DRAFT ICCVAM RECOMMENDATIONS

In Vitro Acute Toxicity Test Methods

March 17, 2006

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1.0 Draft ICCVAM Recommendations for *In Vitro* Acute Toxicity Test Methods

1.1 Draft Recommended Test Method Uses

This independent validation study evaluated two *in vitro* neutral red uptake (NRU) basal cytotoxicity assays: the BALB/c 3T3 (clone A31) mouse fibroblast NRU (hereafter referred to as the 3T3 NRU) test method and the normal human keratinocyte NRU (hereafter referred to as NHK NRU) test method. The objective of the study was to determine their ability to estimate rodent acute oral toxicity LD_{50} values to be used in a weight-of-evidence approach to set the starting dose for *in vivo* acute oral toxicity tests. Based on the results of this validation study, ICCVAM proposes the following draft recommendations:

1. The 3T3 and NHK NRU test methods are not sufficiently accurate to predict the acute oral toxicity of substances for the purposes of hazard classification (see Section 6 of the *In Vitro* Acute Toxicity Test Methods Background Review Document).
2. For the purposes of acute oral toxicity testing, the 3T3 and NHK NRU test methods may be used in a weight-of-evidence approach to determine the starting dose for the current acute oral *in vivo* toxicity protocols (i.e., the Up-and-Down Procedure [UDP] and Acute Toxic Class [ATC])
3. Consistent with the U.S. Government Principles on the Use of Animals in Research, Testing, and Education (National Research Council 1996), and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002)[1], *in vitro* basal cytotoxicity test methods as part of a weight-of-evidence approach to estimate the starting dose for acute oral *in vivo* toxicity test methods should be considered and used where appropriate before testing is conducted using animals. For some types of substances, this approach will reduce the number of animals needed. In some testing situations, the approach may also reduce the numbers of animals that die or need to be humanely killed.

[1] National Research Council. 1996. Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academy Press.

PHS. 2002. Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals.

4. Substances with specific toxic mechanisms that are not expected to be active in 3T3 or NHK cells (e.g., those that are neurotoxic, cardiotoxic, interfere with energy utilization, or alkylate proteins and other macromolecules) will likely be underpredicted by these *in vitro* basal cytotoxicity test methods. Therefore, until such time as a more predictive testing approach is developed, the results from basal cytoxicity testing with such substances may not be appropriate.
5. The regression formula used to determine starting doses should be the revised Registry of Cytotoxicity (RC) regression line [with IC_{50} values in μg/mL and LD_{50} values in mg/kg] developed with the RC chemicals using rat LD_{50} data only and excluding chemicals with mechanisms of action that are not expected to be active in *in vitro* basal cytotoxicity test methods.
6. The performance of other *in vitro* basal cytotoxicity test methods that are based on similar scientific principles and that measure or predict the same biological response (i.e., basal cytotoxicity and the rat acute oral LD_{50} value, respectively) should be demonstrated to meet or exceed the accuracy and reliability of the 3T3 and NHK NRU test methods.
7. Compared to the NHK NRU test method, the 3T3 NRU test method appears to be less labor intensive and less expensive to conduct; therefore, the 3T3 NRU cytotoxicity test method is recommended for general use.

1.2 Draft Recommended Future Studies

1. Additional data should be collected using the 3T3 and/or the NHK NRU test methods to evaluate their usefulness for predicting the *in vivo* acute oral toxicity of chemical mixtures.
2. Additional high quality comparative *in vitro* basal cytotoxicity data should be collected in tandem with *in vivo* rat acute oral toxicity test results to supplement the high quality validation database started by this study. Periodic evaluations of the expanded database should be conducted to further characterize the usefulness and

limitations of using *in vitro* cytotoxicity data as part of a weight-of-evidence approach to estimate starting doses.

3. Additional efforts should be conducted to identify additional *in vitro* tests and other methods necessary to achieve accurate acute oral hazard classification; specifically, studies should be conducted to investigate the potential use of *in vitro* cell-based test methods that incorporate mechanisms of action and evaluations of ADME (absorption, distribution, metabolism, excretion) to provide improved estimates of acute toxicity hazard categories.
4. The *in vivo* database of reference substances used in this validation study should be used to evaluate the utility of other nonanimal approaches to estimate starting doses for acute oral systemic toxicity tests (e.g., widely available software that uses quantitative structure-activity relationships [QSAR]).
5. Standardized procedures to collect information pertinent to an understanding of the mechanisms of lethality should be included in future *in vivo* rat acute oral toxicity studies. Such information will likely be necessary to support the further development of predictive mechanism-based *in vitro* methods.
6. An expanded list of reference substances with estimated rat LD_{50} values substantiated by high quality *in vivo* data should be developed for use in future *in vitro* test method development and validation studies.

Appendix A provides *Draft Performance Standards for In Vitro Acute Toxicity Methods* that are based on ICCVAM guidelines (ICCVAM 2003[2]). **Appendix B** provides two draft recommended *in vitro* NRU basal cytotoxicity protocols that are revised versions of the Phase III protocols used in the validation study. **Appendix B-1** is the *Test Method Protocol for the BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test.* **Appendix B-2** is the *Test Method Protocol for the Normal Human Epidermal Keratinocyte (NHK) Neutral Red Uptake (NRU) Cytotoxicity Test.*

[2] ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No. 03-4508. National Institute for Environmental Health Sciences, Research Triangle Park, NC. Available: http://iccvam.niehs.nih.gov/. [accessed 2 June 2005].

Appendix A

Draft Performance Standards for *In Vitro* Acute Toxicity Methods

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
ATC	Acute Toxicity Class
BRD	Background review document
CASRN	Chemical Abstracts Service Registry Number
CPSC	U.S. Consumer Product Safety Commission
CS	Calf serum
CV	Coefficient of Variation
^{o}C	Degrees Celsius
DMSO	Dimethyl sulfoxide
DOT	U.S. Department of Transportation
DPBS	Dulbecco's Phosphate Buffered Saline
ECVAM	European Center for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
ETOH	Ethanol
FDA	U.S. Food and Drug Administration
FDP	Fixed Dose Procedure
FL	Fluorescein leakage
FR	Federal Register
GHS	Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).
IC_{50}	Test substance concentration producing 50% inhibition of the endpoint measured
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
LD_{50}	Lethal dose that produces lethality in 50% of test animals
LDH	Lactate dehydrogenase

MTT	[3-(4,5,dimethylthiazol-2yl)2,5-diphenyl tetrazolium bromide]
NCS	Newborn calf serum
NHK	Normal human epidermal keratinocytes
NICEATM	National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIOSH	U.S. National Institute for Occupational Safety and Health
NR	Neutral red
NRR	Neutral red release
NRU	Neutral red uptake
NTP	U.S. National Toxicology Program
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
OSHA	U.S. Occupational Safety & Hazards Administration
RC	Registry of Cytotoxicity
RTECS	Registry of Toxic Effects for Chemical Substances
3T3	BALB/c mouse fibroblasts, clone A31
UDP	Up and Down Procedure
UN	United Nations
XTT	[Sodium 3,3,-[(Phenylamino)carbonyl]-3,4-Tetrazolium-Bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate]
ZEBET	German Center for Documentation and Evaluation of Alternative Methods to Animal Experiments

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PREFACE

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) collaborated with the European Centre for the Validation of Alternative Methods (ECVAM), a component of the European Commission's Joint Research Centre, to further characterize the usefulness of *in vitro* basal cytotoxicity test methods as predictors of starting doses for acute oral systemic toxicity (i.e., lethality) assays. NICEATM and ECVAM designed a multi-laboratory validation study to evaluate the performance of two standardized *in vitro* basal cytotoxicity test methods using 72 reference substances with the ZEBET approach of using the Registry of Cytotoxicity (RC) regression model to estimate starting doses for acute oral systemic toxicity tests. Based on the procedures described in the *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity* (hereafter referred to as *Guidance Document* (ICCVAM 2001), the validation study used two mammalian cell types (i.e., BALB/c 3T3 mouse fibroblasts [3T3] and primary normal human epidermal keratinocytes [NHK]) with a neutral red uptake (NRU) cell viability endpoint.

NICEATM developed draft performance standards that could be used to evaluate the acceptability of test methods that are based on similar scientific principles and that measure or predict the same biological or toxic effect as the *in vitro* basal cytotoxicity test methods. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and its Acute Toxicity Working Group (ATWG) reviewed the Background Review Document (BRD) for the NICEATM/ECVAM validation study and the draft performance standards. After commenting and recommending revisions, ICCVAM and the ATWG recommended that these standards be presented to and reviewed by an Expert Peer Panel convened for evaluation of the validation study. ICCVAM, in collaboration with NICEATM, subsequently proposed and sought public comment on draft performance standards for these types of test methods. Following consideration of public and advisory committee comments, ICCVAM will finalize recommended performance standards for *in vitro* basal cytotoxicity test methods.

Performance standards provide the basis by which a validated and accepted proprietary (i.e., copyrighted, trademarked, registered) or non-proprietary test method has been determined to have sufficient accuracy and reliability for a specific testing purpose. In addition, performance standards should assist other test developers in the validation of test methods that are similar in structure and function, and facilitate acceptance of test methods that adhere to the applicable performance standards.

This document is available online at http://iccvam.niehs.nih.gov; printed copies are available on request from the NICEATM (NIEHS, P.O. Box 12233, MD EC-17, Research Triangle Park, NC 27709; telephone: 919-541-3398, fax: 919-541-0947, e-mail: iccvam@niehs.nih.gov).

We gratefully acknowledge the significant contributions of the ICCVAM agency representatives and members of the ICCVAM Acute Toxicity Working Group (ATWG) in the preparation of this document, and the NICEATM staff that assisted throughout the process. We also appreciate the constructive suggestions from interested stakeholders in response to the *Federal Register* notice.

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EXECUTIVE SUMMARY

The purpose of performance standards is to communicate the basis by which validated new proprietary (e.g., copyrighted, trademarked, registered) and nonproprietary test methods have been determined to have sufficient accuracy and reliability for specific testing purposes. Performance standards can then be used to evaluate the accuracy and reliability of other test methods that are based on similar scientific principles and that measure or predict the same biological or toxic effect. The three elements of performance standards are essential test method components (i.e., structural, functional, and procedural elements of a validated test method that a proposed, mechanistically and functionally similar test method should adhere to), a minimum list of reference chemicals for assessing the accuracy and reliability of the proposed test method, and the accuracy and reliability values that should be achieved by the proposed test method using the minimum list of reference chemicals.

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has reviewed and evaluated information presented in the Background Review Document (BRD) for a validation study that evaluated the performance of two standardized *in vitro* basal cytotoxicity test methods for predicting starting doses for acute oral systemic toxicity tests. The study, a collaboration of the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Centre for the Validation of Alternative Methods (ECVAM), tested 72 reference substances using *in vitro* neutral red uptake (NRU) test methods with two mammalian cell types (i.e., BALB/c 3T3 mouse fibroblasts [3T3] and primary normal human epidermal keratinocytes [NHK]). The 3T3 and NHK NRU test methods are not sufficiently accurate to predict the acute oral toxicity of substances for the purposes of hazard classification. However, for the purposes of acute oral toxicity testing, the 3T3 and NHK NRU test methods may be used in a weight-of-evidence approach to determine the starting dose for the current acute oral *in vivo* acute systemic toxicity protocols for the Up-and-Down Procedure (UDP; OECD 2001a; EPA 2002a) and Acute Toxic Class (ATC; OECD 2001b) method. The performance of other *in vitro* basal cytotoxicity test methods that are based on similar scientific principles and that measure or predict the same biological response (i.e.,

basal cytotoxicity and the rat acute oral LD_{50} [median lethal dose], respectively) should be demonstrated to meet or exceed the accuracy and reliability of the 3T3 and NHK NRU test methods.

ICCVAM, the ICCVAM Acute Toxicity Working Group (ATWG), and NICEATM have drafted proposed performance standards for the 3T3 and NHK NRU test methods. This document describes those proposed performance standards. The extent to which proposed *in vitro* basal cytotoxicity test methods should demonstrate comparable performance to these two *in vitro* NRU cytotoxicity test methods should be considered on a case-by-case basis. While it would be desirable for proposed test methods to have reliability and accuracy values at least as good as that of these two NRU test methods, some flexibility might be acceptable to the extent that it would not compromise the ultimate protection of human and animal health.

To demonstrate technical proficiency with the validated test method, ICCVAM recommends that the user evaluate his/her ability to calculate IC_{50} values (i.e., the test concentration that produces 50% inhibition of the endpoint measure) for at least 12 of the 25 reference substances (minimum of two unclassified chemicals and two from each from the five GHS hazard categories) listed in **Table 2-1.** The resulting IC_{50} values should be within 2.5 standard deviations of the reported RC IC_{50} values in the table and a linear regression calculated with the IC_{50} values from the proposed test method and LD_{50} values in the table should not be different from a linear regression calculated using the IC_{50} and LD_{50} values from the table. Intralaboratory Coefficient of Variation (CV) for the IC_{50} of the reference test substances should not exceed 129% for reference substance test chemicals and the mean for the substances tested should not exceed 30%. The proposed *in vitro* basal cytotoxicity test method should provide correct predictions of GHS acute oral toxicity category using the recommended IC_{50}-LD_{50} regression for at least 38% of the substances tested.

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1.0 PURPOSE AND BACKGROUND OF PERFORMANCE STANDARDS

1.1 Introduction

Prior to the acceptance of a new test method for regulatory testing applications, validation studies are conducted to assess its reliability (i.e., the extent of intra- and inter-laboratory reproducibility) and its relevance (i.e., the ability of the test method to correctly predict or measure the biological effect of interest) (ICCVAM 1997, 2003; OECD 1996, 2002). The purpose of performance standards is to communicate the basis by which new proprietary (i.e., copyrighted, trademarked, registered) and nonproprietary test methods have been determined to have sufficient relevance and reliability for specific testing purposes. These 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. Two *in vitro* basal cytotoxicity test methods, the BALB/c 3T3 mouse fibroblast (3T3) neutral red uptake (NRU) assay and the normal human keratinocyte (NHK) NRU assay, underwent a validation process to evaluate the correlation between *in vitro* cytotoxicity and acute lethality and the feasibility of using *in vitro* NRU assays to predict starting doses for *in vivo* acute oral systemic toxicity assays.

This section describes the three elements of performance standards identified by ICCVAM (ICCVAM 2003) and the ICCVAM process used to develop performance standards during a test method evaluation, These test method performance standards are proposed as initial standards that can be used to evaluate future *in vitro* basal cytotoxicity test methods. If other *in vitro* basal cytotoxicity test methods are adequately validated and demonstrate significantly improved performance, then the test method performance standards may be revised accordingly.

1.2 Elements of ICCVAM Performance Standards

Performance standards are standards based on a validated test method that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar (ICCVAM 2003). The three elements of performance standards are:

- **Essential test method components:** These consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed, mechanistically and functionally similar test method. Essential test method components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components will help to assure that a proposed test method is structurally and functionally similar to the corresponding validated test method.
- **A minimum list of reference substances:** Reference substances are used to assess the accuracy and reliability of a proposed, mechanistically and functionally similar test method. These substances are a representative subset of those used to demonstrate the reliability and the accuracy of the validated test method. To the extent possible, this subset of substances should:
  - be representative of the range of responses that the validated test method is capable of measuring or predicting
  - have produced consistent results in the validated test method and in the *in vivo* reference test method and/or the species of interest
  - have well-defined chemical structures
  - be readily available
  - not be associated with excessive hazard or prohibitive disposal costs
  - have performance characteristics (accuracy, sensitivity, specificity, false negative and false positive rates) of the validated test method for the subset of reference chemicals should approximate the performance values obtained during the validation process for all appropriate substances

These reference substances are the minimum number that should be used to evaluate the performance of a proposed, mechanistically and functionally similar test method. Reference substances should not be used to develop the prediction model for the proposed test method. However, if reference substances have been used for this purpose, then they should be replaced with other substances of the same chemical class and biological activity for the endpoint of interest and for which adequate reference data are available. Similarly, if any of the recommended reference substances are unavailable, other substances for which adequate reference data are available could be substituted. Again, to the extent possible, the substituted substance(s) should be of the same chemical class as the original reference substance(s). If desired, additional substances representing other chemical or product classes and for which adequate reference data are available can be used to more comprehensively evaluate the accuracy of the proposed test method. However, none of these additional substances should have been used to develop the proposed test method.

- **Accuracy and reliability values:** These are the accuracy and reliability characteristics that the proposed test method should be comparable to when evaluated using the minimum list of reference chemicals.

1.3 ICCVAM Process for the Development of Performance Standards

The process followed by ICCVAM for developing performance standards for new test methods is as follows:

- NICEATM and the appropriate ICCVAM working group develop proposed performance standards for consideration during the ICCVAM evaluation process. If performance standards are proposed by a test method sponsor, they will be considered by ICCVAM at this stage. Generally, the proposed performance standards are based on the information and data provided in the test method submission or on other available applicable data.
- The ICCVAM/NICEATM Peer Review Panel evaluates the proposed performance standards for completeness and appropriateness during its

evaluation of the validation status of the proposed test method. The proposed performance standards, as well as the test method submission, are made available to the public for comment prior to and during the Peer Review Panel meeting.

- The appropriate ICCVAM working group, with the assistance of NICEATM, prepares the final performance standards for ICCVAM approval, taking into consideration the recommendations of the Peer Review Panel and public comments.

Performance standards recommended by ICCVAM are incorporated into ICCVAM test method evaluation reports, which are then provided to U.S. Federal agencies and made available to the public. Regulatory authorities can then reference the performance standards in the ICCVAM report when they communicate their acceptance of a new test method. In addition, performance standards adopted by U.S. Federal regulatory authorities can be provided in guidelines issued for new test methods. Availability of ICCVAM test method evaluation reports are announced routinely in the *Federal Register*, NTP Newsletters, and by e-mail to ICCVAM/NICEATM listserve groups.

1.4 ICCVAM Development of Recommended Performance Standards for *In Vitro* Acute Toxicity Test Methods

1.4.1 Current Regulatory Testing Requirements for Acute Systemic Toxicity

The major regulatory requirement for acute systemic toxicity testing is for the hazard classification and labeling of products, which is intended to protect handlers and consumers from toxic hazards. The LD_{50} results (i.e., median lethal dose) from acute systemic toxicity tests are used to place substances in various toxicity categories that, in turn, invoke the associated hazard phrases to be used on product labels. **Table 1-1** shows the current U.S. legislation requiring the use of acute systemic toxicity testing for product labeling and the substances regulated. **Table 1-2** shows the statutory protocol requirements and classification systems used by each U.S. regulatory agency. Also included is an international guideline for labeling, the Harmonized Integrated Classification System for Human Health and

Environmental Hazards of Chemical Substances and Mixtures (OECD 2001c), which provides guidance to regulatory agencies on the use of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS; UN 2005) as a method for an internationally comprehensible system for hazard communication.

