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11.0 PRACTICAL CONSIDERATIONS

The 3T3 and NHK NRU test methods are proposed as adjuncts, rather than replacements for, *in vivo* acute oral toxicity assays. Data from these *in vitro* basal cytotoxicity test methods are used with a linear regression model to predict the rat acute oral LD_{50} of the test substance, which is then used to determine the starting dose for subsequent rat acute oral toxicity tests, as described in **Sections 10.2.2** and **10.3.2**. This section discusses practical issues involved in using these two *in vitro* NRU test methods for predicting starting doses for rat acute oral toxicity tests. Practical issues that need to be considered with respect to the implementation of these cell culture methods include the need for, and availability of, specialized equipment, personnel training and expertise requirements, cost considerations, and time expenditures.

11.1 Transferability of the 3T3 and NHK NRU Test Methods

Transferability of a test method is defined as the ability of a method or procedure to be accurately and reliably performed in different, competent laboratories (ICCVAM 2003). Accuracy and reliability of these NRU test methods are discussed in **Sections 6** and **7**, respectively.

Protocols for the 3T3 and NHK NRU test methods, including solubility testing, and prequalification of keratinocyte growth medium, have been optimized and are available on the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov/methods/invitro.htm). The protocols were designed with GLP-compliance in mind and can be easily implemented or adapted by scientists with the appropriate technical experience.

Although the *in vitro* and *in vivo* test methods require some similar, general laboratory skills (e.g., preparation of solutions and test substance doses, record keeping), *in vitro* testing requires skills specific to cell culture systems (e.g., aseptic techniques, microscopic evaluation of cell cultures, propagation of cells in medium) but not to the maintenance, handling, or treatment of rodents.

11.1.1 Facilities and Major Fixed Equipment

The following lists of facility requirements, equipment and supplies, and training and expertise are common to most *in vitro* mammalian cell culture laboratories. Required equipment and supplies are also described in detail in the validation study 3T3 and NHK protocols (**Appendices B** and **C**), the *Guidance Document* (ICCVAM 2001b), and Hartung et al. (2002).

11.1.1.1 *Facility Requirements*

The testing facility should be appropriate for operating a scientific laboratory (e.g., laboratory space, air handling procedures, access to utilities, shipping/receiving department [for appropriate receipt and handling of cell culture materials], etc.). Each facility should provide:

- Adequate facilities, equipment, and supplies
- Proper health and safety guidelines
- Satisfactory quality assurance procedures

Each facility should conform to all appropriate statutes (i.e., local, state, provincial, federal, national, international) concerning safety guidelines (e.g., general workplace safety

guidelines, chemical handling and disposal guidelines, biohazard guidelines). Hartung et al. (2002) provides recommended safety guidelines for working with potentially infectious materials (e.g., HIV, hepatitis B, hepatitis C) and human materials (e.g., cells, tissues, fluids).

11.1.1.2 *Cell Culture Laboratory*

The testing facility should have a designated cell culture laboratory to ensure that *in vitro* cytotoxicity assays are performed under clean and proper aseptic conditions. The dedicated laboratory should be located such that through traffic is minimal to reduce possible disturbances that can lead to contamination which could compromise the cell culture assays. The room temperature of the laboratory should be regulated, monitored, and documented. Access to the laboratory and its supplies and test chemicals should be restricted to appropriate personnel.

11.1.1.3 *Major Equipment*

Each testing facility should have at a minimum the following equipment:

- Incubator (37ºC ± 1ºC, 90% ± 10% humidity, 5.0% ± 1% CO_2/air)
- Laminar flow clean bench/cabinet (standard: "biological hazard")
- Inverted phase contrast microscope
- 96-well plate spectrophotometric plate reader equipped with 540 nm ± 10 nm filter (if testing in 96-well plates)
- Autoclave
- Refrigerator
- Freezer (-70ºC)
- Cryogenic (liquid nitrogen) freezer/storage unit
- Computer

Equipment maintenance and calibration should be routinely performed and documented according to GLP guidelines and testing facility SOPs.

