maibach (1974)						
+													
+	ECPA LLNA Project Report submitted by BASF												
+	ECPA LLNA Project Report submitted by Bayer												
+	ECPA LLNA Project Report submitted by Dow Chemical												
+	ECPA LLNA Project Report submitted by Dupont												
+	ECPA LLNA Project Report submitted by Syngenta/RCC												
-	Submitted by Dow AgroSciences							-	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences							+	NA	NA	NA	NA	NT

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	CASRN	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain
Formulation 11		OD	0.4, 2, 10	1.2, 1.2, 3.2	9.2	1% L92	BALB/c
Formulation 12		EC	0.2, 1, 5	1.2, 3, 11.6	1	1% L92	BALB/c
Formulation 13		EC	1, 5, 25	1.2, 1.3, 10.4	8.7	1% L92	BALB/c
Formulation 14		CS	0.1, 1, 10	0.7, 0.7, 1.3	NC	1% L92	BALB/c
Formulation 15		CS	0.2, 1, 5	0.8, 1.4, 3.2	4.6	1% L92	BALB/c
Formulation 16		EC	1, 5, 25	1.3, 2.2, 12.3	6.6	1% L92	BALB/c
Formulation 17		SL	5, 25, 75	1.7, 9.3, 18.5	8.4	1% L92	BALB/c
Formulation 19		EC	1, 10, 25, 50	4.9, 7.9, 20, 50.5	0.23	1% L92	BALB/c
Formulation 2		SE	5, 20, 80	2, 3.4, 15.8	15.7	1% L92	BALB/c
Formulation 20		SE	2, 10, 50	1.1, 1.4, 3.3	43.7	1% L92	BALB/c
Formulation 21		TK	5, 25, 100	1.3, 1.2, 1.9	NC	1% L92	BALB/c
Formulation 22		ME	5, 25, 100	1.2, 1.4, 5.8	52.3	1% L92	BALB/c
Formulation 23		SL	5, 25, 100	0.8, 1, 1	NC	1% L92	BALB/c
Formulation 24		OD	2, 10, 50	1.4, 4.1, 11.7	6.7	1% L92	BALB/c
Formulation 25		EC	1, 5, 25	1.8, 2.6, 14.7	5.6	1% L92	BALB/c
Formulation 26		EC	1, 5, 25	1, 1, 4	18	1% L92	BALB/c
Formulation 27		EC	1, 5, 25	2.3, 2.5, 11.2	6.1	1% L92	BALB/c
Formulation 28		SC	5, 25, 100	1, 1, 1.1	NC	1% L92	BALB/c
Formulation 29		SC	5, 25, 100	1.8, 1.6, 1.5	NC	1% L92	CBA/J
Formulation 3		SC	5, 20, 80	1, 1.2, 1.7	NC	1% L92	BALB/c
Formulation 30		EW	5, 25, 100	1.8, 7.2, 13.6	9.4	1% L92	CBA/J
Formulation 31		CS	5, 25, 100	1, 1.9, 1.8	NC	1% L92	CBA/J
Formulation 32		EC	5, 25, 100	6.5, 44.7, 69.3	4.3	1% L92	CBA/J
Formulation 33		SL	5, 25, 100	0.7, 1.4, 1.3	NC	1% L92	CBA/J
Formulation 34		SL	5, 25, 100	1.9, 1.4, 1.5	NC	1% L92	CBA/J
Formulation 35		SL	5, 25, 100	1.1, 1.2, 1.3	NC	1% L92	CBA/J
Formulation 37		EC	1, 5, 15	1.4, 2.7, 7.5	5.6	1% L92	CBA/J
Formulation 38		EC	5, 25, 100	1.1, 4.6, 12.7	15.9	1% L92	CBA/J
Formulation 39		OD	1, 5, 25	1.7, 2.5, 3.3	17.5	1% L92	CBA/J

LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	GP Call <sup>2</sup>	GP Test	GP Reference	Human Call	Human References
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	-	NA	Submitted by Dow AgroSciences	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	-	NA	Submitted by Dow AgroSciences	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	CASRN	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain
Formulation 4		SL	5, 20, 80	1.4, 1.1, 1.2	NC	1% L92	BALB/c
Formulation 40		OD	1, 5, 25	1.8, 2.8, 5.7	6.4	1% L92	CBA/J
Formulation 41		SE	5, 25, 100	1.9, 1.9, 4.7	54.5	1% L92	CBA/J
Formulation 42		SL	10, 50, 100	1.2, 2.0, 3.1	95.5	1% L92	CBA/J
Formulation 43		CS	5, 25, 75	NA	NC	1% L92	CBA/J
Formulation 44		SC	5, 25, 100	NA	NC	1% L92	CBA/J
Formulation 45		SC	5, 25, 100	NA	NC	1% L92	CBA/J
Formulation 46		SC	5, 25, 100	NA	NC	1% L92	CBA/J
Formulation 47		EW	5, 25, 100	2.1, 2.1, 6.0	42.3	1% L92	CBA/J
Formulation 49		AL	5, 25, 100	0.7, 1.4, 4.7	61.4	1% L92	CBA/J
Formulation 5		EC	3, 10, 30	1.4, 4, 11.5	7.3	1% L92	BALB/c
Formulation 50		SL	5, 25, 100	1.2, 1.2, 14.7	35	1% L92	CBA/J
Formulation 51		OD	5, 25, 100	1.6, 4.5, 2.9	14.7	1% L92	CBA/J
Formulation 53		EW	2.5, 7.5, 15	1.5, 3.2, 6.7	6.9	1% L92	CBA/J
Formulation 54		SL	5, 25, 100	1.3, 1.2, 2.3	NC	1% L92	CBA/J
Formulation 55		EW	5, 25, 100	1.5, 2.5, 3.7	56.3	1% L92	CBA/J
Formulation 56		SL	5, 25, 100	3.3, 6.1, 3.9	4.2	1% L92	CBA/J
Formulation 6		EW	5, 20, 80	1.3, 2.7, 11.6	23.7	1% L92	BALB/c
Formulation 7		SC	20, 80, 100	1, 1.9, 3.2	96.9	1% L92	BALB/c
		SC	5, 20, 80	2.6, 1.4, 3.2	73.3	1% L92	BALB/c
Formulation 8		EC	1, 5, 25	0.9, 1.1, 7.3	11.1	1% L92	BALB/c
Formulation 9		SC	4, 20, 80	1.1, 1.7, 1.3	NC	1% L92	BALB/c
Fx + Me EW 69		EW	5.0, 10.0, 25.0, 50.0	0.8, 1.6, 3.0, 8.6	25.2	1% L92	CBA/J
Glutaraldehyde	111-30-8		3.1, 6.2, 12.5	9.8, 21.4, 22.9	2.1	DMF/H2O (1/1)	

LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	GP Call <sup>2</sup>	GP Test	GP Reference	Human Call	Human References
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	-	NA	Submitted by Dow AgroSciences	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
+	Submitted by Dow AgroSciences	+	-	BT	Submitted by Dow AgroSciences	NA	NT
NA						NT	
+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
+	Bayer Crop Science, submitted by E. Debruyne	+	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
+	Gerberick et al. (1992)	+	NA	NA	NT	NA	NT

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	CASRN	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain
Hexyl cinnamic aldehyde	101-86-0		3, 10, 30	1.2, 4.6, 18	6.7	1% Pluronic L92/H2O	
			3, 10, 30	1.9, 4.2, 9.2	7	1% Pluronic L92/H2O	
			3, 10, 30	1.9, 2.2, 10.3	12	1% Pluronic L92/H2O	
			3, 10, 30	1.1, 2.5, 15.6	10.8	1% Pluronic L92/H2O	
			3, 10, 30	1.3, 2.2, 4.3	17.6	1% Pluronic L92/H2O	
Methyl 4-hydroxybenzoate	99-76-3		10, 25, 50	0.8, 0.9, 0.8	NC	80% ETOH	
Methyl 2-nonynoate	111-80-8		5, 10, 20	10.4, 17.7, 24.4	2.5	80% ETOH	
			NA	NA	2.5	80% ETOH	
Neomycin sulfate	1405-10-3		0.5, 1, 2	0.9, 0.9, 0.9	NC	25% ETOH	
Oxyfluorfen	42874-03-3	EC	1, 7, 33	0.81, 1.4, 4.9	30.8	1% L92	CBA/Ca
			1, 7, 33	0.9, 1.4, 2.8	NC	1% L92	CBA/J
			1, 7, 33	0.3, 0.9, 2.3	NC	1% L92	CBA/J
			1, 7, 33	1.1, 1.5, 3.1	30.8	1% L92	CBA/JHsd
			1, 7, 33	1.2, 1.2, 5.4	18.1	1% L92	CBA/ CaOlaHsd
Pluronic L92®	NA		1, 2.5, 5, 10, 25, 50	1.3, 1.0, 1.0, 0.8, 0.8, 2.0	NC	H2O	
Propylene glycol	57-55-6		50, 100	1.2, 1.6	NC	H2O	

LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	GP Call <sup>2</sup>	GP Test	GP Reference	Human Call	Human References
+	ECPA LLNA Project Report submitted by BASF	+	NA	NA	NT	NA	NT
+	ECPA LLNA Project Report submitted by Bayer						
+	ECPA LLNA Project Report submitted by Dow Chemical						
+	ECPA LLNA Project Report submitted by Dupont						
+	ECPA LLNA Project Report submitted by Syngenta/RCC						
-	Ryan et al. (2000)	-	NA	NA	NT	NA	Ryan et al. (2000)
+	Ryan et al. (2000); Basketter et al. (2005); Gerberick et al. (2005)	+	NA	NA	NT	+ <sup>7</sup>	Ryan et al. (2000); Basketter et al. (2005)
+							
-	Basketter et al. (1994); Basketter et al. (1999a); Gerberick et al. (1992); Schneider and Akkan (2004)	-	+	BT	Gad et al. (1986); Basketter et al. (1999a)	+ <sup>7,8</sup>	Basketter et al. (1994); Kligman (1966); Magnusson and Kligman (1969); Marzulli and Maibach (1974); Schneider and Akkan (2004)
+	ECPA LLNA Project Report submitted by BASF	+	-	GPMT	ECPA LLNA Project Report submitted by: Dow Chemical	NA	NT
-	ECPA LLNA Project Report submitted by Bayer						
-	ECPA LLNA Project Report submitted by Dow Chemical						
+	ECPA LLNA Project Report submitted by Dupont						
+	ECPA LLNA Project Report submitted by Syngenta/RCC						
-	Ryan et al. (2002)	-	NA	NA	NT	NA	NT
-	Basketter et al. (1998); Basketter et al. (1999a); Gerberick et al. (2005)	-	-	GPMT	Guillot et al. (1983); Wahlberg and Boman (1985); Gad et al. (1986); Basketter et al. (1999a)	+ <sup>8</sup>	Kligman (1966); Basketter et al. (1998); Basketter et al. (1999a)

ICCVAM LLNA Applicability Domain Evaluation Report

Substance Name	CASRN	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain
Quinoxifen	124495-18-7	SC	7, 33, 100	1.1, 0.7, 0.8	NC	1% L92	CBA/J
Quinoxifen/ Cyproconazole	124495-18-7 / 113096-99-4	NA	7, 33, 100	2.1, 10.7, 20.3	9.8	1% L92	CBA/Ca
			7, 33, 100	1.2, 7.2, 12.4	14.8	1% L92	CBA/J
			7, 33, 100	0.4, 3.8, 2.0	26.9	1% L92	CBA/J
			7, 33, 100	1.4, 2.0, 6.2	49.8	1% L92	CBA/JHsd
			7, 33, 100	1.3, 6.5, 13.6	15.5	1% L92	CBA/ CaOlaHsd
			12.5, 25, 50, 75, 100	2, 2.3, 8.6, 15.8, 30.1	27.8	1% L92	CBA/J
Saturated diglycerin	NA		25, 50, 100	1.4, 2.1, 1.9	NC	ETOH/H2O	
Sodium lauryl sulfate	151-21-3		5, 10, 25	3.0, 4.8, 8.5	4.9	1% Pluronic L92/H2O	
Sodium metasilicate	6834-92-0		2, 4, 6	0.9, 1.4, 1.3	NC	15% ETOH	
Trifluralin	1582-09-8	EC	7, 33, 100	6.0, 30.0, 75.2	5.8	1% L92	CBA/Ca
			7, 33, 100	1.9, 8.7, 25.7	11.2	1% L92	CBA/J
			7, 33, 100	3.1, 26.3, 61.5	7	1% L92	CBA/J
			7, 33, 100	1.0, 7.0, 16.1	15.6	1% L92	CBA/JHsd
			7, 33, 100	1.8, 8.2, 20.5	11.9	1% L92	CBA/ CaOlaHsd

<sup>1</sup> Overall LLNA result based on the majority and/or most severe result: "+" = sensitizer; "-" = nonsensitizer.

<sup>2</sup> BT or GPMT result

<sup>3</sup> Data from GlaxoSmithKline (GSK) were submitted by M.J. Olson

<sup>4</sup> Netherlands Organisation for Applied Scientific Research (TNO) Report was submitted by the Comité Européen des Agents de Surface et de leurs Intermédiaires Organiques (European Committee of Organic Surfactants and Their Intermediates) submitted by K. Skirda.

<sup>5</sup> Berufsgenossenschaftliches Institut für Arbeitsschutz (BGIA - German Institute for Occupational Safety and Health) Report was submitted by H.W.Vohr.

<sup>6</sup> National Toxicology Program (NTP) data were submitted by D. Germolec.

LLNA Result <sup>1</sup>	LLNA Reference	Overall LLNA Result <sup>1</sup> (Majority)	GP Call <sup>2</sup>	GP Test	GP Reference	Human Call	Human References
-	ECPA LLNA Project Report submitted by Dow Chemical	-	-	BT	ECPA LLNA Project Report submitted by: Dow Chemical	NA	NT
+	ECPA LLNA Project Report submitted by BASF	+	+	BT	ECPA LLNA Project Report submitted by Dow Chemical	NA	NT
+	ECPA LLNA Project Report submitted by Bayer						
+	ECPA LLNA Project Report submitted by Dow Chemical						
+	ECPA LLNA Project Report submitted by Dupont						
+	ECPA LLNA Project Report submitted by Syngenta/RCC						
+	ECPA LLNA Project Report submitted by Dow Chemical						
-	TNO Report <sup>4</sup>	-	NA	NA	NT	NA	NT
+	BGIA Project FP251 <sup>5</sup>	+	NA	NA	NT	NA	Kligman (1966)
-	NTP Study <sup>6</sup>	-	NA	NA	NT	NA	NT
+	ECPA LLNA Project Report submitted by BASF	+	-	BT	ECPA LLNA Project Report submitted by Dow Chemical	NA	NT
+	ECPA LLNA Project Report submitted by Bayer						
+	ECPA LLNA Project Report submitted by Dow Chemical						
+	ECPA LLNA Project Report submitted by Dupont						
+	ECPA LLNA Project Report submitted by Syngenta/RCC						

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**Annex IV-3**

**Medical Device Eluates Tested in Aqueous Solutions in the LLNA – Comparative Data**

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Project #	NS Negative Control (dpm) <sup>1</sup>	NS Extract <sup>2</sup> (dpm) <sup>1</sup>	SI	LLNA Result <sup>4</sup>	NS Extract (spiked) <sup>3</sup> (dpm) <sup>1</sup>	SI	LLNA Result <sup>4</sup>	NS Positive Control <sup>5</sup> (dpm) <sup>1</sup>	SI	LLNA Result <sup>4</sup>
1	133.3	221.6	1.7	-	1,704.1	12.8	+	20,206.3	151.6	+
2	165.2	236.3	1.4	-	2,209.5	13.4	+	5,703.7	34.5	+
3	331.7	376.7	1.1	-	895.1	2.7	+	4,101.7	12.4	+
4	197.8	186.9	0.9	-	1,056.8	5.3	+	2,664.1	13.5	+
5	244.3	195.1	0.8	-	1,311.0	5.4	+	1,851.8	7.6	+
6	381.3	375.0	1.0	-	1,125.5	3.0	+	3,920.6	10.3	+
7	233.7	234.6	1.0	-	456.7	2.0	+	2,396.6	10.3	+
8	314.5	329.4	1.0	-	1,515.1	4.8	+	3,397.2	10.8	+
9	420.6	191.9	0.5	-	1,261.8	3.0	+	2,479.5	5.9	+
10	215.3	194.3	0.9	-	1,822.0	8.5	+	3,736.4	17.4	+
11	175.6	170.9	1.0	-	1,259.9	7.2	+	2,124.1	12.1	+
12	726.6	424.6	0.6	-	1,940.8	2.7	+	8,907.2	12.3	+
13	285.6	377.3	1.3	-	1,586.3	5.6	+	2,819.0	9.9	+
14	390.9	329.7	0.8	-	3,296.0	8.4	+	8,521.3	21.8	+
15	789.2	304.5	0.4	-	1,577.9	2.0	+	4,331.8	5.5	+
16	379.3	849.0	2.2	-	3,824.0	10.1	+	10,466.7	27.6	+
17	461.9	603.9	1.3	-	1,075.3	2.3	+	4,774.0	10.3	+
18	871.9	945.0	1.1	-	8,875.3	10.2	+	10,247.9	11.8	+
19	332.8	316.4	1.0	-	2,719.8	8.2	+	4,534.5	13.6	+
20	198.5	224.4	1.1	-	790.1	4.0	+	3,101.7	15.6	+
21	759.2	902.9	1.2	-	2,323.1	3.1	+	5,725.8	7.5	+
22	261.7	276.9	1.1	-	3,604.0	13.8	+	4,531.7	17.3	+
23	1,513.3	992.2	0.7	-	3,788.0	2.5	+	11,505.5	7.6	+
24	1,453.9	865.9	0.6	-	7,543.1	5.2	+	9,564.9	6.6	+
25	825.3	438.1	0.5	-	5,262.8	6.4	+	9,808.9	11.9	+
26	777.5	893.8	1.1	-	5,173.9	6.7	+	11,150.1	14.3	+
27	595.5	503.9	0.8	-	5,840.9	9.8	+	7,727.1	13.0	+
28	370.4	601.3	1.6	-	7,842.8	21.2	+	13,347.0	36.0	+

ICCVAM LLNA Applicability Domain Evaluation Report

Project #	NS Negative Control (dpm) <sup>1</sup>	NS Extract <sup>2</sup> (dpm) <sup>1</sup>	SI	LLNA Result <sup>4</sup>	NS Extract (spiked) <sup>3</sup> (dpm) <sup>1</sup>	SI	LLNA Result <sup>4</sup>	NS Positive Control (dpm) <sup>5</sup>	SI	LLNA Result <sup>4</sup>
29	1,318.8	1,475.9	1.1	-	5,706.1	4.3	+	12,477.5	9.5	+
30	1,177.9	2,268.3	1.9	-	7,555.7	6.4	+	9,089.1	7.7	+
31	558.6	784.5	1.4	-	4,850.6	8.7	+	6,124.0	11.0	+
32	944.5	1,018.5	1.1	-	6,922.7	7.3	+	10,209.2	10.8	+
33	1,243.8	691.6	0.6	-	3,475.9	2.8	+	8,882.2	7.1	+
34	872.1	867.8	1.0	-	11,532.6	13.2	+	10,109.2	11.6	+
35	1,009.6	525.4	0.5	-	4,753.8	4.7	+	7,112.1	7.0	+
36	684.3	1,224.8	1.8	-	6,559.5	9.6	+	9,624.1	14.1	+
37	1,282.0	1,258.5	1.0	-	16,400.3	12.8	+	19,533.0	15.2	+
38	529.0	1,003.9	1.9	-	3,588.5	6.8	+	8,043.5	15.2	+
39	207.7	443.4	2.1	-	2,016.1	9.7	+	4,094.1	19.7	+
40	518.5	904.9	1.7	-	2,755.1	5.3	+	4,874.7	9.4	+
41	862.9	877.3	1.0	-	4,171.6	4.8	+	7,437.7	8.6	+
42	599.8	808.0	1.3	-	3,174.3	5.3	+	7,399.7	12.3	+
43	1,134.8	852.4	0.8	-	8,424.8	7.4	+	10,621.8	9.4	+
44	769.5	636.2	0.8	-	4,422.1	5.7	+	10,450.4	13.6	+
45	389.2	600.8	1.5	-	3,677.9	9.4	+	9,347.1	24.0	+
46	674.1	662.3	1.0	-	2,292.3	3.4	+	3,332.9	4.9	+
47	269.1	584.0	2.2	-	1,557.4	5.8	+	5,865.7	21.8	+
48	602.8	930.0	1.5	-	4,184.8	6.9	+	10,186.1	16.9	+

Abbreviations: dpm = disintegrations per minute; NS = normal saline; SI = stimulation index.

<sup>1</sup> Values are an average of dpms from 5 individual animals.

<sup>2</sup> Eluate mixed 5:1 with Pluronic L92

<sup>3</sup> Eluate spiked with 20% dinitrobenzenesulfonic acid (DNBS) (1:1)

<sup>4</sup> (+) = sensitizer; (-) = nonsensitizer

<sup>5</sup> Positive control is 20% DNBS.

**Annex V**

**Supplementary Analysis of Pesticide Formulations in the LLNA**

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***Testing of Pesticide Formulations: LLNA vs. GP with Available Reference Data for the Entire Formulation***

For the 23 formulations that had associated GP data for the formulation itself, 13% (3/23) were classified as sensitizers and 87% (20/23) as nonsensitizers according to the GP results (**Figure D-V-1-1**). These results are based on a positive overall GP call for formulation EXP 10810.<sup>1</sup> The LLNA classified 59% (13/22) of the formulations as sensitizers and 41% (9/22) as nonsensitizers (**Figure D-V-1-1**). All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA. The LLNA also identified an additional six substances as sensitizers that were classified as nonsensitizers in the GP test (**Table D-V-1-1**). There were no comparative human data with which to determine the actual human sensitization potential.

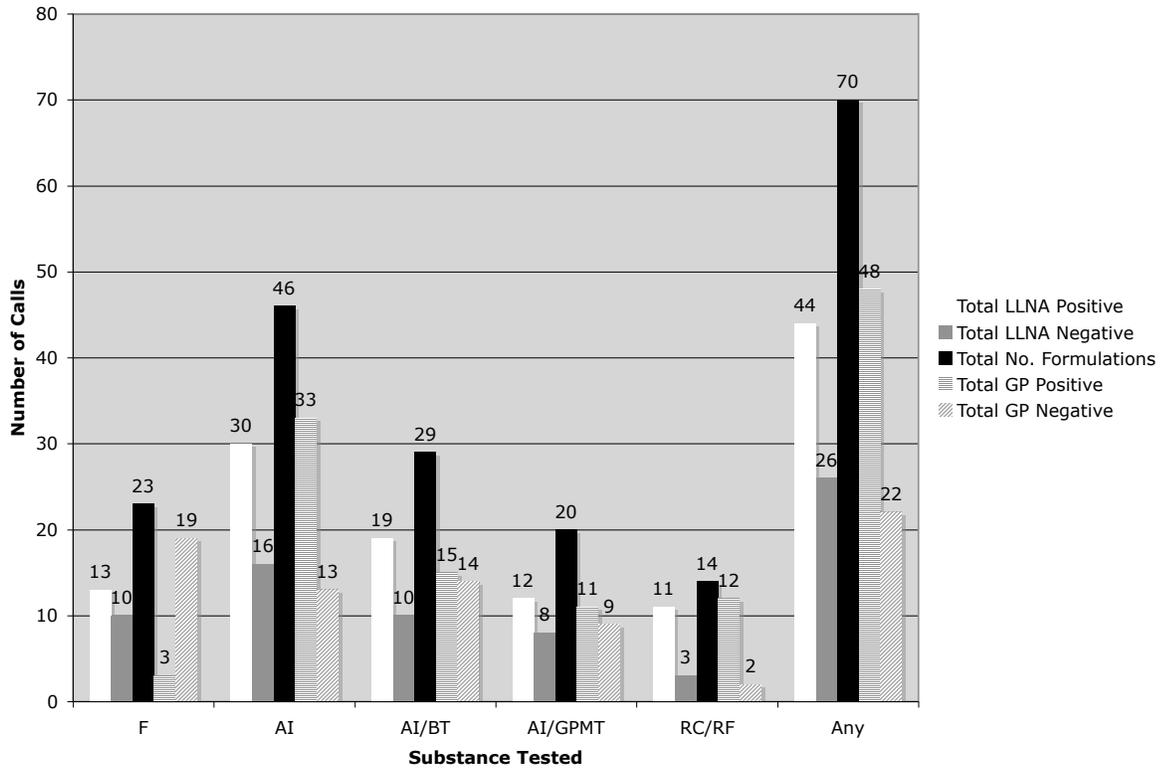
***Testing of Pesticide Formulations: LLNA vs. GP with Any Available Reference Data for Relevant Substances***

Of the 70 formulations, 69% (48/70) were classified as sensitizers and 31% (22/70) as nonsensitizers on the basis of various types of GP data (**Figure D-V-1-1**). To assign these classifications, a most conservative approach was used. That is, if a GP result for the formulation, any active ingredient, a substance related to an active ingredient, or a related formulation indicated sensitization, the formulation was classified as a sensitizer. Additionally, a GP result for the formulation itself was given priority over a result for an active ingredient. A result for an active ingredient was given priority over results for a substance related to an active ingredient, or a related formulation. Based on the LLNA result with the entire formulation for these same 70 pesticide formulations, 63% (44/70) were classified as sensitizers and 37% (26/70) as nonsensitizers (**Figure D-V-1-1**). Sixty-five percent (31/48) of the pesticide formulations classified as sensitizers by a GP test, based on the criteria given above, would also have been classified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified an additional 14 formulations as sensitizers that would have been classified as nonsensitizers by a GP test based on these criteria. However, the LLNA failed to identify as sensitizers an additional 36% (17/48) of formulations that would have been classified as such by a GP test, based on the criteria given above.

---

<sup>1</sup> Formulation EXP 10810 A (submitted by E. Debruyne, Bayer Crop Science), the only formulation for which there was data in both the GPMT and the BT, showed equivocal results in the guinea pig. This formulation tested positive in the GPMT (sensitization incidence 100%), and negative in the BT (sensitization incidence 10%). The patch concentration in the GPMT was the same as the induction concentration in the BT (50%).

**Figure D-V-1-1 Numbers of Positive and Negative LLNA (All Mouse Strains) and GP Calls for Pesticide Formulations**



Abbreviations: AI = Active Ingredient Test; BT = Buehler Test; F = Formulation Test; GP = guinea pig; GPMT = Guinea Pig Maximization Test; RC/RF = Related Substance or Related Formulation Test

**Table D-V-1-1 Evaluation of the Performance of the LLNA in Testing Pesticide Formulations**

Comparison <sup>1</sup>	n <sup>2</sup>	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>	%	No. <sup>3</sup>
LLNA vs. GP <sup>4</sup> (Formulation <sup>5</sup> )	23	57	12/23	100	3/3	50	10/20	50	10/20	0	0/3
LLNA vs. GP <sup>4</sup> (Any <sup>6</sup> )	70	56	39/70	65	31/48	36	8/22	64	14/22	35	17/48
LLNA vs. GP <sup>4</sup> (Active Ingredient <sup>7</sup> )	46	72	33/46	76	25/33	62	8/13	38	5/13	24	8/33
LLNA vs. BT (Active Ingredient <sup>7</sup> )	29	59	17/29	73	11/15	43	6/14	57	8/14	27	4/15
LLNA vs. GPMT (Active Ingredient <sup>7</sup> )	20	55	11/20	64	7/11	44	4/9	56	5/9	36	4/11
LLNA vs. GP <sup>4</sup> (Related Substance or Formulation <sup>8</sup> )	14	64	9/14	75	9/12	0	0/2	100	2/2	25	3/12
<i>ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data<sup>9</sup></i>											
LLNA vs. GP <sup>4</sup>	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human <sup>10</sup>	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP <sup>4</sup> vs. Human <sup>10</sup>	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = Local Lymph Node Assay; No. = number.

*Accuracy* (concordance) = the proportion of correct outcomes (positive and negative) of a test method

*Sensitivity* = the proportion of all positive substances that are classified as positive

*Specificity* = the proportion of all negative substances that are classified as negative

*False negative rate* = the proportion of all positive substances that are falsely identified as negative

*False positive rate* = the proportion of all negative substances that are falsely identified as positive

<sup>1</sup> This accuracy analysis is only for formulations that have LLNA data and some type of associated GP data. None of the pesticide formulations analyzed had human data, so a comparison between LLNA vs. human and LLNA vs. GP is not included.

<sup>2</sup> n = number of substances included in this analysis

<sup>3</sup> The data on which the percentage calculation is based

<sup>4</sup> GP refers to outcomes obtained by studies conducted using either the Guinea Pig Maximization Test, the Buehler Test, or the McGuire Test.

<sup>5</sup> *Formulation* refers to associated GP data for the formulation itself.

<sup>6</sup> *Any* refers to associated GP data for the formulation itself, any active ingredient in the formulation, a substance related to an active ingredient, or a related formulation.

<sup>7</sup> *Active ingredient* refers to associated GP data for any active ingredient in the formulation.

<sup>8</sup> *Related substance or formulation* refers to associated GP data for a substance related to an active ingredient, or a related formulation.

<sup>9</sup> For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**) showing the overall performance of the LLNA vs. GP and human, and GP vs. human is included here.

<sup>10</sup> *Human* refers to outcomes obtained by studies conducted using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

### ***Testing of Pesticide Formulations: LLNA vs. GP with Available Reference Data for Active Ingredients***

Of the 46 formulations that had associated GP data for one or more of the active ingredients, 72% (33/46) were classified as sensitizers and 28% (13/46) as nonsensitizers on the basis of an active ingredient in a GP test. Based on the LLNA result with the entire formulation for these same 46 pesticide formulations, 65% (30/46) were classified as sensitizers and 35% (16/46) as nonsensitizers (**Figure D-V-1-1**). Seventy-six percent (25/33) of the pesticide formulations identified as sensitizers based on a GP test on an active ingredient were identified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified as sensitizers an additional five substances that were classified as nonsensitizers in the GP test. However, the LLNA failed to identify 24% (8/33) of the formulations as sensitizers that would have been classified as such by a GP test on an active ingredient (**Table D-V-1-1**).

Among these same 46 formulations with available GP data for one or more of the active ingredients, 29 had BT data and 20 had GPMT data (**Figure D-V-1-1**).

Of the 29 pesticide formulations with BT data for the active ingredient, 52% (15/29) were classified as sensitizers and 48% (14/29) as nonsensitizers. By comparison, LLNA results with the complete formulation for each of these products identified 66% (19/29) as sensitizers and 34% (10/29) as nonsensitizers (**Figure D-V-1-1**). Eleven of the pesticide formulations identified as sensitizers based on a BT of an active ingredient were identified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified as sensitizers an additional eight substances that would have been classified as nonsensitizers in a BT on an active ingredient. However, the LLNA failed to identify 27% (4/15) formulations as sensitizers that would have been classified as such by a BT on an active ingredient.

Similarly, of the 20 pesticide formulations with GPMT data for the active ingredient, 55% (11/20) were classified as sensitizers and 45% (9/20) as nonsensitizers. The proportion of formulations classified as sensitizers was similar to the proportion classified as sensitizers by the BT on an active ingredient. By comparison, LLNA results with the complete formulation for each of these products identified 60% (12/20) as sensitizers and 40% (8/20) as nonsensitizers. Sixty-four percent (7/11) of the pesticide formulations identified as sensitizers based on a GPMT of an active ingredient were identified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified as sensitizers an additional five formulations that would have been classified as nonsensitizers by a GPMT on an active ingredient. However, the LLNA failed to identify as sensitizers 36% (4/11) of formulations that would have been classified as such by a GPMT based on an active ingredient (**Table D-V-1-1**).

### ***Testing of Pesticide Formulations: LLNA vs. GP with Available Reference Data for a Related Substance***

Of the 14 formulations that had associated GP data for a substance related to an active ingredient, or a related formulation, 86% (12/14) were classified as sensitizers and 14% (2/14) as nonsensitizers on the basis of the related substance or formulation in a GP test. By comparison, LLNA results with the complete formulation identified 79% (11/14) as sensitizers and 21% (3/14) as nonsensitizers (**Figure D-V-1-1**). Nine of the pesticide formulations identified as sensitizers based on a GP test on a substance related to an active ingredient, or a related formulation, were identified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified as sensitizers an additional two formulations that would have been classified as nonsensitizers by a GP test on a substance related to an active

ingredient, or a related formulation. However, the LLNA failed to identify as sensitizers an additional three formulations that would have been classified as such by a GP test on a substance related to an active ingredient, or a related formulation (**Table D-V-1-1**).

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## Appendix E

### Independent Scientific Peer Review Panel Assessment

E1	Summary Minutes from the Independent Scientific Peer Review Panel Meeting on March 4-6, 2008.....	E-3
E2	Peer Review Panel Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products .....	E-33
E3	Summary Minutes from the Independent Scientific Peer Review Panel Meeting on April 28-29, 2009.....	E-73
E4	Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.....	E-91

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**Appendix E1**

**Summary Minutes from the Independent Scientific Peer Review Panel Meeting on  
March 4-6, 2008**

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## Summary Minutes

### Independent Scientific Peer Review Panel Meeting

#### Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

Consumer Product Safety Commission (CPSC), Headquarters

Bethesda, MD

March 4 – 6, 2008

8:30 a.m. – 5:30 p.m.

*Peer Review Panel Members:*

Michael Luster, Ph.D. (Peer Review Panel Chair)	Senior Consultant to the NIOSH Health Effects Laboratory, Morgantown, WV, U.S.
Nathalie Alépée, Ph.D.	Associate Research Fellow, Pfizer PDRD MCT Laboratory, France
Anne Marie Api, Ph.D.	Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ, U.S.
Nancy Flournoy, M.S., Ph.D.	Professor and Chair, Dept. of Mathematics and Statistics, University of Missouri-Columbia, Columbia, MO, U.S.
Thomas Gebel, Ph.D.	Regulatory Toxicologist, Federal Institute for Occupational Safety and Health, Dortmund, Germany
Kim Headrick, B. Admin., B.Sc.	International Harmonization Senior Policy Advisor, Health Canada, Ottawa, Ontario, Canada
Dagmar Jírová, M.D., Ph.D.	Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic
David Lovell, Ph.D.	Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.
Howard Maibach, M.D.	Professor, Dept. of Dermatology, University of California-San Francisco, San Francisco, CA, U.S.

***Peer Review Panel Members:***

James McDougal, Ph.D.	Professor and Director of Toxicology Research, Dept. of Pharmacology and Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, OH, U.S.
Michael Olson, Ph.D.	Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, RTP, NC, U.S.
Raymond Pieters, Ph.D.	Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Jean Regal, Ph.D.	Professor, Dept. of Pharmacology, University of Minnesota Medical School, Duluth, MN, U.S.
Peter Theran, V.M.D.	Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA, U.S.
Stephen Ullrich, Ph.D.	Dallas/Ft. Worth Living Legends Professor & Professor of Immunology, Graduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.
Michael Woolhiser, Ph.D.	Technical Leader - Immunotoxicology, Toxicology and Environmental Research and Consulting Immunology, Dow Chemical, Midland, MI, U.S.
Takahiko Yoshida, M.D., Ph.D.	Professor, Dept. of Health Science, Asahikawa Medical College, Hokkaido, Japan

***ICCVAM and ICCVAM IWG Members:***

Paul Brown, Ph.D.	FDA, Silver Spring, MD, U.S.
Ruth Barratt, Ph.D., D.V.M.	FDA, Rockville, MD, U.S.
Karen Hamernik, Ph.D.	EPA, Washington, DC, U.S.
Masih Hashim, Ph.D.	EPA, Washington, DC, U.S.
Abigail Jacobs, Ph.D. (IWG Co-Chair)	FDA, Silver Spring, MD, U.S.
Kristina Hatlelid, Ph.D.	CPSC, Bethesda, MD, U.S.
Joanna Matheson, Ph.D. (IWG Co-Chair)	CPSC, Bethesda, MD, U.S.
Tim McMahon, Ph.D.	EPA, Washington, DC, U.S.

***ICCVAM and ICCVAM IWG Members:***

Amy Rispin, Ph.D. EPA, Washington, DC, U.S.

William Stokes, D.V.M., DACLAM NIEHS, RTP, NC, U.S.

Raymond Tice, Ph.D. NIEHS, RTP, NC, U.S.

Ron Ward, Ph.D. EPA, Washington, DC, U.S.

Marilyn Wind, Ph.D. (ICCVAM  
Chair) CPSC, Bethesda, MD, U.S.

Jiaqin Yao, Ph.D. FDA, Silver Spring, MD, U.S.

***ECVAM Observer:***

David Basketter, Ph.D. DABMEB Consultancy Ltd., Bedfordshire, U.K.

***Invited Experts:***

George DeGeorge, Ph.D., DABT MB Research Laboratories, Spinnerstown, PA, U.S.

Kenji Idehara, Ph.D. Daicel Chemical Industries, Hyogo, Japan

Masahiro Takeyoshi, Ph.D. Chemicals Evaluation and Research Institute, Saitama, Japan

***Public Attendees:***

Odette Alexander Syngenta Crop Protection, Inc., Greensboro, NC, U.S.

Nancy Beck, Ph.D. PCRM, Washington, DC, U.S.

Ann Blacker, Ph.D. Bayer CropScience, RTP, NC, U.S.

Stuart Cagan, Ph.D. Shell Oil Company, Houston, TX, U.S.

Joan Chapdelaine, Ph.D. Calvert Laboratories, Inc., Olyphant, PA, U.S.

Adriana Doi, Ph.D. BASF Corporation, RTP, NC, U.S.

Carol Eisenmann, Ph.D. Personal Care Products Council, Washington, DC, U.S.

Charles Hastings, Ph.D. BASF Corporation, RTP, NC, U.S.

Kailash Gupta, D.V.M., Ph.D. Retired CPSC, Bethesda, MD, U.S.

John Lyssikatos Hill Top Research, Miamiville, OH, U.S.

Laurence Musset, Ph.D. OECD, Paris, France

Carol O'Neil NuPathe, Conshohocken, PA, U.S.

***Public Attendees:***

Kui Lea Park, Ph.D.	National Institute of Toxicological Research, KFDA, Seoul, Korea
Rafael Rivas	AFRRI/USHUS, Bethesda, MD, U.S.
Terri Sebree	NuPathe, Conshohocken, PA, U.S.
Libby Sommer	EPA, Washington, DC, U.S.
Merrill Tisdell	Syngenta Crop Protection Inc., Greensboro, NC, U.S.
Jeffrey Toy, Ph.D.	FDA, Rockville, MD, U.S.

***NICEATM:***

William Stokes, D.V.M., DACLAM	Director
Raymond Tice, Ph.D.	Deputy Director
Debbie McCarley	Special Assistant to the Director
Support Contract Staff— Integrated Laboratory Systems, Inc. (ILS)	
David Allen, Ph.D.	Michael Paris
Thomas Burns, M.S.	Eleni Salicru, Ph.D.
Linda Litchfield	Judy Strickland, Ph.D., DABT
Douglas Winters, M.S.	

**Abbreviations:**

AFFRI = Armed Forces Radiobiology Research Institute

CPSC = U.S. Consumer Product Safety Commission

ECVAM = European Centre for the Validation of Alternative Methods

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems

IWG = Immunotoxicology Working Group

KFDA = Korea Food and Drug Administration

NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS = National Institute of Environmental Health Sciences  
NIOSH = National Institute of Occupational Safety and Health  
OECD = Organisation for Economic Co-operation and Development  
PCRM = Physicians Committee for Responsible Medicine  
USDA = U.S. Department of Agriculture  
USHUS = Uniformed Services University of the Health Sciences

## **TUESDAY, MARCH 4, 2008**

### **Call to Order and Introductions—**

Dr. Michael Luster (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (hereafter Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Immunotoxicity Working Group (IWG) members, the European Centre for the Validation of Alternative Methods (ECVAM) observer, and members of the public to also introduce themselves. Dr. Luster stated that there would be opportunity for public comments during each of the seven local lymph node assay (LLNA)-related topics. He asked that all those interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Luster emphasized that the comments would be limited to seven minutes per individual and that, while an individual would be welcome to make comments during each commenting period, repeating the same comments at each comment period would be inappropriate. He further stated that the meeting was being recorded and that Panel members should speak directly their microphone. Finally, Dr. Luster noted that if the Panel finished early with the assigned topics on the agenda for that day, they would proceed to the next day's topics if time permitted.

### **Welcome from the ICCVAM Chair—**

Dr. Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to CPSC and to the Panel meeting. Dr. Wind stressed the importance of this Panel's efforts especially considering recent reports that allergies and asthma have increased markedly over the past number of years and that contact dermatitis is the most common occupational illness in the United States. Dr. Wind thanked the Panel members for giving their expertise, time, and effort and acknowledged their important role to the ICCVAM test method evaluation process. Dr. Wind also emphasized the important role of the public and their comments in this process.

### **Welcome from the Director of NICEATM, and Conflict of Interest Statements—**

Dr. William Stokes, Director of NICEATM, stated the Panel meeting was being convened as a National Institutes of Health (NIH) special emphasis panel and was being held in accordance with the Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would serve as the Designated Federal Official for this public meeting. He reminded the Panel that they had signed a conflict-of-interest statement when they were selected for the Panel, in which they identified any potential conflicts of interest. He then read this statement to provide another opportunity for members of the Panel to identify any conflicts not previously declared. Dr. Luster asked the Panel members to declare any direct or indirect conflicts based on Dr. Stokes statements and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict. None of the Panel members declared a conflict of interest.

### **Overview of the ICCVAM Test Method Evaluation Process**

Dr. Stokes provided an overview of the ICCVAM test method evaluation process. He stated that the Panel was made up of 19 different scientists from eight different countries (Canada, Czech Republic, France, Germany, Japan, The Netherlands, United Kingdom, and the United States). Dr. Stokes thanked the Panel members for the significant amount of time and effort that they had devoted to prepare for and attend the meeting. He explained that the purpose of the Panel was to assist ICCVAM by carrying out an independent scientific peer review of the information provided on a series of proposed new versions of the LLNA and some expanded applications of the assay. Dr. Stokes

mentioned that the original LLNA peer review panel in 1998 considered the LLNA a valid substitute for the guinea pig-based test in most testing situations, but not all. He mentioned that three Panel members from the 1998 review are also on the current Panel (i.e., Drs. Howard Maibach, Jean Regal, and Stephen Ullrich). Dr. Stokes also reviewed the nomination that was received from CPSC in January 2007,<sup>1</sup> which provides the basis for the current evaluation.

Dr. Stokes then identified the 15 Federal agencies that comprise ICCVAM and summarized ICCVAM's mission. He noted that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out the critical scientific evaluation of proposed test methods with regard to their usefulness and limitations for regulatory testing and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes provided a brief review of ICCVAM's history and summarized the ICCVAM Authorization Act of 2000,<sup>2</sup> detailing the purpose and duties of ICCVAM. He noted that one of ICCVAM's duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available on the NICEATM-ICCVAM website or can be obtained upon request from NICEATM. He also mentioned that ICCVAM provides guidance on test method development, validation criteria, and processes, and helps to facilitate not only the acceptance of scientifically valid alternative methods, but also encourages international harmonization.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public. Given sufficient regulatory applicability, sufficient data, resources, and priority, a test method will move forward into a formal evaluation. A draft background review document (BRD), which provides a comprehensive review of all available data and information, is prepared by NICEATM, in conjunction with an ICCVAM working group designated for the relevant toxicity testing area (e.g., the IWG). In addition, ICCVAM considers all of the available information and makes draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future studies. The BRD and the draft ICCVAM test method recommendations are made available to the Panel and the public for review and comment. The Panel peer reviews the BRD and evaluates the extent to which it supports the draft ICCVAM test method recommendations. A Panel report is published, which is then considered along with public and SACATM comments by ICCVAM in making final recommendations. These final recommendations are forwarded to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines.

Dr. Stokes reviewed the ICCVAM criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance. He concluded by summarizing the timeline of the review activities beginning with CPSC's nomination in January 2007 and ending with the present Panel meeting.

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<sup>1</sup> [http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf)

<sup>2</sup> [http://iccvam.niehs.nih.gov/docs/about\\_docs/PL106545.pdf](http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf)

## ICCVAM Charge to the Panel

Dr. Stokes reviewed the charge to the Panel, which was to: (1) review the draft BRDs, the draft Addendum to the traditional<sup>3</sup> LLNA, and the draft performance standards for completeness and identify any errors or omissions; (2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been addressed for the proposed revised or modified versions of the LLNA; and (3) consider and provide comment on the extent to which the ICCVAM draft test method recommendations including the proposed use, standardized protocols, performance standards, and additional studies are supported by the information provided in the draft BRDs and draft Addendum.

Dr. Stokes thanked the IWG and ICCVAM for their contributions to this project, and acknowledged the contributions from the participating liaisons from ECVAM and JaCVAM (Japanese Center for the Validation of Alternative Methods). He also acknowledged the NICEATM staff for their support and assistance in organizing the Panel meeting and preparing the materials being reviewed.

## Current Regulatory Testing Requirements and Hazard Classification Schemes for Allergic Contact Dermatitis and the Traditional LLNA Procedure

Dr. Joanna Matheson, Chair of the IWG, briefly reviewed the regulatory testing requirements of U.S. Federal agencies for skin-sensitization hazard identification and provided a brief description of the LLNA protocol.

## Overview of the Agenda

Dr. Luster provided a brief synopsis of the agenda. He stated that there were six test methods and applications along with the draft LLNA performance standards for review and that the same agenda would be followed for each: (1) introductory summary of the draft ICCVAM recommendations from one of the NICEATM staff members; in addition, test method developers would provide a brief description of the methodology for each of the three nonradioactive tests, (2) presentation of the Evaluation Group draft comments by the Evaluation Group leader, (3) Panel discussion, (4) public comments, (5) recommendations and conclusions by the Panel.

## Overview of the Draft LLNA Limit Dose Procedure<sup>4</sup> BRD and Draft ICCVAM Test Method Recommendations

Dr. David Allen, Integrated Laboratory Systems, Inc., the NICEATM support contractor, presented an overview of the draft ICCVAM BRD for the LLNA limit dose procedure. He mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA limit dose procedure. The method was reviewed for its accuracy in correctly identifying sensitizers and non-sensitizers, when compared to the traditional LLNA.

NICEATM published a series of *Federal Register* (FR) notices, including an FR notice (72 FR 27815, May 17, 2007) requesting original data from the LLNA. This FR notice was also sent to over 100 potentially interested stakeholders for their input and comment. As a result, data on 255 substances tested in the LLNA were received. The resulting LLNA database consisted of 471 studies of 466 unique substances, 211 of which were included in the original ICCVAM 1999 evaluation. Dr. Allen briefly summarized the performance characteristics of the LLNA limit dose procedure test

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<sup>3</sup> For the purposes of this document, the radioactive LLNA test method, which was first evaluated by ICCVAM in 1999, and subsequently recommended to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances, is referred to as the traditional LLNA.

<sup>4</sup> Also known as the reduced LLNA (rLLNA).

method, which is detailed in the draft ICCVAM BRD,<sup>5</sup> and briefly summarized the draft ICCVAM test method recommendations for the LLNA limit dose procedure.<sup>6</sup>

**Panel Evaluation:**

Dr. Michael Olson led the Panel discussion on the LLNA limit dose procedure and specifically thanked the members of his Evaluation Group (i.e., Drs. James McDougal, Raymond Pieters, Jonathan Richmond [not present], and Takahiko Yoshida) for their collegial review of the information presented in the draft ICCVAM LLNA Limit Dose Procedure BRD. Dr. Olson also thanked the NICEATM staff for their technical support during the BRD review process. He then presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. The focus was on review of the BRD for errors and omissions, assessment of the validation status of the test method, and review of draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD and recommendations are reflected in the *Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products*, published in May 2008 (hereafter, the Panel report<sup>7</sup>).

During the Panel's evaluation, discussion arose regarding what might have resulted in the inverted-U-shaped dose response that was seen with the false-negative substances in the LLNA limit dose procedure. Dr. Olson responded that although it was difficult to understand what the cause might have been, he speculated that the top dose was either toxic at a systemic-effect level or that those substances were immunosuppressive at the highest dose level. He also stated that there did not seem to be any structural features of the substances that could be attributed for the false negative response in the LLNA limit dose procedure.

The Panel also discussed the use of concurrent versus intermittent positive controls in the LLNA limit dose procedure. Dr. Olson indicated that the Evaluation Group had discussed the possibility to allow intermittent positive controls for laboratories that exhibited repeatable and adequate performance with the LLNA but he indicated that it would be important to describe a set of performance criteria that would determine when this practice would be acceptable. Clearly, if the laboratory was not performing the assay routinely or if there were other reasons to suspect variability in response with any substance, the positive control would be necessary. Dr. Stokes indicated that this discussion was pertinent and indicated that the Panel's suggestions for what the performance criteria might be for intermittent positive control testing would be of interest to the IWG. Dr. Stokes also wanted to clarify that the OECD TG is consistent with the EPA TG and the ICCVAM-recommended test method protocol for the LLNA although the OECD TG allows additional latitude in how tests are run (i.e., four animals per dose group, use of pooled data, and the option to not run a positive concurrent positive).

**Public Comments:**

**Dr. Amy Rispin, EPA**

Dr. Rispin stated that the ICCVAM LLNA report (1999<sup>8</sup>) and standardized protocol (2001<sup>9</sup>) recommends the use of a concurrent positive control in addition to the concurrent negative control required for each study. Subsequently, the OECD (Organisation for Economic Co-operation and Development) Test Guideline (TG) 429 (Skin Sensitisation: Local Lymph Node Assay) was finalized (2002). She said that originally, OECD TG 429 was drafted without a concurrent positive control but that language was added to include the recommended use of a concurrent positive control until

<sup>5</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/LLNAldBRD07Jan08FD.pdf>

<sup>6</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/IWGrecLLNA-LD07Jan08FD.pdf>

<sup>7</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)

<sup>8</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/llnarep.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf)

<sup>9</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/LLNAProt.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf)

laboratories demonstrate competence. Subsequent to that, EPA put forth its LLNA guideline for sensitization,<sup>10</sup> which states that concurrent positive and negative controls are to be included in each study. Dr. Rispin then added that U.S. Federal regulatory agencies, most notably the EPA and FDA, received LLNA data from studies in which the positive control did not achieve the appropriate limits of performance (i.e., the control values were not in the appropriate range) and therefore the studies were deemed unacceptable, underscoring the importance of a concurrent positive control for regulatory acceptance in the United States.

In response to Dr. Rispin's public comment, Drs. Ullrich and Theran asked how competence is determined and if laboratories have difficulties reaching a level of competence, respectively. Dr. Abby Jacobs responded by stating that the FDA has seen large data variations in laboratories that conduct the LLNA. It is often difficult to determine what the variations might be due to (e.g., new technicians, tail vein injection, lymph node removal) and these variations have been seen both in laboratories that are established and those that are not.

**Dr. David Basketter, ECVAM Observer**

Dr. Basketter said that the main point he wanted to address is that efforts should be made to harmonize the LLNA protocol with that described in OECD TG 429. He stated that although there is referral to the "ICCVAM protocol" throughout the BRDs under consideration, OECD TG 429 is more globally recognized for regulatory use of the LLNA and therefore should be the referenced protocol. Dr. Basketter further stated that if the LLNA limit dose procedure followed the ICCVAM protocol using five animals per group instead of following OECD TG 429, which allows using four animals per group, there would only be a savings of one animal for substances that were negative. He stated that the goal of ECVAM was actually to halve the number of animals by omitting the mid- and low-dose groups and that this would achieve significant animal savings since the likely prevalence of non-sensitizers is approximately two-thirds of chemicals tested and non-sensitizers would not require further testing even if dose response information for sensitizers was needed.

Dr. Basketter also mentioned that the retrospective evaluation of the LLNA being presented to the Panel analyzed whether the top dose could identify a substance as a sensitizer and how that compares to the traditional LLNA's performance. Since the traditional LLNA assay was determined to be positive or negative based on a stimulation index (SI) of three, it is problematic if the focus is on statistics when using the five-animal model as this would require also going back and re-evaluating all the preceding data using the statistical approach.

Dr. McDougal responded to Dr. Basketter's comment by stating that one wouldn't have to go back and retrospectively re-evaluate previous data but that new data generated could be analyzed statistically. This approach would include determining if the treatment group was statistically different from the vehicle control group and then determining the biological relevance. This might help to eliminate irritants.

**Panel Conclusions and Recommendations:**

Dr. Luster asked the Panel to review the conclusions and recommendations for the LLNA limit dose procedure they had discussed earlier and to make any revisions, if necessary. One particular question that was asked during the Panel's conclusions and recommendations was whether an OECD TG existed for the LLNA limit dose procedure. Dr. Stokes indicated that the OECD TG would need to be updated to allow for the provision of a limit dose procedure and that's why the Panel's conclusions and recommendations are even more relevant. Dr. Stokes indicated that ICCVAM has already submitted a proposal to update the OECD TG based on the outcome of these deliberations and recommendations from the IWG.

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<sup>10</sup>[http://www.epa.gov/opptsfrs/publications/OPPTS\\_Harmonized/870\\_Health\\_Effects\\_Test\\_Guidelines/Revised/870r-2600.pdf](http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf)

The Panel agreed to use the term weight-of-evidence to refer to existing information that would aid the LLNA limit dose procedure in identifying a substance as a sensitizer or a non-sensitizer. The Panel also discussed the use of concurrent positive controls and recommended that a laboratory that is proficient at conducting the limit dose procedure can test a positive control at routine intervals rather than concurrently (although the Panel did not identify what constituted routine intervals). The Panel also discussed the use of individual versus pooled data and agreed with the ICCVAM-recommended protocol that individual animal data should always be collected. The Panel concluded that individual animal response data are necessary in order to allow for statistical analyses of any differences between treated and control data. In addition, having data from individual animals also allows for identification of technical problems and outlier animals within a dose group. Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised. The Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA limit dose procedure are included in their final Panel report.<sup>11</sup>

## **Overview of the Draft Addendum for the Applicability Domain of the LLNA and Draft ICCVAM Test Method Recommendations**

Dr. Eleni Salicru, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), summarized the information provided in the draft ICCVAM Addendum to the ICCVAM LLNA report (1999). This Addendum provided an updated assessment of the validity of the LLNA for testing the sensitizing potential of mixtures, metals, and aqueous solutions. The database used for this evaluation contained traditional LLNA data submitted as part of the original LLNA evaluation (ICCVAM 1999), data extracted from peer-reviewed articles published after the original evaluation, and data submitted to NICEATM in response to the FR notice (72 FR 27815, May 17, 2007) requesting such data. Dr. Salicru then summarized the performance characteristics of the LLNA when used to test mixtures, metals, and aqueous solutions,<sup>12</sup> as well as the draft ICCVAM test method recommendations for each of the three categories of test substances.<sup>13</sup>

### **Panel Evaluation:**

Dr. McDougal, on behalf of his Evaluation Group, presented for consideration by the entire Panel the draft responses to the questions asked of the Panel by ICCVAM. The Panel then discussed the completeness of the draft ICCVAM Addendum, identified any errors and omissions, and reviewed the draft ICCVAM test method recommendations with regard to the ability of the LLNA to be used to test the sensitizing potential of mixtures, metals, and aqueous solutions. The Panel discussion and their recommended revisions to each section of the draft ICCVAM Addendum are reflected in the Panel report, published in May 2008.<sup>14</sup> During the Panel's evaluation of the LLNA's applicability domain, the difficulty of testing metals in the LLNA was discussed and Dr. Woolhiser asked if testing metals was also problematic in the guinea pig. Dr. Api indicated that with the metals, most of the data has come from the clinical experience because animal studies are not predicting accurately what is happening in the clinic. Dr. Maibach indicated that metals have been tested in the guinea pig and that they are sensitized easily. Dr. Maibach further commented that metals in man need to be patch-tested for clinical relevance at a level close to the irritant dose and that a thoughtful series of algorithms is necessary to determine this. He also pointed out that patch test results to some metals (e.g., nickel, palladium) may indicate that a cell mediated reaction is occurring (i.e., contact allergy) but it needs to be sorted out if this cell mediated reaction actually results in a disease (i.e., allergic contact dermatitis) and this is where the LLNA could prove useful.

<sup>11</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)

<sup>12</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-app/LLNAappADD19Jan08FD.pdf>

<sup>13</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-app/LLNAappRecs19Jan08FD.pdf>

<sup>14</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)

With regard to mixtures, Dr Api commented that based on her experience, when the mixture tested in the LLNA contains a predominant material (loosely defined that as greater than 70 percent) then the LLNA for the mixture mirrors what occurs for that one material. When evidence indicates that the substance is a true mixture, some times the LLNA does what is expected and other times the results are unexpected. In those cases, a weight-of-evidence approach (e.g., structure-activity relationships, clinical evidence) is employed.

**Public Comments:**

**Dr. Charles Hastings, BASF Corporation**

Dr. Hastings, representing CropLife America (an industry association of companies in the crop protection business), provided an overview of current activities in industry related to the use of the LLNA to detect dermal sensitizers and the global issues that are of importance. Dr. Hastings mentioned that CropLife America's primary concern is the testing of pesticide mixtures and formulations. He stated that they support the use of the LLNA for testing the dermal sensitization of mixtures and formulations as well as single ingredients.

Dr. Hastings mentioned that in the United States, EPA OPPTS (Office of Prevention, Pesticides and Toxic Substances) Guideline 870.2600<sup>15</sup> allows for the use of the LLNA as the preferred alternative to the standard guinea pig test. Based on this recommendation, member companies of CropLife America conducted a large number of LLNA studies for both active ingredients and formulations in the European Union (E.U.) and were at the point of submitting data in the United States, as well. Then, in early 2007, they were informed that EPA had concerns about the validity of using the LLNA to test mixtures and formulations, and were advised to discontinue using this test method for that purpose until it had been adequately validated. Dr. Hastings stated that, in contrast to the EPA, E.U. regulators consider the LLNA acceptable for testing pesticide formulations and actually prefer it to a guinea pig test.

Dr. Pieters asked if the E.U. has conducted any evaluations of the validity of the LLNA for testing mixtures and formulations. Dr. Hastings replied that he was not certain if they had performed an extensive evaluation or not but that the E.U. considered the LLNA a validated method and therefore likely considered it appropriate to test not only the active ingredient but also the formulation or mixture.

Dr. Hastings mentioned that one concern in terms of using the LLNA for testing mixtures or formulations, particularly in the E.U., is the testing of aqueous substances. Many of the industry formulations are aqueous-based and may be incompatible with traditional LLNA vehicles. The European Crop Protection Association sponsored a study that evaluated the use of an aqueous vehicle known as Pluronic L92, which helps adhere the test material to the mouse ear. In the study, they tested three aqueous pesticide formulations that contained known sensitizers, using Pluronic L92 as the vehicle. As expected, the test results demonstrated sensitizing activity. Regarding global considerations, Dr. Hastings mentioned that if the LLNA is not accepted for mixture/formulation testing in the United States, industry will have no choice but to conduct both the LLNA, with 18 to 24 animals, and a guinea pig test, with 20 to 30 animals, for each formulation they may develop for global distribution. This scenario counters the ICCVAM goal of "reducing, refining, and replacing" animal use in regulatory safety testing.

Dr. Hastings ended with the following conclusions:

- CropLife America believes the LLNA test can be used for pesticide formulations.

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<sup>15</sup>[http://www.epa.gov/opptsfrs/publications/OPPTS\\_Harmonized/870\\_Health\\_Effects\\_Test\\_Guidelines/Revised/870r-2600.pdf](http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf)

- CropLife America supports the efforts of EPA and ICCVAM to confirm the validity of the LLNA for testing mixtures/formulations and encourages a quick evaluation.
- CropLife America is willing to help, as needed.
- If and, when, it is determined that the LLNA is acceptable, CropLife America requests that EPA notify them so they can then begin conducting the LLNA again for the United States.

Dr. Api asked if CropLife America has data comparing pesticides that have been evaluated in the LLNA and in guinea pigs and/or humans. Dr. Hastings replied that they do and that generally there is not much discrepancy with guinea pig test results. Occasionally they might see a false positive compared to a guinea pig test, but he did not recall ever seeing a false negative. In most cases, they would feel comfortable accepting an occasional false positive because human health is still protected.

**Dr. David Basketter, ECVAM Observer**

Dr. Basketter stated that he had personal reservations about testing complex mixtures and formulations in assays that were designed for testing substances (e.g., the LLNA) since no single test has ever been validated for testing mixtures. On another point, he stated that most of the metals of importance have been tested in both the guinea pig and the LLNA and the “right” answers have been generated. Thus, it does not seem worthwhile to produce new tests with revised protocols for hazard and potency categorization for testing metals.

**Panel Conclusions and Recommendations:**

Dr. Luster asked the Panel if they agreed with the comments and recommendations that were made earlier during the Panel discussion. The Panel agreed with the draft ICCVAM recommendation for continued collection of information from traditional LLNA evaluations of mixtures, metals, and aqueous solutions with comparative data for guinea pig (i.e., guinea pig maximization test [GPMT] or Buehler test [BT]) and human (i.e., human maximization test [HMT] or human repeat insult patch test [HRIPT]) tests. However, the Panel suggested that, given resource limitations, it would be important to organize the recommendations based on relative priority. Dr. Luster asked the Panel if they agreed with this suggestion about prioritization of activities; all members of the Panel agreed with one abstention. Dr. Howard Maibach abstained from voting stating that he hoped this public meeting and the subsequent Panel report would emphasize to industry the need for them to submit more data on mixtures, metals, and aqueous substances in order to provide a clearer evidence of the validity of the LLNA in testing these types of substances. The Panel’s detailed recommendations and conclusions on the applicability domain of the LLNA are included in their final Panel report.<sup>16</sup>

**Method Description and Overview of the LLNA: Daicel Adenosine Triphosphate (LLNA: DA) Test Method**

Dr. Kenji Idehara, Daicel Chemical Industries, Ltd. (private limited company), summarized the technical aspects of the LLNA: DA test method. He described the LLNA: DA as a non-radioisotopic version of the LLNA method in which lymph node adenosine triphosphate (ATP) content is used as a measure of cell proliferation instead of radiolabeled thymidine incorporation. Dr. Idehara indicated that the LLNA: DA was developed six years ago at Daicel Chemical Industries, Ltd., and that they use the test method regularly for in-house assessments of the skin-sensitization potential of chemical materials, intermediates, or products. He summarized the protocol differences between the LLNA: DA and the traditional LLNA. In the LLNA: DA, the application site is treated with 1% sodium lauryl sulfate (SLS) one hour before each test substance (or vehicle control) application, and the test substance is applied to the test site on day 7 as well as on days 1, 2, and 3. The auricular lymph nodes are excised from individual animals on day 8 rather than on day 6 and the amount of ATP in the

<sup>16</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPREpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPREpt2008.pdf)

lymph nodes is measured with a luciferin-luciferase assay. Dr. Idehara mentioned that these modifications (i.e., 1% SLS pretreatment and additional application on day 7) enhance lymph node cell proliferation in order to achieve an SI = 3 in the LLNA: DA, which allows for a more direct comparison to the traditional LLNA.

Dr. Idehara mentioned that after excision, ATP content gradually decreased with time. Therefore, the overall assay time for measuring ATP content needs to be similar (i.e., within approximately 30 minutes) among all test animals. He noted that this was an important point for this method and recommended that the LLNA: DA be conducted by at least two persons. Dr. Idehara mentioned that ATP content assays are conducted using commercially available kits, and his laboratory has experience with two different commercial sources in Japan, Kikkoman and Lonzar.

## **Overview of the Draft LLNA: DA BRD and Draft ICCVAM Test Method Recommendations**

Dr. Allen then presented an overview of the draft ICCVAM BRD for the LLNA: DA test method. He mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: DA to distinguish between sensitizers and non-sensitizers, compared to the traditional LLNA. The objective of the BRD was to describe the current validation status of the LLNA: DA test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Allen mentioned that the data analyzed in the BRD included data provided by Daicel Chemical Industries, Ltd., on 31 substances tested at their laboratories. In addition, data for 14 different coded substances were generated from a two-phased interlaboratory validation study that included 17 total labs. Taken together, the total database represented in the LLNA: DA BRD included 33 different substances. Dr. Allen briefly summarized the performance characteristics of the LLNA: DA test method, which is detailed in the draft ICCVAM BRD.<sup>17</sup> Dr. Allen concluded by briefly summarizing the draft ICCVAM test method recommendations for the LLNA: DA test method.<sup>18</sup>

### **Panel Evaluation:**

Dr. Michael Woolhiser thanked the Panel members of his Evaluation Group (i.e., Drs. Nathalie Alépeé, Thomas Gebel, Sidney Green [not present], and Jean Regal) for their tireless efforts in reviewing their Evaluation Group's assigned documents. He also thanked the NICEATM staff for their technical support during the review process. Dr. Woolhiser then presented the draft responses to ICCVAM's questions about this test method for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.<sup>19</sup>

### **Adjournment—**

The meeting was adjourned for the day at 5:03 p.m., to reconvene at 8:30 a.m., Wednesday, March 5, 2008.

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<sup>17</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/LLNA-DABrd07Jan08FD.pdf>

<sup>18</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/LLNA-DAREcs07Jan08FD.pdf>

<sup>19</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

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## WEDNESDAY, MARCH 5, 2008

### Reconvening of the Panel Meeting

Dr. Luster reconvened the Panel Meeting at 8:30 a.m. He introduced himself and then asked that all Panel members, followed by all others in attendance, introduce themselves as well.

### Overview of the Draft LLNA: DA BRD and Draft ICCVAM Test Method Recommendations

#### Panel Evaluation:

Dr. Woolhiser continued his presentation from the previous day of the draft responses to ICCVAM's questions to the Panel, for consideration by the entire Panel. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.<sup>20</sup> Dr. Woolhiser indicated that the Evaluation Group had two main concerns with the LLNA: DA test method. The first concern related to pretreatment with 1% SLS and understanding how this impacted the biology of the response. Second, the time course of the study was different than the traditional LLNA because it extended the study by one day and included an additional challenge. This brought forth a question about the immunology of the response as it relates to the potential for elicitation and whether or not that is a significant change from the traditional LLNA, which is purely an induction model.