Table 1-1 Summary of Current U.S. Legislation for Using Acute Systemic Toxicity Data for Product Labeling

Legislation (Year of Initial Enactment)	U.S. Regulatory Agency	Substance
Federal Insecticide, Fungicide and Rodenticide Act (1947)	EPA	Pesticides
Federal Hazardous Substances Act (1964)	CPSC	Household products
Occupational Safety and Health Act (1970)	OSHA	Occupational materials
Federal Hazardous Material Transportation Act (1975)	DOT	Transported substances

Abbreviations: EPA = U.S. Environmental Protection Agency; CPSC = U.S. Consumer Product Safety Commission; OSHA = U.S. Occupational Safety and Health Administration; DOT = U.S. Department of Transportation. [Note: The U.S. Food and Drug Administration (FDA) does not require data for acute lethality testing, and in fact, discourages the use of animals for such testing (FDA 1993).]

1.4.2 Test Methods for Assessing Acute Systemic Toxicity

The current internationally recognized test methods for acute systemic toxicity testing are the UDP (OECD 2001a; EPA 2002a), the ATC method (OECD 2001b), and the Fixed Dose Procedure (FDP; OECD 2001d). Information on signs of acute toxicity and target organs can be obtained using any of the three test methods. All three methods are sequential tests in which the outcome of testing one or more animals at the first dose is used to determine the second dose that should be tested. The FDP differs from the UDP and ATC in that it involves testing more animals per dose and the primary endpoint of interest is evident toxicity[3] rather than lethality. The ATC method provides a range for the LD_{50} for classification purposes. The UDP generally provides a point estimate of the LD_{50} with a confidence interval (EPA 2002a).

[3] *Evident toxicity* is a general term describing clear signs of toxicity following administration of test substance, such that an increase to the next highest fixed dose would result in the development of severe toxic signs and probably mortality (ICCVAM 2000).

Table 1-2 Regulatory Classification Systems for Acute Oral Toxicity

Regulatory Agency (Authorizing Act)	Animals	Endpoint	Classification
EPA (Federal Insecticide, Fungicide and Rodenticide Act)	Use current EPA or OECD protocol	Death[1]	I – $LD_{50} \leq 50$ mg/kg II – $50 < LD_{50} \leq 500$ mg/kg III – $500 < LD_{50} \leq 5000$ mg/kg IV – $LD_{50} > 5000$ mg/kg
CPSC (Federal Hazardous Substances Act)	White rats, 200-300 g	Death[1] within 14 days for ≥ half of a group of ≥ 10 animals	Highly toxic – $LD_{50} \leq 50$ mg/kg Toxic – 50 mg/kg $< LD_{50} < 5$ g/kg
OSHA (Occupational Safety and Health Act)	Albino rats, 200-300 g	Death[1], duration not specified.	Highly toxic - $LD_{50} \leq 50$ mg/kg Toxic – $50 < LD_{50} < 500$ mg/kg
DOT (Federal Hazardous Material Transportation Act)	Male and female young adult albino rats	Death[1] within 14 days of half the animals tested. Number of animals tested must be sufficient for statistically valid results.	*Packing Group I* $LD_{50} \leq 5$ mg/kg *Packing Group II* $5 < LD_{50} \leq 50$ mg/kg *Packing Group III* $LD_{50} < 500$ mg/kg (liquid) $LD_{50} < 200$ mg/kg (solid)
OECD Guidance for Use of GHS (2001a)	Protocol not specified	Protocol not specified	I - $LD_{50} \leq 5$ mg/kg II - $5 < LD_{50} \leq 50$ mg/kg III - $50 < LD_{50} \leq 300$ mg/kg IV - $300 < LD_{50} \leq 2000$ mg/kg V - $2000 < LD_{50} \leq 5000$ mg/kg Unclassified - $LD_{50} > 5000$ mg/kg

[1]*Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation* calls for humane killing of moribund animals (OECD 2000). Moribund animals that are humanely euthanized are accepted as deaths.

Abbreviations: EPA = U.S. Environmental Protection Agency; CPSC = U.S. Consumer Product Safety Commission; OECD = Organisation for Economic Co-operation and Development; OSHA = U.S. Occupational Safety and Health Administration; DOT = U.S. Department of Transportation; GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005)

1.4.3 Intended Regulatory Uses for *In Vitro* Cytotoxicity Test Methods

In vitro cytotoxicity test methods are not recommended for the replacement of acute oral toxicity tests in animals. Rather, such test methods are intended to serve as adjuncts for *in vivo* acute systemic toxicity test methods. To select a starting dose for a test substance, the current test guidelines for acute oral systemic toxicity recommend using information on structurally-related substances and the results of any other toxicity tests, including *in vitro* cytotoxicity results (OECD 2001a, b, d; EPA 2002a). *In vitro* basal cytotoxicity test methods are intended to be used as part of the weight-of-evidence approach to select starting doses for the UDP and ATC assays in order to reduce and refine the use of animals for *in vivo* acute

toxicity testing. Since the estimation of the true LD_{50} is irrelevant to setting doses for measuring evident toxicity, the use of *in vitro* basal cytotoxicity test methods for setting starting doses for the FDP was not considered in the NICEATM/ECVAM validation study.

1.4.4 Similarities and Differences in the Endpoints of *In Vitro* Cytotoxicity Test Methods and *In Vivo* Acute Oral Toxicity Test Methods

The endpoint measured in the *in vitro* NRU cytotoxicity test methods is cell death (neutral red [NR] is taken up only by live cells) and the major endpoint of interest is the concentration at 50% inhibition of NRU (i.e., the IC_{50}). The endpoint measured in acute systemic toxicity assays is usually animal death. Cell death and animal death may be similar since animals are comprised of organ systems consisting of tissues, which are comprised of cells. All cells, regardless of whether they are in animals or *in vitro* cell cultures, have similar cellular mechanisms of energy production and utilization and maintenance of cell membrane integrity. Animal death and death of cells in culture due to toxicity are similar in that both involve some type of cellular injury. For the animal, the cellular injury produces tissue and organ injury to the most sensitive target organ, which may then cause the death of the whole organism. Cell death in a culture system involves the death of a single cell type. Cell death and animal death may be produced by the same mechanisms, such as disruption of membrane structure or function, inhibition of mitochondrial function, disturbance of protein turnover, disruption of energy production, etc. (Gennari et al. 2004).

Animal and cell culture systems are different with respect to how a substance or toxin is delivered to the cell and how it is distributed, metabolized, and excreted. After oral administration, animals must absorb the toxin from the gastrointestinal tract, which involves the passage of membranes. The toxin may or may not be heavily bound to serum proteins; this would reduce the availability of the toxin to the target organ. The toxin may then be metabolized during and/or after distribution to the target organs and then the toxin or its metabolites are excreted. In a cell culture system, the only membranes that must be passed are those of the target cell and cellular organelles. No absorption and distribution by other cellular systems is required. Cell culture systems may or may not include serum proteins, which could reduce the availability of toxin to act as its target site. Excretion from the cell

culture milieu cannot occur since cell culture systems have no excretory system. The cultured cells are exposed to substances for the entire duration of exposure in the test system.

Animal and cell culture systems may also be different with respect to the target on which a toxin acts. If a toxin acts in a specialized organ system in a whole animal, it may not produce a toxic effect by the same mechanism in cultured cells that are derived from tissue different from the target organ. For example, a neurotoxin that acts by a neuroreceptor-mediated pathway in animals, would be expected to produce toxicity by a different mechanism in 3T3 or NHK cells, which are derived from fibroblasts, and skin cells, respectively. Even if a neurotoxin were applied to neuronal cells in culture, the cultured cells may not respond in the same way as neuronal cells in a whole animal. Cultured cells may not retain the same functionality as cells *in vivo*.

2.0 IN VITRO ACUTE TOXICITY TEST METHODS

2.1 Background

Pre-validation and validation studies have been completed to evaluate the ability of the 3T3 and NHK NRU test methods to be used in a weight of evidence approach (and not as stand-alone tests) to estimate acute rat oral LD_{50} values, which are to be used as the basis for selecting the starting dose for acute systemic toxicity (i.e., lethality) studies. This section briefly describes the principles of *in vitro* basal cytotoxicity test methods followed by the recommended performance standards that would be used to evaluate test methods that are functionally and mechanistically similar to the 3T3 and NHK NRU test methods. The performance standards consist of 1) essential test method components, 2) reference substances, and 3) the comparable accuracy and reliability that should be achieved. Theoretically, any *in vitro* test capable of determining basal cytotoxicity could be used to estimate the starting dose for acute systemic toxicity testing using the UDP (EPA 2002c, OECD 2001a) or the ATC method (OECD 2001b).

2.2 Principles of *In Vitro* Basal Cytotoxicity Assays to Predict Starting Doses for Acute Oral Toxicity Tests

The basic steps of an *in vitro* basal cytotoxicity assay are as follows:

- the test substance is dissolved in an appropriate solvent and applied as a solution to a sub-confluent cell culture monolayer or to exponentially growing cultures of non-adherent cells
- the test substance is incubated with the cells for a specified period of time
- the test substance is removed and an endpoint indicative of cell viability or cytotoxicity is measured
- the IC_{50} value is calculated (i.e., the concentration at which cell viability or growth is inhibited by 50% compared to control values)

Many different *in vitro* basal cytotoxicity methods can be used to estimate the acute rat oral LD_{50} value (the calculated value of the oral dose that produces lethality in 50% of test animals) and, thus, to predict the starting dose for an acute lethality assay. *In vitro* basal cytotoxicity data determined in various primary cells, as well as in various permanent non-differentiated finite or transformed cell lines, generally exhibit the same dose response cytotoxicity relationship in response to the same xenobiotic, regardless of the type of toxic endpoints investigated. The following endpoints are sufficiently characteristic of basal cytotoxicity (Spielmann et al. 1999; Halle 1998):

- Inhibition of cell proliferation: cell number, cell protein, DNA content, DNA synthesis, colony formation
- Cell viability - metabolic markers: metabolic inhibition test, mitochondrial reduction of tetrazolium salts
- Decreased cell viability - membrane markers: NRU into cell lysosomes, Trypan Blue exclusion, cell detachment for monolayer cultures
- Differentiation markers: functional differentiation within cell clusters, morphological differentiation within cell clusters, intracellular morphology

Markers of the release of intracellular components, such as the enzyme lactate dehydrogenase (i.e., LDH release test), or of dye introduced into the cells previous to chemical exposure, as occurs, for example, in the fluorescein leakage [FL] test or the Neutral Red Release [NRR] test, are not considered to be characteristic for basal cytotoxicity because they specifically detect damage of the outer cell membrane and generally are associated with short-term chemical exposure (ICCVAM 2001). A chemical that specifically damages only cell membranes, however, will be detected correctly in one of the tests for basal cytotoxicity listed above.

Investigators using an *in vitro* basal cytotoxicity system for prediction of the *in vivo* starting dose for acute oral toxicity/lethality studies must be able to demonstrate that the assay is valid for its intended use. This includes demonstrating that any modification to the existing validated reference test method does not adversely affect its performance characteristics. *In vitro* systems may be used to test solids, liquids, and emulsions of any chemical or product class. The liquids can be aqueous or nonaqueous; solids can be soluble or insoluble in water. The samples may be pure chemicals, dilutions, formulations, or waste. Test substances must be soluble in cell culture medium, dimethyl sulfoxide (DMSO), or ethanol (ETOH). The test method endpoint (i.e., percent of control values) will be used to generate an IC_{50} value in μg/mL and this value will be used in the regression formula developed to estimate the LD_{50} value in mg/kg.

2.3 Essential Test Method Components for In Vitro Basal Cytotoxicity Assays to Predict Starting Doses for Acute Oral Toxicity (Lethality) Tests

Essential test method components consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a mechanistically and functionally similar proposed test method. These components include unique characteristics of the test method, critical procedural details, and quality control

measures. Adherence to these components will help assure that a proposed test method is based on the same concepts as the corresponding validated test method.

The following is a description of the essential test method components for *in vitro* basal cytotoxicity assays to predict starting doses for acute oral toxicity/lethality tests.

2.3.1 In Vitro Cell Culture Conditions

- Use a mammalian cell line (or primary cells) that divides rapidly with doubling times of less than 30 hours under standard culture conditions, preferably with calf serum [CS], newborn calf serum [NCS]), or serum-free medium (ICCVAM 2001).
- Propagate cells in sterile tissue culture vessels (e.g., flasks) and then subculture cells to other sterile tissue culture vessels (e.g., 96 well-plates) for use in testing. Initial cell seeding should be done at a density that allows rapid growth throughout the exposure period. However, cell density should not reach confluency by the end of the test exposure period.
- Maintain appropriate cell culture growth conditions throughout the testing period (e.g., 37°C ± 1°C, 90% ± 10% humidity, 5.0% ± 1% CO_2/air). The cell cultures should be free of contamination with bacteria, mycoplasma, or fungi.

Cell culture media should be prequalified by the testing laboratory via a standardized protocol before initiating the test to guarantee that the media provide cells with appropriate nutrients to meet the growth criteria needed for the test method.

2.3.2 Application of the Test Substances

Test Substance Preparation

- Test substance solutions should be prepared in cell culture medium within one hour (unless otherwise known stability conditions of the substance require different parameters) before application to the cell cultures.
- Standard protocol methods for solubility procedures can include mixing the test substance by vortexing, sonication, warming, and stirring. Test substances

should be fully solubilized (i.e., no visual observation of test substance in the dosing solution) before application.

- One inherent limitation to *in vitro* cytotoxicity is the testing of volatile chemicals since the material may evaporate before application to the cells or may not remain in the test vessel when incubated. If volatility is predicted or identified for a test substance (e.g., by detection of cross-contamination of the high concentrations of test substance in culture with lower concentrations or controls in the test vessel), measures can be employed to test moderately volatile chemicals (e.g., cover the test plate with a CO_2 permeable plastic film cover/sealer).

Cytotoxicity Test

- Each cytotoxicity test should contain a range of test substance concentrations such that the IC_{50} value can be determined with at least one cytotoxic point between 0 – 50% viability and at least one cytotoxic point between 50 – 100% viability.
- A minimum of three adequate data points should be collected for each test substance concentration. Note: The NICEATM/ECVAM validation study required the testing of six replicates for each test substance concentration with at least four successful replicates].
- Blanks (i.e., culture vessels without cells) should be available for assessing background interference when measuring the endpoint.
- Cell monolayers in tissue culture vessels should be adequately covered (e.g., a minimum of 100 μL of test substance solution per well in a 96-well test plate).
- The chemical exposure period should be at least the duration of one cell cycle, i.e., 24 – 72 hours (Riddell et al. 1986). [Note: The NICEATM/ECVAM validation study required an exposure period of 48 hours for 3T3 and NHK cells; the cell cycle duration for these cells is generally 17-19 and 10-22 hours in the log phase, respectively.]
- At the end of the exposure period, most endpoints require washing the test substance from the cells with an appropriate buffering solution (e.g., Dulbecco's Phosphate Buffered Saline [DPBS]) before applying the endpoint material (e.g.,

neutral red dye). Washing cells to remove the test substance is the default recommendation unless it is known that washing would interfere with measurement of the endpoint.

2.3.3 Control Substances

Vehicle Controls: The vehicle controls provide the reference for 100% cell growth in the test vessel and, thus, the vehicle (or solvent) must be compatible with the cell culture system (i.e., not cause cytotoxicity or reduce cell growth through other mechanisms) and should not alter the properties of the test substance. The vehicle controls should contain the solvent at the concentration applied to the cells. For example, DMSO and ethanol at a final concentration ≤ 0.5% [v/v] were demonstrated to be compatible with cell growth for 3T3 and NHK cells in the NICEATM/ECVAM validation study. If the compatibility of the solvent with the cell culture system is unknown, cultures with and without the solvent must be included in each experiment.

Positive Controls: The purpose of a positive control chemical is to demonstrate that the cell culture system is responding with adequate sensitivity to a cytotoxic agent for which the magnitude of the cytotoxic response is well characterized. The positive control chemical should be tested concurrently with (and independent of) the test substance. The positive control should be well characterized for its cytotoxicity potential and each test should generate a response that is comparable to the historic IC_{50} range generated by the laboratory. A laboratory should perform a minimum of 10 cytotoxicity tests using the positive control over a number of days to develop a minimum historical database of IC_{50} data. Typically, for biologically based test methods, suggested acceptable ranges for the positive control response are within two to three standard deviations of the historical mean response, but developers of proprietary test methods may establish tighter ranges. Sodium lauryl sulfate (SLS) is an effective positive control substance for use in *in vitro* basal cytotoxicity test methods. [Note: The NICEATM/ECVAM validation study used SLS as the positive control and required 2.5 standard deviations of the historical mean response as the acceptable range.]

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

- consistent and reliable source(s) for the chemical
- 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 in the range of response (including moderate response)

2.3.4 Viability Measurements

- Only standardized, quantitative methods should be used to measure cell viability. The protocol should be compatible with laboratory apparatus such as spectrophotometers that allow a quick and precise measurement of the endpoint.
- Non-specific dye binding must not interfere with the viability measurement. A measurement endpoint that is well established and that has good interlaboratory reproducibility should be used (ICCVAM 2001).
- A detailed concentration-response experiment should be conducted using a progression factor that yields graded effects between no effect and total cytotoxicity. Any desired toxicity measure can be derived from a well-designed concentration-response experiment.
- Preference should be given to endpoints that determine either cell proliferation or cell viability (e.g., NRU, MTT [3-(4,5,dimethylthiazol-2yl)2,5-diphenyl tetrazolium bromide], XTT [Sodium 3,3,-[(Phenylamino)carbonyl]-3,4-Tetrazolium-Bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate]) (ICCVAM 2001).
- Simple endpoints such as total protein content are not recommended, as they may under-predict the toxicity of certain test chemicals by including protein from dead cells.

- A lack of information and a low level of accuracy characterize experiments that seek only to identify the highest tolerated dose or the lowest cytotoxic dose.

Colorimetric endpoints (e.g., NRU) should have the optical density (OD) spectrascopically-measured at the appropriate wavelength (e.g., 540 nm for NRU) and OD values for blanks should be subtracted from the vehicle control and test substance ODs.