11.1.2 <u>Availability of Other Necessary Equipment and Supplies</u>

11.1.2.1 *General Equipment*

Each testing facility should have at a minimum the following equipment:

- Low speed centrifuge
- Adjustable temperature waterbath
- Pipettors
- Balance
- pH meter
- Cell counting system
- Water bath sonicator
- Magnetic stirrer
- Vortex mixer
- Antistatic bar ionizer (for reduction of static on tissue culture plates)

Equipment maintenance and calibration should be routinely performed and documented as per GLP guidelines and testing facility SOPs. The types of equipment listed in this section are available from scientific and laboratory supply companies (e.g., Thomas Scientific - http://www.thomassci.com/index.jsp; Fisher Scientific - https://www.fishersci.com/).

11.1.2.2 *Cell Culture Materials and Supplies*

The following supplies are needed for the *in vitro* NRU test methods. Specific product and private company names are provided either as an identification of actual materials/brands used in the validation study or as examples. Mention of these names does not imply endorsement of the product or company.

- Tissue culture plasticware (flasks [e.g., 25 cm^2, 75-80 cm^2], 96-well plates, disposable pipettes)
- Laboratory glassware (e.g., flasks, bottles, graduated cylinders)
- Adhesive film plate sealers (e.g., Excel Scientific SealPlate™)
- Sterile filtration systems (e.g., vacuum filtration units with 0.22 µm and 0.45 µm sterile filters)
- Culture medium and supplements (e.g., DMEM; prequalified NHK medium)
- NCS (bovine)
- Balanced salt solutions (e.g., HBSS, D-PBS)

Cell culture supplies are generally available through the major scientific and laboratory supply companies and through specialty companies (e.g., GIBCO, SIGMA-Aldrich, CAMBREX/Biowhittaker, Becton Dickinson). Compositions of culture media, supplements/additives, salt solutions, NRU assay chemicals, and the volumes of each needed for each test method, should be defined. All tissue culture flasks and dishes needed to assure proper cell propagation should be identified.

11.1.2.3 *Cell Cultures*

3T3 Mouse Fibroblasts: BALB/c 3T3 cells, clone 31, can be obtained from national/international cell culture repositories (e.g., American Type Culture Collection [ATCC], Manassas, VA, product # CCL-163).

NHKs: These non-transformed keratinocyte cells from cryopreserved primary or secondary cells can be obtained from national/international cell culture repositories (e.g., CAMBREX Bio Science, 8830 Biggs Ford Road, Walkersville, MD), or isolated from donated tissue using proper collection, preparation, and propagation techniques. It may be difficult, at times, to obtain adequate supplies of keratinocytes; the preparation of a pool of cells depends on the availability of tissue donors. It is recommended that testing laboratories procure of a commercially available stock pool of cells and store them indefinitely in a cryogenic freezer.

All cell stock and cultures used for testing must be certified as free of contamination by mycoplasma and bacteria.

11.1.3 <u>Problems Specific to the NHK NRU Test Method</u>

FAL had difficulty obtaining an adequate supply of NHK medium during the validation study. Communication between the UK distributor and the laboratory was uneven and the SMT attempted to resolve the supply issue on several occasions. The other laboratories periodically had difficulties in obtaining NHK medium and supplements that adequately supported keratinocyte growth. Although the purchased medium and supplements met the manufacturer's QA/QC standards, certain lots of the medium and supplements did not support the growth of NHK cells to the extent needed in the test protocol. To deal with these problems, an NHK medium prequalification protocol was incorporated into the study to

avoid unnecessarily repeating studies because of medium and supplements that did not adequately support cell growth. These experiences illustrate the need for multiple sources of keratinocyte cell culture medium. They also suggest that the NHK results could be more variable than the 3T3 results because of the batch-to-batch differences in NHK growth medium and supplements.

11.2 3T3 and NHK NRU Test Method Training Considerations

The ECVAM Good Cell Culture Practice Task Force Report 1 (Hartung et al. 2002) encouraged the establishment of practices and principles that will reduce uncertainty in the development and application of *in vitro* test methods. Training in good cell culture practices, in conjunction with good laboratory practices, are essential for all *in vitro* cytotoxicity testing and should be employed to ensure that data produced from the 3T3 and NHK NRU test methods are reproducible, credible, and acceptable.

In vitro cytotoxicity test methods require personnel trained specifically in sterile tissue/cell culture techniques and general laboratory procedures. Personnel should have mandatory training in good cell culture practices, in the specialized culture procedures needed for these assays, and in safety and handling practices appropriate to the types of substances that may be tested in the laboratory (Hartung et al. 2002).