#### Public Comments:

##### **Dr. George DeGeorge, MB Research Laboratories**

In response to a question raised during the Panel discussion, Dr. DeGeorge commented that using lymph node weight as the readout to differentiate between sensitizers and non-sensitizers in the LLNA is problematic because although there are more lymph node cells packed into a node, each cell has less cytoplasm. The lymph nodes swell to a point, and then excrete water and become smaller lymphocytes that are countable. He cited examples from his laboratory with several different sensitizers, which demonstrate that lymphocytes in the node are smaller when a large SI (e.g., SI = 25) is obtained relative to when a smaller SI (e.g., SI = 3) is obtained.

Dr. DeGeorge also commented that he agreed with a point made during the Panel discussion that the LLNA: DA method and the LLNA: Bromodeoxyuridine Detected by ELISA (LLNA: BrdU-ELISA) method should be considered separately, because they are so dissimilar.

In his final comment, Dr. DeGeorge stated that in the traditional LLNA, in the LLNA: Bromodeoxyuridine Detected by Flow Cytometry (LLNA: BrdU-FC), and probably also in the LLNA: DA, strong sensitizing substances do not need to be administered three times. For instance, if one administers a single, moderately high dose of dinitrochlorobenzene (DNCB) (i.e., one that would induce an SI of 20 to 40) and then measures lymph node cell proliferation on day 1, 2, 3, or 4, an increase in the number of cells in the node and the number of cells that are positive for BrdU would likely be observed. Thus, administrations of additional applications have the potential to cause cumulative irritation. Dr. DeGeorge stated that the LLNA: DA method, which extends the assay to eight days instead of six days, should evaluate what happens to lymph node cell number at earlier sample times. In addition, if the animals receive just one application using a high dose, with or without the SLS, is there an increase in the SI? If so, that would lead to the possibility that the extra applications are not necessary and might lead to cumulative irritation.

##### **Dr. David Basketter, ECVAM Observer**

Dr. Basketter made a statement that from a clinical perspective, substances are typically described as

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<sup>20</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

significant sensitizers or not significant sensitizers, and within that latter group some of the substances may indeed be non-sensitizing. Thus, just because a substance has been shown in an isolated case report to be a human sensitizer does not mean that there is sufficient evidence to consider it as positive for comparison with outcomes of predictive assays. It has to be of sufficient importance (i.e., potency) to trigger a positive classification. Dr. Basketter mentioned SLS, methyl salicylate, and isopropanol, as substances which will always be positive in some human cases although they shouldn't be positive in a predictive assay.

Dr. Basketter also commented that caution should be given to making sensitization assumptions based on chemical class references. As an example, eugenol and isoeugenol are structurally similar and have similar physical properties, but they act by different chemical reaction mechanisms and could fit into distinctly different chemical classes.

Dr. Basketter's last comment acknowledged that much work has been done in terms of validating the traditional LLNA. If one makes minor changes to the LLNA in terms of a different readout for proliferation, then they benefit from all the experience generated in validating the traditional LLNA and less effort is needed to prove that the minor modification is valid. In contrast, if more significant modifications are made, one cannot rely on that same experience. Dr. Basketter cautioned that more importance should be placed on distinguishing whether something has changed substantially enough such that you can no longer rely on the traditional LLNA as a reference.

#### **Dr. Masahiro Takeyoshi, Chemicals Evaluation and Research Institute**

Dr. Takeyoshi made a short presentation about differences in LLNA sensitization responsiveness among different strains of mice. He mentioned that this was an important issue when evaluating the modified LLNA methods being developed in Japan. He showed differences in responsiveness among three different mouse strains commonly used in Japan (i.e., BALB/cAnN, CBA/JN, and CD-1) tested with parabenzquinone in his group's non-radioactive LLNA (i.e., LLNA: BrdU-ELISA). The data indicated that the CBA/JN mouse strain exhibited a higher responsiveness, as indicated by an increased SI, to parabenzquinone than the other two mouse strains tested. Based on these results, CBA/JN mice were chosen for testing substances in the LLNA: BrdU-ELISA test method.

Dr. Takeyoshi also indicated that based on evaluating different SI cutoffs in the LLNA: BrdU-ELISA, 2-mercaptobenzothiazole, 3-(4-isopropylphenyl)isobutyraldehyde, and hydroxycitronellal had low responsiveness (i.e., SI values). He noted that 2-mercaptobenzothiazole is an OECD TG 429 recommended positive control for the LLNA; however, repeat tests could not detect this substance as positive when using an SI value of 1.7 or more. Dr. Takeyoshi suggested that a substance-specific lower response might exist in the test system. Dr. Takeyoshi also summarized LLNA data by Dr. Ullmann and coworkers with the contract lab RCC, Ltd. in which they investigated the responsiveness of six different mouse strains (CBA/CaOlaHsd, CBA/Ca (CruBR), CBA/Jlbn (SPF), CBA/JNcrlj, BALB/c and NMRI) to 25% 2-mercaptobenzothiazole. The data indicated that CBA/JNcrlj mice showed markedly lower responsiveness compared to the other strains tested. These studies indicate that strain related differences would not be negligible with regard to measuring different endpoints of cellular proliferation in the LLNA because depending on the chemicals tested, responsiveness might be potentially impacted. For instance, some of the discordance seen in the LLNA: DA test method (e.g., 2-mercaptobenzothiazole) could be a strain specific effect.

#### **Panel Conclusions and Recommendations:**

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel viewed the difference in treatment schedule between the LLNA: DA and the traditional LLNA to potentially be significant if the treatment schedule for the LLNA: DA corresponds to entering the elicitation phase of skin sensitization. The Panel was concerned that the 1% SLS pretreatment step in the LLNA: DA might modify the inherent sensitivity of the LLNA. They recommended that the test method developer (Daicel Chemical Industries, Ltd.) justify the use of 1% SLS or consider an alternative decision criterion (i.e., an SI threshold other than three) such

that the 1% SLS pretreatment is no longer necessary. Dr. Luster asked the Panel if they agreed with the recommendations and conclusions that the Panel made along with the revisions; unanimously, the Panel agreed. The Panel's detailed recommendations and conclusions on the LLNA: DA test method are included in their final Panel report.<sup>21</sup>

### **Method Description and Overview of the LLNA: BrdU-FC Test Method**

Dr. George DeGeorge, MB Research Laboratories, presented an overview of the LLNA: BrdU-FC test method. He stated that mice are dosed topically on the ears once daily for three consecutive days (i.e., days 1, 2, and 3), just like the traditional LLNA protocol. On day 6, the mice receive an intraperitoneal injection with bromodeoxyuridine (BrdU), and five hours later, the auricular lymph nodes are removed. The lymph nodes from individual animals are processed and, using flow cytometry, the number of BrdU-positive cells are counted from treated animals and compared to control animals as a measure of lymph node cell proliferation.

Dr. DeGeorge described in detail how the cells are processed and gated for flow cytometric analysis. He mentioned that the cells are also permeabilized and treated with propidium iodide which allows gates to be drawn around the G<sub>0</sub>, G<sub>1</sub>, S, and G<sub>2</sub>M phases of the cell cycle. Dr. DeGeorge projected specific examples of flow cytometry plots and histograms for DNCB, hexyl cinnamic aldehyde (HCA), and positive and negative control data.

Dr. DeGeorge also described the tiered protocol for the assessment of sensitization potential using the LLNA: BrdU-FC and how ear swelling measurements and additional immunophenotypic endpoints (i.e., the enhanced LLNA: BrdU-FC) aid in distinguishing skin irritants from an irritating sensitizer.

### **Overview of the Draft LLNA: BrdU-FC BRD and Draft ICCVAM Test Method Recommendations**

Dr. Judy Strickland, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), presented an overview of the draft ICCVAM BRD for the LLNA: BrdU-FC test method. She stated that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: BrdU-FC test method. Specifically, the test method was reviewed for its ability to distinguish between sensitizers and non-sensitizers compared with the traditional LLNA. The objective of the BRD was to describe the current validation status of the LLNA: BrdU-FC test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Strickland indicated that MB Research Laboratories submitted data to NICEATM for the 48 substances analyzed in the BRD in response to an FR notice (72 FR 27815, May 17, 2007) that requested such data. Dr. Strickland briefly summarized the performance characteristics of the LLNA: BrdU-FC test method, which is detailed in the draft ICCVAM BRD,<sup>22</sup> and the draft ICCVAM test method recommendations for the LLNA: BrdU-FC test method.<sup>23</sup>

#### **Panel Evaluation:**

Dr. Raymond Pieters, on behalf of his Evaluation Group, presented the Evaluation Group's review of the draft BRD and the draft test method recommendations for the LLNA: BrdU-FC test method. Specifically, he presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of this test method, and their comments on the draft ICCVAM test

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<sup>21</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)

<sup>22</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/FC-LLNAbrd07Jan08FD.pdf>

<sup>23</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/FCLLNAREcs07Jan08FD.pdf>

method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.<sup>24</sup> The applicability of the draft ICCVAM-recommended LLNA performance standards to the LLNA: BrdU-FC test method was discussed, particularly with regard to the number of substances tested in the LLNA: BrdU-FC method and whether more data would be necessary for review before the validation status of the assay could be determined. Dr. Stokes reminded the Panel that the proposed LLNA performance standards didn't exist when the studies for the LLNA: BrdU-FC test method were performed. The questions should be whether the adequacy of the substances that have been tested is sufficient or if more studies need to be done to cover any gaps that might exist (e.g., range of potencies or activity, chemical classes).

### **Public Comments**

#### **Dr. David Basketter, ECVAM Observer**

Dr. Basketter commented on the statement that Dr. DeGeorge made during his overview of the LLNA: BrdU-FC test method that HCA is irritating. He said that he is not convinced it is a significant irritant. Based on previous data, they had to use 50% HCA in a 48 hour occlusive application in the guinea pig in order to produce a mildly irritating response. Dr. Api added to Dr. Basketter's comment by stating that RIFM has also not found HCA to be an irritant when tested up to 20% in humans.

Dr. Basketter also commented that in the draft BRD for the LLNA: BrdU-FC, resorcinol was noted to be negative in the traditional LLNA and this is not correct. Dr. Basketter's group published results in 2007 in the journal *Contact Dermatitis* that resorcinol is clearly positive in the traditional LLNA when tested at higher concentrations and therefore this should be corrected for the record.

#### **Dr. George DeGeorge, MB Research Laboratories**

Dr. DeGeorge wanted to clarify that the LLNA: BrdU-FC test method was compared to the traditional LLNA to determine if the LLNA: BrdU-FC was more predictive of skin-sensitization potential. He stated that in some cases it was better while in others it wasn't, but overall, using human data as the gold standard reference, the LLNA: BrdU-FC exceeded the traditional LLNA predictivity values and accuracy. He also noted that the additional endpoints included in the LLNA: BrdU-FC allow for them to distinguish irritating substances that typically are considered false positives in the LLNA.

Dr. DeGeorge also noted that since the LLNA: BrdU-FC is so similar to the traditional LLNA the issue of refinement and reduction in animal use is not immediately apparent but if the assay is done in as few as four mice per group with a periodic positive control (e.g., every six months) this represents a significant decrease in animal numbers compared to guinea pig tests. Furthermore, there is a refinement since mice are phylogenetically lower than guinea pigs, and undergo less pain and distress during the assay than guinea pigs undergo.

With regard to the discussion of coefficients of variation (CVs) and the 0.5x to 2.0x EC3 (i.e., the estimated concentration needed to produce a stimulation index of three) range, Dr. DeGeorge suggested that a larger range might be more reasonable because the current range is likely too restrictive.

Dr. George also noted that ICCVAM requires interlaboratory validation if a test method is to be transferred to other laboratories. With regard to the LLNA: BrdU-FC, it is a "me-too" assay and only has "minor" changes from the traditional LLNA and is currently only used in one laboratory. Therefore, the current dataset should suffice for determining the validity of the LLNA: BrdU-FC. In response to Dr. DeGeorge's comment, Dr. Stokes stated that if a method is only proposed to be used by one laboratory, having only intralaboratory data certainly would suffice but if it was proposed for broader use (e.g., adopted or endorsed by regulatory authorities), then other laboratories would have to demonstrate

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<sup>24</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

interlaboratory reproducibility. Dr. Luster asked if there was any mechanism available so that a company or small laboratory could apply for funding to help support an interlaboratory validation. Dr. Stokes indicated that they could nominate the test method for additional validation studies to ICCVAM. It would go through a nomination review process and a prioritization would be given to that. The nomination would then be considered by the member agencies as to whether funding would be provided.

**Panel Conclusions and Recommendations:**

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel suggested that the utility of ear swelling or other methods to detect inflammation appeared warranted for inclusion in every variation of the LLNA (including the traditional LLNA), but should be further investigated before routine inclusion in the protocol is recommended. The Panel further agreed that the draft ICCVAM test method recommendations for future studies highlighted the unanswered questions raised by the available data set. Specifically, conducting interlaboratory studies as a part of the validation process is important.

The Panel considered the immunological markers suggested for the LLNA: BrdU-FC to be appropriate, but noted that other immunological markers for discrimination of irritant versus sensitization phenomena were also available. In general, for any future work, efforts should be made to decrease the variability and to thereby increase the power of the test in order to ensure that more animals were not needed relative to the traditional LLNA or other modified LLNA protocols.

Dr. Luster asked the Panel to indicate if they agreed with the recommendations and conclusions that the Panel made along with the revisions; the Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA: BrdU-FC test method are included in their final Panel report.<sup>25</sup>

**Method Description and Overview of the LLNA: BrdU-ELISA Test Method**

Dr. Masahiro Takeyoshi, Chemicals Evaluation and Research Institute, presented an overview of the LLNA: BrdU-ELISA test method. He stated that the LLNA: BrdU-ELISA test method is very similar to the traditional LLNA test method. Unique to the LLNA: BrdU-ELISA test method, after test substance applications on days 1, 2, and 3, BrdU is injected interperitoneally on day 5. Approximately 24 hours after the BrdU injection, lymph nodes are collected, and detection of the amount of BrdU incorporated into the DNA of lymph node cells is conducted with an ELISA.

In the development process of this method, experiments were conducted to detect the most efficient injection schedule of BrdU. Based on the various injection schedules tested, a single injection protocol on day four was identified as the optimal injection schedule for BrdU administration.

Dr. Takeyoshi then showed a video of laboratory personnel preparing the lymph node cells for BrdU detection by ELISA. He went on to describe data for the LLNA: BrdU-ELISA compared to the traditional LLNA and how performance could be improved using alternative decision criteria (i.e., an SI other than three as the threshold for a positive response).

**Overview of the Draft LLNA: BrdU-ELISA BRD and Draft ICCVAM Test Method Recommendations**

Dr. Salicru presented an overview of the draft ICCVAM BRD for the LLNA: BrdU-ELISA test method. She noted that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: BrdU-ELISA test method. Specifically, the test method was reviewed for its ability to distinguish between sensitizers

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<sup>25</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)

and non-sensitizers compared with the traditional LLNA and guinea pig test methods. The objective of the BRD was to describe the current validation status of the LLNA: BrdU-ELISA test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Salicru stated that data from a total of 29 substances were considered in the accuracy analysis for the LLNA: BrdU-ELISA, and they were all tested in one laboratory. Dr. Salicru briefly summarized the performance characteristics of the LLNA: BrdU-ELISA test method, which are detailed in the draft ICCVAM BRD,<sup>26</sup> and the draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method.<sup>27</sup>

### **Panel Evaluation:**

Ms. Kim Headrick presented her Evaluation Group's (Drs. Anne Marie Api, Howard Maibach, Peter Theran, and Stephen Ullrich) review of the draft BRD and draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method. Specifically, she presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.<sup>28</sup>

### **Public Comments:**

#### **Dr. David Basketter, ECVAM Observer**

Dr. Basketter noted that when the traditional LLNA was first suggested as an alternative to the guinea pig tests, it went through a comprehensive validation process, and one of the concerns was that it should perform reliably and distinctly better than the guinea pig assays. He emphasized that this point should be kept in mind when thinking about the modified LLNA protocols with alternative endpoints that are currently being reviewed. He stated that the current rigor of examination for the modified LLNA protocols being reviewed for validation is higher than that for the traditional LLNA. He speculated that in the not-too-distant future, *in vitro* alternatives are likely to be going through a similar review process and it is going to become ever more difficult to put these alternatives in place, not because there is ill-will against the selections but because of the high standard of being good scientists. Thus, it is important that pragmatic decisions are made using the tools that are available.

#### **Dr. George DeGeorge, MB Research Laboratories**

Dr. DeGeorge commented that he agreed with Dr. Basketter's statements. He said that based on his experience in this peer review process, it is unlikely that he would bring any of the three *in vitro* test methods that MB Research Laboratories is developing for consideration by ICCVAM, given the many high hurdles that have to be negotiated.

In response to the comments by Drs. Basketter and DeGeorge, Dr. McDougal commented that it does not seem unreasonable to raise the bar for what is expected of new or modified tests. Dr. Luster added that understandably, the focus on animal refinement and reduction is paramount, but that as scientists we have to ensure that the bar is maintained sufficiently high so that as the years go by scientific quality is not compromised.

### **Panel Conclusions and Recommendations:**

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel concluded that the available data and test method performance for

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<sup>26</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/BrdUELISAbrd07Jan08.pdf>

<sup>27</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/BrdUELISAREcs07Jan08FD.pdf>

<sup>28</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPREpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPREpt2008.pdf)

the LLNA: BrdU-ELISA support the draft ICCVAM test method recommendations that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that more information and existing data must be made available before the LLNA: BrdU-ELISA can be recommended for use. The Panel also stated that a detailed protocol was needed, in addition to sufficient quantitative data for broader analysis on a larger set of balanced reference substances that take into account physicochemical properties and sensitization potency, as well as an appropriate evaluation of interlaboratory reproducibility.

The Panel's main concern with this test method was that the accuracy of the LLNA: BrdU-ELISA at  $SI \geq 3$  was inadequate and not equivalent to the traditional LLNA. Furthermore, although using a decision criterion of  $SI \geq 1.3$  improved the test's performance in identifying sensitizers from non-sensitizers, it did not resolve concerns about the test method, particularly considering that power calculations suggest a much larger number of animals per group would be required to identify a positive response. Thus, the Panel also concluded that it might be more appropriate to use a statistically based decision criterion rather than a stimulation index to classify substances as sensitizers, and that this should be further investigated. Dr. Luster asked the Panel to indicate if they agreed with the recommendations and conclusions that the Panel made along with the revisions; unanimously, the Panel agreed. The Panel's detailed recommendations and conclusions on the LLNA: BrdU-ELISA test method are included in their final Panel report.<sup>29</sup>

### **Overview of the Draft ICCVAM Performance Standards for the LLNA**

Dr. Allen presented an overview of the draft ICCVAM Performance Standards for the LLNA. He briefly summarized the overall purpose of performance standards (i.e., to provide a basis for evaluating the performance of a proposed test method that is mechanistically and functionally similar to the validated test method) and the three elements encompassed within such performance standards (i.e., essential test method components, a minimum list of reference substances, and accuracy/reliability values). He noted that the proposed applicability of these draft ICCVAM LLNA performance standards is for the evaluation of LLNA protocols that deviate from the ICCVAM-recommended LLNA protocol only with respect to the method for assessing lymphocyte proliferation (e.g., using non-radioactive instead of radioactive reagents). Dr. Allen then provided an overview of the essential test method components, the minimum list of reference substances, and the accuracy/reliability values as detailed in the draft ICCVAM LLNA Performance Standards.<sup>30</sup>

#### **Panel Evaluation:**

Dr. Woolhiser, on behalf of his Evaluation Group, presented the Evaluation Group's responses to the ICCVAM questions asked about the draft ICCVAM LLNA Performance Standards for the entire Panel to consider. The overall question for the Panel was whether these performance standards were considered adequate for assessing the accuracy and reliability of test method protocols that were based on similar scientific principles and that measured the same biological effect as the traditional LLNA. The Panel discussion and their recommended revisions to the draft ICCVAM LLNA Performance Standards are reflected in the Panel report published in May 2008.<sup>31</sup>

#### **Adjournment—**

The meeting was adjourned at 5:42 p.m., to reconvene at 8:30 a.m., Thursday, March 6, 2008.

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<sup>29</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)

<sup>30</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/PerfStds/LLNAPerfStd07Jan08FD.pdf>

<sup>31</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)

## **THURSDAY, MARCH 6, 2008**

### **Reconvening of the Panel Meeting**

Dr. Luster reconvened the Panel Meeting at 8:30 a.m. He introduced himself and then asked that all Panel members and all others in attendance introduce themselves as well.

### **Overview of the Draft ICCVAM LLNA Performance Standards**

#### **Panel Evaluation:**

Dr. Woolhiser reviewed some of the important points highlighted during the previous day's discussion on this topic, and then continued to summarize the remaining comments of his Evaluation Group on the questions asked by ICCVAM on the draft ICCVAM LLNA Performance Standards for consideration by the entire Panel. As mentioned above, the Panel discussion and their recommended revisions to the draft ICCVAM LLNA Performance Standards are reflected in the Panel report published in May 2008.<sup>32</sup>

Dr. Woolhiser noted that there were general comments on the topic order for the Panel's review. He asked if Dr. Stokes would comment on the rationale for the topic order. Dr. Stokes indicated that as the IWG deliberated the order of topics for this review, consideration was given to the fact that the three non-radioactive methods had undergone validation studies prior to the creation of LLNA performance standards. Thus, the non-radioactive test methods were reviewed before the performance standards, so as to not bias the Panel's assessment of each test method's performance. The performance standards could then be considered for their application to future test methods.

#### **Public Comments:**

##### **Dr. Amy Rispin, EPA**

Dr. Rispin stated that her intent was to provide some additional regulatory perspective on some of the points that have been discussed. When Federal agencies evaluate the validation status of a test method under ICCVAM, they conduct a comprehensive analysis of overall performance (i.e., accuracy and reliability) in the context of making regulatory decisions with data from the test method. Thus, in a regulatory situation, equal or greater accuracy compared to the reference test method is the expectation. If the number of animals can be decreased only at the expense of accuracy, the acceptability of such a test method for the particular regulatory purpose would need to be carefully considered. Certain methods, instead of being complete replacements, might have to be relegated to the role of screens, where positives would be accepted, but negatives would require further testing - a less than ideal situation.

Dr. Rispin commented that performance standards are the regulating agencies' basis for the acceptability of variations of accepted test methods. If an agency receives data from a modified LLNA method that has not been reviewed and validated in the ICCVAM process, there is unlikely to be a comprehensive peer review of it within the agency, given resource limitations. Therefore, the question of major versus minor departures from the functional criteria is important to ICCVAM and its member agencies. One cannot anticipate that there will be anything other than these performance standards to adequately evaluate the usefulness and limitations of a new method.

##### **Dr. David Basketter, ECVAM Observer**

Dr. Basketter first commented on a point that Dr. Thomas Gebel alluded to during the Panel's discussion of the draft ICCVAM LLNA Performance Standards, which was that if a new laboratory performed the traditional LLNA to assess 18 or 22 chemicals, they probably wouldn't get a complete match. Dr. Basketter disagreed with Dr. Gebel's statement and viewed that a competent laboratory performing the LLNA would get it 100% correct.

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<sup>32</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

Dr. Basketter then provided some comments that he stated were "from the ECVAM perspective." He stated that the ECVAM performance standards tried to address adhering to a standard protocol and that any change to the protocol other than the method for evaluating lymph node proliferation (e.g., strain, species, number of applications, time) was considered not to be minor, and therefore such a protocol would not be applied to these performance standards. By restricting the performance standards to minor changes, ECVAM was trying to minimize the number of chemicals required to evaluate sensitivity. Furthermore, the EC3 value could be used to see if the test method could classify substances in the appropriate range of sensitization potency.

ECVAM initially chose their reference substances in order to determine whether a modified method (differing only in the method for measuring cell proliferation) would give the same answer as the traditional LLNA. Thus, there was no intent to compare to the guinea pig or human data.

Dr. Basketter speculated that it is doubtful that data from multiple LLNA studies on the same substance are available and therefore it is unlikely that much larger sample sizes from which to calculate mean EC3 values and associated ranges will be obtained.

Dr. Basketter concluded by stating that ECVAM will not include more false positives and false negatives in its list. It has included one false positive and false negative in order to harmonize with ICCVAM but they don't see an added statistical value of just having one more false positive and false negative.

**Karen Hamernik, EPA**

Dr. Hamernik concurred with the comments that Dr. Rispin made previously, that performance standards, if developed such that they are too generalized with respect to minor versus major changes, would be problematic for regulatory agencies when they are reviewing submissions that include data from a modified LLNA protocol. Dr. Hamernik also asked for clarification from the Panel on a statement made during their discussions that a test for concordance for measuring the accuracy of classification (i.e., yes/no answer) should be done and that a chemical-for-chemical match is not necessary. Dr. Flournoy responded that concordance is not absolute but a continuum. Dr. Luster further clarified that the Panel discussion was based on the fact that the traditional LLNA is not a perfect match when compared to the guinea pig tests. Because there are false negatives and false positives compared to the guinea pig, there should be some flexibility so that an absolute chemical-by-chemical match is not required. In addition, a scientifically valid explanation can be provided for any discordance. Dr. Stokes emphasized that this was an important point and that additional clarity on the differences between a chemical-by-chemical match and overall accuracy need to be carefully considered before the final test method accuracy requirements are defined.

**Panel Conclusions and Recommendations:**

Dr. Luster asked the Panel to review the conclusions and recommendations for the ICCVAM LLNA performance standards they had discussed earlier and to make any revisions, if necessary. The Panel indicated that modified LLNA protocols that are undergoing validation should contain essential test method components that follow the ICCVAM-recommended protocol,<sup>33</sup> unless adequate scientific rationale for deviating from this protocol was provided. The Panel also identified aspects of the LLNA that should be required as part of the test method validation process, if more extensive changes to the protocol are being considered: (1) application of the test substance to the skin with sampling of the lymph nodes draining that site, (2) measurement of cell proliferation in the draining lymph node, (3) absence of a skin reaction that could be indicative of the onset of the elicitation phase of skin sensitization, (4) data collected at the level of the individual animal to allow for an estimate of the

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<sup>33</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/LLNAProt.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf)

variance within control and treatment groups,<sup>34</sup> and (5) if dose response information is needed, there are an adequate number of dose groups ( $n \geq 3$ ) with which to accurately characterize the dose response for a given test substance.

The Panel also recommended that statistical tests to analyze the data might allow for a more accurate interpretation. They recommended that a suitable variance-stabilizing transformation (e.g., log transformation, square root transformation) be applied in all statistical analyses and in reporting summary standard deviations. The Panel also recommended that a more rigorous evaluation be conducted of what would be considered an appropriate range of EC<sub>t</sub> values (i.e., estimated concentration needed to produce a stimulation index that is indicative of a positive response) to include as a requirement. This would be a statistical evaluation that considers the variability of EC<sub>t</sub> values generated among the sensitizers included on the performance standards reference substances list and the statistical multiple comparisons problem.

Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised. The members of the Panel agreed with one abstention; Dr. McDougal abstained from voting stating that he still had a concern about what constitutes a “major/minor” change. The Panel’s detailed recommendations and conclusions on the ICCVAM LLNA performance standards are included in their final Panel report.<sup>35</sup>

## **Overview of the Draft LLNA Potency Determinations BRD and Draft ICCVAM Test Method Recommendations**

Dr. Strickland presented an overview of the draft ICCVAM BRD for the use of the LLNA to determine skin-sensitization potency. She mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA as a stand-alone assay for hazard categorization of skin-sensitization potency. In the BRD, the LLNA was evaluated for its ability to categorize substances for skin-sensitization potency using EC<sub>3</sub> values.

Dr. Strickland noted that the analyses conducted in the BRD were based on LLNA studies obtained from ICCVAM (1999), the published literature, and data received in response to an FR notice (72 FR 27815, May 17, 2007) requesting original data from the LLNA. As a result, the analyzed data included 170 substances with LLNA, human, and/or guinea pig data. Dr. Strickland noted that three sets of data were analyzed and briefly summarized the results which are detailed in the draft ICCVAM BRD.<sup>36</sup> Dr. Strickland also briefly summarized the draft ICCVAM test method recommendations for potency determinations.<sup>37</sup>

### **Panel Evaluation:**

Ms. Headrick presented her Evaluation Group’s draft responses to ICCVAM’s questions to the Panel for consideration by the entire Panel. These included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD and recommendations are reflected in the Panel report published in May 2008.<sup>38</sup>

During the course of the discussion on the potency applicability of the LLNA, Dr. Woolhiser asked what the basis for the human threshold concentration cutoff values of 250 and 500  $\mu\text{g}/\text{cm}^2$  were. Dr.

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<sup>34</sup> Individual animal data will allow the application of a formal statistical test, if deemed necessary, and will also allow power calculations associated with the modified LLNA test.

<sup>35</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

<sup>36</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/LLNAPotency18Jan08FD.pdf>

<sup>37</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/LLNAPotencyRecs18Jan08FD.pdf>

<sup>38</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

Wind replied that a number of experts and clinicians from throughout the world went back and looked at what, in their countries, they demarcated as strong sensitizers. The proposed Globally Harmonized System of Classification and Labeling of Chemicals (GHS) subcategory guidance values for the LLNA, guinea pig tests (GPMT, BT) and human data (HMT and HRIPT) were made on the basis of an impact analysis of 175 chemicals. In addition, the two proposed cut-offs were evaluated by the GHS Expert Group on Sensitization based upon chemicals already regulated as strong sensitizers to ensure their inclusion within the GHS categorization scheme. Clinical members of the Expert Group also confirmed relevance of the cut-off values such that clinically important skin sensitizers fell into the appropriate subcategory. The proposed guidance values were also in line with the European Commission's Expert Working Group recommendations.

### **Public Comments:**

#### **Dr. David Basketter, ECVAM Observer**

Dr. Basketter commented that reviewing the potency data by splitting it into pooled and unpooled groups could be interesting but might be difficult since the majority of available data likely comes from pooled groups. Furthermore, much of the deliberation concluding that individual animal data must be used was derived from analyses based only or largely on pooled data from four animals.

Dr. Basketter further stated that he viewed the analyses, which make the assumption that the human threshold data is the gold standard, as fundamentally flawed. Human data comes from studies conducted at different times, with different protocols, according to varying quality standards, and by different people. Therefore, there is no definitive knowledge of the reproducibility of the data. However, he considers the analyses adequate for recommending the LLNA as a part of a weight-of-evidence decision on human sensitization potency categorizations.

#### **Dr. Amy Rispin, EPA**

Dr. Rispin noted that there has been much discussion about various ways of handling the potency data. The OECD expert task force on skin sensitization needs to see an analytical comparison of what is considered to be the most appropriate approach for evaluating the data. The question for categorization purposes is, *What is the ideal testing modality for separating strong versus weak sensitizers for potency categorization?* A regulator who must assign a categorization is going to be confronted with all available test data and must know which data should be given the greatest weight in their evaluation.

Dr. Rispin noted that the OECD task force also reviewed the draft BRD on potency determinations and sent a list of several questions to the Panel, some of which have been answered, many of which have not been. One of the questions is, can the LLNA protocols be refined (e.g., by selection of solvents or choice of other test parameters) to improve correlation? She concluded by noting that she hopes that the additional analyses that the Panel has suggested will bring some clarity to the matter.

### **Panel Conclusions and Recommendations:**

Dr. Luster asked the Panel to review the conclusions and recommendations for the LLNA potency determinations they had discussed earlier and to make any revisions, if necessary. The Panel agreed with the draft ICCVAM recommendation that the LLNA should not be used as a stand-alone assay for categorizing skin sensitizers as strong versus weak, but that it could be used as part of a weight-of-evidence evaluation (e.g., along with quantitative structure-activity relationships, peptide reactivity, human evidence, historical data from other experimental animal studies) for this purpose. The Panel also agreed with ICCVAM's recommendation that any LLNA studies conducted for the purpose of evaluating skin-sensitization potency should use the ICCVAM-recommended LLNA protocol. In addition, the Panel stated that the relevant testing guidelines for the traditional LLNA should be revised to include the procedure for calculating an EC3 value. Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised; the Panel unanimously agreed. The Panel's detailed

recommendations and conclusions on the LLNA potency determinations are included in their final Panel report.<sup>39</sup>

### **Concluding Remarks—**

Dr. Luster, on behalf of the Panel, thanked the NICEATM-ICCVAM staff for their continued assistance during the review process and the Panel meeting. He also thanked Drs. Joanna Matheson and Abby Jacobs, the IWG co-chairs, and Dr. Marilyn Wind, ICCVAM Chair and IWG member, for the hard work they put into the project. Dr. Luster also thanked the Panel and the Panel Chairs for their involvement in the huge task of reviewing seven topics. He commented that, for future reference for ICCVAM, the Panel in their individual groups were able to do a good job in reviewing the materials, but because they were so focused on their particular topics due to serious time constraints, there may not have been the full benefit of their expertise for other topics in all cases.

Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked the invited test method developers for their excellent summaries of their method for the benefit of the Panel, and CPSC for hosting the Panel meeting. He mentioned that there has been discussion about obtaining additional existing data (i.e., on mixtures, on one or more of the non-radiolabeled test methods), and that should these data become available in a timely manner and if NICEATM is able to assimilate and analyze the data, the Panel might be reconvened by teleconference to review the data. Dr. Stokes concluded by saying he looked forward to further working with the Panel members to complete their Panel report.

### **Adjournment—**

The meeting was adjourned and concluded at 3:20 p.m.

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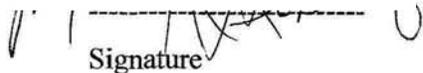
<sup>39</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPREpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPREpt2008.pdf)

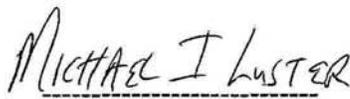
William S. Stokes, D.V.M.  
NIEHS  
P.O. Box 12233  
MD-EC17  
Research Triangle Park, NC 27709

Dear Dr. Stokes,

The Meeting Summary Minutes, Independent Scientific Peer Review Panel Meeting, Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products, accurately summarizes the Peer Review Panel meeting of March 4-6, 2008, in Bethesda, MD.

Sincerely,

  
Signature

  
Printed Name

  
Date

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## **Appendix E2**

### **Peer Review Panel Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products**

**The full document is available electronically on the enclosed CD-ROM or at:  
[http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPrept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPrept2008.pdf)**

**The document is also available on request from NICEATM:**

#### **NICEATM**

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**Independent Scientific Peer Review Panel Report:  
Validation Status of New Versions and Applications of the  
Murine Local Lymph Node Assay: A Test Method for Assessing  
the Allergic Contact Dermatitis Potential of Chemicals and  
Products**

**May 2008**

**Interagency Coordinating Committee on the Validation of Alternative  
Methods (ICCVAM)**

**National Toxicology Program (NTP) Interagency Center for the  
Evaluation of Alternative Toxicological Methods (NICEATM)**

**National Institute of Environmental Health Sciences (NIEHS)  
National Institutes of Health  
U.S. Public Health Service  
Department of Health and Human Services**

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**This document is available electronically at:  
[http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf)**

**The findings and conclusions of this report are those of the  
Independent Scientific Peer Review Panel and should not be construed  
to represent the official views of ICCVAM or its member agencies.**

## Table of Contents

<b>List of Tables .....</b>	<b>iii</b>
<b>List of Abbreviations and Acronyms.....</b>	<b>iv</b>
<b>Members of the Independent Scientific Peer Review Panel .....</b>	<b>vi</b>
<b>Preface .....</b>	<b>ix</b>
<b>Executive Summary .....</b>	<b>xiii</b>
<b>1.0 Murine Local Lymph Node Assay (LLNA) Limit Dose Procedure .....</b>	<b>1-1</b>
1.1 Comments on the Draft Background Review Document (BRD) for Completeness, Errors, and Omissions .....	1-1
1.2 Comments on the Validation Status of the LLNA Limit Dose Procedure .....	1-4
1.3 Comments on the Draft ICCVAM Test Method Recommendations on the LLNA Limit Dose Procedure .....	1-8
<b>2.0 LLNA for Testing Aqueous Solutions, Metals, and Mixtures.....</b>	<b>2-1</b>
2.1 Comments on the Draft Addendum for Completeness, Errors, and Omissions.....	2-1
2.2 Comments on the Validation Status of the Traditional LLNA for Testing Aqueous Solutions, Metals and Mixtures .....	2-1
2.3 Comments on the Draft ICCVAM Test Method Recommendations on the Traditional LLNA for Testing Aqueous Solutions, Metals, and Mixtures .....	2-3
<b>3.0 Non-Radioactive LLNA Protocol - The LLNA: Daicel Adenosine Triphosphate (LLNA: DA) Test Method .....</b>	<b>3-1</b>
3.1 Comments on the Draft BRD for Completeness, Errors, and Omissions.....	3-1
3.2 Comments on the Validation Status of the LLNA: DA .....	3-2
3.3 Comments on the Draft ICCVAM Test Method Recommendations on the LLNA: DA .....	3-6

**4.0 Non-Radioactive LLNA Protocol - The LLNA: Bromodeoxyuridine Detected by Flow Cytometry (BrdU-FC) Test Method .....4-1**

4.1 Comments on the Draft BRD for Completeness, Errors, and Omissions ..... 4-1

4.2 Comments on the Validation Status of the LLNA: BrdU-FC ..... 4-3

4.3 Comments on the Draft ICCVAM Test Method Recommendations on the LLNA: BrdU-FC ..... 4-6

**5.0 Non-Radioactive LLNA Protocol - The LLNA: Bromodeoxyuridine Detected by ELISA (BrdU-ELISA) Test Method.....5-1**

5.1 Comments on the Draft BRD for Completeness, Errors, and Omissions ..... 5-1

5.2 Comments on the Validation Status of the LLNA: BrdU-ELISA ..... 5-3

5.3 Comments on the Draft ICCVAM Test Method Recommendations on the LLNA: BrdU-ELISA ..... 5-7

**6.0 Draft ICCVAM LLNA Performance Standards.....6-1**

6.1 Comments on the Proposed Purpose and Applicability ..... 6-1

6.2 Comments on the Essential Test Method Components..... 6-2

6.3 Comments on the Proposed Reference Substances..... 6-3

6.4 Comments on the Test Method Accuracy Standards ..... 6-5

6.5 Comments on the Test Method Reliability Standards..... 6-7

6.6 Summary..... 6-8

6.7 Additional Statistical Comments ..... 6-9

**7.0 Use of the LLNA for Potency Determinations.....7-1**

7.1 Comments on the Draft BRD for Completeness, Errors, and Omissions ..... 7-1

7.2 Comments on the Validation Status of the Traditional LLNA to Determine Skin Sensitization Potency ..... 7-1

7.3 Comments on the Draft ICCVAM Test Method Recommendations for the Use of the LLNA for Potency Determination ..... 7-5

**8.0 References .....8-1**

**Appendix A: Peer Review Panel Member Biosketches .....A-1**

**Appendix B: Questions for the Peer Review Panel..... B-1**

**List of Tables**

<b>Table 1-1</b>	<b>Power Calculations for the Traditional LLNA .....</b>	<b>1-5</b>
<b>Table 3-1</b>	<b>Power Calculations for the LLNA: DA .....</b>	<b>3-3</b>
<b>Table 4-1</b>	<b>Power Calculations for the LLNA: BrdU-FC .....</b>	<b>4-8</b>
<b>Table 5-1</b>	<b>Power Calculations for the LLNA: BrdU-ELISA.....</b>	<b>5-2</b>

### List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ANOVA	Analysis of variance
AOO	Acetone: olive oil (4:1)
BRD	Background Review Document
BrdU	Bromodeoxyuridine
BT	Buehler Test
CD4	Cluster of differentiation 4
CPSC	U.S. Consumer Product Safety Commission
CRO	Clinical research organization
CV	Coefficient of variation
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNCB	Dinitrochlorobenzene
EC3	Estimated concentration needed to produce a stimulation index of three
ECt	Estimated concentration needed to produce a stimulation index that is indicative of a positive response
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme Linked Immunosorbent Assay
eLLNA: BrdU-FC	Enhanced LLNA with BrdU detected by flow cytometry
EPA	U.S. Environmental Protection Agency
FC	Flow cytometry
FR	<i>Federal Register</i>
GLP	Good Laboratory Practice
GPMT	Guinea Pig Maximization Test
GSK	GlaxoSmithKline
HCA	Hexyl cinnamic aldehyde
HMT	Human Maximization Test
HRIPT	Human Repeat Insult Patch Test
HTdR	<sup>3</sup> H-Methyl Thymidine
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for Validation of Alternative Methods
LLNA	Local Lymph Node Assay
LLNA: BrdU-ELISA	LLNA with BrdU detected by ELISA
LLNA: BrdU-FC	LLNA with BrdU detected by FC
LLNA: DA	LLNA: Daicel Adenosine Triphosphate
LNC	Lymph node cells
LOEL	Lowest observed effect level
MEK	Methyl ethyl ketone
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NOEL	No observed effect level
NTP	National Toxicology Program
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
QSAR	Quantitative structure-activity relationship
REACH	Registration, Evaluation, and Authorisation of Chemicals
rLLNA	Reduced LLNA
SAR	Structure-activity relationship
SD	Standard deviation
SI	Stimulation index
SDS	Sodium dodecyl sulfate
SLS	Sodium lauryl sulfate
TG	Test Guideline
Th	T-helper
vs.	Versus

### Members of the Independent Scientific Peer Review Panel

**Michael Luster, Ph.D. (Panel Chair)**, Senior Consultant to the National Institute of Occupational Safety and Health (NIOSH) Health Effects Laboratory, Morgantown, WV

**Nathalie Alépée, Ph.D.**, Associate Research Fellow, Pfizer PDRD MCT Laboratory, France

**Anne Marie Api, Ph.D.**, Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ

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<sup>1</sup> Drs. Green and Richmond were unable to attend the public meeting on March 4-6, 2008. However, they were involved in the review of the background review documents and concur with the conclusions and recommendations included in this report.

*Independent Peer Review Panel Report*

*May 2008*

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*Independent Peer Review Panel Report*

*May 2008*

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## Preface

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine local lymph node assay (LLNA) to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation of the validation status of the LLNA that included an assessment by an international independent scientific peer review panel (hereafter, Panel). The Panel report and the ICCVAM LLNA test method recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website.<sup>1</sup> The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (OECD 2002; ISO 2002; EPA 2003). For this Panel report, this LLNA will be referred to as the “traditional” LLNA.

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally requested through NICEATM that ICCVAM assess the validation status of:<sup>2</sup>

- The traditional LLNA as a stand-alone assay for potency determinations (including severity) for the purpose of hazard classification
- Three modifications of the traditional LLNA not requiring the use of radioactive materials
- The LLNA limit dose procedure (also referred to as the "reduced" LLNA)
- The ability of the traditional LLNA to test mixtures, metals, and aqueous solutions (i.e., to re-evaluate the applicability domain for the traditional LLNA)

NICEATM, in coordination with ICCVAM and the ICCVAM Immunotoxicity Working Group, prepared a comprehensive draft background review document (BRD) for each modified version of the traditional LLNA test method being evaluated, as well as a draft applicability domain addendum to the final BRD published previously on the traditional LLNA. Each draft BRD and the draft addendum detailed the available data and information from the published literature and submissions received in response to a 2007 *Federal Register (FR)* notice that had requested data related to CPSC’s nomination (*FR* notice Vol. 72, No. 95, p. 27815-27817, May 17, 2007). In addition, ICCVAM developed draft LLNA Performance Standards intended for use in validating alternative test methods that are functionally and mechanistically similar to the traditional LLNA. Finally, ICCVAM, based on the information contained in each of the draft BRDs and the draft addendum, developed draft test method recommendations.

The various supporting documents and the draft ICCVAM recommendations were provided to a new international Panel for an independent scientific review. In addition, NICEATM announced the availability of these documents on the NICEATM-ICCVAM website

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<sup>1</sup> The 1999 ICCVAM Panel report and recommendations can be obtained at: [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/llnarep.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf)

<sup>2</sup> The CPSC nomination can be obtained at: [http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf)

(<http://iccvam.niehs.gov>) for public comment in a *FR* notice (Vol. 73, No. 5, p. 1360-1362, January 8, 2008) and via the ICCVAM listserv. The *FR* notice also announced the public Panel meeting, to be convened at the CPSC Headquarters in Bethesda, MD on March 4–6, 2008.

The Panel was charged with:

- Reviewing each ICCVAM draft BRD and the draft addendum for completeness and identifying any errors or omissions of existing relevant data or information
- Evaluating the information in each draft BRD and the draft addendum to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM 2003) had been appropriately addressed for the recommended use of the new versions and applications of the traditional LLNA
- Considering the ICCVAM draft test method recommendations for the following and commenting on the extent to which they are supported by the information provided in the draft BRDs and the draft addendum:
  - proposed test method uses
  - proposed recommended standardized protocols
  - proposed test method performance standards
  - proposed additional studies
- Evaluating the draft ICCVAM LLNA Performance Standards and considering whether they were adequate for assessing the accuracy and reliability of alternative test methods that are functionally and mechanistically similar to the traditional LLNA

During our public meeting in March 2008, the Panel discussed each charge, listened to public comments, and developed conclusions and recommendations for ICCVAM on each of the nominated activities. The Panel wished to emphasize that they were to consider two overall questions. They were to consider: (1) whether the validation status of each of the above proposed modifications or alternative uses of the LLNA had been adequately characterized for its intended purpose according to established ICCVAM validation criteria (available on the NICEATM-ICCVAM website, <http://iccvam.niehs.gov>), and (2) whether proposed modifications or alternative uses of the LLNA are sufficiently accurate and reliable to be used for the identification of sensitizing substances and non-sensitizing substances in place of the traditional LLNA procedure.

This report details the Panel's independent conclusions and recommendations. ICCVAM will consider this report, along with all relevant public comments, as it develops final test method recommendations. The final ICCVAM test method recommendations will be forwarded to U.S. Federal agencies for their consideration in accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545).

The Panel gratefully acknowledges the efforts of NICEATM staff in coordinating the logistics of the peer review Panel meeting and in preparing materials for their review. The

*Independent Peer Review Panel Report*

*May 2008*

Panel also thanks each of the test method developers, Drs. George DeGeorge (LLNA: BrdU-FC), Kenji Idehara (LLNA: DA), and Masahiro Takeyoshi, (LLNA: BrdU-ELISA) for providing summaries and additional clarifications of the non-radioactive test methods under review. Finally, as Panel Chair, I want to thank each Panel member for her or his thoughtful and objective review of these LLNA-related activities.

Michael Luster, Ph.D.  
Chair, LLNA Peer Review Panel  
May 2008

*Independent Peer Review Panel Report*

*May 2008*

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## Executive Summary

This report describes the conclusions and recommendations of an international independent scientific peer review panel (hereafter, Panel). This Panel was charged by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) with evaluating the validation status of new versions and applications of the murine local lymph node assay (LLNA) for assessing the allergic contact dermatitis (ACD) potential of chemicals and products. The LLNA, which was first evaluated in 1999 by ICCVAM, is hereafter referred to as the “traditional LLNA” to distinguish it from other versions considered by the Panel. The new versions and applications considered include:

- The LLNA limit dose procedure (also referred to as the "reduced" LLNA<sup>1</sup>)
- The ability of the traditional LLNA to test mixtures, metals, and aqueous solutions (i.e., a re-evaluation of the applicability domain for the traditional LLNA)
- Three modifications of the traditional LLNA not requiring the use of radioactive materials:
  - LLNA: DA (Local Lymph Node Assay: Daicel Adenosine Triphosphate)
  - LLNA: BrdU-FC (Local Lymph Node Assay: Bromodeoxyuridine detected by flow cytometry)
  - LLNA: BrdU-ELISA (Local Lymph Node Assay: Bromodeoxyuridine detected by ELISA)
- The traditional LLNA as a stand-alone assay for potency determinations (including severity) for the purpose of hazard classification

The Panel also evaluated the draft ICCVAM LLNA Performance Standards and considered whether they were adequate for assessing the accuracy and reliability of alternative test methods that are functionally and mechanistically similar to the traditional LLNA.

### LLNA Limit Dose Procedure

The Panel agreed that the LLNA limit dose procedure, which normally allows for testing at one dose level, should be routinely recommended for hazard identification when used for testing purposes which do not require dose response information, because it would offer time, cost, throughput and logistical benefits as well as using fewer animals. In instances when a necessity to measure relative skin sensitization potency for the purpose of risk assessment was present, then the traditional LLNA should be used in order to generate dose response information. Still, the Panel recommended use of the LLNA limit dose procedure as

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<sup>1</sup> As described in this report, the Panel agreed that consideration should be given to applying the same term to the LLNA limit dose procedure since in various places throughout the draft BRD it was referred to differently as either the “cut-down”, the “limit dose”, or the “reduced LLNA” (i.e., “rLLNA”). Since the European Centre for the Validation of Alternative Methods (ECVAM) has already established a naming convention of “rLLNA”, the Panel recommended adopting the ECVAM terminology to harmonize the terminology used among the international validation agencies. However, because the ICCVAM documents that were reviewed use “LLNA limit dose procedure” that term is retained in this report.

the initial testing procedure to identify sensitizers and non-sensitizers before conducting the traditional LLNA even when dose response information *is* required since if the test substance were negative in the limit dose procedure, it would not be necessary to conduct a multiple-dose LLNA test.

The draft background review document (BRD) for the LLNA limit dose procedure provides a comprehensive review of available data and information for assessing the usefulness and limitations of this modified version of the LLNA for the purpose of skin sensitization hazard classification. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that it be updated to reflect their suggestions/corrections relating to general, statistical, and specific editorial issues. In particular, the Panel noted that the differences in terminology used for this procedure caused confusion and recommended that an internationally harmonized term be adopted. They suggested referring to the procedure as the “reduced LLNA” (i.e. “rLLNA”) since that is being used by the European Centre for the Validation of Alternative Methods (ECVAM).

The Panel concluded that the stimulation index (SI) based on the ratio of 3.0 as the cutoff value was indicative of a response that was sufficiently greater than the control and would be considered an immunologically relevant response, but recommended that statistical analyses be used to definitively establish that a response induced by a test substance is significantly different from the vehicle control. The Panel agreed that the LLNA protocol recommended by ICCVAM (ICCVAM 1999; Dean et al. 2001) should be the standard protocol for all future LLNA limit dose studies using the traditional LLNA protocol. Specifically, prospective LLNA limit dose procedure studies should require that lymph nodes be collected from individual animals instead of pooling them with other animals in a treatment group, which is also currently permitted by the Organisation for Economic Co-operation and Development (OECD) Test Guideline 429 (OECD 2002). Individual animal response data are necessary in order to statistically analyze for differences between treated and control data. In addition, having data from individual animals also allows for identification of technical problems and outlier animals within a dose group. Based on power calculations provided as supplemental information, the Panel agreed that five animals per dose group is an appropriate number to recommend for LLNA limit dose studies following the traditional LLNA protocol. It should be noted that the Panel’s analysis of the LLNA limit dose dataset was not restricted to studies with confirmed individual animal data, and that the Panel considered data known to have been generated using pooled group data. The Panel stated that, internationally, both individual and pooled animal data have likely been used both for regulatory decisions and for in-house decisions relating to product development and risk management. In addition, the fact that the retrospective data analysis set out in the draft LLNA limit dose procedure BRD did not distinguish between individual or pooled animal data suggested that both met the quality standards for inclusion in the draft BRD.

Although they did not reach consensus, the Panel suggested that for laboratories in which the LLNA is “routinely” performed and have demonstrated the ability to consistently obtain positive results, hexyl cinnamic aldehyde (HCA) or another positive control (e.g., a substance that matches the chemical class of the test substances) could be run at intervals for quality control purposes rather than concurrent with each experiment. The Panel cited Kimber et al. (2006), which describes “routine” use of the “rLLNA” utilizing only a vehicle and a high-dose group, as a rationale for this suggestion. However, the Panel does not recommend

omitting the concurrent positive control in laboratories that perform the LLNA only “occasionally”.

Based on the analyses presented in the draft BRD, the Panel considered the accuracy of the LLNA limit dose procedure to have been adequately evaluated and compared to the traditional LLNA, mindful of the limitations associated with a retrospective evaluation. For instance, it cannot be assumed that the compounds tested in the retrospective studies were always tested at the highest possible dose unless such information was explicitly indicated. In this regard, the Panel recommended that a more detailed description of what is considered “*avoidance of excessive irritation*” and “*evidence of systemic toxicity*” be included in any LLNA protocol in order to aid in choosing the most appropriate high (i.e., limit) dose, although specific indicators of “*systemic toxicity or excessive irritation*” were not formally discussed.

The Panel agreed that it was appropriate to assume that the intra- and inter-laboratory reproducibility of the LLNA limit dose procedure and the traditional LLNA would be similar, because reproducibility is more dependent on the method than on the number of dose groups. However, reducing the number of test substances dose groups from three to one might reduce the sensitivity of the assay. The traditional LLNA may have a greater chance of correctly identifying a sensitizer even in the presence of one or more technical errors since data from three dose groups are being considered and an  $SI \geq 3.0$  at any dose group would result in the substance being classified as a sensitizer. However, for the purpose of adopting an assay that uses fewer animals and provides increased throughput for testing purposes, these hypothetical considerations are not a sufficient reason to argue against use of the limit dose LLNA procedure.

#### **LLNA for Testing Aqueous Solutions, Metals, and Mixtures**

The draft ICCVAM recommendations state that, although more data are needed to assess the use of the LLNA for testing for mixtures and aqueous solutions before a recommendation can be made, the traditional LLNA appears to be useful for the testing of metal compounds, with the exception of nickel. The Panel agreed with these draft ICCVAM recommendations. Regarding the use of the LLNA for testing mixtures, the Panel acknowledged that the ability of ICCVAM to develop draft test method recommendations was limited not only by the amount of data available, but the relatively poor concordance of traditional LLNA outcomes in comparison to those obtained in guinea pig tests, and recommended that this be noted in the final ICCVAM recommendations. The term “mixtures” can represent an infinite number of materials and it would be more beneficial to specify types or formulations of mixtures that are being examined.

Regarding metals, the Panel concluded that the accuracy statistics for the traditional LLNA when compared to results obtained from evaluation in humans supported use of the traditional LLNA as a hazard identification tool for metals, with the exception of nickel, which produces variable responses. One minority opinion stated that the results for nickel compounds were not entirely questionable and that the traditional LLNA might also be suitable for testing nickel compounds. Thus, the Panel recommended further evaluation of the variable results obtained for nickel in the context of the available literature on allergic contact dermatitis to nickel in humans.

Regarding substances tested in aqueous solutions, the Panel suggested expanding the brief section of the draft test method recommendations discussing the test method protocol for the traditional LLNA to specifically point out how the conclusions of the applicability domain evaluation may affect the standard traditional LLNA protocol. For instance, it could be suggested that aqueous test solutions be avoided due to problems associated with skin application. It would be preferable for a hierarchy of organic solvents to be considered as dosing vehicles, with emphasis on using a vehicle to which humans may actually be exposed in circumstances linked to occupational sensitization.

The Panel agreed with the draft ICCVAM recommendation for continued accrual of information from traditional LLNA evaluations of mixtures, metals, and aqueous solutions with comparative data for guinea pig (i.e., guinea pig maximization test [GPMT] or Buehler test [BT]) and human (i.e., human maximization test [HMT] or human repeat insult patch test [HRIPT]) tests. However, the Panel suggested that, given resource limitations, it would be important to organize the recommendations based on relative priority.

The draft Addendum to the original validation report for the traditional LLNA (ICCVAM 1999) provided a comprehensive review of currently available data and information for evaluating the usefulness and limitations of the traditional LLNA for assessing the skin sensitization potential of mixtures, metal compounds, and substances tested in aqueous solutions. The Panel evaluated the draft Addendum for completeness, errors, and omissions and concluded that there were no apparent errors or omissions, although they did state that the term “mixtures” was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations of mixtures that are being examined.

The Panel did not identify any classes of chemicals missing from the dataset used to review the utility of the traditional LLNA for testing aqueous solutions. However, while they did not propose an alternative, the Panel expressed concern over the most appropriate definition for an aqueous solution (defined in the draft Addendum as any solution containing  $\geq 20\%$  water). For the mixtures included in the analysis, the Panel noted that quantitative compositions had not been provided and therefore they could not comment on whether these mixtures were representative of the types of mixtures typically tested in the traditional LLNA. With respect to metals (none of which are mixtures), there was a paucity of important representatives of commercially useful metals such as platinum, palladium, iron, zinc, manganese and silver in the data set. The Panel suggested that to enlarge the group of metal non-sensitizers, substances used as cosmetic ingredients (e.g., titanium dioxide) and aluminum compounds currently used in antiperspirants might be considered.

The Panel agreed that, although it was important to identify data obtained according to GLP guidelines, data obtained from non-GLP studies should not be excluded automatically from this retrospective analysis. The Panel concluded that other factors could be used to identify high quality data. Examples include data published in peer-reviewed journals or obtained from a study conducted in a laboratory that has GLP capabilities.

The Panel concluded that, considering the limited comparative data that were available, particularly for mixtures and aqueous solutions, the accuracy assessment of the traditional LLNA for testing mixtures, metals, and aqueous solutions when compared to available human and/or guinea pig test results was as comprehensive as was possible. The limited

amount of comparative data made it unfeasible to draw definitive conclusions for mixtures and aqueous solutions from the available accuracy statistics.

#### **Non-Radioactive LLNA Protocol - The LLNA: DA Test Method**

The Panel concluded that the available data and test method performance support the ICCVAM draft recommendations for the LLNA: Daicel Adenosine Triphosphate test method (LLNA: DA), and that the test method may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that this recommendation is contingent upon receipt, review, and analyses of additional existing data and information from the test method developer. Therefore, this non-radioactive version of the traditional LLNA cannot currently be recommended for the hazard identification of skin sensitizing substances, regardless of whether or not there are restrictions on the use of radioactive materials, until such time as this existing data has been received and confirmed.

The draft LLNA: DA BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: DA test method to assess the allergic contact dermatitis potential of chemicals and other substances. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that their suggestions/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The Panel agreed that five animals per dose group should be recommended for validation of modified LLNA test methods. The Panel, however, noted that supplemental power calculations for the LLNA: DA test method indicated that the power for detecting a three-fold increase in the treatment group was estimated to be 95% for a sample size of three mice per dose group. Thus, the Panel identified the use of three animals per dose group as a potential opportunity to reduce animal number when using modified assays in the future, assuming all essential validation requirements can be successfully met. A minority opinion expressed by five Panel members was that if laboratories were operating under OECD guidance (OECD 2002) and a reliable validation dataset had been generated, then pooled data from a least four animals per dose group could be considered.

Generally, the Panel viewed the difference in treatment schedule between the LLNA: DA and the traditional LLNA to be potentially significant if the LLNA: DA induced the elicitation phase of skin sensitization. The Panel was concerned that the 1% sodium lauryl sulfate (SLS) pretreatment step in the LLNA: DA might modify the inherent sensitivity of the LLNA. They recommended that the test method developer (Daicel Chemical Industries, Ltd.) justify the use of 1% SLS or consider an alternative decision criterion (i.e., an SI threshold other than 3.0) such that the 1% SLS pretreatment is no longer necessary.

The Panel considered the database of substances tested in the LLNA: DA to be representative of a sufficient range of chemicals expected to be tested for skin sensitization potential, and concluded that the accuracy analysis had made appropriate comparisons to the traditional LLNA, guinea pig tests, and human data/experience. The Panel could not identify specific characteristics associated with the one false negative (i.e., 2-mercaptobenzothiazole) or the one false positive (i.e., benzalkonium chloride), but reemphasized that the potential impact of pretreatment with 1% SLS in this context needed to be considered.

With regard to test method reliability, the Panel concluded the intralaboratory reproducibility of the LLNA: DA had not been adequately evaluated. They noted that the two sensitizers tested had similar chemical structures (i.e., eugenol and isoeugenol) and that it was unclear if the tests were truly independent. The Panel also noted that the interlaboratory reproducibility of the assay could not be adequately evaluated given the lack of original laboratory data and limitations in the study design. In particular, they cited the use of pooled lymph nodes from the mice in each treatment group and the testing of each substance at predetermined dose levels established by the lead laboratory as study design limitations. Still, a Panel minority considered pooled data acceptable and the setting of dose levels for all laboratories based on results from the lead laboratory to be reasonable.

The Panel also commented that ideally, test substances should be coded during the validation of a new assay, although they did not feel that a lack of coding constituted a reason for rejecting the current LLNA: DA dataset. The Panel also commented that although GLP compliance is highly recommended for validation studies, the current studies should not be rejected solely on the basis of a lack of GLP compliance. However, the Panel considered it important to obtain the original records for all validation studies (which have been requested by NICEATM) in order to confirm that the reported data were the same as the data recorded in the laboratory notebooks.

With regard to the 5% (1/19) false negative and 10% (1/10) false positive rates obtained with the LLNA: DA, the Panel commented that it was important to identify reasons why the substances gave “false” results, taking into consideration factors such as intended use of the substances and the target population. They agreed that it might be useful to follow a suspected inaccuracy with an investigation of the mechanistic basis for the discordance since it may help to establish a biologically-based rationale for the discordance.

The Panel noted that the available LLNA: DA data did not support all of the ICCVAM draft recommendations in the proposed test method standardized LLNA: DA protocol. First, although the Panel agreed with the ICCVAM protocol that recommends five animals per dose group, they noted that supplemental statistical information provided for the LLNA: DA test method implied that using less than five animals per dose group was acceptable (e.g., a 3.0-fold increase in the SI value would likely be detected with 99% confidence when using four animals per dose group). In addition, the Panel considered it important to adequately characterize the effect of the 1% SLS pretreatment step in the LLNA: DA, and it should be demonstrated that the day 8 applications do not induce a skin reaction that could be indicative of the onset of the elicitation phase of skin sensitization. Keeping these points in mind, the Panel agreed that if the limit dose procedure was applicable to the traditional LLNA, then it would also be applicable to the LLNA: DA in order to further reduce the number of animals used.

The Panel also stated that the available data supported the ICCVAM draft recommendations for the LLNA: DA in terms of future studies, which included performing a more comprehensive evaluation using more non-sensitizers within and across laboratories. A minority opinion stated by one Panel member was that although testing more sensitizers might be warranted for interlaboratory validation studies, a sufficient number of non-sensitizers (n=11) had already been tested within the same laboratory.

The Panel also commented that the protocol differences between the LLNA: DA and the traditional LLNA could not clearly be constituted as “major” or “minor” changes. However, they considered this issue largely irrelevant if a test method was able to correctly predict the dermal sensitization potential of a test substance. Consequently, the Panel concluded that the current draft ICCVAM Performance Standards could be applicable to the LLNA: DA as a mechanistically and functionally similar test method.

#### **Non-Radioactive LLNA Protocol - The LLNA: BrdU-FC Test Method**

Overall, the Panel concluded that the available data and test method performance of the LLNA with bromodeoxyuridine (BrdU) detected by flow cytometry (LLNA: BrdU-FC) supported the draft ICCVAM recommendations that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that more information and existing data must be made available before the LLNA: BrdU-FC can be recommended for routine use. The Panel concluded that the test method usefulness and limitations identified in the draft ICCVAM recommendations accurately summarized the limits of the information supplied and the additional information that would need to be generated or provided for further consideration of the test method. As a result, the Panel concluded that the LLNA: BrdU-FC could not currently be considered as a scientifically valid replacement alternative to the traditional LLNA. Still, the Panel suggested that the test method recommendation should clearly state that the test method was not “invalid”, but simply that there was currently not sufficient evidence and information to state that it had been adequately validated.

The draft LLNA: BrdU-FC BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: BrdU-FC test method to assess the ACD-inducing potential of substances. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that their recommendations/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The LLNA: BrdU-FC included routine measurements of ear swelling as an indicator of excessive skin irritation. The Panel viewed that this, or any other quantitative measurement of skin irritation, should be carefully considered for inclusion in all LLNA protocols. The Panel considered inclusion of optional quantification of immunophenotypic markers as an additional mechanism for distinguishing irritants from sensitizers to be useful, as it might reduce the frequency of false positives (i.e., substances which are actually skin irritants) and improve comparisons with human data. However, they considered application of immunological markers too detailed and costly for routine LLNA use (i.e., for hazard classification purposes) and more suited for research purposes.

The Panel noted that the substances tested in the LLNA: BrdU-FC seemed representative of a sufficient range of chemical classes and physical chemical properties, and thus that the test method appeared applicable to many of the types of chemicals and products that are typically tested for skin sensitization potential. However, the Panel considered the total database available for evaluation of the validation status of the LLNA: BrdU-FC to be relatively small compared to the large number of substances assessed in the traditional LLNA. Therefore, the Panel recommended caution when making conclusions related to its concordance with the

traditional LLNA. Still, the accuracy of the LLNA: BrdU-FC was considered adequately evaluated and comparable to the traditional LLNA.

The Panel concluded that intralaboratory reproducibility was not adequately assessed and it should be better evaluated in order to support the validation of this test method. The Panel suggested that although the studies evaluated in the draft BRD were not GLP-compliant, this should not affect acceptance of the data for an evaluation of the validation status of this test method. However, some sources of variability in the intralaboratory data, such as failure to appreciate differences in composition of dosing solutions between experiments caused by test article instability or other phenomena, might be obscured if not in complete compliance with GLP guidelines. Thus, the Panel suggested that any additional studies undertaken to validate the test method should ideally be GLP-compliant.

The Panel agreed that the available data supported the ICCVAM draft recommendations for the LLNA: BrdU-FC procedure in terms of the proposed test method standardized protocol. They suggested that the utility of ear swelling or other methods to detect inflammation appeared warranted in every variation of the LLNA (including the traditional LLNA), but should be further investigated before routine inclusion in the protocol is recommended. The Panel also concluded that the traditional LLNA limit dose procedure could be applied to the LLNA: BrdU-FC, keeping in mind the limitations associated with a “limit dose” procedure.

The Panel further agreed that the ICCVAM draft recommendations for future studies highlighted the unanswered questions raised by the available data set. Specifically, conducting interlaboratory studies as a part of the validation process is important. The Panel considered the immunological markers suggested for the LLNA: BrdU-FC to be acceptable, but that additional immunological markers for discrimination of irritant versus sensitization phenomena were also possible. In general, for any future work, efforts should be made to decrease the variability and to thereby increase the power of the test in order to ensure that more animals were not needed relative to the traditional LLNA or other alternative LLNA protocols.

The Panel considered the protocol differences between the LLNA: BrdU-FC and the traditional LLNA to be “minor” changes, and therefore concluded that assessment of the validity of this test method could be based on the draft ICCVAM LLNA Performance Standards. The Panel also cautioned, however, that a clear definition of what constituted a “major” versus a “minor” change, or a different protocol altogether could be better addressed once the recommendations for the current draft ICCVAM LLNA Performance Standards were finalized.