2.3.5 Interpretation of Results

IC_{50} Determination

The endpoint values obtained for each test sample can be used to calculate the percentage of cell viability or growth relative to the negative (vehicle) control, which is arbitrarily set at 100%. The cell viability criteria used to determine an IC_{50} value must be clearly defined and documented, and be shown to be appropriate. In general, such criteria are established during test optimization, tested during a prevalidation phase, and confirmed in a validation study.

Regression Formula

The regression formula for *in vitro* acute toxicity test methods in the NICEATM/ECVAM validation study was determined from the chemical IC_{50} values (µg/mL) and rat LD_{50} values (mg/kg) from the Registry of Cytotoxicity (RC) database. The RC contains acute oral LD_{50} values for rats and mice obtained from the Registry of Toxic Effects for Chemical Substances (RTECS®) and IC_{50} values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints for chemicals with known molecular weights (Halle 1998). Of the 347 chemicals in the RC, 282 chemicals have rat LD_{50} values. Those chemicals without rat data were excluded. Chemicals with mechanisms of action not expected to be active in culture cells used for basal cytotoxicity test methods were excluded from the database prior to deriving the regression line. The regression formula based upon rat data and excluding chemicals with specific mechanisms other than cytotoxicity was developed from the data for 232 chemicals.

$$\textbf{log (LD}_{\textbf{50}}\textbf{ in mg/kg) = 0.357 x log (IC}_{\textbf{50}}\textbf{ in } (\mu g/mL)\textbf{ + 2.194}$$

Before using a candidate *in vitro* basal cytotoxicity test to predict starting doses, the correlation between the *in vitro* test and the *in vivo* test must be established quantitatively. This can be accomplished by testing in the new assay a minimum of 12 reference substances from **Table 2-1** that cover all six hazard classification categories (i.e., the entire range of cytotoxicity) and that produce the same regression formula as the total database. After testing, the IC_{50} data are used to calculate a regression formula (least square method) for the selected reference substances using the corresponding LD_{50} values provided in **Table 2-1.** The resulting regression is compared against the original regression. If the regressions are not statistically significantly different (at $p < 0.05$; e.g., comparison of slope and intercept), then the test is considered suitable to generate IC_{50} data to use with the recommended regression formula for estimating starting doses for acute oral systemic toxicity/lethality tests.

2.3.6 Test Report

The test report should include the following information, if relevant to the conduct of the study:

Test Substances and Control Substances

- Chemical name(s) such as Chemical Abstracts Service Registry Number (CASRN) and molecular weight (if known), followed by other names, if known
- Formulation (if available) of the test substance if the material is a mixture
- Purity and composition of the substance or preparation (in percentage[s] by weight)
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., vortexing, sonication, warming; solvent used)
- Stability, if known

Justification of the In Vitro Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time
- If the test method employs proprietary components, documentation on the procedure used to ensure their integrity from "lot-to-lot" and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent positive control ranges based on historical data
- Acceptable negative and solvent/vehicle control data

Test Conditions

- Cell system used
- Calibration information for measuring device used for measuring cell viability (e.g., spectrophotometer)
- Details of test procedure used
- Test doses used
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of evaluation criteria used

Results

- Tabulation of data from individual test samples (e.g., OD values and calculated percentage cell viability data for the test substance and the positive, negative, and benchmark controls, reported in tabular form, including data from replicate repeat experiments as appropriate, and means and ± the standard deviation for each trial)
- Calculated IC_{50} value
- Calculated starting dose (i.e., LD_{50} value) using IC_{50} value in regression formula
- Regression formula (prediction model) used

Description of Other Effects Observed

Discussion of the Results

Conclusion

2.4 Reference Substances for In Vitro Basal Cytotoxicity Assays to Predict Starting Doses for Acute Oral Toxicity Tests

Reference substances are used to assess the accuracy and reliability of a proposed, mechanistically and functionally similar test method and are a representative subset of those used to demonstrate the reliability and the accuracy of the validated test method. These substances are:

- representative of the range of responses that the validated test method is capable of measuring or predicting
- have produced consistent results in the validated test method
- reflect the accuracy of the validated test method
- have well-defined chemical structures
- are readily available
- are not associated with excessive hazard or prohibitive disposal costs

To demonstrate technical proficiency with the validated test method, the user should evaluate his/her ability calculating IC_{50} values of at least 12 of the reference substances (minimum of two unclassified chemicals and two from each from the five GHS hazard categories) listed in **Table 2-1.** The **resulting** IC_{50} values should be within 2.5 standard deviations of the reported RC IC_{50} values in the table.

This subset of 25 reference substances was chosen from the 72 reference substances used in the NICEATM/ECVAM *in vitro* acute toxicity test methods validation study and are common to the RC database. Criteria used for selecting the substances in this subset are:

- they must be in common with the RC database and have rat LD_{50} data
- they cannot be the same substances as those that were removed from the revised RC regression formula because of mechanism of action
- they cannot be volatile or insoluble (determined by the NICEATM/ECVAM study)

The low number of substances in the $5 < LD_{50} \leq 50$ mg/kg and $300 < LD_{50} \leq 2000$ mg/kg GHS categories is due to the exclusionary criteria described above. Reference substances that exhibited solubility difficulties (with 3T3 medium) or were volatile are included as a secondary subset and are recommended for investigational purposes only.

The substances in this list represent the following types of chemical classes: alcohols; amides; boron compounds; cadmium compounds; carboxylic acids; copper compounds; cyclic hydrocarbons; heterocyclics; mercury compounds; organometalics; potassium compounds; sodium compounds; and sulfur compounds. Chemicals that exhibited solubility difficulties (with 3T3 medium) or were volatile are included as a secondary subset and are to be used for investigational purposes only.

Table 2-1 Recommended Reference Substances for Evaluation of In Vitro Basal Cytotoxicity Methods for Predicting Starting Dose for Acute Systemic Toxicity Tests

Chemical	CASRN[a]	LD50[b] (mg/kg)	IC50x[c] (µg/mL)
$LD_{50} \leq 5$ mg/kg			
Mercury II chloride	7487-94-7	1	4.07
Triethylenemelamine	51-18-3	1	0.159
Cycloheximide	66-81-9	2	0.166
Busulfan	55-98-1	2	11.3
Phenylthiourea	103-85-5	3	82.2
$5 < LD_{50} \leq 50$ mg/kg			
Triphenyltin hydroxide	76-87-9	44	0.0180
Sodium bichromate	10588-01-9	50	0.244
$50 < LD_{50} \leq 300$ mg/kg			
Hexachlorophene	70-30-4	61	3.21
Cadmium II chloride	10108-64-2	88	1.17
Sodium oxalate	62-76-0	155	59.0
Sodium fluoride	7681-49-4	180	77.7
Diquat dibromide	85-00-7	231	55.1
Cupric sulfate pentahydrate	7758-99-8	300	82.4
$300 < LD_{50} \leq 2000$ mg/kg			
Acetylsalicylic acid	50-78-2	1000	409.0
Propranolol HCl[3]	350-60-90	470	35.5
$2000 < LD_{50} \leq 5000$ mg/kg			
Acetaminophen	103-90-2	2404	409.7
Potassium chloride	7447-40-7	2602	6113
Dimethylformamide	68-12-2	2800	8335
Sodium chloride	7647-14-5	2998	4436
Chloramphenicol	56-75-7	3393	255.3
Lactic acid	50-21-5	3730	5946
Trichloroacetic acid	76-03-9	4999	1338
$LD_{50} > 5000$ mg/kg			
Ethylene glycol	107-21-1	8567	34454
Dibutyl phthalate	84-74-2	11998	211.6
Glycerol	56-81-5	12691	57477

Chemical	CASRN[a]	LD50[b] (mg/kg)	IC50x[c] (μg/mL)
Secondary Subset			
Precipitating Chemicals[1]			
$LD_{50} \leq 5$ mg/kg			
Arsenic trioxide	1327-53-3	20	1
Parathion	56-38-2	2	27.1
$300 < LD_{50} \leq 2000$ mg/kg			
Giberrellic Acid	77-06-5	6305	797
Volatile Chemicals[2]			
$300 < LD_{50} \leq 2000$ mg/kg			
Phenol	108-95-2	414	283.3
$LD_{50} > 5000$ mg/kg			
Ethanol	64-17-5	14008	17464
2-Propanol	67-63-0	5843	10038

[a]Chemical Abstracts Service Registry Number
[b]The calculated value of the oral dose that produces lethality in 50% of test animals (rats and mice).
[c]Reference substance concentration (geometric mean) producing 50% inhibition of the endpoint measured (i.e., cell viability).
[1]Reference substances expected to precipitate at cytotoxic concentrations.
[2]Reference substances expected to contaminate neighboring wells at high concentrations.
[3]Propranol HCl data were not used in developing the regression formula because the compound lacked rat data (RC LD_{50} value = 466 mg/kg [from mouse]). NICEATM found a rat LD_{50} value of 470 mg/kg in the literature and decided that this reference substance would be an acceptable representative of the $300 < LD_{50} \leq 2000$ mg/kg category.

2.5 Accuracy and Reliability

The third element of the performance standards is the determination of accuracy (also known as relevance) and reliability values. The proposed test method, functionally and mechanistically similar to the 3T3 NRU test method, will use selected reference substances to assess accuracy and reliability.

2.5.1 Accuracy

When evaluated using the minimum list of recommended reference substances (**Table 2-1**), the proposed test method should have performance characteristics that are comparable to the performance of the validated 3T3 NRU test method. Accuracy is defined as the closeness of agreement between a test method result and an accepted reference value (ICCVAM 2003). Substances, ranging in toxicity activity from strong to weak, and representing relevant chemical classes across all GHS hazard categories are included so that the performance of the

proposed test method can be determined and compared to that of the validated reference test method.

Although the *in vitro* basal cytotoxicity test methods are not intended as replacements for acute systemic toxicity assays, the accuracy of these assays to predict LD_{50} values in the correct GHS acute oral toxicity hazard classification category (UN 2005) was evaluated. This accuracy evaluation characterizes the extent that additional test methods will be necessary to achieve accurate *in vitro* predictions of acute oral toxicity hazards for regulatory classification and labeling purposes.

The log IC_{50} data from the 3T3 and NHK NRU test methods and the corresponding reference log LD_{50} values (mg/kg) values were used to calculate linear regressions for each validation study laboratory. The slopes for all regressions were statistically significant ($p < 0.0001$), which indicated a significant relationship between IC_{50} and LD_{50} values. The higher adjusted R^2 values for the 3T3 regressions, compared with the NHK regressions indicated that the 3T3 IC_{50} data provided a better fit to the LD_{50} data than the corresponding NHK data (see **Table 2-2).**

Table 2-2 Linear Regression Analyses of *In Vitro* and *In Vivo* Results[1]

Laboratory	Weight Unit Regressions[2]		
	Slope	Intercept	Adjusted R^2
3T3 NRU Test Method			
Lab 1	0.509	1.552	0.420
Lab 2	0.453	1.513	0.307
Lab 3	0.515	1.542	0.421
Combined[3]	0.516	1.498	0.409
NHK NRU Test Method			
Lab 1	0.425	1.679	0.319
Lab 2	0.375	1.798	0.276
Lab 3	0.424	1.704	0.318
Combined[3]	0.424	1.720	0.322

[1]Data for 70 reference substances in the 3T3 assay and 71 reference substances in the NHK assay.
[2]log IC_{50} in μg/mL; log LD_{50} in mg/kg.
[3]Single regression for the test method using the geometric mean of the laboratory-specific IC_{50} values for each reference substance.

Table 2-3 shows that the accuracy of the 3T3 and NHK NRU test methods for predicting GHS acute oral toxicity categories was similar. The accuracy for the 3T3 regression was 46% while the accuracy for the NHK regression was 38%.

2.5.2 Reliability

Reliability is the degree to which a test method can be performed reproducibly within and among laboratories over time (ICCVAM 2003) and was assessed in the NICEATM/ECVAM validation study by determining both intra- and inter-laboratory reproducibility. Intralaboratory reproducibility is the agreement of results produced when qualified people within the same laboratory perform the test method using the same test protocol at different times. Interlaboratory reproducibility is the agreement of results from different qualified laboratories using the same protocol and reference substances. Interlaboratory reproducibility indicates the extent to which a test method can be transferred successfully among laboratories. Intra- and inter-laboratory reproducibility of the 3T3 and NHK NRU test methods were determined using Analysis of Variance (ANOVA) and Coefficient of Variation (CV) analysis. Interlaboratory reproducibility of the 3T3 and NHK NRU test methods was also assessed by comparing the laboratory-specific *in vitro-in vivo* regressions (for each test method) to one another.

Table 2-3 Prediction of GHS Toxicity Category[1] by RC Rat-Only Weight Regression Excluding Chemicals with Specific Mechanisms of Toxicity

Reference Rodent LD_{50}[2]	3T3 NRU-Predicted Toxicity Category						Total	Accuracy	Toxicity Overpredicted	Toxicity Underpredicted
	< 5	5 – 50	50 – 300	300-2000	2000-5000	> 5000				
< 5	0	0	2	2	0	0	4[3]	0%	100%	0%
5 – 50	0	1	4	2	0	0	7[4]	14%	86%	0%
50 – 300	0	0	4	1	0	0	5[5]	80%	20%	0%
300 – 2000	0	1	1	7	0	0	9[6]	78%	0%	22%
2000 – 5000	0	0	0	3	6	0	9[7]	67%	0%	33%
> 5000	0	0	0	5	4	3	12[8,9]	25%	0%	75%
Total	0	2	11	20	10	3	46	46%	24%	30%
Predictivity	0%	50%	36%	35%	60%	100%				
Category Underpredicted	0%	50%	9%	40%	40%	0%				
Category Overpredicted	0%	0%	55%	25%	0%	0%				
Reference Rodent LD_{50}[2]	**NHK NRU-Predicted Toxicity Category**						**Total**	**Accuracy**	**Toxicity Overpredicted**	**Toxicity Underpredicted**
	< 5	**5 – 50**	**50 – 300**	**300 – 2000**	**2000 – 5000**	**> 5000**				
< 5	0	0	2	2	0	0	4[3]	0%	100%	0%
5 – 50	0	1	4	2	0	0	7[4]	14%	86%	0%
50 – 300	0	1	3	1	0	0	5[5]	60%	20%	20%
300 – 2000	0	1	0	8	0	0	9[6]	89%	0%	11%
2000 – 5000	0	0	0	5	4	0	9[7]	44%	0%	56%
> 5000	0	0	0	4	7	2	13[9]	15%	0%	85%
Total	0	3	9	22	11	2	47	38%	23%	38%
Predictivity	0%	33%	33%	36%	36%	100%				
Category Underpredicted	0%	67%	0%	41%	64%	0%				
Category Overpredicted	0%	33%	67%	23%	0%	0%				

[1]GHS-Globally Harmonized System of Classification and Labelling of Chemicals with LD_{50} in mg/kg (UN 2005). The RC rat-only weight regression excluding chemicals with specific mechanisms of toxicity is log LD_{50} (mg/kg) = log IC_{50} (μg/mL) X 0.357 + 2.194.

< 5:	$LD_{50} \leq 5$ mg/kg
5 – 50:	$5 < LD_{50} \leq 50$ mg/kg
50 – 300:	$50 < LD_{50} \leq 300$ mg/kg
300 – 2000:	$300 < LD_{50} \leq 2000$ mg/kg
2000 – 5000:	$2000 < LD_{50} \leq 5000$ mg/kg
> 5000:	$LD_{50} > 5000$ mg/kg

Numbers in table represent number of reference substances.

[2]*In vivo* reference LD_{50} values

Goodness of fit F-tests indicated that the laboratory-specific regressions for both test methods were not significantly different from one another ($p = 0.796$ for comparison of the 3T3 regressions and $p = 0.985$ for comparison of the NHK regressions).

The reliability of the proposed test method for the reference substances should be comparable to or better than that of the validated 3T3 NRU test method. **Table 2-4** illustrates intra- and inter-laboratory CV results obtained from the NICEATM/ECVAM *in vitro* acute toxicity test methods validation study.

Table 2-4 Summary of CV Results for the 3T3 and NHK NRU Test Methods

	3T3 NRU Test Method	**N**	**NHK NRU Test Method**	**N**
Intralaboratory CV				
Range	1-122%	202	1-129%	208
Mean CV (and Range)	26%	202	26%	208
Lab 1	23% (2-95%)	68	23% (2-76%)	69
Lab 2	33% (1-98%)	66	42%(1-129%)	69
Lab 3	21% (1-122%)	68	14% (1-38%)	70
Interlaboratory CV				
Range	2-135%	68	1-99%	69
Mean	46%	68	28%	69

CV - coefficient of variation; N - number of values

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Appendix B

Draft Recommended Test Method Protocols

B-1 Test Method Protocol for the BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test B-1-3

B-2 Test Method Protocol for the Normal Human Epidermal Keratinocyte (NHK) Neutral Red Uptake (NRU) Cytotoxicity Test..... B-2-3

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Appendix B-1

Test Method Protocol for the BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test

This draft recommended 3T3 NRU test method protocol is substantially the same as the protocol used in Phase III of the NICEATM/ECVAM validation study. Revisions were made based on recommendations from NICEATM and the study directors involved in the study. The changes are as follows:

- Explanations and directions for the use of the revised Hill function for determining IC50 values are included in the protocol.
- The range for relative humidity values for the cell culture incubators was changed from 90 % ± 5 % humidity to 90 % ± 10 % humidity.
- An additional step was added to the test substance solubility protocol to allow testing of higher concentrations of test material.
- The spreadsheet templates used in the NICEATM/ECVAM validation study are incorporated into this protocol as an annex (ANNEX I).
- The stand-alone solubility protocol is incorporated into this protocol as an annex (ANNEX II).

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Test Method Protocol

The BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test A Test for Basal Cytotoxicity

I. PURPOSE

This test method is used to evaluate the cytotoxicity of test substances using the BALB/c 3T3 Neutral Red Uptake (NRU) *in vitro* cytotoxicity test. The data generated from the *in vitro* cytotoxicity assays are used to evaluate the effectiveness of the assay to predict the starting doses for rodent acute oral systemic toxicity assays. This test method protocol outlines the procedures for performing the basal cytotoxicity test and is the result of the *in vitro* validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM).

If changes or modifications are made to this protocol, the testing laboratory should prove that the results are comparable to those obtained when using the original protocol.

II. TEST SYSTEM

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.

Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after chemical exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

III. KEY PERSONNEL

A. Laboratory

1) Study Director
2) Laboratory Technician(s)

B. Testing Facility

1) Scientific Advisor
2) Quality Assurance Director
3) Safety Manager
4) Facility Management

IV. DEFINITIONS

3 ***Hill function***: a four parameter logistic mathematical model relating the concentration of test substance to the response being measured in a sigmoidal shape.

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{logEC50} - X)\text{HillSlope}}}$$

where Y= response, X is the logarithm of dose (or concentration), Bottom is the minimum response, Top is the maximum response, logEC50 is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve. When Top = 100 and Bottom = 0, the EC_{50} is the concentration at 50% viability (i.e., the IC_{50}).

4 ***Documentation***: all methods and procedures will be noted in a Study Workbook; logs will be maintained for general laboratory procedures and equipment (e.g., media preparation, test substance preparation, incubator function); all optical density data obtained from the spectrophotometer plate reader will be saved in electronic and paper formats; all calculations of ICx values and other derived data will be in electronic and paper format; all data will be archived.

5 ***IC_{50}***: test substance concentration producing 50% inhibition of the endpoint measured (i.e., cell viability).

V. IDENTIFICATION OF CONTROL SUBSTANCES

A. Positive Control (PC)
Sodium Lauryl Sulfate (SLS)

B. Vehicle Control (VC)
Assay medium (DMEM containing 5% NBCS, 4 mM L-Glutamine, 100 IU/mL Penicillin, 100 µg/mL Streptomycin)

C. Solvent Control
VC control with solvent (i.e., assay medium, dimethyl sulfoxide [DMSO], or ethanol [ETOH])

(DMSO is the preferred solvent for substances that are not water [i.e., assay medium] soluble.)

VI. PROCEDURES

A. Materials

1. Cell Line

BALB/c 3T3 cells, clone A31
(e.g., CCL-163, American Type Culture Collection [ATCC], Manassas, VA, USA)

2. Technical Equipment

[Note: Suggested brand names/vendors are listed in parentheses. Equivalents may be used.]

4 Incubator: 37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air
5 Laminar flow clean bench/cabinet (standard: "biological hazard")
6 Waterbath: 37ºC ± 1ºC
7 Inverse phase contrast microscope
8 Sterile glass tubes with caps (e.g., 5 mL)
9 Centrifuge
10 Laboratory balance
11 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm ± 10 nm filter
12 Shaker for microtiter plates
13 Cell counter or hemocytometer
14 Pipetting aid
15 Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette), dilution block
16 Cryotubes
17 Tissue culture flasks (e.g., 75 - 80 cm^2, 25 cm^2)
18 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Falcon tissue culture-treated)
19 pH paper (wide and narrow range)
20 Multichannel reagent reservoir
21 Waterbath sonicator
22 Magnetic stirrer
23 Antistatic bar ionizer/antistatic gun (optional for neutralizing static on 96-well plates)
24 Dry heat block (optional)
25 Adhesive film plate sealers (e.g., Excel Scientific SealPlate™,Cat # STR-SEAL-PLT or equivalent)
26 Vortex mixer
27 Filters/filtration devices

[Note: Tissue culture flasks and microtiter plates should be prescreened to ensure that they adequately support the growth of 3T3 cells. Multi-channel repeater pipettes may be used for plating cells in the 96-well plates, dispensing plate rinse solutions, NR medium, and desorb solution. Do not use the repeater pipette for dispensing test substances to the cells.]

3. Chemicals, Media, and Sera

a) Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine; should have high glucose [4.5gm/l] (e.g., ICN-Flow Cat. No. 12-332-54)
b) L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49)
c) New Born Calf Serum (NCS) (e.g., Biochrom # SO 125)
d) 0.05 % Trypsin/0.02 % EDTA solution (e.g., SIGMA T 3924, ICN-Flow, # 16891-49)
e) Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}(for trypsinization)
f) Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}(CMF-HBSS)
g) Dulbecco's Phosphate Buffered Saline (D-PBS) [formulation containing calcium and magnesium cations; glucose optional] (for rinsing)
h) Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49)
i) Neutral Red (NR) Dye – tissue culture-grade; liquid form (e.g., SIGMA N 2889); powder form (e.g., SIGMA N 4638)
j) DMSO, U.S.P. analytical grade (Store under nitrogen @ -20ºC)
k) ETOH, U.S.P. analytical grade (100 %, non-denatured for test substance preparation; 95 % can be used for the desorb solution)
l) Glacial acetic acid, analytical grade
m) Distilled H_2O or any purified water suitable for cell culture and NR desorb solution (sterile)
n) Sterile/non-sterile paper towels (for blotting 96-well plates)

[Note: Due to lot variability of NCS, first check a lot for growth stimulating properties with 3T3 cells (approximately 20-24 hours doubling time) and then reserve a sufficient amount of NCS.]

B. Preparations of Media and Solutions

[Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures will be adequately documented.]

1. Media

DMEM (buffered with sodium bicarbonate) supplemented with (final concentrations in DMEM are quoted):

a) Freeze Medium; contains 2X concentration of NCS and DMSO of final freezing solution

40 %	NCS
20 %	DMSO

b) Routine Culture Medium

10 %	NCS
4 mM	Glutamine

c) Chemical Dilution Medium

4 mM	Glutamine
200 IU/mL	Penicillin
200 µg/mL	Streptomycin

d) NR Dilution Medium

5 %	NCS
4 mM	Glutamine
100 IU/mL	Penicillin
100 µg/mL	Streptomycin

[Note: The Chemical Dilution Medium with test substance will dilute the serum concentration of the Routine Culture Medium in the test plate to 5 %. Serum proteins may mask the toxicity of the test substance, but serum cannot be totally excluded because cell growth is markedly reduced in its absence.]

Completed media formulations should be kept at approximately 2-8°C and stored for no longer than two weeks.

2. Neutral Red (NR) Stock Solution

The liquid tissue culture-grade stock NR Solution is the first choice (e.g., SIGMA #N2889, 3.3 mg/mL). Store liquid tissue culture-grade NR Stock Solution at the storage conditions and shelf-life period recommended by the manufacturer.

A stock solution can be made with powder NR dye and water (e.g., 0.25 g NR Dye powder in 100 mL H_2O) if the liquid stock form is not available. The stock should be stored in the dark at room temperature for up to two months.

3. Neutral Red (NR) Medium

EXAMPLE:

0.758 mL (3.3 mg NR dye/mL sol.)	NR Stock Solution
99.242 mL	NR Dilution Medium (pre-warmed to 37°C)

The final concentration of the NR Medium is **25 µg NR dye/mL** and aliquots will be prepared on the day of application.

[Note: The NR Medium shall be filtered (e.g., Millipore filtering, 0.2 – 0.45 µm pore size) to reduce NR crystals. Aliquots of the NR Medium should be maintained at 37°C (e.g., in a waterbath) before adding to the cells and used within 60 minutes of preparation but also used within 15 minutes after removing from 37°C storage. Examine the solution for crystals.]

4. ETOH/Acetic Acid Solution (NR Desorb)

1 %	Glacial acetic acid solution
50 %	ETOH
49 %	H_2O

C. Methods

1. Cell Maintenance and Culture Procedures

BALB/c 3T3 cells are routinely grown as a monolayer in tissue culture grade flasks (e.g., 75 - 80 cm^2) at 37ºC ± 1ºC, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO_2/air. The cells should be examined on a daily (i.e., on workdays) basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted in a Study Workbook.

2. Receipt of Cryopreserved BALB/c 3T3 Cells

Upon receipt of cryopreserved BALB/c 3T3 cells, the vial(s) of cells shall be stored in a liquid nitrogen freezer until needed.

3. Thawing Cells

Thaw cells by putting ampules into a waterbath at 37°C ± 1ºC. Leave for as brief a time as possible.

3 Resuspend the cells in pre-warmed Routine Culture Medium and transfer into pre-warmed Routine Culture Medium in a tissue-culture flask.
4 Incubate at 37ºC ± 1ºC, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO_2/air.
5 When the cells have attached to the bottom of the flask (within 4 to 24 hours), decant the supernatant and replace with fresh pre-warmed (37ºC) medium. Culture as described above.
6 Passage at least two times before using the cells in a cytotoxicity test.

A fresh batch of frozen cells from the stock lot of cells should be thawed out and cultured approximately every two months. This period resembles a sequence of about 18

passages.

4. Routine Culture of BALB/C 3T3 Cells

When cells exceed 50 % confluence (but less than 80 % confluent) they should be removed from the flask by trypsinization:

3 Decant medium, briefly rinse cultures with 5 mL PBS or Hanks' BSS (without Ca^{2+}, Mg^{2+}) per 25 cm^2 flask (15 mL per 75 cm^2 flask). Wash cells by gentle agitation to remove any remaining serum that might inhibit the action of the trypsin.
4 Discard the washing solution. Repeat the rinsing procedure and discard the washing solution.
5 Add 1-2 mL trypsin-EDTA solution per 25 cm^2 to the monolayer for a few seconds (e.g., 15-30 seconds).
6 Remove excess trypsin-EDTA solution and incubate the cells at room temperature.
7 After 2-3 minutes, lightly tap the flask to detach the cells into a single cell suspension.

5. Cell Counting

After detaching the cells, add 0.1-0.2 mL of pre-warmed (37ºC) Routine Culture Medium/cm^2 to the flask (e.g., 2.5 mL for a 25 cm^2 flask). Disperse the monolayer by gentle trituration to obtain a single cell suspension for exact counting. Count a sample of the cell suspension obtained using a hemocytometer or cell counter (e.g., Coulter counter).

6. Subculture of Cells

After determination of cell number, the culture can be sub-cultured into other flasks or seeded into 96-well microtiter plates (see **Section VI.E.1** for 96-well test plate configuration). BALB/c 3T3 cells are routinely passaged at suggested cell densities as listed in the table (approximate doubling time is 20-24 hours). Laboratories must determine and adjust the final density to achieve appropriate growth.

Table 1. Cell Density Guidelines for Subculturing

Days in Culture	Seeding Density (cells/cm^2)	Total Cells per 25 cm^2 flask	Total Cells per 75 cm^2 flask
2	16800	4.2×10^5	1.26×10^6
3	8400	2.1×10^5	6.3×10^5
4	4200	1.05×10^5	3.15×10^5

[Note: It is important that cells have overcome the lag growth phase when they are used for the test.]

7. Freezing Cells (procedure required only if current stock of cells is depleted)

Stocks of BALB/c 3T3 cells can be stored in sterile, freezing tubes in a liquid nitrogen freezer. DMSO is used as a cryoprotective agent.

4 Centrifuge trypsinized cells at approximately 200 x g.
5 Suspend the cells in cold Routine Culture Medium (half the final freezing volume) so a final concentration of 1-5x10^6 cells/mL can be attained.
6 Slowly add cold Freeze Medium to the cells so that the solvent will equilibrate across the cell membranes. Bring the cell suspension to the final freezing volume. The final cell suspension will be 10 % DMSO. Aliquot the cell suspension into freezing tubes and fill to 1.8 mL.
7 Place the tubes into an insulated container (e.g., styrofoam trays) and place in a freezer (-70 to -80°C) for 24 hours (~freezing rate of 1°C/minutes). The laboratory needs to ensure that the freezing protocol is applicable to the 3T3 cells and that the cells are viable when removed from cryopreservation.
8 Place the frozen tubes into liquid nitrogen for storage.

8. Preparation of Cells for Assays

- Cultured cells that will be used in seeding the 96-well plates should be fed fresh medium the day before subculturing to the plates. On the day of plate seeding, prepare a cell suspension of 2.0 – 3.0x10^4 cells/mL in Routine Culture Medium. Using a multi-channel pipette, dispense 100 µl Routine Culture Medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate (See **Section VI.E.1**). In the remaining wells, dispense 100 µl of a cell suspension of 2.0 – 3.0x10^4 cells/mL (= 2.0 – 3.0x10^3 cells/well). The seeding density should be noted to ensure that the cells in the control wells are not overgrown after three days (i.e., 24 hour incubation in step **b** and 48 hour exposure to test substances). Prepare one plate per substance to be tested.
- Incubate cells for 24 hours ± 2 hours (37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air) so that cells form a less than half (< 50%) confluent monolayer. This incubation period assures cell recovery and adherence and progression to exponential growth phase.
- Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook.

9. Determination of Doubling Time

a) Establish cells in culture and trypsinize cells as per **Section VI.C.4** for subculture. Resuspend cells in NR Dilution Medium (5 % NBCS/NCS). Seed cells at 4200 cells/cm^2.

b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air).

c) After 4 - 6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Study Director may determine cell viability by dye exclusion (e.g., Trypan Blue; Nigrosin). Use appropriate size exclusion limits if using a Coulter counter. Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop.

d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase).

D. Preparation of Test Substances

[Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.]

Test substance solubility should be determined by following the procedures outlined in **ANNEX II** of this protocol.

1. Test Substances in Solution

a) Allow test substances to equilibrate to room temperature before dissolving and diluting.

b) Prepare test substance immediately prior to use rather than preparing in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in future substance analyses.

c) For substances dissolved in DMSO or ETOH, the final DMSO or ETOH concentration for application to the cells must be 0.5 % (v/v) in the vehicle controls and in all of the eight test concentrations.

d) The stock solution for each test substance should be prepared at the highest concentration found to be soluble in the solubility test conducted per **ANNEX II**: *Test Method Procedure - Solubility Determination of Test Substances*. Thus, the highest test concentration applied to the cells in each range finding experiment is:

4 0.5 times the highest concentration found to be soluble in the solubility test, if the substance was soluble in Chemical Dilution Medium, or

5 1/200 the highest concentration found to be soluble in the solubility test if the substance was soluble in ETOH or DMSO.

e) The seven lower concentrations in the range finding experiment are prepared by successive dilutions that decrease by one log unit each. The following example illustrates the preparation of test substance in solvent and the dilution of dissolved test substance in Chemical Dilution Medium before application to 3T3 cells.

Example: Preparation of Test Substance in Solvent Using a Log Dilution Scheme

If DMSO is determined to be the preferred solvent at Tier 3 of the solubility test (i.e., 200,000 μg/mL), dissolve the substance in DMSO at 200,000 μg/mL for the chemical stock solution.

1) Label eight tubes 1 – 8. Add 0.9 mL solvent (e.g., DMSO) to tubes 2 -- 8.
2) Prepare stock solution of 200,000 μg test substance/mL solvent in tube # 1.
3) Add 0.1 mL of 200,000 μg/mL dilution from tube #1 to tube #2 to make a 1:10 dilution in solvent (i.e., 20,000 μg/mL).
4) Add 0.1 mL of 20,000 μg/mL dilution from tube #2 to tube #3 to make another 1:10 dilution (i.e., 1:100 dilution from stock solution) in solvent (i.e., 2,000 μg/mL)
5) Continuing making serial 1:10 dilutions in the prepared solvent tubes.
6) Since each concentration is 200 fold greater than the concentration to be tested, make a 1:100 dilution by diluting 1 part dissolved test substance in each tube with 99 parts of Chemical Dilution Medium (e.g., 0.1 mL test substance in DMSO + 9.9 mL Chemical Dilution Medium) to derive the eight 2X concentrations for application to 3T3 cells. Each 2X test substance concentration will then contain 1 % v/v solvent. The 3T3 cells will have 0.05 mL Routine Culture Medium in the wells prior to application of the test substance. By adding 0.05 mL of the appropriate 2X test substance concentration to the appropriate wells, the test substance will be diluted appropriately (e.g., highest concentration in well will be 1,000 μg/mL) in a total of 0.1 mL and the solvent concentration in the wells will be 0.5% v/v.
7) A test substance prepared in Chemical Dilution Medium, DMSO, or ETOH may precipitate upon transfer into the Routine Culture Medium. The 2X dosing solutions should be evaluated for precipitates and the results recorded in the Study Workbook. It is permissible to test all of the dosing solutions in the dose range finding assay and main experiments. However, doses containing test substance precipitates should be avoided because it creates doubt about the concentration of test substance exposed to the cells.

Document all test substance preparations in the Study Workbook.

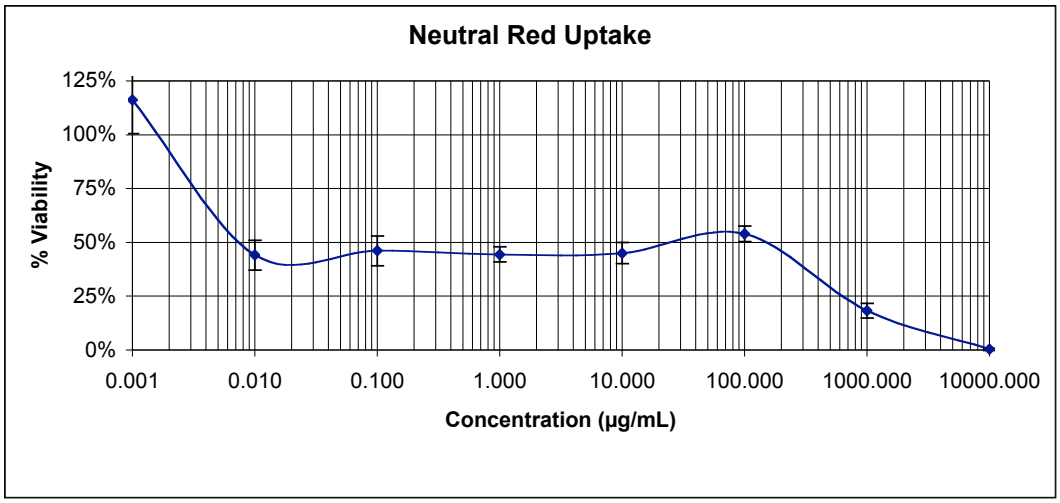
2. pH of Test Substance Solutions

Prior to or immediately after application of the test substance to the 96-well plate, measure the pH of the highest 2X dosing concentration of the test substance (i.e., C1 in the test plate, see **Figure 1**) in culture medium. Use pH paper (e.g., pH 0 - 14 to estimate and pH 5 – 10 to determine more precise value; or Study Director's discretion) for measurements. The pH paper should be in contact with the solution for approximately one minute. Document the pH and note the color of the 2X concentration medium (i.e., in the Microsoft Excel ® template; see **ANNEX 1** for an example template). Medium color for all dosing dilutions should be noted in the Study Workbook. Do not adjust the pH.