The facility management should establish scientific guidelines and procedures, train and supervise professional and technical staff, and evaluate results and performance within their discipline area relative to the testing requirements. Performance of the tests requires a moderate degree of technical capability and a high degree of skill in monitoring and maintaining appropriate cell growth conditions, troubleshooting the potential and real problems in culture systems, and analyzing and interpreting *in vitro* cytotoxicity data. Each individual engaged in the conduct of a study, or responsible for its supervision, shall have education, training, and experience, or combination thereof, to enable that individual to perform the assigned duties. The NRU test methods do not require that personnel be trained to perform *in vivo* testing.

11.2.1 Required Training and Expertise

Personnel performing *in vitro* testing should have training in basic cell culture aspects such as: sterile technique, handling culture media, feeding cultures, cell counting, subculture (trypsinization), detection and elimination of contamination, cell growth and measurement of growth curves, viability assays, and storage and freezing/thawing of cells. Additionally, training is encouraged for special culture procedures such as primary cell and tissue cultures, toxicity testing, and viability assays. Laboratory personnel should be trained in the application of GLP requirements (see **Section 8.1.1**), and in the safe storage, handling, and disposal of toxic substances.

11.2.1.1 *Specific Training and Expertise Needed*

Personnel performing the *in vitro* cytotoxicity test methods should be well experienced in general cell culture techniques and should be able to:

- Work with cryogenic freezing apparatus
- Pipette solutions with large volume pipettors and multi-channel pipettors

- Establish cells in culture vessels under aseptic conditions and monitor growth; recognize normal and abnormal cell growth characteristics; and document observations of cell cultures throughout all aspects of the procedure
- Perform the *in vitro* assays by following the protocols to grow the cells, count, transfer, and feed the cells, treat the cells with test substances, perform application of adhesive plate sealers to culture plates for control of volatile substances, perform the NRU assay, perform optical density measurements, transfer data to electronic templates
- Operate equipment necessary for maintaining cell culture laboratories (e.g., incubators, biohazard hoods, spectrophotometric microtiter plate readers)

11.2.1.2 *General Laboratory Expertise Needed*

Personnel should also be able to understand and perform basic laboratory techniques and laboratory management:

- Prepare cell culture solutions (e.g., culture medium, NRU solutions), measure pH, know proper storage conditions, and maintain proper documentation
- Prepare test substances for application to cell cultures, follow solubility protocols to adequately prepare test chemicals in solution, recognize solubility issues (e.g., insolubility nature of chemical, precipitation), and implement procedures for dissolving the test chemicals
- Monitor and control laboratory environment (e.g., temperature, humidity, lighting, traffic), maintain equipment to support cell cultures (e.g., temperature, humidity, gas flow, calibrations)

11.2.2 Training Requirements to Demonstrate Proficiency

Laboratories establish their own criteria for proficiency but, over the course of training, laboratory personnel should be able to understand the protocol, perform the protocol with guidance from an experienced supervisor/trainer and, eventually, perform the protocol with minimal or no supervision. An experienced supervisor determines when a technician is adequately trained because there are no standardized criteria or tasks that can be used to accurately measure competence. After the technician demonstrates competence in executing all the aspects of the test protocols(s), it is appropriate to perform routine assessments of technical competence using a benchmark, coded control test substance (e.g., SLS). It is essential that the laboratory staff be certified as proficient in using the test methods to test unknowns.

The laboratories in the validation study were selected because of their experience in performing *in vitro* cytotoxicity assays but were required to develop additional skills through Phases I and II (e.g., data collection and transfer to Excel® and PRISM® templates). Inexperienced laboratory personnel were trained by having them perform “training” assays using SLS. In the early phases of the validation study, the laboratories continued training by testing coded reference substances of various toxicities, and performing solubility testing on substances of varying solubilities. These procedures helped improve proficiency among the laboratories for the final phase of the validation study.

11.2.2.1 *Proficiency With GLP-Compliance*

Results from these test methods will be submitted to regulatory agencies that will, for the most part, require GLPs. Laboratories should work toward attaining GLP compliance. GLP compliance in each laboratory is determined by its independent QA unit. ECBC and IIVS conducted this validation study in compliance with GLP (see **Section 8.1.1**). Their respective QA units (as per GLPs) reviewed the various aspects of the study and issued QA statements that addressed whether the test methods and the results described in the Final Report accurately followed the test protocol and reflected the raw data produced during the study, and provided assurance that all testing was done under according to GLP. FAL (which was non-GLP-adherent) followed the GLP standards referenced in **Section 8.1.1** as guidelines for conducting this study. FAL had no QA unit to judge GLP compliance.