#### **Non-Radioactive LLNA Protocol - The LLNA: BrdU-ELISA Test Method**

The Panel concluded that the available data and test method performance for the LLNA with BrdU detected by enzyme-linked immunosorbent assay (LLNA: BrdU-ELISA) support the ICCVAM draft recommendations that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that more information and existing data must be made available before the LLNA: BrdU-ELISA can be recommended for use. The Panel also stated that a detailed protocol was needed, in addition to sufficient quantitative data for broader analysis on a larger set of balanced reference substances that take into account

physicochemical properties and sensitization potency, as well as an appropriate evaluation of interlaboratory reproducibility.

The draft LLNA: BrdU-ELISA BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: BrdU-ELISA test method to assess the ACD-inducing potential of chemicals and other substances. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that their suggestions/corrections relating to general, statistical and specific editorial issues be incorporated into the final document.

The Panel's main concern with the test method was that the accuracy of the LLNA: BrdU-ELISA at  $SI \geq 3.0$  was inadequate and not equivalent to the traditional LLNA. Furthermore, although using a decision criterion of  $SI \geq 1.3$  improved the test's performance in identifying sensitizers from non-sensitizers, it did not resolve concerns about the test method. Based on a power analysis for the LLNA: BrdU-ELISA, which was provided to the Panel as supplemental information, the Panel concluded that it was difficult to justify using a  $SI \geq 1.3$  as the cutoff value, given the much larger number of animals that would be required to detect a 1.3-fold increase above vehicle controls with similar power to the traditional LLNA when five animals per dose group are used. For a three-fold increase, the supplemental statistical analyses indicated that a sample size of four was sufficient. Still, the Panel agreed with the ICCVAM recommendation to use five animals per dose group and to collect individual animal data. They concluded that this would allow for more robust calculations in the event that an outlier prevented some of the data from being included in the analysis. A minority opinion by five Panel members was stated that if laboratories were operating under OECD guidance (OECD 2002) and a reliable validation dataset had been generated, then pooled data from a least four animals could be considered.

The Panel noted that in organizations where the use or disposal of radioactive materials was restricted, the potential to use the LLNA: BrdU-ELISA could reduce the number of animals needed per test compared to the traditional LLNA and would result in less pain and suffering compared to using traditional guinea pig test methods. However, if the  $SI \geq 1.3$  was chosen as the decision criterion because of its improved accuracy compared to  $SI \geq 3.0$ , the Panel stated that the number of mice needed to perform the LLNA: BrdU-ELISA test should be compared to the number of guinea pigs that would be needed for skin sensitization tests in order to assess if the LLNA: BrdU-ELISA actually reduced overall animal use for skin sensitization testing.

In general, the Panel considered the number of substances tested in the LLNA: BrdU-ELISA too few, and that data from more substances tested using the traditional LLNA, guinea pig tests, and human tests should have been included. The Panel also did not consider the available data from the LLNA: BrdU-ELISA to be representative of a sufficient range of chemical classes and physical chemical properties. The limited dataset prevents an evaluation of whether the test method would be considered applicable to any of the types of chemicals and products typically tested for skin sensitization potential.

However, the Panel concluded that the appropriate comparisons between the traditional LLNA, guinea pig test and human data had been made. The Panel agreed that the false negative rate for hazard identification using the  $SI \geq 3.0$  in the LLNA: BrdU-ELISA was excessive (i.e., using this SI threshold value, the LLNA: BrdU-ELISA misclassified 29% and

39% of the substances classified as sensitizers in the traditional LLNA or in humans, respectively).

The Panel also considered that the intralaboratory reproducibility of the LLNA: BrdU-ELISA was not adequately evaluated and compared to the traditional LLNA. The Panel indicated that the number of substances was too few, and in some cases there was a wide variation in repeat tests of the same substance. The Panel recommended a more comprehensive evaluation of the intralaboratory reproducibility of the test method, using different SI values, and that the analysis of the variability of the estimated concentration needed to produce a positive SI value (EC<sub>t</sub> values) be conducted on a log scale.

The Panel also noted that interlaboratory reproducibility for the LLNA: BrdU-ELISA could not be evaluated because neither the design of the study sponsored by the Japanese Center for Validation of Alternative Methods nor any of the resulting data had been provided in advance of their evaluation. The Panel agreed that a multi-laboratory validation study using a balanced set of chemicals would adequately characterize the interlaboratory reproducibility of the LLNA: BrdU-ELISA.

In general, the Panel agreed that the available data support the ICCVAM draft recommendations for the LLNA: BrdU-ELISA procedure in terms of the proposed test method standardized protocols. However, as noted above, a minority opinion by five Panel members was that there could be circumstances in which pooled data from at least four animals could also be acceptable. The Panel also stated that if the LLNA: BrdU-ELISA was found to be equivalent to the traditional LLNA in the future that it would be appropriate to apply the LLNA limit dose procedure to the test. The Panel also agreed with ICCVAM's test method recommendations for future studies and emphasized that more data were needed in order to determine the appropriate threshold value for the decision criterion. The Panel concluded that it might be more appropriate to use a statistically-based decision criterion rather than a stimulation index to classify substances as sensitizers, and that this should be further investigated.

The Panel agreed that the LLNA: BrdU-ELISA protocol differed from the traditional LLNA only in the method used to assess lymphocyte proliferation and as such concluded that this represented a "minor" change (as defined in the current draft ICCVAM LLNA Performance Standards) and separate performance standards for the LLNA: BrdU-ELISA were not needed.

#### **Draft ICCVAM LLNA Performance Standards**

The draft ICCVAM LLNA Performance Standards are intended to evaluate the acceptability of proposed test methods that are mechanistically and functionally similar to the traditional LLNA. ICCVAM proposed that the applicability of the draft ICCVAM LLNA Performance Standards be restricted to protocols that incorporate "minor" modifications to the traditional LLNA procedure, defined as changes only to the method for measuring lymphocyte proliferation. The Panel agreed that different methods of measuring lymphocyte proliferation represent "minor" modifications, but recommended that, instead of trying to define "minor" modifications, a better strategy might be to define criteria that would need to be satisfied in order to ensure that the alternative test method was mechanistically and functionally similar to the traditional LLNA (e.g., only measure cell proliferation associated with the induction

phase of a skin sensitization reaction). The Panel considered that the draft performance standards were also appropriate for evaluating other modifications. Examples of acceptable modifications included test animal sex, strain, the use of rats rather than mice, the number of animals per group, and timing of test article treatment. One minority opinion considered the potential impact of changes to protocol components other than the method of measuring lymphocyte proliferation to be significant and therefore would require more extensive validation, which was not defined.

The Panel indicated that alternative LLNA protocols that are undergoing validation should contain essential test method components that follow the ICCVAM-recommended protocol (ICCVAM 1999; Dean et al. 2001), unless adequate scientific rationale for deviating from this protocol was provided.

The Panel also identified aspects of the LLNA that should be required as part of the test method validation process: (1) application of the test substance to the skin with sampling of the lymph nodes draining that site, (2) measurement of cell proliferation in the draining lymph node, (3) absence of a skin reaction that could be indicative of the onset of the elicitation phase of skin sensitization, (4) data collected at the level of the individual animal to allow for an estimate of the variance within control and treatment groups (using this variance, a power analysis needs to be conducted to demonstrate that the modified method is utilizing a sufficient number of animals per treatment group to permit hazard identification with at least 95% power), and (5) if dose response information is needed, there are an adequate number of dose groups ( $n \geq 3$ ) with which to accurately characterize the dose response for a given test substance.

The Panel noted that the list of substances included in the draft ICCVAM LLNA Performance Standards was sufficiently representative of the types of materials that are likely to be tested for skin sensitization. However, among the 13 sensitizers in the list of "required" substances, only five were considered to have robust data (i.e., traditional LLNA data based on at least three independent studies).

To evaluate performance for use in hazard identification, the Panel concluded that all 22 substances in the draft ICCVAM-recommended list should be tested and accuracy statistics calculated (Note: this list of substances includes "required" substances as well as "optional" false negative and false positive substances, of which only 8/22 have "robust" datasets [ $n \geq 3$  as defined by the Panel]). To the extent possible, a rationale for any discordant results should be provided. However, the most potent sensitizers (e.g., dinitrochlorobenzene [DNCB]) should always be identifiable. Also, considerable weight should be given to the balance between animal welfare and human safety when considering the adequacy of test method accuracy. Based on the limited data available for the sensitizers on the list and the lack of standardization of test methods from which the results were obtained, the current database does not support inclusion of EC<sub>t</sub> values as a component of the accuracy evaluation.

The Panel agreed with the draft ICCVAM recommendations for evaluating test method reliability. These recommendations included obtaining EC<sub>t</sub> values that are generally within 0.5x to 2.0x of the mean historical EC<sub>3</sub> (i.e., estimated concentrations needed to produce an SI of 3) values for hexyl cinnamic aldehyde (HCA) (intralaboratory, n=4 experiments in one laboratory), or HCA and DNCB (interlaboratory, n=1 experiment in three laboratories). However, the Panel recommended that the criteria for independent tests should be specified

(e.g., different animal shipment, different reagents, different operator). The Panel concluded that the proposed criteria for acceptability appeared to be appropriate in this case, because only one or two substances were being evaluated (i.e., a statistical multiple comparisons<sup>2</sup> problem does not exist). The Panel also suggested that historical control data using HCA and DNCB in the same vehicle could be used to demonstrate adequate intra- and/or inter-laboratory reproducibility.

The Panel also recommended that statistical tests to analyze the data might allow for a more accurate interpretation. They recommended that a suitable variance-stabilizing transformation (e.g., log transformation, square root transformation) be applied in all statistical analyses and in reporting summary standard deviations. The Panel also recommended that a more rigorous evaluation be conducted of what would be considered an appropriate range of EC<sub>t</sub> values to include as a requirement. This would be a statistical evaluation that considers the variability of EC<sub>t</sub> values generated among the sensitizers included on the performance standards reference substances list and the statistical multiple comparisons problem.

#### **Use of the LLNA for Potency Determinations**

The Panel agreed with the draft ICCVAM recommendation that the LLNA should not be used as a stand-alone assay for categorizing skin sensitizers as strong vs. weak, but that it could be used as part of a weight-of-evidence evaluation (e.g., along with quantitative structure-activity relationships, peptide reactivity, human evidence, historical data from other experimental animal studies) for this purpose. The Panel also agreed with the draft ICCVAM recommendation that any LLNA studies conducted for the purpose of evaluating skin sensitization potency should use the ICCVAM-recommended LLNA protocol. In addition, the Panel viewed that the relevant testing guidelines for the traditional LLNA should be revised to include the procedure for calculating an EC<sub>3</sub> value.

A draft BRD was compiled by ICCVAM that provided a comprehensive review of available data and information and an evaluation of the usefulness and limitations of the traditional LLNA for the categorization of substances with regard to skin sensitization potency. The Panel evaluated the draft BRD for completeness, errors, and omissions and noted alternative analyses that would allow for a more complete evaluation of the use of the traditional LLNA for skin sensitization potency categorizations (see below).

The Panel agreed that the database of substances evaluated for potency determinations was sufficient and represented a range of chemical classes and physicochemical properties applicable to products typically tested for skin sensitization potential. The Panel also concluded that since the database was compiled from existing data, the lack of substance coding likely had no impact on the retrospective evaluation presented in the draft BRD. Still, the Panel recommended the coding of test substances in any future validation studies. The

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<sup>2</sup> When multiple experiments are conducted and multiple observations, comparisons, or hypothesis tests are conducted, the chance of observing rare events increases. Suppose, for example, that an interval is established such that 5% of observations from a particular population of data are outside that interval. Then if  $k$  independent experiments generate data from this population (e.g., a standard normal distribution), the chances that all 20 results will lie inside the interval is  $(1.0 - 0.05)^k$  (N. Flournoy, personal communication).

Panel generally agreed that potency determinations based on traditional LLNA results should ideally be limited to data from studies that evaluated lymph node proliferation in individual animals so that outliers and technical errors could be identified. However, they also agreed that pooled animal data should not be excluded automatically from a retrospective analysis.

The Panel indicated that the relevance of the LLNA for potency determinations had been adequately compared and evaluated to human (i.e., HMT or HRIPT) and guinea pig (i.e., GPMT or BT) data. A minority opinion stated by one Panel member was that the relevance of the traditional LLNA to human clinical observations had not been sufficiently determined.

In general, the Panel agreed that the proposed two-level categorization scheme (weak vs. strong sensitizers) for both human and guinea pig data was appropriate. However, a minority opinion stated by two Panel members was that a moderate category should be included since certain compounds might be on the border between weak and strong sensitizers. Thus, they suggested that the five-category scheme proposed by Kimber et al. (2003), which includes non-sensitizers, might be recommended.

The Panel concluded that the decision criteria providing the best overall performance was the use of  $<250 \mu\text{g}/\text{cm}^2$  to distinguish between strong and weak sensitizers in humans and the use of an LLNA EC3  $\leq 9.4\%$  to distinguish between strong and weak sensitizers in the LLNA. The Panel stated that more data would be needed to determine if values different from these two would be more appropriate. The Panel also recommended that safety factors other than 10 for the lowest observed effect level (LOEL) be evaluated to determine if improved results could be obtained. The Panel also suggested an analysis that directly compares the LOEL values without using a safety factor (i.e., using LOEL data only) and an analysis that only uses no observed effect level data. The Panel further stated that traditional LLNA tests based on pooled or individual lymph nodes for a dose group should be evaluated independently to assess the impact of using pooled data on the accuracy analysis for skin sensitization potency. Finally, the Panel stated that the effect of different vehicles should be recognized as a limitation in the current data analysis and a likely contributor to the variability observed within and across laboratories.

The Panel stated that data from studies that could not be confirmed as being GLP-compliant, but that were from peer-reviewed literature or sources with high-quality laboratory management practices, were still appropriate to include in the accuracy analysis. However, the Panel stated that, ideally, GLP compliance should be the standard, as it is clearly the only objective way to judge the credibility of the data.

The Panel recommended that more data should be collected to determine the optimal threshold in humans for distinguishing between strong and weak sensitizers. In addition, the Panel discouraged conducting additional animal studies unless such studies would be expected to lead to an overall reduction in animal use. The Panel recommended that the LOELs from Akkan et al. (2003) be used instead of the  $\text{DSA}_{05}$  (i.e., the dose per skin area leading to a sensitization incidence of 5%) values from Schneider and Akkan (2004) in all of the potency analyses. A minority opinion by one Panel member stated that it was acceptable to use the  $\text{DSA}_{05}$  values from Akkan et al. (2003) as LOEL values in the evaluation. This panelist mentioned that the  $\text{DSA}_{05}$  value is a LOEL value adjusted to 5% incidence of induction in order to correct for human studies leading to different inductions. Furthermore, the panelist stated that because the  $\text{DSA}_{05}$  is corrected for an induction rate of 5%, it would

*Independent Peer Review Panel Report*

*May 2008*

be better to compare with the traditional LLNA EC3 than to use the default uncorrected LOEL.

**Appendix A**  
**Peer Review Panel Member Biosketches**

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## Panel Member Biosketches

### **Nathalie Alépée, Ph.D.**

Dr. Alépée performed research leading to a Ph.D. in Medical Virology and Microbiology at the Centre National de la Recherche Scientifique research institute, Gif sur Yvette, France. She is currently the Global Pfizer Leader for photosafety, including the global portfolio support and Associate Research Fellow in Investigative Toxicology, at Pfizer Global Research and Development, Amboise, France. As a laboratory manager in the Molecular and Cellular Toxicology Group with Pfizer, she implemented the Local Lymph Node Assay (LLNA) in the laboratory. She serves on the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC), representing the European Federation of Pharmaceutical Industries Associations (EFPIA). She is also the Pfizer representative to the European Partnership on Alternative to Animal Testing (EPAA), in two working groups; Identification of Opportunities, Including R&D (working group 2), and Validation and Acceptance (working group 5). She served as a peer reviewer of the reduced LLNA test protocol and prediction model for ESAC in 2007 and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA.

### **Anne Marie Api, Ph.D.**

Dr. Api received a Ph.D. from Aston University in Birmingham, England and is currently Vice President of Human Health Sciences at the Research Institute for Fragrance Materials (RIFM), as well as the Scientific Director. She is responsible for the human health scientific program, and the investigation and initiation of new research and testing projects for RIFM. She is also Adjunct Assistant Professor at the University of Medicine and Dentistry of New Jersey. She is a member of 10 professional organizations, including the American Contact Dermatitis Society, the European Society of Contact Dermatitis, and the Society of Investigative Dermatology. She participated in the World Health Organization (WHO) International Workshop in Skin Sensitization in Chemical Risk Assessment held in Berlin, Germany in 2006. She is author of over 100 publications and presentations relevant to dermatology and dermatotoxicology.

### **Nancy Flournoy, M.S., Ph.D.**

Dr. Flournoy received a M.S. degree in Biostatistics from the University of California at Los Angeles, and a Ph.D. in Biomathematics from the University of Washington. She is Professor and Chair of the Department of Statistics at the University of Missouri-Columbia. Her research interests include adaptive designs, bioinformatics, chemometrics, clinical trials, and environmetrics. She has an extensive list of edited volumes and papers on statistical theory, statistical genetics and immunology, epidemiology in immune suppressed subjects, clinical trials for prevention and treatment of viral infection, transplantation biology and its effects on digestion, lungs, eyes, mouth, and central nervous system, optimization of statistical processing, and additional papers, interviews, and technical reports. She has editorial responsibilities for numerous statistical journals, serves on numerous advisory boards, and nominating committees. She is a member and past Chair of the Council of Sections of the American Statistical Association, and served in various other statistical, medical and toxicological societies or programs as Chair or as a member of the Board of Directors. She is a former member of the Scientific Advisory Committee on Alternative Toxicological

Methods. She also served on the Expert Panels for the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) that evaluated the Revised Up-and-Down Procedure; the Current Validation Status of *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants; and Five *In Vitro* Pyrogen Test Methods.

**Thomas Gebel, Ph.D.**

Dr. Gebel received a Ph.D. in Toxicology from the University of Mainz and is certified as a toxicologist by the German Society of Toxicology. His scientific interests are in biomonitoring, genetic toxicology, environmental hygiene, and occupational toxicology. He has published over 40 papers in peer-reviewed scientific journals. He is employed by the German Federal Institute for Occupational Safety and Health, and is an Associate Professor at the University of Goettingen. Dr. Gebel is currently a member of the Organisation for Economic Co-operation and Development (OECD) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) expert group on sensitization and head of the German advisory committee on classification and labeling of existing substances and biocides. Dr. Gebel also is head of the German Delegations to the United Nations Economic and Social Council Sub-Committee of Experts on the GHS, and to the OECD Task Force on Harmonisation of Classification and Labeling. He participated in the WHO International Workshop in Skin Sensitization in Chemical Risk Assessment held in Berlin, Germany in 2006.

**Sidney Green Ph.D., F.A.T.S.**

Dr. Green received a Ph.D. in Biochemical Pharmacology from Howard University. His research interests include toxicology, mutagenic assay systems, and alternatives to animals in toxicology. He is currently Graduate Professor of Pharmacology at Howard University and a faculty member at the Centers for Alternatives to Animal Testing at the Johns Hopkins University School of Public Health. Previously, he has been Director of the Department of Toxicology at Covance Laboratories Inc. and the Director of the Division of Toxicological Research at the U.S. Food and Drug Administration (FDA). Dr. Green is a Fellow of the Academy of Toxicological Sciences (F.A.T.S.). He has served on numerous expert panels and committees. He was a participant in an International Workshop organized by ICCVAM and NICEATM on *In Vitro* Methods for Assessing Acute Systemic Toxicity in 2000. He served on the ICCVAM/NICEATM Expert Panels that evaluated the Corrositex® Test Method for Assessing Dermal Corrosivity Potential of Chemicals, and *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants. He is a former member of the ICCVAM Advisory Committee on Alternative Toxicological Methods (ACATM) and of SACATM. He has authored over 60 publications for peer-reviewed journals.

**Kim Headrick, B.Admin., B.Sc.**

Kim Headrick received Bachelor of Administration and B.Sc. degrees from the University of Ottawa, Canada. She is currently International Harmonization and Senior Policy Advisor for Health Canada, and Chair of the UN Sub-Committee of Experts on GHS. She manages the overall strategy for the implementation of the GHS in Canada. She was awarded the Queen Elizabeth Commemorative Golden Jubilee Medal in 2002, which focuses on the achievements of people who, over the past 50 years, have created the Canada of today. She is

a member of the OECD Task Force on Harmonization of Classification and Labelling and the OECD Expert Group Meeting on Sensitization Hazards.

**Dagmar Jírová, M.D., Ph.D.**

Dr. Jírová received a Ph.D. from the Medical Faculty of Hygiene at Charles University in Prague. She is currently the Head of the Reference Center for Cosmetics, and Head of National Reference Laboratory for Experimental Immunotoxicology at the National Institute of Public Health in the Czech Republic. Her main responsibilities include safety assessment of consumer products, particularly cosmetics and their ingredients, performance of toxicological methods *in vivo* in animals, human patch testing for local toxicity assessment, and introduction of *in vitro* techniques for screening of toxicological endpoints using cell and tissue cultures. She represents the Czech Republic in the Standing Committee on Cosmetics of the European Commission. She is an ESAC-ECVAM member and was involved in Peer Review Panel for Skin Irritation Validation Study and LLNA test protocol and prediction model. She is author of more than 100 publications and presentations relevant to dermatotoxicology including a recent presentation at the 6th World Congress on Alternatives & Animal Use in the Life Sciences, held in Tokyo, 2007, titled “Comparison of Human Skin Irritation and Photoirritation Patch Test Data with Cellular *in vitro* Assays and Animal *in vivo* data”.

**David Lovell, Ph.D., B.Sc. (Hons), F.S.S., FIBiol, CStat, CBiol**

Dr. Lovell received a Ph.D. from the Department of Human Genetics and Biometry, University College, London. He is currently Reader in Medical Statistics at the Postgraduate Medical School at the University of Surrey. Previously, he was Associate Director and Head of Biostatistics support to Clinical Pharmacogenomics at Pfizer Global Research and Development in Sandwich, Kent providing data management and statistical support to pharmacogenetics and genomics. He joined Pfizer in 1999 as the Biometrics Head of Clinical Pharmacogenetics. Before joining Pfizer, Dr. Lovell was the Head of the Science Division at BIBRA International, Carshalton, which included Molecular Biology, Genetic Toxicology, Biostatistics and Computer Services. At BIBRA, Dr. Lovell managed the statistical and computing group providing specialized statistical support to BIBRA’s Clinical Unit and contract research work. He conducted and managed research programs on genetics, statistics and quantitative risk assessment for the European Union (EU) and U.K. Government Departments. His research interests at BIBRA were in the use of mathematical and statistical methods together with genetic models in the understanding of toxicological mechanisms and risk assessment problems. Dr. Lovell had previously been a Senior Research Officer with the U.K. Medical Research Council (MRC) Experimental Embryology and Teratology Unit, a visiting Postdoctoral Fellow at the National Institute of Environmental Health Sciences (NIEHS) in North Carolina, U.S., a Geneticist at the MRC Laboratories, Carshalton, and a Research Assistant in Cytogenetics at Birmingham University. He has acted as a consultant to a number of organizations, has considerable experience of working with Regulatory Authorities, has many publications related to his work and has wide experience of making presentations to a wide range of audiences. He is a member of the U.K. Government’s advisory Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) and the Independent Scientific Advisory Committee for Medicines and Healthcare Products Regulatory Agency database research. He served on the NICEATM-ICCVAM Expert Panels

that evaluated the Frog Embryo Teratogenesis Assay - Xenopus, *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro* Pyrogen Test Methods.

**Michael Luster, Ph.D.**

Dr. Luster received a Ph.D. in Immunology from Loyola University of Chicago. He was formerly Chief, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health (NIOSH), and currently serves as a senior advisor to the Director of the Health Effects Laboratories and the staff of Toxicology and Molecular Biology Branch at NIOSH. Program areas include neuroscience, dermatology, molecular carcinogenesis, molecular epidemiology, molecular toxicology, molecular epidemiology, and inflammation/immunotoxicology. In addition, Dr. Luster conducts basic and applied research in immunotoxicology including its application in risk assessment. Current research activities include molecular epidemiology studies of genetic polymorphism involved in workplace-related diseases and experimental studies involving occupational allergic rhinitis. Dr. Luster is also working with various staff at the U.S. Environmental Protection Agency (EPA) through the Risk Assessment Forum to develop immunotoxicity testing guidelines. He also directed two studies for the NTP on the Toxicology and the Carcinogenesis of Promethazine and Ortho-phenylphenol, in 1990 and 1986, respectively. He is a co-author of over 300 publications in peer-reviewed journals.

**Howard Maibach, M.D.**

Dr. Maibach received an M.D. from Tulane University. He is currently a professor in the Department of Dermatology at the University of California, San Francisco (UCSF), where he is also Chief of the Occupational Dermatology Clinic. In his 35 years at UCSF, Dr. Maibach has written and lectured extensively on dermatotoxicology and dermatopharmacology. His current research programs include defining the chemical-biologic faces of irritant dermatitis and the study of percutaneous penetration. Dr. Maibach served on the 1998 ICCVAM Peer Panel that evaluated the Murine LLNA. Dr. Maibach has been on the editorial boards of over 30 scientific journals and is a member of 19 professional societies including the American Academy of Dermatology, San Francisco Dermatological Society, and the International Commission on Occupational Health. He has co-authored over 1500 publications related to dermatology.

**James McDougal, Ph.D., F.A.T.S.**

Dr. McDougal earned a Ph.D. in Pharmacology/Toxicology at the University of Arizona. He is currently Professor and Director of Toxicology Research in the Department of Pharmacology and Toxicology at Wright State University's Boonshoft School of Medicine. Prior to his appointment at Wright State, he worked in the Air Force toxicology research organization for about 17 years. He has active skin research programs related to dermal pharmacokinetics, molecular biology of skin irritation, dermal risk assessment, and biologically-based mathematical modeling. He has served on many national committees, published more than 75 manuscripts, and consults for a wide variety of government and industry organizations. Dr. McDougal is a member of the National Academy of Sciences (National Research Council) Committee on Toxicology and the American Congress of Governmental Industrial Hygienists Threshold Limit Value Committee for Chemical substances. Dr. McDougal is also past president of the Dermal Toxicology Specialty Section of the Society of Toxicology.

**Michael Olson, Ph.D., A.T.S.**

Dr. Olson received a Ph.D. in Toxicology from the University of Arkansas for Medical Sciences, with dissertation research conducted at the FDA National Center for Toxicological Research. Following graduate training, he served as NIEHS National Research Service Award Post-doctoral Fellow in the Department of Pharmacology, School of Medicine - University of North Carolina. Currently he is Director, Occupational Toxicology, Corporate Environment Health and Safety for GlaxoSmithKline. Dr. Olson is a Fellow of the Academy of Toxicological Sciences (A.T.S.). His research interests include mechanisms of chemically-induced toxicity; genetic toxicity; xenobiotic metabolism; alternative methods in toxicology; hazard evaluation, risk assessment, and communication. Dr. Olson has authored a number of peer-reviewed manuscripts and book chapters in these areas as well as preparing many occupational health effects reviews for pharmaceutical active ingredients, isolated intermediates, and associated chemicals. He has served as an editorial board member and *ad hoc* referee for numerous toxicology and biosciences journals. In addition, he has worked as a Visiting Scientist, EPA, as well as advisor to EPA Risk Assessment Forum, U.S. National Institutes of Health (NIH) (Toxicology Study Section I), U.S. Air Force, Transportation Research Board, and the National Research Council - National Academy of Sciences. A member of several biomedical professional societies, Dr. Olson has served in elective and appointed positions in the Society of Toxicology, including Chairman of the Society of Toxicology (SOT) Occupational Health Specialty Section.

**Raymond Pieters, Ph.D.**

Dr. Pieters received a Ph.D. at Utrecht University and is currently an Associate Professor at the Institute for Risk Assessment Sciences, and Group Leader for Immunotoxicology at that institution. In 2007, he presented a paper on Development of Strategies to Assess Drug Hypersensitivity at the Congress of the European Societies of Toxicology. He was involved in the development of the Reporter Antigen Popliteal Lymph Node Assay, an assay to assess the immunomodulating potential of chemicals, which enables differentiation between immunosensitizing chemicals (sensitizers), immunostimulating chemicals (irritants), and chemicals that have no apparent immunological effects. He has published over 70 papers on sensitization and other subjects in immunotoxicology in peer-reviewed journals, including a review article, *Murine Models of Drug Hypersensitivity*, in 2005.

**Jean Regal, Ph.D.**

Dr. Regal received a Ph.D. in Pharmacology from the University of Minnesota. She is currently a Professor in the Department of Pharmacology, Department of Biochemistry & Molecular Biology and Associate Dean of Faculty Affairs, Medical School Duluth, University of Minnesota. Her current research is focused on respiratory allergy, especially asthma. She has served on multiple NIH review panels regarding asthma, as an immunotoxicologist in 2000 for an Institute of Medicine Committee on Health Effects Associated with Exposures Experienced during the Persian Gulf War, as well as on the 1998 ICCVAM Peer Panel that evaluated the Murine LLNA. In 2007 she served as an *ad hoc* reviewer for the NTP Board of Scientific Counselors for two nominations: Artificial Butter Flavoring Mixture & O-phthalaldehyde, at NIEHS. Also in 2007, she served on an NIEHS Center in Environmental Toxicology pilot project program for the University of Texas Medical Branch at Galveston. She is currently Vice-President-elect of the Immunotoxicology

Specialty Section of SOT and Associate Editor of the Journal of Immunotoxicology. Dr. Regal has authored over 50 research articles and reviews in peer-reviewed journals and holds two patents on pulmonary administration of sCR1 and other complement inhibitory proteins.

**Jonathan Richmond, B.Sc. (Hons) Med.Sci., MB ChB, FRCSEd, FRMS**

Dr. Richmond received a Bachelor of Science in Medical Science with Honors (B.Sc. [Hons] Med.Sci.) and Bachelor of Medicine and Bachelor of Surgery (MB ChB) degrees with Distinction in Medicine and Therapeutics from Edinburgh University. Presently, he is head of the Animals Scientific Procedures Division at the Home Office. He is a Fellow of the Royal College of Surgeons of Edinburgh (FRCSEd) and a Fellow of the Royal Society of Medicine (FRMS). Other appointments include convener of the U.K. interdepartmental group on the 3Rs, board member U.K. National Centre for the 3Rs, convener of the International Standards Organization Technical Corrigendum 194/Working Group 3 (*Biocompatibility of Medical Device Materials*), and member of related expert working groups. He is a former member of the EU Committee on Scientific and Technical Progress and past Chairman of the European Commission Technical Expert Working Group on ethical review. He served as chair of the peer review panel for the reduced LLNA test protocol and prediction model for ESAC in 2007 and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA. He served on the ICCVAM/NICEATM Expert Panel that evaluated Five *In Vitro* Pyrogen Test Methods. He has a variety of publications in peer-reviewed journals and national and international meetings, on the principles and practice of surgery, regulation of biomedical research, principles of humane research, bioethics, and public policy.

**Peter Theran, V.M.D.**

Dr. Theran holds a Doctor of Veterinary Medicine degree from the University of Pennsylvania. He has had many years of experience both as a veterinary internal medicine specialist at the Massachusetts Society for Prevention of Cruelty to Animals' Angell Memorial Animal Hospital in Boston, and as the director of Boston University Medical Center's Laboratory Animal Science Center. He presently serves on a number of government committees as an animal welfare member, and is a member of the Board of Directors of the Institute for *In Vitro* Sciences in Gaithersburg, MD and Chimp Haven in Shreveport, Louisiana. He served on the NICEATM-ICCVAM Expert Panels that evaluated the *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro* Pyrogen Test Methods. He is a former member of ACATM and SACATM. He is presently working as a consultant.

**Stephen Ullrich, Ph.D.**

Dr. Ullrich received a Ph.D. in Microbiology from Georgetown University. He is currently the Dallas/Fort Worth Living Legends Professor, and Professor of Immunology at the University of Texas, M.D. Anderson Cancer Center, where he is also Associate Director, The Center for Cancer Immunology Research. He is also a member of the Animal Research Strategic Advisory Committee. He has served numerous national review committees and panels, including the 1998 ICCVAM Peer Panel that evaluated the Murine LLNA. Dr. Ullrich has authored over 75 peer-reviewed publications, over 30 invited articles, and he holds four patents in the U.S., E.U., and Australia for a UV-induced Immunosuppressive Substance. He is the past President of the American Society for Photobiology.

**Michael Woolhiser, Ph.D.**

Dr. Woolhiser received a Ph.D. in Pharmacology and Toxicology from the Medical College of Virginia at Virginia Commonwealth University. He is a specialist in immunotoxicology and is currently a toxicologist for the Dow Chemical Company where he serves as a Technical Leader for Immunotoxicology, and Polyurethane Business Toxicology Consultant. Dr. Woolhiser is also an Adjunct Professor at the Center for Integrative Toxicology, Michigan State University. He is a member of the Program Committee of the Society of Toxicology's Immunotoxicology Specialty Section. He has served on numerous working groups, including an LLNA Expert Working Group under the European Crop Protection Agency's Toxicology Expert Group, a European Centre for Ecotoxicology and Toxicology of Chemicals LLNA Task Force. He has authored 29 peer-reviewed publications.

**Takahiko Yoshida, M.D., Ph.D.**

Dr. Yoshida earned his M.D. and a Ph.D. in Medical Science from Tokai University. He is currently Professor in the Department of Health Science at Asahikawa Medical College. Prior to this appointment, he held the posts of Instructor, Assistant Professor and Associate Professor at the Tokai University School of Medicine. He has also been a Guest Researcher at NIEHS. He has also worked as an occupational physician for major Japanese corporations, including Toyota and Sony. Dr. Yoshida's research interests include occupational health, public health, environmental health and preventative medicine. He is a member of the International Congress of Occupational Health, the Japanese Society of Hygiene, the Japanese Society of Immunotoxicology, the Japanese Society of Clinical Ecology, and the SOT.

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**Appendix E3**

**Summary Minutes of Independent Scientific Peer Review Panel Meeting on April 28-29,  
2009**

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## Summary Minutes

### Independent Scientific Peer Review Panel Meeting

#### Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA)

William H. Natcher Conference Center

National Institutes of Health

Bethesda, MD

April 28 - 29, 2009

8:30 a.m. - 5:30 p.m.

*Peer Review Panel Members:*

Michael Luster, Ph.D. (Peer Review Panel Chair)	Senior Consultant to the NIOSH Health Effects Laboratory, Morgantown, WV
Nathalie Alépée, Ph.D.	Scientific Coordinator on Alternatives Methods in Life Science, L'Oréal Research and Development, Aulnay sous Bois, France
Anne Marie Api, Ph.D.	Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ
Nancy Flournoy, M.S., Ph.D.	Professor and Chair, Dept. of Mathematics and Statistics, University of Missouri – Columbia, Columbia, MO
Dagmar Jírová, M.D., Ph.D.	Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic
David Lovell, Ph.D.	Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.
Howard Maibach, M.D.	Professor, Dept. of Dermatology, University of California – San Francisco, San Francisco, CA
Michael Olson, Ph.D.	Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, Research Triangle Park, NC

**Peer Review Panel Members:**

Raymond Pieters, Ph.D. <sup>1</sup>	Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Jean Regal, Ph.D.	Professor, Dept. of Pharmacology, University of Minnesota Medical School, Duluth, MN
John Richmond, MB ChB, FRCSEd	Head, Animals Scientific Procedures Division, Home Office, London, U.K.
Peter Theran, V.M.D.	Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA
Stephen Ullrich, Ph.D.	Dallas/Ft. Worth Living Legends Professor and Professor of Immunology, Postgraduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX
Michael Woolhiser, Ph.D.	Science and Technology Leader – Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI
Takahiko Yoshida, M.D., Ph.D.	Professor, Dept. of Health Science, Asahikawa Medical College, Hokkaido, Japan

**ICCVAM and ICCVAM Immunotoxicity Working Group Members:**

Paul Brown, Ph.D.	FDA, Center for Drug Evaluation and Research, Silver Spring, MD
Masih Hashim, Ph.D.	EPA, Office of Pesticide Programs, Washington, DC
Ying Huang, Ph.D.	FDA, Center for Biologics Evaluation and Research, Silver Spring, MD
Abigail Jacobs, Ph.D. (IWG Co-Chair)	FDA, Center for Drug Evaluation and Research, Silver Spring, MD
Jodie Kulpa-Eddy, D.V.M.	USDA, Animal and Plant Health Inspection Service, Riverdale, MD
Elizabeth Margosches, Ph.D.	EPA, Office of Pollution Prevention and Toxics, Washington, DC
Joanna Matheson, Ph.D. (IWG Co-Chair)	CPSC, Bethesda, MD

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<sup>1</sup> Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the review of the revised draft background review documents and the revised draft LLNA applicability domain Addendum.

***ICCVAM and ICCVAM Immunotoxicity Working Group Members:***

Deborah McCall	EPA, Office of Pesticide Programs, Washington, DC
Tim McMahon, Ph.D.	EPA, Office of Pesticide Programs, Washington, DC
John Redden, M.S.	EPA, Office of Pesticide Programs, Washington, DC
R. Adm. William Stokes, D.V.M., DACLAM	NIEHS, Research Triangle Park, NC
Ron Ward, Ph.D.	EPA, Office of Pollution Prevention and Toxics, Washington, DC
Marilyn Wind, Ph.D. (ICCVAM Chair)	CPSC, Bethesda, MD

***Invited Experts:***

George DeGeorge, Ph.D., DABT	MB Research Labs, Spinnerstown, PA
Kenji Idehara, Ph.D.	Daicel Chemical Industries, Ltd., Hyogo, Japan
Masahiro Takeyoshi, Ph.D.	Chemicals Evaluation and Research Institute, Saitama, Japan

***JaCVAM Observer:***

Hajime Kojima, Ph.D.	National Institute of Health Sciences, Tokyo, Japan
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***Public Attendees:***

Joan Chapdelaine, Ph.D.	Calvert Laboratories, Inc., Olyphant, PA
Merrill Tisdell	Syngenta Crop Protection Inc., Greensboro, NC
Gary Wnorowski, M.B.A, L.A.T.	Eurofins Product Safety Labs

***NICEATM:***

R. Adm. William Stokes, D.V.M., DACLAM	Director
Debbie McCarley	Special Assistant to the Director
Contract Support Staff – Integrated Laboratory Systems, Inc. (ILS)	
David Allen, Ph.D.	Eleni Salicru, Ph.D.
Thomas Burns, M.S.	Frank Stack

**NICEATM:**

Linda Litchfield

Judy Strickland, Ph.D., DABT

Greg Moyer, M.B.A.

Abbreviations:

CPSC = U.S. Consumer Product Safety Commission

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems

IWG = Immunotoxicity Working Group

NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS = National Institute of Environmental Health Sciences

NIOSH = National Institute of Occupational Safety and Health

USDA = U.S. Department of Agriculture

**Tuesday, April 28, 2009**

### **Call to Order and Introductions**

Dr. Michael Luster (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (hereafter Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Immunotoxicity Working Group (IWG) members, and members of the public to also introduce themselves. Dr. Luster stated that there would be opportunity for public comments during each of the four murine local lymph node assay (LLNA)-related topics. He asked that all those interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Luster emphasized that the comments would be limited to seven minutes per individual and that, while comments from one individual would be welcomed during each commenting period, repeating the same comments at each comment period would be inappropriate.

### **Welcome from the ICCVAM Chair**

Dr. Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to the National Institutes of Health and to the Panel meeting. Dr. Wind thanked the ICCVAM IWG and NICEATM staff for their efforts in preparing the draft documents being reviewed and for arranging the logistics of the meeting. Dr. Wind thanked the Panel members for dedicating their time, effort, and expertise to this review and acknowledged their important role to the ICCVAM test method evaluation process. Dr. Wind also emphasized the important role of the public and their comments in this process.

### **Welcome from the Director of NICEATM, and Conflict of Interest Statements**

Dr. William Stokes, Director of NICEATM, stated the Panel meeting was being convened as an NIH Special Emphasis Panel and was being held in accordance with applicable U.S. Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would be serving as the Designated Federal Official for this public meeting. He reminded the Panel that they signed a conflict of interest (COI) statement during the Panel selection process, in which they identified any potential real or perceived COI. He read the COI statement and then Dr. Luster asked that panelists again declare any potential direct or indirect COI and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict.

Dr. Michael Woolhiser declared a COI regarding the Panel's review of the LLNA Applicability Domain, because The Dow Chemical Company, Dr. Woolhiser's employer, submitted much of the data that were being considered. He indicated that he would recuse himself from the Panel's evaluation of the applicability domain, but would remain available to answer any questions that the Panel might have about the test substances or the data.

### **Overview of the ICCVAM Test Method Evaluation Process**

Dr. Stokes began by thanking the 15 Panel scientists from six different countries (Czech Republic, France, Japan, The Netherlands, United Kingdom, and the United States) for their significant commitment of time and effort preparing for and attending the meeting. He explained that the purpose of the Panel was to conduct an independent scientific peer review of the information provided on a series of proposed new versions of the LLNA and proposed expanded applications of the assay. The Panel is then asked to comment on the extent that the available information supports the draft ICCVAM recommendations. Dr. Stokes indicated that the original LLNA peer review panel in 1998 considered the LLNA a valid substitute for the guinea pig-based test in most but not all testing

situations. He noted that three Panel members from the 1998 review are also on the current Panel (i.e., Drs. Howard Maibach, Jean Regal, and Stephen Ullrich). Dr. Stokes also reviewed the nomination that was received from CPSC in January 2007,<sup>2</sup> which provides the basis for the current evaluation.

Dr. Stokes then identified the 15 Federal agencies that comprise ICCVAM and summarized ICCVAM's mission. He noted that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out the critical scientific evaluation of the results of validation studies for proposed test methods to assess their usefulness and limitations for regulatory testing, and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes provided a brief review of ICCVAM's history and summarized the ICCVAM Authorization Act of 2000,<sup>3</sup> including the purpose and duties of ICCVAM. He noted that one of ICCVAM's primary duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available on the NICEATM-ICCVAM website or can be obtained upon request from NICEATM. He also mentioned that ICCVAM provides guidance on test method development, validation processes, and helps to facilitate not only the acceptance of scientifically valid alternative test methods, but also encourages internationally harmonized recommendations on the usefulness and limitations of alternative test methods.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public and determines whether the test method should move forward into a formal evaluation. If so, a draft background review document (BRD), which provides a comprehensive review of all available data and information, is prepared by NICEATM in conjunction with an ICCVAM working group designated for the relevant toxicity testing area (e.g., the IWG). In addition, ICCVAM considers all available information and develops draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future optimization/validation studies. The draft BRD and the draft ICCVAM test method recommendations are made available to the Panel and the public for review and comment. The Panel peer reviews the draft BRD and evaluates the extent to which it supports the draft ICCVAM test method recommendations. A Panel report is published, which is then considered along with public and SACATM comments by ICCVAM in developing final recommendations. These final recommendations are forwarded to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines. Agencies have 180 days to respond to the ICCVAM recommendations.

Dr. Stokes reviewed the ICCVAM criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance. He concluded by summarizing the timeline of the review activities beginning with CPSC's nomination in January 2007 and ending with the present Panel meeting.

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<sup>2</sup> [http://iccvam.niehs.nih.gov/methods/immunotox/llnadoocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadoocs/CPSC_LLNA_nom.pdf)

<sup>3</sup> [http://iccvam.niehs.nih.gov/docs/about\\_docs/PL106545.pdf](http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf)

## **ICCVAM Charges to the Panel**

Dr. Stokes reviewed the charges to the Panel: (1) review the draft BRDs and the draft Addendum to the traditional<sup>4</sup> LLNA for completeness and identify any errors or omissions; (2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been appropriately addressed for the proposed revised or modified versions of the LLNA; and (3) comment on the extent to which the ICCVAM draft test method recommendations including the proposed usefulness and limitations, standardized test method protocols, performance standards, and additional studies are supported by the information provided in the draft BRDs and draft Addendum.

## **Overview of the Agenda**

Dr. Luster then reviewed the agenda and the order of presentations. He stated that for each review topic, the test method developer would present an overview of the test method protocol, followed by a presentation by NICEATM staff summarizing each revised draft BRD, and lastly a member of the IWG would present the draft ICCVAM recommendations. Following presentations, the Panel Evaluation Group Leader for the topic under consideration would present the group's draft recommendations, followed by Panel discussion. Public comments would then be presented, followed by the opportunity for additional Panel discussion in consideration of the public comments. The Panel would then vote to accept the Panel consensus, with any minority opinions being so noted with the rationale provided for the minority opinion.

## **Current Regulatory Testing Requirements and Hazard Classification Schemes for Allergic Contact Dermatitis (ACD) and the Traditional LLNA Procedure**

Dr. Matheson presented an overview of ACD and relevant regulatory requirements. She briefly discussed the ICCVAM final recommendations for the LLNA Performance Standards, the updated ICCVAM LLNA test method protocol, and the reduced LLNA (rLLNA), all of which were reviewed by the Panel at their meeting in March 2008.

The Panel questioned who was responsible for conducting the future studies referred to in the revised draft ICCVAM test method recommendations. Dr. Stokes replied that these recommendations are provided for consideration by the stakeholder community. Those organizations with appropriate resources can use this information to guide their research, development, and validation activities.

A question arose from the Panel as to why pooled data (as opposed to individual animal data) are collected for the LLNA.

Dr. Matheson replied that, pooled data are often collected since OECD Test Guideline 429 allows the use of a minimum of four animals per treatment group when collecting pooled data, but requires a minimum of five animals per treatment group when collecting individual animal data. Legislation in some countries, and many Animal Care and Use Committees, require that the test method to be used is the one requiring the fewest animals. Dr. Matheson also noted that the ICCVAM LLNA test method protocol has recently been revised to allow the use of a minimum of four animals per treatment group when collecting individual animal data, so there is now no reason not to collect individual animal data. At the Panel meeting in March 2008, the Panel stated that all future LLNA studies should require that lymph nodes be collected from individual animals instead of pooling them

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<sup>4</sup> For the purposes of this document, the radioactive LLNA test method, which was first evaluated by ICCVAM in 1999, and subsequently recommended to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances, is referred to as the traditional LLNA.

with other animals in a treatment group since individual animal response data allows for identification of technical problems and outlier animals within a dose group.<sup>5</sup>

A question arose as to whether the U.S. Environmental Protection Agency (EPA) prefers LLNA or guinea pig data for submission. Dr. Matheson ceded the floor to Ms. Debbie McCall of EPA Office of Pesticide Programs, who was in attendance. Ms. McCall said that EPA prefers LLNA data, but will accept either guinea pig maximization test (GPMT) or Buehler test (BT) data.

## **Overview of the Revised Draft LLNA: DA Test Method Procedure BRD and Revised Draft ICCVAM Test Method Recommendations**

The first test method reviewed was the LLNA: DA test method. This test method measures the ATP content of lymph node cells by the luciferin/luciferase method, as an index of lymphocyte proliferation, after exposure to a test substance.

Dr. Kenji Idehara of Daicel Chemical Industries, Ltd., Japan (the test method developer) presented a synopsis of the test method to the Panel.

A Panelist asked about the half-life of ATP in the lymph node cells after the mouse is sacrificed. Dr. Idehara replied that the ATP concentration declines 20 to 30% in an hour, with a half-life of about 2 to 2.5 hours. The assay time from animal sacrifice to complete measurement of ATP content for each individual animal is maintained as similar as possible, within approximately 30 min. He also said that the time between sacrifice and ATP assay is not a problem when collecting individual animal data, if the time between the excision of the lymph nodes, the preparation of the cell suspensions, and the measurement of the ATP concentrations is kept relatively constant between animals.

A Panelist asked if the lymph node samples were randomized before the ATP assays were conducted. Dr. Idehara replied that the samples were not randomized.

On behalf of NICEATM, Dr. Salicru presented an overview of the revised draft LLNA: DA BRD to the Panel.

A question arose about NICEATM's use of different decision criteria for the accuracy analysis, and the reproducibility analyses in the revised draft BRD. Dr. Salicru noted that a decision criterion of  $SI \geq 2.5$  was used for the reproducibility analyses because it was found to be the optimal decision criterion for identifying sensitizers (i.e., it resulted in a 0% false positive rate).

Dr. Wind presented the revised draft ICCVAM test method recommendations for the LLNA: DA test method to the Panel. She noted that ICCVAM favored the multiple decision criteria to eliminate any false positives or false negatives. A Panelist commented that, as more data are accumulated using the test method, false positives and false negatives might appear.

A Panelist asked, if the true stimulation index (SI) value for a compound was 2.0, if that compound would be classified as a sensitizer or a nonsensitizer. Dr. Wind replied that, as described in the revised draft ICCVAM recommendations, other information would be necessary to definitively answer that question.

Dr. Kojima presented the results of the Japanese Society for Alternatives to Animal Experiments (JSAAE) interlaboratory validation studies of the LLNA: DA and the LLNA: BrdU-ELISA test methods to the Panel. In the presentation, he noted that the JaCVAM Regulatory Acceptance Board has examined the results of the studies for both test methods and accepted the LLNA: DA as a replacement for the traditional LLNA. The JaCVAM Regulatory Acceptance Board has requested additional data for the LLNA: BrdU-ELISA.

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<sup>5</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2008.pdf)

**Panel Evaluation:**

Dr. Woolhiser presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: DA test method. The Panel agreed that the available data and test method performance support the use of the LLNA: DA to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. They concurred with ICCVAM's proposal that, based on the current validation database, the multiple SI decision criteria should be used to identify sensitizers and nonsensitizers (i.e.,  $SI \geq 2.5$  for sensitizers,  $SI \leq 1.7$  for nonsensitizers). The Panel also noted that the limitation of these test methods when using the proposed multiple decision criteria is the indeterminate classification of substances that fall in the range of SI values for which a classification is uncertain (i.e.,  $1.7 < SI < 2.5$ ). The Panel recommended that when such results are obtained, users should carefully interpret the results using an integrated decision strategy in conjunction with all other available information (e.g., dose response and quantitative structure-activity relationship [QSAR] information, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for an accurate sensitization hazard classification or if additional testing is necessary. The Panel emphasized that, from an animal welfare perspective, retesting should not be undertaken until all other available information is evaluated, and a determination is made that such testing is required to fill a data gap. The Panel also recommended that more detailed guidance be developed for regulatory agencies on how the multiple decision criteria could be used in practice.

Subsequent Panel discussions focused on ICCVAM's recommendation to use multiple decision criteria to identify sensitizers and nonsensitizers. In general, the Panel preferred the multiple decision criteria to a single decision criterion for identifying sensitizers and nonsensitizers. A Panelist recommended that graphs showing the maximum SI obtained with the modified test method (the LLNA: DA, in this case) plotted against the maximum SI obtained with the traditional LLNA, for each test substance, be included in the final BRD. This was a general recommendation for both test methods that use multiple decision criteria (i.e., the LLNA: DA and LLNA: BrdU-ELISA). It was also pointed out that, as more data are accumulated for these test methods, the cut-off SI values for sensitizers and nonsensitizers would likely change.

Bootstrapping analysis was mentioned as a means to provide some measure of variability of the chosen cut-off values. It was also mentioned that the tables in Section 7.0 of the revised draft BRD provide no measurement of variation for the data. It was suggested that all of these tables include treatment means, standard deviations, and the mean squares, so that F-values can be calculated for between and among laboratory means. However, the Panel agreed that, while this information would be useful for inclusion in the final BRD, it would not impact the Panel's overall conclusions about the test method.

Some discussion followed about variations in the LLNA: DA test method protocol from the updated ICCVAM-recommended traditional LLNA test method protocol (i.e., sodium lauryl sulfate pretreatment prior to test substance application and an additional test substance application on day 7). The Panel agreed that despite these variations, the LLNA: DA was still mechanistically and functionally similar to the traditional LLNA.

**Public Comments:**

At the conclusion of the Panel discussion, Dr. Luster called for public comments. None were presented.

**Panel Conclusions and Recommendations:**

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated Evaluation Group presentation as modified during the discussions. The Panel approved unanimously.

## **Applicability Domain of the LLNA and Revised Draft ICCVAM Test Method Recommendations**

NICEATM provided an overview of the revised draft Addendum on the LLNA applicability domain. Subsequent to the 2008 Panel consideration of this topic, new data were obtained for pesticide formulations, dyes, essential oils, and substances tested in aqueous solution, but none were obtained for metals. Since the Panel previously considered the use of the term *mixtures* too broad, data were separately evaluated by product subgroups in the revised draft Addendum, and they were identified in general terms as pesticide formulations and other products. Dr. Wind presented the revised draft ICCVAM test method recommendations for the LLNA applicability domain to the Panel.

Subsequent to Dr. Wind's presentation, Dr. Luster asked Ms. McCall of EPA to clarify EPA's position on the use of LLNA data for pesticide formulations. Ms. McCall replied that EPA accepted positive or negative LLNA data on single substance technical grade additives. Between 2003 and 2007, EPA received few LLNA studies on pesticide formulations. Positive LLNA results were accepted, but for negative results, EPA required a confirmatory test. The majority of sensitization data submitted to EPA for pesticide formulations are from the guinea pig BT. There are limited human data available on pesticides due to the ethics limitations for conducting human studies, and applicants provide all of EPA's data.

A Panelist commented that the GPMT is more sensitive than the BT; he said that, in his experience, the GPMT showed roughly 60% positive results versus 20% positive results for the BT, for the same group of formulations. He said that the LLNA is more concordant with the GPMT than it is with the BT. He said that the GPMT is the preferred test in Europe. The Panel agreed that this should be reflected in the comparisons of LLNA and guinea pig results.

### **Panel Evaluation:**

Dr. Olson presented the draft position developed by Evaluation Group A, which was charged with primary review of the LLNA applicability domain, to the Panel. While the Panel agreed that there were too few data in the revised draft Addendum for some of the test substance classes (e.g., dyes, essential oils) to make a firm statement about concordance of the LLNA with other test methods for these classes, the Panel stated that any material should be suitable for testing in the LLNA unless there is a biologically-based rationale for exclusion, such as unique physicochemical properties that might affect their ability to interact with immune processes. The Panel therefore agreed that the LLNA should be considered appropriate for testing pesticide formulations and other products, unless there is a biologically-based rationale for exclusion.

The Panel also concurred that, while studies done with BALB/c mice should not be excluded from the evaluations in the revised draft Addendum, CBA should remain the preferred strain for the updated ICCVAM-recommended LLNA test method protocol, and that the use of any other strain, or of male rather than female mice, should be justified by the investigator.

The Panel did not agree that Pluronic L92 should be added to the list of preferred vehicles for the LLNA, but it did agree that studies done with Pluronic L92 should not be excluded from the evaluations in the revised draft Addendum.

While the concordance of LLNA results for essential oils was properly compared with human results, the Panel noted that the revised draft Addendum neglected to consider information that showed LLNA results were more concordant with human results when the major component was  $\geq 70\%$ , compared to the concordance for the essential oil itself. The Panel also commented that the term *natural complex substances* was more appropriate for these types of substances than *essential oils*, because this is the terminology used for the Registration, Evaluation, Authorisation and Restriction of Chemical substances program now in force in the European Union (EU).

In reference to the data for the medical device eluates in the revised draft Addendum, the Panel commented that ISO Standard 1099 requires the chemical analysis of such materials before skin sensitization testing is undertaken, and therefore agreed that the data provided were of little use for evaluating the performance of the LLNA for testing these types of substances.

**Public Comments:**

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

**Mr. Gary Wnorowski, Eurofins Product Safety Labs**

Mr. Gary Wnorowski said he had registered to make a public comment, but that Ms. McCall of EPA had already addressed his question by her answer to Dr. Luster's question regarding acceptability of pesticide formulation data.

**Panel Conclusions and Recommendations:**

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated presentation. The Panel approved unanimously.

**Adjournment**

At the conclusion of the discussion on the applicability domain, Dr. Luster adjourned the Panel for the day at 5:30 p.m., to reconvene at 8:30 a.m. on Wednesday, April 29, 2009.

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**Wednesday, April 29, 2009**

**Overview of the Draft LLNA: BrdU-ELISA Test Method Revised Draft BRD and Revised Draft ICCVAM Test Method Recommendations**

Dr. Luster called for Panel consideration of the LLNA: BrdU-ELISA test method. This test method measures bromodeoxyuridine (BrdU), a thymidine analog, instead of radioactive thymidine, incorporated into the DNA of proliferating lymphocytes, via an enzyme-linked immunosorbent assay (ELISA).

Dr. Masahiro Takeyoshi of Chemicals Evaluation and Research Institute, Japan (the test method developer) presented a synopsis of the test method to the Panel.

On behalf of NICEATM, Dr. Strickland presented an overview of the revised draft ICCVAM LLNA: BrdU-ELISA BRD to the Panel.

A Panelist asked why ICCVAM proposes an SI value of 2.0 as the cutoff value for a sensitizer instead of a value of 2.5, since the data indicated that no false positives would result if either value were used. Dr. Strickland replied that the value of 2.0 was chosen because this was the lowest value that resulted in a 0% false positive rate, thus minimizing the range of uncertainty.

Dr. Jacobs presented the revised draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method to the Panel.

**Panel Evaluation:**

Dr. Ullrich presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: BrdU-ELISA test method, to the Panel.

The Panel agreed that the LLNA: BrdU-ELISA test method was mechanistically and functionally similar to the traditional LLNA, and the ICCVAM LLNA Performance Standards could be used to evaluate it. The Panel also concurred that the available data and test method performance support the use of the LLNA: BrdU-ELISA to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. They agreed with ICCVAM's proposal that, based on the current validation database, the multiple SI decision criteria should be used to identify sensitizers and nonsensitizers

(i.e.,  $SI \geq 2.0$  for sensitizers,  $SI > 1.3$  for nonsensitizers). The Panel also noted that the limitation of these test methods when using the proposed multiple decision criteria is the indeterminate classification of substances that fall in the range of SI values for which a classification is uncertain (i.e.,  $2.0 > SI \geq 1.3$ ). The Panel recommended that when such results are obtained, users should carefully interpret the results in an integrated decision strategy in conjunction with all other available information (e.g., dose-response and QSAR information, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for an accurate sensitization hazard classification or if additional testing is necessary. The Panel emphasized that, from an animal welfare perspective, retesting should not be undertaken until all other available information is evaluated, and a determination is made that such testing is required to fill a data gap. The Panel also recommended that more detailed guidance be developed for regulatory agencies on how the multiple decision criteria could be used in practice.

Subsequent Panel discussions focused on ICCVAM's recommendation to use multiple decision criteria to identify sensitizers and nonsensitizers. In general, the Panel preferred the multiple decision criteria to a single decision criterion for identifying sensitizers and nonsensitizers. The Panel agreed that all of the comments for the LLNA: DA test method regarding the graphs and tables in the revised draft BRD, and the provision of measures of variation for interlaboratory reproducibility data, apply to the BrdU-ELISA also.

A Panelist commented that the use of interpolation for determining EC<sub>t</sub> values presupposed a monotonic increase in SI values and that isotonic regression might be more appropriate in cases in which a monotonic increase does not occur. More Panel discussion occurred regarding the practical usefulness of the multiple decision criteria. It was agreed that the term *integrated assessment* was more appropriate than *weight-of-evidence* to describe the approach taken to classify substances that fell into the uncertainty range.

The Panel discussed when it was appropriate to rely on hypothesis testing (as opposed to decision criteria based on a cutoff SI value) to classify substances. The Panel commented that, in some cases, statistical significance might not indicate a biological effect. The Panel agreed with the language regarding hypothesis testing in the current ICCVAM LLNA Performance Standards (Appendix A - Section 3.0).

#### **Public Comments:**

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

#### **Dr. George De George, MB Research Labs**

Dr. De George raised the following points:

- The data evaluated for the 1999 ICCVAM evaluation of the LLNA were statistically analyzed.
- As a result of that analysis, the optimum SI cutoff for a sensitizer was determined as 3.16.
- The Panel for the 1999 evaluation chose 3.0 as the SI cutoff to provide an added level of confidence.
- Routine statistical analysis of LLNA data to classify test substances was not recommended in the 1999 evaluation. In Dr. DeGeorge's opinion, the best reason to collect individual animal data was so that, in the future, studies could be done to determine an optimum method for hypothesis testing of LLNA data.
- Newer variant LLNA tests should be subjected to the same level (and not held to a higher level) of requirements for validation as the traditional LLNA.

**Panel Conclusions and Recommendations:**

At the conclusion of the public comments, Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated presentation. The Panel approved unanimously.

**Overview of the Revised Draft LLNA: BrdU-FC Test Method BRD and Revised Draft ICCVAM Test Method Recommendations**

Dr. Luster called for Panel consideration of the LLNA: BrdU-FC test method. This test method measures bromodeoxyuridine (BrdU), a thymidine analog, instead of radioactive thymidine, incorporated into the DNA of proliferating lymphocytes, via flow cytometric analysis. The test method also allows for the measurement of immunophenotypic markers in the lymphocyte population, ostensibly aiding in discrimination between irritants and sensitizers.

Dr. George DeGeorge of MB Research Labs, Spinnerstown, PA (the test method developer) presented a synopsis of the test method to the Panel. In addition to a brief description of the test method protocol, Dr. DeGeorge made the following points:

- The test method protocol was based on the ICCVAM-recommended LLNA test method protocol, using  $SI \geq 3.0$  as the decision criterion for a sensitizer.
- Test substances were chosen to include those tested in the traditional LLNA.
- Guinea pig data and human results are considered less reliable.
- The LLNA: BrdU-FC uses lower doses of test substances than the traditional LLNA to avoid irritating concentrations.
- The LLNA: BrdU-FC makes correct calls for some substances for which the traditional LLNA does not.
- All of the data generated by MB Research Labs using the LLNA: BrdU-FC are available for review at the laboratory (although not all data are available electronically).
- MB Research Labs is currently attempting to find other laboratories interested in participating in an interlaboratory validation study.

Following Dr. De George's presentation, a Panelist asked the following questions:

- Does MB Research Labs conduct LLNA: BrdU-FC studies according to GLP? Dr. De George said yes.
- What is the treatment group size? Dr. DeGeorge responded that five animals per treatment group were used.
- Can measurement of ear swelling be added to any LLNA variant test method as an additional endpoint? Dr. DeGeorge replied that it could, and that it could help resolve which doses to test.

On behalf of NICEATM, Dr. Allen presented a summary of the revised draft LLNA: BrdU-FC BRD to the Panel. At the conclusion of Dr. Allen's presentation, Dr. DeGeorge pointed out that an in-house flow cytometer and trained operators weren't necessary to conduct the test method, because the lymphocytes were fixed as part of the test method protocol, and the flow cytometry analysis could be outsourced.

Dr. Jacobs then presented the revised draft ICCVAM test method recommendations for the LLNA: BrdU-FC test method to the Panel.

**Panel Evaluation:**

Dr. Richmond presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: BrdU-FC test method, to the Panel.

The Panel agreed that the LLNA: BrdU-FC test method was mechanistically and functionally similar to the traditional LLNA, and the ICCVAM LLNA Performance Standards could be used to evaluate it. The Panel also concurred that the database of more than 45 representative test substances yielded adequate accuracy based on results from one laboratory, and that intralaboratory reproducibility also had been adequately demonstrated. However, the Panel agreed with the ICCVAM proposal to defer a formal recommendation on the validity of the LLNA: BrdU-FC until an independent audit of all data supporting the analysis has been conducted and until transferability has been demonstrated in an interlaboratory validation study. The Panel recommended that ICCVAM should work with NICEATM to support and facilitate the independent audit and interlaboratory validation study. The Panel recommended that upon completion of these tasks and determination of satisfactory data quality, power, and interlaboratory reproducibility, that the LLNA: BrdU-FC could be considered to have adequate validation and performance to support its consideration for regulatory use.

Much Panel discussion about the necessary statistical power of the test method occurred. Power is defined as the probability that the test method would determine that a test group showing a positive result is different from the negative control (i.e., that a sensitizer would be detected as such). Data presented to the Panel during their 2008 evaluation indicated that the test method would require nine animals per treatment group to achieve 95% power; the power with five animals per group was estimated at 80% in that evaluation. The Panel agreed that, before an interlaboratory validation study was begun, it should be verified that the LLNA: BrdU-FC test method has power at least equal to that of the traditional LLNA using five animals per treatment group.

**Public Comments:**

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

**Dr. George De George, MB Research Labs**

Dr. De George raised the following points:

- Power calculations on a subset of the data are not as reliable as accuracy statistics calculated from the entire dataset for 45 chemicals.
- Power calculations are a new requirement for validation, and not contained in the ICCVAM LLNA Performance standards.
- It was Dr. De George's opinion that it would be difficult, if not impossible, to get three qualified testing laboratories to participate in an interlaboratory validation study.

**Panel Conclusions and Recommendations:**

Subsequent to the public comments, the Panel commented that the flow cytometric analysis for samples from all three laboratories in an interlaboratory study could be done at MB Research Labs. Power calculations could be done by NICEATM on the most recent data generated by the LLNA: BrdU-FC test method.

The Panel decided to make a nomination to ICCVAM, with high priority, that NICEATM organize and supervise an interlaboratory validation study for the LLNA: BrdU-FC test method.

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report. The Panel approved unanimously.

### **Concluding Remarks**

Dr. Luster, on behalf of the Panel, thanked the NICEATM-ICCVAM staff for their continued assistance during the review process and the Panel meeting. He also thanked Drs. Joanna Matheson and Abby Jacobs, the IWG co-chairs, and Dr. Marilyn Wind, ICCVAM Chair and IWG member, for the hard work they put into the project. Dr. Luster also thanked the Panel, the Evaluation Group Chairs, and the experts on the test methods, who presented them to the Panel.

Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked the invited test method developers for their excellent summaries of their test method for the benefit of the Panel. Dr. Stokes concluded by saying he looked forward to further working with the Panel members to complete their Panel report.

### **Adjournment**

Dr. Luster adjourned the Panel at 11:30 a.m., concluding the meeting.

William S. Stokes, D.V.M., D.A.C.L.A.M.  
NIEHS  
P.O. Box 12233  
Mail Stop: K2-16  
Research Triangle Park, NC 27709

Dear Dr. Stokes,

The Meeting Summary Minutes, Independent Scientific Peer Review Panel Meeting, Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Updated Evaluation of the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA), accurately summarizes the Peer Review Panel meeting of April 28-29, 2009, in Bethesda, MD.