3. Concentrations of Test Substance

a) Range Finder Experiment
- Test eight concentrations of the test substance by diluting the stock solution using log dilutions (e.g., 1:10, 1:100, 1:1000, etc.).
- If a range finder experiment does not generate enough cytotoxicity, then higher doses should be attempted. If cytotoxicity is limited by solubility, then more stringent solubility procedures to increase the stock concentration (to the maximum concentration specified in **Section VI.D.3.b**.) should be employed.
- Place the test substance concentration into an incubator (37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air) and stir or rock for up to 3 hours, if necessary, to facilitate dissolution. For stocks prepared in medium, vessel caps should be loose to allow for CO_2 exchange. Proceed with dosing solution preparation and dosing.
- If a range finding test produces a biphasic curve, then the doses selected for the subsequent main experiments should cover the most toxic dose-response range (see **Example 1** – the most toxic range is 0.001 – 0.1 μg/mL) that reduces viability to 50 %.

Example 1 – Biphasic Curve



b) Main Experiment (Definitive Assay)

- Depending on the slope of the concentration-response curve estimated from the range finder, the dilution/progression factor in the concentration series of the main experiment should be smaller (e.g., dilution factor of $\sqrt[6]{10} = 1.47$). Cover the relevant concentration range around the IC_{50} (> 0 % and < 100 % effect) preferably with several points of a graded effect, but with a minimum of two points, one on each side of the estimated IC_{50} value, avoiding too many non-cytotoxic and/or 100 %-cytotoxic concentrations.
- Determine which test substance concentration is closest to the IC_{50} value. Use that value as a central concentration and adjust dilutions higher and lower in equal steps for the definitive assay.

Maximum Doses to be Tested in the Main Experiments

If minimal or no cytotoxicity was measured in the dose range finding assay, a maximum dose for the main experiments will be established as follows:

For test substances prepared in Chemical Dilution Medium

- The highest test substance concentration that may be applied to the cells in the main experiments will be either 100 mg/mL, or the maximum soluble dose.
- Test substance will be weighed into a glass tube and the weight will be documented. A volume of Chemical Dilution Medium will be added to the vessel so that the concentration is 200,000 µg/mL (200 mg/mL).
- The solution is mixed using the mechanical procedures that produced solubility when performing the solubility test (See **ANNEX II**). If complete solubility is achieved in medium, then 7 additional serial stock dosing solutions may be prepared from the 200 mg/mL 2X stock. If the test substance is insoluble in medium at 200 mg/ml, proceed by adding medium, in small incremental amounts, to attempt to dissolve the substance by using the sequence of mechanical procedures specified in **ANNEX II**.

- More stringent solubility procedures may be employed if needed based on results from the range finder experiment (**Section VI.D.3.a.**). The highest soluble stock solution will be used to prepare the 7 additional serial stock dosing solutions.

<u>For test substances prepared in either DMSO or ETOH</u>

- The highest test substance concentration that may be applied to the cells in the main experiments will be ≤ 2.5 mg/mL or less, depending upon the maximum solubility in solvent.
- Weigh the test substance into a glass tube and document the weight. Add the appropriate solvent (determined from the original solubility test) to the vessel so that the concentration is 500,000 μg/mL (500 mg/mL).
- Mix the solution using the sequence of mechanical procedures specified in **ANNEX II**. If complete solubility is achieved in the solvent, then seven additional serial stock dosing solutions may be prepared from the 500 mg/mL 200X stock. If the test substance is insoluble in solvent at 500 mg/ml, proceed by adding solvent, in small incremental amounts, to attempt to dissolve the substance by again using the sequence of mixing procedures. The highest soluble stock solution will be used to prepare the seven additional serial stock dosing solutions.

If precipitates are observed in the 2X dilutions, continue with the experiment and make the appropriate observations and documentation.

c) Test Substance Dilutions

The dosing factor of 3.16 (= $\sqrt[2]{10}$) divides a log into two equidistant steps, 2.15 (= $\sqrt[3]{10}$) into three steps, 1.47 (= $\sqrt[6]{10}$) into six steps, 1.78 ($\sqrt[4]{10}$) into four steps, and 1.21 (= $\sqrt[12]{10}$) into 12 steps.

EXAMPLE:

10						31.6						100
10				21.5				46.4				100
10		14.7		21.5		31.6		46.4		68.1		100
10	12.1	14.7	17.8	21.5	26.1	31.6	38.3	46.4	56.2	68.1	82.5	100

An example of decimal geometric concentration series for factor 1.47:
Dilute 1 volume of the highest concentration by adding 0.47 volumes of diluent. After equilibration, dilute 1 volume of this solution by adding 0.47 volumes of diluent...(etc.).

E. Test Procedure

1. 96-Well Plate Configuration

The 3T3 NRU assay for test substances will use the 96-well plate configuration as shown in **Figure 1**.

Figure 1. 96-Well Plate Configuration for Positive Control (PC) and Test Substance Assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb
B	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
C	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
D	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
E	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
F	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
G	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
H	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb

VC1 and VC2 = VEHICLE CONTROL
$C_1 – C_8$ = Test Substances or PC (SLS) at eight concentrations (C1 = highest, C8 = lowest)
b = BLANKS (Test substance or PC, but contain **no** cells)
VCb = VEHICLE CONTROL BLANK (contain **no** cells)

2. Application of Test Substance

6.0 Two optional methods for rapidly applying the 2X dosing solutions onto the 96-well plates may be utilized.

1) Add each of the 2X dosing solutions into labeled, sterile reservoirs (e.g., Corning/Costar model 4870 sterile polystyrene 50 mL reagent reservoirs; or Corning/Transtar model 4878 disposable reservoir liners, 8-channel; or other multichannel reservoirs).
2) Use a "dummy" plate (i.e., an empty sterile 96-well plate) prepared to hold the dosing solutions immediately prior to treatment of the test plate (with cells). The test substance and control dosing solutions should be dispensed into the dummy plate in the same pattern/order as will be applied to the plate containing cells. More volume than needed for the test plate (i.e., greater than 50 µl/well) should be in the wells of the dummy plate.

At the time of treatment initiation, a multi-channel micropipettor is used to transfer the 2X dosing solutions, from the reservoirs or dummy plate, to the appropriate wells on the treatment plate (as described in step c. below). These methods will ensure that the dosing solutions can be transferred rapidly to the appropriate wells of the test plate to initiate treatment times and to minimize the range of treatment initiation times across a large number of treatment plates, and to prevent "out of order" dosing. Do not use a multichannel repeater pipette for dispensing test substance to the plates.

7.0 After 24 hours ± 2 hours incubation of the cells, remove Routine Culture Medium from the cells by careful inversion of the plate (i.e., "dump") over an appropriate receptacle. Gently blot the plate on a sterile paper towel so that the monolayer is minimally disrupted. Do not use automatic plate washers for this procedure nor vacuum aspiration.

8.0 Immediately add 50 µL of fresh pre-warmed Routine Culture Medium to all of the wells, including the blanks. Fifty microliters (50 µL) of dosing solution will be rapidly transferred from the 8-channel reservoir (or dummy plate) to the appropriate wells of the test plate using a single delivery multi-channel pipettor. For example, the VC may be transferred first (into columns 1, 2, 11, and 12), followed by the test substance dosing solutions from lowest to highest dose, so that the same pipette tips on the multi-channel pipettor can be used for the whole plate. [The Vehicle Control blank (VCb) wells (column 1, column 12, wells A2, A11, H2, H11) will receive the Vehicle Control dosing solutions (which should include any solvents used)]. Blanks for wells A3 – A10 and H3 – H10 shall receive the appropriate test substance solutions for each concentration (e.g., wells A3 and H3 receive C_1 solution).

9.0 Incubate cells for 48 hours ± 0.5 hours (37ºC ± 1ºC, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO_2/air).

10.0 **Positive Control**: For each set of test substance plates used in an assay, prepare a separate plate of positive control concentrations. If multiple sets of test substance plates are set up, then clearly designate the positive control plates for each set; each set will be an individual entity. The Study Director will decide how many test substance plates will be run with a positive control plate. This plate will follow the same schedule and procedures as used for the test substance plates (including appropriate test substance concentrations in the appropriate wells and meeting test acceptance criteria – see **sections VI.E.1, E.2, and E.5**).

3. Microscopic Evaluation

After at least 46 hours of treatment, examine each plate under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record any changes in morphology of the cells due to the cytotoxic effects of the test substance, but do not use these records for any quantitative measure of cytotoxicity. Undesirable growth characteristics of

control cells may indicate experimental error and may be cause for rejection of the assay. Use the following Visual Observations Codes in the description of cell culture conditions. Numerical scoring of the cells (see **Section VI.E.3**) should be determined and documented in the Study Workbook and in the appropriate section of the Microsoft Excel® template.

Visual Observations Codes

Note Code	Note Text
1	Normal Cell Morphology
2	Low Level of Cell Toxicity
3	Moderate Level of Cell Toxicity
4	High level of Cell Toxicity
1P	Normal Cell Morphology with Precipitate
2P	Low Level of Cell Toxicity with Precipitate
3P	Moderate Level of Cell Toxicity with Precipitate
4P	High level of Cell Toxicity with Precipitate
5P	Unable to View Cells Due to Precipitate

4. Measurement of NRU

a) Carefully remove (i.e., "dump") the medium with test substance and rinse the cells very carefully with 250 µL pre-warmed D-PBS. Remove the rinsing solution by dumping and remove excess by gently blotting on paper towels. Add 250 µL NR medium (to all wells including the blanks) and incubate (37ºC ± 1ºC, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO_2/air) for 3 hours ± 0.1 hour. Observe the cells briefly during the NR incubation (e.g., between 2 and 3 hours – Study Director's discretion) for NR crystal formation. Record observations in the Study Workbook. Study Director can decide to reject the experiment if excessive NR crystallization has occurred.

b) After incubation, remove the NR medium, and carefully rinse cells with 250 µl pre-warmed D-PBS.

c) Decant and blot D-PBS from the plate.

d) Add exactly 100 µl NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.

e) Shake microtiter plate rapidly on a microtiter plate shaker for 20 – 45 minutes to extract NR from the cells and form a homogeneous solution. Plates should be protected from light by using a cover during shaking.

f) Plates should be still for at least five minutes after removal from the plate shaker (or orbital mixer). If any bubbles are observed, assure that they have been ruptured prior to reading the plate. Measure the absorption (within 60 minutes of adding NR Desorb solution) of the resulting colored solution at 540 nm ± 10 nm in a microtiter plate reader (spectrophotometer), using the blanks as a reference.

[Note: A mean $OD_{540 \pm 10nm}$ of 0.031 - 0.065 for the VC blanks is a target range of ODs but not a test acceptance criterion (range = mean OD ± 2.5 standard deviations; mean = 0.048; SD = 0.007; N = 233).] Save raw data in the Microsoft Excel® template.

5. Quality Check of 3T3 NRU Assay

a) Test Acceptance Criteria
All acceptance criteria (i.e., criteria 1, 2, and 3) must be met for a test to be acceptable.

1) The PC (SLS) IC_{50} must be within ± two and a half (2.5) standard deviations (SD) of the historical mean established by the Test Facility, and must meet criteria 2 and 3, and must have an r^2 (coefficient of determination) value calculated for the Hill model fit (i.e., from PRISM® software) ≥ 0.85. NICEATM/ECVAM study generated the following PC data:
   - IC_{50} mean = 41.5 µg/mL; SD = 4.8 (n = 233)
   - range for IC_{50} mean ± 2.5 SD = 29.5 µg/mL – 53.5 µg/mL
2) The left and right mean of the VCs do not differ by more than 15% from the mean of all VCs.
   - At least one calculated cytotoxicity value > 0 % and ≤ 50 % viability and at least one calculated cytotoxicity value > 50 % and < 100 % viability must be present.

Exception: If a test has only one point between 0 and 100 % **and** the smallest dilution factor (i.e., 1.21) was used **and** all other test acceptance criteria were met, then the test will be considered acceptable.

Stopping Rule for Insoluble Substances: If the most rigorous solubility procedures have been performed and the assay cannot achieve adequate toxicity to meet the test acceptance criteria after three definitive trials, then the Study Director may end all testing for that particular substance.

[Note: A corrected mean $OD_{540 \pm 10nm}$ of 0.183 - 0.769 for the VCs is a target range of ODs but not a test acceptance criterion (range = mean OD ± 2.5 standard deviations; mean = 0.476; SD = 0.117; N = 233).]

b) Checks for Systematic Cell Seeding Errors
To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row 2) and the right side (row 11 for the test plates) of the 96-well plate. Aberrations in the cell monolayer for the VCs may reflect a volatile and toxic test substance present in the assay. If volatility is suspected, then proceed to **Section VI.E.6**. Checks for cell seeding errors may also be performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent.

6. Testing Volatile Substances

Although this test method is not suitable for highly volatile substances, mildly volatile substances may be tested with some success. Volatile test substances may generate vapors from the treatment medium during the test substance treatment incubation period. These vapors may become resorbed into the treatment medium in adjacent wells, such that culture wells nearest the highest doses may become contaminated by exposure. If the test substance is particularly toxic at the doses tested, the cross contamination may be evident as a significant reduction in viability in the VC cultures (i.e., VC1) adjacent to the highest test substance doses.

If potential test substance volatility is suspected (e.g., for low density liquids) or if the initial range finder test (non-sealed plate) results show evidence of toxic effects in the control cultures (i.e., > 15 % difference in viability between VC1 [column 2] and VC2 [column 11]), then seal the subsequent test plates using the following procedure.

a) Plates and substances will be prepared as usual according to **Sections VI.D and VI.E**.
b) Immediately after the 96-well culture plate has been treated with the suspected volatile substance (**Section VI.E.2.b**), apply the adhesive plate sealer (e.g., using a hand, microplate roller, etc.) directly over the culture wells. Assure that the sealer adheres to each culture well (well tops should be dry). Place the 96-well plate cover over the sealed plate and incubate the plate under specified conditions (**Section VI.E.2.b**). [Note: Do not jam the plate lid over the film to avoid deforming the sealer and causing the sealer to detach from culture wells. Loose fit of the plate lid is acceptable.]
c) At the end of the treatment period, the plate sealer should be carefully removed to avoid spillage. Continue with the NRU assay as per **Section VI.E.4**.

F. Data Analysis

- The Study Director will use good biological/scientific judgment for determining "unusable" wells that will be excluded from the data analysis and provide explanations for the removal of any data from the analysis.
- A calculation of cell viability expressed as NRU is made for each concentration of the test substance by using the mean NRU of the six replicate values (minimum of four acceptable replicate well) per test concentration (blanks will be subtracted). This value is compared with the mean NRU of all VC values. Relative cell viability is then expressed as percent of untreated VC. If achievable, the eight concentrations of each substance tested will span the range of no effect up to total inhibition of cell viability.
- Data from the microtiter plate reader should be transferred to a spreadsheet template (e.g., Microsoft Excel®) that will automatically determine cell

viability, calculate IC_{50} values by linear interpolation, and perform statistical analyses (including statistical identification of outliers) (see **ANNEX 1** for an example spreadsheet template).

- A Hill function analysis should be performed using statistical software (e.g., GraphPad PRISM® 3.0) and a template to calculate IC_{20}, IC_{50}, and IC_{80} values (and the associated confidence limits) for each test substance.
- Dose-responses for which the toxicity plateaus as concentration increases do not fit the Hill function well when Bottom =0. To obtain a better model fit, unconstrain the Bottom parameter so that the model calculates the Bottom value. However, when Bottom ≠ 0, the EC_{50} reported by the Hill function ≠ 50% viability since the Hill function defines EC_{50} as the point midway between Top and Bottom. To obtain the appropriate IC_{50} when Bottom≠ 0, use the following rearranged Hill function:

$$X = \log EC_{50} - \frac{\log\left(\frac{Top - Bottom}{Y - Bottom} - 1\right)}{HillSlope}$$

- X is the logarithm of concentration at 50% response, logEC_{50} is logarithm of concentration at the response midway between Top and Bottom, Top is the maximum response, Bottom is the minimum response, Y = 50 (i.e., 50% response), and HillSlope describes the steepness of the curve.

[Note: IC_{50} values are used in a regression formula to predict the LD_{50} value of a test substance as an estimate of the starting dose for an acute oral toxicity test.]