11.2.3 Personnel Needed to Perform the *In Vitro* NRU Test Methods

The facility management will be responsible for determining which qualified personel meet the criteria (e.g., scientific knowledge, specialized training) for the following positions needed for adequate performance of the *in vitro* NRU test methods and oversight of the testing.

- Study Director: the individual with the overall responsibility for the technical conduct of the testing (e.g., is familiar with the test procedures, provides SOPs and ensures GLP compliance, analyzes and interprets the data, determines test acceptance, oversees recordkeeping procedures, and produces the test reports.
- Quality Assurance Officer: monitors the testing to assure conformance with GLP requirements; must be independent of the Study Director.
- Laboratory Technician(s): individuals trained in sterile tissue/cell culture techniques and general laboratory procedures and who are capable of performing the test methods according to GLPs.

11.3 Cost Considerations

11.3.1 3T3 and NHK NRU Test Methods

11.3.1.1 *Equipment Costs*

Major instruments and equipment needed to implement the *in vitro* cytotoxicity test methods are described in **Section 11.1.1**. Ranges of costs for some of the equipment were obtained from on-line catalogues for two major scientific equipment and supplies companies (Thomas Scientific - http://www.thomassci.com/index.jsp; Fisher Scientific - https://www.fishersci.com/). These prices are for equipment that will meet the minimum needs of the NRU test methods (see **Table 11-1**). These costs were researched in August 2006.

11.3.1.2 *Costs for Cell Cultures and Supplies*

Supplies such as cell culture chemicals, the reagents used to measure NRU, and cell culture plasticware are available from numerous suppliers, and are not cost prohibitive.

Table 11-1 Costs for Major Laboratory Equipment

Equipment	Range of Costs[1]
Class II Biological Safety Cabinet	$7,300 – $12,200
CO_2 Incubator	$5,100 – $16,400
Spectrophotometer Microplate Reader	$5,000 – $7,500
Freezer (capable of -70°C)	$8,000 – $15,300
Refrigerator	$1,300 – $9,800
Centrifuge (benchtop model)	$2,100 – $8,500
Microscope (inverse phase contrast)	$3,000 – $14,500
Coulter Counter[2, 3]	$3,000 – $9,000
Autoclave (benchtop model)[2]	$3,500 – $15,400
Cryogenic (liquid nitrogen) Storage	$1,000 – $3,700

[1]From on-line scientific equipment catalogues (Thomas Scientific - http://www.thomassci.com/index.jsp; Fisher Scientific - https://www.fishersci.com/). [searched August 2006]
[2]May be useful, but not required for performing the tests.
[3]Other automatic cell counters may be used.

The 3T3 NRU test method is generally less expensive to perform than the NHK NRU test method. One vial of the immortalized 3T3 cells (~$200 [ATCC]) can be propagated indefinitely by passaging cells and periodically cryopreserving batches of cells. The NHK NRU test method requires a fresh sample of primary cells for each test run (~$380 per vial [CAMBREX]). Because primary NHK cells are passaged only once after initiating the culture, there are no cells available to cryopreserve a stock batch of cells. The DMEM medium used for the 3T3 cells is less expensive, more "generic", and more readily available than keratinocyte-specific NHK medium. (See **Table 11-2.**)

11.3.1.3 *Commercial Testing*

The following price quotes are provided as examples of test costs and were acquired from commercial laboratories through Internet contact or through personal communication. Use of information from these specific laboratories does not imply endorsement of them.

A representative of MB Research Laboratories (Spinnerstown, PA, http://www.mbresearch.com/) provided a quote (personal communication, 2005) for an *in vitro* 24-hr cytotoxicity test (but not a 48-hour test period) of $1050 (USP standards[1]) or $1950 (ISO standards[1]) for a set of three test chemicals. The lead laboratory for the NICEATM/ECVAM study, IIVS (Gaithersburg, MD, http://www.iivs.org/) provides commercial laboratory GLP-compliant testing using this study's protocols (48-hour test

[1] USP=United States Pharmacopeia; ISO=International Standards Organization. These organizations provide international standard testing requirements for products that require high quality for public use.

period) at a cost of $1120 - $1850 per chemical/sample for one cell type (personal communication 2005) (see **Table 11-2**).