Sincerely,

-----  
Signature

*MICHAEL LUSTER*  
-----  
Printed Name

*8/21/09*  
-----  
Date

## **Appendix E4**

### **Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products**

**The full document is available electronically on the enclosed CD-ROM or at:  
[http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRRept2009.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRRept2009.pdf)**

**The document is also available on request from NICEATM:**

#### **NICEATM**

**National Institute of Environmental Health Sciences**

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**Independent Scientific Peer Review Panel Report:  
Updated Validation Status of New Versions and Applications of  
the Murine Local Lymph Node Assay:  
A Test Method for Assessing the Allergic Contact Dermatitis  
Potential of Chemicals and Products**

**June 2009**

**Interagency Coordinating Committee on the  
Validation of Alternative Methods**

**National Toxicology Program Interagency Center for the  
Evaluation of Alternative Toxicological Methods**

**National Institute of Environmental Health Sciences  
National Institutes of Health  
U.S. Public Health Service  
Department of Health and Human Services**

**National Toxicology Program  
P.O. Box 12233  
Research Triangle Park, NC 27709**

**This document is available electronically at:  
[http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRept2009.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf)**

**The findings and conclusions of this report are those of the  
Independent Scientific Peer Review Panel and should not be construed  
to represent the official views of ICCVAM or its member agencies.**

**When referencing this document, please cite as follows:**

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Independent Scientific Peer Review Panel Report:  
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Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of  
Chemicals and Products. Research Triangle Park, NC: National Institute of Environmental  
Health Sciences.

## Table of Contents

<b>List of Abbreviations and Acronyms</b> .....	<b>iii</b>
<b>Members of the Independent Scientific Peer Review Panel</b> .....	<b>v</b>
<b>Preface</b> .....	<b>vii</b>
<b>Executive Summary</b> .....	<b>xi</b>
<b>1.0 Nonradioactive LLNA Protocol – The Murine Local Lymph Node Assay (LLNA): Daicel Adenosine Triphosphate (LLNA: DA) Test Method</b> .....	<b>1-1</b>
1.1 Review of the Revised Draft Background Review Document for Completeness, Errors, and Omissions .....	1-1
1.2 Review of the Validation Status of the LLNA: DA .....	1-1
1.3 Comments on the Revised Draft ICCVAM Test Method Recommendations on the LLNA: DA.....	1-9
<b>2.0 Nonradioactive LLNA Protocol – The LLNA: Bromodeoxyuridine Detected by Flow Cytometry (BrdU-FC) Test Method</b> .....	<b>2-1</b>
2.1 General Comments .....	2-1
2.2 Review of the Revised Draft BRD for Completeness, Errors and Omissions .....	2-2
2.3 Review of the Validation Status of the LLNA: BrdU-FC .....	2-3
2.4 Comments on the Revised Draft ICCVAM Test Method Recommendations on the LLNA: BrdU-FC.....	2-7
<b>3.0 Nonradioactive LLNA Protocol – The LLNA: Bromodeoxyuridine Detected by ELISA (BrdU-ELISA) Test Method</b> .....	<b>3-1</b>
3.1 Review of the Revised Draft BRD for Completeness, Errors, and Omissions .	3-1
3.2 Review of the Validation Status of the LLNA: BrdU-ELISA .....	3-1
3.3 Comments on the Revised Draft ICCVAM Test Method Recommendations on the LLNA: BrdU-ELISA.....	3-8
<b>4.0 Use of the LLNA for Testing Pesticide Formulations and Other Products, Aqueous Solutions, and Metals</b> .....	<b>4-1</b>
4.1 Review of the Revised Draft Addendum for Completeness, Errors, and Omissions .....	4-1
4.2 General Questions.....	4-2
4.3 Pesticide Formulations.....	4-5
4.4 Dyes .....	4-8
4.5 Natural Complex Substances.....	4-9
4.6 Substances Tested in Aqueous Solutions.....	4-10

4.7 Comments on the Revised Draft ICCVAM Test Method Recommendations on  
the Traditional LLNA Applicability Domain ..... 4-12

**5.0 References..... A-1**

**Appendix A: Peer Review Panel Member Biosketches ..... A-1**

**Appendix B: Questions for the Peer Review Panel..... B-1**

### List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ANOVA	Analysis of variance
AOO	Acetone: olive oil (4:1)
ATP	Adenosine triphosphate
BIBRA	British Industrial Biomedical Research Association
BRD	Background review document
BrdU	Bromodeoxyuridine
CV	Coefficient of variation
DNCB	2,4-Dinitrochlorobenzene
EC2	Estimated concentration of a substance needed to produce a stimulation index of 2 (value is expressed as a percentage)
EC2.5	Estimated concentration of a substance needed to produce a stimulation index of 2.5 (value is expressed as a percentage)
EC3	Estimated concentration of a substance needed to produce a stimulation index of 3 (value is expressed as a percentage)
ECt	Estimated concentration of a substance needed to produce a stimulation index that is indicative of a positive response (value is expressed as a percentage)
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme-linked immunosorbent assay
eLLNA: BrdU-FC	Enhanced LLNA: BrdU detected by flow cytometry
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
E.U.	European Union
FR	<i>Federal Register</i>
GLP	Good Laboratory Practice
GPMT	Guinea pig maximization test
HCA	Hexyl cinnamic aldehyde
HMT	Human maximization test
HRIPT	Human repeat insult patch test
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
IWG	ICCVAM Immunotoxicity Working Group
JSAAE	Japanese Society for Alternatives to Animal Experiments
LLNA	Murine local lymph node assay

LLNA: BrdU-ELISA	LLNA: BrdU detected by ELISA
LLNA: BrdU-FC	LLNA: BrdU detected by flow cytometry
LLNA: DA	LLNA: Daicel adenosine triphosphate
MRC	U.K. Medical Research Council
NAS	National Academy of Sciences
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NIOSH	National Institute of Occupational Safety and Health
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
QSAR	Quantitative structure–activity relationship
REACH	Registration, Evaluation, and Authorisation of Chemicals
RIFM	Research Institute for Fragrance Materials
rLLNA	Reduced LLNA
SACATM	Scientific Advisory Committee for the Validation of Alternative Toxicological Methods
SD	Standard deviation
SI	Stimulation index
SLS	Sodium lauryl sulfate
SOT	Society of Toxicology
UCSF	University of California, San Francisco

### Members of the Independent Scientific Peer Review Panel

**Michael Luster, Ph.D. (Panel Chair)**, Senior Consultant to the National Institute of Occupational Safety and Health (NIOSH) Health Effects Laboratory, Morgantown, WV

**Nathalie Alépée, Ph.D.**, Scientific Coordinator on Alternatives Methods in Life Science, L'Oréal Research and Development, Aulnay sous Bois, France

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**Dagmar Jírová, M.D., Ph.D.**, Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic

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**Stephen Ullrich, Ph.D.**, Dallas/Ft. Worth Living Legends Professor & Professor of Immunology, Postgraduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX

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**Takahiko Yoshida, M.D., Ph.D.**, Professor, Department of Health Science, Asahikawa Medical College, Hokkaido, Japan

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<sup>1</sup> Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the review of the revised draft background review documents and the revised draft Addendum and concurs with the conclusions and recommendations included in this report.

*Independent Peer Review Panel Report*

*June 2009*

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## Preface

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine local lymph node assay (LLNA) to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation of the validation status of the LLNA that included an assessment by an international independent scientific peer review panel (hereafter, Panel). The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (OECD 2002; ISO 2002; EPA 2003). (This LLNA will be referred to hereafter as the “traditional” LLNA.)

In January 2007, the U.S. Consumer Product Safety Commission formally requested that ICCVAM assess the validation status of:<sup>2</sup>

- The traditional LLNA as a stand-alone assay for potency determinations (including severity) for the purpose of hazard classification
- Three modifications of the traditional LLNA not requiring the use of radioactive materials
- The reduced LLNA (rLLNA; also referred to as the LLNA limit dose procedure)
- The ability of the traditional LLNA to test mixtures, metals, and aqueous solutions (i.e., to re-evaluate the applicability domain for the traditional LLNA)

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), in coordination with ICCVAM and the ICCVAM Immunotoxicity Working Group (IWG), prepared comprehensive draft background review documents (BRDs) for each modified version of the traditional LLNA test method being evaluated, as well as a draft applicability domain addendum to the final BRD published previously on the traditional LLNA. In addition, ICCVAM developed draft LLNA performance standards intended for use in validating alternative test methods that are functionally and mechanistically similar to the traditional LLNA. Finally, ICCVAM, based on the information contained in each of the draft BRDs and the draft addendum, developed draft test method recommendations.

The supporting documents and the draft ICCVAM recommendations were provided to a new international Panel for an independent scientific review. This Panel met in public session in

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<sup>2</sup> The U.S. Consumer Product Safety Commission nomination can be obtained at:  
[http://iccvam.niehs.nih.gov/methods/immunotox/llnadoocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadoocs/CPSC_LLNA_nom.pdf).

March 2008.<sup>3</sup> Subsequent to the Panel review, finalized recommended performance standards for the LLNA and ICCVAM recommendations for the rLLNA were published.<sup>4</sup> The final documents considered the comments of the Panel, the public, and ICCVAM's scientific advisory panel.

The Panel concluded in March 2008 that more information and data were required for the three modified nonradioactive LLNA test methods before recommendations could be made regarding their use for regulatory safety testing (ICCVAM 2008). Similarly, the Panel concluded that more data would be needed before a recommendation on the usefulness and limitations of the current applicability domain of the traditional LLNA could be made. Subsequent to the Panel meeting, NICEATM received additional LLNA data for pesticide formulations and other products, as well as new data for the three modified nonradioactive LLNA test methods.

Using the additional information and working in coordination with the IWG, NICEATM revised the BRDs for each of these modified test methods and new applications of the LLNA. The revised draft BRDs provide the data and analyses supporting the scientific validity of the modified test methods and proposed applications. ICCVAM also prepared revised draft test method recommendations regarding proposed usefulness and limitations, standardized protocols, and future studies.

The revised draft BRDs, the revised draft applicability domain addendum, and revised draft ICCVAM recommendations were provided to the Panel for independent scientific review. In addition, NICEATM announced the availability of these documents on the NICEATM – ICCVAM website for public comment in a *Federal Register* (FR) notice (74 FR 8974) and via the ICCVAM email list. The FR notice also announced the public Panel meeting, to be convened at the National Institutes of Health in Bethesda, Maryland, on April 28 – 29, 2009.

The Panel was charged with:

- Reviewing each revised draft BRD and the revised draft addendum for completeness, and identifying any errors or omissions of existing relevant data or information
- Evaluating the information in each revised draft BRD and the revised draft addendum to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM 2003) had

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<sup>3</sup> The conclusions and recommendations of the Panel are included in its report, which is available at: [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPREpt2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPREpt2008.pdf).

<sup>4</sup> The *Recommended LLNA Performance Standards* document is available at: [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/lina-ps/LLNAPerfStds.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/lina-ps/LLNAPerfStds.pdf); the ICCVAM

- been appropriately addressed for the recommended use of the new versions and applications of the traditional LLNA
- Considering the ICCVAM revised draft test method recommendations for the following, and commenting on the extent to which they are supported by the information provided in the revised draft BRDs and the revised draft addendum:
    - Proposed test method uses
    - Proposed recommended standardized protocols
    - Proposed test method performance standards
    - Proposed additional studies

During its public meeting in April 2009, the Panel discussed each charge, listened to public comments, and developed conclusions and recommendations for ICCVAM. The Panel emphasizes that it was asked to consider two overall questions. The Panel was to consider: (1) whether the validation status of each of the above proposed modifications or alternative uses of the LLNA had been adequately characterized for its intended purpose according to established ICCVAM validation criteria,<sup>5</sup> and (2) whether proposed modifications or alternative uses of the LLNA are sufficiently accurate and reliable to be used for the identification of sensitizing substances and nonsensitizing substances in place of the traditional LLNA procedure.

This report details the Panel's independent conclusions and recommendations. ICCVAM will consider this report, along with all relevant public comments, as it develops final test method recommendations. The final ICCVAM test method recommendations will be forwarded to U.S. Federal agencies for their consideration in accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545).

The Panel gratefully acknowledges the efforts of NICEATM staff in coordinating the logistics of the peer review Panel meeting and in preparing materials for the Panel's review. The Panel also thanks each of the test method developers, Drs. George DeGeorge (LLNA: bromodeoxyuridine detected by flow cytometry test method), Kenji Idehara (LLNA: Daicel adenosine triphosphate test method), and Masahiro Takeyoshi, (LLNA: bromodeoxyuridine detected by ELISA) for providing summaries and additional clarifications of the

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recommendations for the rLLNA are in the *ICCVAM Test Method Evaluation Report*, available at: [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNA-LD/TMER.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNA-LD/TMER.pdf).

<sup>5</sup> ICCVAM validation criteria are detailed in the document, *Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods*, available at [http://iccvam.niehs.nih.gov/docs/about\\_docs/validate.pdf](http://iccvam.niehs.nih.gov/docs/about_docs/validate.pdf).

*Independent Peer Review Panel Report*

*June 2009*

nonradioactive test methods under review. Finally, as Panel Chair, I thank each Panel member for her or his thoughtful and objective review of these LLNA-related activities.

Michael Luster, Ph.D.  
Chair, LLNA Peer Review Panel  
June 2009

## Executive Summary

This report describes the conclusions and recommendations of an international independent scientific peer review panel (hereafter, Panel). This Panel was charged by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) with evaluating the validation status of new versions and applications of the murine local lymph node assay (LLNA) for assessing the allergic contact dermatitis (ACD) potential of chemicals and products. The LLNA which was first evaluated in 1999 by ICCVAM is hereafter referred to as the “traditional LLNA” to distinguish it from other versions considered by the Panel. The new versions and applications considered include:

- The application of the traditional LLNA for evaluating pesticide formulations and other products, metals, and substances in aqueous solutions
- Three modified versions of the traditional LLNA not requiring the use of radioactive markers:
  - LLNA: DA (LLNA: Daicel adenosine triphosphate)
  - LLNA: BrdU-FC (LLNA: bromodeoxyuridine detected by flow cytometry)
  - LLNA: BrdU-ELISA (LLNA: bromodeoxyuridine detected by ELISA)

### Nonradioactive LLNA Protocol – The LLNA: DA Test Method

The Panel concluded that the available data and performance support the revised draft ICCVAM recommendations on usefulness and limitations for the LLNA: DA test method. They agreed that the test method could be used for identifying substances as potential skin sensitizers and nonsensitizers. On the basis of the available data, accuracy is optimized if a stimulation index (SI)  $\geq 2.5$  is used to identify sensitizers, and an SI  $\leq 1.7$  is used to identify nonsensitizers. A limitation of the LLNA: DA involves the indeterminate identification of substances with SI values between 1.7 and 2.5 (exclusive). Thus, when an SI between 1.7 and 2.5 is obtained in the LLNA: DA, users should carefully interpret the results in an integrated decision strategy in conjunction with all available and relevant information (e.g., dose response information, statistical analyses, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for a definitive skin sensitization identification or if additional testing is necessary. The Panel noted that because the decision criteria chosen to identify sensitizers and nonsensitizers were based on a post hoc analysis, prospective testing with the test method might affect the proposed model. For this reason, data generated should be routinely evaluated to determine if the proposed model is still optimal with regard to the decision criteria. Even with these limitations, the LLNA: DA provides opportunities to reduce animal usage (e.g., use of guinea

pigs) in those regions in which guinea pig tests rather than the traditional LLNA are performed because radioisotope use is not permitted. In addition, the use of two decision criteria allows for a more definitive identification of sensitizers and nonsensitizers, which also provides animal welfare benefits by reducing further tests that might be required in instances where the hazard classification of a substance is not as clear.

The revised draft LLNA: DA background review document (BRD) was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: DA test method to assess the ACD-inducing potential of chemicals and other products. The Panel evaluated the revised draft BRD for completeness, errors, and omissions, and recommended that its suggestions/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The Panel agreed that the data supported the revised draft ICCVAM recommendations for the proposed standardized protocol for the LLNA: DA. The recommendations for maintaining a positive control database reflect current evidence and best practice. The Panel agreed that four animals per dose group should be recommended for the LLNA: DA.

The Panel considered the substances tested in the LLNA: DA to be representative of a sufficient range of chemicals expected to be tested for skin sensitization potential, and concluded that the accuracy analysis had made appropriate comparisons to the traditional LLNA, guinea pig tests, and human data/experience. The Panel indicated that the number of substances in the range of uncertainty was too few to determine if specific characteristics (e.g., chemical class, physical form, molecular weight, peptide reactivity, etc.) associated with those substances could be used for definitive skin sensitization identification.

With regard to test method reliability, the Panel concluded that the interlaboratory reproducibility of the LLNA: DA had been adequately evaluated. The Panel noted that five of the 10 laboratories that participated in the first phase of the interlaboratory validation study exceeded the performance standards' acceptable range for EC<sub>t</sub> values (estimated concentration of a substance needed to produce an SI that is indicative of a positive response) for 2,4-dinitrochlorobenzene (DNCB). The Panel indicated that this was understandable since DNCB is a strong sensitizer and the LLNA: DA has a different dosing regimen and time course than the traditional LLNA, which might extend into the elicitation phase of skin sensitization. However, all the laboratories that participated in the first and second phase of the interlaboratory validation study obtained EC<sub>2.5</sub> values (estimated concentration of a substance needed to produce an SI of 2.5) within the concentration range indicated for hexyl cinnamic aldehyde (HCA), which documents the test method's favorable reproducibility and performance.

The Panel stated that the available data supported the revised draft ICCVAM recommendations for the LLNA: DA in terms of future studies, which included performing more LLNA: DA studies on metals, irritants, and formulations with comparative traditional LLNA, guinea pig, and human data. Regarding irritants, the proposed future studies might help explain why results obtained using the LLNA: DA were discordant with the traditional LLNA and may even provide general insight into the problematic nature of discriminating irritants in the LLNA. The Panel also recommended that additional decision criteria and guidance should be identified for substances with SI greater than 1.7 but less than 2.5, and that the additional decision criteria be reassessed as additional discriminators and data become available (e.g., high-quality human ACD data). The Panel recommended that a protocol for defining and reevaluating the SI decision criteria for sensitizers and nonsensitizers be developed. Further, future interlaboratory validation studies should simultaneously evaluate intralaboratory reproducibility, using the appropriate statistics, to evaluate variation both within a laboratory and between laboratories. Additionally, the Panel strongly recommended that a statistician actively participate in the preparation of future BRDs and formulation of ICCVAM recommendations.

The Panel disagreed with the revised draft ICCVAM recommendation that separate performance standards be developed to assess modified versions of the LLNA: DA test method. Although the test methods differ in the dosing regimen and in the timing of the assay, the Panel viewed the LLNA: DA as mechanistically similar to the traditional LLNA, in that both methods measure cellular stimulation in the draining lymph nodes. Consequently, the Panel concluded that the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) are applicable to the LLNA: DA as a mechanistically and functionally similar test method. Generally, the Panel viewed the difference in treatment schedule between the LLNA: DA and the traditional LLNA to be potentially significant if the LLNA: DA test progressed through the elicitation phase of skin sensitization, which is associated with a localized skin reaction. Thus, the Panel was concerned that if the duration of the test involved the elicitation phase of ACD development, this would produce undue discomfort and distress in the animals. The Panel also recommended that the test method developer (Daicel Chemical Industries, Ltd.) justify the use of 1% sodium lauryl sulfate (SLS) (i.e., determine whether the 1% SLS pretreatment is necessary).

#### **Nonradioactive LLNA Protocol – The LLNA: BrdU-FC Test Method**

The Panel concluded that the data and test method performance of the LLNA: BrdU-FC supported the revised draft ICCVAM recommendations that the test method may be useful for identifying substances as potential skin sensitizers or nonsensitizers, and agreed that formal recommendations should be deferred until original study records are received for an

independent audit and interlaboratory transferability and reproducibility have been assessed. The final test method recommendations should highlight those items of highest priority for further validation consideration: (1) a review of the original data at the individual animal level with appropriate positive and negative controls, (2) an evaluation, based on the data from the intralaboratory study data, of the minimum number of animals required per test group to ensure test performance is as good as or better than the traditional LLNA, then (3) an interlaboratory reproducibility study conducted and evaluated according to the specifications in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) and with appropriate quality control systems. The Panel agreed that, subsequently, less critical items (e.g., methodological specifics, immunophenotypic endpoints, alternative decision criteria for identifying materials as sensitizers and nonsensitizers) should then be evaluated.

The revised draft LLNA: BrdU-FC BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: BrdU-FC test method to assess the ACD-inducing potential of chemicals and other products. The Panel evaluated the revised draft BRD for completeness, errors, and omissions, and recommended that its recommendations/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The Panel agreed that the available data supported the revised draft ICCVAM recommendations for the proposed test method protocol for the LLNA: BrdU-FC procedure. Also, revised power calculations should be performed using the data provided for the intralaboratory performance to determine the minimum group size required to provide a level of test performance equivalent to or better than the traditional LLNA. The minimum group size in the protocol should then be adjusted, if necessary. The ICCVAM recommendation for maintaining a positive control database reflects current evidence and best practice. The Panel considered the measurement of ear swelling and the use of immunophenotypic markers as potentially valuable adjuncts to the traditional LLNA and other modified LLNA protocols.

The Panel noted that since the 2008 Panel evaluation no new data for additional test substances were added to the analyses in the revised draft BRD, although new data for intralaboratory reproducibility were properly integrated into the assessment. As such, similar to 2008, the substances tested in the LLNA: BrdU-FC seemed representative of a sufficient range of chemical classes and physical chemical properties, and thus the test method appeared applicable to many of the types of chemicals and products that are typically tested for skin sensitization potential. The results of the revised concordance assessments of the LLNA: BrdU-FC against the traditional LLNA test method suggest that the LLNA: BrdU-FC (as performed at the originating facility) can be developed as a reliable alternative to the

traditional LLNA, with the same applicability domain. Both the LLNA: BrdU-FC and the eLLNA: BrdU-FC (“enhanced” LLNA: BrdU-FC), on the basis of the information available, performed equally well compared with the traditional LLNA in a single laboratory.

The Panel concluded that compared to the 2008 review, intralaboratory reproducibility was adequately assessed and fit for the intended purpose. This was based on additional studies submitted for HCA and DNCB. The Panel agreed that the assessment of interlaboratory reproducibility described in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) can be appropriately applied to the LLNA: BrdU-FC test method.

The Panel affirmed that the revised draft ICCVAM recommendations for future studies highlighted the unanswered questions raised by the available data set. The Panel specifically recommended: (1) that an independent audit of the original data should be performed to establish the validity of the data relied upon in the revised draft BRD, (2) that revised power calculations should be performed using the data provided for the intralaboratory validation so that the number of animals needed to provide performance equivalent to, or better than, the traditional LLNA can be determined, (3) that an interlaboratory study is an absolute requirement for validation to determine the transferability and reliability of the test method when used in different laboratories, (4) that alternate prediction models (e.g., multiple SIs similar to those recommended for the LLNA: DA and LLNA: BrdU-ELISA test methods) should be considered, and (5) that the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) should be followed in this future work. The Panel recommended that ICCVAM should work with NICEATM to support and facilitate these activities. The Panel also considered that an emphasis should be given to the use of ear swelling measurements to identify local irritants as a means of improving the traditional LLNA and modified LLNA test methods. This is particularly relevant when considering the challenges associated with discriminating irritants from sensitizers in the LLNA and ultimately emphasizes the need to better understand the correlation between mouse ear data and human data/experience.

It is the view of the Panel that this test method can be considered to have been scientifically validated and to be ready for regulatory consideration if the following requirements are satisfactorily met: (1) an independent data audit should be conducted confirming the acceptable quality of the data relied upon in the revised draft BRD, (2) a revised evaluation of the minimum number of animals required should be conducted; then, if  $n = 4$  or  $5$  yields statistical power that is equivalent to or better than the traditional LLNA, an interlaboratory evaluation should be performed using the test, (3) the interlaboratory study should produce results that satisfy the requirements in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009).

The Panel considered the LLNA: BrdU-FC and the traditional LLNA to be mechanistically and functionally similar. Thus, the studies proposed by the ICCVAM-recommended LLNA performance standards are sufficient to establish the intra- and interlaboratory performance of the LLNA: BrdU-FC. The Panel commented that for regulatory data submissions, a laboratory (either with flow cytometry experience and/or following training and certification of personnel) should demonstrate proficiency by repeating the evaluation of the same substance (i.e., four independent tests) to allow an assessment of intralaboratory reproducibility before using the test for regulatory purposes. Results should be evaluated for both a known strong and known moderate sensitizer (i.e., DNCB and HCA, respectively). The inclusion of a known, reproducible weak sensitizer and a negative control is also essential to confirm that the full range of appropriate responses can be reproduced.

Additional considerations would include development of a standard test method protocol, standard operating procedure, and other documentation, and adherence to recognized quality assurance/quality control systems for flow cytometry and associated data acquisition equipment.

#### **Nonradioactive LLNA Protocol – The LLNA: BrdU-ELISA Test Method**

The Panel concluded that the data and performance for the LLNA: BrdU-ELISA test method supported the revised draft ICCVAM recommendations that it can be used for identifying substances as potential skin sensitizers and nonsensitizers. An  $SI \geq 2.0$  should be used to identify substances as sensitizers and  $SI < 1.3$  should be used to identify nonsensitizers. A limitation of the LLNA: BrdU-ELISA involves the indeterminate identification of substances that produce an SI greater than or equal to 1.3 but less than 2.0. When such a result is obtained in the LLNA: BrdU-ELISA, users should carefully interpret the results in an integrated decision strategy in conjunction with all available and relevant information (e.g., dose response information, statistical analyses, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for definitive skin sensitization identification or if additional testing is necessary. The Panel noted that because the decision criteria chosen to identify sensitizers and nonsensitizers were based on post hoc analysis, prospective testing with the test method might affect the proposed model. For this reason, data generated should be routinely evaluated to determine if the proposed model is still optimal with regard to the decision criteria. Even with these limitations, the LLNA: BrdU-ELISA provides opportunities to reduce animal usage (e.g., use of guinea pigs) in those regions that are not permitted to use radioisotopes and thus perform guinea pig tests rather than the traditional LLNA. In addition, using two decision criteria allows for a more definitive identification of sensitizers and

nonsensitizers, which also provides animal welfare benefits by reducing further tests that might be required in instances where the hazard classification of a substance is not as clear.

The revised draft LLNA: BrdU-ELISA BRD was compiled to provide a comprehensive review of available data and information evaluating the usefulness and limitations of the LLNA: BrdU-ELISA test method to assess the ACD-inducing potential of chemicals and other products. The Panel evaluated the draft BRD for completeness, errors, and omissions and recommended that its suggestions/corrections relating to general, statistical and specific editorial issues be incorporated into the final document.

The Panel agreed that the available data supported the revised draft ICCVAM recommendations for the proposed standardized test method protocol for the LLNA: BrdU-ELISA test method. The recommendations for maintaining a positive control database reflect current evidence and best practice. The Panel agreed that four animals per dose group should be recommended for the LLNA: BrdU-ELISA.

The Panel considered the database of substances tested in the LLNA: BrdU-ELISA to be representative of a sufficient range of chemicals expected to be tested for skin sensitization potential, and concluded that the accuracy analysis had made appropriate comparisons to the traditional LLNA, guinea pig tests, and human data/experience. The Panel indicated that the number of substances in the range of uncertainty (i.e.,  $1.3 \leq SI < 2.0$ ) was too few to determine if specific characteristics (e.g., chemical class, physical form, molecular weight, peptide reactivity, etc.) associated with those substances could be used for definitive skin sensitization identification.

In 2008, the Panel did not find sufficient power for using  $SI \geq 1.3$  as the decision criterion. Even with a group size of eight animals, the power was only 50% (ICCVAM 2008). Power calculations might be necessary to determine if the sample size used is sufficient for those substances that are not definitively identified as sensitizers or nonsensitizers (i.e., substances in the range of uncertainty of  $1.3 \leq SI < 2.0$ ).

With regard to test method reliability, the Panel concluded that the interlaboratory reproducibility had been adequately evaluated and that the test is reproducible. Considering that the radioisotope measurement in the traditional LLNA is more sensitive than the technique for the LLNA: BrdU-ELISA, and that the analysis of EC3 values (estimated concentration of a substance needed to produce a stimulation index of 3) in the traditional LLNA was based on a larger dataset, it is appropriate to adjust the acceptability range of the two positive control substances tested, dependent on the method used for measurement of the endpoint. Although the qualitative performance was acceptable in the interlaboratory study, the quantitative data for two of the laboratories suggests a relatively high degree of variability, which justifies the routine use of appropriate positive and negative controls.

The Panel stated that the available data supported the revised draft ICCVAM recommendations for the LLNA: BrdU-ELISA in terms of future studies, which included performing more LLNA: BrdU-ELISA studies on metals, irritants, and formulations with comparative traditional LLNA, guinea pig, and human data. Regarding irritants, the proposed future studies might help explain why results obtained using the LLNA: BrdU-ELISA and traditional LLNA were discordant, and further address the general challenge of discriminating irritants in the traditional LLNA itself. The Panel also recommended that additional decision criteria and guidance should be identified for substances that produce an SI greater than or equal to 1.3 but less than 2.0, and that the additional decision criteria be reassessed as additional discriminators and data become available (e.g., high-quality human ACD data). The Panel recommended that a protocol for defining and reevaluating the SI decision criteria for sensitizers and nonsensitizers be developed. Further, future interlaboratory validation studies should simultaneously evaluate intralaboratory reproducibility, using the appropriate statistics, to evaluate variation both within a laboratory and between laboratories. As stated previously, the Panel strongly recommended that a statistician actively participate in the preparation of future BRDs and formulation of ICCVAM recommendations.

The Panel agreed with the revised draft ICCVAM recommendation that separate performance standards should not be developed to assess modified versions of the LLNA: BrdU-ELISA test method. The LLNA: BrdU-ELISA is mechanistically and functionally similar to the traditional LLNA, such that the ICCVAM-recommended LLNA performance standards (ICCVAM 2009) could be used to evaluate future modifications of the LLNA: BrdU-ELISA.

#### **LLNA for Testing Pesticide Formulations and Other Products, Aqueous Solutions, and Metals**

The Panel comprises experts with knowledge in the evaluation of a range of test materials, but it is by no means expert in all of the product classes for which skin sensitization potential should be evaluated. The Panel also acknowledges that information and data gaps exist which prevent a full understanding of ACD epidemiology in humans. The test materials for which data are provided in the revised draft Addendum cover only a subset of the active ingredients used in each of the relevant product classes, and their frequency of use within those product classes is not noted in the revised draft Addendum. The Panel recommends that Federal agencies considering the results of this validation process assess how representative the test materials and findings in the revised draft Addendum are relative to substances of interest. In particular, the agencies should assess the chemical classes used in, and the range of biological effects of, the materials and products in which they have an interest.

The revised draft ICCVAM recommendations state that, although the database is limited, the traditional LLNA appears to be useful for evaluating substances tested in aqueous solutions or pesticide formulations provided the potential for overclassification (i.e., false positives) is not a limitation. The Panel agreed with these revised draft ICCVAM recommendations noting that the high rate of false positive substances may be inherent to the product and/or chemical class, testing of substances at concentrations that produced skin irritation, and to the fact that the LLNA detects the induction phase of skin sensitization. Furthermore, where comparative data were available, the LLNA identified more sensitizers than did guinea pig tests (predominantly Buehler tests which are considered to be less sensitive than the guinea pig maximization test [Basketter et al. 1993; Frankild et al. 2000]) but missed no materials that the guinea pig tests classified as sensitizers.

The Panel further suggested that, unless there are unique physiochemical properties associated with a material that might affect its ability to interact with immune processes, it should be a candidate for LLNA testing. An example of a material class that may possess such unique properties is some nanomaterials that are incapable of recognition by dendritic cells. Along these lines, the Panel also disagreed with the revised draft ICCVAM recommendation that a definitive recommendation on the usefulness of the LLNA for testing natural complex substances and dyes could not be made until more data were accrued. The Panel considered these classes of materials suitable for testing in the LLNA unless there are unique physiochemical properties associated with these materials that might affect their ability to interact with immune processes.

The Panel expressed a strong desire to avoid revalidation of the LLNA for new classes/types of test substances unless there is a biologically-based rationale. For new classes of test materials (e.g., nanomaterials), an integrated assessment of all available and relevant information should be conducted. This should include computer-assisted structure-activity relationships, prediction/measurement of biotransformation to potential reactive species, and possibly peptide, protein, or lipid binding. The Panel agreed that if any variant of the LLNA is validated for use to test novel classes, then the findings should be relevant to the family of validated LLNA tests and that similar uncertainties would surround the use of guinea pig models to evaluate novel classes of test materials.

The revised draft Addendum to the original validation report for the traditional LLNA (ICCVAM 1999) provided a comprehensive review of currently available data and information for evaluating the usefulness and limitations of the traditional LLNA for assessing the skin sensitization potential of pesticide formulations and other products, substances tested in aqueous solutions, and metals. The Panel evaluated the revised draft Addendum for completeness, errors, and omissions and concluded that there were no apparent errors. However, a Panel member did

note during the public meeting an omission regarding the natural complex substances; the relationship between the LLNA, guinea pig, and human data for major constituents (substances constituting at least 70%) of some of the natural complex substances and the LLNA results of the natural complex substances themselves was omitted. The Panel recommended that its suggestions/corrections relating to general, statistical, and specific editorial issues be incorporated into future revisions.

The Panel stated in its 2008 review (ICCVAM 2008) that the term *mixtures* was used too broadly (i.e., can represent an infinite number of materials), and this concern was addressed in the revised draft Addendum by dividing the substances considered into pesticide formulations, dyes, natural complex substances, and substances tested in aqueous solutions (this group included pesticide formulations tested in aqueous solutions), and analyzing the data for each group separately. The Panel agreed that the terms used to classify information submitted for the revised analysis are sensible and help to divide the dataset into useful categories for analysis, and that the product categories selected fit well with the nature and range of materials in the database. Such categories indicate classes of materials for which there exist, or do not exist, LLNA data and thus provide useful information for industry and regulatory agencies.

The Panel noted that the revised draft Addendum does not consider many classes of formulations to which humans may be exposed, by intention or by accident, such as: metalworking fluids, fuels, petroleum products used as lubricants, detergents and other cleaning agents, enzymes used in cleaning products, chemical household products, chemical (low molecular weight) pharmaceutical products, medical device materials (chemically characterized extracts), and nanomaterials (e.g., titanium oxide). Available data for substances within these classes may prove informative for human health.

Regarding pesticide formulations, the Panel concluded that the performance characteristics, reproducibility, and reliability of the LLNA had been adequately assessed and that the methods of data analysis were appropriate. The Panel indicated that the analysis for dyes, natural complex substances, and substances tested in aqueous solutions reflected the available information and the appropriate concordance statistics.

With regard to future studies, the Panel agreed with the ICCVAM recommendation for continued accumulation of information in the targeted areas. The Panel also indicated that solubility data should ideally be provided so that thermodynamic activity can be computed and compared to maximum theoretical percutaneous penetration. This information should be considered when comparing the data from LLNA studies in lipophilic delivery systems compared to that in aqueous systems. The Panel also suggested that, before additional animal testing is conducted, consideration should be given to product use and whether this renders a need to test the substance for skin sensitization potential.

**Appendix A**  
**Peer Review Panel Member Biosketches**

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## Panel Member Biosketches

### **Nathalie Alépée, Ph.D.**

Dr. Alépée performed research leading to a Ph.D. in Medical Virology and Microbiology at the Centre National de la Recherche Scientifique institute, Gif sur Yvette, France. She is currently the scientific coordinator on Alternatives Methods in Life Science Department at L'Oréal Research and Development, Aulnay sous Bois, France. She is the L'Oréal representative to the European Partnership on Alternative to Animal Testing, and serves on two working groups: Identification of Opportunities, Including R&D (working group 2), and Validation and Acceptance (working group 5). She is also the representative in the eye irritation working group to the European Cosmetics Association and in the French Groupement d'Intérêt Scientifique Platform on Alternatives. She has served on the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC), representing the European Federation of Pharmaceutical Industries Associations, and was nominated as Organisation for Economic Co-operation and Development expert for eye and skin irritation. As a manager in Investigative Toxicology with Pfizer Global Research and Development, Amboise, France, she implemented the murine local lymph node assay (LLNA) in the laboratory. She served as a peer reviewer of the reduced LLNA test protocol and prediction model for ESAC in 2007, and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA.

### **Anne Marie Api, Ph.D.**

Dr. Api received a Ph.D. from Aston University in Birmingham, England and is currently Vice President of Human Health Sciences at the Research Institute for Fragrance Materials (RIFM). She is responsible for the human health scientific program and for the investigation and initiation of new research and testing projects for RIFM. She is a member of 10 professional organizations, including the American Academy of Dermatology, American Contact Dermatitis Society, the European Society of Contact Dermatitis, and the Society of Investigative Dermatology. She participated in the World Health Organization International Workshop in Skin Sensitization in Chemical Risk Assessment held in Berlin, Germany, in 2006, and a BfR International Workshop on Contact Dermatitis in October 2008. She is author of over 100 publications and presentations relevant to dermatology and dermatotoxicology.

### **Nancy Flournoy, M.S., Ph.D.**

Dr. Flournoy received B.S. and M.S. degrees in Biostatistics from the University of California at Los Angeles, and a Ph.D. in Biomathematics from the University of Washington. She is Professor and Chair of the Department of Statistics at the University of

Missouri. Her research interests include adaptive designs, bioinformatics, chemometrics, clinical trials, and environmetrics. She has an extensive list of edited volumes and papers on statistical theory, statistical genetics and immunology, epidemiology in immune-suppressed subjects, clinical trials for prevention and treatment of viral infection, transplantation biology and its effects on digestion, lungs, eyes, mouth, and central nervous system, optimization of statistical processing, and additional papers, interviews, and technical reports. She has editorial responsibilities for numerous statistical journals and serves on numerous advisory boards and nominating committees. She is a member and past Chair of the Council of Sections of the American Statistical Association, and served in various other statistical, medical and toxicological societies or programs as Chair or as a member of the Board of Directors. She is a former member of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). She also served on the Expert Panels for the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) that evaluated the Revised Up-and-Down Procedure; the Current Validation Status of *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants; and Five *In Vitro* Pyrogen Test Methods.

**Dagmar Jírová, M.D., Ph.D.**

Dr. Jírová received a Ph.D. from the Medical Faculty of Hygiene at Charles University in Prague. She is currently the Head of the Department of Toxicology and Veterinary Services and the Reference Center for Cosmetics at the National Institute of Public Health in the Czech Republic. Her main responsibilities include safety assessment of consumer products, particularly cosmetics and their ingredients, performance of toxicological methods *in vivo* in animals, human patch testing for local toxicity assessment, and introduction of *in vitro* techniques for screening of toxicological endpoints using cell and tissue cultures. She represents the Czech Republic in the Standing Committee on Cosmetics of the European Commission. She is an ESAC-ECVAM member and was involved in the Peer Review Panel for Skin Irritation Validation Study and LLNA test protocol and prediction model. She is author of more than 100 publications and presentations relevant to dermatotoxicology, including a recent presentation at the Sixth World Congress on Alternatives and Animal Use in the Life Sciences, held in Tokyo, 2007, titled “Comparison of Human Skin Irritation and Photoirritation Patch Test Data with Cellular *in vitro* Assays and Animal *in vivo* data”.

**David Lovell, Ph.D., B.Sc. (Hons), F.S.S., FIBiol, CStat, CBiol**

Dr. Lovell received a Ph.D. from the Department of Human Genetics and Biometry, University College, London. He is currently Reader in Medical Statistics at the Postgraduate Medical School at the University of Surrey. Previously, he was Associate Director and Head

of Biostatistics support to Clinical Pharmacogenomics at Pfizer Global Research and Development in Sandwich, Kent, providing data management and statistical support to pharmacogenetics and genomics. He joined Pfizer in 1999 as the Biometrics Head of Clinical Pharmacogenetics. Before joining Pfizer, Dr. Lovell was the Head of the Science Division at British Industrial Biological Research Association (BIBRA) International, Carshalton, which included Molecular Biology, Genetic Toxicology, Biostatistics and Computer Services. At BIBRA, Dr. Lovell managed the statistical and computing group providing specialized statistical support to BIBRA's Clinical Unit and contract research work. He conducted and managed research programs on genetics, statistics and quantitative risk assessment for the European Union and U.K. Government Departments. His research interests are the use of mathematical, statistical, and bioinformatic methods together with genetic models in the understanding of toxicological mechanisms and risk assessment problems. Dr. Lovell had previously been a Senior Research Officer with the U.K. Medical Research Council (MRC) Experimental Embryology and Teratology Unit, a visiting Postdoctoral Fellow at the U.S. National Institute of Environmental Health Sciences (NIEHS), a Geneticist at the MRC Laboratories, Carshalton, and a Research Assistant in Cytogenetics at Birmingham University. He has acted as a consultant to a number of organizations, has considerable experience of working with Regulatory Authorities, has many publications related to his work and has wide experience of making presentations to a wide range of audiences. He is a member of the Scientific Committee of the European Food Safety Authority, the U.K. Government's advisory Committees on Mutagenicity and Carcinogenicity of Chemicals in Food, Consumer Products and the Environment and the Independent Scientific Advisory Committee for the U.K. Medicines and Healthcare Products Regulatory Agency database research. He served on the NICEATM-ICCVAM Expert Panels that evaluated the Frog Embryo Teratogenesis Assay - Xenopus, *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, and Five *In Vitro* Pyrogen Test Methods.

**Michael Luster, Ph.D.**

Dr. Luster received a Ph.D. in Immunology from Loyola University of Chicago. He was formerly Chief, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health (NIOSH), and currently serves as a senior advisor to the Director of the Health Effects Laboratories and the staff of Toxicology and Molecular Biology Branch at NIOSH. Program areas include neuroscience, dermatology, molecular carcinogenesis, molecular epidemiology, molecular toxicology, molecular epidemiology, and inflammation/immunotoxicology. In addition, Dr. Luster conducts basic and applied research in immunotoxicology including its application in risk assessment. Current research activities include molecular epidemiology studies of genetic polymorphism involved in workplace-related diseases and experimental studies involving

occupational allergic rhinitis. Dr. Luster is also working with various staff at the U.S. Environmental Protection Agency (EPA) through the Risk Assessment Forum to develop immunotoxicity testing guidelines. He also directed two studies for the NTP on the Toxicology and the Carcinogenesis of Promethazine and Ortho-phenylphenol, in 1990 and 1986, respectively. He is a co-author of over 300 publications in peer-reviewed journals.

**Howard Maibach, M.D.**

Dr. Maibach received an M.D. from Tulane University. He is currently a professor in the Department of Dermatology at the University of California, San Francisco (UCSF), where he is also Chief of the Occupational Dermatology Clinic. In his 35 years at UCSF, Dr. Maibach has written and lectured extensively on dermatotoxicology and dermatopharmacology. His current research programs include defining the chemical-biologic faces of irritant dermatitis and the study of percutaneous penetration. Dr. Maibach served on the 1998 ICCVAM Peer Panel that evaluated the LLNA. Dr. Maibach has been on the editorial boards of over 30 scientific journals and is a member of 19 professional societies including the American Academy of Dermatology, San Francisco Dermatological Society, and the International Commission on Occupational Health. He has co-authored over 1500 publications related to dermatology.

**Michael Olson, Ph.D., A.T.S.**

Dr. Olson received a Ph.D. in Toxicology from the University of Arkansas for Medical Sciences, with dissertation research conducted at the U.S. Food and Drug Administration National Center for Toxicological Research. Following graduate training, he served as NIEHS National Research Service Award Postdoctoral Fellow in the Department of Pharmacology, School of Medicine - University of North Carolina. Currently he is Director, Occupational Toxicology, Corporate Environment Health and Safety for GlaxoSmithKline. Dr. Olson is a Fellow of the Academy of Toxicological Sciences (A.T.S.). His research interests include mechanisms of chemically-induced toxicity; genetic toxicity; xenobiotic metabolism; alternative methods in toxicology; hazard evaluation, risk assessment, and communication. Dr. Olson has authored a number of peer-reviewed manuscripts and book chapters in these areas as well as preparing many occupational health effects reviews for pharmaceutical active ingredients, isolated intermediates, and associated chemicals. He has served as an editorial board member and *ad hoc* referee for numerous toxicology and biosciences journals. In addition, he has worked as a Visiting Scientist, EPA, as well as advisor to EPA Risk Assessment Forum, U.S. National Institutes of Health (NIH) (Toxicology Study Section I), U.S. Air Force, Transportation Research Board, and the National Research Council - National Academy of Sciences (NAS). A member of several biomedical professional societies, Dr. Olson has served in elective and appointed positions in

the Society of Toxicology, including Chairman of the Society of Toxicology (SOT) Occupational Health Specialty Section.

**Raymond Pieters, Ph.D.**

Dr. Pieters received a Ph.D. at Utrecht University and is currently an Associate Professor at the Institute for Risk Assessment Sciences, and Group Leader for Immunotoxicology at that institution. In 2007, he presented a paper on Development of Strategies to Assess Drug Hypersensitivity at the Congress of the European Societies of Toxicology. He was involved in the development of the Reporter Antigen Popliteal Lymph Node Assay, an assay to assess the immunomodulating potential of chemicals, which enables differentiation between immunosensitizing chemicals (sensitizers), immunostimulating chemicals (irritants), and chemicals that have no apparent immunological effects. He has published over 70 papers on sensitization and other subjects in immunotoxicology in peer-reviewed journals, including a review article, *Murine Models of Drug Hypersensitivity*, in 2005.

**Jean Regal, Ph.D.**

Dr. Regal received a Ph.D. in Pharmacology from the University of Minnesota. She is currently a Professor in the Department of Pharmacology, Department of Biochemistry and Molecular Biology, University of Minnesota Medical School, Duluth. Her current research is focused on respiratory allergy, especially asthma. She has served on multiple NIH review panels regarding asthma, as an immunotoxicologist in 2000 for an Institute of Medicine Committee on Health Effects Associated with Exposures Experienced during the Persian Gulf War, as well as on the 1998 and 2008 ICCVAM Peer Panel that evaluated the LLNA. In 2007 she served as an ad hoc reviewer for the NTP Board of Scientific Counselors for two nominations: Artificial Butter Flavoring Mixture & O-phthalaldehyde, at NIEHS. She is currently President of the Immunotoxicology Specialty Section of SOT and Associate Editor of the Journal of Immunotoxicology. Dr. Regal has authored over 50 research articles and reviews in peer-reviewed journals.

**Jonathan Richmond, B.Sc. (Hons) Med.Sci., MB ChB, FRCSEd, FRMS**

Dr. Richmond received a Bachelor of Science in Medical Science with Honors (BSc [Hons] Med.Sci.) and Bachelor of Medicine and Bachelor of Surgery (MB ChB) degrees with Distinction in Medicine and Therapeutics from Edinburgh University. Presently, he is head of the Animals Scientific Procedures Division at the Home Office. He is a Fellow of the Royal College of Surgeons of Edinburgh (FRCSEd) and a former Fellow of the Royal Society of Medicine (FRMS). Other appointments include convener of the U.K. Interdepartmental Group on the 3Rs, convener of the International Standards Organization Technical Corrigendum 194/Working Group 3 (*Biocompatibility of Medical Device*

*Materials*), and member of related expert working groups. He is a former member of the European Union (E.U.) Committee on Scientific and Technical Progress and past Chairman of the European Commission Technical Expert Working Group on ethical review, and former board member of the U.K. National Centre for the 3Rs. He served as chair of the peer review panel for the reduced LLNA test protocol and prediction model for ESAC in 2007 and has been designated as an ESAC peer reviewer for ECVAM's performance standards for the standard LLNA. He served on the NICEATM-ICCVAM Expert Panel that evaluated Five *In Vitro* Pyrogen Test Methods, and developed performance standards for minor variations on the test method. He has a variety of publications in peer-reviewed journals and national and international meetings, on the principles and practice of surgery, regulation of biomedical research, principles of humane research, bioethics, and public policy.

**Peter Theran, V.M.D.**

Dr. Theran holds a Doctor of Veterinary Medicine degree from the University of Pennsylvania. He has had many years of experience both as a veterinary internal medicine specialist at the Massachusetts Society for the Prevention of Cruelty to Animals' Angell Memorial Animal Hospital in Boston, and as the director of Boston University Medical Center's Laboratory Animal Science Center. He has served on NIH and NAS committees as an animal welfare member, and is a member of the Board of Directors of the Institute for *In Vitro* Sciences in Gaithersburg, MD, and Chimp Haven in Shreveport, LA. He served on the NICEATM-ICCVAM Expert Panels that evaluated the *In Vitro* Test Methods for Identifying Ocular Corrosives and Severe Irritants, LLNA and *In Vitro* Pyrogen Test Methods. He is a former member of SACATM. He is presently working as an animal welfare consultant.

**Stephen Ullrich, Ph.D.**

Dr. Ullrich received a Ph.D. in Microbiology from Georgetown University. He is currently the Dallas/Fort Worth Living Legends Professor and Professor of Immunology at the University of Texas, M.D. Anderson Cancer Center, where he is also Associate Director, The Center for Cancer Immunology Research. He is also a member of the Animal Research Strategic Advisory Committee. He has served numerous national review committees and panels, including the 1998 ICCVAM Peer Panel that evaluated the Murine LLNA. Dr. Ullrich has authored over 75 peer-reviewed publications, over 30 invited articles, and he holds four patents in the U.S., E.U., and Australia for a UV-induced Immunosuppressive Substance. He is the past President of the American Society for Photobiology.

**Michael Woolhiser, Ph.D.**

Dr. Woolhiser received a Ph.D. in Pharmacology and Toxicology from the Medical College of Virginia at Virginia Commonwealth University. He is a specialist in immunotoxicology

and is currently a toxicologist for the Dow Chemical Company, where he serves as a Technical Leader for Immunotoxicology and Polyurethane Business Toxicology Consultant. Dr. Woolhiser is also an Adjunct Assistant Professor at the Center for Integrative Toxicology, Michigan State University. He has served on numerous working groups, including an LLNA Expert Working Group under the European Crop Protection Agency's Toxicology Expert Group, a European Centre for Ecotoxicology and Toxicology of Chemicals LLNA Task Force. He has authored 32 peer-reviewed publications.

**Takahiko Yoshida, M.D., Ph.D.**

Dr. Yoshida earned his M.D. and a Ph.D. in Medical Science from Tokai University. He is currently Professor in the Department of Health Science at Asahikawa Medical College. Prior to this appointment, he held the posts of Instructor, Assistant Professor, and Associate Professor at the Tokai University School of Medicine. He has also been a Guest Researcher at NIEHS. He has also worked as an occupational physician for major Japanese corporations, including Toyota and Sony. Dr. Yoshida's research interests include occupational health, public health, environmental health, and preventative medicine. He is a member of the International Congress of Occupational Health, the Japanese Society of Hygiene, the Japanese Society of Immunotoxicology, the Japanese Society of Clinical Ecology, and the SOT.

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## Appendix F

### *Federal Register* Notices and Public Comments

F1	<i>Federal Register</i> Notices .....	F-3
F2	Public Comments Received in Response to <i>Federal Register</i> Notices .....	F-23
F3	Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments: SACATM Meeting on June 18-19, 2008 .....	F-107
F4	Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments: SACATM Meeting on June 25-26, 2009 .....	F-121

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## Appendix F1

### *Federal Register* Notices

72 FR 27815 (May 17, 2007) The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data .....	F-5
72 FR 52130 (September 12, 2007) Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments .....	F-8
73 FR 1360 (January 8, 2008) Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments .....	F-10
73 FR 25754 (May 7, 2008) Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) .....	F-13
73 FR 29136 (May 20, 2008) Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments.....	F-15
74 FR 8974 (February 27, 2009) Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments.....	F-17
74 FR 19562 (April 29, 2009) Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) .....	F-19
74 FR 26242 (June 1, 2009) Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for	

Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of  
Availability and Request for Public Comments ..... F-21

(NIEHS), National Institutes of Health (NIH).

**ACTION:** Request for comments, submission of relevant data, and nominations of scientific experts.

**SUMMARY:** The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the “cut-down” or “limit dose” LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated). ICCVAM reviewed the nomination, assigned it a high priority, and proposed that NICEATM and ICCVAM carry out the following activities in its evaluation: (1) Initiate a review of the current literature and available data, including the preparation of a comprehensive background review document, and (2) convene a peer review panel to review the various proposed LLNA uses and procedures for which sufficient data and information are available to adequately assess their validation status. ICCVAM also recommends development of performance standards for the LLNA. At this time, NICEATM requests: (1) Public comments on the appropriateness and relative priority of these activities, (2) nominations of expert scientists to consider as members of a possible peer review panel, and (3) submission of data for the LLNA and/or modified versions of the LLNA.

**DATES:** Submit comments, data, and nominations by June 15, 2007. Relevant data will also be accepted after this date and considered when feasible.

**ADDRESSES:** Dr. William S. Stokes, NICEATM Director, NIEHS, P.O. Box 12233, MD EC-17, Research Triangle Park, NC 27709, (fax) 919-541-0947, (e-mail) [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov). Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709. Responses can be submitted electronically at the ICCVAM-NICEATM Web site: [http://iccvam.niehs.nih.gov/contact/FR\\_pubcomment.htm](http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm) or by e-mail, mail, or fax.

**FOR FURTHER INFORMATION CONTACT:** Other correspondence should be

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**DEPARTMENT OF HEALTH AND HUMAN SERVICES**

**National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); the Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data**

**AGENCY:** National Institute of Environmental Health Sciences

directed to Dr. William S. Stokes (919-541-2384 or [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov)).

**SUPPLEMENTARY INFORMATION:**

**Background**

ICCVAM previously evaluated the validation status of the LLNA as a stand-alone alternative method to the Guinea Pig Maximization Test (GPMT) and the Buehler Assay (NIH publication No. 99-4494; available at <http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm>). Based on this evaluation, ICCVAM recommended the LLNA as a valid substitute for the guinea pig methods for most testing situations. The Environmental Protection Agency, Food and Drug Administration, and the CPSC subsequently accepted the method as a valid substitute. The OECD also adopted the LLNA as OECD Test Guideline 429.

In January 2007, the CPSC submitted a nomination to NICEATM (<http://iccvam.niehs.nih.gov/SuppDocs/submission.htm>) requesting that ICCVAM assess the validation status of:

- The LLNA as a stand-alone test for potency determinations (including severity) for the purpose of hazard classification.
- LLNA protocols that do not require the use of radioactive materials.
- The LLNA “cut-down” or “limit dose” procedure.
- The ability of the LLNA to test mixtures, aqueous solutions, and metals.
- The current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been determined to be useful).

Since 2003, ICCVAM has routinely developed performance standards for test methods; however, they were not developed for the LLNA, which was reviewed in 1999. Accordingly, ICCVAM proposes to now develop performance standards for the LLNA. Performance standards communicate the basis by which new proprietary and nonproprietary test methods have been determined to have sufficient relevance and reliability for specific testing purposes. Performance standards based on test methods accepted by regulatory agencies can be used to evaluate the reliability and relevance of other test methods that are based on similar scientific principles and measure or predict the same biological or toxic effect. On January 24, 2007, ICCVAM unanimously endorsed with a high priority: (1) Developing performance standards for the LLNA and (2) initiating a review of the available data and information associated with the CPSC nominated activities. A determination of which (if any) of the

nominated activities will move forward will be made subsequent to this review and after consideration of comments by the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). If a decision is made to proceed with evaluation of these test methods, ICCVAM and NICEATM propose convening a peer review panel to review the usefulness and limitations of each of the LLNA methods listed above. The panel would also formulate conclusions on the adequacy of draft ICCVAM performance standards, any proposed future validation studies, and draft ICCVAM-proposed standardized test method protocols.

**Request for Public Comments and Nominations of Scientific Experts**

NICEATM requests public comments on the appropriateness and relative priority of the nominated activities. NICEATM also requests the nominations of scientists with relevant knowledge and experience to serve on the panel if a panel meeting occurs. Areas of relevant expertise include, but are not limited to: physiology, pharmacology, immunology, skin sensitization testing in animals, development and use of in vitro methodologies, biostatistics, knowledge about the use of chemical datasets for validation of toxicity studies, and hazard classification of chemicals and products. Each nomination should include the person’s name, affiliation, contact information (i.e., mailing address, e-mail address, telephone and fax numbers), curriculum vitae, and a brief summary of relevant experience and qualifications.

**Request for Data**

NICEATM invites the submission of data from standard LLNA testing (i.e., OECD TG 429) with mixtures, aqueous solutions, and/or metals, as well as corresponding data from human and other animal studies. In addition, NICEATM invites the submission of data supporting the use of (1) the LLNA as a stand-alone test for determining potency (including severity) for the purpose of hazard classification, (2) the LLNA “cut-down” or “limit dose” procedure, and (3) LLNA protocols that do not require the use of radioactivity. Although data can be accepted at any time, data submitted by June 15, 2007, will be considered during the ICCVAM evaluation process. Submitted data will be used to further evaluate the usefulness and limitations of the LLNA and may be incorporated into future NICEATM and ICCVAM reports and publications as appropriate. The data

will also be included in a database to support the investigation of other test methods for assessing skin sensitization.

When submitting chemical and protocol information/test data, please reference this **Federal Register** notice and provide appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, as applicable).

NICEATM prefers data to be submitted as copies of pages from study notebooks and/or study reports, if available. Raw data and analyses available in electronic format may also be submitted. Each submission for a chemical should preferably include the following information, as appropriate:

- Common and trade name.
- Chemical Abstracts Service Registry Number (CASRN).
- Chemical class.
- Product class.
- Commercial source.
- LLNA protocol used.
- Individual animal responses.
- The extent to which the study complied with national or international Good Laboratory Practice (GLP) guidelines.
- Date and testing organization.
- Sensitization data from other test methods.

**Consideration by SACATM**

On June 12, 2007, SACATM will meet at the Marriott Bethesda North Hotel and Conference Center in Bethesda, Maryland. The agenda includes consideration of the nominated LLNA activities, priorities, and proposed activities <http://ntp.niehs.nih.gov/go/7441>) and an opportunity for oral public comments. The SACATM meeting was announced in a separate **Federal Register** notice (**Federal Register** Vol. 72, No. 83, pp. 23831–32, May 1, 2007).

**Background Information on ICCVAM and NICEATM**

ICCVAM is an interagency committee composed of representatives from 15 federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285l–3, available at <http://iccvam.niehs.nih.gov/about/PL106545.htm>) establishes ICCVAM as a permanent interagency committee of the

NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of federal agencies. Additional information about ICCVAM and NICEATM is available on the following Web site: <http://iccvam.niehs.nih.gov>.

Dated: May 8, 2007.

**David A. Schwartz,**  
*Director, National Institute of Environmental Health Sciences and National Toxicology Program.*

[FR Doc. E7-9544 Filed 5-16-07; 8:45 am]

**BILLING CODE 4140-01-P**

**DEPARTMENT OF HEALTH AND HUMAN SERVICES**

**National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments**

**AGENCY:** National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

**ACTION:** Request for comments.

**SUMMARY:** The Murine Local Lymph Node Assay (LLNA) is the first alternative test method evaluated and recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). It was subsequently accepted by regulatory authorities to determine the allergic contact dermatitis potential of chemicals and products. In January 2007, the U.S. Consumer Product Safety Commission (CSPC) submitted a nomination requesting that NICEATM and ICCVAM assess the validation status of (1) The LLNA as a stand-alone assay for potency determination for hazard classification purposes; (2) modified LLNA protocols; (3) the LLNA limit test; (4) the use of LLNA to test mixtures, aqueous solutions, and metals; and (5) the applicability domain for LLNA. In order to facilitate the review of the modified LLNA protocols, ICCVAM proposed developing performance standards for the LLNA. In May 2007, a **Federal Register** notice was published (Vol. 72, No. 95, pages 27815–27817, May 17, 2007) requesting comments and data relevant to these nominated activities. In June 2007, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed the nominated activities as high priorities for ICCVAM. In response to SACATM comments, along with those provided by the public in response to the previous **Federal Register** notice, ICCVAM also endorsed these activities as high priorities. ICCVAM subsequently prepared draft performance standards for the LLNA and now requests public comments on this draft document, which is available on the NICEATM/ICCVAM Web site at: (<http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm>) or by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** below).

**DATES:** Submit comments on or before October 29, 2007.

**ADDRESSES:** Dr. William S. Stokes, NICEATM Director, NIEHS, P.O. Box

12233, MD EC-17, Research Triangle Park, NC 27709, (fax) 919-541-0947, (e-mail) [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov). Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709. Responses can be submitted electronically at the ICCVAM-NICEATM Web site: [http://iccvam.niehs.nih.gov/contact/FR\\_pubcomment.htm](http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm) or by e-mail, mail, or fax.

**FOR FURTHER INFORMATION CONTACT:** Other correspondence should be directed to Dr. William S. Stokes (919-541-2384 or [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov)).

**SUPPLEMENTARY INFORMATION:**

**Background**

The LLNA is an alternative test method used for skin sensitization testing that reduces the number of animals needed, reduces the time required for testing, and can substantially reduce or avoid pain and distress associated with traditional guinea pig testing methods. The LLNA was the first alternative test method evaluated and recommended by ICCVAM and based on the recommendations of ICCVAM and an independent scientific peer review panel, the LLNA has been accepted by U.S. and international regulatory authorities as an alternative to the guinea pig maximization test and Buehler test for assessing allergic contact dermatitis (EPA 2003; ISO 2002; OECD 2002). Since 2003, ICCVAM has routinely developed performance standards for test methods; however, because the concept of performance standards was not developed by ICCVAM until 2003, they were not developed during the ICCVAM evaluation of the LLNA in 1998 (NIH Publication No. 99-4494, available: ([http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/llnarep.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf))).

In January 2007, CSPC submitted a nomination requesting that NICEATM and ICCVAM assess the validation status of (1) The LLNA as a stand-alone assay for potency determination for classification purposes; (2) modified LLNA protocols; (3) the LLNA limit test; (4) the use of LLNA to test mixtures, aqueous solutions, and metals; and (5) the applicability domain for LLNA. ICCVAM endorsed the nomination and also decided to develop performance standards to facilitate evaluation of modified LLNA protocols to the traditional LLNA. In May 2007, a **Federal Register** notice was published requesting comments and data relevant to these activities (Vol. 72, No. 95, pages 27815–27817, May 17, 2007; available,

[http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_9544.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf)). In June 2007, SACATM endorsed these activities as high priorities for ICCVAM. In response to SACATM comments, along with those provided by the public in response to the previous **Federal Register** notice, ICCVAM endorsed these activities, including the development of performance standards, as high priorities. ICCVAM subsequently prepared draft performance standards for the LLNA, which are available on the NICEATM/ICCVAM Web site at: (<http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm>).

These draft test method performance standards are proposed to evaluate the performance of LLNA test methods that incorporate specific modifications to the measurement of lymphocyte proliferation in the traditional LLNA. These modifications focus specifically on incorporating non-radioactive procedures to evaluate lymphocyte proliferation in the draining auricular lymph nodes rather than incorporation of radioactivity (i.e., <sup>3</sup>H-thymidine), which is used in the traditional LLNA.

Public comments received in response to the draft LLNA performance standards will be considered by ICCVAM during development of a revised draft version of this document. A public meeting is planned for early 2008 where an international, independent, peer review panel will evaluate the revised draft LLNA performance standards and review the other nominated LLNA related activities. Following this meeting, the recommendations of the peer review panel will be made available for public and SACATM comment. ICCVAM will consider the panel report and public and SACATM comments in preparing final LLNA performance standards.

#### **Request for Public Comments**

NICEATM invites the submission of written comments on the draft LLNA performance standards. When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). All comments received by the deadline listed above will be placed on the NICEATM/ICCVAM Web site (<http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm>) and made available to the peer review panel and ICCVAM.

#### **Background Information on ICCVAM and NICEATM**

ICCVAM is an interagency committee composed of representatives from 15 federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285l-3, available at <http://iccvam.niehs.nih.gov/about/PL106545.htm>) establishes ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of federal agencies. Additional information about ICCVAM and NICEATM is available on the following Web site: <http://iccvam.niehs.nih.gov>.

Dated: September 5, 2007.

#### **Samuel H. Wilson,**

*Acting Director, National Institute of Environmental Health Sciences and National Toxicology Program.*

[FR Doc. E7-18011 Filed 9-11-07; 8:45 am]

**BILLING CODE 4140-01-P**

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**DEPARTMENT OF HEALTH AND  
HUMAN SERVICES**

**National Institutes of Health**

**National Toxicology Program (NTP);  
NTP Interagency Center for the  
Evaluation of Alternative Toxicological  
Methods (NICEATM); Announcement  
of an Independent Scientific Peer  
Review Panel Meeting on the Murine  
Local Lymph Node Assay; Availability  
of Draft Background Review  
Documents; Request for Comments**

**AGENCY:** National Institute of  
Environmental Health Sciences  
(NIEHS), National Institutes of Health  
(NIH).

**ACTION:** Meeting announcement and  
request for comments.

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**SUMMARY:** NICEATM in collaboration  
with the Interagency Coordinating  
Committee on the Validation of  
Alternative Methods (ICCVAM)  
announces an independent scientific  
peer review panel meeting to evaluate  
modifications and new applications for  
the Murine Local Lymph Node Assay  
(LLNA). The LLNA is an alternative test  
method that can be used to determine  
the allergic contact dermatitis potential  
of chemicals and products. The panel  
will review the following:

- The validation status of three  
modified LLNA test method protocols  
that use non-radioactive probe  
chemicals.
- The validation status of a LLNA  
limit dose procedure.
- The use of the LLNA to test  
mixtures, aqueous solutions, and metals  
(applicability domain for the LLNA).
- The use of the LLNA to determine  
potency (potential for causing allergic  
contact dermatitis).
- Revised draft recommended  
performance standards for the LLNA.

At this meeting, the panel will peer  
review the draft background review  
documents and revised draft LLNA  
performance standards for each topic  
and evaluate the extent that established  
validation and acceptance criteria have  
been appropriately addressed. The  
panel will also comment on the extent

that the review documents support draft ICCVAM recommendations on proposed test method protocols, proposed uses of the LLNA, and the revised draft LLNA performance standards.

NICEATM invites public comments on the draft background review documents, draft ICCVAM test recommendations, draft test method protocols, and revised draft LLNA performance standards. All documents will be available on the NICEATM–ICCVAM Web site at <http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm> by January 8, 2008.