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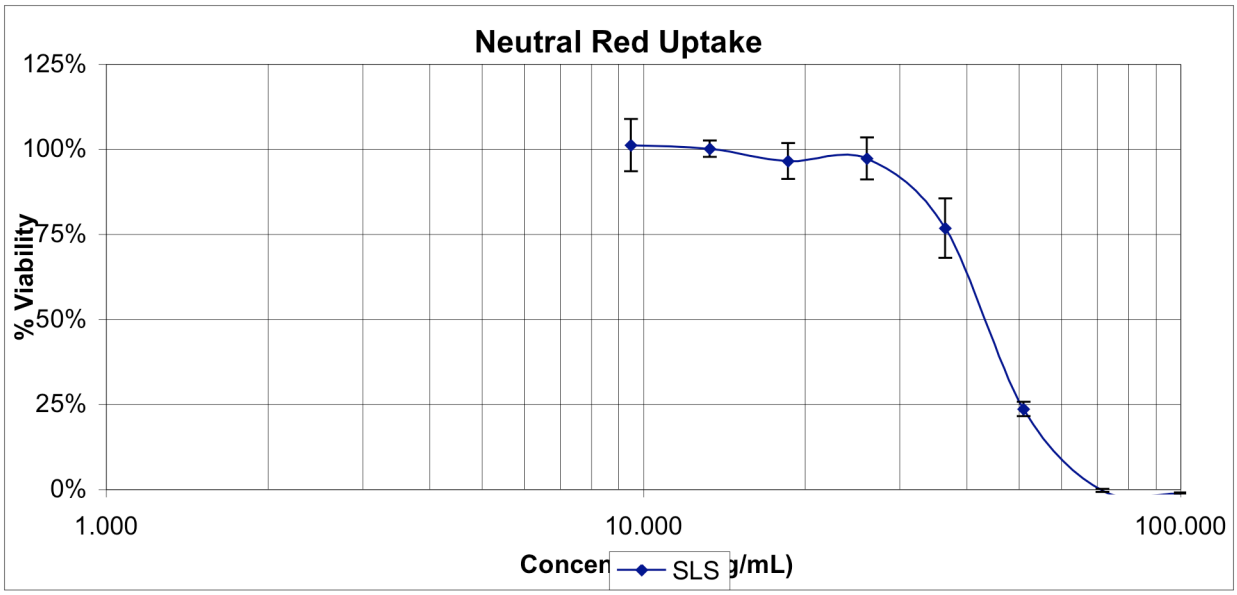
ANNEX 1
Microsoft EXCEL® Example Spreadsheet Template

Test Facility :	A					Study Number.:	A1					
Chemical Code :	SLS					96-Well Plate ID :	A11					
2nd Chem. Code*:	11					Experiment ID :	XX					
					96-WELL PLATE MAP							
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
C	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
D	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
E	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
F	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
G	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
					RAW ABSORBANCE DATA (OD_{550})							
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.044	0.044	0.045	0.045	0.045	0.046	0.051	0.057	0.057	0.043	0.041	0.044
B	0.042	0.456	0.043	0.043	0.130	0.300	0.395	0.414	0.418	0.402	0.401	0.042
C	0.043	0.407	0.042	0.041	0.130	0.294	0.383	0.382	0.413	0.375	0.385	0.044
D	0.043	0.438	0.042	0.043	0.147	0.337	0.409	0.404	0.438	0.436	0.391	0.047
E	0.044	0.448	0.041	0.045	0.132	0.321	0.429	0.414	0.416	0.420	0.441	0.042
F	0.045	0.411	0.040	0.042	0.127	0.375	0.397	0.402	0.422	0.447	0.403	0.043
G	0.041	0.405	0.043	0.040	0.124	0.361	0.444	0.442	0.425	0.448	0.405	0.044
H	0.041	0.041	0.048	0.042	0.042	0.044	0.042	0.042	0.040	0.044	0.041	0.041
Max	0.045	0.456	0.043	0.045	0.147	0.375	0.444	0.442	0.438	0.448	0.441	0.047
Min	0.041	0.405	0.040	0.040	0.124	0.294	0.383	0.382	0.413	0.375	0.385	0.041
Next Max	0.044	0.448	0.042	0.043	0.132	0.361	0.429	0.414	0.425	0.447	0.405	0.044
Next Min	0.042	0.407	0.041	0.041	0.127	0.300	0.395	0.402	0.416	0.402	0.391	0.042
Rmax	-0.250	-0.157	-0.333	-0.400	-0.652	-0.173	-0.246	-0.467	-0.520	-0.014	-0.643	-0.500
Rmin	0.250	0.039	0.333	0.200	0.130	0.074	0.197	0.333	0.120	0.370	0.107	0.167
					CORRECTED ABSORBANCE (Sample OD_{550} - Mean Blank OD_{550})							
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.001	-0.002	0.002	0.002	0.001	0.005	0.008	0.009	-0.001	-0.002	0.001
B	-0.001	0.413	-0.004	-0.001	0.087	0.255	0.349	0.365	0.370	0.359	0.358	-0.001
C	0.000	0.364	-0.005	-0.003	0.087	0.249	0.337	0.333	0.365	0.332	0.342	0.001
D	0.000	0.395	-0.005	-0.001	0.104	0.292	0.363	0.355	0.390	0.393	0.348	0.004
E	0.001	0.405	-0.006	0.002	0.089	0.276	0.383	0.365	0.368	0.377	0.398	-0.001
F	0.002	0.368	-0.007	-0.001	0.084	0.330	0.351	0.353	0.374	0.404	0.360	0.000
G	-0.002	0.362	-0.004	-0.004	0.081	0.316	0.398	0.393	0.377	0.405	0.362	0.001
H	-0.002	-0.002	0.002	-0.001	-0.001	-0.001	-0.005	-0.008	-0.009	0.001	-0.002	-0.002
Mean Blank =	0.043		0.047	0.044	0.044	0.045	0.047	0.050	0.049	0.044		
					RELATIVE VIABILITY (% OF VEHICLE CONTROL)							
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		110.7%	-0.9%	-0.1%	23.2%	68.4%	93.4%	97.7%	99.0%	96.1%	96.0%	
C		97.6%	-1.2%	-0.7%	23.2%	66.7%	90.2%	89.1%	97.7%	88.9%	91.7%	
D		105.9%	-1.2%	-0.1%	27.7%	78.3%	97.2%	95.0%	104.4%	105.2%	93.3%	
E		108.6%	-1.5%	0.4%	23.7%	74.0%	102.5%	97.7%	98.5%	100.9%	106.7%	
F		98.7%	-1.7%	-0.4%	22.4%	88.5%	94.0%	94.5%	100.1%	108.2%	96.5%	
G		97.1%	-0.9%	-0.9%	21.6%	84.7%	106.5%	105.2%	100.9%	108.4%	97.1%	
H												

Test Facility :	A				Study Number.:	A1				
Chemical Code :	SLS				96-Well Plate ID :	A11				
2nd Chem. Code*:	11				Experiment ID :	XX				
	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2
Conc. (µg/mL) :	0.0	100	71.4	51.0	36.4	26.0	18.6	13.3	9.49	0.0
Mean Corr. OD :	0.385	-0.005	-0.001	0.088	0.286	0.363	0.360	0.374	0.378	0.361
SD :	0.023	0.001	0.002	0.008	0.033	0.023	0.020	0.009	0.029	0.020
Mean Vehicle Control :	0.373									
Mean Blank :	0.043									
% of Vehicle Control :	103.1%	-1.3%	-0.3%	23.6%	76.8%	97.3%	96.5%	100.1%	101.3%	96.9%
SD :	6.0%	0.3%	0.5%	2.1%	8.7%	6.2%	5.3%	2.4%	7.7%	5.2%
% CV :	5.86%	-25.1%	-150%	9.09%	11.4%	6.33%	5.47%	2.39%	7.59%	5.41%
Mean VC - VC1 (%) :	-3.1%									
Mean VC - VC2 (%) :	3.1%									
Mean Absolute OD :	0.416									
					Visual Observations					
	VC	C1	C2	C3	C4	C5	C6	C7	C8	
ENTER CODES:	1	4	4	3	2	1	1	1	1	
Interpolated IC_{50} :					**4.32E+01**		**µg/mL**			



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TEST CHEMICAL								
Test Facility :	A			Study Number.:	A1			
Chemical Code :	SLS			96-Well Plate ID :	A11			
2nd Chem. Code*:	11			Experiment ID :	XX			
* Testing Facility Accession Code, if applicable								
PREPARATION OF TEST CHEMICAL								
Solvent:	Medium					Dilution factor:	1.4	
Solvent Conc. (%, v/v) in dosing solutions :	N/A				Highest Stock Conc.:	20,000	µg/mL	
Aids used to dissolve :	☐ Vortexing	☐ sonication	☐ heating to 37C					
pH (highest medium stock or 2X dosing solution) :	8.0							
Medium Clarity/Color (highest 2X dosing solution):	clear red				If ppt, note lowest conc.:			
Concentration Series (µg/mL)								
C1	C2	C3	C4	C5	C6	C7	C8	
100	71.4	51.0	36.4	26.0	18.6	13.3	9.49	
Positive Control (SLS)	100 - 9.49	µg/mL						
CELL LINE/TYPE								
Name:	BALB/c 3T3		Supplier:	ATCC		Lot No.	not provided	
Passage No.:	69		Passage No. in Assay:	75		Proliferating/frozen	24-May-02	
CELL CULTURE CONDITIONS								
Medium:	DMEM		Supplier:			Lot No.:		
Serum:	NCS		Supplier:			Lot No.:		
Serum Conc.:			Growth Medium:	10%		Treatment Medium:	0%	
TEST ACCEPTANCE CRITERIA								
No. of values >50% and <100%:	3		No. of values >0% and ≤50%:	1		Accept?	YES	
VC: % Difference between Col 2 and mean VC.:	-3%					Accept?	YES	
PC: Hill Function R^2 Value of SLS:	0.99					Accept?	YES	
PC: IC_{50} of SLS:	43.2	µg/mL				Accept?	YES	
TIMELINE								
Cell Seeding Date			Dose Application Date			OD_{550} Determination Date		
TEST RESULTS								
VC: Mean Corrected OD_{550}:	0.373					Hill Function R^2 Value:	0.9869	
log IC20 :	1.551E+00	µg/mL	log IC50 :	1.635E+00	µg/mL	log IC80 :	1.718E+00	µg/mL
IC20 :	3.56E+01	µg/mL	IC50 :	4.32E+01	µg/mL	IC80 :	5.22E+01	µg/mL
Test Chemical F.W. :	288.4							
IC_{20} :	0.12331183 mM		IC_{50} :	0.1496252 mM		IC_{80} :	0.18113599 mM	

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ANNEX II

TEST METHOD PROCEDURE
Solubility Determination of Test Substances

PROPOSAL

This procedure was designed to identify the solvent that would provide the highest soluble concentration of a test substance so there would be uniform availability of the substance to cells used for *in vitro* basal cytotoxicity testing. The solubility exercises can be performed in a routine and repeatable manner and provide guidelines to effectively prepare test substances for toxicity testing in the NRU test methods.

TEST SYSTEM

The solubility test procedure is based on attempting to dissolve substances in various solvents with increasingly rigorous mechanical techniques. The solvents to be used, in the order of preference, are cell culture medium, DMSO, and ETOH. Determination of whether a test substance has dissolved is based entirely on visual observation for the purposes of this protocol. A test substance has dissolved if the solution is clear and shows no signs of cloudiness or precipitation.

PROCEDURES

Preparation of the 3T3 medium will follow all procedures in the 3T3 NRU protocol.

Materials – see Section VI.A

Preparations of Media and Solutions – see Section VI.B

All solutions glassware, pipettes, etc., should be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures should be adequately documented.

Determination of Solubility

- Solubility should be determined in a step-wise procedure that involves attempting to dissolve a test substance at a relatively high concentration with the sequence of mechanical procedures specified in **Mechanical Procedures**. **Table 1** and **Figures 1** and **2** illustrate the step-wise procedures. The hierarchy of preference of solvent for dissolving test substances is medium, DMSO, and then ETOH. If the substance does not dissolve in the solvent, the volume of solvent is increased so as to decrease the test substance concentration by a factor of 10, and then the sequence of mechanical procedures are repeated in an attempt to solubilize the substance at the lower concentrations.
- For testing solubility in medium, the starting concentration is 200,000 μg/ml (i.e., 200 mg/mL) in Tier 1, but for DMSO and ETOH the starting concentration is 200,000 μg/ml (i.e., 200 mg/mL) in Tier 3.

Methods

A. Tier 1 begins with testing 200 mg/mL in Chemical Dilution Medium (see **Table 1**).
   1. Weigh approximately 100 mg (100,000 μg) of the test substance into a glass tube. Document the test substance weight.
   2. Add approximately 0.5 mL of medium into the tube so that the concentration is 200,000 μg/ml (200 mg/mL).
   3. Mix the solution as specified in **Mechanical Procedures**. If complete solubility is achieved, then additional solubility procedures are not needed.

B. If the test substance is insoluble in Tier 1 at 200 mg/mL, then proceed to Tier 2.
   1. Weigh approximately 10 mg (10,000 μg) of the test substance into a glass tube. Document the substance weight.
   2. Add approximately 0.5 mL of medium into the tube so that the concentration is 20,000 μg/ml (20 mg/mL).
   3. Mix the solution as specified in **Mechanical Procedures**. If complete solubility is achieved, then additional solubility procedures are not needed.

C. If the test substance is insoluble in Chemical Dilution Medium, proceed to Tier 3.

   1. Add enough medium, approximately 4.5 mL, to attempt to dissolve the substance at 2 mg/mL by using the sequence of mixing procedures. If the test substance dissolves in medium at 2 mg/mL, no further procedures are necessary.
   2. If the test substance does NOT dissolve in medium, weigh out approximately 100 mg test substance in a second glass tube and add enough DMSO to make the total volume approximately 0.5 mL (for 200 mg/mL) and mix the solution as specified in **Mechanical Procedures**.
   3. If the test substance does not dissolve in DMSO, weigh out approximately 100 mg test substance in another glass tube and add enough ETOH to make the total volume approximately 0.5 mL (for 200 mg/mL) and mix the solution as specified in **Mechanical Procedures**.
   4. If the substance is soluble in either solvent, no additional solubility procedures are needed.

D. If the substance is NOT soluble in Chemical Dilution Medium, DMSO, or ETOH at Tier 3, then continue to Tier 4 in **Table 1**.
   1. Add enough solvent to increase the volume of the three (or four) Tier 2 solutions by 10 and attempt to solubilize again using the sequence of mixing procedures. If the test substance dissolves, no additional solubility procedures are necessary.
   2. If the test substance does NOT dissolve, continue with Tier 5 and, if necessary, Tier 6 using DMSO and ETOH.
   3. Tier 5 begins by diluting the Tier 4 samples with DMSO or ETOH to bring the total volume to 50 mL. The mixing procedures are again followed to attempt to solubilize the substance.

4. Tier 6 is performed, if necessary, by weighing out another two samples of test substance at ~10 mg each and adding ~50 mL DMSO or ETOH for a 200 μg/mL solution, and following the mixing procedures.

Example

- If complete solubility is not achieved at 20,000 μg/mL in Chemical Dilution Medium at Tier 2 using the mixing procedures, then the procedure continues to Tier 3 by diluting the solution to 5 mL with medium and mixing again.
- If the substance is not soluble in Chemical Dilution Medium, two samples of ~ 100 mg test substance are weighed to attempt to solubilize in DMSO and ETOH at 200,000 μg/mL (i.e., 200 mg/mL). Solutions are mixed following the sequence of procedures prescribed in **Mechanical Procedures** in an attempt to dissolve.
- If solubility is not achieved at Tier 3, then the solutions prepared in Tier 3 are diluted by 10 so as to test 200 μg/mL in media, and 20,000 μg/mL in DMSO and ETOH. This advances the procedure to Tier 4. Solutions are again mixed in an attempt to dissolve.
- If solubility is not achieved in Tier 4, the procedure continues to Tier 5, and to Tier 6 if necessary (see **Figures 1** and **2** and **Table 1**).

MECHANICAL PROCEDURES

A. The following hierarchy of mixing procedures will be followed to dissolve the test substance:

1. Add test substance to solvent as in Tier 1 of **Table 1**. (Test substance and solvent should be at room temperature.)
2. Gently mix at room temperature. Vortex the tube (1 –2 minutes).
3. If test substance has not dissolved, use waterbath sonication for up to 5 minutes.
4. If test substance is not dissolved after sonication, then warm solution to 37°C for 5 - 60 minutes. This can be performed by warming tubes in a 37°C waterbath or in a CO_2 incubator at 37°C. The solution may be stirred during warming (stirring in a CO_2 incubator will help maintain proper pH).
5. Proceed to Tier 2 (and Tiers 3-6, if necessary of **Table 1** and repeat procedures 2-4).

B. The preference of solvent for dissolving test substances is Chemical Dilution Medium, DMSO, and then ETOH. Thus, if all solvents for a particular tier are tested simultaneously and a test substance dissolves in more than one solvent, then the choice of solvent follows this hierarchy. For example, if, at any tier, a substance were soluble in Chemical Dilution Medium and DMSO, the choice of solvent would be medium. If the substance were insoluble in medium, but soluble in DMSO and ETOH, the choice of solvent would be DMSO.

936 **Table 1 Determination of Solubility in Chemical Dilution Medium, DMSO, or**
937 **ETOH**

938

Tier	1	2	3	4	5	6
Total Volume Chemical Dilution Medium	0.5 mL	0.5 mL	5 mL	50 mL		
Concentration of Test Substance Tier 1: Add ~ 100 mg to a tube. Add enough medium to equal Tier 1 volume. If insoluble, go to Tier 2. Tier 2: Add ~10 mg to another tube. Add enough medium to equal the first volume. Dilute to subsequent volumes if necessary.	200,000 µg/mL (200 mg/mL)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	200 µg/mL (0.20 mg/mL)		
Total Volume DMSO/ETOH			0.5 mL	5 mL	50 mL	
Concentration of Test Substance (Add ~100 mg to a large tube. Add enough DMSO or ETOH to equal the first volume. Dilute with subsequent volumes if necessary.)			200,000 µg/mL (200 mg/mL)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	
Total Volume DMSO/ETOH						50 mL
Concentration of Test Substance (Add ~10 mg to a large tube. Add enough DMSO or ETOH to equal 50 mL.)						200 µg/mL (0.2 mg/mL)
EQUIVALENT CONCENTRATION ON CELLS	100,000 µg/mL (100 mg/mL)	10,000 µg/mL (10 mg/mL)	1000 µg/mL (1 mg/mL)	100 µg/mL (0.1 mg/mL)	10 µg/mL (0.01 mg/mL)	1 µg/mL (0.001 mg/mL)

939

940 [NOTE: The amounts of test substance weighed and Chemical Dilution Medium added may
941 be modified from the amounts given above, provided that the targeted concentrations
942 specified for each tier are tested.]
943

943 **FIGURE 1 SOLUBILITY STEP-WISE (TIERED) PROCEDURE**

TIER 1

STEP 1:	200 mg/mL test substance (TS) in 0.5 mL Chemical Dilution Medium • if TS soluble in medium, then **STOP**. • if TS insoluble in medium, then go to STEP 2.

TIER 2

STEP 2:	20 mg/mL TS in 0.5 mL Chemical Dilution Medium • if TS soluble, then **STOP**. • if TS insoluble, then go to STEP 3.

TIER 3

STEP 3:	200 mg/mL TS in DMSO 4 if TS soluble, then **STOP**. 5 if TS insoluble, test at 200 mg/mL in ETOH. 6 if TS soluble, then **STOP.** 7 If TS insoluble, go to STEP 4.

TIER 4

STEP 4:	0.2 mg/mL TS in medium (one or both) – increase volume from STEP 2 by 10 (i.e., to 50 mL) • if TS soluble in both media, then **STOP**. • if TS insoluble in one medium, test at 20 mg/mL in DMSO – increase volume from STEP 3 by 10 (i.e., to 5 mL). • if TS soluble, then **STOP.** • if TS insoluble, test at 20 mg/mL in ETOH – increase volume from STEP 3 by 10 (i.e., to 5 mL). • if TS soluble, then **STOP.** • if TS insoluble, then go to STEP 5.

TIER 5

STEP 5:	2 mg/mL TS in DMSO – increase volume from STEP 4 by 10 (i.e., to 50 mL) • if TS soluble, then **STOP.** • if TS insoluble, test at 2 mg/mL in ETOH – increase volume from STEP 4 by 10 (i.e., to 50 mL). • if TS soluble, then **STOP.** • if TS insoluble, then go to STEP 6.