Table 11-2 Costs for Cell Culture Materials and Commercial Laboratory *In Vitro* Cytotoxicity Testing

Item	Cost (approximate)	Number of Tests Possible	Other
3T3 Cells	~$200/vial[1]	indefinite	One vial can produce an indefinite supply of cells by propagating the cells in culture and periodically freezing a pool of cells.
NHK Cells	~$380/vial[2]	~5 (96-well plates)	Since cells are passaged only once beyond cryopreservation, new vials should be thawed as needed to maintain continuous testing.
Dulbeccos' Minimum Essential Medium (D-MEM) with supplements	~$20/500mL[3]	~15 (96-well plates)	Establish cells in culture (~20 mL/vial of cells; 60 mL/3 vials), seed cells in 96-well plates (12 mL/plate; 180 mL/15 plates); prepare stock solution and eight concentration dilutions (~20 mL/chemical; 300 mL/15 plates).
NHK Medium with supplements	~$80/500 mL[2]	~15 (96-well plates)	Same as DMEM (above)
Commercial Laboratory Testing (MB Research Laboratories [GLP-compliant])	$1050/$1950 (USP/ISO) per 3 test materials[4]	1 test/material	*in vitro* NRU cytotoxicity test (24-hour test period)
Commercial Laboratory Testing (Institute for *In Vitro* Sciences [GLP-compliant])	$1120 (GLP) per test material (minimum of 5 materials tested simultaneously)[4]	1 range finder, 2 definitive tests per test material	*in vitro* NRU cytotoxicity test (48-hour test period)
Commercial Laboratory Testing (Institute for *In Vitro* Sciences)	$1850 (GLP) per single test material (tested individually)[4]	1 range finder, 2 definitive tests per test material	*in vitro* NRU cytotoxicity test (48-hour test period)

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; USP/ISO= United States Pharmacopeia/International Standards Organization GLP=Good Laboratory Practices

[1]Catalogue price from American Type Culture Collection (ATCC) (http://www.atcc.org/)
[2]Catalogue price from CAMBREX (http://www.cambrex.com/Welcome.asp)
[3]Catalogue price from INVITROGEN (http://www.invitrogen.com/content.cfm?pageid=1)
[4]Personal communication (Raabe 2005)

11.3.2 Rodent Acute Oral Toxicity Testing

As stated in **Section 11.3.1.3**, presentation of price quotes from commercial laboratories provides examples of test costs and does not imply an endorsement of that laboratory. **Table 11-3** provides some commercial prices for acute oral systemic toxicity testing. MB Research Laboratories performs the UDP test at a cost of $750 for three rats and charges $250 for each additional rat needed. In the best-case scenario, the UDP test needs only three rats ($750). In the worst-case scenario, this test would need an additional 12 rats (15 maximum for the test); the total cost of the test would be $3,750. In this costing strategy, $250 is saved for each rat not used by an accurate prediction of the starting dose by the 3T3 or NHK NRU test method. Because the *in vitro* cytotoxicity test costs from $350 to $1850 per chemical, there is no net savings in animal costs if fewer than two to six animals are saved.

Table 11-3 Commercial Prices for Conducting *In Vivo* Acute Rat Toxicity Testing

Test	GLP-Compliant	Non GLP-Compliant	Company
Acute Oral Toxicity UDP: Limit Test - 2000 mg/kg	$1200	$1000	Product Safety Laboratories
Acute Oral Toxicity UDP: Limit Test - 5000 mg/kg	$800	$650	Product Safety Laboratories
Acute Oral Toxicity UDP: LD_{50}	$2700	$2200	Product Safety Laboratories[1]
Acute Oral Rat Toxicity: single dose[2]	$950	NA	Bio Research Laboratories
Acute Oral Rat Toxicity: two doses[2]	$1500	NA	Bio Research Laboratories
Acute Oral Rat Toxicity: LD_{50}	$3000	NA	Bio Research Laboratories
Acute Oral Toxicity – UDP	$730 for the first 3 animals; $250 each additional animal	NA	MB Research Laboratories[1]