**DATES:** The meeting is scheduled for March 4–6, 2008, from 8:30 a.m. to 5 p.m. each day. The meeting is open to the public free of charge, with attendance limited only by the space available. In order to facilitate planning for this meeting, persons wishing to attend are asked to register by February 20, 2008, via the NICEATM–ICCVAM Web site ([http://iccvam.niehs.nih.gov/contact/reg\\_LLNAPanel.htm](http://iccvam.niehs.nih.gov/contact/reg_LLNAPanel.htm)). The deadline for written comments is February 22, 2008.

**ADDRESSES:** The meeting will be held at the U.S. Consumer Product Safety Commission (CPSC) Headquarters, Bethesda Towers Bldg., 4330 East West Highway, Bethesda, MD.

**FOR FURTHER INFORMATION CONTACT:** Comments may also be submitted via the NICEATM–ICCVAM Web site at [http://iccvam.niehs.nih.gov/contact/FR\\_publiccomment.htm](http://iccvam.niehs.nih.gov/contact/FR_publiccomment.htm). Comments or other correspondence can be sent to Dr. William S. Stokes, NICEATM Director, NIEHS, P.O. Box 12233, MD EC-17, Research Triangle Park, NC, 27709, (phone) 919-541-2384, (fax) 919-541-0947, (e-mail) [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov). Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709.

#### SUPPLEMENTARY INFORMATION:

##### Background

The LLNA is a reduction and refinement alternative test method for skin sensitization testing because it reduces the number of animals needed and can substantially reduce or avoid pain and distress compared to traditional guinea pig testing methods for sensitization. The LLNA was the first alternative test method evaluated and recommended by ICCVAM (NIH Publication No. 99-4494, available at: [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/llnarep.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf)). Based on the recommendations of ICCVAM and an independent scientific peer review panel, U.S. and international regulatory authorities have

accepted the LLNA as an alternative to the guinea pig maximization test and Buehler test for assessing allergic contact dermatitis (ISO 2002; OECD 2002; EPA 2003). This review will evaluate the potential for broader use of the LLNA for regulatory testing of chemicals and products for allergic contact dermatitis potential, enabling further reduction and refinement (less pain and suffering) of animal use for this purpose. In January 2007, the CPSC submitted a nomination requesting that NICEATM and ICCVAM assess the validation status of (1) the LLNA as a stand-alone assay for potency determination for hazard classification purposes; (2) modified LLNA protocols; (3) the LLNA limit test; (4) the use of the LLNA to test mixtures, aqueous solutions, and metals; and (5) the applicability domain for the LLNA. In June 2007, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed these activities as high priorities for ICCVAM. NICEATM on behalf of ICCVAM also sought input from the public on these activities (**Federal Register**: Vol. 72, No. 95, pages 27815–27817, May 17, 2007). After considering these inputs, ICCVAM endorsed these activities as high priorities. ICCVAM is also developing performance standards to facilitate evaluation of modified LLNA protocols compared to the traditional LLNA. Although ICCVAM has routinely developed performance standards for test methods since 2003, they were not developed as part of the ICCVAM evaluation of the LLNA in 1998. These draft performance standards for the LLNA were made public and comments were requested via the **Federal Register** (Vol. 72, No. 176, pages 52130–52131, Sept. 12, 2007). The May 2007 **Federal Register** notice requested data from studies using the LLNA or modified versions of the LLNA.

Drawing on the submitted data and literature sources, ICCVAM and NICEATM drafted background review documents for each of the modifications and new applications of the LLNA. ICCVAM has also developed draft test method recommendations regarding the proposed usefulness, limitations, and validation status of these test methods. ICCVAM will convene an independent scientific panel to peer review the draft background review documents for the test methods and determine whether the data and analyses in the draft documents support the draft ICCVAM test method recommendations. The panel will also be asked to comment on the adequacy of the revised draft performance standards, proposed future

studies, draft standardized test method protocols, and recommended reference substances. NICEATM will ask the panel to consider all available information, including the scientific studies cited in the draft review documents, public comments, and any new information identified during the peer review, for developing their conclusions and recommendations.

#### Peer Review Panel Meeting

The purpose of this meeting is to conduct a scientific peer review of the revised draft performance standards and an evaluation of modifications and new applications for the LLNA. The LLNA is an alternative test method that can be used to determine the allergic contact dermatitis potential of chemicals and products. The panel will review the following:

- The LLNA as a stand-alone assay for potency determination for hazard classification purposes
- Modified LLNA protocols
- The LLNA limit test
- The use of the LLNA to test mixtures, aqueous solutions, and metals (applicability domain for the LLNA)
- The use of the LLNA to determine potency (potential for causing allergic contact dermatitis).

The panel will consider the draft background review documents for each of these methods and evaluate the extent that established validation and acceptance criteria are appropriately addressed for each test method (as described in the ICCVAM document, *Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods*, NIH Publication No. 97-981, available at [http://iccvam.niehs.nih.gov/docs/about\\_docs/validate.pdf](http://iccvam.niehs.nih.gov/docs/about_docs/validate.pdf)). The panel will then comment on the extent to which the draft ICCVAM recommendations are supported by the information provided in the background review document for each topic. It is anticipated that the panel will address the topics in the following order:

1. The LLNA limit test.
2. The applicability domain of the LLNA including its suitability for mixtures, aqueous solutions, and metals.
3. The LLNA as a stand-alone assay for potency determination for hazard classification.
4. The revised draft performance standards for the LLNA.
5. The modified LLNA test method protocols using non-radioactive materials.

Additional information about the meeting, including a roster of the panel members and the draft agenda, will be made available two weeks prior to the meeting on the NICEATM-ICCVAM Web site (<http://iccvam.niehs.nih.gov>). This information will also be available after that date by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

**Attendance and Registration**

This public meeting will take place March 4–6, 2008, at the CPSC Headquarters, Bethesda Towers Bldg., 4330 East West Highway, Bethesda, MD (an area map, driving directions, and CPSC contact information are available at <http://www.cpsc.gov/about/contact.html>). The meeting will begin at 8:30 a.m. and is scheduled to conclude at approximately 5 p.m. each day, although adjournment on March 6 may occur earlier or later depending upon the time needed for the expert panel to complete its work. It is also possible that the panel may conclude its deliberations on March 5 and not need to meet on March 6. Persons needing special assistance in order to attend, such as sign language interpretation or other reasonable accommodation, should contact 919–541–2475 (voice), 919–541–4644 TTY (text telephone, through the Federal TTY Relay System at 800–877–8339), or e-mail [niehsoeeo@niehs.nih.gov](mailto:niehsoeeo@niehs.nih.gov). Requests should be made at least seven days in advance of the event.

**Availability of the Draft Background Review Documents and Draft ICCVAM Recommendations**

NICEATM prepared draft background review documents on each of these modifications or applications of the LLNA that describe the current validation status of the modified test methods and applications and contain all of the data and analyses supporting this proposed validation status. The draft background review documents, draft ICCVAM test method recommendations, draft test method protocols, and revised draft test method performance standards are available from the NICEATM-ICCVAM Web site (<http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm>) or by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

**Request for Public Comments**

NICEATM invites the submission of written comments on the draft background review documents, draft ICCVAM test method recommendations, draft test method protocols, and revised draft test method performance

standards. Written comments should be submitted preferably electronically via the NICEATM-ICCVAM Web site or by e-mail ([niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov)); the deadline for submission of written comments is February 22, 2008. When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). Written comments may also be sent by mail, fax, or e-mail to Dr. William Stokes (see **FOR FURTHER INFORMATION CONTACT** above). All comments received will be placed on the NICEATM-ICCVAM Web site (<http://iccvam.niehs.nih.gov>) and identified by the individual's name and affiliation or sponsoring organization (if applicable). Comments will also be sent to the panel and ICCVAM agency representatives and made available at the meeting.

This meeting is open to the public, and time will be provided for the presentation of oral comments by the public at designated times during the peer review. Members of the public who wish to present oral statements at the meeting should contact NICEATM (see **FOR FURTHER INFORMATION CONTACT** above) no later than February 20, 2008, and provide contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). Up to seven minutes will be allotted per speaker, one speaker per organization. Persons registering to make comments are asked to provide NICEATM a written copy of their statement by February 27, 2008, so that copies can be distributed to the panel prior to the meeting. If this is not possible, please bring 40 copies of your comments to the meeting for distribution and to supplement the record. Written statements can supplement and expand the oral presentation.

Summary minutes and the panel's final report will be available following the meeting on the NICEATM-ICCVAM Web site (<http://iccvam.niehs.nih.gov>). ICCVAM will consider the panel's conclusions and recommendations and any public comments received when finalizing their test method recommendations and performance standards for these methods.

**Background Information on ICCVAM and NICEATM**

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use or generate toxicological information. ICCVAM conducts technical evaluations of new, revised,

and alternative methods with regulatory applicability, and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, or replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 2851–3, available at [http://iccvam.niehs.nih.gov/docs/about\\_docs/PL106545.pdf](http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf)) establishes ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of Federal agencies. Additional information about ICCVAM and NICEATM is available on the NICEATM-ICCVAM Web site at <http://iccvam.niehs.nih.gov>.

**References**

EPA. 2003. EPA OPPTS 870.2600 Test Guideline—Skin Sensitization. Available: [http://www.epa.gov/opptsfrs/publications/OPPTS\\_Harmonized/870\\_Health\\_Effects\\_Test\\_Guidelines/Drafts/870-2600.pdf](http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Drafts/870-2600.pdf).  
 ISO. 2002. ISO 10993–10 Biological evaluation of medical devices—Part 10: Tests for irritation and delayed-type hypersensitivity. Geneva: International Organization for Standardization.  
 OECD. 2002. OECD Guideline for the Testing of Chemicals—Test Guideline 429: Skin Sensitization: Local Lymph Node Assay (adopted 24 April 2002). Paris: Organisation for Economic Co-operation and Development.

Dated: December 19, 2007.

**Samuel H. Wilson,**  
*Acting Director, National Institute of Environmental Health Sciences and National Toxicology Program.*

[FR Doc. E7–25553 Filed 1–7–08; 2:42 pm]

**BILLING CODE 4140-01-P**

25754

Federal Register / Vol. 73, No. 89 / Wednesday, May 7, 2008 / Notices

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**DEPARTMENT OF HEALTH AND  
HUMAN SERVICES**

**National Toxicology Program (NTP);  
Office of Liaison, Policy and Review;  
Meeting of the Scientific Advisory  
Committee on Alternative  
Toxicological Methods (SACATM)**

**AGENCY:** National Institute of  
Environmental Health Sciences  
(NIEHS), National Institutes of Health  
(NIH).

**ACTION:** \*COM057\* Meeting  
announcement and request for  
comment.

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**SUMMARY:** Pursuant to section 10(a) of  
the Federal Advisory Committee Act, as  
amended (5 U.S.C. Appendix 2), notice  
is hereby given of a meeting of  
SACATM on June 18–19, 2008, at the

Radisson Hotel Research Triangle Park, 150 Park Drive, Research Triangle Park, NC 27709. The meeting is scheduled from 8:30 a.m. to 5:30 p.m. on June 18 and 8:30 a.m. until adjournment on June 19. The meeting is open to the public with attendance limited only by the space available. SACATM advises the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Director of the NIEHS and NTP regarding statutorily mandated duties of ICCVAM and activities of NICEATM.

**DATES:** The SACATM meeting will be held on June 18 and 19, 2008. All individuals who plan to attend are encouraged to register online at the NTP Web site (<http://ntp.niehs.nih.gov/go/7441>) by June 10, 2008. In order to facilitate planning, persons wishing to make an oral presentation are asked to notify Dr. Lori White, NTP Executive Secretary, via online registration, phone, or email by June 10, 2008 (see **ADDRESSES** below). Written comments should also be received by June 10 to enable review by SACATM and NIEHS/NTP staff before the meeting.

**ADDRESSES:** The SACATM meeting will be held at the Radisson Hotel Research Triangle Park, 150 Park Drive, Research Triangle Park, NC 27709 [hotel: (919) 549-8631]. Public comments and other correspondence should be directed to Dr. Lori White (NTP Office of Liaison, Policy and Review, NIEHS, P.O. Box 12233, MD A3-01, Research Triangle Park, NC 27709; telephone: 919-541-9834 or e-mail: [whiteltd@niehs.nih.gov](mailto:whiteltd@niehs.nih.gov)). Courier address: NIEHS, 111 T.W. Alexander Drive, Room A326, Research Triangle Park, NC 27709. Persons needing interpreting services in order to attend should contact 301-402-8180 (voice) or 301-435-1908 (TTY). Requests should be made at least 7 days in advance of the meeting.

**SUPPLEMENTARY INFORMATION:**

**Preliminary Agenda Topics and Availability of Meeting Materials**

Preliminary agenda topics include:

- NICEATM-ICCVAM Update;
- Overview of NICEATM-ICCVAM 5-Year Plan;
- NRC Report: Toxicity Testing in the 21st Century;
- Presentations from Federal Agencies on Research, Development, Translation, and Validation Activities Relevant to the NICEATM-ICCVAM Five-Year Plan;
- Report on the ICCVAM-NICEATM Independent Scientific Peer Review Meeting: Validation Status of New

Versions and Applications of the Murine Local Lymph Node Assay (LLNA), a Test Method for Assessing the Contact Dermatitis Potential of Chemicals and Products;

- Report on the ICCVAM-NICEATM-ECVAM-JACVAM Scientific Workshop on Acute Chemical Safety Testing: Advancing In Vitro Approaches and Humane Endpoints for Systemic Toxicity Evaluations;
- Nominations to ICCVAM: NTP Rodent Bioassay for Carcinogenicity;
- Proposal for International Cooperation on Alternative Test Methods;
- Update from the Japanese Center for the Validation of Alternative Methods;
- Update from the European Center for the Evaluation of Alternative Methods,

A copy of the preliminary agenda, committee roster, and additional information, when available will be posted on the NTP Web site (<http://ntp.niehs.nih.gov/go/7441>) or available upon request (see **ADDRESSES** above). Following the SACATM meeting, summary minutes will be prepared and available on the NTP website or upon request.

**Request for Comments**

Both written and oral public input on the agenda topics is invited. Written comments received in response to this notice will be posted on the NTP Web site. Persons submitting written comments should include their name, affiliation (if applicable), and sponsoring organization (if any) with the document. Time is allotted during the meeting for presentation of oral comments and each organization is allowed one time slot per public comment period. At least 7 minutes will be allotted for each speaker, and if time permits, may be extended up to 10 minutes at the discretion of the chair. Registration for oral comments will also be available on-site, although time allowed for presentation by on-site registrants may be less than for pre-registered speakers and will be determined by the number of persons who register at the meeting.

Persons registering to make oral comments are asked to do so through the online registration form (<http://ntp.niehs.nih.gov/go/7441>) and to send a copy of their statement to Dr. White (see **ADDRESSES** above) by June 10 to enable review by SACATM, NICEATM-ICCVAM, and NIEHS/NTP staff prior to the meeting. Written statements can supplement and may expand the oral presentation. If registering on-site and reading from written text, please bring 40 copies of the statement for

distribution and to supplement the record.

**Background Information on ICCVAM, NICEATM, and SACATM**

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the development, scientific validation, regulatory acceptance, implementation, and national and international harmonization of new, revised, and alternative toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 [42 U.S.C. 2851-3] established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found on their Web site (<http://iccvam.niehs.nih.gov>).

SACATM was established in response to the ICCVAM Authorization Act [Section 2851-3(d)] and is composed of scientists from the public and private sectors. SACATM advises ICCVAM, NICEATM, and the Director of the NIEHS and NTP regarding statutorily mandated duties of ICCVAM and activities of NICEATM. SACATM provides advice on priorities and activities related to the development, validation, scientific review, regulatory acceptance, implementation, and national and international harmonization of new, revised, and alternative toxicological test methods. Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at <http://ntp.niehs.nih.gov/go/167>.

Dated: April 28, 2008.

**Samuel H. Wilson,**

*Acting Director, National Institute of Environmental Health Sciences and National Toxicology Program.*

[FR Doc. E8-10010 Filed 5-6-08; 8:45 am]

**BILLING CODE 4140-01-P**

**DEPARTMENT OF HEALTH AND HUMAN SERVICES****National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments**

**AGENCY:** National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

**ACTION:** Request for comments.

**SUMMARY:** NICEATM, in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), convened an independent international scientific peer review panel on March 4–6, 2008 to evaluate new versions and applications of the LLNA for assessing the allergic contact dermatitis potential of chemicals and products. The peer review panel (“the Panel”) report from this meeting is now available. The report contains (1) the Panel’s evaluation of the validation status of the methods and (2) the Panel’s comments and conclusions on draft ICCVAM test method recommendations. NICEATM invites public comment on the Panel’s report. The report is available on the NICEATM–ICCVAM Web site at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm) or by contacting NICEATM at the address given below.

**DATES:** Written comments on the Panel report should be received by July 7, 2008.

**ADDRESSES:** Comments should be submitted preferably electronically via the NICEATM–ICCVAM Web site at [http://iccvam.niehs.nih.gov/contact/FR\\_pubcomment.htm](http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm). Comments can also be submitted by e-mail to [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov). Written comments can be sent by mail or fax to Dr. William S. Stokes, Director, NICEATM, NIH/NIEHS, P.O. Box 12233, MD EC–17, Research Triangle Park, NC 27709, (phone) 919–541–2384, (fax) 919–541–0947. Courier address: NICEATM, 79 T.W. Alexander Drive, Building 4401, Room 3128, Research Triangle Park, NC 27709.

**FOR FURTHER INFORMATION CONTACT:** Dr. William S. Stokes, Director, NICEATM (919–541–2384 or [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov)).

**SUPPLEMENTARY INFORMATION:****Background**

In January 2007, the Consumer Product Safety Commission submitted a nomination to NICEATM and ICCVAM to assess the validation status of (1) The use of the LLNA to determine potency for hazard classification purposes; (2) LLNA protocols using non-radioactive procedures; (3) the LLNA limit dose procedure; and (4) the use of the LLNA to test mixtures, aqueous solutions, and metals (*i.e.*, an updated assessment of the applicability domain of the LLNA). In June 2007, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed these activities as high priorities for ICCVAM. NICEATM, on behalf of ICCVAM, also sought input from the public on these activities and requested data from studies using the LLNA or modified versions of the LLNA (**Federal Register** Vol. 72, No. 95, pages 27815–27817, May 17, 2007). After considering all comments received, ICCVAM endorsed carrying out these activities as high priorities. ICCVAM also developed draft LLNA performance standards to facilitate evaluation of modified LLNA protocols that are functionally and mechanistically similar to the traditional LLNA. These draft LLNA performance standards were made public and comments were requested via the **Federal Register** (Vol. 72, No. 176, pages 52130–52131, Sept. 12, 2007).

ICCVAM and NICEATM prepared draft background review documents (BRDs) that provided comprehensive reviews of available data and relevant information for each of the modifications and new applications of the LLNA. ICCVAM also developed draft test method recommendations regarding the proposed usefulness and limitations, standardized protocols, and future studies. Both the draft BRDs and draft recommendations were made available for public comment, and a public peer review meeting was announced in the **Federal Register** (Vol. 73, No. 5, pages 1360–1362, Jan. 8, 2008).

The Panel met in public session on March 4–6, 2008. The Panel reviewed the draft ICCVAM BRDs for completeness, errors, and omissions of any existing relevant data or information. The Panel evaluated the information in the BRDs to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM, 2003) had been appropriately addressed. The Panel then considered the ICCVAM draft test method

recommendations (*i.e.*, proposed test method uses, proposed recommended standardized protocol, proposed test method performance standards, and proposed additional studies) and commented on whether the recommendations were supported by the information provided in the draft BRDs.

The Panel's conclusions and recommendations are detailed in the *Peer Review Panel Final Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products* (available at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm)). The draft BRDs, draft ICCVAM test method recommendations, and the draft LLNA Performance Standards are available at <http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm>.

**Request for Comments**

NICEATM invites the submission of written comments on the Panel's report. When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). All comments received will be made publicly available on the NICEATM-ICCVAM Web site at <http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm>. In addition, there will be an opportunity for oral public comments on the Panel's report during an upcoming meeting of SACATM scheduled for June 18-19, 2008. Information concerning the SACATM meeting will be published in a separate **Federal Register** notice and available on the SACATM Web site at <http://ntp.niehs.nih.gov/go/7441>.

ICCVAM will consider the Panel report along with SACATM and public comments when finalizing test method recommendations. An ICCVAM test method evaluation report, which will include the final ICCVAM recommendations, will be forwarded to relevant Federal agencies for their consideration. The evaluation report will also be available to the public on the NICEATM-ICCVAM Web site and by request from NICEATM (see **ADDRESSES** above).

**Background Information on ICCVAM, NICEATM, and SACATM**

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate

toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes scientific validation, regulatory acceptance, and national and international harmonization of toxicological test methods that more accurately assess safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285I-3, available at [http://iccvam.niehs.nih.gov/docs/about\\_docs/PL106545.pdf](http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf)) established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of Federal agencies. Additional information about ICCVAM and NICEATM can be found at the NICEATM-ICCVAM Web site (<http://iccvam.niehs.nih.gov>).

Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at <http://ntp.niehs.nih.gov/go/167>.

**References**

ICCVAM, 2003, ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508, Research Triangle Park, NC: NIEHS. Available at: <http://iccvam.niehs.nih.gov>.

Dated: May 8, 2008.

**Samuel H. Wilson,**

*Acting Director, National Institute of Environmental Health Sciences and National Toxicology Program.*

[FR Doc. E8-11195 Filed 5-19-08; 8:45 am]

**BILLING CODE 4140-01-P**

**DEPARTMENT OF HEALTH AND HUMAN SERVICES****National Institutes of Health****National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments**

**AGENCY:** National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

**ACTION:** Meeting announcement and request for comments.

**SUMMARY:** NICEATM, in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), announces a second meeting of an independent scientific peer review panel (hereafter, Panel) to evaluate three non-radioactive modified versions and new applications for the Murine Local Lymph Node Assay (LLNA). The LLNA is an alternative test method that can be used to determine the allergic contact dermatitis potential of chemicals and products.

The Panel will consider additional data and information for the three non-radioactive modified versions and new applications of the LLNA obtained by NICEATM subsequent to the original Panel meeting in March 2008. Based on this new information, the Panel will review the following:

- The validation status of three modified LLNA test methods.
- The proposed applicability domain of the LLNA.

The Panel will peer review revised draft BRDs for each topic and evaluate the extent that established validation and acceptance criteria have been appropriately addressed. The Panel also will be asked to comment on the extent to which draft ICCVAM test method recommendations are supported by the data analyses provided in the BRDs.

NICEATM invites public comments on the draft BRDs and draft ICCVAM test recommendations. All documents will be available on the NICEATM–ICCVAM Web site at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm) by March 3, 2009.

**DATES:** The meeting is scheduled for April 28–29, 2009 from 8:30 a.m. to 5 p.m. each day. The deadline for

registration and submission of written comments is April 14, 2009.

**ADDRESSES:** The meeting will be held at the Natcher Conference Center, National Institutes of Health, 45 Center Drive, Bethesda, MD 20892. Persons needing special assistance in order to attend, such as sign language interpretation or other reasonable accommodation, should contact 301–402–8180 (voice) or 301–435–1908 TTY (text telephone). Requests should be made at least seven business days in advance of the event.

**FOR FURTHER INFORMATION CONTACT:** Dr. William S. Stokes, Director, NICEATM, NIEHS, P.O. Box 12233, Mail Stop: K2–16, Research Triangle Park, NC 27709; (telephone) 919–541–2384; (fax) 919–541–0947; (e-mail) [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov). Courier address: NICEATM, NIEHS, 530 Davis Drive, Room 2035, Mail Stop: K2–16, Durham, NC 27713.

**SUPPLEMENTARY INFORMATION:****Background**

In January 2007, the U.S. Consumer Product Safety Commission (CPSC) submitted a nomination to NICEATM ([http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf)) requesting that ICCVAM assess the validation status of (1) the LLNA limit dose procedure; (2) three modified LLNA test method protocols that use non-radioactive probe chemicals; (3) the use of the LLNA to test mixtures, aqueous solutions, and metals (applicability domain for the LLNA); and (4) the use of the LLNA to determine potency (potential for causing allergic contact dermatitis). NICEATM compiled draft BRDs that provided comprehensive reviews of the available data and relevant information, which were used as the basis for draft ICCVAM test method recommendations. These documents were released to the Panel and the public for review and comment in January 2008 (73FR1360).

In March 2008, NICEATM and ICCVAM convened the public Panel meeting during which the Panel concluded that more information and data were required for the three modified LLNA test methods before recommendations could be made regarding their use for regulatory safety testing. Similarly, the Panel concluded that more data would be needed before a recommendation on the usefulness and limitations on the current applicability domain of the traditional LLNA could be made. The Panel's conclusions are detailed in a report, which was made available in May 2008 (73FR29136), and includes

consideration of public comments made prior to and during their deliberations.

Subsequent to the Panel meeting, NICEATM received additional LLNA data for pesticide formulations and other products, as well as new data for the three modified LLNA test methods. Using the additional information, NICEATM revised the BRDs for each of these modified test methods and new applications of the LLNA. The revised draft BRDs provide all of the data and analyses supporting the scientific validity of the modified test methods and proposed applications. ICCVAM prepared revised draft test method recommendations regarding the proposed usefulness and limitations, standardized protocol, and future studies. NICEATM will reconvene the Panel to consider the additional information and revised recommendations.

**Peer Review Panel Meeting**

This meeting will take place April 28–29, 2009, at the Natcher Conference Center, National Institutes of Health, 45 Center Drive, Bethesda, Maryland, 20892. It will begin at 8:30 a.m. and is scheduled to conclude at approximately 5 p.m. on each day. The meeting is open to the public at no charge, with attendance limited only by the space available. The Panel will consider the revised draft BRDs for each of these modified versions and new applications of the LLNA and evaluate the extent that established validation and acceptance criteria are appropriately addressed for each test method and application (as described in the ICCVAM document, *Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods*, NIH Publication No. 97–3981, available at [http://iccvam.niehs.nih.gov/docs/about\\_docs/validate.pdf](http://iccvam.niehs.nih.gov/docs/about_docs/validate.pdf)). The Panel will then comment on the extent to which each of the revised draft ICCVAM test method recommendations is supported by the information provided in the corresponding revised draft BRDs. The Panel is expected first to review the three modified LLNA test methods, and then review the use of the LLNA for testing pesticide formulations and other products.

Additional information about the Panel meeting, including a roster of the Panel members and the draft agenda, will be made available two weeks prior to the meeting on the NICEATM–ICCVAM Web site (<http://iccvam.niehs.nih.gov>). This information will also be available after that date by

contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

**Attendance and Registration**

In order to facilitate planning for this meeting, persons wishing to attend are asked to register by April 14, 2009, via the NICEATM–ICCVAM Web site ([http://iccvam.niehs.nih.gov/contact/reg\\_LLNAPanel.htm](http://iccvam.niehs.nih.gov/contact/reg_LLNAPanel.htm)). Visitor parking is located in the multi-level parking garage accessible via NIH Gateway Drive. All visitors should proceed to the Gateway Center to receive a visitor badge. Note: parking is limited and a government-issued ID is required for access (an area map, driving directions, and NIH contact information are available at <http://www.nih.gov/about/visitor/index.htm>).

**Availability of the Revised Documents**

The revised draft BRDs and revised draft ICCVAM test method recommendations will be available from the NICEATM–ICCVAM Web site ([http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm)) by March 3, 2009, or by contacting NICEATM (see **FOR FURTHER INFORMATION CONTACT** above).

**Request for Public Comments**

NICEATM invites the submission of written comments on the revised draft BRDs and revised draft ICCVAM test method recommendations and prefers that comments be submitted by April 14, 2009, electronically via the NICEATM–ICCVAM Web site [http://iccvam.niehs.nih.gov/contact/FR\\_pubcomment.htm](http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm) or via e-mail at [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov). Written comments may also be sent by mail, fax, or e-mail to Dr. William Stokes, Director of NICEATM, at the address listed above (see **FOR FURTHER INFORMATION CONTACT**). When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). All comments received will be placed on the NICEATM–ICCVAM Web site (<http://iccvam.niehs.nih.gov>), and identified by the individual’s name and affiliation or sponsoring organization (if applicable). Comments will also be provided to the Panel and ICCVAM agency representatives, and made available at the meeting.

Time will be provided for the presentation of oral comments by the public at designated times during the peer review. Members of the public who wish to present oral statements at the meeting (one speaker per organization) should contact NICEATM (see **FOR**

**FURTHER INFORMATION CONTACT** above) by April 14, 2009 and provide a written copy of their comments. Each speaker is asked to provide contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable) when registering to make oral comments. Up to seven minutes will be allotted per speaker. If this is not possible, please bring 40 copies of your comments to the meeting for distribution and to supplement the record. Written statements can supplement and expand the oral presentation. Please provide NICEATM with copies of any supplementary written statement using the guidelines outlined above.

Summary minutes and the Panel’s final report will be available following the meeting on the NICEATM–ICCVAM Web site (<http://iccvam.niehs.nih.gov>). ICCVAM will consider the Panel’s conclusions and recommendations and any public comments received in finalizing their test method recommendations and performance standards for these methods.

**Background Information on ICCVAM, NICEATM, and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)**

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285l–3) established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found on their Web site (<http://iccvam.niehs.nih.gov>).

SACATM was established January 9, 2002, and is composed of scientists from the public and private sectors (67 FR 11358). SACATM provides advice to the Director of the NIEHS, to ICCVAM, and to NICEATM regarding the statutorily-mandated duties of ICCVAM and activities of NICEATM. Additional

information about SACATM, including the charter, roster, and records of past meetings, can be found at <http://ntp.niehs.nih.gov/>; see “Advisory Board & Committees” (or directly at <http://ntp.niehs.nih.gov/go/167>).

Dated: February 19, 2009.

**John R. Bucher,**

*Associate Director, NTP.*

[FR Doc. E9–4280 Filed 2–26–09; 8:45 am]

**BILLING CODE 4140-01-P**

**DEPARTMENT OF HEALTH AND  
HUMAN SERVICES****National Toxicology Program (NTP);  
Office of Liaison, Policy and Review;  
Meeting of the Scientific Advisory  
Committee on Alternative  
Toxicological Methods (SACATM)**

**AGENCY:** National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

**ACTION:** Meeting announcement and request for comments.

**SUMMARY:** Pursuant to section 10(a) of the Federal Advisory Committee Act, as amended (5 U.S.C. Appendix 2), notice is hereby given of a meeting of SACATM on June 25–26, 2009, at the Hilton Arlington Hotel, 950 North Stafford Street, Arlington, VA 22203. The meeting is open to the public with attendance limited only by the space available. SACATM advises the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and the Director of the NIEHS and NTP regarding statutorily mandated duties of ICCVAM and activities of NICEATM.

**DATES:** The SACATM meeting will be held on June 25 and 26, 2009. The meeting is scheduled from 8:30 a.m. to 5 p.m. on June 25 and 8:30 a.m. until adjournment on June 26, 2009. All individuals who plan to attend are encouraged to register online at the NTP Web site (<http://ntp.niehs.nih.gov/go/7441>) by June 17, 2009. In order to facilitate planning, persons wishing to make an oral presentation are asked to notify Dr. Lori White, NTP Executive Secretary, via online registration, phone, or e-mail by June 17, 2009 (see **ADDRESSES** below). Written comments should also be received by June 17, 2009, to enable review by SACATM and NIEHS/NTP staff before the meeting.

**ADDRESSES:** The SACATM meeting will be held at the Hilton Arlington Hotel, 950 North Stafford Street, Arlington, VA 22203 [hotel: (703) 528-6000]. Public comments and other correspondence should be directed to Dr. Lori White (NTP Office of Liaison, Policy and Review, NIEHS, P.O. Box 12233, MD K2-03, Research Triangle Park, NC 27709; telephone: 919-541-9834 or e-mail: [whitel@niehs.nih.gov](mailto:whitel@niehs.nih.gov)). Courier address: NIEHS, 530 Davis Drive, Room 2136, Durham, NC 27713. Persons needing interpreting services in order to attend should contact 301-402-8180 (voice) or 301-435-1908 (TTY).

Requests should be made at least 7 days in advance of the meeting.

**SUPPLEMENTARY INFORMATION:**

**Preliminary Agenda Topics and Availability of Meeting Materials**

- Preliminary agenda topics include:
- NICEATM–ICCVAM Update.
  - Regulatory Acceptance of ICCVAM–Recommended Alternative Test Methods.
  - NRC Report *Recognition and Alleviation of Pain in Laboratory Animals*.
  - Implementation of NICEATM–ICCVAM Five-Year Plan.
  - Federal Agency Research, Development, Translation, and Validation Activities Relevant to the NICEATM–ICCVAM Five-Year Plan (EPA and USDA).
  - Report on second meeting of Independent Peer Review Panel: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.
  - Report on the Independent Scientific Peer Review Panel on Alternative Ocular Safety Testing Methods.
  - Update from the Japanese Center for the Validation of Alternative Methods.
  - Update from the European Centre for the Evaluation of Alternative Methods.
  - Update from Health Canada.
- A copy of the preliminary agenda, committee roster, and additional information, when available, will be posted on the NTP Web site (<http://ntp.niehs.nih.gov/go/7441>) or available upon request (see **ADDRESSES** above). Following the SACATM meeting, summary minutes will be prepared and available on the NTP Web site or upon request.

**Request for Comments**

Both written and oral public input on the agenda topics is invited. Written comments received in response to this notice will be posted on the NTP Web site. Persons submitting written comments should include their name, affiliation (if applicable), and sponsoring organization (if any) with the document. Time is allotted during the meeting for presentation of oral comments and each organization is allowed one time slot per public comment period. At least 7 minutes will be allotted for each speaker, and if time permits, may be extended up to 10 minutes at the discretion of the chair. Registration for oral comments will also be available on-site, although time

allowed for presentation by on-site registrants may be less than for pre-registered speakers and will be determined by the number of persons who register at the meeting.

Persons registering to make oral comments are asked to do so through the online registration form (<http://ntp.niehs.nih.gov/go/7441>) and to send a copy of their statement to Dr. White (see **ADDRESSES** above) by June 17, 2009, to enable review by SACATM, NICEATM–ICCVAM, and NIEHS/NTP staff prior to the meeting. Written statements can supplement and may expand the oral presentation. If registering on-site and reading from written text, please bring 40 copies of the statement for distribution and to supplement the record.

**Background Information on ICCVAM, NICEATM, and SACATM**

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability and promotes the development, scientific validation, regulatory acceptance, implementation, and national and international harmonization of new, revised, and alternative toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 [42 U.S.C. 285l–3] established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found on their Web site (<http://iccvam.niehs.nih.gov>).

SACATM was established in response to the ICCVAM Authorization Act [Section 285l–3(d)] and is composed of scientists from the public and private sectors. SACATM advises ICCVAM, NICEATM, and the Director of the NIEHS and NTP regarding statutorily mandated duties of ICCVAM and activities of NICEATM. SACATM provides advice on priorities and activities related to the development, validation, scientific review, regulatory acceptance, implementation, and national and international harmonization of new, revised, and

alternative toxicological test methods. Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at <http://ntp.niehs.nih.gov/go/167>.

Dated: April 22, 2009.

**John R. Bucher,**

*Associate Director, National Toxicology Program.*

[FR Doc. E9–9845 Filed 4–28–09; 8:45 am]

**BILLING CODE 4140-01-P**

meeting is now available. The report contains (1) the Panel's evaluation of the updated validation status of the methods and (2) the Panel's comments on the updated draft ICCVAM test method recommendations. NICEATM invites public comment on the Panel's report. The report is available on the NICEATM-ICCVAM Web site at [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRRRept2009.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRRRept2009.pdf) or by contacting NICEATM at the address given below.

**DATES:** Written comments on the Panel report should be received by July 15, 2009.

**ADDRESSES:** Comments should be submitted preferably electronically via the NICEATM-ICCVAM Web site at [http://iccvam.niehs.nih.gov/contact/FR\\_pubcomment.htm](http://iccvam.niehs.nih.gov/contact/FR_pubcomment.htm). Comments can also be submitted by e-mail to [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov). Written comments can be sent by mail or fax to Dr. William S. Stokes, Director, NICEATM, NIEHS, P.O. Box 12233, Mail Stop: K2-16, Research Triangle Park, NC 27709; (fax) 919-541-0947. *Courier address:* NIEHS, NICEATM, 530 Davis Drive, Room 2035, Durham, NC 27713.

**FOR FURTHER INFORMATION CONTACT:** Dr. William S. Stokes (telephone) 919-541-2384, (fax) 919-541-0947 and (e-mail) [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov).

**SUPPLEMENTARY INFORMATION:**

**Background**

In January 2007, the Consumer Product Safety Commission submitted a nomination to NICEATM and ICCVAM to assess the validation status of (1) the use of the LLNA to determine potency for hazard classification purposes, (2) LLNA protocols using non-radioactive procedures, (3) the LLNA limit dose procedure, and (4) the use of the LLNA to test mixtures, aqueous solutions, and metals (*i.e.*, an updated assessment of the applicability domain of the LLNA). In June 2007, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) endorsed these activities as high priorities for ICCVAM. NICEATM, on behalf of ICCVAM, also sought input from the public on these activities and requested data from studies using the LLNA or modified versions of the LLNA (72 FR 27815). After considering all comments received, ICCVAM endorsed carrying out these activities as high priorities. ICCVAM also developed draft LLNA performance standards to facilitate evaluation of modified LLNA protocols that are functionally and mechanistically similar to the traditional LLNA. These draft LLNA performance standards were made

public and comments were requested in September 2007 (72 FR 52130).

ICCVAM and NICEATM prepared draft background review documents (BRDs) that provided comprehensive reviews of available data and relevant information for each of the modifications and new applications of the LLNA. ICCVAM also developed draft test method recommendations regarding the proposed usefulness and limitations, standardized protocols, and future studies. NICEATM announced availability of the draft BRDs and draft recommendations for public comment and the public peer review meeting in January 2008 (73 FR 1360).

The Panel met in public session on March 4-6, 2008, to review these topics, and their report was made available in May 2008 (73 FR 29136). The draft BRDs and draft test method recommendations, the draft ICCVAM LLNA test method performance standards, the Panel's report, and all public comments were made available to SACATM for comment at their meeting on June 18-19, 2008 (73 FR 25754).

As a result of additional data received by ICCVAM subsequent to the March 2008 Panel meeting, the draft BRDs for the following were updated:

- The validation status of three modified LLNA test method protocols that do not require the use of radioactive substances.
- The use of the LLNA for testing pesticide formulations, other products, and aqueous solutions.

**Second Meeting of the Peer Review Panel**

The Panel met again in public session on April 28-29, 2009 (74 FR 8974). The Panel reviewed the revised draft ICCVAM documents for completeness, errors, and omissions of any existing relevant data or information. The Panel evaluated the information in the revised draft documents to determine the extent to which each of the applicable criteria for validation and acceptance of toxicological test methods (ICCVAM, 2003) had been appropriately addressed. The Panel then considered the ICCVAM draft recommendations for test method uses and limitations, proposed standardized protocol, proposed plans for development of test method performance standards, and proposed additional studies, and commented on the extent that the recommendations were supported by the information provided in the draft BRDs.

**Availability of the Peer Panel Report**

The Panel's conclusions and recommendations are detailed in the

**DEPARTMENT OF HEALTH AND HUMAN SERVICES**

**National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments**

**AGENCY:** National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH).

**ACTION:** Request for comments.

**SUMMARY:** NICEATM, in collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), convened an independent, international, scientific peer review panel (hereafter, Panel) on April 28-29, 2009, to evaluate three non-radioactive modified versions and new applications for the Murine Local Lymph Node Assay (LLNA). The LLNA is an alternative test method that can be used to determine the allergic contact dermatitis potential of chemicals and products. The Panel report from this

*Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products* (available at [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRpt2009.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRpt2009.pdf)). The revised draft documents reviewed by the Panel and the draft ICCVAM test method recommendations are available at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm).

**Request for Public Comments**

NICEATM invites the submission of written comments on the Panel's report. When submitting written comments, please refer to this **Federal Register** notice and include appropriate contact information (name, affiliation, mailing address, phone, fax, e-mail, and sponsoring organization, if applicable). All comments received will be made publicly available via the NICEATM-ICCVAM Web site at [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm). In addition, there will be an opportunity for oral public comments on the Panel's report during an upcoming meeting of SACATM scheduled for June 25–26, 2009 (74 FR 19562). Information concerning the SACATM meeting is available at <http://ntp.niehs.nih.gov/go/7441>. ICCVAM will consider the Panel report along with SACATM and public comments when finalizing test method recommendations. An ICCVAM test method evaluation report, which will include the final ICCVAM recommendations, will be forwarded to relevant Federal agencies for their consideration. The evaluation report will also be available to the public on the NICEATM-ICCVAM Web site at <http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm> and by request from NICEATM (see **ADDRESSES** above).

**Background Information on ICCVAM, NICEATM, and SACATM**

ICCVAM is an interagency committee composed of representatives from 15 Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with

regulatory applicability and promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety and hazards of chemicals and products and that refine, reduce, and replace animal use. The ICCVAM Authorization Act of 2000 (42 U.S.C. 285I–3) established ICCVAM as a permanent interagency committee of the NIEHS under NICEATM. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM and ICCVAM work collaboratively to evaluate new and improved test methods applicable to the needs of U.S. Federal agencies. Additional information about ICCVAM and NICEATM can be found on their Web site (<http://iccvam.niehs.nih.gov>).

SACATM was established January 9, 2002, and is composed of scientists from the public and private sectors (67 FR 11358). SACATM provides advice to the Director of the NIEHS, ICCVAM, and NICEATM regarding the statutorily mandated duties of ICCVAM and activities of NICEATM. Additional information about SACATM, including the charter, roster, and records of past meetings, can be found at <http://ntp.niehs.nih.gov>/ see "Advisory Board & Committees" (or directly at <http://ntp.niehs.nih.gov/go/167>).

**Reference**

ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03–4508. Research Triangle Park, NC: NIEHS. Available at: <http://iccvam.niehs.nih.gov>.

Dated: May 19, 2009.  
**John R. Bucher**,  
 Associate Director, NTP.  
 [FR Doc. E9–12360 Filed 5–29–09; 8:45 am]  
**BILLING CODE 4140–01–P**


## Appendix F2

### Public Comments Received in Response to *Federal Register* Notices

72 FR 27815 (May 17, 2007)

The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

- Dr. Eric Debruyne (BAYER CropScience) ..... F-27
- Dr. H.-W. Vohr (Bayer HealthCare AG) ..... F-29
- Dr. H.-W. Vohr (Bayer HealthCare AG) ..... F-34
- Dr. H.-W. Vohr (Bayer HealthCare AG) ..... F-39
- Dr. Kirill Skirda (CESIO) ..... F-44
- Mark S. Maier, Ph.D., DABT (CropLife America) ..... F-45
- Dr. Phil Botham (European Crop Protection Association) ..... F-46
- Peter Ungeheuer (European Federation for Cosmetic Ingredients) ..... F-49
- Dori Germolec (NIEHS) ..... F-51
- Dori Germolec (NIEHS) ..... F-52
- Robert L. Guest (Safeparm Laboratories Ltd) ..... F-53
- Daniel R. Cerven, M.S. and Melissa K. Kirk, Ph.D. (MB Research Laboratories) ..... F-55
- Daniel Marsman, D.V.M., Ph.D. (Procter & Ganble) ..... F-59
- Michael J. Olson, Ph.D. (GlaxoSmithKline) ..... F-60
- Anne Marie Api, Ph.D. (Research Institute for Fragrance Manufacturers) ..... F-62
- Peter S. Thorne, Ph.D. (The University of Iowa) ..... F-64
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Sara Amundson (Humane Society Legislative Fund), Dr. Martin Stephens (Humane Society of the United States), Kristie Stoick, M.P.H. (Physicians Committee for

Responsible Medicine), Sue A. Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society) .....	F-65
72 FR 52130 (September 12, 2007)	
Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments	
• Ann-Therese Karlberg (Goteborg University) .....	F-70
• Dr. Jon Richmond.....	F-71
• Prof. dr. Henk Van Loveren (National Institute of Public Health and the Environment, the Netherlands).....	F-73
• Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Sara Amundson (Humane Society Legislative Fund), Dr. Martin Stephens (Humane Society of the United States), Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine), Sue A. Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society) .....	F-75
73 FR 1360 (January 8, 2008)	
Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments	
• Dr. David Basketter .....	F-78
• Dr. David Basketter .....	F-79
• Kenneth T. Bogen, Dr.P.H., DABT (Exponent).....	F-80
• G. Frank Gerberick, Ph.D. (The Procter & Gamble Company) .....	F-81
• Laurence Musset (OECD) .....	F-90
• B. Schau.....	F-93
• Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals) and Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine) .....	F-94
73 FR 25754 (May 7, 2008)	
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	
• B. Sachau .....	F-99
73 FR 29136 (May 20, 2008)	
Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	
• No responses received	
74 FR 8974 (February 27, 2009)	
Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments	
• Nancy Douglas, Ph.D. and Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Kristie Stoick, M.P.H. (Physicians Committee for Responsible	

Medicine), Martin Stephens, Ph.D. (The Humane Society of the United States), Sara Amundson (Humane Society Legal Fund, Doris Day Animal League), Sue Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society) .....F-100

74 FR 19562 (April 29, 2009)

Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

- No responses received

74 FR 26242 (June 1, 2009)

Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

- Brian E. Harvey, M.D., Ph.D. (Sanofi Aventis) .....F-105

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Date: Fri, 29 Jun 2007 09:56:56 +0200  
To: Neepa Choksi  
Cc: David Allen, Doug Winters  
Subject: Re: RE ICCVAM/NICEATM FR Notice: LLNA Nomination and Request for Data

Dear Neepa,

In response to the NICEATM request published in the Federal Register notice, vol. 72, N° 95, on May 17th, 2007 and further to the e-mails we have exchanged on this subject, Bayer CropScience is submitting data from a number of studies conducted using the LLNA assay with different types or pesticide formulations. This data is submitted specifically to address the following questions: (1) the evaluation of the LLNA as a stand-alone assay for determining potency for the purpose of hazard classification, and (2) the ability of the LLNA for testing mixtures and aqueous solutions”.

In our studies, the LLNA study protocol includes the addition of a positive control spiked into the tested formulation in order to demonstrate the ability of the assay to detect sensitizer in such formulations and thus the validity of the results.

The data is submitted in two forms in the attached zipped file:

1. detailed summaries of the data obtained with several formulations using both the LLNA and another validated method for evaluation of the sensitization potential of the pesticide formulation (Buehler tests with 3 or 9 inductions, Maximized M&K test) are provided for 11 different pesticide formulations (EC, SL, EW, OF, WG, SC).
2. full reports of most of the studies from the above list where the LLNA assay showed a positive response while the classical methods were negative.

Please note that, for confidentiality reasons, the names of the active ingredients contained in the different formulations have been blinded.

We hope that this data will be useful to the evaluation conducted by the NICEATM. Please do not hesitate to use me as your contact for any queries or questions on our data and studies.

Cordialement / Best regards / Mit freundlichen Grüßen

Eric

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6/20/07

**Concerns:**

**Data package 1, submitted to NICEATM and ICCVAM for further evaluation of the LLNA and modifications of it**

In 2001 experts of several institutes (authority, academia, industry) in Europe decided to initiate a catch-up validation of a modification of the standard - radioactive - LLNA as described before by Homey et al. and Vohr et al. [Ref. 1.1 and 1.2.]. From the very beginning the studies were supported by the VCI (Verband der Chemischen Industrie e.V. (German chemical industry federation)).

It was decided to test 3 (first round) and 9 (second round) international standards out of a list of 26 standards under full GLP compliance. The substances should be submitted blinded by an independent coordinator to the participating labs. A well-known expert from the Swiss authority Swissmedic, T. Maurer, accepted to supervise the study, to select the test substances including submission of the test items as well as to organize the data submission to an independent statistician (J. Hüsler, University of Bern, Switzerland).

It was decided to start with a pilot study using HCA as test substance to finally harmonize the protocol used by the participating labs. In addition, a new evaluation scheme was agreed on which takes the assessment of skin reaction due to irritation into account [Ref. 1.3.].

Afterwards a first round with 3 test substances and two strains of mice (BALB/c and NMRI outbred) had been carried out. The test items were not only blinded but also labeled differently for each participating lab for this first part of the study by the coordinator. An intermediate assessment of the still blinded test substances served as a milestone to continue or not, and to

select one of the mouse strains for the second round of the study. Because of extremely good correlation of the data between labs it was decided to continue with another 9 standards in a second round with BALB/c.

All 9 participating labs measured weights and cell counts of the draining lymph nodes, and for acute skin reaction ear weights (8mm punch). Ear thickness was measured in some labs in addition. One lab used radioactive labeling as well, and one lab used NMRI also with all standards.

All raw data were sent to T. Maurer who forwarded these to J. Hüsler for statistical evaluation [Ref.1.4.]. Only after the overall evaluation the codes were de-blinded by T. Maurer.

Evaluation based on cell count indices turned out to be as sensitive as the radioactive method. The cut-off concentrations (EC values) were very similar for both methods (cf. also publications of the catch-up validation).

The additional determination of acute ear (skin) reaction by ear weight/ear thickness turned out to be very useful for further assessment of the lymph node reaction, i.e. to exclude false positive results. Results of this catch-up validation have been published in peer reviewed papers [Ref 1.5. and 1.6.] and at different meetings in poster sessions.

With respect to the cut-off values (EC (Effective Concentration) values) it is obvious that each parameter (end point) requires its own specific cut-off value. This is accepted since decades for example in guinea pig assays:  $\geq 30\%$  positive reactions in M&K tests or  $\geq 15\%$  positive reactions in Bühler tests.

For the radioactive labeling the cut-off value has been fixed to that concentration of test substance that induces a 3 times increase in stimulation index, i.e. the so-called EC3 value. For cell count indices such cut-off values are much lower, for example 1.5 times increase of stimulation index. This is understandable by the facts that cell count indices have i) lower individual variances compared to 3H-Thymidine incorporation, and ii) lower maximum stimulation indices compared to radioactive labeling. For example, a strong sensitizing substance may easily induce indices about 30-50 by 3H-Thymidine incorporation but only indices about 4-5 by cell counting. However, crucial for the assessment are not impressive high stimulation indices, but reliable determination of a safe and accurate cut-off value, so the reasonable and reliable determination of the concentration of a test substance exceeding it. These concentrations exceeding the thresholds can then be compared between methods and modifications, and are indeed comparable as it has been shown by our catch-up validation! In [Ref. 1.7.] the results of EC1.5 values of all participating labs are averaged and the classification range of potency given as calculated in the different labs. Statistically significant increases were taken into account just as all stimulation indices exceeding the cut-off value. i.e. EC1.5, without being of statistical significance.

Interestingly, there was an extremely good correlation between statistically significant increases in stimulation indices and the exceeding of thresholds or cut-off values. Similar finding have already been published by Gerberick et al. in 1992 [Ref. 1.8.] as can be taken from the attached table (statistically significant indices in red):

Table 1 (modified after Gerberick et al., 1992) showing significant stimulation indices of two different endpoints, i.e. cell counting or radioactive labeling obtained with international standards.

Compound	Cell counts	3H.Thymidine
Benzalkonium chloride 0,5%	2,70	9,00
1%	4,08	11,10
2%	2,93	7,60
Benzocaine 5%	1,39	1,30
10%	0,99	1,00
20%	1,12	1,30
DCNB 0,001%	0,94	0,80
0,05%	2,06	10,70
0,10%	2,83	21,10
Ethylendiamine 1%	1,06	1,10
5%	1,07	1,10
10%	1,77	2,20
Eugenol 25%	2,72	5,40
50%	2,70	10,60
75%	2,72	10,50
Glutaraldehyde 3,1%	2,54	9,80
6,20%	4,52	21,40
12,50%	5,35	22,90
MCI/MI 50ppm	3,04	8,10
500ppm	5,68	27,80
1000ppm	4,59	48,20
Nickel chloride 2,5%	0,98	1,30
5%	1,50	2,60
10%	1,96	6,60
Oxazolone 0,0001%	0,94	1,60
0,005%	1,62	8,70
0,05%	4,52	55,20
TNCB 0,01%	3,02	18,00
0,05%	6,62	80,30
0,10%	7,23	103,30

Beside all references mentioned here in the text two reports with all standards tested in one lab with BALB/c or NMRI (outbred) mice are also included in this package 1. Of course, the test substances are called in both reports A to L, but A to C were differently named in each participating lab.

The actual identity of these standards can be taken from the following Table 2:

**Round I**

<b>Code</b>	<b>Compound</b>	<b>Proposed classification</b>	<b>Reference</b>
HCA	Hexylcinnamaldehyde	Sensitiser	Dearman 2001
A	p-hydroquinone	Sensitiser	Kimber 1998
B	SDS	Irritant	Basketter 1992
C	4-aminobenzoic acid	Negative	Basketter 1992

**Round II**

<b>Code</b>	<b>Compound</b>	<b>Proposed classification</b>	<b>Test concentrations</b>	<b>Reference</b>
D	Xylene	Irritant	10, 30, 100%	Kligman 1966
E	Octanoic acid	Weak Irritant	1, 3, 10%	ECETOC 1995
F	MCI	Sensitiser	0.03, 0.1, 0.3%	Botham 1991
G	Mercaptobenzothiazole*	Sensitiser	3, 10, 30%	Scholes 1992
H	Isoeugenol	Sensitiser	3, 10, 30%	Basketter 1992
I	Potassium dichromate	Sensitiser	0.3, 1, 3%	Basketter 1992
K	Hydroxycitronellal	Sensitiser	6, 20, 60%	Basketter 1992 Montelius 1994
L	Tween 80	Irritant	10, 30, 100%	Magnusson 1969

Kind regards,

H.-W. Vohr

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6/20/07

**Concerns:**

**Data package 2, submitted to NICEATM and ICCVAM for further evaluation of the LLNA and modifications of it**

In 2005 the BG Institute for Occupational Safety and Health of the German Social Accident Insurance - BGIA (Berufsgenossenschaftliches Institut für Arbeitsschutz) initiated a meeting about skin sensitization, and the experiences so far with the Local Lymph Node Assay. Experts from different institutes (authority, academia, industry) in Germany discussed the data. There was a concern about the increase in positive results with LLNA compared to the years of experiences with guinea pig assays. This is also illustrated by the peer reviewed paper of Vohr and Ahr, 2005 [Ref. 2.1.]. During this meeting it was decided to compare the "standard" - radioactive - LLNA with a non-radioactive modification, i.e. cell counting, with 13 related compounds (epoxy resin components); most of which are classified as skin sensitizers based on guinea pig data. HCA was chosen as positive control. In accordance with the exemplary described method in OECD 429 mouse strain CBA was used for this study. For further information about the compounds and protocol see also Ref 2.2. and 2.3., and Table 1 below. Although both PP presentations are in German the main messages are clear and self-explanatory.

One of the goals was to correlate stimulation indices of both methods as well as cut-off concentrations evaluated by them, i.e. the effective or estimated concentrations of test items exceeding the cut-off lines defined for both methods. These EC values correspond to EC3 for the radioactive labeling or EC1.5 for the cell counting as also described previously [Ref. 2.4.].

Another aim of this study was to classify the test substances according to their potency to induce cell proliferation in the draining lymph nodes. This classification was based on the ECETOC

criteria described before [Ref. 2.5.]. Due to the fact that applications of moderate to strong irritants could result in false positive reactions ear weight was measured in addition to balance the influence of such non-specific cell activation. It has to be mentioned, however, that here skin reactions were measured three days after the last application (on day 6) while the "acute" skin reaction has reasonably to be measured one day after the last application on day 4. In case of 6 days protocols this parameter could be determined by measuring ears swelling at day 4 which was unfortunately not possible during this study. However, this has no influence on the overall assessment of the results, esp. on the comparison of estimated concentrations and stimulation indices.

Following 13 related test substances have been chosen for the comparison (Table 1):  
Acetone was used as vehicle to reach acceptable solubility for all test items. Therefore, the positive control HCA was also tested in acetone.

---

Bisphenol A, resin, Bakelite EPR 164 (CAS-Nr. 25068-38-6)  
Bisphenol A, resin, distilled, Bakelite EPR 162 (CAS-Nr.1675-54-3)  
Bisphenol F, resin, Bakelite EPR 161 (CAS-Nr. 9003-36-5)

1,6-Hexanediol Diglycidyl Ether (CAS-Nr. 16096-31-4)  
P-Tertbutylphenyl Glycidyl Ether (CAS-Nr. 3101-60-8)  
Trimethylolpropane triglycidyl ether (CAS-Nr. 3454-29-3)  
Dodecyl/tetradecyl glycidyl ether (CAS-Nr. 68609-97-2)

m-Xylylenediamine (CAS-Nr. 1477-55-0)  
3-Aminomethyl-3,5,5-trimethylcyclohexylamine (CAS-Nr. 2855-13-2)  
Bis(3-aminopropyl)amine (CAS-Nr. 56-18-8)  
2,2,4(2,4,4)-Trimethyl-1,6-hexanediamine (CAS-Nr. 25620-58-0)  
N-(2-Hydroxyethyl)ethylenediamine (CAS-Nr. 111-41-1)  
1,2-Diaminocyclohexane (CAS-Nr. 694-83-7)

---

All the studies have been conducted at BASF AG, Ludwigshafen, Germany, under full GLP compliance. Data were presented by the study director, Dr. A.O. Gamer, and discussed in a similar panel as before.

#### Conclusions:

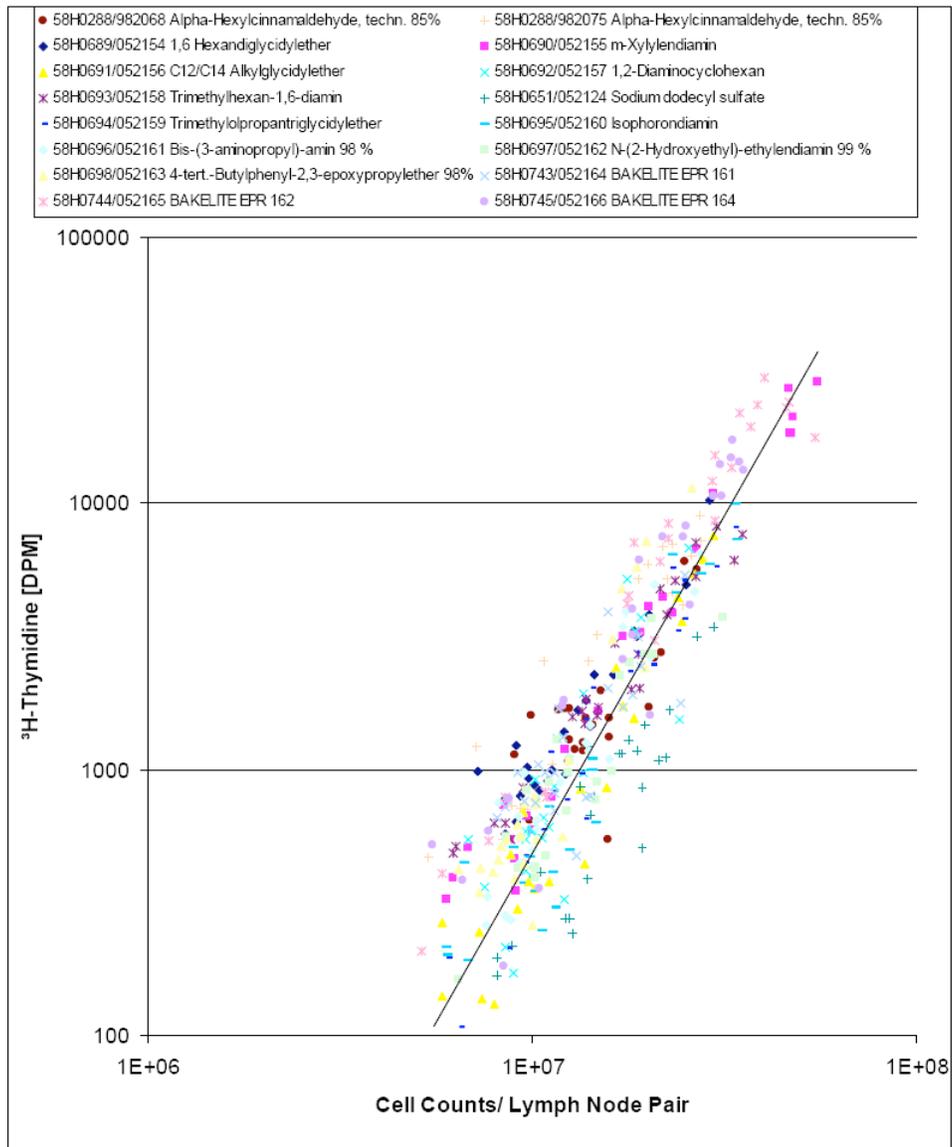
--- There was an extremely good correlation between stimulation indices obtained by radioactive labeling and non-radioactive cell counting [see also Fig. 1 below and Ref. 2.6.].

--- Therefore, the effective concentrations calculated are very similar for both endpoints [see also Table 2 below and Ref. 2.6.].

--- The vehicle (acetone) may have an impact in the relatively low effective doses (i.e. relative high potency) determined for the test substances. This may easily be recognized by the results obtained with HCA diluted in acetone alone or acetone:olive oil (AOO 4:1).

--- Taken the irritant potential also into account will improve the assessment of the overall sensitizing potency. However, optimal time point for the determination of acute skin reaction is one day after last application, i.e. day 4 in standard protocol.

**Figure 1:** Comparison of lymph node cell count and <sup>3</sup>H-thymidine incorporation taken from the Report by AO Gamer and R Landsiedel [Ref 2.6.]



**Table 2:** Tested concentrations and “Estimated Concentrations<sup>1</sup>” of skin sensitising threshold of epoxy resin components from Ref. 2.6.

Substance name	Concentrations tested	Results			
		EC3	EC1.5	EC3lg	EC1.5lg
	% (w/w)				
1,6-Bis(2,3-epoxypropoxy)hexane	3, 1, 0.3	1.9	1.7	1.6	1.5
m-Phenylenebis(methylamine)	3, 1, 0.3	0.4	0.4	0.4	0.3
Oxirane, mono((C12-14-alkyloxy)methyl)derivs	3, 1, 0.3	0.6	0.7	0.5	0.6
1,2-Diaminocyclohexane	1, 0.3, 0.1	0.4	0.6	0.4	0.5
Trimethylhexamethylene diamine	10, 3, 1	1.9	0.5	1.7	0.8
1-(2,3-Epoxypropoxy)-2,2-bis[(2,3-epoxypropoxy)methyl]butane	10, 3, 1	1.4	1.7	1.3	1.4
3-Aminomethyl-3,5,5-trimethylcyclohexylamine	3, 1, 0.3	1.0	1.2	1.0	1.1
Dipropylene triamine 98%	3, 1, 0.3	0.9	1.0	0.8	1.0
N-(2-Hydroxyethyl)-ethylendiamine 99%	30, 10, 3	15.2	14.4	13.3	12.7
p-tert-Butylphenyl 1-(2,3-epoxy)propyl ether 98%	1, 0.3, 0.1	0.4	0.5	0.3	0.4
Bakelite EPR 161	1, 0.3, 0.1	0.7	0.6	0.6	0.5
Bakelite EPR 162	3, 1, 0.3	-	-	0.2	0.1
Bakelite EPR 164	3, 1, 0.3	0.1	-	0.2	0.1
$\alpha$ -Hexyl cinnamic aldehyde/AOO	10, 5, 2.5	10.5	6.9	10.7	6.5
$\alpha$ -Hexyl cinnamic aldehyde/Acetone	30, 10, 3	-	0.1	1.2	1.8

- no meaningful calculation possible

<sup>1</sup> Estimated concentration that leads to the respective stimulation index  
 EC was estimated by linear regression  
 EClg was estimated by linear regression using a log transformation of the concentration

Kind regards,

H.-W. Vohr

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6/28/07

**Concerns:**

**Data package 3, submitted to NICEATM and ICCVAM for further evaluation of the LLNA and modifications of it**

The principle of the method had been published in 1989, and a first collaborative validation study in 1991. In these first trials the stimulation of the lymph nodes, i.e. cell proliferation, was measured by <sup>3</sup>H-Thymidin incorporation. In 1999 the principle of the LLNA had been stated as valid alternative to guinea pig assays by the ICCVAM, although the need for further modifications was also noted. Concerns focused on false positive results caused by strong irritants or negative results based on the use of aqueous formulations.

In 2002 the method has been published in guideline OECD 429, and 2003 in EPA guideline OPPTS 870.2600 as a stand-alone test. Corresponding to the concerns mentioned above the use of "wholly aqueous vehicles are to be avoided.". As published by Ryan et al. in 2002 1% Pluronic PE 9200 (L92) may be chosen for using aqueous vehicles in the Local Lymph Node Assay [Ref.3.1.]. As can be taken from the information in this paper it is possible to achieve positive results by the addition of this surfactant to aqueous formulations of test items. However, the cut-off concentrations (EC3 values) increased significantly compared to vehicles recommended in the guidelines. Apart from that the data impressively show the influence of vehicles on the cut-off concentrations determined by the LLNA exemplary illustrated by Table 1 (primordial Table 3 in the paper of Ryan et al.).

Table 1 (taken from publication Ryan et al., 2002)

Effect of vehicle on the relative skin sensitization potency of DNBS, formaldehyde, potassium dichromate and nickel sulfate

Chemical	Vehicle	EC3 Value
DNBS	Water	16%
	1% L92	6.4%
	DMSO	2.0%
	DMF	< 1.0%
Formaldehyde	Water	14.5%
	1% L92	4.2%
	DMSO	< 1.0%
	DMF	< 1.0%
Potassium dichromate	1% L92	0.17%
	DMSO	0.05%
	DMF	0.0327%
Nickel sulfate	1% L92	2.5%
	DMSO	4.8%
	DMF	> 5.0%

To examine the use of surfactants on the ability to test aqueous formulations in the Local Lymph Node Assay we started with aqueous formulations of HCA. The test item was formulated immediately before each administration in Pluronic PE 9200 / 0.9% NaCl solution, 1% v/v or Cremophor / 0.9% NaCl solution, 2% v/v [cf. also Ref 3.3.].

In a first trial we compared HCA in different vehicles with 2% Cremophor. Results are shown in the Table below (Table 2).

Table 2: Modified LLNA using NMRI and HCA as positive control. Cut-off cell count index is set to 1.4, i.e. EC1.4 should be used [Ref. 3.2.].