TIER 6

STEP 6:	0.2 mg/mL TS in 50 mL DMSO • if TS soluble, then **STOP.** • if TS insoluble, test at 0.2 mg/mL in 50 mL ETOH • **STOP**

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Figure 2 Solubility Flow Chart

Tier	1		2		3		4		5		6
Concentration in Medium	**Start Here** 200 mg/mL	Incomplete solubility →	20 mg/mL	Incomplete solubility →	2 mg/mL		0.20 mg/mL				
					Incomplete solubility ↓		Incomplete solubility ↓				
Concentration in DMSO					200 mg/mL		20 mg/mL		2 mg/mL		0.2 mg/mL
					Incomplete solubility ↓		Incomplete solubility ↓		Incomplete solubility ↓		Incomplete solubility ↓
Concentration in ETOH					200 mg/mL	→ Incomplete solubility	20 mg/mL	→ Incomplete solubility	2 mg/mL	→ Incomplete solubility	0.2 mg/mL **End**
Concentration on Cells	100 mg/mL		10 mg/mL		1 mg/mL		0.1 mg/mL		0.01 mg/mL		0.001 mg/mL

Notes: 3T3 Medium - Dulbecco's Modification of Eagle's Medium, with supplements, for 3T3 mouse fibroblasts

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Appendix B-2

Test Method Protocol for the Normal Human Epidermal Keratinocyte (NHK) Neutral Red Uptake (NRU) Cytotoxicity Test

This draft recommended NHK NRU test method protocol is substantially the same as the protocol used in Phase III of the NICEATM/ECVAM validation study. Revisions were made based on recommendations from NICEATM and the study directors involved in the study. The changes are as follows:

- Explanations and directions for the use of the revised Hill function for determining IC50 values are included in the protocol.
- The range for relative humidity values for the cell culture incubators was changed from 90 % ± 5 % humidity to 90 % ± 10 % humidity.
- An additional step was added to the test substance solubility protocol to allow testing of higher concentrations of test material.
- The spreadsheet templates used in the NICEATM/ECVAM validation study are incorporated into this protocol as an annex (ANNEX I).
- The stand-alone solubility protocol is incorporated into this protocol as an annex (ANNEX II).
- The stand-alone NHK media prequalification protocol is incorporated into this protocol as an annex (ANNEX III).

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TEST METHOD PROTOCOL

THE NORMAL HUMAN KERATINOCYTE (NHK) NEUTRAL RED UPTAKE (NRU) CYTOTOXICITY TEST
A Test for Basal Cytotoxicity

I. PURPOSE

This test method is used to evaluate the cytotoxicity of test substances using the Normal Human Keratinocyte (NHK) Neutral Red Uptake (NRU) *in vitro* cytotoxicity test. The data generated from the *in vitro* cytotoxicity assays are used to evaluate the effectiveness of the cytotoxicity assay to predict the starting doses for rodent acute oral systemic toxicity assays. This test method protocol outlines the procedures for performing the basal cytotoxicity test and is the result of the *in vitro* validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM).

If changes or modifications are made to this protocol, the testing laboratory should prove that the results are comparable to those obtained when using the original protocol.

II. TEST SYSTEM

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.

Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic substance, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after substance exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

III. KEY PERSONNEL

A. Laboratory

1) Study Director
2) Laboratory Technician(s)

B. Testing Facility

1) Scientific Advisor
2) Quality Assurance Director
3) Safety Manager
4) Facility Management

IV. DEFINITIONS

A. ***Hill function:*** a four parameter logistic mathematical model relating the concentration of test substance to the response being measured in a sigmoidal shape.

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{logEC50} - X)\text{HillSlope}}}$$

where Y= response, X is the logarithm of dose (or concentration), Bottom is the minimum response, Top is the maximum response, logEC50 is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve. When Top = 100 and Bottom = 0, the EC_{50} is the concentration at 50% viability (i.e., the IC_{50}).

B. ***Documentation***: all methods and procedures will be noted in a Study Workbook; logs will be maintained for general laboratory procedures and equipment (e.g., media preparation, test substance preparation, incubator function); all optical density data obtained from the spectrophotometer plate reader will be saved in electronic and paper formats; all calculations of ICx values and other derived data will be in electronic and paper format; all data will be archived.

C. ***IC_{50}***: test substance concentration producing 50% inhibition of the endpoint measured (i.e., cell viability).

V. IDENTIFICATION OF CONTROL SUBSTANCES

A. Positive Control (PC)
Sodium Lauryl Sulfate (SLS)

B. Vehicle Control (VC)
Keratinocyte assay medium

C. Solvent Control
VC control with solvent (i.e., keratinocyte assay medium, dimethyl sulfoxide [DMSO], or ethanol [ETOH])
(DMSO is the preferred solvent for substances that are not water [i.e., assay medium] soluble.)

VI. PROCEDURES

A. Materials

1. Cell Line

Normal Human Epidermal Keratinocytes (NHK)
Non-transformed cells; from cryopreserved primary or secondary cells (e.g., **Clonetics #CC-2507 or equivalent** - *Cambrex* [Cambrex Bio Science, 8830 Biggs Ford Road, Walkersville, MD**).** Cells will be Clonetics NHK cells.

2. Technical Equipment

[Note: Suggested brand names/vendors are listed in parentheses. Equivalents may be used.]

8 Incubator: 37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air
9 Laminar flow clean bench (standard: "biological hazard")
10 Waterbath: 37ºC ± 1ºC
11 Inverse phase contrast microscope
12 Sterile glass tubes with caps (e.g., 5 mL)
13 Centrifuge
14 Laboratory balance
15 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm ± 10 nm filter
16 Shaker for microtiter plates
17 Cell counter or hemocytometer
18 Pipetting aid
19 Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette), dilution block
20 Cryotubes
21 Tissue culture flasks (75 - 80 cm^2, 25 cm^2)
22 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Corning/COSTAR tissue culture-treated)
23 pH paper (wide and narrow range)
q) Multichannel reagent reservoir
r) Waterbath sonicator
28 Magnetic stirrer
29 Antistatic bar ionizer/antistatic gun (optional for neutralizing static on 96-well plates)
30 Dry heat block (optional)
31 Adhesive film plate sealers (e.g., Excel Scientific SealPlate™,Cat # STR-SEAL-PLT or equivalent)
32 Vortex mixer
33 Filters/filtration devices

[Note: Tissue culture flasks and microtiter plates should be prescreened to ensure that they adequately support the growth of NHK. Multi-channel repeater pipettes may be used for plating cells in the 96-well plates, dispensing plate rinse solutions, NR medium, and desorb solution. Do not use the repeater pipette for dispensing test substances to the cells.]

3. Chemicals, Media, and Sera

9 Keratinocyte Basal Medium without Ca^{++} (e.g., KBM®, Clonetics CC-3104) that is completed by adding supplements (e.g., KBM® SingleQuots®, Clonetics CC-4131) to achieve the proper concentrations of epidermal growth factor, insulin, hydrocortisone, antimicrobial agents, bovine pituitary extract, and calcium (e.g., Clonetics Calcium SingleQuots®, 300 mM $CaCl_2$, Clonetics CC-4202).

10 HEPES Buffered Saline Solution (HEPES-BSS) (e.g., Clonetics # CC-5022)

11 0.025 % Trypsin/EDTA solution (e.g., Clonetics # CC-5012)

12 Trypsin Neutralizing Solution (TNS) (e.g., Clonetics # CC-5002)

13 Phosphate Buffered Saline (PBS)

14 Dulbecco's Phosphate Buffered Saline (D-PBS) [formulation containing calcium and magnesium cations; glucose optional] (for rinsing)

15 Neutral Red (NR) Dye – tissue culture-grade; liquid form (e.g., SIGMA N 2889); powder form (e.g., SIGMA N 4638)

16 DMSO, U.S.P analytical grade (Store under nitrogen @ -20ºC)

17 ETOH, U.S.P. analytical grade (100 %, non-denatured for test substance preparation; 95 % can be used for the desorb solution)

18 Glacial acetic acid, analytical grade

19 Hanks' Balanced Salt Solution without Ca^{2+} or Mg^{2+} (CMF-HBSS) (e.g., Invitrogen # 14170)

20 Distilled H_2O or any purified water suitable for cell culture and NR desorb solution (sterile)

21 Sterile/non-sterile paper towels (for blotting 96-well plates)

B. Preparations of Media and Solutions

[Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures will be adequately documented.]

1. Media

[Note: This protocol is based on the use of Clonetics KBM® medium and supplements. Other media may be acceptable if proper cell growth conditions can be maintained as per this protocol. Prequalify candidate media by using the keratinocyte medium prequalification in **ANNEX III**.]

a) Routine Culture Medium/Treatment Medium

KBM® (Clonetics CC-3104) supplemented with KBM® SingleQuots® (Clonetics CC-4131) and Clonetics Calcium SingleQuots® (CC-4202) to make 500 mL medium. Final concentrations of supplements in medium are:

0.0001 ng/mL	Human recombinant epidermal growth factor
5 μg/mL	Insulin
0.5 μg/mL	Hydrocortisone
30 μg/mL	Gentamicin
15 ng/mL	Amphotericin B
0.10 mM	Calcium
30 μg/mL	Bovine pituitary extract

Complete media should be kept at 2-8°C and stored for no longer than two weeks.

NOTE: KBM® SingleQuots® contain the following stock concentrations and volumes:

0.1 ng/mL	hEGF	0.5 mL
5.0 mg/mL	Insulin	0.5 mL
0.5 mg/mL	Hydrocortisone	0.5 mL
30 mg/mL	Gentamicin, 15 μg/mL Amphotericin-B	0.5 mL
7.5 mg/mL	Bovine Pituitary Extract (BPE)	2.0 mL

Clonetics Calcium SingleQuots® are 2 mL of 300 mM calcium.

165 μl of solution per 500 mL calcium-free medium equals 0.10 mM calcium in the medium.

2. Neutral Red (NR) Stock Solution

The liquid tissue culture-grade stock NR Solution will be the first choice for performing the assay (e.g., SIGMA #N2889, 3.3 mg/mL). Store liquid tissue culture-grade NR Stock Solution at the storage conditions and shelf-life period recommended by the manufacturer.

A stock solution can be made with powder NR dye and water (e.g., 0.33 g NR Dye powder in 100 mL H_2O) if the liquid stock form is not available. The stock should be stored in the dark at room temperature for up to two months.

3. Neutral Red (NR) Medium

EXAMPLE:
1.0 mL (3.3 mg NR dye/mL) NR Stock Solution

99 ml	99.0 mL	Routine Culture Medium (pre-warmed to 37°C)

The final concentration of the NR Medium is **33 µg NR dye/mL** and aliquots will be prepared on the day of application.

[Note: The NR Medium shall be filtered (e.g., Millipore filtering, 0.2 – 0.45 µm pore size) used to reduce NR crystals. Aliquots of the NR Medium should be maintained at 37°C (e.g., in a waterbath) before adding to the cells and used within 60 minutes of preparation but also used within 15 minutes after removing from 37°C storage. Examine the solution for crystals.]

4. ETOH/Acetic Acid Solution (NR Desorb)

1 %	Glacial acetic acid solution
50 %	ETOH
49 %	H_2O

C. Methods

1. Cell Maintenance and Culture Procedures

NHK cells are routinely grown as a monolayer in tissue culture grade flasks (e.g., 25 cm^2) at 37ºC ± 1ºC, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO_2/air. The cells should be examined on a daily (i.e., on workdays) basis under a phase contrast microscope, and any changes in morphology or their adhesive properties must be noted in a Study Workbook.

2. Receipt of Cryopreserved Keratinocytes

Upon receipt of cryopreserved keratinocytes, the vial(s) of cells shall be stored in a liquid nitrogen freezer until needed.

3. Thawing Cells and Establishing Cell Cultures

4 Thaw cells by putting ampules into a water bath at 37°C for as brief a time as possible. Do not thaw cells at room temperature or by hand. Seed the thawed cells into culture flasks as quickly as possible and with minimal handling.

5 Slowly (taking approximately 1-2 minutes) add 9 mL of pre-warmed Routine Culture Medium to the cells suspended in the cryoprotective solution and transfer cells into flasks containing pre-warmed Routine Culture Medium (See **Table 1**).

6 Incubate the cultures at 37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air until the cells attach to the flask (within 4 to 24 hours), at which time the Routine Culture Medium should be removed and replaced with fresh Routine Culture Medium.

7 Unless otherwise specified, the cells should be incubated at 37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air and fed every 2-3 days until they exceed 50 % confluence (but less than 80 % confluent).

Table 1. Guidelines for Establishing Cell Cultures

Cells/25 cm^2 flask (in approximately 5 mL) 1 flask each cell concentration	6.25 x 10^4 (2500/cm^2)	1.25 x 10^5 (5000/cm^2)	2.25 x 10^5 (9000/cm^2)
Approximate Time to Subculture	96+ hours	72 - 96 hours	48 - 72 hours
Cells to 96-Well Plates	6 – 8 plates	6 – 8 plates	6 – 8 plates

Cell growth guidelines – actual growth of individual cell lots may vary.

4. Subculture of NHK Cells to 96-Well Plates

[Note: It is important that cells have overcome the lag growth phase when they are used for the test. Keratinocytes will be passaged only into the 96-well plates and will not be subcultured into flasks for use in later assays]

8 When the keratinocyte culture in a 25 cm^2 flask exceeds 50 % confluence (but less than 80 % confluent), remove the medium and rinse the culture twice with 5 mL HEPES-BSS. The first rinse may be left on the cells for up to 5 minutes and the second rinse should remain on the cells for approximately 5 minutes. Discard the washing solutions.

9 Add 2 mL trypsin/EDTA solution to each flask and remove after 15 to 30 seconds. Incubate the flask at room temperature for 3 to 7 minutes. When more than 50 % of the cells become dislodged, rap the flask sharply against the palm of the hand.

10 When most of the cells have become detached from the surface, rinse the flask with 5 mL of room temperature TNS. If more than one flask is subcultured, the same 5 mL of TNS may be used to rinse a total of up to two flasks.

11 Then rinse the flask with 5 mL CMF-HBSS and transfer the cell suspension to a centrifuge tube.

12 Pellet the cells by centrifugation for 5 minutes at approximately 220 x g. Remove the supernatant by aspiration.

13 Resuspend the keratinocyte pellet by gentle trituration (to have single cells) in Routine Culture Medium. It is important to obtain a single cell suspension for exact counting. Count a sample of the cell suspension using a hemocytometer or cell counter.

14 Prepare a cell suspension –1.6 – 2.0 x10^4 cells/mL in Routine Culture Medium. Using a multi-channel pipette, dispense 125 µl Routine Culture Medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate. In the remaining wells, dispense 125 µl of the cell suspension (2x10^3 – 2.5x10^3 cells/well). Prepare one plate per substance to be tested (see **Figure 1**, **Section VI.E.1**).

15 Incubate cells (37ºC ± 1ºC, 90 % ± 10 % humidity, and 5 % ± 1 % CO_2/air) so that cells form a 20+ % monolayer (~48-72 hours). This incubation period

assures cell recovery and adherence and progression to exponential growth phase.

16 Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook.

5. Determination of Doubling Time

a) Establish cells in culture and trypsinize cells as per **Section VI.C.4** for subculture. Resuspend cells in appropriate culture medium. Use **Table 1** to determine seeding densities.

b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air).

c) After 4-6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document. Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop.

d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase).

D. Preparation of Test Substances

[Note: Preparation under red or yellow light is recommended to preserve substances that degrade upon exposure to light.]

Test substance solubility should be determined by following the procedures outlined in **ANNEX II** of this protocol.

1. Test Substance in Solution

- Allow test substances to equilibrate to room temperature before dissolving and diluting.
- Prepare test substance immediately prior to use and not in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The Study Director may store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility substances) in a freezer (e.g., -70°C) for use in future substance analyses.

- For substances dissolved in DMSO or ETOH, the final DMSO or ETOH concentration for application to the cells must be 0.5 % (v/v) in the vehicle controls and in all of the eight test concentrations.

- The stock solution for each test substance should be prepared at the highest concentration found to be soluble in the solubility test (**ANNEX II**: *Test Method Procedure - Solubility Determination of Test Substances*). Thus, the highest test concentration applied to the cells in each range finding experiment is:
  - 0.5 times the highest concentration found to be soluble in the solubility test, if the substance was soluble in medium, or
  - 1/200 the highest concentration found to be soluble in the solubility test if the substance was soluble in ETOH or DMSO.
- The seven lower concentrations in the range finding experiment are prepared by successive dilutions that decrease by one log unit each. The following example illustrates the preparation of test substance in solvent and the dilution of dissolved test substance in medium before application to NHK cells.

Example: Preparation of Test Substance in Solvent Using a Log Dilution Scheme
If DMSO is determined to be the preferred solvent at Tier 3 of the solubility test (i.e., 200,000 μg/mL), dissolve the substance in DMSO at 200,000 μg/mL for the substance stock solution.

8) Label eight tubes 1 – 8. Add 0.9 mL solvent (e.g., DMSO) to tubes 2 -- 8.
9) Prepare stock solution of 200,000 μg test substance/mL solvent in tube # 1.
10) Add 0.1 mL of 200,000 μg/mL dilution from tube #1 to tube #2 to make a 1:10 dilution in solvent (i.e., 20,000 μg/mL).
11) Add 0.1 mL of 20,000 μg/mL dilution from tube #2 to tube #3 to make another 1:10 dilution (i.e., 1:100 dilution from stock solution) in solvent (i.e., 2,000 μg/mL)
12) Continuing making serial 1:10 dilutions in the prepared solvent tubes.
13) Since each concentration is 200 fold greater than the concentration to be tested, make a 1:100 dilution by diluting 1 part dissolved substance in each tube with 99 parts of culture medium (e.g., 0.1 mL of test substance in DMSO + 9.9 mL culture medium) to derive the eight 2X concentrations for application to NHK cells. Each 2X test substance concentration will then contain 1 % v/v solvent. The NHK cells will have 0.125 mL of culture medium in the wells prior to application of the test substance. By adding 0.125 mL of the appropriate 2X test substance concentration to the appropriate wells, the test substance will be diluted appropriately (e.g., highest concentration in well will be 1,000 μg/mL) in a total of 0.250 mL and the solvent concentration in the wells will be 0.5% v/v.
14) A test substance prepared in DMSO or ETOH may precipitate upon transfer into the Routine Culture Medium. The 2X dosing solutions should be evaluated for precipitates and the results recorded in the Study Workbook. It

will be permissible to test all of the dosing solutions in the dose range finding assay and main experiments. However, doses containing test substance precipitates should be avoided because it creates doubt about the concentration of test substance exposed to the cells.

Document all test substance preparations in the Study Workbook.