Abbreviations: UDP=Up-and-Down Procedure; GLP=Good Laboratory Practices; NA=Not available.
[1]Personal communication (Wnorowski 2005).
[2]Washington State Biological Testing Methods #80-12 For the Designation of Dangerous Waste; Part B: Acute Oral Rat Toxicity Test [http://www.ecy.wa.gov/pubs/80012.pdf]. This test method is an adaptation of the EPA Health Affects Test Guidelines OPPTS 870.110 Acute Oral Toxicity and American Society for Testing and Materials (ASTM) methods E 1163-90 (Standard test method for estimating acute oral toxicity in rats) and E 1372-90 (Standard test method for conducting a 90-day oral toxicity study in rats).

The President of Product Safety Laboratories, Gary Wnorowski, (Dayton, NJ, http://www.productsafetylabs.com/), provided a cost quote of $2700 for determination of a rat LD_{50} value using the UDP test; the cost is independent of the number of rats that are needed. Each test dose is administered ~24-48 hours after the previous dose and each animal test generally does not exceed four days. The time involved in providing the LD_{50} value is approximately three months (initiation of the test to provision of the final report). Having the estimated LD_{50} value would not affect the cost of the *in vivo* test but could reduce the number of animals needed.

Bio Research Laboratories performs the rat acute oral toxicity test using a test method that determines lethality and signs of acute toxicity from a waste sample administered in a single dose, by gavage, to a limited number of rats. The bioassay determines if the test sample

produces an LD_{50} either greater than or less than a regulatory threshold corresponding to a hazardous waste designation (i.e., 5000, 500, 50 mg/kg). A minimum of 10 rats is used at the tested dose for the regulatory threshold value that is relevant to the test sponsor. In this testing scenario, knowledge of the estimated LD_{50} would not reduce animal use or test costs if a single predetermined dose is tested.

11.4 Time Considerations for Performing the 3T3 and NHK NRU Tests

11.4.1 The 3T3 NRU Test Method

Approximately one week is needed to thaw cryopreserved 3T3 cells, propagate them, and passage them at least two times before subculturing them into the 96-well test plates. After subculture into 96-well plates, the cells are incubated another 24 hours to reach the proper confluence, and then exposed to test chemical for 48 hours. The initial 3T3 NRU test (range finder or definitive test) takes approximately 10 days. However, after the cells are established in culture, they can be passaged for approximately two months before having to go back to the cryopreserved cells to start a new culture. A 3T3 NRU test can be completed in less than four consecutive days when started from an established stock culture. Multiple substances can be tested at the same time, and different tests can overlap each other; thus, many substances can be tested in a relatively short time.

11.4.2 The NHK NRU Test Method

Approximately one week is needed to thaw cryopreserved NHK cells, propagate them, and passage them into the 96-well test plates. After subculture into 96-well plates, the cells are incubated another 48-72 hours to reach the proper confluence and then exposed to test chemical for 48 hours. The entire NHK NRU test (range finder or definitive test) requires approximately 11-12 days. Cells can be seeded at different densities from one starter vial in the culture flasks so that passaging the cultures can take place on different days. Once the cells are established in culture, they are passaged once to the 96-well test plates and an NHK NRU test can usually be completed in five to six consecutive days. Multiple substances can be tested at the same time, and different tests can overlap each other; thus, many substances can be tested in a relatively short time.

11.4.3 Prequalification of NHK Medium

The protocol for the prequalification of NHK medium requires nearly identical steps, and similar time-line (i.e., 11-12 days), as required for the NHK rangefinder and definitive tests. **Table 11-2** provides an estimate of how many tests could be performed using one 500 mL bottle of medium with supplements (~15 tests in 96-well plates).

11.4.4 *In Vivo* Testing

According to guidelines for acute oral toxicity testing, single animals or groups of animals are dosed in sequence, usually at 2-4 day intervals, and observations are generally made for up to 14 days (for animals that are not moribund) for the main test and limit dose test (EPA 2002a; OECD 2001a; OECD 2001b, OECD 2001c). The addition of 3T3 or NHK NRU testing to estimate a starting dose prior to the implementation of the UDP main test or limit dose test would take 10-12 days, but could save up to 14 days of observation for every animal not used.