HCA					Statist. Signific.		
	Vehicle	3%	10%	30%		EC1.4	Potency*
	MEK	1,22	1.42	1.99	*	9.3	moderate
	AOO (4:1)	1.15	1.28	1.79	*	14.7	weak
	DMF	0.87	1.13	1.77	*	18.4	weak
	PEG400	0.81	1.04	1.69	*	21.1	weak
	Cremophor	0.71	0.98	1.37		(31.5)	(weak)

\* Potency classification according to ECETOC technical Report No. 87, 2003

Although an improvement, addition of Cremophor alone did not reach the EC values between 5% and 20% as normally determined with standard (guideline) vehicles. Therefore, we included an additional infrared irradiation (about 20 min. before treatment) of the animals to enhance the blood flow in the skin and by this enhance penetration. This additional treatment by infrared irradiation caused indeed higher, and statistically significant stimulation indices as can be taken from the Table below.

<b>Vehicle</b>	<b>3%</b>	<b>10%</b>	<b>30%</b>	<b>.</b>	<b>EC1.4</b>	<b>Potency#</b>
Cremophor (2%)	0.71	0.98	1.37		(31.5)	(weak)
Cremo. (2%) + IR	0.82	1.34	1.45	*	20.9	weak

\*: Statistically significant

#: Potency classification according to ECETOC technical Report No. 87, 2003

Similar studies were then conducted with L92 and infrared irradiation in combination with aqueous HCA formulations. In each case HCA has been classified by this method as weak sensitizer within a range of EC values comparable to those obtained with other (guideline) vehicles. Such positive control studies with aqueous formulations are done in regular intervals in our lab (Bayer HealthCare AG, Immunotoxicology) since years. Results of these studies are also included in the Excel file attached to this data package [Ref. 3.3.].

It has to be mentioned here that based on all our experiences so far with Cremophor or Pluronic it seems that Pluronic (L92) enhances the intrinsic irritant properties of test compounds while Cremophor does not! This property of L92 may be problematic for correct classification of test items when radioactive labeling without discrimination of irritation and sensitization is used for measuring cell proliferation. One example of such a positive control study report with HCA in 1% Pluronic is attached as Ref. 3.4., which is equal to data of Ref. 3.3., "Tabelle 4, 2005/2".

Because sponsors did not want us to submit data with aqueous formulations all we can provide are data from a pre-validation study with HCA as positive controls and three aqueous formulations (A-C) from which one had been tested positive in GPMT before (A as weak sensitizer; B unknown; C tested negative before). The results are given in Ref. 3.5. including all controls with 2% Cremophor or 1% L92 plus infrared irradiation.

The overall conclusion from these studies is that stimulation index induced by formulation A at the highest concentration (50%) just reached the cut-off level of EC1.4, statistically significant. Hence, formulation A would be classified as a weak sensitizing formulation while the other two formulations turned out to be negative.

Conclusions:

--- There is some differences in stimulation indices obtained with various vehicles. EC value may vary by a factor of +/- 2 of overall mean. A change in classification of potency by this factor is possible [cf. also review article by McGarry, 2007; Ref. 3.6.].

--- Aqueous formulations may be tested by adding 1% L92 or 2% Cremophor to the formulation to increase adherence to the skin. Skin irradiation with infrared will accessorily improve the outcome, i.e. test sensitivity.

--- By this modifications (surfactant + infrared irradiation) it is possible to test aqueous formulations with nearly the same sensitivity as with vehicles recommended in the guidelines.

--- However, there is no profound validation study of the LLNA or a modification of it with aqueous formulations or mixtures down to the present day.

--- It seems as if Pluronic enhances the irritant properties of test compounds applied, and by this increase the non-specific activation of lymph node cells which may be a problem for classification according to potency by radioactive methods.

Kind regards,

H.-W. Vohr

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Toxicology online, 2007 (doi:10.1016/j.tox.2007.06.002)

European Committee of  
Organic Surfactants and  
their Intermediates



A Sector Group of **Cefic**

12 June 2007

Dr. William S. Stokes  
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NIEHS  
Research Triangle Park, NC 27709  
Via E-mail: niceatm@niehs.nih.gov

Re. Federal Register 72 (95), May 17, 2007, pages 27815-27817

Dear Dr. Stokes

I am writing to you on behalf the CESIO Local Lymph Node Assay Task Force (CESIO is the Sector Group of CEFIC dealing with organic surfactants and their intermediates). This Task Force was established in 2006 with the aim to exchange the experiences of the different Industry Sectors using the Local Lymph Node Assay (LLNA) for sensitisation testing.

The Task Force noted that several Industry sectors experienced positive results in the LLNA that were unexpected on the basis of the structure activity relationships (SAR's) or considered false positive results on the basis of guinea pig tests, human experience or other information.

The experience of the Task Force with the LLNA has been summarised in the following report: A. Penninks (2006): Limitations of the Local Lymph Node Assay (LLNA) as preferred test for skin sensitisation: concerns about false positive and false negative test results, TNO report V7217).

CESIO would appreciate if this report were included in the ICCVAM evaluation process of the LLNA.

CESIO has encouraged its membership to submit data discussed in the Penninks (2006) review to NICEATM.

Yours sincerely,

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(originally signed – sent electronically)



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15 June 2007

Dr. William S. Stokes  
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**RE: Nomination of LLNA peer review panel members**

Dear Dr. Stokes:

On behalf of CropLife America the national trade association representing the crop protection industry, I respectfully nominate Dr. Gregory S. Ladics and Dr. Mike Woolhiser to sit on the Interagency Coordinating Committee of the Validation of Alternative Methods (ICCVAM) local lymph node assay (LLNA) peer review panel to review proposed LLNA uses and procedures.

Drs. Ladics and Woolhiser have extensive experience in toxicology, specifically in the field of immunotoxicology. These scientists bring a high degree of expertise in immunotoxicology and scientific objectivity that will contribute greatly to the charge of ICCVAM regarding its review of the LLNA.

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Attached with this letter, please find a curriculum vitae and brief summary of relevant experience and qualifications for Dr. Woolhiser. Dr. Ladics' curriculum vitae and relevant experience will become available early next week and I will forward those documents to you when they are received.

Best Regards,

A handwritten signature in black ink, appearing to read "M. Maier".

Mark S. Maier, PhD, DABT  
Health Science Policy Leader

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202-296-1585

**Date:** Tue, 12 Jun 2007 16:48:59 +0200

**Subject:** Local Lymph Node Assay Data with Aqueous Products

On behalf of the European Crop Protection Association, I'm forwarding with this e-mail full reports of ECPA's 2006 study on the use of the mouse local lymph node assay with aqueous-based plant protection products (formulations). I also attach a summary of the study in the form of a poster presented at the March 2007 Society of Toxicology meeting.

This submission is in response to the NICEATM request published in the Federal Register on May 17th, 2007 specifically to address the question on "the ability of the LLNA to test mixtures and aqueous solutions"

SoT poster showing overview of the ECPA study:-

Paper by Ryan et al (2002) which includes evidence of the suitability of Pluronic L92 as a vehicle for aqueous materials in the LLNA - this was the basis of the ECPA "validation study"

Individual lab reports testing 3 positive control chemicals and 4 pesticide formulations in the LLNA with Pluronic L92 as vehicle:-

Dow

BASF

Bayer

Dupont

Syngenta (conducted at RCC, Switzerland)

We also intend to forward reports of the guinea pig studies conducted on the four plant protection products at Dow. These will follow shortly.

Please use me as your contact point for any queries or questions on our data and study. My coordinates are:-

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Frankfurt, June 14 2007

Dear Madam / Sir

**Re. LLNA: Request for Comments, Nominations of Scientific Experts, and Submission of Data (Federal Register 72 (95), May 17, 2007, page 27815)**

The European Federation for Cosmetic Ingredients (EFFCI) appreciates a.m. request of NICEATM and the opportunity to contribute with comments and available data relevant to the term of references. EFFCI member companies have experienced over the last years more and more unexpected and unexplainable positive findings in the murine local lymph node assay. Most of these materials are in consumer use for decades without exhibiting any indication of skin sensitizing properties on the basis of guinea pig tests (M+K, Buehler), human data and/or experience. Based hereupon, EFFCI installed a LLNA working group to consider the scientific accuracy of LLNA results with cosmetic raw materials. Beside mechanistic considerations also experimental work was initiated by this working group with materials which apparently are not adequately represented in the existing validation trials of the LLNA.

In this respect EFFCI sponsored the following comparative experimental test with cosmetic raw materials and which we would like to share with NICEATM. EFFCI would appreciate if this report will be included in the ICCVAM evaluation process of the LLNA:

“Comparative Experimental Study  
on the Skin Sensitising Potential of  
Selected Unsaturated Chemicals as  
Assessed by the Murine Local Lymph Node Assay (LLNA)  
and the Guinea Pig Maximisation Test (GPMT)”  
(Annex)

/2

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In this study, eight unsaturated substances and one saturated substance - that were assumed to have low or no sensitisation potentials - were subjected to comparative testing in the LLNA and the Guinea Pig Maximisation Test (GPMT). The aim of this project was to investigate the justification or the potential limitations of the LLNA as a stand-alone method by comparing the sensitizing potential data obtained with these two different tests in strict adherence to their respective OECD guidelines.

EFfCI is also willing to actively participate in the evaluation and review process of this exercise and nominates Dr. Reinhard Kreiling, Chair of the EFfCI Toxicology Working Group as potential member of a possible peer review panel. Dr. Reinhard Kreiling is a Senior Toxicologist and Deputy Head of the Toxicology Department of Clariant GmbH, Sulzbach, Germany. A CV would be available if necessary.

We are at your disposal should you need further clarification or if you wish to discuss the results.

Yours sincerely

A handwritten signature in blue ink, appearing to read 'Peter Ungeheuer', is positioned above the printed name and title.

Peter Ungeheuer  
Secretary General

**Annex**

On 5/21/07 2:09 PM, "Dori Germolec" <germolec@niehs.nih.gov> wrote:

I would suggest Dr. Mary Jane Selgrade at USEPA. I would suggest Dr. Jean Regal at the University of Minnesota and Dr. Michael Luster. Mike has recently retired from NIOSH and is now a consultant. I am sure that you have Drs. Kimber, Basketter and Gerberick as part of the sponsors. I would also suggest Dr. Kimber White from Virginia Commonwealth University, who is our ITOX contractor. Please let me know if you need any additional names. I am not sure if you are looking for government or extramural panelists or both.

Dori

**From:** Dori Germolec  
**Date:** Fri, 29 Jun 2007 11:52:56 -0400  
**To:** "Stokes, William (NIH/NIEHS) [E]", "Choksi, Neepa (NIH/NIEHS) [C]"  
**Cc:** "Tice, Raymond (NIH/NIEHS) [E]"  
**Conversation:** LLNA data for ICCVAM review

Based on the request for data from standard LLNA testing announced in the Federal Register, Thursday, May 17, 2007 (FR\_E7\_9544), I would like to submit 20 reports from the National Toxicology Program's effects to assess the potential for chemicals to induce hypersensitivity, which include standard LLNA testing. Because these are large files I will copy them to a CD-ROM and hand deliver the disc to the ICCVAM office. A majority of these reports also include other studies such as the Mouse Ear Swelling test. I have an additional 17 reports evaluating chemical-induced hypersensitivity that do not include the LLNA, as these studies were conducted before the development of the standard protocol. Please let me know if these reports would also be informative for your data review.

Dori Germolec  
Integrative Toxicology Group  
NIEHS  
79 Alexander Drive  
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Research Triangle Park, NC 27709  
T: (919) 541-3230  
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Page 1 of 2

**Subject:** FR Notice Comments - 72FR27815 - LLNA

**Date:** Friday, June 15, 2007 1:43 PM

Dear Dr Stokes,

Safepharm Laboratories Ltd., UK (SPL) has conducted Local lymph node assays on behalf of sponsoring companies since 1997. The assays have been conducted on a wide variety of chemicals and chemical preparations. Since August 2002 the use of other animal models for evaluation of skin sensitisation potential for regulatory purposes (e.g. methods that require the use of guinea pigs) has been permissible in the UK only if a valid scientific reason can be provided as to why a LLNA cannot be conducted. In effect, the LLNA is the only method that can be used in the UK for assessment of skin sensitisation potential for regulatory purposes. We therefore support the proposed activities of ICCVAM-NICEATM as detailed in the Federal Register vol. 72, No. 95, p.27815-27817, 17 May 2007 in response to the U.S. CPSC nomination of January 10, 2007.

We have witnessed concerns in some areas of the chemical industry, with regard to the applicability of the LLNA for testing of preparations, mixtures and irritant substances, and also with regard to the fact that the LLNA has not always provided results consistent with existing knowledge of the test substance or related test substances. We do not know if all of these concerns are justified, but they can only serve to reduce confidence in the predictive capability of the assay. This is not desirable when the assay offers significant scientific and animal welfare advantages over guinea pig models for many product types, and in a country where the assay is effectively the only available method for evaluation of skin sensitisation potential for regulatory purposes. An assessment of the applicability domain of the assay in its current form and the use of the assay for testing mixtures, preparations, aqueous solutions, irritant substances and metals is therefore very much welcomed. It seems very appropriate to initiate a review of the current peer-reviewed literature and available data, in order to prepare a comprehensive background review document, conduct a review of the validation status of the LLNA for its various uses and to develop relevant performance standards.

It is noted that at its 26th meeting held on 26-27th April 2007 at the European Centre for the Validation of Alternative Methods (ECVAM), the non-commission members of ECVAM Scientific and Advisory Committee (ESAC) considered the reduced version of the LLNA (rLLNA) to be scientifically validated, but only when used as a screening test to distinguish between sensitisers and non-sensitiser and with due regard to the conditions set forth in the official ESAC statement of 27th April 2007. This statement was based on the outcome of a review of LLNA data for 211 chemicals<sup>1</sup>. The review of existing and newly-provided LLNA data proposed by ICCVAM-NICEATM therefore presents an ideal opportunity to assess further the validity of the rLLNA for screening purposes.

As a contract research organisation, SPL is unable to provide data for review by ICCVAM-NICEATM without the permission of its Sponsors, although we

Page 2 of 2

consider it may be possible to provide a summary of study outcomes, coupled to general product type, should this be of interest to ICCVAMNICEATM.

In conclusion, Safeparm Laboratories Ltd. welcomes the proposed activities of ICCVAM-NICEATM in response to the U.S. CPSC nomination of January 10, 2007, and will be pleased to explore ways in which our experience may be of use in the process.

Yours sincerely,  
Robert L. Guest  
Head of Alternative and Acute Toxicology  
Safeparm Laboratories Ltd.

<sup>1</sup> I Kimber, RJ Dearman, CJ Betts, GF Gerberick, CA Ryan, PS Kern, GY Patlewicz, DA Basketter (2006). The local lymph node assay and skin sensitization: a cut-down screen to reduce animal requirements? Contact Dermatitis 2006: 54:181-185



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mbinfo@mbresearch.com

June 4, 2007

Dr. Mary Wolfe  
Director, NTP Liaison and Scientific Review Office  
NIEHS/NIH  
P.O. Box 12233, MD A3-01  
111 TW Alexander Drive  
Research Triangle Park, NC 27709

RE: Nominations to ICCVAM, Non-Radioactive Murine Local Lymph Node Assays, Request for Comment, *Federal Register*, Vol. 72, No.83, pages 23831-23832, May 17, 2007

Dear Dr. Wolfe and Honorable Committee Members:

In response to the Consumer Product Safety Commission's request to NICEATM and ICCVAM to evaluate non-radioactive versions of the Local Lymph Node Assay (LLNA), MB Research Laboratories would like to offer its support for this nomination and extend our assistance and available information towards the validation of non-radioactive LLNA methods.

MB Research Laboratories has developed and routinely performs a commercial research protocol for the assessment of acute dermal sensitization using a **Flow Cytometry-based Local Lymph Node Assay – FC-LLNA**. In contrast to the radioactive LLNA, the FC-LLNA assesses proliferation by determining incorporation of the thymidine analog bromodeoxyuridine (BrdU) into the DNA of lymph node cells, along with evaluation of lymph node cell number, using flow cytometric methods. It is safer to conduct because of the elimination of hazardous radioactive material, and with added endpoints, is able to better identify true sensitizers and false positive irritants.

The FC-LLNA is a direct result of a three-year SBIR grant project (R44-ES-10234-02). The goal of the project was to develop a commercially viable assay that would be a significant improvement over the standard radioactive LLNA while maintaining high levels of accuracy, sensitivity, specificity, and predictivity. During the conduct of our internal validation studies, over 50 chemicals, including sensitizers, nonsensitizers and irritants were tested. Since 2001, more than 80 FC-LLNA studies have been conducted by clients in the chemical, pharmaceutical, and consumer product industries for safety evaluations and potential submission to regulatory agencies.

The FC-LLNA is very similar to the ICCVAM-validated LLNA protocol but adapted for flow cytometric evaluation. Specifically, the dosing method, assay schedule, vehicles and positive controls are identical. Of the similarities, most notably both assays evaluate lymphocyte proliferation and designate a cut off value of stimulation index (SI) = 3 as a positive indication of sensitization.

The significant difference between the two protocols is that in the radioactive LLNA mice are injected by tail vein with <sup>3</sup>H thymide, while in the FC-LLNA mice are injected intraperitoneally with BrdU. Additionally, because the cells are not radioactively labeled, an aliquot can also be stained for immunophenotyping and activation marker analysis, thus reducing the need for additional animal groups. Profiling of immunophenotypic markers such as B220, CD3, I-A<sup>K</sup> and CD69 can be added to our basic protocol to distinguish between sensitizers and false positive irritants. Ear swelling measurements have also been included to the basic FC-LLNA test to evaluate irritation of test articles and screen for possible false positives.

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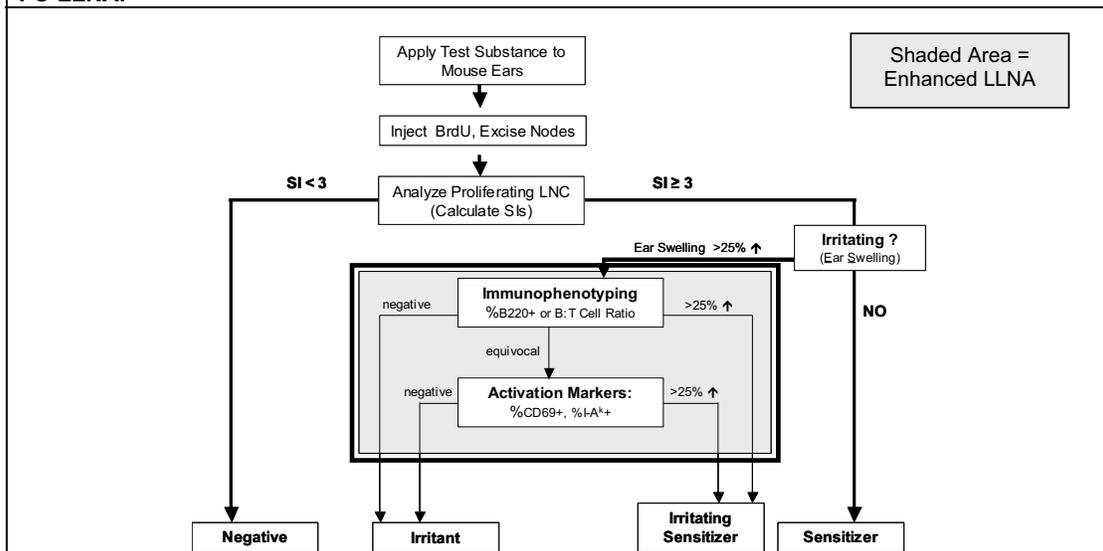
Nominations to ICCVAM, Non-Radioactive Murine Local Lymph Node Assays, Request for Comment  
June 1, 2007

In the FC-LLNA, proliferation of lymph node cells is measured by a combination of BrdU incorporation and total lymph node cell number. As with the radioactive version of the LLNA, an SI of 3 or greater indicates a positive sensitizing response. Each treatment group consists of five mice. Each mouse is evaluated independently by multiplying the total number of lymphocytes by the percentage of lymph node cells that are positive for BrdU incorporation. The total number of proliferating cells in the test group is divided by the total number of proliferating cells in the vehicle group to give a stimulation index. The FC-LLNA yields SI's similar to those in the ICCVAM validation report as well as other published results for the radioactive LLNA. The estimated concentration of chemical required to induce an SI of 3 (EC3), can be used to determine the potency of sensitizers. EC3 values obtained in the cytometric LLNA are quite comparable to those found in the radioactive LLNA, and in most cases fall within the range of values obtained for chemicals tested in the radioactive assay. (See Table 1)

For our validation, immunophenotype analysis of the nodal cells was conducted using the marker combinations B220/CD3 to determine the ratio of B cells to T cells and I-A<sup>k</sup>/CD69 to determine the activation state of the nodal lymphocytes. More specifically, to investigate activation state, the murine MHC class II alloantigen (IA) surface marker was evaluated and the percentage of the total nodal percentage of I-A<sup>k</sup>+ cells that were also positive for the CD69 marker was determined. A major advantage of the FC-LLNA is that immunophenotype analysis can be performed on an aliquot of the cells harvested for SI analysis and no additional animals need be used.

An illustration of the FC-LLNA multi-tiered approach to evaluate sensitizers and eliminate false-positive irritants is shown in Figure 1.

**Figure 1. Multi-tiered Testing Strategy for the Assessment of Sensitization Potential using the FC-LLNA.**



In the first tier, an SI<3 indicates a non sensitizer. For chemicals that elicit an SI>3, ear thickness measurements can be utilized as an indication of irritancy, since CBA mice are brown, thus erythema cannot be evaluated. In the second tier of our FC-LLNA, positive ear swelling flags possible false positive irritants due to the fact that irritants dramatically increase the thickness of the ear, while contact allergens induce a minimal increase in skin thickness due to low inflammatory response. In the last tier, immunophenotyping markers are used to distinguish between true sensitizers and false positive irritants. These markers strongly correlate to positive sensitization potential. Additionally, we have found that some irritants do not increase ear swelling, but can be distinguished from sensitizers because of a lack of immunophenotypic response.

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Nominations to ICCVAM, Non-Radioactive Murine Local Lymph Node Assays, Request for Comment  
June 1, 2007

Table 1 is a list of compounds tested in the FC-LLNA compared to the radioactive LLNA based on SI alone. Also included in the table are a group of equivocal compounds, which were not included in contingency table evaluations.

**Table 1: LLNA Compound List Comparing MB Research Flow Cytometry (FC) LLNA Results with ICCVAM Validation Radioactive (R) LLNA Results**

Positive by Radioactive LLNA			FC	R	Negative by Radioactive LLNA			FC	R
2,4-dinitrochlorobenzene	+	+			6-methyl coumarin	-	-		
Aminophenol HCL	+	+			Benzoic acid	-	-		
Benzoyl peroxide	+	+			Chlorobenzene	-	-		
Chlorpromazine +UVR	+	+			Glycerol	-	-		
Citral	+	+			Hexane	-	-		
Cobalt chloride	+	+			Hydrocortisone	-	-		
Copper chloride	+	+			Isopropanol	-	-		
Croton Oil	+	+			Lactic acid	-	-		
Diethylenetriamine	+	+			Methyl salicylate	-	-		
Diphenylcyclopropanone	+	+			Nickel chloride	-	-		
Ethylene glycol dimethacrylate <sup>#</sup>	+	+			p-aminobenzoic acid	-	-		
Eugenol	+	+			Propylene glycol	-	-		
Fluorescein isothiocyanate	+	+			Propylparaben	-	-		
Formaldehyde	+	+			Resorcinol	+	-		
Hexylcinnamaldehyde	+	+			Sulfanilamide	-	-		
IsoEugenol	+	+			Tween 80	+	-		
Isopropyl Myristate	+	+							
Linalool	+	+							
Oxazolone	+	+							
Potassium dichromate	+	+							
p-phenylenediamine	+	+							
Pyridine	+	+							
Sodium lauryl sulfate <sup>#</sup>	+	+							
Tetrachlorosalicylanilide	+	+							
Trimellitic anhydride	+	+							
Xylene	+	+							

Equivocal			FC	R
Aniline	-	+/-		
Benzalkonium chloride <sup>#</sup>	+	+/-		
Benzocaine	+/-	+/-		
Ethylenediamine	+	+/-		
MBT	+/-	+		
Salicylic acid	+/-	-		

\* = HSE contract research report 399, 2001. Development of the Local Lymph Node Assay for Risk Assessment of Chemicals and Formulations, Rebecca J. Dearman and Ian Kimber, Syngenta Central Toxicology Laboratory, UK, 2001, p.12.

# = Classify as irritants but not sensitizers using the enhanced FC-LLNA with immunophenotype endpoints.

We have also provided in Table 2, a comparative evaluation of data from the flow cytometric assay (FC), the radioactive assay (R), guinea pig results (GP) and human data (H). The cytometric assay has 95% accuracy to the radioactive assay, as well as 93% sensitivity and 100% specificity. Moreover, while the FC-LLNA is less accurate than the radioactive assay when compared to the guinea pig assay (79% vs. 89%) it is more accurate than the radioactive test when compared to human data (88% vs. 72%).

**Table 2: Comparative Evaluation of the Flow Cytometric LLNA**

Comparison of Method	Total #	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity	
		%	#	%	#	%	#	%	#	%	#
<b>FC-LLNA vs. R-LLNA</b>	42	<b>95%</b>	40/42	<b>93%</b>	26/28	<b>100%</b>	14/14	<b>100%</b>	26/26	<b>88%</b>	14/16
FC-LLNA vs. Human	26	88%	22/25	90%	18/20	83%	5/6	95%	18/19	71%	5/7
R-LLNA vs. Human	74	72%	53/74	72%	49/68	67%	4/6	96%	49/51	17%	4/23
FC-LLNA vs. Guinea Pig*	29	79%	23/29	74%	14/19	90%	9/10	93%	14/15	64%	9/14
R-LLNA vs. Guinea Pig*	97	89%	86/97	91%	62/68	83%	24/29	93%	62/67	80%	24/30

Radioactive LLNA results obtained from ICCVAM Validation of the LLNA<sup>b</sup>

\* = Results from Guinea Pig Maximization Test and/or Beuhler Assay

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Nominations to ICCVAM, Non-Radioactive Murine Local Lymph Node Assays, Request for Comment  
June 1, 2007

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Augmentation of the original LLNA with the flow cytometry endpoints increases the sensitivity and discriminating power of the LLNA, while (1) complying with the Animal Welfare Act by directly addressing the reduction in animal number; (2) increasing the quality and quantity of data generated when compared to existing methods; and (3) substantially reducing the cost of analysis and waste disposal by avoiding the use of radioactivity.

In conclusion, MB Research Laboratories fully supports the Consumer Product Safety Commission's nomination to ICCVAM for the evaluation of the non-radioactive LLNA methods for classifying sensitizers and offers to assist ICCVAM by offering the FC-LLNA protocol, validation data and methods for consideration as a direct substitute to the Guinea Pig Sensitization Test.

/s/

Daniel R. Cerven, MS  
Director of Laboratories  
MB Research Laboratories

/s/

Melissa K. Kirk, Ph.D.  
Study Director/Lab Supervisor  
MB Research Laboratories

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June 14, 2007

Dr. William S. Stokes  
Director, NICEATM and  
Executive Director, ICCVAM  
National Institute of Environmental Health Sciences  
PO Box 12233, MD EC-17  
Research Triangle Park, NC 27709

Re: FR notice dated May 17, 2007 (CPSC nomination of Local Lymph Node Assay) -  
response to call for nominations for potential Expert Panel [72 FR 23832].

Dear Dr. Stokes,

This letter is in response to the request for comments on the US CPSC proposal to ICCVAM-NICEATM for an updated evaluation of the validation status of the murine local lymph node assay. I am pleased to submit the nomination of Dr. G. Frank Gerberick to serve on the proposed expert panel to review an updated LLNA Background Review Document and (a.) the validity of proposed modifications to the LLNA (eg. non-radioactive protocols), (b.) as a stand-alone assay for potency determination for classification purposes, and (c.) to explore applicability domains to address Regulatory concerns over the LLNA's validity for testing mixtures, aqueous solutions, and metals. It is expected that this Expert Panel would also review any proposed ICCVAM recommendations for: (e.) current uses and/or limitations for above methods, (f.) test method protocols and/or decision criteria, (g.) performance standards, and (h.) future/additional studies.

Dr. Gerberick is an esteemed colleague at the Procter & Gamble Company in our corporate research division overseeing the global Skin Irritation/Contact Sensitization program. His extensive work over the past two decades in the field of dermal irritation/contact sensitization has made him one of the world's foremost authorities on contact allergy and dermal sensitization. This work has included his pioneering work with the LLNA assay and *in vitro* and *in silico* test methodologies for better scientific understanding of the risk factors for dermal sensitization. It is my opinion that he would be a substantial asset to ICCVAM/NICEATM in the evaluation of new information and proposed applications for the assay he helped pioneer.

Thank you for your thoughtful consideration,

Dan

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Page 1 of 2

**Date:** Thu, 7 Jun 2007 09:00:44 -0400

**Subject:** NTP NICEATM Nomination of experts and response to call for data - LLNA

**Ref.:** Federal Register vol. 72 no. 95, p. 27815, 17 May 2007

Dr. Stokes -

Responding to the request for comment on the US CPSC proposal to ICCVAM-NICEATM for evaluation of the validation status of the murine local lymph node assay, I am pleased to submit the following information for consideration. (The views expressed in item 1.) below are solely my own and do not necessarily reflect the corporate position of GSK.)

1.) Appropriateness and relative priority of items comprising the proposed review of the status of the LLNA: It seems entirely justified that the proposed review should be undertaken based on the large volume of high quality peer-reviewed information published on performance, data evaluation and proposed protocol modifications of the LLNA in the period since the original ICCVAM-sponsored LLNA validation exercise. As proposed by US CPSC, ICCVAM-NICEATM preparation of a comprehensive background review should precede activation of a study panel. Regarding the priority of items for the background review as presented in the Federal Register notice, I suggest that the priority sequence should be slightly rearranged to highlight items 1, 5, 4, 2 and 3 (as identified in the Fed. Reg. notice) in priority sequence. Thus, from most to least pressing: 1. development of data to allow the LLNA to be used as a stand-alone tool in determining potency / severity of sensitising potential of chemicals; 2. evaluation and extension of the domain of applicability of the LLNA; 3. use of the LLNA for testing mixtures, aqueous solutions, and metals; 4. development of an animal-sparing cut-down approach to the LLNA focused on use of untreated vs. single high-concentration test group; and 5. assessment of the status of LLNA methods using non-radiolabeled tracer for end-point analysis.

2.) Nomination of expert scientists to serve on a possible LLNA review panel: I am pleased to offer the name of my GSK colleague Frederick J. Guerriero as a possible panel member. Mr Guerriero is a key member of the GSK Occupational Toxicology working group and in this capacity has had the responsibility of protocol development, study contracting and evaluation of a large number of LLN assays over the past 7-8 years. In addition, Mr Guerriero has previously served on the NICEATM study panel which evaluated *in vitro* alternatives for evaluation of ocular irritant/corrosion effects of chemicals. As a secondary potential candidate for the study panel, I would also be pleased to volunteer my service which is based in similar experience to that of Mr. Guerriero.

3.) Submission of LLNA data: Over the past 5 years GSK has transitioned to sole use of the LLNA as a means for evaluating the sensitising potential of a wide variety of chemical materials used in the synthesis of pharmaceuticals. The spectrum of substances which have been evaluated includes commodity chemicals used as starting materials, proprietary synthetic intermediates of varying structural complexity, and active pharmaceutical entities. All of these

Page 2 of 2

assessments have been conducted by the "traditional" control + 3 concentration protocol using 3H-thymidine label. A small proportion of materials also have companion data evolved with the M&K or Beuhler dermal sensitisation protocol. Although the composite data are not presently in a readily transmitted form, I believe that we could be in position to share results of assessment of ca.190 chemicals if materials from the pharmaceutical sector would be of interest in the assessment which NICEATM is planning.

I will send this letter in print form with mailing today. I look forward to your reply in due course.

Sincerely yours -  
Michael J. Olson, Ph.D.  
Director, Occupational Toxicology  
Corporate Environment, Health and Safety  
GlaxoSmithKline



**Research Institute for Fragrance Materials, Inc.**

50 Tice Boulevard  
Woodcliff Lake, New Jersey 07677 USA  
Phone: 201-689-8089 FAX: 201-689-8090

June 15, 2007

Dr. William S. Stokes  
NICEATM Director  
NIEHS  
P.O. Box 12233  
MD EC-17  
Research Triangle Park, NC 27709

Dear Dr. Stokes:

This letter is in response to the NICEATM request for data on the murine local lymph node assay that appeared in the Federal Register on Thursday May 17, 2007 (Volume 72, No. 95, p. 27815).

The Research Institute for Fragrance Materials, Inc. (RIFM), the international scientific authority for the safe use of fragrance materials, is the most comprehensive source of toxicology data, literature and information on the safety evaluation of fragrance materials. Through extensive research and testing and constant monitoring of all scientific literature available, RIFM maintains a database of fragrance and flavor materials considered the largest repository of this type of information in the world. All of RIFM's scientific findings are evaluated by an independent, scientific Expert Panel—an international group of dermatologists, pathologists, toxicologists and environmental scientists who are completely unbiased with no connection to the fragrance industry. More information about RIFM can be found on the RIFM web site at [www.rifm.org](http://www.rifm.org).

The murine Local Lymph Node Assay (LLNA) has provided toxicologists with a tool that provides both a reduction in the use of animals and a refinement over traditional assays for hazard identification and potency classification of contact sensitizers. Since 2000, RIFM has used the LLNA almost exclusively for this purpose. The data that RIFM has generated in the LLNA has been incorporated into several publications that aim to provide a standardized data set for the development of alternative methodologies.

RIFM has explored the use of the LLNA in various essential oils. Mr. Jon Lalko, RIFM Senior Test Program Specialist managed this project, which had two goals: 1) to investigate the potential of individual essential oils to induce dermal sensitization and to determine the relative potency of the oil; and 2) to examine any difference in sensitization potential for the major components arising from their exposure. The initial work was published in *Food and Chemical Toxicology* (2007), Volume 44, pp. 739-746). A copy of the publication is attached. RIFM has continued to investigate the use of the LLNA in various essential oils. Enclosed is a summary of the LLNA data RIFM has sponsored on several essential oils.

Stokes Letter  
June 15, 2007

2

Much work has been done to correlate the dose-response data obtained in the mouse LLNA with what is known about potency in humans. The EC3 value has recently been demonstrated to closely correlate with the NOEL from human sensitization tests designed to confirm lack of induction. RIFM has compared the relationship between the LLNA EC3 value and the NOEL for sensitization in humans. A detailed analysis of the dermal sensitization data for 31 fragrance ingredients that have exhibited dermal sensitization potential revealed that for the majority of the materials, there is a very good correlation between the EC3 or predicted NOEL from the LLNA and the NOEL in confirmatory human tests. This preliminary analysis was presented at the World Health Organization/International Program On Chemical Safety International Workshop On Skin Sensitization In Chemical Risk Assessment last October. The abstract, which is in press, is attached.

We hope that these data are useful. If there is any more information or details that we can provide, please feel free to contact me.

Best regards.

Sincerely,



Anne Marie Api, Ph.D.  
Vice President,  
Human Health Sciences

AMA/caj

cc: Jon F. Lalko  
Ladd W. Smith

From: "Thorne, Peter S"  
Date: Mon, 21 May 2007 06:50:09 -0500  
To: Neepa Choksi  
Subject: RE: ICCVAM/NICEATM FR Notice: LLNA Nomination and Request for Data

Dear Dr. Choksi:

As you may know, I served on the panel that reviewed the LLNA as the first ICCVAM method. At that time the process was new and less developed than now. One of the challenges we faced was comparing somewhat limited data that were derived from non-uniform methodology. It certainly seems appropriate to take another look at the LLNA at this time and to develop performance standards. I suspect that a new data set will be richer, more methodologically uniform, and likely will include a wider range of compounds for consideration. Thus, it is an appropriate activity and deserves this further attention.

Sincerely,

Peter S. Thorne, PhD  
Professor and Director  
The University of Iowa  
Environmental Health Sciences Research Center

June 15, 2007

Dr William S Stokes  
Director, NICEATM  
National Institute of Environmental Health Sciences  
PO Box 12233, MD EC-17  
Research Triangle Park, NC 27709

**Re: 72 FR 27815; May 17, 2007; National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); the Murine Local Lymph Node Assay (LLNA): Request for Comments, Nominations of Scientific Experts, and Submission of Data**

Dear Dr. Stokes:

These comments are submitted on behalf of the Alternatives Research and Development Foundation, the American Anti-Vivisection Society, Humane Society Legislative Fund, The Humane Society of the United States, People for the Ethical Treatment of Animals and the Physicians Committee for Responsible Medicine. The parties to this submission are national animal protection, health, and scientific advocacy organizations with a combined constituency of more than 10 million Americans who share the common goal of promoting reliable and relevant regulatory testing methods and strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

In January, 2007, (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the “cut-down” or “limit dose” LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated).

ICCVAM reviewed the nomination, assigned it a high priority, and proposed that NICEATM and ICCVAM carry out the following activities in its evaluation: (1) Initiate a review of the current literature and available data, including the preparation of a comprehensive background review document, and (2) convene a peer review panel to review the various proposed LLNA uses and procedures for which sufficient data and information are available to adequately assess their validation status. ICCVAM also recommends development of performance standards for the LLNA. At this time, NICEATM requests: (1) Public comments on the appropriateness and relative priority of these activities, (2) nominations of expert scientists to consider as members of a possible peer review panel, and (3) submission of data for the LLNA and/or modified versions of the LLNA.

At the meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) on June 12, 2007, several comments were made that suggested ICCVAM was assuming a relatively rapid review of these issues. However, this is not borne out by the CSPEC

nomination which does not mention an expedited process. In addition, ICCVAM has recommended the creation of a background review document (BRD) and review by an expert peer review panel, with no mention of an expedited process. The cost/benefit of this LLNA review has not been evaluated, and SACATM was asked to vote to accept or reject NICEATM/ICCVAM's decision to proceed without offering any alternatives. Doubts about the cost/benefit of this project caused one SACATM member to vote against proceeding.

Despite the fact that ICCVAM documents, including the Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods,<sup>1</sup> mention the possibility of an expedited review process, it would appear that this process has only been used in one case. Despite repeated critiques of ICCVAM for failing to act expeditiously, we are still unable to locate a description of the expedited review process in ICCVAM literature and the parameters for applying it.

In light of the fact that the LLNA has been used by regulatory agencies for classifying skin sensitizers for years and both research data and regulatory use of the LLNA have been extensively reviewed in the literature, yet another review of this widely accepted method is unwarranted. The only circumstance under which this proposal is acceptable is if ICCVAM quickly reviews the existing literature and makes an expedited evaluation regarding the relevance of this information to Agency regulatory needs. ICCVAM's limited resources should be spent validating and promoting for regulatory acceptance any of the number of non-animal methods for skin sensitization that are currently in development.

In March 1999, ICCVAM published a final peer review report concluding that the LLNA is a valid alternative to currently accepted guinea pig test methods.<sup>2</sup> The U.S. EPA, FDA, and OSHA announced their acceptance of the LLNA as an alternative to the guinea pig maximization test for assessing allergic contact dermatitis in October 1999. That same year, ESAC, the Scientific Advisory Committee of the European Centre for the Validation of alternative Methods (ECVAM), also endorsed the LLNA for regulatory use.

In September 2000, the European Centre for Ecotoxicology and Toxicity of Chemicals (ECETOC) published a comprehensive review of sensitization test methods with respect to hazard identification and labeling, (and?) to determine whether the various methods are appropriate for determining relative potency and risk assessment.<sup>3</sup> The conclusions from this review included: (1) the LLNA is a viable and complete alternative to traditional guinea pig test methods for the purposes of skin sensitization hazard identification, and (2) the LLNA is suitable for the determination of relative skin sensitizing potency and the adaptation of this method for derivation of comparative criteria such as EC3 values provides an effective and quantitative basis for such measurements. This report further recommends that "the LLNA is the recommended method for new assessments of relative potency and/or for the investigation of the influence of vehicle or formulation on skin sensitizing potency."

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<sup>1</sup> [http://iccvam.niehs.nih.gov/SuppDocs/SubGuidelines/SD\\_subg034508.pdf](http://iccvam.niehs.nih.gov/SuppDocs/SubGuidelines/SD_subg034508.pdf)

<sup>2</sup> <http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm>

<sup>3</sup> ECETOC. 2000. Skin Sensitization Testing for the Purpose of Hazard Identification and Risk Assessment.

More recent work has further verified the use of the LLNA as a stand-alone method for estimating potency for regulatory purposes, including a 2005 study that concludes that there is a “clear linear relationship between LLNA-derived EC3 values and historical human skin patch data.”<sup>4</sup> A 2007 review concludes that “The LLNA, when conducted according to published guidelines, provides a robust method for skin sensitization testing that not only provides reliable hazard identification information but also data necessary for effective risk assessment and risk management.” In addition, a retrospective analysis of the regulatory use of the LLNA in the EU was published in 2006 and concluded that “the LLNA is satisfactory for routine regulatory use.”<sup>5</sup> We acknowledge that the LLNA must be validated for determining sensitization potency for regulatory use; however, we urge ICCVAM to take an abbreviated test validation approach, as was recommended by the recent International Programme on Chemical Safety Workshop on Skin Sensitization in Chemical Risk Assessment:<sup>6</sup> “An abbreviated test validation approach may be appropriate to assess the validity of potency assessment based on the LLNA and its appropriateness for predicting sensitizing induction potency in humans.”

The “cut-down” or “limit dose” LLNA approach (reduced, or rLLNA) has recently been reviewed by an ECVAM peer review panel. In April, 2007, ESAC issued a statement supporting the use of the rLLNA “within tiered-testing strategies to reliably distinguish between chemicals that are skin sensitizers and non-sensitizers “thereby reducing animal use by as much as 50%.”<sup>7</sup> The statement also notes the following limitations: that “the test results provided by the rLLNA do not allow the determination of the potency of a sensitising chemical,” and that “negative test results associated with testing using concentrations of less than 10% should undergo further evaluation”

The applicability and limitations of this modification of the LLNA have been clearly established. Therefore, in lieu of a lengthy review of this method, ICCVAM should expeditiously review and endorse the ESAC peer review and circulate harmonized testing recommendations regarding this assay to US agencies before year’s-end and NICEATM should collaborate with ECVAM to address the question of concentration threshold.

Other recent work has included the development of several applications of non-radioactive detection methods for the LLNA, including BrdU incorporation, methods measuring the release of various cytokines, and methods using fluorescent markers and quantification by flow cytometry. In many cases, these methods have been shown to be as sensitive as protocols involving radio-labeling.<sup>8</sup> In addition, in NIH-sponsored and contract work, MB Research has shown that “for a large range of chemicals, the FC-LLNA EC3 values were consistent with

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<sup>4</sup> Basketter et al. Predictive identification of human skin sensitization thresholds. *Contact Dermatitis*. 2005; 53 (5): 260-267.

<sup>5</sup> Cockshott et al., The local lymph node assay in practice: a current regulatory perspective. *Hum Exp Toxicol* 2006; 25 (7): 387-394.

<sup>6</sup> [http://www.who.int/ipcs/methods/harmonization/areas/sensitization\\_summary.pdf](http://www.who.int/ipcs/methods/harmonization/areas/sensitization_summary.pdf)

<sup>7</sup> [http://ecvam.jrc.it/publication/ESAC26\\_statement\\_rLLNA\\_20070525-1.pdf](http://ecvam.jrc.it/publication/ESAC26_statement_rLLNA_20070525-1.pdf)

<sup>8</sup> Takeyoshi et al. Advantage of using CBA/N strain mice in a non-radioisotopic modification of the local lymph node assay. *J Appl Toxicol*. 2006. 26:5-9. Takeyoshi et al. Novel approach for classifying chemicals according to skin sensitizing potency by non-radioisotopic modification of the local lymph node assay. *J Appl Toxicol*. 2005. 25:120-134. Suda et al. Local lymph node assay with non-radioisotope alternative endpoints. *J Toxicol Sci*. 2002. 27:205-218.

those reported in ICCVAM LLNA validation studies.”<sup>9</sup> Both ECVAM and Japanese Center for the Validation of Alternative Methods (JaCVAM) are currently reviewing these methods and, rather than initiate a full independent review, ICCVAM must collaborate with these ongoing efforts.

With regard to the assessment of the LLNA for aqueous mixtures and metals, the information that is currently available should allow ICCVAM to make a rapid determination of the applicability and limitations of the LLNA for these classes of chemicals and, if it cannot, we do not endorse further validation efforts in this regard, but recommend the pursuit of *in vitro* methods for this purpose.

Several non-animal methods for estimating sensitivity are under development, including quantitative structure activity relationship (QSAR) modeling that shows a high concordance with both guinea pig and LLNA data,<sup>10</sup> quantification of peptide reactivity, which also shows a high concordance with LLNA data,<sup>11</sup> and human cell cultures.<sup>12</sup> We urge ICCVAM to secure an interagency grant from the CPSC to fund the validation of one or more of these non-animal methods. Clearly, ICCVAM and the CPSC both benefit from the sharing of resources, as the CPSC nominated the method and ICCVAM will be tasked with the final work product.

ICCVAM should consider taking an approach similar to the European Sens-it-iv project,<sup>13</sup> which involves the coordinated efforts of more than two dozen groups from industry, academia and other organizations, all working toward the common goal of developing *in vitro* methods to assess immunotoxicity. ICCVAM should consider facilitating the creation of such a goal-oriented task force.

To summarize, given the fact that the LLNA has been used by regulatory agencies for classifying skin sensitizers for years and both research data and regulatory use of the LLNA have been extensively reviewed in the literature and by other countries, yet another lengthy review of this widely accepted method is clearly unwarranted. Instead, we urge ICCVAM to perform an expedited review of the existing information regarding the LLNA’s performance and limitations and to issue recommendations to US agencies with all due speed. In the interest of eventual replacement of animals in sensitization testing, ICCVAM must spend its time and resources promoting the development and regulatory use of non-animal methods, which it can do by engaging in integrated approaches to *in vitro* immunotoxicity.

Sincerely,

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<sup>9</sup> <http://www.mbresearch.com/TOXNOTE/TOXNOTE-LLNA.pdf>

<sup>10</sup> Fedorowicz et al., Structure-activity models for contact sensitization. *Chem Res Toxicol.* 2005; 18(6): 954-969.

<sup>11</sup> Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. 2007; 97(2): 417-427.

<sup>12</sup> Schoeters et al. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced skin sensitization. 2007; 44(12): 3222-3233.

<sup>13</sup> <http://www.sens-it-iv.eu/>

/s/

Catherine Willett, PhD  
Science Policy Advisor  
Regulatory Testing Division  
People for the Ethical Treatment of Animals

/s/

Sara Amundson  
Executive Director  
Humane Society Legislative Fund

/s/

Dr. Martin Stephens  
Vice President for Animal Research Issues  
Humane Society of the United States

/s/

Kristie Stoick, MPH  
Research Analyst  
Physicians Committee for Responsible Medicine

/s/

Sue A. Leary  
President  
Alternatives Research & Development Foundation

/s/

Tracie Letterman, Esq.  
Executive Director  
American Anti-Vivisection Society

**Subject:** LLNA evaluation

**Date:** Wednesday, October 24, 2007 3:38 AM

**From:** Ann-Therese Karlberg

Dear Dr Allen,

Since my group is one of the groups in academia that performs the Local lymph node assay most frequently (one a week for many years) as part of our research program I received your mail from a Danish college.

The thing that I want to comment on is the lack of thorough chemical considerations in the choice of the substances used for testing. The substances chosen for testing should be pure, with conclusive structures and no mixtures in different ways. I will give you two examples among the substances discussed in the lists: 1. Abietic acid is considered a moderate sensitizer. In our investigations of abietic acid we found it extremely easily oxidized when exposed to oxygen in air. Abietic acid itself is not an allergen but is activated by air exposure on normal storage and handling so that allergenic oxidation products are formed in a complex mixture. The most prominent allergens identified are the hydroperoxides which as such also are unstable. In fact it is not possible to keep abietic acid pure and non-oxidized unless it is stored under argon. This makes abietic acid an unsuitable compound for evaluation of LLNA since the activity can vary depending on storage conditions of the substance. 2. Citral consists of the two stereoisomers geranial and neral which are both moderate allergens according to LLNA in our hands. Whether the results obtained in the tests with citral are due to reactions to geranial or neral or both have never been discussed. What can be said is that the dose estimated is not conclusive. Since both geranial and neral are available on the market there is no need to test them in a mixture and get non-conclusive results.

Furthermore, I think it is important that substances with an allergenic activity based on different types of reactive sites should be included to eliminate that only certain types of reactive chemicals are tested. If there are thing that you want to discuss more in detail I would be happy to discuss with you.

Best regards,

Ann-Therese Karlberg  
Professor  
Dermatochemistry and Skin Allergy  
Department of Chemistry  
Göteborg University  
SE-412 96 Gothenburg  
Sweden

**Subject:** FR Notice Comments - 72FR52130: LLNA Performance Standards

**Date:** Monday, September 24, 2007 11:39 AM

Below is the result of your feedback form. It was submitted by  
( ) on Monday, September 24, 2007 at 11:39:19

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Comment\_date: 24 September 2007

Prefix: Dr.

FirstName: Jon

LastName: Richmond

Degree: BSc MB ChB FRDSEd FRMS

onBehalfOf: no

Title:

Department:

Company:

Country: UK

Phone:

EMail:

Comments: This is welcome development, the general approach seems sound, and I have only a few constructive comments at this stage.

1. The document does not set out the need for or divers of a need to develop and validate alternative indices of lymphocyte proliferation.
2. At 2.3.2.2, after line 419, details of all audits and copies of all audit reports should be included.
3. Appendix A, animal selection and preparation, animal species selection: lines 633/634: ths gender and strain are separate consideration and should be listed as separate bullet points.

4. Annex A, animal preparation, line 647 - the acclimitization period whould be AT LEAST 5 days. Also it is not clear if ear-punching or -notching or ear-clips are acceptable means of marking/identifying animals.

5. Annex A, selection of doses, line 699. Whilst it is reasonably clear to those familair with the key reference documents what is in tended, strictly speaking in plain English consecutive doses wold by 100%, 99%, 98% etc.

**Subject:** FW: ICCVAM/NICEATM FR Notice related to the murine LLNA

**Date:** Monday, October 22, 2007 11:34 AM

**From:** Henk van Loveren <Henk.van.Loveren@rivm.nl>

Dear dr. Allen

Thank you for giving us the opportunity to respond to the draft ICCVAM performance standards for the murine LLNA: methods for assessing lymphocyte proliferation.

We have discussed the draft in my group (Janine Ezendam, Rob vandebriel, Wim de Jong) and have the following comments:

Add to line 316 after LLNA: "Especially for the latter category of products to be investigated adaptations may be possible to overcome this problem. See ASTM protocol F2148-01.

ASTM F2148-01. Standard practice for evaluation of delayed contact hypersensitivity using the murine local lymph node assay. ASTM F2148-01, West Conshohocken, PA, USA.

Add to line 337 after proliferation: Should perhaps possible other endpoints be mentioned here? In any case, also modifications in determination of cellular proliferation exist that use ex vivo DNA labeling with tritium-thymidine and should be mentioned here (Kimber and Weisberger 1989, Van Och et al 2000).

Kimber, I., Weisenberger, C. A murine local lymph node assay for the identification of contact allergens. Assay development and results of an initial validation study. Arch. Toxicol. 63, 274-282, 1989.

Van Och, F.M.M., Slob, W., De Jong, W.H., Vandebriel, R.J., Van Loveren, H. A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local lymph node assay: employment of a regression method that includes determination of the uncertainty margins. Toxicology 146, 49-59, 2000.

Add to note 5 at page 6: An alternative mice strain that is frequently used is the BALB/c strain which shows similar responses as the CBA mice (Woolhiser et al 2000).

Woolhiser MR, Munson AE, Meade BJ. Comparison of mouse strains using

the local lymph node assay. Toxicology 146, 221-227, 2000.

Line 441: Delete 20. This gives the impression that you need to validate each alternative assay with these 20 compounds. Or is this the intention?

Line 532:

Why is the CV limited to 30% ? This looks reasonable but in table 2-3 for DNCB two out of 6 laboratories have a CV above 30%, of 35 and 46% respectively.

Line 636: A comparison of the performance of several mouse strains in the LLNA is presented in Woolhiser et al 2000.

Line 659: An example is presented in ASTM protocol F2148-01.

Line 717: The pooling approach should be discouraged as a statistic evaluation is not possible and non responding outliers cannot be detected. Also in the ICCVAM evaluation and proposed protocol pooling is not recommended. Include in text preference for individual sampling and determination of cell proliferation.

Line 743: Add text: For this reason individual sampling should be recommended.

\*\*\*\*\*

Prof. dr. Henk Van Loveren  
National Institute of Public Health and the Environment  
PO Box 1  
3720 BA Bilthoven  
the Netherlands  
tel.....+31(0)302742476  
mobile.... +31(0)646166122  
fax.....+31(0)302744437

\*\*\*\*\*

Page 1 of 3

**Subject:** FR Notice Comments - 72FR52130: LLNA Performance Standards

**Date:** Monday, October 29, 2007 4:31 PM

Dr William S Stokes  
Director, NICEATM  
National Institute of Environmental Health Sciences  
PO Box 12233, MD EC-17  
Research Triangle Park, NC 27709

Re: 72 FR 52130; September 12, 2007; National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments.

Dear Dr. Stokes:

These comments are submitted on behalf of the Alternatives Research and Development Foundation, the American Anti- Vivisection Society, Humane Society Legislative Fund, The Humane Society of the United States, People for the Ethical Treatment of Animals, and the Physicians Committee for Responsible Medicine. The parties to this submission are national animal protection, health, and scientific advocacy organizations with a combined constituency of more than 10 million Americans who share the common goal of promoting reliable and relevant regulatory testing methods and strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

In January, 2007, (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the "cut-down" or "limit dose" LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated). The development of these performance standards is an initial response to this nomination, and ICCVAM is requesting comment on these performance standards.

Although we fully support the development of performance standards that expedite the validation of new protocols that are similar to previously validated methods, we reiterate our disappointment that ICCVAM/ NICEATM has chosen to apply its limited resources to the lengthy process of developing performance standards for such a narrow scope of applicability. These performance standards apply only to modifications of the "standard LLNA" that involve incorporation of non-radioactive methods of detecting lymphocyte proliferation.

A major aspect of the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 U.S.C. 285l-3) is the charge to "reduce, refine, and/or replace the use of

Page 2 of 3

animals in testing where feasible." The performance standards described in this FR notice apply to modifications of the standard LLNA that do not affect the number of animals used in this method. The only conceivable reduction could occur if the availability of accepted non-radioactive methods of detection would allow more laboratories to perform the LLNA, and if they then choose the LLNA over the Guinea Pig Maximization test or the Buehler Test. The issue of how this exercise (development of performance standards with this limited applicability) addresses ICCVAM's mandate of reducing, refining or replacing the use of animals is not currently mentioned in the draft document and needs to be adequately explained.

In addition, the draft performance standards require the use of a minimum of 20 reference compounds. The criteria by which the compounds were chosen and the characteristics of the compounds are described; however, there is no justification for the requirement of such a large number of compounds for this particular method modification. The methods to which these performance standards apply will differ from the "standard LLNA" only in the method of detection of lymphocyte proliferation; therefore the element of concern is sensitivity of the detection method. All other aspects of the methods to be evaluated will be identical to the standard LLNA, including delivery and biological response. It is therefore not necessary to test representatives for every chemical class or every solvent that has been tested in the standard LLNA. The important characteristic of the reference compound is the magnitude of proliferation response that is generated, and the list of reference compounds chosen should be limited to those that represent the range of response seen with the standard LLNA.

Finally, it is the belief of the parties to this submission that the limited resources available to ICCVAM/NICEATM would be better spent on activities that would have greater impact on the reduction, refinement or replacement of animal use, such as evaluating the use of human cell lines or one of the available in vitro skin models as a replacement for the LLNA.

Sincerely,

Catherine Willett, PhD  
Science Policy Advisor  
Regulatory Testing Division  
People for the Ethical Treatment of Animals

Sara Amundson  
Executive Director  
Humane Society Legislative Fund

Dr. Martin Stephens  
Vice President for Animal Research Issues  
Humane Society of the United States

Page 3 of 3

Kristie Stoick, MPH  
Research Analyst  
Physicians Committee for Responsible Medicine

Sue A. Leary  
President  
Alternatives Research & Development Foundation

Tracie Letterman, Esq.  
Executive Director  
American Anti-Vivisection Society

**Subject:** FR Notice Comments - 73FR1360 - LLNA Peer Panel Meeting  
**Date:** Monday, January 28, 2008 9:33 AM

Below is the result of your feedback form. It was submitted by  
( ) on Monday, January 28, 2008 at 09:33:10

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Comment\_date: January 28th 2008

Prefix: Dr.

FirstName: David

LastName: Basketter

Degree: BSc DSc FRCPATH

onBehalfOf: no

Title:

Department:

Company:

Country: UK

Comments: Looking at the very detailed work that has been done on reviewing potency assessments in the LLNA, I am moved to observe that we have here a wealth of information which indicates that relative human potency can be assessed well. The scientific PRP needs to keep in mind that toxicologists working on just about all other endpoints have very much less data. Despite this, decisions on safe exposure limits are made, on a daily basis, for endpoints such as chronic tox etc, solely based on thresholds observed in rat feeding studies (or similar), where there is no validation, no correlation with human effects/potency etc., and if these were subjected to the type of rigorous review being applied to the LLNA, all of them would, without question, fail dismally. Despite limitations, the LLNA offers a good step forward in assessing skin sensitisers. Good toxicologists are those who understand the limitations of an assay, as well as its strengths.

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## **Dr David A Basketter, BSc, DSc, CBIol, FIBiol, FRCPath**

### **Comments on ICCVAM draft document on skin sensitisation potency**

1. A very considerable body of good work has been undertaken and well documented.
2. However, human data on skin sensitisation thresholds has been given undue status as an accurate gold standard. The threshold data (no effect/lowest effect) levels are actually subject to a number of problems. These are outlined below.
3. Human threshold data for an individual allergen often (perhaps the majority of the time) represents the result of a single determination, thus there is very little information on accuracy/reproducibility.
4. As a single determination, one has no idea whether a no/low effect level is close to, or far away from, the true human threshold.
5. The protocols used to generate these human threshold data points are distinctly variable, with clear evidence of differing sensitivities between tests, most notably when comparing the human repeated insult patch test (HRIPT) with the human maximisation test. The HRIPT itself is not a standard procedure, but rather a generic name for a class of test.
6. The protocols are not always fully described, thus assumptions have to be made about certain details, notably the dosimetry (including dose per unit area and time of application, both of which are important determinants of the sensitivity of the assay).
7. The human tests use a highly outbred species, further increasing the variability of these predictive assays.

All of these points are variously made in the publications which compare directly human predictive test and LLNA skin sensitisation thresholds, but I do not see this reflected adequately in the ICCVAM document. I suppose the key point is that LLNA EC3 values, as the document indicates, do show a correlation with human thresholds, but they cannot be expected to predict the historic human data with great accuracy because that historic data is not of itself particularly precise and certainly is very far from representing a gold standard. No amount of statistical/mathematical agonising will tell us more, we just have to live with it and recognise that the human data might be good enough to indicate there is a correlation, but is not good enough to inform us about the quality of that correlation.

Please do not hesitate to ask if you have any questions.

*/s/*

DABMEB Consultancy Ltd, 2 Normans Road, Sharnbrook, Bedfordshire, MK44 1PR, UK  
Tel/Fax: +44-1234782944; Mobile: +44-7788726937; email: david.basketter@ukonline.co.uk

**Subject:** MW of xylene = 107.18, or 106.12?

**Date:** Wednesday, May 6, 2009 8:38 PM

**From:** Kenneth Bogen

**To:** NIEHS NICEATM <niceatm@niehs.nih.gov>

*(attached document: Appendix B of the Nonradioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol - Draft Background Review Document - January 2008)*

Dear ICCVAM Staff:

Your attached draft document lists the molecular weight of xylene as being 107.18, whereas most sources list this as 106.165 or 106.17. What is your source for the 107.18 number, and is it correct?

Best regards,

**Ken**

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*Kenneth T. Bogen, DrPH DABT*

**Exponent**

Oakland, CA 94607

[www.exponent.com](http://www.exponent.com)



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February 22, 2008

William S. Stokes, D.V.M., DAACLAM  
RADM, U.S. Public Health Service  
Director, National Toxicology Program Interagency Center for the  
Evaluation of Alternative Toxicological Methods  
Executive Director, Interagency Coordinating Committee on  
the Validation of Alternative Toxicological Methods  
National Institute of Environmental Health Sciences, NIH, DHHS  
P.O. Box 12233  
Research Triangle Park, NC 27709

Dear Dr. Stokes

Thank you for the opportunity to review and comment on the documents prepared by ICCVAM and NICEATM related to a number of the modifications/proposed uses for the traditional LLNA that will be considered by an independent international expert panel in early March.

The teams have done a great job summarizing the available data on the LLNA and for the most part we are in agreement with the conclusions and recommendations outlined in the documents. What makes the LLNA such a valuable tool for skin sensitization hazard identification and risk assessment is that the strengths and limitations of the assay are recognized so well. I am not sure there is another toxicological test that is more understood and evaluated than the LLNA. I am certain that most experts in the field of skin allergy would agree that the older guinea pig skin sensitization test methods are considerably less understood, specifically related to their lack of evaluation through a formal validation process. Our hope is that this peer review of the LLNA will lead to a better appreciation of the LLNA and more important help researchers develop non-animal test methods for evaluating potential skin sensitizing chemicals by using the robust and quantitative nature of the LLNA as a foundation to compare new alternative methods.

For your review and consideration our LLNA experts (Cindy Ryan, Pierre Aeby, Petra Kern and myself) have prepared comments on the LLNA documents posted on the website. I hope you will find them useful and please let us know if you need any additional information.

Sincerely,

G. Frank Gerberick, Ph.D.  
Research Fellow Victor Mills Society

## **DRAFT ICCVAM Recommendations: LLNA Potency**

### *Comparison of LLNAEC3 values to human data:*

An evaluation of the ability of the LLNA to predict the relative sensitization potency of chemicals in humans necessitates the use of human sensitization data for comparative purposes. In order for such a comparison to provide meaningful information, one must be aware of and understand the limitations in each of the datasets. The human data used in the comparison are derived from either HRIPT or HMT studies in which single test concentrations, expressed as  $\mu\text{g}/\text{cm}^2$ , were used for the induction phase of the test protocol. Therefore, a test concentration could be defined as the NOEL, when in reality it may just be the highest concentration tested to date which did not induce sensitization and there is a probability that higher levels would also fail to induce. This certainly could be the case if a LOEL for the particular chemical has not been identified. Indeed, it is difficult to compare LLNA EC3 concentrations against a human NOEL or an arbitrary value of the LOEL/10 (which is intended to represent an estimation of a probable induction threshold value). On one side, the LLNA data were generated using a test protocol designed to produce quantitative values with dose response information which permit the calculation of the LLNA EC3 and on the other side, the human data were generated by a variety of different human repeated insult patch test and human maximization test protocols which, by design are more qualitative in nature, and unless a series of studies were conducted, provide limited if any information on an induction dose response.

It is concerning that in the evaluation of the LLNA to predict skin sensitization potency in humans key values for the comparison are “pragmatically determined”, as is indicated in lines 335-337 of the background review document “Next, the optimal EC3 value that maximized obtaining the correct skin sensitization calls for strong and weak sensitizers (using one or the other proposed decision criterion) was pragmatically determined.” Similar wording is used in lines 801-804. The method or rationale for this “pragmatic determination” are not clearly evident in the document. A sound statistical approach should have been used instead and would have provided a more scientifically robust comparison.

### *Comparison of LLNA EC3 values to guinea pig data:*

To assess the ability of the LLNA to predict skin sensitization potency in Guinea Pigs is not relevant to the purpose of this review. Guinea pig tests such as the Buehler (BT) and Guinea Pig Maximization tests (GPMT) were designed for the purpose of hazard identification and are poorly suited for potency estimations. While the ECETOC Technical Report No. 87, Contact Sensitisation: Classification According to Potency proposes methods to categorize allergenic potency based on BT and GPMT data, it demands that the study was conducted in full accord with OECD TG 406 and advises judicious interpretation of the data as does a similar European Union commission expert review. While the BT and GPMT have served the toxicology community well for many years as predictive skin sensitization hazard methods, it is important to recognize that, unlike the LLNA, neither of these tests has been formally validated by a recognized organization nor has the inter-laboratory variability been adequately investigated.

In several sections of the background review document, for examples Lines 321-324 and lines 714-717, it is indicated that for each substance with comparative LLNA and guinea pig data, potency was evaluated by comparing the LLNA EC3 concentration against the percentage of responding guinea pigs in the BT or GPMT and the associated induction concentration. Comparing LLNA EC3 concentration against the percentage of responding guinea pigs is not appropriate in our opinion and resulting data are of very different natures; the LLNA measures events associated with the induction of skin sensitization and provides objective, quantitative dose response information whereas data derived from the guinea pig tests are based on a subjective evaluation of skin responses occurring at the elicitation phase of sensitization and provides no dose response information on the induction phase.

It appears that the authors understand the difficulty of comparing LLNA EC3 values with potency classifications based on guinea pig data. In line 395 of the background review document it states that "...for substances that had more than one EC3 or guinea pig response, the geometric mean EC3 value and the weight of evidence GP classification category was used. Although the data generated by the GPMT and the BT is categorical, using the weight of evidence categorization provided some measure of a mean response across multiple studies." Considering the admitted difficulties encountered in dealing with multiple sets of guinea pig-derived data, the authors should be consistent and not make any conclusion based on such comparison.

*Proposed classification categories for sensitization:*

While cut-off values for potency classification are proposed based on either Buehler test and GPMT responses (Table 1-1) we would caution the use of such data in the absence of any other supporting data due to the nature of the test design. In addition, the proposed scheme uses the intradermal induction dose of the GPMT along with the % responders as the basis for classification. We believe that the topical induction concentration should be considered as it is the more relevant route of exposure and the concentration used for intradermal injection is often limited by the addition of Freund's Complete Adjuvant.

