Internationally Harmonized Performance Standards for the Murine Local Lymph Node Assay (LLNA)

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Introduction

- United States and international regulatory authorities accept the murine local lymph node assay (LLNA) as an alternative test method for allergic contact dermatitis testing (ICCVAM 1999; OECD 2002).
- The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Centre for the Validation of Alternative Methods (JaCVAM), has developed LLNA performance standards (PS) that can be used to evaluate modified versions of the LLNA that are mechanistically and functionally similar to the LLNA.
- These performance standards consist of:
  - Essential test method components
  - Reference substances
  - The accuracy and reliability that should be achieved or exceeded by a modified test method

Essential Test Method Components

Essential test method components are structural, functional, and procedural elements of a validated test method, necessary for it to be evaluated using the PS. For the LLNA, these include:

- The test substance is applied topically to both ears of mice.
- Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.
- Lymphocyte proliferation must be measured during the induction phase of skin sensitization.
- The highest dose selected must be the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation.
- A vehicle control must be included in each study and, where appropriate, a positive control should be used.
- A minimum of four animals per dose group is required.

The essential test method components have been internationally harmonized for the validation of modifications to the traditional LLNA. However, certain national regulatory authorities might have requirements that differ for the prospective use of a modified LLNA test method in support of regulatory submissions.

Development of ICCVAM Performance Standards for the LLNA: Timeline

Date	Event
January 10, 2007	ICCVAM nomination from the CPSC.
January 24, 2007	ICCVAM endorses development of LLNA performance standards. Immunotoxicology Working Group is reactivated.
May 17, 2007	*Federal Register* notice (72 FR 27815) – *The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts for Peer Review Panel, and Submission of Data to Consider for LLNA PS.*
September 12, 2007	Draft ICCVAM LLNA Performance Standards released for public comment (72 FR 52130).
September 25-27, 2007	ECVAM Workshop on an evaluation of performance standards and alternative endpoints for the LLNA. ECVAM Workshop Report 65. Basketter *et al.* (2008). *ATLA* 36, 243-257.
October 30-31, 2007	ECVAM Scientific Advisory Committee (ESAC) considers both ICCVAM and ECVAM drafts of LLNA performance standards.
January 8, 2008	Revised draft PS published for public comment (73 FR 1360).
March 4-6, 2008	Review of draft PS by international independent Peer Review Panel, CPSC, Bethesda, MD; public meeting with opportunity for oral public comments.
May 20, 2008	Announcement of public availability of the *Peer Review Panel Report on the Validation Status of New Versions and Applications of the LLNA* (73 FR 29136)
June 18-19, 2008	SACATM public meeting comments on Panel report.
September 23-24, 2008	ECVAM/JACVAM/ICCVAM meeting on internationally harmonized LLNA PS.
October 29, 2008	ICCVAM endorses harmonized LLNA PS.
November 4-5, 2008	ESAC endorses harmonized LLNA PS.
March 2009	Final ICCVAM LLNA performance standards published.
July 2009	Circulation of revised OECD TG 429 with LLNA PS.

Abbreviations: CPSC = U.S. Consumer Product Safety Commission; ECVAM = European Centre for the Validation of Alternative Methods; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; IWG = ICCVAM Immunotoxicity Working Group; LLNA = murine local lymph node assay; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; SACATM = Scientific Advisory Committee on Alternative Toxicological Methods

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Updated ICCVAM-recommended Test Method Protocol for the LLNA

ICCVAM's LLNA performance standards include the updated ICCVAM-recommended test method protocol (**see Poster 580**).

Key Elements

- The highest dose tested should be the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation.
- Individual animal data is collected.
- A concurrent positive control is included in each LLNA study.
- A minimum of four individual animals rather than five individual animals per group is required.

Criteria for Selection of Reference Substances

- Commercial availability
- Available LLNA, guinea pig, and (where possible) human data/experience
- Range of LLNA responses from negative to weak to strong
- Range of chemistry and chemical classes
- Range of physical properties (e.g., solids vs. liquids)

Performance Standards Reference Substances for the LLNA

- 18 required substances and
- 4 "optional substances" that are either false positive or false negative in the LLNA when compared to either human or guinea pig results.