11.4.5 The Limit Test

The *in vitro* NRU test methods can provide a savings of time when used to determine if an *in vivo* acute oral toxicity limit test can be employed as the initial test for a substance with unknown *in vivo* toxicity. If the IC_{50} value from an *in vitro* NRU test could accurately predict an LD_{50} that is greater than, or equal to, the limit dose (i.e., 2000 mg/kg or 5000 mg/kg), the *in vivo* test could start at the limit test dose. This approach has the potential to eliminate the need to do the main test and could result in a net savings of six days for the UDP test method and about one day for the ATC test method. **Table 11-4** illustrates the following:

- Time needed to perform the 3T3 and NHK NRU test
- Time needed to reach the limit test starting dose when initiating the *in vivo* main test using the default starting doses (UDP and ATC)

The times presented in **Table 11-4** use the following assumptions:

- 3T3 cells reach ≤50% confluence in approximately 24 hours
- NHK cells reach >20% confluence in approximately 48 hours
- Animals show no evident toxicity 48 hours post-dosing, and additional animals are dosed at the next higher default dose
- Limit test dose = 5000 mg/kg for the UDP and 2000 mg/kg for the ATC method

Table 11-4 Comparison of Time Needed for *In Vitro* and *In Vivo* Testing

Time	3T3 NRU Test Method	NHK NRU Test Method	UDP (5000 mg/kg upper limit)	ATC (2000 mg/kg upper limit)
Day 1	Seed cells in 96-well plate Incubate for 24 ±2 hr	Seed cells in 96-well plate Incubate for approximately 48 to 72 hr	Dose 1 animal at default dose (175 mg/kg) Observe for 48 hr	Dose 3 animals at default dose (300 mg/kg) Observe for 48 hr
Day 2	Apply test substance Incubate for 48 ±0.5 hr	Incubate	Observe	Observe
Day 3	Incubate	Apply test substance Incubate for 48 ±0.5 hr	No death Dose 1 animal at next default dose (550 mg/kg) Observe 48 hr	0 – 1 animal dies Dose 3 animals at default dose (300 mg/kg) Observe 48 hr
Day 4	NRU: 3 ±0.1 hr Elute NR: 0.33 to 0.75 hr OD_{540} measurement Calculate IC_{50} Estimate LD_{50} and Starting Dose*	Incubate	Observe	Observe
Day 5		NRU: 3 ±0.1 hr Elute NR: 0.33 to 0.75 hr OD_{540} measurement Calculate IC_{50} Estimate LD_{50} and Starting Dose*	No death Dose 1 animal at next default dose (1750 mg/kg) Observe 48 hr	0 – 1 animal dies Dose 3 animals at next default dose (2000 mg/kg) **Starting Point for the Limit Test**
Day 6			Observe	
Day 7			No death Dose 1 animal at next default dose (5000 mg/kg) **Starting Point for the Limit Test**	

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; UDP=Up-and-Down Procedure; ATC=Acute Toxic Class method; hr=Hour; NR=Neutral red; OD_{540}=Optical density at 540 nm.

11.5 Summary

- All equipment and supplies should be readily commercially available. During the validation study, direct communication with the NHK medium supplier insured that specific lots of medium were available to the laboratories. The test methods are expected to be transferable to laboratories experienced with mammalian cell culture methods.

- Much of the training and expertise needed to perform the 3T3 and NHK NRU test methods are common to mammalian cell culture procedures. Additional technical training would not be extensive because these test methods are similar to other *in vitro* mammalian cell culture assays, and no extraordinary techniques are necessary. GLP training should be provided to technicians to ensure proper adherence to protocols and documentation procedures.
- Prices for commercial testing for one chemical are $1,120 to $1,850 (**Table 11-2**) for *in vitro* cytotoxicity testing in the 3T3 and NHK test methods, respectively, to determine the IC_{50} (Raabe 2005, personal communication). In contrast, the *in vivo* rat acute oral testing for LD_{50} determination could cost from $750 - $3,750 (**Table 11-3**), depending on the test method used and the toxicity of the test substance. Comparison of costs of *in vitro* testing to *in vivo* testing is difficult because the *in vitro* NRU test methods are not replacements for the animal testing, and animal testing would be performed regardless of the responses of the 3T3 or NHK cells. The use of these *in vitro* NRU test methods may not reduce the overall cost of the *in vivo* rat acute oral toxicity test, but has the potential to reduce the number of animals needed for a study.

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