The proposed classification (as well as the one proposed by ECETOC TR No. 87) considers only data from guinea pig tests which are defined as 'positive' by the accepted TG 406 definition of a sensitizing chemical (i.e. induces 30% or 15% positive responses in the GPMT or BT respectively). It is possible that a weakly sensitizing chemical tested in a guinea pig test could elicit positive responses in 20% or 25% of the test animals in a GPMT or 10% in the BT, and would be considered as a non-sensitizer and thus would not be classified according to the proposed scheme while a chemical with any LLNA EC3 value would be assigned to one of the 2 proposed categories. Data obtained through the LLNA allows for a continuous spectrum of EC3 values and thus provides a rank ordering of relative potencies which offer more opportunities for categorization beyond two categories. And on the other side, Human and GP tests which are designed to provide yes/no answers have various threshold values creatively proposed in order to force results in the same two categories.

In the proposed two level classification scheme for sensitization potency (Table 1-1), the criteria for classification for category 1 are given as "A high frequency of occurrence...." OR "A probability of occurrence of a high sensitization rate in humans..." and for category 2 are given

as “A low or moderate frequency ....” OR “A probability of occurrence of a low to moderate sensitization rate in humans...”. The frequency of sensitization or the sensitization rate within an exposed population concerns the **prevalence** of allergic contact sensitization to a particular chemical, which is entirely different from the inherent **potency** of the chemical. Therefore the use of such criteria to classify potency is not appropriate. The likelihood of a chemical inducing skin sensitization within an exposed population (i.e. the probable sensitization rate) depends on two key elements: the intrinsic allergenic potency of the chemical AND the conditions and extent of the allergen exposure (e.g. frequency, duration, exposure conditions, etc.). Clinically, the nature, extent and duration of exposure are commonly the predominant determinants of prevalence. The relative potency of a chemical concerns the amount of chemical required to induce sensitization. In general, the more potent the allergen, the lower the dose per unit area required to induce sensitization. Prevalence data are derived from diagnostic patch testing of patients with suspect allergic contact dermatitis, often presenting with clinical disease, in dermatology clinics. The diagnostic patch test itself is designed to detect the weakest degrees of allergy by using occluded exposure conditions for 48 hours and highest allergen concentrations possible to elicit a reaction. For example, the standard patch test concentration for nickel sulfate is 2.5%. Applied in a diagnostic patch test using an 8 mm Finn chamber delivers a dose per unit area of 750  $\mu\text{g}/\text{cm}^2$ , well above the identified human induction threshold of 154  $\mu\text{g}/\text{cm}^2$  (see Table 2 of Appendix A of the LLNA potency background review documents). Many times the nature of the exposure conditions leading to the induction of allergy for these patients is not clearly defined. At best the published results of thousands of such diagnostic patch tests can be used to evaluate trends in patch test reactions.

One example often used to illustrate the difference between potency and prevalence is nickel. It is a very common contact allergen with a relatively high sensitization rate in the US and Europe. However, experimental evidence indicates that nickel is a relatively weak contact allergen, with LLNA EC3 of 140  $\mu\text{g}/\text{cm}^2$  and a human induction threshold of 154  $\mu\text{g}/\text{cm}^2$  for nickel sulfate. The high prevalence is due to the wide distribution, frequent exposure and the nature of exposure, often through ‘compromised’ skin such as body piercing.

Conversely, the preservative methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) is a well known contact allergen considered to be of strong to extreme potency with LLNA EC3 of 2.25  $\mu\text{g}/\text{cm}^2$  and a human induction NOEL of 1.25  $\mu\text{g}/\text{cm}^2$ . In Europe, the prevalence rate of allergy to MCI/MI is stable at 1-3% of patch-tested patients. Considering the number of MCI/MI-containing cosmetics and toiletries that are on the market, the opportunities for exposure and the allergenic potency of the preservative one would expect a much higher incidence rate. The prevalence rate for this potent allergen is kept low because of regulatory guidelines/limits on the level of MCI/MI permissible in certain products, thus limiting the dose per unit area of the exposure. Thus, the clinical prevalence of the strong allergen MCI/MI is low whereas for nickel, a known weak allergen, the prevalence is considerably higher which is opposite of what would be expected if only looking at potency and not considering exposure.

The proposed two level classification scheme for sensitization potency (Table 1-1) does not accurately reflect the range of allergenic potencies that have been demonstrated by both animal and human data. LLNA EC3 values and human induction thresholds clearly span several orders of magnitude as shown by the data in Table 2 of Appendix A of the LLNA potency background

review documents. Human threshold values range from 1.25  $\mu\text{g}/\text{cm}^2$  for MCI/MI, to 250  $\mu\text{g}/\text{cm}^2$  for isoeugenol, to 2755  $\mu\text{g}/\text{cm}^2$  for farnesol, to 20,690  $\mu\text{g}/\text{cm}^2$  for benzyl benzoate. Clinical experience with allergic contact dermatitis would also indicate that discrete classes of sensitizing potency exist (Contact Derm, 2000, 42:344-348).

## **DRAFT ICCVAM Recommendations: LLNA Applicability Domain**

### *Draft Recommendations – Use of the LLNA to Test Mixtures:*

A dataset of 18 mixtures was evaluated, 15 of which had guinea pig data and none had human data. As a result, the LLNA data were compared to the guinea pig data. Since the database is severely limited due to the lack of human data, there is no proof that the guinea pig data would be representative of the human response. Thus, using the guinea pig data as the standard to which the LLNA data should be compared is not appropriate.

In addition, the usefulness of these data is limited further by the fact that information on the ingredients is known for only one of the 15 mixtures and 11 were tested in the LLNA in an aqueous vehicle, the performance of which is also being assessed in this same report.

High quality LLNA mixture data is published in Lalko et al. (2006), cited in section 7.6 of Addendum No. 1 to the ICCVAM report. This publication concerns the evaluation of essential oils and includes analytical data on the composition of the oils as well as LLNA data on the identified major constituents. These data should have been included in the evaluation and not just mentioned as other available scientific reports.

Since the database is severely limited due to the lack of human data, we agree with the recommendation that an assessment of the suitability of the LLNA for testing mixtures should not be conducted until a sufficient quantity of quality data become available. A similar logic of course also applies to guinea pig test methods.

### *Draft Recommendations – Use of the LLNA to Test Metal Compounds:*

The reference dataset contains human data for 17 metal compounds representing 13 different metals. Since the allergenic potential in humans of most all of the known metals has been established, one questions the importance of or need for an assessment of the LLNA's ability to detect metal allergens. However, we agree with the recommendation that the LLNA is useful for the testing of metal compounds. Whether or not the LLNA is useful for testing nickel compounds is of limited importance as nickel is a well known human contact allergen.

In addition, since only 1 of the 14 metal compounds with LLNA and human data was tested in both in an aqueous vehicle, the comparison does not add much value to the assessment, especially in light of the fact that the performance of the LLNA using aqueous vehicles is being assessed in this same report.

### *Draft Recommendations – Use of the LLNA to Test Substances in Aqueous Solutions:*

A dataset of 21 substances tested in aqueous solutions was evaluated, 4 of which had had human data. Since the database is severely limited due to the lack of human data, we agree with the recommendation that an assessment of the suitability of the LLNA for testing substance in

aqueous solutions should not be conducted until a sufficient quantity of quality data become available.

### **DRAFT ICCVAM Recommendations: LLNA Limit Dose Procedure**

#### *Draft Recommendations – Limit Dose Procedure:*

We agree with the recommendation that the LLNA limit dose procedure is appropriate for hazard identification purposes.

We must point out that a 10% concentration threshold for defining non-sensitizing chemicals is not, as suggested in line 44 of the recommendation, proposed by Kimber et al. (2006) as the absolute cut-off. In the discussion section of that same paper, Kimber et al. indicate that for the purposes of that article the 10% threshold was used and that that figure “should not be regarded as inviolable.” They go on to say that a case could be made for using, for instance, either 15% or 20%. In the 2005 Gerberick et al. paper (Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis*, 16(4):157-202), compounds that did not induce a positive response at any concentration tested, with the highest concentration being at least 20% or greater, were categorized as non-sensitizing.

In addition, the 10% threshold concentration at which all which all negative results would be considered valid did not originate in the cited Kimber et al 2006 publication. The original reference is Cockshott et al., 2006, *Human and Experimental Toxicology*, 25:387-394 in which the performance of the LLNA was evaluated in a regulatory context. In that paper, a negative result obtained with the highest concentration tested at 10% would be considered a valid result if the positive control, a mild to moderate sensitizer, gave a positive response. In other words, a chemical which is negative at a top concentration of 10% does not represent a significant human sensitization hazard. This is similar to the definition of a non-sensitizing chemical in the Guinea Pig Maximization Test (GPMT) or Buehler test as one which induces less than 30% or 15% positive responses respectively. Therefore, if a chemical elicits positive responses in 20% or 25% of the test animals in a GPMT, it would be considered as a non-sensitizer from a regulatory perspective.

### **Comments on DRAFT ICCVAM Recommendations: LLNA Non-Radioactive Methods**

#### *DRAFT ICCVAM Recommendations: LLNA BrdU ELISA Procedure*

We agree with the recommendation that more information and data are needed on this method in order to conduct a meaningful assessment of the BrdU ELISA procedure’s performance relative to the traditional LLNA. It is especially important to have information regarding the inter-laboratory performance of this assay.

We do have one suggestion for consideration. Table 6-2 of the Background Review Documents shows a comparison of standard LLNA EC3 values and 0.5x-2x range for the performance standard chemicals and EC3 values calculated from the BrdU ELISA LLNA. Since an alternative SI cutoff for the BrdU ELISA LLNA was identified that provides greater accuracy

than an SI = 3 cutoff i.e., SI = 1.3, a comparison of BrdU ELISA EC1.3 values to standard LLNA EC3 values would be helpful.

***DRAFT ICCVAM Recommendations: LLNA BrdU FC Procedure***

We agree with the recommendation that more information and data are needed on this method in order to conduct a meaningful assessment of the BrdU-FC procedure's performance relative to the traditional LLNA. While the total number of chemicals tested (45) is sufficient, it is especially important to have information regarding the inter-laboratory performance of this assay. The background review document speculates that the transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC would be similar to the traditional LLNA. However, we do not think that will be the case. Flow cytometry is not a trivial technique. It is certainly more error prone than scintillation counting and often the quality of the results is very dependant on trained personnel and precise procedures.

Only 13 of the 18 minimum performance standard reference chemicals have been tested in the LLNA BrdU-FC procedure. This may not be sufficient to assess the test performance according to the ICCVAM Performance Standards for the LLNA. In addition, rather than focusing on the number of chemicals for which the BrdU-FC procedure produced equivocal results or did not obtain 100% concordance with the ICCVAN LLNA performance standard reference chemicals, we believe that it would be of greater value to investigate potential causes for those results. Such information would provide some understanding of the limitations of the methods.

Since the purpose of this evaluation of the LLNA BrdU-FC procedure is to assess its ability to be a non-radioactive alternative to the traditional LLNA, is a comparison with Guinea Pig data justified?

The provided test protocol indicates that at least 6 mice be employed for an irritation prescreen and a possible 12 more be used for the optional quantitative irritation test. Therefore, this method has the potential to use more mice than the traditional LLNA. This requirement for greater animal usage must be taken into consideration when evaluating the BrdU-FC Procedure and it must be determined that the quality or quantity of information provided by this method exceeds that which would be obtained with the traditional LLNA. In other words, are the additional mice required by the BrdU-FC worth any possible additional information that would be gained compared to conducting a traditional LLNA?

***DRAFT ICCVAM Recommendations: LLNA DA Procedure***

Beyond the method to assess lymph node cell proliferation, the test protocol for the LLNA DA contains several key deviations from the OECD Test Guideline 429 recommended protocol and the Essential Test Method Components as described in the Draft ICCVAM Performance Standards for the LLNA . As indicated in the recommendation document (lines 77-79), the LLNA DA has made major modification to the traditional LLNA in both the test substance treatment and sampling schedule. Therefore, this method is outside of the requirements of the draft ICCVAM Performance Standards for the LLNA and should not be consider for validation as an LLNA alternative at this time.

**Subject:** New Form Results 2

**Date:** Tuesday, February 12, 2008 3:07 AM

Below is the result of your feedback form. It was submitted by  
( ) on Tuesday, February 12, 2008 at 03:07:25

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FirstName: Laurence

LastName: Musset

Company: OECD

Title: Principal Administrator

Phone-AreaCode:

Phone-Local3:

Phone-Last4:

Phone-Ext:

QuestionsComments: Questions from the OECD Expert Group on Sensitization

I. The approach by ICCVAM to validate the LLNA for the prediction of strong and weak skin sensitizers poses a methodological challenge. The reason is that the possibility of misclassification in humans of a substance's potency may negatively influence the outcome of the validation; i.e., it is possible that available HRIPT and HMT data may lead to a false human skin sensitization potency categorization. It is often difficult to correctly interpret the total dose used in the human tests due to insufficient documentation of total area dosed or possible prior patient exposure history.

In their analysis, Schneider and Akkan (2004) used the chemicals included in the 1999 ICCVAM validation as a starting point for a literature search to identify skin sensitizers for which quantitative human data on induction doses were available expressed as dose per unit area ( $\mu\text{g}/\text{cm}^2$ ). They were able to identify and assess 46 substances. They were not able to identify more substances as relevant uncertainties are related to limitations in the human data, which mostly come from older studies. First, the reporting of size of the skin area to which the test substance has been applied and of the volume of test solution used is often insufficient. In some cases, skin area and test solution volume could be deduced from information given on types of patches and application systems used. Moreover, in human HRIPT and HMT studies observed incidences for sensitization reactions depend on the concentrations applied

during both the induction and elicitation phase. Often, but not in all cases, the same concentration was applied for both phases. Otherwise, the overall outcome of the test may have been influenced by different elicitation concentrations, a factor not considered in the regression analysis.

In the evaluation performed by ICCVAM in 2008, 76 substances with quantitative human data among them 16 with negative LLNA results have been included. With respect to the points raised by Schneider and Akkan, it is important that it is described why it was possible in the current analysis to include more substances with both positive human and LLNA data (n=60) than Schneider and Akkan (n=46). Therefore, detailed information on ICCVAM's assessment of human dose per unit area is needed and the possibility of misclassification arising from such approach needs to be described. This is important with respect to the assessment of the rate of putative misclassification of strong/weak skin sensitizers using the human data in order to interpret the outcome of the validation study.

- Should the HMT and HRIPT data be treated as equivalent?
- Is a correction factor/uncertainty factor/safety factor of 10 the most appropriate for the extrapolation of LOAEL values to NOAEL values? Schneider & Akkan (2004) used arithmetic means for human and LLNA data except when there were discordant results with varying vehicles. The authors interpolated linearly from the LOEL to a dose corresponding to an estimated sensitization incidence of 5% (DSA05). Griem et al (2003) used LOAELs which were divided by an arbitrary factor in cases of high observed incidences.
- ICCVAM analyzed 250 ug/cm<sup>2</sup> and 500 ug/cm<sup>2</sup> as the cut-off values for a stronger sensitizer. Has the reverse analysis been performed where the LLNA (e.g., at EC3 1% or 2%) and the GP data have been set as the standard and an optimal human cut-off calculated (does it vary between the LLNA and the GP data)?

II. Once criteria are determined for acceptability and use of human data, questions arise about the data from LLNA studies:

- Can the LLNA protocols be narrowed, e.g., by selection of solvents or choice of other test parameters to improve correlation coefficients? Is it meaningful to combine results for different solvents?
- For repeat LLNA studies for a chemical substance, which EC3 value should be selected? Should the geometric mean or the most conservative value be used?

III. How representative of sensitizers may the selection of chemicals with human data be? Does the set of chemicals analyzed by ICCVAM emphasize strong sensitizers?

IV. What are the differences between the validation approach used by Basketter, Gerberick and Kimber (BRD Appendix A) with the approach taken by ICCVAM?

V. With regard to Table 6-2, please compare and contrast the approaches taken by the various investigators represented. That is, analyze the possible sources of variability in the various approaches.

VI. Note that ICCVAM presents the variability among EC3 values for repeat LLNA tests. Can the panel estimate variability for human data points?

VII. When weighing evidence in human or animal data, what are the critical parameters to be considered?

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**Subject:** public comment on federal register of 1/8/08 vol 73 #5 pg 1360 dhs nih  
**Date:** Tuesday, January 8, 2008 7:05 AM

murine local lymph node assay llna test method - attn dr william stokes and sam wilson

use zero animals, not fewer animals. the testing of these materials on animals started in medieval times -1500 a.d. and we should be using more modern, more accurate methods today than torturing animals in labs. use people to test skin sensitization -- then you will get real information on the sensitization. what you are doing is torturing animals. i am sick of that torture of animals.

b. schau

February 22, 2008

Dr William S Stokes  
Director, NICEATM  
National Institute of Environmental Health Sciences  
PO Box 12233, MD EC-17  
Research Triangle Park, NC 27709

**Re: 73 FR 25553; January 8, 2008; National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments**

Dear Dr. Stokes:

These comments are submitted on behalf of People for the Ethical Treatment of Animals and the Physicians Committee for Responsible Medicine. The parties to this submission are national animal protection, health, and scientific advocacy organizations with a combined constituency of more than two million Americans who share the common goal of promoting reliable and relevant regulatory testing methods and strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

Please take note of the following thoughts and transmit them to the Peer Review Panel (PRP) accordingly.

In January, 2007, (ICCVAM) received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification; (2) the “cut-down” or “limit dose” LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the current applicability domain (i.e., the types of chemicals and substances for which the LLNA has been validated).

Now more than a year later, ICCVAM is preparing for a peer review meeting to evaluate its recommendations and findings on these four items. It is unclear when final recommendations will be transmitted to federal agencies, but if ICCVAM’s review of *in vitro* pyrogenicity methods is any indication, it may be at least another year.

Since this review of the LLNA and the proposed recommendations contained therein will lead to little reduction or refinement of animal use in sensitization, the resources that ICCVAM devote to this exercise should be kept to a minimum, and any forthcoming recommendations should be transmitted to agencies immediately following the Peer Review.

We have divided our comments into sections following the FR Notice:

**LLNA limit dose procedures (the reduced or rLLNA) —draft Background Review Document (BRD) and other related documents**

In April, 2007, ESAC issued a statement supporting the use of the rLLNA “within tiered-testing strategies to reliably distinguish between chemicals that are skin sensitizers and non-sensitizers “thereby reducing animal use by as much as 50%.”<sup>1</sup>

In spite of the ESAC recommendations, ICCVAM has conducted its own data call in and data review. The reviewed database is comprehensive and contains a broad cross-section of the chemical universe. The performance characteristics were all above 95% (false negative and positive rates are very low or zero). Even though this additional review was largely unnecessary, we are pleased that ICCVAM’s draft recommendations concluded favorably for the rLLNA procedure and urge the Peer Review Panel to concur. ICCVAM should forward recommendations regarding the use of the rLLNA to federal agencies immediately following the Peer Review.

**Mixtures, metals, and aqueous solutions—draft Updated Assessment of the Validity of the LLNA for Mixtures, Metals, and Aqueous Solutions and related documents**

ICCVAM has evaluated available data with respect to the use of LLNA in predicting the skin sensitization potential of mixtures, metals, and aqueous solutions. In all cases, the limited availability of data prevented a conclusive recommendation for the use of the LLNA; for metals, the LLNA is recommended only as part of a weight-of-evidence approach, which does not significantly promote a reduction in the use of animals.

Clearly this approach to expanding the applicability domain of the LLNA has not proved terribly fruitful, and we do not endorse further validation efforts in this regard, but recommend all resources are directed towards the pursuit of *in vitro* methods for this purpose.

**Potency—draft BRD and related documents**

Once again, ICCVAM has reviewed all availed data and come to a conclusion that is in opposition to that of other experts in the field. For more than 10 years data has been accumulating indicating the potential for the LLNA to make a determination of the sensitization potency of a chemical.<sup>2</sup> Several publications by Basketter and others (many of which are referenced in the BRD) as well as the eloquent argument by Basketter et al. presented in Appendix A, conclude that LLNA is appropriate for determining potency. In September 2000, the European Centre for Ecotoxicology and Toxicity of Chemicals (ECETOC) published a comprehensive review of sensitization test methods with respect to hazard identification and labeling, to determine whether the various methods are appropriate for determining relative potency and risk assessment.<sup>3</sup> The conclusions from this review included: (1) the LLNA is a viable and complete alternative to traditional guinea pig test

<sup>1</sup> [http://ecvam.jrc.it/publication/ESAC26\\_statement\\_rLLNA\\_20070525-1.pdf](http://ecvam.jrc.it/publication/ESAC26_statement_rLLNA_20070525-1.pdf)

<sup>2</sup> Kimber I, Basketter D A. Contact sensitization: A new approach to risk assessment. *Human and Ecological Risk Assessment* 1997; 3: 385 - 395.

<sup>3</sup> ECETOC. 2000. *Skin Sensitization Testing for the Purpose of Hazard Identification and Risk Assessment*.

methods for the purposes of skin sensitization hazard identification, and (2) the LLNA is suitable for the determination of relative skin sensitizing potency and the adaptation of this method for derivation of comparative criteria such as EC3 values provides an effective and quantitative basis for such measurements. This report further recommends that “the LLNA is the recommended method for new assessments of relative potency and/or for the investigation of the influence of vehicle or formulation on skin sensitizing potency.”

More recent work has further verified the use of the LLNA as a stand-alone method for estimating potency for regulatory purposes, including a 2005 study that concludes that there is a “clear linear relationship between LLNA-derived EC3 values and historical human skin patch data.”<sup>4</sup> A 2007 review concludes that “The LLNA, when conducted according to published guidelines, provides a robust method for skin sensitization testing that not only provides reliable hazard identification in formation but also data necessary for effective risk assessment and risk management.” In addition, a retrospective analysis of the regulatory use of the LLNA in the EU was published in 2006 and concluded that “the LLNA is satisfactory for routine regulatory use.”<sup>5</sup>

Despite all of this, ICCVAM’s review of the LLNA for potency determination does not support such a finding, even though, according to the BRD, the LLNA was better overall at predicting sensitization potency than guinea pig data. It is clear from the BRD that different data treatments result in different R<sup>2</sup> values, and the BRD should more clearly discuss the reasons those analysis decisions were made. Further, the BRD should explain in detail why conclusions were drawn that are opposite to that of the evidence they reference.

We urge the PRP to take into account the submission in Appendix A of the draft LLNA-potency BRD, which details why the LLNA is a scientifically appropriate method of potency determination, and the subsequent submitted comment by Dr. David Basketter, a recognized expert in the field of skin sensitization, when making its final report to ICCVAM.

#### **Non-radioactive methods—draft BRDs and related documents**

Three new methods of measuring lymphocyte proliferation have been proposed. Unlike the traditional LLNA, these new methods do not use a radioactive indicator, which could increase the use of the LLNA in facilities that cannot use radioactive material. The new methods include two variants of a bromodioxymidine system [BrdU: ELISA and BrdU: Flow Cytometry (FC)] and the LLNA: DA.

When compared to human data, the LLNA: BrdU-FC had a higher accuracy rate, higher sensitivity, the same specificity, the same false positive rate, and a lower false negative rate than the traditional LLNA. Despite this performance, the assay does not achieve complete concordance with the proposed LLNA Performance Standards the PRP will be evaluating. This is also the case with for the LLNA-DA method, which compares identically to human data, yet

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<sup>4</sup> Basketter et al. Predictive identification of human skin sensitization thresholds. *Contact Dermatitis*. 2005; 53 (5): 260-267.

<sup>5</sup> Cockshott et al., The local lymph node assay in practice: a current regulatory perspective. *Hum Exp Toxicol* 2006; 25 (7): 387-394.

falls short when compared to the traditional LLNA. While reasons for this are not clear, it is worth an examination of whether we should compare new methods to the methods they are replacing or to the endpoint of actual interest.

The BrdU: ELISA has been recommended for use by ICCVAM pending receipt of additional information and using alternative decision criteria. We support this finding. Because of the incomplete concordance between these methods and the traditional LLNA, ICCVAM qualified their acceptance and recommends a “weight-of-evidence” approach. While it is usually good scientific practice to evaluate any test method results in weight-of-evidence manner, qualifications such as these undercut the recommendations and introduce undue confusion to the reader. In our view, this gives a company a clear incentive to conduct more testing, when in reality the methods evaluated have acceptable performance and should simply be recommended.

### **Performance Characteristics**

Although we fully support the development of performance standards that expedite the validation of new protocols that are similar to previously validated methods, we reiterate our disappointment that ICCVAM/ NICETAM has chosen to apply its limited resources to the lengthy process of developing performance standards for such a narrow scope of applicability. These performance standards apply only to modifications of the “standard LLNA” that involve incorporation of non-radioactive methods of detecting lymphocyte proliferation.

In addition, the draft performance standards require the use of a minimum of 22 reference compounds. The criteria by which the compounds were chosen and the characteristics of the compounds are described; however, there is no justification for the requirement of such a large number of compounds for this particular method modification. The methods to which these performance standards apply will differ from the “standard LLNA” only in the method of detection of lymphocyte proliferation; therefore the element of concern is sensitivity of the detection method. All other aspects of the methods to be evaluated will be identical to the standard LLNA, including delivery and biological response. It is therefore not necessary to test representatives for every chemical class or every solvent that has been tested in the standard LLNA. The important characteristic of the reference compound is the magnitude of proliferation response that is generated, and the list of reference compounds chosen should be limited to those that represent the range of response seen with the standard LLNA.

In addition, a major criterion for the selection of the above compounds is that there are Guinea pig data available; more appropriately, chemicals should be chosen on the basis of available human data.

### **Conclusions and Future directions**

This exercise is a good example of actions undertaken by ICCVAM which result in frustration in the animal protection community. In the future we hope that ICCVAM will take a more holistic approach to determine the ways in which it spends its limited time and resources so as to ensure maximum benefit for animals in laboratories.

Several non-animal methods for estimating sensitivity are under development, including quantitative structure activity relationship (QSAR) modeling that shows a high concordance with guinea pig and LLNA data,<sup>6</sup> quantification of peptide reactivity, which also shows a high concordance with LLNA data,<sup>7,8</sup> and human cell cultures.<sup>9,10</sup> We urge ICCVAM to secure an interagency grant from the CPSC to fund the validation of one or more of these non-animal methods. Clearly, ICCVAM and the CPSC both benefit from the sharing of resources, as the CPSC nominated the method and ICCVAM will be tasked with the final work product.

ICCVAM should consider taking a more pro-active approach similar to the European Sens-it-iv project,<sup>11</sup> which involves the coordinated efforts of more than two dozen groups from industry, academia and other organizations, all working toward the common goal of developing *in vitro* methods to assess immunotoxicity.

Sincerely,

/s/

Catherine Willett, PhD  
Science Policy Advisor  
Regulatory Testing Division  
People for the Ethical Treatment of Animals

/s/

Kristie Stoick, MPH  
Scientific and Policy Advisor  
Physicians Committee for Responsible Medicine

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<sup>6</sup> Fedorowicz et al. Structure-activity models for contact sensitization. *Chem Res Toxicol.* 2005; 18(6): 954-969.

<sup>7</sup> Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. *Toxicol. Sci.* 2007; 97(2): 417-427.

<sup>8</sup> Natsch and Emter. Skin sensitizers induce antioxidant response element dependent genes: Application to the *in vitro* testing of the sensitization potential of chemicals. *Tox Sci.* 2008; 102(1): 110-119.

<sup>9</sup> Sakaguchi, et al., Development of an *in vitro* skin sensitization test using human cell lines; huna Cell Line Activation Test (h-CLAT) II. An inter-laboratory study of the h-CLAT. *Toxicol. In vitro.* 2005; 20 (5): 774-784.

<sup>10</sup> Schoeters et al. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced skin sensitization. *Mol. Immunol.* 2007; 44(12): 3222-3233.

<sup>11</sup> <http://www.sens-it-iv.eu/>

**Subject:** page 53 of your five year plan  
**Date:** Monday, May 12, 2008 6:56 PM  
**From:** jean public  
**To:** <niceatm@niehs.nih.gov>, (others)

membership of sac

drug industry profiteers  
other industries profiteers  
1 national animal protection organizaion (who is this?)  
representatives selected by nih from a college, another govt agency, intl regulatory  
body or other corporate profiteers

i note that the revolving door from industry pervades what is going on at this agency.  
and i do not believe this membership is at all a cross section of the american public. i  
urge that you change the membership to more clearly reflect the american public,  
rather than corporate profiteers.

b. sachau

**Subject:** 74 FR 8974; February 27, 2009  
**Date:** Tuesday, April 14, 2009 7:31 PM  
**From:** Kate Willett  
**To:** NIEHS NICEATM <niceatm@niehs.nih.gov>

April 14, 2009

Dr William S Stokes  
Executive Director, ICCVAM  
Director, NICEATM  
National Institute of Environmental Health Sciences  
PO Box 12233, MD EC-17  
Research Triangle Park, NC 27709

**Re: 74 FR 8974; February 27, 2009; National Toxicology Program (NTP); NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM); Announcement of the second meeting of the Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments**

Dear Dr. Stokes:

These comments are submitted on behalf of Physicians Committee for Responsible Medicine, People for the Ethical Treatment of Animals, the Humane Society of the United States, the Alternatives Research & Development Foundation, the American Anti-Vivisection Society, and the Doris Day Animal League. These organizations represent more than ten million Americans who share the common goal of promoting regulatory testing strategies that protect human health and the environment while reducing, and ultimately eliminating, the use of animals.

In January, 2007, ICCVAM received a nomination from the U.S. Consumer Product Safety Commission (CPSC) to evaluate the validation status of: (1) The murine local lymph node assay (LLNA) for determining potency for hazard classification; (2) the “reduced” or “limit dose” LLNA approach; (3) non-radiolabeled LLNA methods; (4) the use of the LLNA for testing mixtures, aqueous solutions, and metals; and (5) the applicability domain of the LLNA.

More than a year later, ICCVAM’s Peer Review Panel reviewed findings on these five items and concluded that insufficient data existed to make recommendations about non-radioactive LLNA methods or the use of the LLNA to test mixtures,

aqueous solutions and metals. The second review panel meeting scheduled for April, 2009, is intended to reevaluate these issues in light of more recent and more complete data.

The draft recommendations resulting from this second review of the LLNA have the potential to lead to reduction or refinement of animal use in sensitization in some sectors, particularly for pesticide formulations and increased use of non-radioactive detection methods. However, we are still concerned that the time and resources that ICCVAM has devoted to this exercise has detracted from serious focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

### **Proposed applicability domain of the LLNA - mixtures, metals, and aqueous solutions**

The limited availability of data or the lack of clear definition of the test substance prevented a conclusive recommendation from the previous ICCVAM review for the use of the LLNA. Draft recommendations from the current review of formulation and aqueous solutions offer a potential for expanded use, if over-classification is accepted (presumably by both the manufacturer and the regulatory Agency). In the interim, little has changed in the availability of comparative human data and we support the review's observation that there is a need to identify relevant human data and human experience in order to continue to evaluate the applicability of LLNA to mixtures and aqueous solutions. As this approach would provide the most valuable information and does not involve further animal testing, it should certainly be a priority at this time.

During this second review, ICCVAM has come to essentially the same conclusion regarding the usefulness of the LLNA for testing metals that it had in May 2008 – that the LLNA may be useful except in the case of nickel-containing compounds.

### **Validation status of three modified (non-radioactive) LLNA test methods**

Three new methods of measuring lymphocyte proliferation have been proposed. Unlike the traditional LLNA, these new methods do not use a radioactive indicator, which could increase the use of the LLNA in facilities that cannot use radioactive material. The new protocols include two methods for detecting bromodioxymidine incorporation [BrdU-ELISA and BrdU-Flow Cytometry (FC)] and a method for detecting ATP content (LLNA: DA).

When compared to human data, the **LLNA: BrdU-FC** had a higher accuracy rate, higher sensitivity, the same specificity, the same false positive rate, and a lower false negative rate than the traditional LLNA. In order to better understand this lack of concordance, the 2008 panel requested original records for all of the studies included in the evaluation. Despite not receiving those original records, ICCVAM proceeded with the re-evaluation of this test method and, not surprisingly, arrived at a similar conclusion; that the method may prove useful; however, recommendations for use are deferred pending release of the requested data. Not only does this represent wasted effort on the part of ICCVAM and the PRP, it continues to beg the larger question of whether it is relevant to be comparing a new method, such as the LLNA: BrdU-FC, to the traditional LLNA rather than to the endpoint of actual interest, human sensitivity.

ICCVAM has concluded that it is now appropriate to recommend the **LLNA: BrdU-ELISA** and **LLNA: DA** methods with specific limitations in the decision criteria. Substances falling within an intermediate stimulation index (SI) specified for each method would be subjected to an “integrated decision strategy in conjunction with all other available information (e.g., dose response information, statistical analyses of treated vs. control animals, peptide-binding activity, molecular weight, results from related chemicals, other testing data).” While we support this finding in general, we believe that it should be made clear that “other testing data” refers to retrospective analyses rather than initiation of additional tests in animals.

The panel also recommends that all three of these alternative detection methods be evaluated for their ability to assess mixtures, metals, and aqueous solutions concurrently with the assessment of these substances in the traditional LLNA. Since the only difference between these methods and the traditional LLNA is the method of detection, it is unlikely that there will be any differences in the applicability of these methods and the traditional LLNA with regard to mixtures, metals and aqueous solutions. Therefore, it would be highly inappropriate to perform these redundant studies, especially since there are no available data for comparison.

### **Conclusions and Future Directions**

If, based on the Draft Recommendations from this second review, the LLNA becomes a standard for pesticides formulations and if recommendations for the non-radioactive methods allow more laboratories to perform the LLNA over the Guinea Pig Maximization test or the Buehler Test, in a best-case scenario, this will

result in a moderate reduction in animal use. ICCVAM has devoted a significant portion of its resources over the past two years to these activities and we feel this is a misappropriation of ICCVAM's limited resources and do not endorse further validation efforts in this regard. Instead, we recommend that ICCVAM's limited resources be directed toward the pursuit of *in vitro* methods for this purpose.

Several non-animal methods for estimating sensitivity are under development, including quantitative structure activity relationship (QSAR) modeling that shows a high concordance with guinea pig and LLNA data [1], quantification of peptide reactivity, which also shows a high concordance with LLNA data [2, 3], *in vitro* skin models [4], and human cell cultures [5, 6]. We urge ICCVAM to secure an interagency grant from the CPSC to fund the validation of one or more of these non-animal methods.

ICCVAM should consider taking a more pro-active approach similar to the European Sens-it-iv project [7], which involves the coordinated efforts of more than two dozen groups from industry, academia and other organizations, all working toward the common goal of developing *in vitro* methods to assess immunotoxicity.

Sincerely,

Nancy Douglas, PhD  
People for the Ethical Treatment of Animals

Catherine Willett, PhD  
People for the Ethical Treatment of Animals

Kristie Stoick, MPH  
Physicians Committee for Responsible Medicine

Martin Stephens, PhD  
The Humane Society of the United States

Sara Amundson  
Humane Society Legal Fund  
Doris Day Animal League

Sue Leary  
Alternatives Research & Development Foundation

Tracie Letterman, Esq  
American Anti-Vivisection Society

[1] Fedorowicz et al. Structure-activity models for contact sensitization. *Chem Res Toxicol.* 2005; 18(6): 954-969.

[2] Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. *Toxicol. Sci.* 2007; 97(2): 417-427.

[3] Natsch and Emter. Skin sensitizers induce antioxidant response element dependent genes: Application to the in vitro testing of the sensitization potential of chemicals. *Tox Sci.* 2008; 102(1): 110-119.

[4] Hayden et al. 2003. *In vitro* skin equivalent modes for toxicity testing. Published in Alternative Toxicological Methods. Editors H. Salem, S.A. Katz. CRC Press LLC, Boca Raton, FL, USA, 229-247.

[5] Sakaguchi, et al., Development of an in vitro skin sensitization test using human cell lines; huna Cell Line Activation Test (h-CLAT) II. An inter-laboratory study of the h-CLAT. *Toxicol. In vitro.* 2005; 20 (5): 774-784.

[6] Schoeters et al. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced skin sensitization. *Mol. Immunol.* 2007; 44(12): 3222-3233.

[7] <http://www.sens-it-iv.eu/>



15-July-2009

Dr. William S. Stokes, Director, NICEATM, NIEHS,  
P.O. Box 12233,  
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**Re: Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products**

Dear Dr. Stokes,

Sanofi-aventis U.S. Inc, a member of the sanofi-aventis Group, appreciates the opportunity to comment on the above-referenced report, the *Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products* and provide the following comments:

General Comments

The document is quite technical and comments will focus on sections 1-3 and section 4, testing of pesticide formulation. Sanofi-aventis acknowledges some positive approaches to the LLNA methods proposed within the report. These approaches include the reduction in the number of animals, the replacement of the guinea pig, and the avoidance of radioactive compounds, and the use of negative and positive controls for the three methodologies. While the report offers three modified methodologies for the LLNA, these methodologies do not highlight significant progress from the classical LLNA.

Specific Comments

Section 1.0 – LLNA-DA

1) In this protocol the justification for replacing the guinea pig is provided. The replacement is not mentioned for the LLNA-BrdU-FC or the LLNA-BrdU-ELISA. It could be mentioned for the other two methodologies.

2) An explanation of the use of sodium lauryl sulfate is need due to ethical reasons.

Section 2.0 – LLNA BrdU-FC

1) In this protocol the ear swelling is recommended to evaluation irritancy. The assessment would be interesting for the LLNA-DA and LLNA BrdU-ELISA or the rationale to incorporate the ear swelling in this method needs to be explained.

2) The difficulties of the LLNA reside in classifying compound based on decision criteria for stimulation index and in discriminating irritancy from sensitization. The LLNA BrdU-FC method might offer the ability to discriminate irritants from sensitizers but might be problematic for weak sensitizers. For this assay, no inter-laboratory studies have been performed so a great deal of work is necessary to validate this approach.

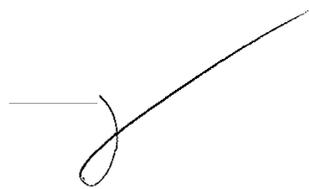
Section 3.0 – LLNA BrdU-ELISA

1) The number of animals is not homogeneous between the three methodologies (LLNA-DA: 4 mice; LLNA BrDU-FC: 4-5 mice; LLNA BrdU-ELISA: 8 mice). The inconsistency might trigger the preference to avoid LLNA-BrdU-ELISA for ethical reason.

2) The validated benchmark for positive effect in the LLNA is a stimulation index of  $\geq 3$ . When a value very close to 3 is observed, standard practice is to repeat the assay to obtain either a definitive result or confirm an equivocal finding. As written, the recommendation by ICCVAM appears to discourage this practice when using the LLNA BrdU-FC. This does not appear to be related to the number of animals needed and therefore there is no obvious explanation.

Sanofi-aventis appreciates the opportunity to comment on the draft ICCVAM report and hopes the comments provided are useful in preparing the final report.

Sincerely,

A handwritten signature in black ink, appearing to read "B. Harvey", is written over a horizontal line.A handwritten signature in black ink, appearing to read "B. Harvey", is written over a horizontal line.

Brian E. Harvey, M.D., Ph.D.  
Vice President  
Regulatory Policy

## **Appendix F3**

### **Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments**

#### **SACATM Meeting on June 18-19, 2008**

The following is excerpted from the final minutes and speaker presentations of the SACATM meeting convened on June 18-19, 2008. The full meeting minutes are available online at:  
<http://ntp.niehs.nih.gov/go/8202>

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Minutes from the June 18 -19, 2008 SACATM Meeting

**IX. VALIDATION STATUS OF NEW VERSIONS AND APPLICATIONS OF THE  
MURINE LOCAL LYMPH NODE ASSAY**

**A. Introduction and Overview of Proposed Methods and Applications**

Minutes from the June 18 -19, 2008 SACATM Meeting

Dr. Marilyn Wind presented the *Report on the Independent Scientific Peer Review Meeting: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA), a Test Method for Assessing the Contact Dermatitis Potential of Chemicals and Products - Introduction and Overview*, on behalf of Dr. Joanna Matheson, Co-chair of the ICCVAM Immunotoxicity Working Group. In 2007, the timeline for the ICCVAM evaluations included the nomination from the CPSC, endorsement by ICCVAM, SACATM's endorsement of the recommended high priority for ICCVAM evaluation, and preparation of six detailed draft background review documents and draft performance standards. In 2008 the LLNA peer review panel met and a report was made available. The new/updated LLNA applications and protocols reviewed by the peer review panel included: LLNA limit dose procedure; LLNA for testing mixtures, metals, and aqueous solutions; non-radioactive LLNA: DA method; non-radioactive LLNA: BrdU-FC method; non-radioactive LLNA: BrdU-ELISA method; draft ICCVAM LLNA performance standards, and use of the LLNA for potency determinations. The documents prepared by NICEATM and the ICCVAM Immunotoxicity Working Group for each new/updated LLNA application included the draft BRD, the draft ICCVAM test method recommendations, and questions for the peer review panel.

Dr. Wind gave an overview of the murine LLNA test method protocol, explaining its initial development in 1986 by Kimber *et al.* (1986), its purpose, the dose levels used, and the stimulation index (SI). The test substance is applied to mouse ears and the mice are then injected through the tail vein with radiolabeled thymidine (or an analogue of thymidine). Lymph nodes are removed and the amount of radiolabel in the lymph node is determined as a measure of lymphocyte proliferation. A test substance with a stimulation index (SI) of 3 is considered a sensitizer.

The LLNA limit dose test method protocol differs from the traditional LLNA protocol in that only a single dose, the highest dose that does not induce systemic toxicity or excessive local irritation, is used. The LLNA limit dose test method database has data from 471 studies, representing 466 unique substances. Results with the LLNA limit dose test method almost always agree with results from the traditional LLNA. The draft ICCVAM recommendation was that the LLNA limit dose procedure should be used for the hazard identification of skin sensitizing substances if dose-response information is not needed.

Dr. Wind explained that there has been a comprehensive update of available data and information regarding the current usefulness and limitations of the LLNA for assessing the skin sensitizing potential of mixtures, metals, and substances tested in aqueous solutions. Substances used for the update included 18 mixtures, 17 metal compounds represented by 13 different metals, and 21 substances tested in aqueous solutions. Evaluating the test method performance for mixtures compared to guinea pig, the LLNA has an accuracy of 53% (8/15), a sensitivity of 50% (3/6), a specificity of 56% (5/9), a false positive rate of 44% (4/9), and a false negative rate of 50% (3/6). There were no comparative data for mixtures tested in humans.

Evaluating the test method performance for substances in aqueous solutions, the LLNA had 50% accuracy, 33% sensitivity, and 100% specificity compared to human data. Comparing guinea pig data, the false positive rate was 67%. The LLNA had 50% accuracy, sensitivity, and specificity. The false positive and false negative rates were high at 50% (n = 6).

## Minutes from the June 18 -19, 2008 SACATM Meeting

Evaluating the test method performance for metal compounds, excluding nickel, the LLNA had 86% accuracy, 100% sensitivity, and 60% specificity compared to human data for all metal compounds (n = 14). The false positive and false negative rates were 40% and 0%, respectively. The LLNA had similar accuracy and sensitivity when compared to guinea pig data (n = 6). Based on one substance tested, the false positive rate was 100%. ICCVAM prepared draft recommendations stating that the LLNA appears useful for the testing of metal compounds, with the exception of nickel. More data are needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures and aqueous solutions will be made.

Dr. Wind reviewed the non-radiolabeled LLNA: DA test method protocol and the data from 31 substances tested by Daicel Chemical Industries. The LLNA: DA had at least 90% accuracy, sensitivity, and specificity when compared to the traditional LLNA. The draft ICCVAM-recommended use was that the LLNA: DA may be useful for identifying substances as potential skin sensitizers and non-sensitizers. The non-radiolabeled LLNA: BrdU-FC test method utilized data from 45 substances submitted by MB Research Labs. The draft ICCVAM-recommended use was that the test might be useful for identifying substances as potential skin sensitizers and non-sensitizers but more information and data are needed. The non-radiolabeled LLNA: BrdU-ELISA test method used data from 29 substances. The draft ICCVAM recommended use was that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but more information and data are needed.

Dr. Wind reviewed the draft LLNA performance standards proposed for the assessment of versions of the LLNA that vary only from the ICCVAM-recommended LLNA by using non-radioactive vs. radioactive methods. The proposed minimum list of reference substances includes 18 substances ranging from strongly positive to strongly negative and for which there are available LLNA, guinea pig, and human data. The proposed accuracy standards are based on a chemical-by-chemical match and a set of four “optional” substances for demonstrating improved performance. She then discussed the proposed intralaboratory reproducibility standards that should be derived on four separate occasions and at least one week between tests to ensure that the tests are independent using two specified chemicals with known skin sensitizing potential.

Use of the LLNA for potency categorization as a stand-alone assay was determined using 170 substances with LLNA, human, and/or guinea pig data. The draft ICCVAM-recommended use was that the LLNA should not be considered a stand-alone test for potency categorization, but could be used in a weight-of-evidence evaluation to discriminate between strong and weak sensitizers. Dr. Wind closed her presentation with a description of the independent scientific peer panel meeting held at CPSC headquarters in March 2008 with attendance of over 50 people from five countries. The panel included experts in dermatology, toxicology, biostatistics, regulatory policy, immunology, and veterinary medicine.

**B. Overview of the Panel Report**

Dr. Luster presented the *Overview of the LLNA Independent Scientific Peer Review Panel Report*, starting with the charge to the panel, which was to review the draft BRDs and evaluate

Minutes from the June 18 -19, 2008 SACATM Meeting

the extent to which applicable validation and acceptance criteria of toxicological test methods have been appropriately addressed. Further they were to consider the ICCVAM draft test method recommendations for proposed method uses and limitations, recommended standardized protocols, test method performance standards, and proposed future studies and was asked to comment on the extent to which they are supported by the information provided in the BRD. LLNA modifications and applications evaluated included: LLNA limit dose procedure; LLNA for testing mixtures, metals, and aqueous solutions; non-radiolabeled LLNA: DA method; non-radiolabeled LLNA: BrdU-FC method; non-radiolabeled LLNA: BrdU-ELISA method; draft ICCVAM LLNA performance standards, and the use of LLNA for potency determinations.

He reported that the panel recommended the LLNA limit dose procedure, or rLLNA, which follows the traditional LLNA protocol except for the number of doses tested, for the hazard identification of skin sensitizing chemicals when dose-response information is not required. The panel also recommended that it could be used as an initial test when dose-response information is required.

The panel agreed with the ICCVAM draft recommendation for the use of the LLNA to test mixtures, metals, and substances tested in aqueous solutions and emphasized the need for the continued accrual of information (i.e., LLNA data, comparative guinea pig and human data) for mixtures, metals, and substances tested in aqueous solutions. The panel agreed with the draft ICCVAM recommendations that the LLNA: DA, LLNA: BrdU-FC, and LLNA: BrdU-ELISA non-radiolabeled test methods may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but this recommendation is contingent upon receipt of additional data and information.

Regarding performance standards, the panel agreed that the use of non-radiolabeled reagents for measuring cell proliferation is a "minor" modification of the traditional LLNA protocol. Other allowable minor modifications include sex, strain, species, animals per group, and timing of test article treatment. The panel emphasized that regardless of the modification, there is the same expectation of performance and that the test method must measure only the induction phase of the immune response. They also recommended that data be collected at the level of the individual animal, that five mice per dose group be used (until reliable power calculations are conducted), and that concurrent positive controls be run until the laboratory has extensive historical data.

Regarding accuracy standards, the current database does not support the inclusion of EC3 values as a component of the accuracy evaluation. For use in hazard identification, a modified method should be evaluated with all 22 substances on the ICCVAM list (including the four optional substances) and accuracy statistics calculated. Regarding reliability standards, the panel considered using the ECt range as appropriate for the intralaboratory reproducibility analysis. They stated that the appropriateness of the 0.5x to 2.0x EC3 range for the reference substances has not been adequately justified.

The panel agreed with ICCVAM that the LLNA should not be considered a stand-alone assay for categorization of skin sensitization potency, but rather it could be used in a weight-of-evidence evaluation to discriminate between strong and weak sensitizers. More data are needed to

## Minutes from the June 18 -19, 2008 SACATM Meeting

determine the optimal threshold in humans for distinguishing between strong and weak sensitizers.

Dr. Fox asked about the dose of BrdU and the sacrifice time following application of the chemical for the LLNA: BrdU-FC and LLNA: BrdU-ELISA test methods. He said it is important because BrdU is cytometric and expensive. Dr. Allen said NICEATM does not have a dose per weight, only a volume, which is 200 µl per mouse, and 5 hours after BrdU administration, the lymph nodes are excised for the LLNA: BrdU-FC protocol and 24 hour post injection collected for the LLNA: BrdU-ELISA. Dr. DeGeorge said the dose is administered by the weight of the animal; it is 20 µl per gram of body weight. The concentration of the BrdU injected is 100 mg/ml. He said the kinetics that were done fall between a 2 and 10-hour range, where 5 hours is the common sacrifice time. Dr. Freeman said at his company they make a standard solution and vary the volume by the weight of the mouse. Dr. DeGeorge said the information is in the BRDs.

**C. Public Comments**

Dr. DeGeorge registered as a public commenter and provided an annotated handout of pages 23, 24, 33, and 34 from Dr. Wind's presentation titled, *Introduction and Overview of the Proposed Methods and Applications*. He stated that although his laboratory conducts the LLNA, he is not specifically representing his lab, but is there on the basis of his experience conducting hundreds of LLNAs with various chemicals. He stated that the IP kinetics/IV dosing of BrdU can be done, though it is technically difficult, and that BrdU is less expensive than radioactive compounds. He asked SACATM to make specific recommendations that were lacking in previous expert reviews and in the tremendous amount of work that has been presented. He noted that originally the list of performance standards included 18 substances, but it was changed to add four more substances. Two tested as false positives and two as false negatives in the original LLNA vs. modified LLNA and he questioned their inclusion as test substances. Dr. DeGeorge said today was the first he had heard that 100% results would not be necessary for the modified LLNAs to be accepted. He cited the BRDs as stating that you should conduct accuracy calculations and statistics. If 18 of 18 chemicals were correct, there would be no reason in seven separate test areas to require calculations of accuracies, selectivity, and sensitivity. That number would always be 100% and anything less would fail. He believed that the true intention is not to hold the modified LLNAs to a higher standard than the original LLNA, which had an accuracy of between 72 and 86%, depending on comparisons to guinea pig or human. With respect to the flow cytometry LLNA, originally it was designed for a wide range of chemicals and included equivocal substances. In the future, picking compounds that are not clearly positive or negative should be discouraged. He stated that now the gold standard has switched. For five of the 13 sensitizers on the performance standards reference substance list, there are data from only one LLNA study for each substance.

He further stated that there would be more data for the modified LLNA than the data to which it is being compared. He called upon SACATM to espouse criteria for validation that specify a minimum accuracy and offered 90% as a reasonable number for concordance accuracy. In the case of specificity and selectivity, he suggested 80%. He considered these values to be well above the original standards and commonly recognized as acceptable. He asked SACATM to address the test method performance standards. He cited the BRDs that discuss the use of

Minutes from the June 18 -19, 2008 SACATM Meeting

substitutes or alternative compounds, as long as they are robust and asked SACATM to allow them. He mentioned proposed additional studies and said it should be explicitly specified whether or not they are required because the BRD says the 18 chemicals need to be tested. Regarding interlaboratory reproducibility, he said you cannot move to interlaboratory validation with animals until intralaboratory validation is completed.

Kate Willett, from PETA, congratulated ICCVAM on the speed at which the review was completed. She recognized the need for development of performance standards for the methods in general, but if the comparison is between radioactive and BrdU, then the number of reference compounds is excessive. In comparing detection methods, she suggested using only a few compounds that have highly reliable data and challenging the ends of the spectrum for testing sensitivity. She then asked ICCVAM and SACATM about plans to deal with follow-up for some of the assays. She said some assays were left with no recommendation pending additional data and it sounded like additional data would be forthcoming. She asked about ICCVAM's schedule or plan for reviewing the data, because she would like to see the review completed and have ICCVAM resources spent elsewhere.

Dr. Wind responded that more data are coming in and when they get all the data ICCVAM intends to reconvene the panel to look at the new data and make recommendations.

**D. SACATM Discussion**

Dr. Ehrich, a lead discussant, provided written comments that Dr. White read into the record. "• LLNA Limit Dose Procedure: 153/153 nonsensitizing agents detected and 308/318 sensitizing agents detected. The numbers make this assay look good.

• LLNA for Testing Aqueous Solutions, Metals and Mixtures: 18 mixtures tested, some without guinea pig data for validation. 17 metals tested, 12/14 sensitizers detected with 2/5 false positives. Not enough products tested to say how good this will be for metals. 21 agents at least 20% water tested but only 4 with human data, which is not enough, so can't offer opinion about this.

• Non-radioactive LLNA protocol – the LLNA DA Test Method: performance >90% for the 19 + 10 sensitizer/nonsensitizers examined, with false positives <10%. Not sure if this would be good enough for mixtures, metals or aqueous solutions.

• Non-radioactive LLNA protocol – the LLNA BrdU-FC Test Method: Flow cytometry used, with 45 test agents. Some gave equivocal results and no multi-lab studies yet. Reference studies need work. This is promising but not ready yet.

• Non-radioactive LLNA protocol – the LLNA BrdU-ELISA Test Method: This is still in progress, 23 compounds tested with an accuracy of 83%. Not detailed protocol yet. Premature to make judgments.

• Draft ICCVAM LLNA Performance Standards: no comment.

• Use of the LLNA for Potency Determinations: Purpose unclear. Was this for a validation study?"

Dr. Brown, a lead discussant, said she was a bit overwhelmed by the amount of material and focused on the final conclusions, relying on the panel and their expertise. She was impressed with the process, the number of individuals, and the thoroughness of the report. She expressed

Minutes from the June 18 -19, 2008 SACATM Meeting

disappointment that more conclusive recommendations could not be made from the material and that data came in too late. She asked if there were a way to make sure the data are available before setting the meeting. Dr. Brown said she shared some of the sentiments expressed by the public, such as what are the next steps. She proposed finishing this evaluation and making concrete recommendations. Tests that do not use radioactivity should get more acceptances and it is important to get the method out and get people using it. She did not find any omissions in the document. She was unclear on the purpose of the performance standards and how they would be used. She thought it should be clear what the gold standard is when asking people to provide data. The platinum standard is really what happens in humans because that is what we are trying to mimic. She said animal data are acceptable as an alternative to human data and that it is sometimes necessary to accept small sample sizes due to the limited use of alternative test methods. Dr. Stokes responded by reiterating that ICCVAM worked very swiftly once the nomination was made. NICEATM had to create the draft BRDs because the test sponsors did not submit them. He said preparing the BRDs was a huge undertaking, and test sponsors submitting complete BRDs would minimize the total review time.

Dr. Stokes said NICEATM and ICCVAM had not anticipated the difficulty in obtaining validation data and scheduled the review expecting that the data would be readily available. He said in other countries data are not provided until there is a peer-reviewed publication. This is not the case in United States and that is why there was a delay in obtaining data. He mentioned Dr. DeGeorge's comment about his data collected over the past eight years. He explained that it was a huge undertaking in terms of time and effort to obtain the original records and they did not have sufficient time or resources. Dr. Stokes said the data have been requested, some have been received, and hopefully they will get the rest. ICCVAM plans to have another expedited peer review meeting to follow up. ICCVAM is aware of the interest in these modified LLNA protocols because of the advantages offered and they are anxious to complete the review. He said agencies use an accepted traditional method in decision-making and when there is a new proposed method they always compare the performance of the new method to the existing approved method. ICCVAM is comparing new methods to both the traditional LLNA and the traditional guinea pig test because they are what the agencies accept right now. The LLNA was accepted, not because it could predict the traditional guinea pig test so well, but because its performance for predicting human sensitizers was comparable to the traditional. They will continue to assess performance of new test methods against both the currently accepted test, as well as against existing human data and/or experience, but it depends upon the data provided. He explained that they were very fortunate in getting the most robust response from industry and mentioned that the current LLNA database includes over 400 substances, compared to 200 for the original review. He acknowledged how pleased NICEATM and ICCVAM were with the willingness of industry to contribute the data, which allowed for a much more thorough evaluation of the limit test.

Dr. Charles, a lead discussant, commended the expert panel for going through the data and coming up with recommendations in the limited timeframe. He concurred regarding the inclusion of a discussion on determining the maximum dose if only a single dose is to be used in a screen process. He said you must be able to define endpoints such as "excessive irritation." He agreed with the panel for a modifying requirement that a concurrent strong positive control not be performed for every single test. The positive control is merely telling you "yes" or "no."

Minutes from the June 18 -19, 2008 SACATM Meeting

He asked about using a couple of animals, instead of five animals, and about doing the tests on a continuous basis. He asked how much additional work is needed to prove that the methodology is consistent and works. For the LLNA, he saw the need for the weak sensitizers, especially with regard to adding in a 1% SLS. He said, even with three animals there is pretty good correlation with the traditional LLNA, so we need further comment from the panel about the need for five animals. He concurred that four are probably needed, especially if there is adequate power in the alternative test systems. He suggested finding alternatives to the radioisotope methods. Regarding the number of chemicals used to validate the test method performance standards, five of them were ones he considered equivocal or only had one test performed on them. He suggested using chemicals with more robust data.

Dr. Dong, a lead discussant, said the panel did a wonderful job. The tables summarizing the power analysis for the modified LLNA methods are not as transparent as they should be. More footnotes or elaborations are needed for Tables 1-1, 3-1, 4-1 and 5-1 in the report. For example, the mean response and the standard deviation (SD) for the control group are not given in each of the tables, although they can be back calculated if one is familiar with the analysis procedure. He said the information is important because the SD of the response for the control group has a direct impact on the power calculations so long as the SD for the control group is assumed as the SD for the treatment group. But more importantly, the SD or variance of the control group seems to be vehicle-driven or vehicle-specific. For example, in the power calculation for the FC LLNA as shown in Table 4-1, the SD is much better when dimethylsulfoxide is used in the control group. Hence the power calculated was much higher, up to 95% with only five animals. If and when the SD or the variability of the response of the control group is vehicle-driven, then it is likely that the accuracy of the method could also be vehicle-driven. Dr. Dong said if it is too late to address this issue for the present analysis, then it should still be something that is worth considering for future studies.

Dr. Barile commended the peer review panel on a tremendous job with the amount of data submitted. He said the evaluation of the data apparently took more time than the deadline allowed. He found that some of the conclusions, statistical analysis, and the data presented from a scientific point of view rather confusing and in some incidences the conclusions were not consistent with the data. He said there were major changes throughout the study as chemicals were added in and out. If chemicals were taken out, that would alter the results of the analysis during the conduct of the studies, especially if the study were ongoing for many years. He found a bigger problem with the reference standards; 10 of the 22 chemicals were performed in only one study and he found them very difficult to compare. Another four had just two performance studies, making the majority of the reference standard done fewer than two times. He found confusing the standards used to describe accuracy, specificity, and sensitivity when comparing between the traditional LLNA and the nonradioactive methods. He also commented on the lack of the human data. He questioned the reporting of false positives in the BrdU-FC and was unclear as to the percentage being used. He questioned the use of optional chemicals and asked if they were false positives and false negatives to get a concordance with the traditional LLNA. He said ICCVAM should make sure that false positives and false negatives with the nonradioactive methods match the traditional LLNA. He questioned what constituted a 100 % concordance. He asked about the cost of the studies, and presumed it was high because of the number of animals and the labs that were asked to do these studies. He asked if it would have

Minutes from the June 18 -19, 2008 SACATM Meeting

been more feasible and cost-effective to wait for the additional information to come in, especially considering the time constraints on the peer review panel. He suggested giving the regional laboratories more time, reducing the number of studies, and getting clarification on the data that have been presented.

Dr. Stokes responded that there had been some confusion about the lack of data available to support the three modified LLNA protocols. ICCVAM did receive summary data for each substance for each test method, but did not receive individual animal data. ICCVAM typically requests quality assurance reports that can also be provided to the peer review panel. ICCVAM had summary data that allowed for calculation of sensitivity and specificity for each method, but not for examination of the variation among animals receiving the same dose of each chemical. With regard to selecting the 22 proposed reference chemicals for performance standards, the Immunotoxicity Working Group spent considerable time selecting the 18 chemicals and four additional optional chemicals. They started out looking at all of the 211 chemicals in the original validation database that were commercially available and applied the different criteria that are listed as to what characteristics the chemicals should have. They selected chemicals that did not produce equivocal responses and that had data using the traditional guinea pig methods as well as human data or experience. When they applied those criteria, it significantly reduced the number of chemicals from which to choose. The working group also wanted to provide a range of diversity in terms of the vehicles used and the chemical characteristics of each of the substances and sought to have a range of potency in terms of responses. So with only 13 positive chemicals and those kinds of criteria being applied, he explained that it was difficult to identify substances that had been evaluated in multiple LLNA studies, and as a result, some substances have only one study. He said ideally it would be better to have multiple studies for each substance. He reminded SACATM that these are draft ICCVAM recommendations and that after the meeting, ICCVAM will be taking the comments into consideration, along with public comments, and the report from the independent peer review panel. He said ICCVAM appreciated the comments, which will help them to revise and finalize the performance standards.

Dr. Barile said he was unsure what “level of accuracy” means. He suggested having numbers associated with accuracy, specificity, and sensitivity. Ninety percent accuracy would be considered acceptable; 80% sensitivity, specificity, also would be scientifically on target. He said it would make this summary and future summaries and evaluations much clearer.

Dr. Fox asked Dr. Luster to provide the biological basis of the assay from a molecular and cellular biology perspective. He said this is a cell-cycle reentry assay and asked whether or not the mitochondrial DNA is being measured at the same time. Dr. Luster responded that the assay is looking at the induction of the response, not the elicitation. The material is applied to the ear and the antigens are picked up by the dendritic cells in the dermis and translocated into the lymph node. If the particular T-cell recognizes a particular antigen, it undergoes cell proliferation. It is a T-lymphocyte proliferation event that eventually leads to the elicitation and the clinical response, hypersensitivity. He added that he does not think the mitochondrial DNA proliferate much and it is mostly nuclear DNA being measured in the assay.

Minutes from the June 18 -19, 2008 SACATM Meeting

Dr. Fox stated that he wanted to know exactly what is detected biologically and then follow up with two other questions. He said in the review for the validation, the panel recommended histopathology, but it was a weak recommendation. He said this recommendation should be considered because it is consistent or parallel with the previous recommendations for five ocular irritants. He suggested establishing histopathology if ICCVAM is going to continue with the LLNA. He thought that there must be a better alternative to the LLNA, i.e., realistically there has to be a way to assess toxicity and skin irritation better than applying a chemical to the guinea pig or mouse ears and looking at them to decide on activation. He saw no mention of any alternative to using whole animals in the report and thought it would be important to discuss an *ex vivo* or non-animal alternative. He said he calculated the dose of BrdU at 2000 µg/kg, which is a huge dose that can damage the nucleus. Dr. Stokes said the dose of BrdU is 5 mg BrdU/mouse. He said a validation study is currently being planned on an *in vitro* method for sensitization that Dr. Kojima would be talking about. ICCVAM is providing input regarding the chemicals to use for the study. Dr. Kojima said it is an *in vitro* sensitization assay being developed with ECVAM and would be ready next year. Dr. Fox asked for information on the biology of the LLNA. Dr. Luster responded that they are looking at activation of dendritic cells by looking at markers of cell division; CD1 and CD86 and several others are activated. He said the panel strongly suggested that there be some histology associated with the reduced LLNA. Dr. Stokes said they could discuss this further at the next advisory meeting.

Dr. McClellan questioned the change in time period and suggested some simpler approaches to comparing BrdU to tritiated thymidine. Dr. Tice responded that in every test method evaluation ICCVAM does, they look at how reliable the method is and how accurate or relevant it is in predicting the particular event that is used for classification. With the reduced LLNA, the question was: does it perform as well as the traditional method given that you are only using one dose level rather than three? In the case of the three alternative methods, each method was compared independently against the original radioactive LLNA. Even taking into account the small changes in protocol, one of the issues to address is whether those changes were considered to be minor changes or major changes, where a major change might have an impact on the performance of the assay. In the ICCVAM guidelines on the LLNA, the OECD test guidelines, and the EPA guidelines, it specifies the use of male CBA mice. Another strain of mouse or another sex of CBA can be used if you demonstrate that it doesn't impact the performance of the assay. Performance is assessed through accuracy and reliability. Performance standards were not available at the time that the original LLNA was evaluated. Performance standards are used to help accelerate the validation of an alternative test method that is functionally and mechanistically similar to an existing test method. Had those performance standards existed, they would have been used, both in the development and evaluation of the non-radioactive methods. Considering that performance standards didn't exist then, ICCVAM is not holding those assays to those standards, but they are looking to see how they perform in that context. The working group also looked at expanding the applicability domain because the traditional LLNA is not considered useful for metals. There weren't enough data on complex mixtures and on aqueous solutions. The use of LLNA for metals was a re-evaluation compared to the radioactive methods, which might have impacted also on the nonradioactive methods. Dr. Tice explained that the panel had to work through a fairly complicated scenario. NICEATM tried to set up the test methods for the panel in sequential fashion to prepare them for what they evaluated later during the meeting.

Minutes from the June 18 -19, 2008 SACATM Meeting

Dr. Wind said she wanted to make sure that everyone understood that ICCVAM knew the methods being developed were nonradioactive test methods. One of the reasons the LLNA wasn't being used more widely is that there are a number of countries where the use of radioactivity is not allowed, and, in addition, there are difficulties associated with using radioactivity. She said ICCVAM thought it was important to look at nonradioactive LLNA methods; however, they did not develop those methods. She said the methods were under development and were brought to the Immunotoxicity Working Group for review. She noted that performance standards make it easier for “me too” assays to be developed and not have to go through the same rigorous validation process as the original assay. She said the Europeans were pushing for the assay to be used as a “stand-alone.” It is possible with the LLNA to make a determination of up to five different potency categories. CPSC staff felt that this was very important, particularly since under the GHS, there was an expert group examining the use of LLNA in determining classification based on potency categories. She explained that the panel addressed numerous questions, which is why the review seems so confusing.

Dr. McClellan expressed concern that such a complex structure has been created for validating new tests. He said it will result in only a few new tests being available in 10 years and suggested occasionally stepping back from the rules.

Dr. Freeman said the discussion illuminated the issue of the roles that ICCVAM, NICEATM, the committee, and the agencies play in terms of promulgating the tests in a way that can impact our society in a regulatory fashion. Dr. McClellan agreed and said he thought this meeting had been one of the best because of the breadth of the agenda and opportunities for SACATM to provide advice.