Substance	Form	Vehicle	Max SI (Conc.)	EC3 (%)[1]	N[2]	LLNA vs. GP	LLNA vs. Human
5-Chloro-2-methyl-4-isothiazolin-3-one	Liq	DMF	22.7 (0.1%)	0.009	1	+/+	+/+
DNCB	Sol	AOO	43.9 (0.025%)	0.049	15	+/+	+/+
4-Phenylenediamine	Sol	AOO	26.4 (1%)	0.11	6	+/+	+/+
Cobalt chloride	Sol	DMSO	7.2 (5%)	0.6	2	+/+	+/+
Isoeugenol	Liq	AOO	31 (5%)	1.5	47	+/+	+/+
2-Mercaptobenzo-thiazole	Sol	DMF	8.6 (10%)	1.7	1	+/+	+/+
Citral	Liq	AOO	20.5 (20%)	9.2	6	+/+	+/+
HCA	Liq	AOO	20 (50%)	9.7	21	+/+	+/+
Eugenol	Liq	AOO	17 (50%)	10.1	11	+/+	+/+
Phenyl benzoate	Sol	AOO	11.1 (25%)	13.6	3	+/+	+/+
Cinnamic alcohol	Sol	AOO	5.7 (90%)	21	1	+/+	+/+
Imidazolidinyl urea	Sol	DMF	5.5 (50%)	24	1	+/+	+/+
Methyl methacrylate	Liq	AOO	3.6 (100%)	90	1	+/+	+/+
Chlorobenzene	Liq	AOO	1.7 (10%)	NA	1	-/-	-/*
Isopropanol	Liq	AOO	1.7 (10%)	NA	1	-/-	-/+
Lactic acid	Liq	DMSO	2.2 (50%)	NA	1	-/-	-/*
Methyl salicylate	Liq	AOO	2.7 (20%)	NA	9	-/-	-/-
Salicylic acid	Sol	AOO	2.5 (25%)	NA	1	-/-	-/-
Optional Substances that Provide an Opportunity to Demonstrate Improved Performance Relative to the Traditional LLNA							
Sodium lauryl sulfate	Sol	DMF	8.9 (10%)	8.1	5	+/-	+/-
Ethylene glycol dimethacrylate	Liq	MEK	7 (50%)	28	1	+/-	+/+
Xylene	Liq	AOO	3.1 (100%)	95.8	1	+/**	+/-
Nickel chloride	Sol	DMSO	2.4 (5%)	NA	2	-/+	-/+

Abbreviations: AOO = acetone: olive oil (4:1); Conc = concentration; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; EC3 = estimated concentration needed to produce a stimulation index of 3; GP = guinea pig test result; HCA = hexyl cinnamic aldehyde; Liq = liquid; LLNA = murine local lymph node assay result; Max SI = maximum stimulation index; MEK = methyl ethyl ketone; NA = not applicable since SI <3; NC = not calculated since data was obtained from a single study; No. = number; Sol = solid.
[1] Mean value where more than one EC3 value was available
[2] Number of LLNA studies from which data were obtained
* = Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.
** = GP data not available

Test Method Performance Standard: Accuracy

- The accuracy of a modified LLNA test method should meet or exceed that of the traditional LLNA when evaluated using the 18 minimum recommended reference substances.

Test Method Performance Standard: Intra- and Interlaboratory Reproducibility

- Intralaboratory reproducibility:
  - Derive ECt values for HCA on 4 separate occasions (minimum 1 week between tests).
    - ECt values between 5 and 20% indicate acceptable intralaboratory reproducibility.
- Interlaboratory reproducibility:
  - Derive ECt values for HCA and DNCB from at least one study in each of 3 separate laboratories.
    - ECt values between 5% and 20% for HCA and between 0.025% and 0.1% for DNCB indicate acceptable interlaboratory reproducibility

ECt: estimated concentration needed to produce a stimulation index with a specific threshold value (an stimulation index value) in order to distinguish between sensitizers and nonsensitizers.

Independent Scientific Peer Review Panel

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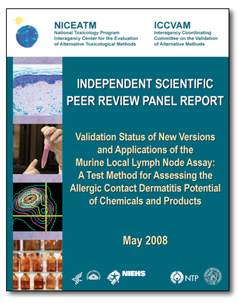

Highlights from the LLNA Peer Review Panel Meeting (ICCVAM 2008)

Essential Test Method Components

- Individual animal data should be collected to allow an estimate of the variance within control and treatment groups.
- These tests should measure the induction phase of the immune response only.
- A concurrent positive control should be used.
  - (Note: if a known sensitizer is being tested during the validation effort, a concurrent positive control might not be needed.)

Accuracy Standards

- Ideally, the performance of an alternative LLNA protocol should be equivalent or better than that of the traditional LLNA, but it may not be necessary to reach the same level of accuracy if appropriate rationale for any discordance is provided.
  - The sensitizers on the list should be weighted such that the strongest sensitizers must always be identified.

Reliability Standards

- Using an ECt range is appropriate for the intra- and inter-laboratory reproducibility analysis because a large database of LLNA studies is available for HCA and DNCB from which to determine the appropriateness of the range.

Conclusions

- Internationally harmonized performance standards for the LLNA provide criteria that can be used to more efficiently and more rapidly evaluate the validity of similar new test methods.
- New versions of the LLNA that provide for improved performance and that offer other advantages, such as not requiring the use of nonradioactive markers, are expected to result in broader use of the LLNA, which will further reduce and refine animal use for allergic contact dermatitis assessments while ensuring human safety.
- Adoption of a revised OECD TG 429 (OECD 2002) that incorporates these performance standards will facilitate more rapid and efficient international validation and acceptance of modified LLNA protocols (e.g., those using nonradioactive markers of lymphocyte proliferation).

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