Dr. Stokes appreciated SACATM's insights and precautionary concerns. ICCVAM has advocated, from the very beginning, communicating and interacting with assay developers. When this occurs, ICCVAM connects them with regulatory scientists who have experience in that particular toxicity endpoint to discuss validation study designs and protocols before they conduct a validation study. This interaction enables ICCVAM to work with them on the appropriate design of the study and selection of the appropriate chemicals that should be used to generate the data needed by regulatory agencies to make decisions on whether that test is acceptable for the purpose that it is proposed for. He said if you look at the number of chemicals and the number of laboratories that have been used for the data for these three methods, if the performance standards had been available for the developers to use, significantly fewer number of animals would have been used at a lot less expense. Laboratories have generated probably three times as much data as ICCVAM has proposed in the draft performance standards. He said this is ICCVAM's attempt to try to get ahead of that curve and get the performance standards out there for use by test method developers. ICCVAM routinely provides performance standards now with every new method. If performance standards had been developed in 1998, it would have benefited and expedited the development and validation of these three non-radioactive LLNA methods.

Dr. Fox concurred with Dr. McClellan in not understanding the 24-hour BrdU vs. the 5-hour BrdU. He said the half-life of BrdU is only 2 hours. He suggested ICCVAM use a different

Minutes from the June 18 -19, 2008 SACATM Meeting

approach in regarding assay reviews, such as bringing the proposed assay to SACATM to get input on whether it's an appropriate assay to review or if the appropriate questions are being asked in its review. Dr. Stokes said the suggestion seemed reasonable as a way to proceed in the future, whenever possible.

## **Appendix F4**

### **Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments**

#### **SACATM Meeting on June 25-26, 2009**

The following is excerpted from the final minutes and speaker presentations of the SACATM meeting convened on June 25-26, 2009. The full meeting minutes are available online at:  
<http://ntp.niehs.nih.gov/go/8202>

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Minutes from the June 25 – 26, 2009 SACATM Meeting

**XI. Report on the Second Meeting of the Independent Peer Review Panel:  
Evaluation of the Updated Validation Status of New Versions and Applications of  
the Murine Local Lymph Node Assay (LLNA)**

**A. Presentations**

Dr. Paul Brown, FDA and member of the Immunotoxicity WG, provided an introduction and overview of the proposed LLNA methods and applications. He said the traditional LLNA was reviewed by ICCVAM in 1998 and again in 2008. He outlined some of the regulatory requirements for skin sensitization evaluation that currently exist and then provided an overview of the LLNA test method protocol. The purpose of the LLNA is to identify chemical sensitizers through quantification of lymphocyte proliferation. A Stimulation Index (SI) is calculated as the ratio of radioactivity incorporated into draining auricular lymph nodes cells of treated animals to that of vehicle control animals. In 2008, the peer review panel agreed with ICCVAM that more data were needed to evaluate three modified versions of the LLNA not requiring radiolabeling and application of the LLNA for pesticide formulations, other products, and substances tested in aqueous solutions. Additional data were submitted to NICEATM and ICCVAM. The Immunotoxicity Working Group (IWG) working with NICEATM revised the draft BRDs, and ICCVAM updated the draft test method recommendations.

Dr. Paul Brown provided overviews of the protocols, some details of the test method data, and a summary of the draft ICCVAM recommendations:

- The LLNA: Daicel Adenosine Triphosphate (DA) test method with specific, defined limitations can be used to identify substances as potential skin sensitizers and nonsensitizers.
- Substances that produce  $SI > 1.7$  and  $< 2.5$  should be evaluated using an integrated decision strategy with all available and relevant information.
- The LLNA: Bromodeoxyuridine enzyme-linked immunosorbent assay (BrdU-ELISA) test method with specific, defined limitations can be used to identify substances as potential skin sensitizers and nonsensitizers. Substances that produced  $1.3 \leq SI < 2.0$  should be evaluated using an integrated decision strategy with all available and relevant information.
- The LLNA: BrdU Flow Cytometry (FC) test method appears useful for identifying substances as potential skin sensitizers or nonsensitizers; however, more information and data are needed before ICCVAM can make a recommendation.

Regarding the applicability domain of the LLNA, Dr. Paul Brown said ICCVAM had comprehensively updated data and information on 104 pesticide formulations, 6 textile dyes, 12 natural complex substances, and 24 substances tested in aqueous solutions. Based on these data, ICCVAM had the following draft recommendations:

- The LLNA is more likely than a guinea pig test to classify a pesticide formulation as a sensitizer.
- More data are needed before a recommendation on the use of the LLNA for testing dyes can be made.
- A definitive recommendation on the use of the LLNA for testing natural, complex substances cannot be made until a larger number of known human sensitizers have been tested.

Minutes from the June 25 – 26, 2009 SACATM Meeting

- LLNA is more likely than a guinea pig test to classify a substance tested in an aqueous solution as a sensitizer. LLNA has utility for hazard classification of substances tested in aqueous solutions provided that the potential for possible over-classification is not a limitation.

Dr. Paul Brown said the ICCVAM Independent Scientific LLNA Peer Review Panel meeting was held April 28-29, 2009, in Bethesda, MD. The panel consisted of 15 experts from six countries.

Dr. Diggs asked about the negative aspects of over-regulation. Dr. Paul Brown said it would depend on the agency. At the Center for Drug Evaluation and Research, where drugs that will be used intentionally for benefit in humans are regulated, over-classification can have negative effects. Dr. Levine said the EPA tries not to over-label because it would dilute the utility of the labeling; people would stop paying attention to the labels. Dr. Freeman said over-classification could have a commercial impact and possibly lead to product deselection when the product has real value.

Dr. Michael Luster, West Virginia University, chaired the panel and provided highlights of the panel report. He thanked the panelists, the Evaluation Group Chairs, Drs. Michael Olson, Stephen Ullrich, and Michael Woolhiser, and the NICEATM staff. He reviewed the ICCVAM charges to the Panel and the modifications and applications to be reviewed.

Dr. Luster then presented the abbreviated highlights of the Panel's report:

- LLNA: DA - The available data and test method performance support its use to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. Based on the current validation database, multiple SI values should be used as decision criteria to identify sensitizers and nonsensitizers.
- LLNA: BrdU-ELISA - The available data and test method performance support its use to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. Based on the current validation database, multiple SI values should be used as decision criteria to identify sensitizers and nonsensitizers.
- LLNA-BrdU-FC - The database of more than 45 representative test substances yielded adequate accuracy based on results from one laboratory; intralaboratory reproducibility had also been adequately demonstrated; however, a recommendation on the validity of this test should be deferred pending an independent audit of the data and an interlaboratory validation study, both of which the Panel recommended. If both of these issues can be successfully addressed, then the assay should be considered scientifically validated as an alternative method for the traditional LLNA.
- All three of the nonradiolabeled LLNA protocols are mechanistically and functionally similar to the traditional LLNA and therefore, do not require separate test method performance standards.
- An emphasis should be made to include ear swelling measurements and/or immunophenotypic markers as an indicator of irritation for the traditional LLNA and for any modified LLNA test methods.

Minutes from the June 25 – 26, 2009 SACATM Meeting

- Any material should be a candidate for testing in the LLNA unless there are unique physicochemical properties associated with the class of test materials that might affect its ability to interact with the normal immune processes. Therefore, the LLNA should be considered applicable to pesticide formulations, other products, and substances in aqueous solutions unless there is a biologically based rationale for exclusion.
- The Panel expressed a strong desire to avoid revalidation of the LLNA for new classes/types of test substances unless there is a biologically based rationale. If any variant of the LLNA is validated for use to test novel classes, then the findings should be relevant to the family of validated LLNA tests.

Dr. Freeman asked about using the lower cut-off values as the thresholds for positive or negative labeling, in order to make decision-making more straightforward. Dr. Luster said it was a small database, the error rate for positives was too high, and it might cause misuse of the methodology. If LLNA results are indeterminate, a guinea pig test may need to be done, but overall, fewer guinea pigs will be used and the end result will benefit animal welfare. The Panel discussed peptide reactivity as a good predictor of the LLNA, but did not make a recommendation on it. Dr. Fox expressed concern for using the adenosine triphosphate (ATP) assay, deeming it a poor assay for measuring proliferation. He questioned the BrdU methodology and suggested some alternatives. Dr. Luster said the Panel did not formally discuss ways to improve the assays.

Dr. Fox said FC is the most sensitive and promising assay and Dr. Luster agreed. Dr. Freeman asked about the cost of the LLNA using FC compared to the other assays. Dr. Luster said costs include the instrument and trained personnel. He said immunophenotyping was used separately to identify irritants from sensitizers, but was not part of the Panel's review. Dr. Freeman asked about accuracy and sensitivity of the FC compared to humans or guinea pigs. Dr. Luster said the results equivocated somewhat, but that only a few chemicals did not show the same results. Dr. Fox said a two-channel fluorescence-activated cell-sorting machine is cheaper and easier to calibrate than a scintillation counter. Dr. Luster agreed due to the cost of disposal of <sup>3</sup>H-thymidine. Dr. Marilyn Brown said it is essential to assess the LLNA in relation to human data when available, and asked about the actual use of LLNA compared to guinea pig tests. Dr. Levine said the EPA is getting a fair number of LLNAs now, which should increase when companies know it is accepted. Dr. Meyer asked about statistical expertise on the panel and about comparing continuous and percentage data. Dr. Luster said there were two statisticians and they did not discuss that issue.

Dr. Hansen asked about tracking the frequency of submissions, acceptances, and revisions by registrants. Dr. Levine said the EPA does not track submissions, but has done rejection analyses on particular studies. She will suggest tracking at EPA. Dr. Luster said the OECD might have tracking information because the original LLNAs, for which they have a large database, were developed in Europe. Dr. Freeman was unsure about the outcome of recommendations once the agencies received them, so it would be good to have such information from agencies made publicly available; it may encourage further use of the methods. Dr. Fitzpatrick asked if drug sponsors might be willing to share that information with ICCVAM. Dr. Paul Brown said the FDA does not

Minutes from the June 25 – 26, 2009 SACATM Meeting

formally track submissions, but a number of LLNA assays have been submitted. In FDA's pre-meeting discussions, sponsors were told that the LLNA is acceptable. Assays have not been rejected unless there is a problem with the particular assay. Dr. Levine asked about mixtures that contain a small component of sensitizing material, creating the possibility of false negatives, and the potential for the interaction of components in a mixture to be a sensitizer when the individual components are not. Dr. Luster said the approach is to test the individual material, the vehicle, and the mixture separately. There are examples of interaction in mixtures that have the potential to destroy the epidermis, so it is important to test the combination. Dr. Levine asked about waiving testing on new formulations if they are fairly similar to existing formulations. Dr. Luster said it would be up to the regulatory agencies, but cautioned that formulations can change between batches and between companies. Dr. Levine suggested more limited testing on pesticide formulations, which are produced in series that vary only in active ingredients. Dr. Charles asked about the use of sodium lauryl sulfate (a potential sensitizer) pretreatment in the DA assay. Dr. Luster said the data had not been obtained, but the Panel did not think it would change the outcome of the recommendation.

**B. SACATM discussion**

Dr. Freeman asked whether SACATM could provide advice about the priority for the inter-laboratory validation studies for the FC assay. Dr. Stokes said ICCVAM accepts nomination for evaluation or validation of test methods and then decides a draft priority, which is presented to SACATM. In this case, SACATM is presented a proposed activity, which it discusses, decides on a priority, and makes a recommendation to ICCVAM.

Regarding false positives, Mr. Wnorowski, a lead discussant, asked if the next step would be guinea pig testing and which test would carry more weight. Dr. Levine said from regulatory point of view, the most conservative tests would be used. If the weight of evidence includes human data and there is a potential for over-prediction by the LLNA, then that would be taken into consideration in the labeling. Dr. Freeman suggested using guinea pig studies for those substances in the indeterminate range. Dr. Levine said a line is included on pesticides stating, "the product may cause allergic reaction in sensitive individuals." Companies developing consumer products may abandon them if they are deemed sensitizers, so the company must make the decision about what testing is done.

Dr. Meyer, a lead discussant, expressed concern regarding the comparison of different statistical analyses between the FC and ELISA methodologies and felt this issue should be addressed. Dr. Stokes responded that before the BRD is finalized, ICCVAM would consult with a statistician to make sure the appropriate analyses were done. Dr. Meyer asked about the behavior of different classes of compounds in different assays, especially the aqueous substances, which should not go through the stratum corneum. She asked if the sodium dodecyl sulfate pre-treatment for permeability had ever been validated. Dr. Luster said it was not included in the data from the sponsors. Dr. Meyer said such treatment might explain why the different classes of compounds performed so

Minutes from the June 25 – 26, 2009 SACATM Meeting

differently in the tests. She asked to see the statistics on the FC test before making a recommendation.

Dr. Ehrich, a lead discussant, expressed strong support for the Panel's report. She said the LLNA DA method looked ready for release. The submitter had done the appropriate steps to meet the recommendations of the 2008 panel. She said the assay is not easy technically, which is why variability is an issue. Inter- and intra-laboratory studies have been done and she supported the Panel's conclusions. The LLNA DA is more sensitive than the ELISA test method, but the intermediate range for both test methods needs to be further defined and reevaluated. No new data were presented for the ELISA beyond the 2008 panel report. Dr. Ehrich supported giving high priority to the FC inter-laboratory studies and agreed that there should not be separate performance standards for the non-radioactive methods. Some intermediate areas still exist, but could be handled on a case-by-case basis. Additional performance standards would only add unnecessary delay to the release. She said it is important to provide non-radioactive tests, since some places do not allow radiation. Testing for mixtures, pesticide formulations, aqueous solutions, and metals is improved since the 2008 report. There are still some substances that are difficult to test, but there is no reason to continue to use radiolabeled testing in guinea pigs.

Dr. Charles, a lead discussant, generally concurred with Panel's recommendations and agreed giving a high priority to the FC testing. The use of dual ranges in the DA and ELISA assay for assessing sensitizers versus non-sensitizers could potentially place many compounds in limbo, so the decision criteria should be reassessed as more data are obtained. He concurred with the suggestion to include evaluation of ear swelling as an indicator of irritation and immunophenotypic marker assessment. The BRD formulations tested included many potential false negatives relative to the guinea pig maximization test (GPMT). He agreed that the GPMT was never fully validated for formulations and possibly under-predicts relative to the LLNA.

Dr. Barile, a lead discussant, suggested including data on accuracy, specificity, sensitivity, and performance standards that were available only in the BRDs from last year. He found it hard to make suggestions on applicability since new substances were added to the test formulations without including the performance data. He approved of the two decision criteria to allow specific cut-off points. He questioned the concern about the lack of human data, which are hard to obtain, and why comparisons with animal data are not enough. He questioned the prohibitions on using radioactivity in other countries and stated that radioactive procedures are very sensitive, though costly, and should not be discarded. He asked about the development of non-animal tests for detecting sensitizers. Dr. Stokes mentioned the human Cell Line Activation Test (h-CLAT) method undergoing validation in Japan and the peptide reactivity assays submitted for validation by Proctor and Gamble. Because of the Cosmetics Directive in Europe, which will completely ban the use of animals for repeat dose studies by 2013, there is much interest in developing non-animals methods to assess allergic contact dermatitis. Dr. Barile said he would like to see more discussion regarding the biology and mechanisms that are the bases of the tests, such as what is being tested by the LLNA, what cell types are proliferating, and which mouse strain is being used. He

Minutes from the June 25 – 26, 2009 SACATM Meeting

suggested making the non-animal testing a priority over the FC tests. Dr. Fox suggested the compounds be tested for photoactivation and photosensitization. He agreed with Dr. Barile that non-animals methods should have the highest priority. Dr. Stokes clarified that ECVAM has the lead on three non-animal validation studies, which are a high priority in Europe. Dr. Kreysa added that ECVAM had received three submissions for non-animal test methods for skin sensitization and are planning validation studies now. Using these three test methods in a testing strategy could possibly serve as a replacement for animal tests.

Dr. Paul Brown said the FDA typically does not do non-clinical testing of drug products for photoallergenicity. Topical products are usually tested in a human photoallergenicity study and a repeat patch test for allergenicity in humans. Those results determine further clinical development and assessment for hypersensitivity reactions; therefore, the FDA would eventually get definitive human data to characterize photosensitivity of a product. Dr. Fox encouraged testing for photoallergenicity and said the assay does not address it. Dr. Luster said there are LLNA data on photosensitization. Dr. Meyer encouraged the development of non-radioactive methods, which are easier to teach, and said ELISAs are easier technically to teach than FC. Dr. Corcoran asked about thresholds and the boundary between positive and non-positive responses in the LLNA and the guinea pig test. Dr. Luster said false positives were an issue with pesticide formulations. In the old GPMT, the substance was just put on the skin. Now, 1% pluronic acid can be used as detergent to increase dermal penetration of water-soluble substances. Mr. Wnorowski said the GPMT is generally considered more conservative and more likely to give false positives than the Buehler test; whereas the Buehler test tends to give a positive response less often. The sensitivity of the human test is intermediate. The LLNA is the most conservative and generates the most false positives. Many registrants consider that unacceptable and would be reluctant to label the product as a sensitizer. Dr. Corcoran hoped to hear that the LLNA identified sub-positive responses, creating a weight of evidence argument against labeling. He thought the LLNA's rate of false positives caused over-classification and could be a disincentive for its use. Mr. Wnorowski concurred. Dr. Levine said from a regulatory perspective, it is possible to eliminate the Buehler test if replaced by another test. Dr. Freeman, a member of the original LLNA review panel, did not recall that the LLNA over-predicts compared to the GPMT. He suggested for complete transparency that the final report should reflect the performance of the various tests. Dr. Stokes said ICCVAM would extract those data from the 1999 TMER. ICCVAM has done all the analyses, and the overall accuracy of ~70% was comparable to the predictivity of the LLNA for existing human data and the combined Buehler-GPMT tests for human data. The overall accuracy of the LLNA for predicting the GPMT was about 88%. The difference of 15 % could be due to over-prediction compared to the GPMT.

Mr. Wnorowski expressed concern about the limited, additional data for the pesticide formulations. Compared to the original assays on pure chemicals, these data show that the pesticide formulations appear to produce false positives in the LLNA compared to the guinea pig-based tests. Dr. Allen clarified the difference in sensitivity between the Buehler test and the GPMT. For the 22 substances for which there were comparative tests, 20 of the guinea pig tests were actually Buehler tests, so there is a question as to

Minutes from the June 25 – 26, 2009 SACATM Meeting

whether they could have been concordant if they had been GPMTs. Strictly comparing the performance of the LLNA and the GPMT for those 22 substances, the accuracy is not great because the trend was to get a positive result more often in the LLNA. The original concern about the use of LLNA for mixtures was that the LLNA would give false negatives, but it is actually more conservative. Mr. Wnorowski agreed and expressed concern that if the LLNA is too conservative, it will not be used unless regulatory agencies require it, because of its impact on the marketing of products.

Dr. Marilyn Brown said laboratories have moved away from using the LLNA because it is the only test that uses radioactivity. Providing a LLNA test that doesn't use radioactivity would increase its use.

Dr. Freeman asked for a vote on whether NICEATM-ICCVAM should set a high priority on the inter-laboratory validation of the FC method because the only currently data are from just one laboratory. Dr. Corcoran said everything cannot be high priority and that doing the FC validation would mean that ICCVAM could not do something else. Dr. Stokes agreed and said the vote would be advice for the NTP and NICEATM to make decisions about competing priorities for limited resources. SACATM has not provided advice on nominations for validation studies for two years, and ICCVAM currently has no new nominations for validation studies. Dr. Diggs seconded the motion. SACATM voted 9 yes, 1 no (Dr. Meyer), 1 abstention (Dr. Barile), and 1 recusal (Dr. Marsman). Dr. Meyer voted against the motion because she was uncomfortable with the statistics and thought the ELISA is a better method to move forward. Dr. Barile abstained because he thought the other two tests should have equal priority and because FC is difficult to use for training and is costly. Dr. Fox suggested lowering the priority of the ATP assay because it is technically flawed. Dr. Stokes said all SACATM comments would be considered in finalizing the recommendations of the IWG and ICCVAM.

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## Appendix G

### Relevant Skin Sensitization Regulations and Testing Guidelines

G1	Table of Relevant Skin Sensitization Test Regulations.....	G-3
G2	EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization (March 2003) .....	G-7
G3	ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Delayed-type Hypersensitivity (2002).....	G-25
G4	OECD Test Guideline 429: Skin Sensitisation – Local Lymph Node Assay (Adopted April 2002) .....	G-27
G5	OECD Test Guideline 406: Skin Sensitisation (Adopted July 1992).....	G-37

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## **Appendix G1**

### **Table of Relevant Skin Sensitization Test Regulations**

Note to the Reader:

Regulations may be updated in the future. It is recommended that users review the most current version of all regulations identified.

Electronic versions of United States Code (U.S.C.) can be obtained at:

<http://www.gpoaccess.gov/uscode/index.html>

Electronic versions of the Code of Federal Regulations (CFR) can be obtained at:

<http://www.gpoaccess.gov/cfr/index.html>

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<b>Skin Sensitization Testing: Relevant US Federal Laws, Regulations, Guidelines, and Recommendations</b>				
<b>Agency, Center, or Office</b>	<b>Regulated Products</b>	<b>Statutory Requirements</b>	<b>Regulations</b>	<b>Guidelines and Recommendations</b>
FDA/CDER	Pharmaceuticals	Federal Food, Drug, and Cosmetic Act (U.S.C. Title 21, Chapter 9)  Public Health Service Act (U.S.C. Title 42, Chapter 6A)	21 CFR 312  21 CFR 314	Guidance for Industry Immunotoxicology Evaluation of Investigational New Drugs (2002)
EPA/OPPTS	Chemicals as defined by Section 5 of the Act  Pesticides	Toxic Substances Control Act (U.S.C. Title 15, Chapter 53)  Federal Insecticide, Fungicide, and Rodenticide Act (U.S.C. Title 7, Chapter 6)	40 CFR 158.50 40 CFR 158.100 40 CFR 158.340 40 CFR 700-799	OPPTS 870.2600 (2003) (see <b>Appendix G2</b> )
CPSC	Consumer Products	Federal Hazardous Substances Act (U.S.C. Title 15, Chapters 1261-1278)	16 CFR 1500.3	No Specific Guidelines, Guidances, or Recommendations
OSHA	Chemicals	Occupational Safety and Health Act of 1970 (U.S.C. Title 29, Chapter 15)	29 CFR 1910.1200	No Specific Guidelines, Guidances, or Recommendations

<b>Relevant Skin Sensitization Regulations and Guidelines Europe</b>			
<b>Agency, Center, or Office</b>	<b>Regulated Products</b>	<b>Regulations and Directives</b>	
EU	Dangerous Preparations (Chemicals and Chemical Mixtures)	Directive 1999/45/EC of the European Parliament and of the Council of 31 May 1999  Annex V to Directive 67/548/EEC of 27 June 1967	
	Pesticides	Directive 91/414/EEC of the European Parliament and of the Council of 15 July 1991	
<b>Relevant Skin Sensitization Regulations and Guidelines International</b>			
<b>Organizations</b>	<b>Regulated Products</b>	<b>Legal Instruments and Recommendations</b>	<b>Guidelines, Guidance, and Recommendations</b>
GHS	Chemicals	GHS Part 3, Chapter 3.4	No Specific Guidelines, Guidances, or Recommendations
ISO	Medical Devices	NA	ISO 10993-10 (2002) (see <b>Appendix G3</b> )
OECD	Chemicals	NA	OECD Test Guideline 429 (2002) (see <b>Appendix G4</b> )  OECD Test Guideline 406 (1992) (see <b>Appendix G5</b> )
ICH	NA	NA	No Specific Guidelines, Guidances, or Recommendations

**Appendix G2**

**EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization  
(March 2003)**

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United States  
Environmental Protection  
Agency

Prevention, Pesticides  
and Toxic Substances  
(7101)

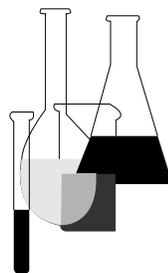
EPA 712-C-03-197  
March 2003



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# Health Effects Test Guidelines

## OPPTS 870.2600 Skin Sensitization



## INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

**Final Guideline Release:** This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512-0132. This guideline is also available electronically in PDF (portable document format) from EPA's Internet Web site at <http://www.epa.gov/opptsfrs/home/guidelin.htm>.

**OPPTS 870.2600 Skin sensitization.**

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPTS Harmonized Test Guidelines Series 870, Guideline 870.2600 Skin Sensitization, dated August 1998; 40 CFR 798.4100 Dermal Sensitization; OECD 406 Skin Sensitization (adopted July 1992); and OECD 429 Skin Sensitization: Local Lymph Node Assay (adopted April 2002).

(b) **Purpose.** The purpose of the selected test is to identify substances with skin sensitization potential. Determination of the potential to cause or elicit skin sensitization reactions (allergic contact dermatitis) is an important element in evaluating a substance's toxicity. Information derived from skin sensitization tests serves to identify possible hazards to a population exposed repeatedly to a test substance. Testing is not required if the test material is a known skin sensitizer. If it is suspected that the test material is a strong dermal irritant, see OPPTS 870.1000, paragraph (d)(2)(iii).

(c) **Definitions.** The following definitions apply to this test guideline. The definitions in Section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) also apply to this test guideline.

*Challenge exposure* is an exposure of a previously treated subject to a test substance following an induction period to elicit a contact hypersensitivity response.

*Induction exposure* is the administration of a test substance to the test subject with the intention of inducing contact sensitization.

*Induction period* is a period of at least 1 week following an induction exposure during which sensitization may develop.

*Skin sensitization (allergic contact dermatitis)* is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterized by pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these. In other mammalian species, the reactions may differ and only erythema and edema may be seen.

*Stimulation index (SI)* is the ratio of <sup>3</sup>H-methyl thymidine or <sup>125</sup>I-iododeoxyuridine (<sup>125</sup>IU) incorporation into test group lymph nodes relative to that recorded for solvent/vehicle control group lymph nodes.

(d) **Test procedures**—(1) **Methods.** Any of the following test methods is considered to be acceptable:

- (i) Local Lymph Node Assay (LLNA) test, or
- (ii) Guinea-Pig Maximization Test (GPMT), or
- (iii) Buehler test.

(2) **Choice of assays.** See OPPTS 870.1000 for a general discussion of factors to be considered prior to performing the test. In addition, the following considerations apply:

(i) The LLNA (see references in paragraphs (g)(1) through (g)(6) of this guideline) is a preferred alternative method, where applicable, to the traditional guinea pig test because it demonstrates an equivalent prediction of human allergic contact dermatitis as compared to the other sensitization tests, provides quantitative data and an assessment of dose-response, gives consideration to animal welfare concerns, and is suitable for testing colored substances. It should be recognized that there are certain testing situations that may necessitate the use of traditional guinea pig tests. The tester should note that the LLNA may not be appropriate for all types of test materials, such as certain metallic compounds, high molecular weight proteins, strong dermal irritants and materials that do not sufficiently adhere to the ear for an acceptable period of time during treatment. When using the LLNA, particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles or test materials and runny liquids are to be avoided. In all instances, the tester must document that appropriate techniques were used to facilitate adherence to the mouse ear for an adequate exposure duration. It may be possible to use the LLNA to test some of these materials if appropriate techniques are used to facilitate adherence.

(ii) In situations for test materials where the LLNA is not applicable or may provide unreliable or problematic results, the GPMT or Buehler tests are recommended (see references in paragraphs (g)(7) through (g)(14) of this guideline).

(iii) Although the LLNA, GPMT, or Buehler tests are considered to be acceptable tests, it is recognized that other tests may give useful results. If other tests are used, the investigator must provide justification/reasoning for use of other procedures and methods and protocols must be provided. A positive and negative control group must be included in each test.

**(e) Test methods—(1) LLNA method—(i) Principle of the method.** The basic principle underlying the LLNA is that skin sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of chemical application. Generally, under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cellular proliferation as a function of *in vivo* radioisotope incorporation

into the DNA of dividing lymphocytes. The LLNA assesses this proliferation in the draining auricular lymph nodes located in the cervical region at the bifurcation of the jugular vein. Lymphocyte proliferation in test groups is compared to that in concurrent solvent/vehicle-treated controls. A positive control is added to each assay to provide an indication of appropriate assay performance.

(ii) **Animal selection**—(A) **Sex and strain of animals.** Young adult female mice (nulliparous and non-pregnant) of the CBA/Ca or CBA/J strain should be used at age 8–12 weeks. All animals are to be age-matched (preferably within a one-week time frame). Females are used because the existing database is predominantly based on this gender. Males and other strains of mice should not be used until it is sufficiently demonstrated that significant strain-specific and/or gender-specific differences in the LLNA response do not exist.

(B) **Housing and feeding.** The temperature of the experimental animal room should be  $21 \pm 3$  °C and the relative humidity 30–70%. When artificial lighting is used, the light cycle should be 12 hours light: 12 hours dark. For feeding, standard laboratory mouse diets are to be used with an unlimited supply of drinking water. The mice must be acclimatized for at least 5 days prior to the start of the test. Animals must be housed individually. Healthy animals are randomly assigned to control and treatment groups having statistically homogeneous body weights. The animals are uniquely identified prior to being placed on study. Although a variety of techniques exist to uniquely mark mice, any method that involves identification via ear marking (e.g., ear tags) must not be used.

(iii) **Test conditions**—(A) **Preparation of doses.** Solid test substances are to be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Stable suspensions might also be acceptable. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance are to be prepared daily unless stability data demonstrate the acceptability of storage.

(B) **Solvent/vehicle.** The solvent/vehicle is to be selected on the basis of maximizing the test concentration while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone/olive oil (4:1 v/v), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide, but others may be used if appropriately justified. The selected solvent/vehicle must not interfere with or bias the test result and should be selected to achieve the maximum concentration/skin exposure of the test substance. Ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

(C) **Controls.** (1) Concurrent negative (solvent/vehicle) and positive controls are to be included in each test. In some circumstances, it may be useful to include a naive control. Except for treatment with the test substance, animals in the control groups are to be handled in an identical manner to animals of the treatment groups.

(2) Positive controls are used to ensure the appropriate performance of the assay. The positive control must produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) of three or greater ( $SI \geq 3$ ) over the solvent or vehicle control group. The positive control dose is to be chosen such that the induction is clear but not excessive. Preferred positive control substances are hexyl cinnamic aldehyde (HCA) and mercaptobenzothiazole. There may be circumstances where, given adequate justification, other positive control substances may be used. However, benzocaine should not be used as a positive control in the LLNA.

(3) The positive control substance is tested in the vehicle that is known to elicit a consistent response (i.e., acetone/olive oil). If a non-standard vehicle (chemically relevant formulation) is used with a positive control, the non-standard vehicle (chemically relevant formulation) must be tested for a local lymph node response prior to the initiation of the study and the results reported.

(iv) **LLNA test procedure—(A) A minimum of five animals are used per dose group.** At least three consecutive doses of the test substance are to be used. A solvent/vehicle control group and a positive control group are also required. Doses are normally selected from within the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.1%. In general, dose selection is based on factors such as toxicity, solubility, irritancy and any other available information such as the results of other testing and structure-activity relationships. To avoid false negatives, test as high a concentration as possible. Generally, the maximum concentration tested is the highest achievable level that avoids overt systemic toxicity and excessive local irritation. To identify the appropriate maximum test substance dose, an initial toxicity test, conducted under identical experimental conditions except for an assessment of lymph node proliferative activity, may be necessary. To support an ability to identify a dose-response relationship, data must be collected on at least three test substance treatment doses, in addition to the concurrent solvent/vehicle control group. Where the LLNA study results are negative, the concurrent positive control must induce a  $SI \geq 3$  relative to its solvent/vehicle-treated control.

(B) **LLNA experimental procedure.** The LLNA experimental procedure is to be performed by appropriately trained staff as follows:

(1) Day 1. Record the body weight of each mouse prior to dermal applications. Apply 25  $\mu$ L/ear of the appropriate dilution of the test sub-

stance, or the positive control, or the solvent/vehicle control alone to the dorsum of both ears. A positive displacement pipettor may facilitate application of the test material.

(2) Days 2 and 3. Repeat the application procedure as carried out on day 1.

(3) Days 4 and 5. No treatment.

(4) Day 6. Record the body weight of each mouse. Inject 250  $\mu$ L of sterile phosphate buffered saline (PBS) containing 20  $\mu$ Ci of  $^3$ H-methyl thymidine or 250  $\mu$ L PBS containing 2  $\mu$ Ci  $^{125}$ IU and  $10^{-5}$  M fluorodeoxyuridine into each experimental mouse via the tail vein. Five hours later, the draining (auricular) lymph node of each ear is excised and pooled in PBS for each animal. A single cell suspension of lymph node cells (LNC) is prepared for each mouse. The single cell suspension is prepared in PBS by either gentle mechanical separation through 200-mesh stainless steel gauze or another acceptable technique for generating a single cell suspension. The LNC are washed twice with an excess of PBS and the DNA precipitated with 5% trichloroacetic acid (TCA) at 4  $^{\circ}$ C for approximately 18h.

(5) For the  $^3$ H-methyl thymidine method, pellets are resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid. Incorporation of  $^3$ H-methyl thymidine is measured by B-scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the  $^{125}$ IU method, the 1 mL TCA pellet is transferred directly into gamma counting tubes. Incorporation of  $^{125}$ IU is determined by gamma counting and also expressed as dpm/mouse.

(C) **Observations.** At a minimum, observe mice once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded, with records being maintained for each individual mouse.

(D) **Measurements and calculation of results.** (1) The proliferative response of lymph node cells from the pooled lymph nodes of each individual animal is expressed as the number of radioactive disintegrations per minute (dpm) per animal, subtracting out any background dpm. Then the group mean dpm, along with an appropriate measure of inter-animal variability (i.e., mean  $\pm$  standard deviation), is calculated for each test group (i.e., positive, solvent/vehicle, and any other control groups) and the solvent/vehicle group. Final results are expressed as the SI which is calculated as a ratio (i.e., SI = mean dpm of test group divided by mean dpm of solvent/vehicle control group).

(2) In addition to an assessment of the magnitude of the ratio estimate, SI, conduct statistical analyses which include both an overall assess-

ment (e.g. ANOVA) of the dose-response relationships and pairwise comparisons of the SIs of the test groups, positive control group and any other control group versus that of the solvent/vehicle control group. In choosing an appropriate method of statistical analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis.

**(v) Data interpretation and reporting for LLNA—(A) Data Interpretation.** (1) A substance is regarded as a skin sensitizer in the LLNA if at least one concentration of the test material results in a 3-fold or greater increase in <sup>3</sup>H-methyl thymidine or <sup>125</sup>IU incorporation in the lymph node cells of test group lymph nodes relative to that recorded for solvent/vehicle control lymph nodes, as indicated by the SI. However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response. A quantitative assessment must be performed by statistical analysis of individual animal data in order to provide a more complete evaluation of the test substance (see paragraph (e)(1)(iv)(D)(2) of this guideline). Factors to be considered in evaluating the biological significance of a response or outcome of the test include the results of the SI determinations, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the solvent/vehicle and positive control responses.

(2) Strong irritants may yield false positive results in the LLNA due to the initiation of a significant lymphocyte proliferation. However, the dose-response information from the assay may help to uncover a strong irritant response since, for instance, it has been shown that the proliferation induced by irritation usually results in a shallow dose-response relationship. Concurrent evaluation of ear swelling may also provide helpful information on differentiating weak sensitizers from strong irritants.

**(B) Test report.** The test report for LLNA must contain the following specific information:

(1) Test substance. (i) Identification data and CAS number, if known, and EPA registration number, if applicable;

(ii) Physical nature and purity;

(iii) Physicochemical properties relevant to the conduct of the study;

(iv) Stability of the test substance, if known; and

(v) Lot number of the test substance.

(2) Solvent/vehicle. (i) Solvent/vehicle used and its purity;

(ii) Justification for choice of solvent/vehicle, if appropriate; and

(iii) Solubility and stability of the test substance in the solvent/vehicle.

(3) Test animals. (i) Strain of mice used;

(ii) Acclimation information;

(iii) Number, age, and sex of mice;

(iv) Source, housing conditions, diet, etc.;

(v) Individual body weight of the animals at the start and end of the test, including body weight range, mean, and associated error term for each group;

(vi) Health and microbiological/pathogen status of the mouse; and

(vii) Details of animal food and water quality;

(4) Test conditions. (i) Details of test substance preparation;

(ii) Details of the administration of the test substance;

(iii) Detailed description of treatment and sampling schedules; and

(iv) Methods for measurement of toxicity.

(5) Results. (i) Positive and negative (solvent/vehicle) control data in tabular form;

(ii) Data from range-finding study, if conducted;

(iii) Doses used;

(iv) Rationale for dose level selection;

(v) Signs of toxicity;

(vi) Dpm/mouse values for each mouse within each treatment group and control group;

(vii) Group mean dpm/mouse and associated error term for each treatment group and control group;

(viii) The SI calculated, compared to the concurrent solvent/vehicle control group, for each test substance treatment dose group, the concurrent positive control group, and any other concurrent control group;

(ix) Individual mouse dpm data must be presented in tabular form, along with the group mean dpm, its associated error term and the SI for each dose group;

(x) Criteria for considering studies as positive or negative (including information on any qualitative or quantitative measure of ear swelling);

- (xi) Dose-response relationship;
  - (xii) Statistical analyses and method applied;
  - (xiii) Concurrent and negative control data as established in the tester's laboratory; and
  - (xiv) Concurrent positive control data.
- (6) Discussion of the results.
- (7) Conclusions.
- (8) The reporting requirements specified under 40 CFR Part 158 (for pesticides) and 40 CFR Part 792, Subpart J (for toxic substances) should be followed.

**(2) GPMT and Buehler Methods—(i) Principle of the test methods.** Following initial exposure to a test substance, the animals are subjected, after a period of not less than 1 week, to a challenge exposure with the test substance to establish whether a hypersensitive state has been induced. Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction with that of the initial induction exposure. The test animals are initially exposed to the test substance by intradermal and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (the induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure is compared with that demonstrated by control animals that undergo sham treatment during induction and then receive the challenge exposure.

**(ii) Animal selection—(A) Species and strain.** The young adult guinea pig is preferred. Young adult commonly used laboratory strains must be employed.

**(B) Housing and feeding.** The temperature of the experimental animal room should be  $20 \pm 3$  °C with the relative humidity 30–70 percent. Where the lighting is artificial, the sequence should be 12 h light/12 h dark. Conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

**(C) Number and sex.** The number and sex will depend on the method chosen. Either sex may be used in the Buehler test and the GPMT. If females are used, they must be nulliparous and not pregnant. The Buehler test recommends using a minimum of 20 animals in the treatment and at least 10 as controls. At least 10 animals in the treatment group and 5 in the control group must be used with the GPMT, with the stipulation that if it is not possible to conclude that the test substance is a sensitizer after using fewer than 20 test and 10 control guinea pigs, the testing of

additional animals to give a total of at least 20 test and 10 control animals is strongly recommended

**(D) Control animals.** (2) Every 6 months, assess the sensitivity and reliability of the experimental technique in naive animals by the use of positive control substances known to have mild-to-moderate skin-sensitizing properties. In a properly conducted test, a response of at least 30 percent in an adjuvant test and at least 15 percent in a nonadjuvant test is expected for mild-to-moderate sensitizers. Preferred substances are hexylcinnamic aldehyde (CAS No.101–86–0), mercaptobenzothiazole (CAS No. 149–30–4), benzocaine (CAS No. 94–09–7), dinitro-chloro-benzene (CAS No. 97–00–7), or DER 331 epoxy resin (CAS No. 25068–38–6). There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used.

(2) To ensure that the response to the challenge reaction in treated animals is truly of allergic origin and not due to skin irritancy, a sham-treated vehicle-only control is included in the test strategy. This sham-treated control group is treated in exactly the same manner as the test animals, except that during the induction phase the test article is omitted. The selected vehicle must not interfere or alter the test results.

**(E) Dose levels.** The dose level will depend on the test method selected. In the Buehler test, select the concentration of the induction dose such that it is high enough to cause mild irritation, and the challenge dose such that it is the highest non-irritating concentration. In the GPMT, the concentration of the induction dose must be well tolerated systemically, and must be high enough to cause mild-to-moderate skin irritation; the GPMT challenge dose must use the highest non-irritating concentration.

**(F) Observation of animals.** (1) Skin reactions are to be graded and recorded after the challenge exposures at the time specified by the methodology selected. This is usually at 24 and 48 hours. Additional notations are to be made as necessary to fully describe unusual responses.

(2) Regardless of the test method selected, initial and terminal body weights must be taken and recorded.

**(G) Procedures.** The procedures to be used are those described by the test method chosen. Brief summaries are given here, but the tester is referred to the original literature for more complete guidance on conducting the Buehler test (see references in paragraphs (g)(7) through (g)(10) of this guideline) or the GPMT (see references in paragraphs (g)(11) through (g)(14) of this guideline).

(1) The Buehler test uses topical administration via a closed patch on days 0, 6–8, and 13–15 for induction, with topical challenge of the untreated flank for 6 hours on day 27–28. Readings are made approximately 24 hours after removing the challenge patch, and again 24 hours

after that. If the results are equivocal, the animals may be rechallenged one week later, using either the original control group or a new control group for comparison.

(2) The GPMT uses intradermal injection with and without Freund's complete adjuvant (FCA) for induction, followed on days 5–8 by topical irritation/induction, followed by topical challenge for 24 hours on day 20–22. Readings are made approximately 24 hours after removal of the challenge dose, and again after another 24 hours. As with the Buehler test, if the results are equivocal, the animals may be rechallenged 1 week later. If only 10 animals were used initially and gave equivocal results, the use of an additional 10 experimental and 5 control animals is strongly recommended.

(3) Blind reading of both test and control animals is recommended.

(4) Removal of the test material is accomplished with water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

(5) Hair is removed from the site of application by clipping, shaving, or possibly by depilation, depending on the test selected.

(iii) **Data and reporting for GPMT and Buehler Methods.** Data must be summarized in tabular form, showing for each individual animal the skin reaction, results of the induction exposure, and the challenge exposure at times indicated by the method chosen. As a minimum, the erythema and edema must be graded and any unusual finding must be recorded.

(A) **Evaluation of the results.** The evaluation of results will provide information on the proportion of each group that became sensitized and the extent (slight, moderate, severe) of the sensitization reaction in each individual animal.

(B) The following specific information is to be reported for the GPMT and Buehler Methods.

(1) A description of the method used and the commonly accepted name.

(2) Information on the positive control study, including the positive control substance used, the method used, and the time conducted.

(3) The number, species, strain, age, source, and sex of the test animals.

(4) Individual body weights of the animals at the start of the test and at the conclusion of the test.

(5) A brief description of the grading system.

- (6) Each reading made on each individual animal.
  - (7) The chemical identification and relevant physicochemical properties of the test substance.
  - (8) Manufacturer, source, purity, and lot number of test substance.
  - (9) Physical nature, and, where appropriate, concentration and pH value for the test substance.
  - (10) The vehicles used for induction and challenge and justification for their use, if other than water or physiological saline. Any material that might reasonably be expected to react with or enhance or retard absorption of the test substance must be reported.
  - (11) The total amount of test substance applied for induction and challenge, and the technique of application in each case.
  - (12) Description of any pre-test conditioning, including diet, quarantine and treatment of disease.
  - (13) Description of caging conditions including number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animals.
  - (14) Histopathological findings, if any.
  - (15) Discussion of results.
  - (16) A list of references cited in the body of the report, i.e., references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results.
  - (17) The reporting requirements as specified under 40 CFR Part 158 (for pesticides) and 40 CFR Part 792, Subpart J (for toxic substances) should be followed
- (f) **Screening tests.** The mouse ear swelling test (MEST) (see references in paragraphs (g)(15) through (g)(18) of this guideline) may be used as a screening test to detect moderate to strong sensitizers. If a positive result is seen in this assay, the test substance may be designated a potential sensitizer, and it may not be necessary to conduct a further test in guinea pigs. If the MEST does not indicate sensitization, the test substance should not be designated a nonsensitizer without confirmation in an accepted test using guinea pigs or LLNA if appropriate.
- (g) **References.** The following references should be consulted for additional background information on this test guideline.

(1) *The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds*. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), National Institutes of Environmental Health Sciences, NIH Publication No. 99-4494.3 (1999). (Document available at <http://iccvam.niehs.nih.gov/methods/llnadocs/llnarep.pdf>.) Description and picture of auricular lymph node dissection available at <http://iccvam.niehs.nih.gov/methods/llnadocs/LLNAProt.pdf>.

(2) Kimber, I. et al. The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. *Contact Dermatitis* 21:215-220 (1989)

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## **Appendix G3**

### **International Organization for Standardization - ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Delayed-type Hypersensitivity (2002)**

Document available from the ISO website:

[http://www.iso.org/iso/iso\\_catalogue/catalogue\\_tc/catalogue\\_detail.htm?csnumber=33364](http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=33364)

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## **Appendix G4**

### **OECD Test Guideline 429: Skin Sensitisation – Local Lymph Node Assay (Adopted April 2002)**

Note: An updated version of this test guideline was approved by OECD's Working Group of National Coordinators for Test Guideline Programme in March 2010 and is expected to be formally updated by September 2010

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OECD/OCDE

429

Adopted:  
24<sup>th</sup> April 2002**OECD GUIDELINE FOR THE TESTING OF CHEMICALS****Skin Sensitisation: Local Lymph Node Assay****INTRODUCTION**

1. The OECD Test Guideline Programme periodically reviews progress in test method development and refinement, both in terms of scientific advances and animal welfare, to determine whether existing Test Guidelines should be updated and whether new Guidelines should be developed. Toward that end, a new assay for the determination of skin sensitisation in the mouse, the Local Lymph Node Assay (LLNA) has been sufficiently validated and accepted to justify its adoption as a new Test Guideline (1)(2)(3). This is the second Guideline to be promulgated for assessing skin sensitisation potential of chemicals in animals. The other Guideline (406) utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (4).

2. The LLNA provides certain advantages with regard to both scientific progress and animal welfare. It studies the induction phase of skin sensitisation and provides quantitative data suitable for dose response assessment. The details of the validation of the LLNA and a review of the associated work have been published (5)(6)(7)(8). In addition, it should be noted that the mild/moderate sensitisers, which are recommended as suitable positive control substances for guinea pig test methods, are also appropriate for use with the LLNA (6)(8)(9).

**INITIAL CONSIDERATIONS**

3. The LLNA provides an alternative method for identifying skin sensitising chemicals and for confirming that chemicals lack a significant potential to cause skin sensitisation. This does not necessarily imply that in all instances the LLNA should be used in place of guinea pig tests, but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation.

4. The LLNA is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of contact sensitising activity. It has, however, the potential to reduce the number of animals required for this purpose. Moreover, the LLNA offers a substantial refinement of the way in which animals are used for contact sensitisation testing. The LLNA is based upon consideration of immunological events stimulated by chemicals during the induction phase of sensitisation. Unlike guinea pig tests the LLNA does not require that challenged-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test. Thus, the LLNA reduces animal distress. Despite the advantages of the LLNA over traditional guinea pig tests, it should be recognised that there are certain limitations that may necessitate the use of traditional guinea pig tests (e.g., false negative findings in the LLNA with certain metals, false positive findings with certain skin irritants)(10).

## 429

## OECD/OCDE

### **PRINCIPLE OF THE TEST**

5. The basic principle underlying the LLNA is that sensitisers induce a primary proliferation of lymphocytes in the lymph node draining the site of chemical application. This proliferation is proportional to the dose applied (and to the potency of the allergen) and provides a simple means of obtaining an objective, quantitative measurement of sensitisation. The LLNA assesses this proliferation as a dose-response in which the proliferation in test groups is compared to that in vehicle treated controls. The ratio of the proliferation in treated groups to that in vehicular controls, termed the Stimulation Index, is determined, and must be at least three before a test substance can be further evaluated as a potential skin sensitiser. The methods described here are based on the use of radioactive labelling to measure cell proliferation. However, other endpoints for assessment of proliferation may be employed provided there is justification and appropriate scientific support, including full citations and description of the methodology.

### **DESCRIPTION OF THE ASSAY**

#### **Selection of animal species**

6. The mouse is the species of choice for this test. Young adult female mice of CBA/Ca or CBA/J strain, which are nulliparous and non-pregnant, are used. At the start of the study, animals should be between 8-12 weeks old, and the weight variation of the animals should be minimal and not exceed 20% of the mean weight. Other strains and males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA response do not exist.

### **HOUSING AND FEEDING CONDITIONS**

7. Animals should be individually housed. The temperature of the experimental animal room should be 22°C ( $\pm$  3°C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

### **PREPARATION OF ANIMALS**

8. The animals are randomly selected, marked to permit individual identification (but not by any form of ear marking), and kept in their cages for at least 5 days prior to the start of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of treatment all animals are examined to ensure that they have no observable skin lesions.

#### **Reliability check**

9. Positive controls are used to demonstrate appropriate performance of the assay and competency of the laboratory to successfully conduct the assay. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI)  $>3$  over the negative control group. The positive control dose should be chosen such that the induction is clear but not excessive. Preferred substances are hexyl cinnamic aldehyde (CAS No 101-86-0) and mercaptobenzothiazole (CAS No 149-30-4). There may be circumstances in which, given adequate justification, other control substances, meeting the above criteria, may be used. While ordinarily a positive control group may be required in each assay, there may be situations in which test laboratories will have available historic positive control data to show consistency of a satisfactory response over a six-month or

## OECD/OCDE

429

more extended period. In those situations, less frequent testing with positive controls may be appropriate at intervals of no greater than 6 months. Although the positive control substance should be tested in the vehicle that is known to elicit a consistent response (e.g., acetone:olive oil), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary. In such situations the possible interaction of a positive control with this unconventional vehicle should be tested.

**TEST PROCEDURE****Number of animals and dose levels**

10. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a negative control group treated only with the vehicle for the test substance, and a positive control, as appropriate. In those cases in which individual animal data are to be collected, a minimum of five animals per dose group are used. Dose and vehicle selection should be based on the recommendations given in reference (2). Doses are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5% etc. Existing acute toxicity and dermal irritation data should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure whilst avoiding systemic toxicity and excessive local skin irritation (2)(11). Except for absence of treatment with the test substance, animals in the control groups should be handled and treated in a manner identical to that of animals in the treatment groups.

11. The vehicle should be selected on the basis of maximising the test concentrations and solubility whilst producing a solution/suspension suitable for application of the test substance. In order of preference, recommended vehicles are acetone/olive oil (4:1 v/v), dimethylformamide, methyl ethyl ketone, propylene glycol and dimethyl sulphoxide (2)(10), but others may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test substance is marketed as an additional control. Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system, which wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

**Experimental schedule**

12. The experimental schedule of the assay is as follows:

- *Day 1:*  
Individually identify and record the weight of each animal. Open application of 25µL of the appropriate dilution of the test substance, the vehicle alone, or the positive control (as appropriate), to the dorsum of each ear.
- *Days 2 and 3:*  
Repeat the application procedure carried out on day 1.
- *Days 4 and 5:*  
No treatment.
- *Day 6:*  
Record the weight of each animal. Inject 250µL of phosphate-buffered saline (PBS) containing 20 µCi (7.4e+5 Bq) of <sup>3</sup>H-methyl thymidine into all test and control mice via the tail vein. Alternatively inject 250 µL PBS containing 2 µCi (7.4e + 4 Bq) of <sup>125</sup>I-iododeoxyuridine and 10<sup>-5</sup>M fluorodeoxyuridine into all mice via the tail vein. Five hours (5 h) later, the animals are killed. The draining auricular lymph nodes from each ear are excised and pooled in PBS for each experimental group (pooled treatment group approach);

## 429

## OECD/OCDE

alternatively pairs of lymph nodes from individual animals may be excised and pooled in PBS for each animal (individual animal approach). Details and diagrams of the node identification and dissection can be found in Annex I of the ICCVAM Immunotoxicology Working Group LLNA Protocol (10).

### **Preparation of cell suspensions**

13. A single cell suspension of lymph node cells (LNC) either from pooled treatment groups or bilaterally from individual animals is prepared by gentle mechanical disaggregation through 200 µm-mesh stainless steel gauze. Lymph node cells are washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4°C for 18h(2). Pellets are either re-suspended in 1 mL TCA and transferred to scintillation vials containing 1.0 mL of scintillation fluid for <sup>3</sup>H-counting, or transferred directly to gamma counting tubes for <sup>125</sup>I-counting.

### **Determination of cellular proliferation (incorporated radioactivity)**

14. Incorporation of <sup>3</sup>H-methyl thymidine is measured by β-scintillation counting as disintegrations per minute (DPM). Incorporation of <sup>125</sup>I-iododeoxyuridine is measured by <sup>125</sup>I-counting and also is expressed as DPM. Depending on the approach used, the incorporation will be expressed as DPM/treatment group (pooled approach) or DPM/animal (individual approach).

## **OBSERVATIONS**

### **Clinical observations**

15. Animals should be carefully observed once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with individual records being maintained for each animal.

### **Body weights**

16. As stated in paragraph 12, individual animal body weights should be measured at the start of the test and at the scheduled kill of the animals.

## **CALCULATION OF RESULTS**

17. Results are expressed as the Stimulation Index (SI). When using the pooled approach, the SI is obtained by dividing the pooled radioactive incorporation for each treatment group by the incorporation of the pooled vehicle control group; this yields a mean SI. When using the individual approach, the SI is derived by dividing the mean DPM /mouse within each test substance group and the positive control group by the mean DPM/mouse for the solvent/vehicle control group. The average SI for vehicle treated controls is then 1.

18. Use of the individual approach to calculate the SI will enable the performance of a statistical analysis of the data. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis. An adequate approach for interpreting the

## OECD/OCDE

429

data is to evaluate all individual data of treated and vehicle controls, and derive from these the best fitting dose response curve, taking confidence limits into account (10)(12)(13). However, the investigator should be alert to possible “outlier” responses for individual animals within a group that may necessitate the use of an alternative measure of response (e.g. median rather than mean) or elimination of the outlier.

19. The decision process with regard to a positive response includes a stimulation index  $\geq 3$ , together with consideration of dose-response and, where appropriate, statistical significance (3)(6)(10)(13)(14).

20. If it is necessary to clarify the results obtained, consideration should be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitisers, whether it causes excessive skin irritation, and the nature of the dose response seen. These and other considerations are discussed in detail elsewhere (7).

**DATA AND REPORTING****Data**

21. Data should be summarised in tabular form showing the mean and individual DPM values and stimulation indexes for each dose (including vehicle control) group.

**Test report**

22. The test report should contain the following information:

## Test substance:

- identification data (e.g. CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (e.g. volatility, stability, solubility);
- if mixture, composition and relative percentages of components.

## Vehicle:

- identification data (purity; concentration, where appropriate; volume used);
- justification for choice of vehicle.

## Test animals:

- strain of mice used;
- microbiological status of the animals, when known;
- number, age and sex of animals;
- source of animals, housing conditions, diet, etc.

## Test conditions:

- details of test substance preparation and application;
- justification for dose selection (including results from range finding study, if conducted);- vehicle and test substance concentrations used, and total amount of substance applied;
- details of food and water quality (including diet type/source, water source).

**429**

**OECD/OCDE**

Reliability check:

- a summary of results of latest reliability check, including information on substance, concentration and vehicle used;
- concurrent and/or historical positive and negative control data for testing laboratory.

Results:

- individual weights of animals at start of dosing and at scheduled kill;
- a table of mean/median (pooled approach) and individual (individual approach) DPM values, as well as the range of values for both approaches, and stimulation indices for each dose (including vehicle control) group;
- statistical analysis, where appropriate;
- time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal.

Discussion of results:

- A brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitiser.

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## OECD/OCDE

429

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**Appendix G5**

**OECD Test Guideline 406: Skin Sensitisation  
(Adopted July 1992)**

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**406**Adopted:  
17.07.92**OECD GUIDELINE FOR TESTING OF CHEMICALS****Adopted by the Council on 17<sup>th</sup> July 1992****Skin Sensitisation****INTRODUCTION**

1. OECD Guidelines for Testing of Chemicals are periodically reviewed in light of scientific progress. In such reviews, special attention is given to possible improvements in relation to animal welfare. This updated version of the original guideline 406, adopted in 1981, is the outcome of a meeting of OECD experts held in Paris in May 1991.

2. Currently, quantitative structure-activity relationships and *in vitro* models are not yet sufficiently developed to play a significant role in the assessment of the skin-sensitisation potential of substances which therefore must continue to be based on *in vivo* models.

3. The guinea pig has been the animal of choice for predictive sensitisation tests for several decades. Two types of tests have been developed: adjuvant tests in which sensitisation is potentiated by the injection of Freund's Complete Adjuvant (FCA), and non-adjuvant tests. In the original guideline 406, four adjuvant tests and three non-adjuvant tests were considered to be acceptable. In this updated version, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman which uses adjuvant (1)(2)(3)(4) and the non-adjuvant Buehler Test (5)(6) are given preference over other methods and the procedures are presented in detail. It is recognised, however, that there may be circumstances where other methods may be used to provide the necessary information on sensitisation potential.

4. The immune system of the mouse has been investigated more extensively than that of the guinea pig. Recently, mouse models for assessing sensitisation potential have been developed that offer the advantages of an endpoint which is measured objectively, short duration and minimal animal treatment. The mouse ear swelling test (MEST) and the local lymph node assay (LLNA) appear to be promising. Both assays have undergone validation in several laboratories (7)(8)(9)(10)(11) and it has been shown that they are able to detect reliably moderate to strong sensitisers. The LLNA or the MEST can be used as a first stage in the assessment of skin sensitisation potential. If a positive result is seen in either assay, a test substance may be designated as a potential sensitiser, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in the LLNA or MEST, a guinea pig test (preferably a GPMT or Buehler Test) must be conducted using the procedure described in this guideline.

5. Definitions used are set out in the Annex.

**GENERAL PRINCIPLE OF SENSITISATION TESTS IN GUINEA PIGS**

6. The test animals are initially exposed to the test substance by intradermal injection and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (induction

## 406

### OCDE / OECD

period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals is compared with that demonstrated by control animals which undergo sham treatment during induction and receive the challenge exposure.

#### **ELEMENTS COMMON TO SENSITISATION TESTS IN GUINEA PIGS**

##### **Sex of animals**

7. Male and/or female healthy young adult animals can be used. If females are used they should be nulliparous and non-pregnant.

##### **Housing and feeding conditions**

8. The temperature of the experimental animal room should be 20°C ( $\pm$  3°C) and the relative humidity 30-70 per cent. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

##### **Preparation of the animals**

9. Animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

##### **Reliability check**

10. The sensitivity and reliability of the experimental technique used should be assessed every six months by use of substances which are known to have mild-to-moderate skin sensitisation properties.

11. In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a non-adjuvant test should be expected for mild/moderate sensitisers. Preferred substances are hexyl cinnamic aldehyde (CAS No. 101-86-0), mercaptobenzothiazole (CAS No. 149-30-4) and benzocaine (CAS No. 94-09-7). There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used.

##### **Removal of the test substance**

12. If removal of the test substance is considered necessary, this should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

#### **DESCRIPTION OF THE GUINEA-PIG MAXIMISATION TEST METHOD**

##### **Number of animals**

13. A minimum of 10 animals is used in the treatment group and at least 5 animals in the control group. When fewer than 20 test and 10 control guinea pigs have been used, and it is not possible to conclude that the test substance is a sensitiser, testing in additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.

**Dose levels**

14. The concentration of test substance used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation. The concentration used for the challenge exposure should be the highest nonirritant dose. The appropriate concentrations can be determined from a pilot study using two or three animals. Consideration should be given to the use of FCA-treated animals for this purpose.

**Induction: Intradermal Injections****Day 0 - treated group**

15. Three pairs of intradermal injections of 0.1 ml volume are given in the shoulder region which is cleared of hair so that one of each pair lies on each side of the midline.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the test substance in an appropriate vehicle at the selected concentration

Injection 3: the test substance at the selected concentration formulated in a 1:1 mixture (v/v) FCA/water or physiological saline.

16. In injection 3, water soluble substances are dissolved in the aqueous phase prior to mixing with FCA. Liposoluble or insoluble substances are suspended in FCA prior to combining with the aqueous phase. The concentration of test substance shall be equal to that used in injection 2.

17. Injections 1 and 2 are given close to each other and nearest the head, while 3 is given towards the caudal part of the test area.

**Day 0 - control group**

18. Three pairs of intradermal injections of 0.1 ml volume are given in the same sites as in the treated animals.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the undiluted vehicle

Injection 3: a 50% w/v formulation of the vehicle in a 1:1 mixture (v/v) FCA/water or physiological saline.

**Induction: Topical Application****Day 5-7 - treated and control groups**

19. Approximately twenty-four hours before the topical induction application, if the substance is not a skin irritant, the test area, after close-clipping and/or shaving is painted with 0.5 ml of 10% sodium lauryl sulphate in vaseline, in order to create a local irritation.

**Day 6-8 - treated group**

20. The test area is again cleared of hair. A filter paper (2 x 4 cm) is fully-loaded with test substance in a suitable vehicle and applied to the test area and held in contact by an occlusive dressing for 48 hours. The choice of the vehicle should be justified. Solids are finely pulverised and

## 406

### OCDE / OECD

incorporated in a suitable vehicle. Liquids can be applied undiluted, if appropriate.

#### **Day 6-8 - control group**

21. The test area is again cleared of hair. The vehicle only is applied in a similar manner to the test area and held in contact by an occlusive dressing for 48 hours.

#### **Challenge: Topical Application**

#### **Day 20-22 - treated and control groups**

22. The flanks of treated and control animals are cleared of hair. A patch or chamber loaded with the test substance is applied to one flank of the animals and, when relevant, a patch or chamber loaded with the vehicle only may also be applied to the other flank. The patches are held in contact by an occlusive dressing for 24 hours.

#### **Observations - treated and control groups**

23. - approximately 21 hours after removing the patch the challenge area is cleaned and closely-clipped and/or shaved or depilated if necessary;
- approximately 3 hours later (approximately 48 hours from the start of the challenge application) the skin reaction is observed and recorded according to the grades shown below;
- approximately 24 hours after this observation a second observation (72 hours) is made and once again recorded.

Blind reading of test and control animals is encouraged.

#### **TABLE: MAGNUSSON AND KLIGMAN GRADING SCALE FOR THE EVALUATION OF CHALLENGE PATCH TEST REACTIONS**

0 = no visible change

1 = discrete or patchy erythema

2 = moderate and confluent erythema

3 = intense erythema and swelling

#### **Rechallenge**

24. If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

#### **Clinical observations**

25. All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures, e.g.

histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

#### **DESCRIPTION OF THE BUEHLER TEST METHOD**

##### **Number of animals**

26. A minimum of 20 animals is used in the treatment group and at least 10 animals in the control group.

##### **Dose levels**

27. The concentration of test substance used for each induction exposure should be the highest to cause mild irritation. The concentration used for the challenge exposure should be the highest non-irritating dose. The appropriate concentration can be determined from a pilot study using two or three animals.

28. For water soluble test materials, it is appropriate to use water or a dilute non-irritating solution of surfactant as the vehicle. For other test materials 80% ethanol/water is preferred for induction and acetone for challenge.

##### **Induction: Topical application**

###### **Day 0 - treated group**

29. One flank is cleared of hair (closely-clipped). The test patch system should be fully loaded with test substance in a suitable vehicle (the choice of the vehicle should be justified; liquid test substances can be applied undiluted, if appropriate). The test patch system is applied to the test area and held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours.

30. The test patch system must be occlusive. A cotton pad is appropriate and can be circular or square, but should approximate 4-6 cm<sup>2</sup>. Restraint using an appropriate restrainer is preferred to assure occlusion. If wrapping is used, additional exposures may be required.

###### **Day 0 - control group**

31. One flank is cleared of hair (closely-clipped). The vehicle only is applied in a similar manner to that used for the treated group. The test patch system is held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours. If it can be demonstrated that a sham control group is not necessary, a naive control group may be used.

###### **Days 6-8 and 13-15 - treated and control groups**

32. The same application as on day 0 is carried out on the same test area (cleared of hair if necessary) of the same flank on day 6-8, and again on day 13-15.

##### **Challenge**

###### **Day 27-29 - treated and control groups**

33. The untreated flank of treated and control animals is cleared of hair (closely-clipped). An occlusive patch or chamber containing the appropriate amount of test substance is applied, at the maximum non-irritant concentration, to the posterior untreated flank of treated and control animals.

## 406

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When relevant, an occlusive patch or chamber with vehicle only is also applied to the anterior untreated flank of both treated and control animals. The patches or chambers are held in contact by a suitable dressing for 6 hours.

#### **Observations - treated and control groups**

34. - approximately 21 hours after removing the patch the challenge area is cleared of hair;
- approximately three hours later (approximately 30 hours after application of the challenge patch) the skin reactions are observed and recorded according to the grades shown in the Guinea-Pig Maximisation Test (see paragraph 23);
- approximately 24 hours after the 30 hour observation (approximately 54 hours after application of the challenge patch) skin reactions are again observed and recorded.

Blind reading of test and control animals is encouraged.

#### **Rechallenge**

35. If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. The rechallenge may also be performed on the original control group.

#### **Clinical observations**

36. All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures, e.g. histopathological examination, measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

#### **DATA AND REPORTING (GPMT and Buehler Test)**

##### **Data**

37. Data should be summarised in tabular form, showing for each animal the skin reactions at each observation.

##### **Test report**

38. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Vehicle:

- justification of choice of vehicle.

Test animals:

- strain of guinea-pig used;

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406

- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start and at the conclusion of the test.

## Test conditions:

- technique of patch site preparation;
- details of patch materials used and patching technique;
- result of pilot study with conclusion on induction and challenge concentrations to be used in the test;
- details of test substance preparation, application and removal;
- vehicle and test substance concentrations used for induction and challenge exposures and the total amount of substance applied for induction and challenge.

## Reliability check:

- a summary of the results of the latest reliability check including information on substance, concentration and vehicle used.

## Results:

- on each animal including grading system;
- narrative description of the nature and degree of effects observed;
- any histopathological findings.

## Discussion of the results.

If a screening assay is performed before the guinea pig test the description or reference of the test, including details of the procedure, must be given together with results obtained with the test and reference substances.

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## 406

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406

ANNEX

**DEFINITIONS**

Skin sensitisation (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterised by pruritis, erythema, oedema, papules, vesicles, bullae or a combination of these. In other species the reactions may differ and only erythema and oedema may be seen.

Induction exposure: an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

Induction period: a period of at least one week following an induction exposure during which a hypersensitive state may develop.

Challenge exposure: an experimental exposure of a previously treated subject to a test substance following an induction period, to determine if the subject reacts in a hypersensitive manner.

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