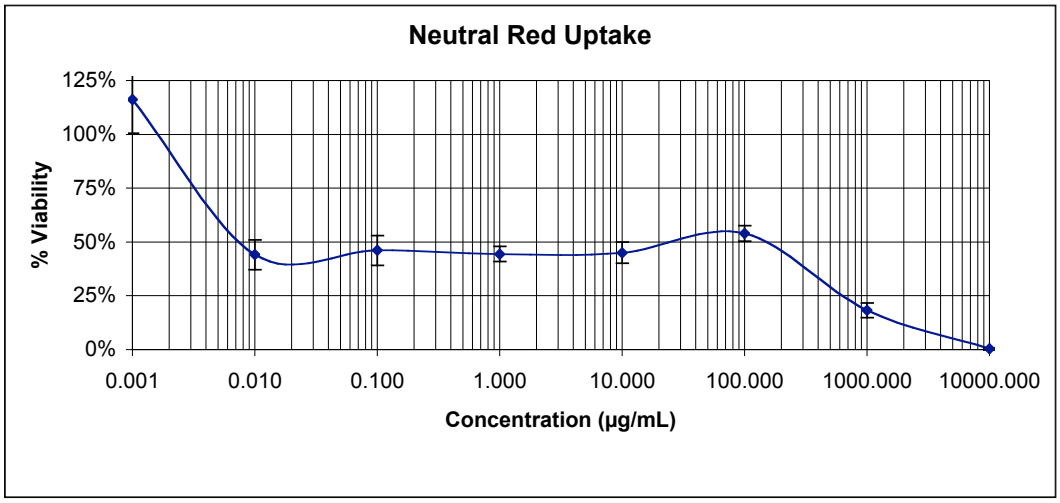
2. pH of Test Substance Solutions

Prior to or immediately after application of the test substance to the 96-well plate, measure the pH of the highest 2X dosing concentration of the test substance (i.e., C1 in the test plate, see Figure 1) in culture medium. Use pH paper (e.g., pH 0 – 14 to estimate and pH 5 – 10 to determine more precise value; or Study Director's discretion). The pH paper should be in contact with the solution for approximately one minute. Document the pH and note the color of the 2X concentration medium (i.e., in the Microsoft Excel® template; see **ANNEX 1** for an example template). Medium color for all dosing dilutions should be noted in the Study Workbook. Do not adjust the pH.

3. Concentrations of Test Substance

a) Range Finder Experiment
- Test eight concentrations of the test substance by diluting the stock solution using log dilutions (e.g., 1:10, 1:100, 1:1000, etc.).
- If a range finder experiment does not generate enough cytotoxicity, then higher doses should be attempted. If cytotoxicity is limited by solubility, then more stringent solubility procedures to increase the stock concentration (to the maximum concentration specified in **Section VI.D.3.b**.) should be employed.
- Place the highest test substance concentration into an incubator (37ºC ± 1ºC, 90 % ± 10 % humidity, 5.0 % ± 1 % CO_2/air) and stir or rock for up to 3 hours, if necessary, to facilitate dissolution. For stocks prepared in medium, vessel caps should be loose to allow for CO_2 exchange. Proceed with dosing solution preparation and dosing.
- If a range finding test produces a biphasic curve, then the doses selected for the subsequent main experiments should cover the most toxic dose-response range (see Example 1 – the most toxic range is 0.001 – 0.1 μg/mL) that reduces viability to 50%.

Example 1 – Biphasic Curve



b) Main Experiment (Definitive Assay)

- Depending on the slope of the concentration-response curve estimated from the range finder, the dilution/progression factor in the concentration series of the main experiment should be smaller (e.g., dilution factor of $\sqrt[6]{10} = 1.47$). Cover the relevant concentration range around the IC_{50} (> 0 % and < 100 % effect) preferably with several points of a graded effect, but with a minimum of two points, one on each side of the estimated IC_{50} value, avoiding too many non-cytotoxic and/or 100 %-cytotoxic concentrations.

- Determine which test substance concentration is closest to the IC_{50} value. Use that value as a central concentration and adjust dilutions higher and lower in equal steps for the definitive assay.

Maximum Doses to be Tested in the Main Experiments

If minimal or no cytotoxicity was measured in the dose range finding assay, a maximum dose for the main experiments will be established as follows:

For test substances prepared in Routine Culture Medium

- The highest test substance concentration that may be applied to the cells in the main experiments will be either 100 mg/mL, or the maximum soluble dose.
- Test substance will be weighed into a glass tube and the weight will be documented. A volume of Routine Culture Medium will be added to the vessel so that the concentration is 200,000 µg/mL (200 mg/mL).
- The solution is mixed using the mechanical procedures specified in **ANNEX II** of this protocol. If complete solubility is achieved in medium, then seven additional serial stock dosing solutions may be prepared from the 200 mg/mL 2X stock. If the test substance is insoluble in medium at 200 mg/ml, proceed by adding medium, in small incremental amounts, to

attempt to dissolve the substance by using the sequence of mixing procedures specified in **ANNEX II**.

- More stringent solubility procedures may be employed if needed based on results from the range finder experiment (**Section VI.D.3.a.**). The highest soluble stock solution will be used to prepare the seven additional serial stock dosing solutions.

For test substances prepared in either DMSO or ETOH

11.0 The highest test substance concentration that may be applied to the cells in the main experiments will be ≤ 2.5 mg/mL, depending upon the maximum solubility in solvent.

12.0 Test substance will be weighed into a glass tube and the weight will be documented. A volume of the appropriate solvent (determined from the original solubility test) will be added to the vessel so that the concentration is 500,000 μg/mL (500 mg/mL).

13.0 The solution is mixed as specified **ANNEX II**. If complete solubility is achieved in the solvent, then seven additional serial stock dosing solutions may be prepared from the 500 mg/mL 200X stock. If the test substance is insoluble in solvent at 500 mg/ml, proceed by adding solvent, in small incremental amounts, to attempt to dissolve the substance by using the sequence of mixing procedures. The highest soluble stock solution will be used to prepare the seven additional serial stock dosing solutions.

If precipitates are observed in the 2X dilutions, continue with the experiment, make the appropriate observations and documentation, and report data to the SMT.

c) Test Substance Dilutions

The dosing factor of 3.16 ($= \sqrt[2]{10}$) divides a log into two equidistant steps, 2.15 ($= \sqrt[3]{10}$) into three steps, 1.47 ($= \sqrt[6]{10}$) into six steps, 1.78 ($\sqrt[4]{10}$) into four steps, and 1.21 ($= \sqrt[12]{10}$) into 12 steps.

EXAMPLE:

10						31.6						100
10				21.5				46.4				100
10		14.7		21.5		31.6		46.4		68.1		100
10	12.1	14.7	17.8	21.5	26.1	31.6	38.3	46.4	56.2	68.1	82.5	100

An example of decimal geometric concentration series for factor 1.47: *Dilute 1 volume of the highest concentration by adding 0.47 volumes of diluent. After equilibration, dilute 1 volume of this solution by adding 0.47 volumes of diluent...(etc.).*

E. Test Procedure

1. 96-Well Plate Configuration

The NHK NRU assay for test substances will use the 96-well plate configuration shown in **Figure 1**.

Figure 1. 96-Well Plate Configuration for Positive Control (PC) and Test Substance Assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb
B	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
C	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
D	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
E	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
F	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
G	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
H	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb

VC1 and VC2 = VEHICLE CONTROL
$C_1 – C_8$ = Test Substances or PC (SLS) at eight concentrations (C1 = highest, C8 = lowest)
b = BLANKS (Test substance or PC, but contain **no** cells)
VCb = VEHICLE CONTROL BLANK (contain **no** cells)

2. Application of Test Substance

1. Two optional methods for rapidly applying the 2X dosing solutions onto the 96-well plates may be utilized.

    1) Add each of the 2X dosing solutions into labeled, sterile reservoirs (e.g., Corning/Costar model 4870 sterile polystyrene 50 mL reagent reservoirs or Corning/Transtar model 4878 disposable reservoir liners, 8-channel; or other multichannel reservoirs).
    2) Use a "dummy" plate (i.e., an empty sterile 96-well plate) prepared to hold the dosing solutions immediately prior to treatment of the test plate (with cells). The test substance and control dosing solutions should be dispensed into the dummy plate in the same pattern/order as will be applied to the plate containing cells. More volume than needed for the test

plate (i.e., greater than 125 µl/well) should be in the wells of the dummy plate.

At the time of treatment initiation, a multi-channel micropipettor is used to transfer the 2X dosing solutions, from the reservoirs or dummy plate, to the appropriate wells on the treatment plate (as described in step c. below). These methods will ensure that the dosing solutions can be transferred rapidly to the appropriate wells of the test plate to initiate treatment times and to minimize the range of treatment initiation times across a large number of treatment plates, and to prevent "out of order" dosing. Do not use a multichannel repeater pipette for dispensing test substance to the plates.

2. After 48 - 72 hours (i.e., after cells attain 20+ % confluency [see **Section VI.C.4(h)**]) incubation of the cells, add 125 µl of the appropriate concentration of test substance, the PC, or the VC (see **Figure 1** for the plate configuration) directly to the test wells. Do not remove Routine Culture Medium for re-feeding the cells. The dosing solutions will be rapidly transferred from the 8-channel reservoir (or dummy plate) to the test plate using a single delivery multi-channel pipettor. For example, the VC may be transferred first (into columns 1, 2, 11, and 12), followed by the test substance dosing solutions from lowest to highest dose, so that the same pipette tips on the multi-channel pipettor can be used for the whole plate. [The Vehicle Control blank (VCb) wells (column 1, column 12, wells A2, A11, H2, H11) will receive the Vehicle Control dosing solutions (which should include any solvents used)]. Blanks for wells A3 – A10 and H3 – H10 shall receive the appropriate test substance solution for each concentration (e.g., wells A3 and H3 receive C_1 solution).] Incubate cells for 48 hours ± 0.5 hours (37ºC ± 1ºC, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO_2/air).
3. **Positive Control**: For each set of test substance plates used in an assay, prepare a separate plate of positive control concentrations. If multiple sets of test substance plates are set up, then clearly designate the positive control plates for each set; each set will be an individual entity. The Study Director will decide how many test substance plates will be run with a positive control plate. This plate will follow the same schedule and procedures as used for the test substance plates (including appropriate substance concentrations in the appropriate wells and meeting test acceptance criteria see **Sections VI.E.1, E.2, and E.5**).

3. Microscopic Evaluation

After at least 46 hours treatment, examine each plate under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record any changes in morphology of the cells due to the cytotoxic effects of the test substance, but do not use these records for any quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells may indicate experimental error and may be cause for rejection of

the assay. Use the following Visual Observations Codes in the description of cell culture conditions. Numerical scoring of the cells (see **Section VI.E.3**) should be determined and documented in the Study Workbook and in the appropriate section of the Microsoft Excel® template.

Visual Observations Codes

Note Code	Note Text
1	Normal Cell Morphology
2	Low Level of Cell Toxicity
3	Moderate Level of Cell Toxicity
4	High level of Cell Toxicity
1P	Normal Cell Morphology with Precipitate
2P	Low Level of Cell Toxicity with Precipitate
3P	Moderate Level of Cell Toxicity with Precipitate
4P	High level of Cell Toxicity with Precipitate
5P	Unable to View Cells Due to Precipitate

4. Measurement of NRU

a) Carefully remove (i.e., "dump") the Routine Culture Medium (with test substance) and rinse the cells very carefully with 250 µL pre-warmed D-PBS. Remove the rinsing solution by dumping and remove excess by gently blotting on paper towels. Add 250 µL NR medium (to all wells including the blanks) and incubate (37ºC ± 1ºC, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO_2/air) for 3 hours ± 0.1 hour. Observe the cells briefly during the NR incubation (e.g., between 2 and 3 hours – Study Director's discretion) for NR crystal formation. Record observations in the Study Workbook. Study Director can decide to reject the experiment if excessive NR crystallization has occurred.

b) After incubation, remove the NR medium, and carefully rinse cells with 250 µL pre-warmed D-PBS.

c) Decant and blot D-PBS from the plate. (Optionally: centrifuge the reversed plate.)

d) Add exactly 100 µL NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.

e) Shake microtiter plate rapidly on a microtiter plate shaker for 20 – 45 minutes to extract NR from the cells and form a homogeneous solution. Plates should be protected from light by using a cover during shaking.

f) Plates should be still for at least five minutes after removal from the plate shaker (or orbital mixer). If any bubbles are observed, assure that they have been ruptured prior to reading the plate. Measure the absorption (within 60 minutes of adding NR Desorb solution) of the resulting colored solution at 540 nm ± 10 nm in a microtiter plate reader (spectrophotometer), using the blanks as a reference.

[Note: A mean $OD_{540 \pm 10nm}$ of 0.043 - 0.059 for the VC blanks is a target range of ODs but not a test acceptance criterion (range = mean OD ± 2.5 standard deviations; mean = 0.054; SD = 0.003; N = 114)]. Save raw data in the Microsoft Excel® template.

5. Quality Check of Assay

a) Test Acceptance Criteria
All acceptance criteria (i.e., criteria 1, 2, and 3) must be met for a test to be acceptable.

1) The PC (SLS) IC_{50} must be within two and a half (2.5) standard deviations (SD) of the historical mean established by the Test Facility and must meet criteria 2 and 3, and must have an r^2 (coefficient of determination) value calculated for the Hill model fit (i.e., from PRISM® software) ≥ 0.85. NICEATM/ECVAM study generated the following PC data:
   - IC_{50} mean = 3.11 µg/mL; SD = 0.72 (n = 114)
   - range for IC_{50} mean ± 2.5 SD = 1.31 µg/mL – 4.91 µg/mL
2) The left and the right mean of the VCs do not differ by more than 15 % from the mean of all VCs.
3) At least one calculated cytotoxicity value > 0 % and ≤ 50 % viability and at least one calculated cytotoxicity value > 50 % and < 100 % viability must be present.

Exception: If a test has only one point between 0 and 100 % **and** the smallest dilution factor (i.e., 1.21) was used **and** all other test acceptance criteria were met, then the test will be considered acceptable.

Stopping Rule for Insoluble Substances: If the most rigorous solubility procedures have been performed and the assay cannot achieve adequate toxicity to meet the test acceptance criteria after three definitive trials, then the Study Director may end all testing for that particular substance.

[Note: A corrected mean $OD_{540 \pm 10nm}$ of 0.205 - 1.645 for the VCs is a target range of ODs but will not be a test acceptance criterion (range = mean OD ± 2.5 standard deviations; mean = 0.685; SD = 0.175; N = 114).]

b) Checks for Systematic Cell Seeding Errors
To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row 2) and the right side (row 11 for the test plates) of the 96-well plate. Aberrations in the cell monolayer for the VCs may reflect a volatile and toxic test substance present in the assay. If volatility is suspected, then proceed to **Section VI.E.6**. Checks for cell seeding errors may also be performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent.

7. Testing Volatile Substances

Although this test method is not suitable for highly volatile substances, mildly volatile substances may be tested with some success. Volatile test substances may generate vapors from the treatment media during the test substance treatment incubation period. These vapors may become resorbed into the treatment medium in adjacent wells, such that culture wells nearest the highest doses may become contaminated by exposure. If the test substance is particularly toxic at the doses tested, the cross contamination may be evident as a significant reduction in viability in the VC cultures (i.e., VC1) adjacent to the highest test substance doses.

If potential test substance volatility is suspected (e.g., for low density liquids) or if the initial range finder test (non-sealed plate) results show evidence of toxic effects in the control cultures (i.e., > 15 % difference in viability between VC1 [column 2] and VC2 [column 11]), then seal the subsequent test plates by the following procedure.

a) Plate Sealer Method

d) Plates and substances will be prepared as usual according to **Sections VI.D and VI.E**.

e) Immediately after the 96-well culture plate has been treated with the suspected volatile substance (**Section VI.E.2.b**), apply the adhesive plate sealer (e.g., using a hand, microplate roller, etc.) directly over the culture wells. Assure that the sealer adheres to each culture well (well tops should be dry). Place the 96-well plate cover over the sealed plate and incubate the plate under specified conditions (**Section VI.E.2.b**). [Note: Do not jam the plate lid over the film to avoid deforming the sealer and causing the sealer to detach from culture wells. Loose fit of the plate lid is acceptable.]

f) At the end of the treatment period, the plate sealer should be carefully removed to avoid spillage. Continue with the NRU assay as per **Section VI.E.4**.

F. Data Analysis

- The Study Director will use good biological/scientific judgment for determining "unusable" wells that will be excluded from the data analysis and provide explanations for the removal of any data from the analysis.
- A calculation of cell viability expressed as NRU is made for each concentration of the test substance by using the mean NRU of the six replicate values (minimum of four acceptable replicates wells) per test concentration. This value is compared with the mean NRU of all VC values. Relative cell viability is then expressed as percent of untreated VC. If achievable, the eight concentrations of each substance tested will span the range of no effect up to total inhibition of cell viability.
- Data from the microtiter plate reader should be transferred to a spreadsheet template (e.g., Microsoft Excel®) that will automatically determine cell viability, calculate IC_{50} values by linear interpolation, and perform statistical analyses

(including statistical identification of outliers). The template should also calculate the concentrations associated with 20 %, 50 %, and 80 % viability using the Hill slope and EC_{50} (i.e., IC_{50}) from the Hill function analysis (see **ANNEX 1** for an example spreadsheet template).

- A Hill function analysis should be performed using statistical software (e.g., GraphPad PRISM® 3.0) and a template to calculate IC_{20}, IC_{50}, and IC_{80} values (and the associated confidence limits) for each test substance.
- Dose-responses for which the toxicity plateaus as concentration increases do not fit the Hill function well when Bottom =0. To obtain a better model fit, unconstrain the Bottom parameter so that the model calculates the Bottom value. However, when Bottom ≠ 0, the EC_{50} reported by the Hill function ≠ 50% viability since the Hill function defines EC_{50} as the point midway between Top and Bottom. To obtain the appropriate IC_{50} when Bottom≠ 0, use the following rearranged Hill function:

$$X = \log EC_{50} - \frac{\log\left(\frac{Top - Bottom}{Y - Bottom} - 1\right)}{HillSlope}$$

- X is the logarithm of concentration at 50% response, $logEC_{50}$ is logarithm of concentration at the response midway between Top and Bottom, Top is the maximum response, Bottom is the minimum response, Y = 50 (i.e., 50% response), and HillSlope describes the steepness of the curve.

[Note: IC_{50} values are used in a regression formula to predict the LD_{50} value of a test substance as an estimate of the starting dose for an acute oral toxicity test.]

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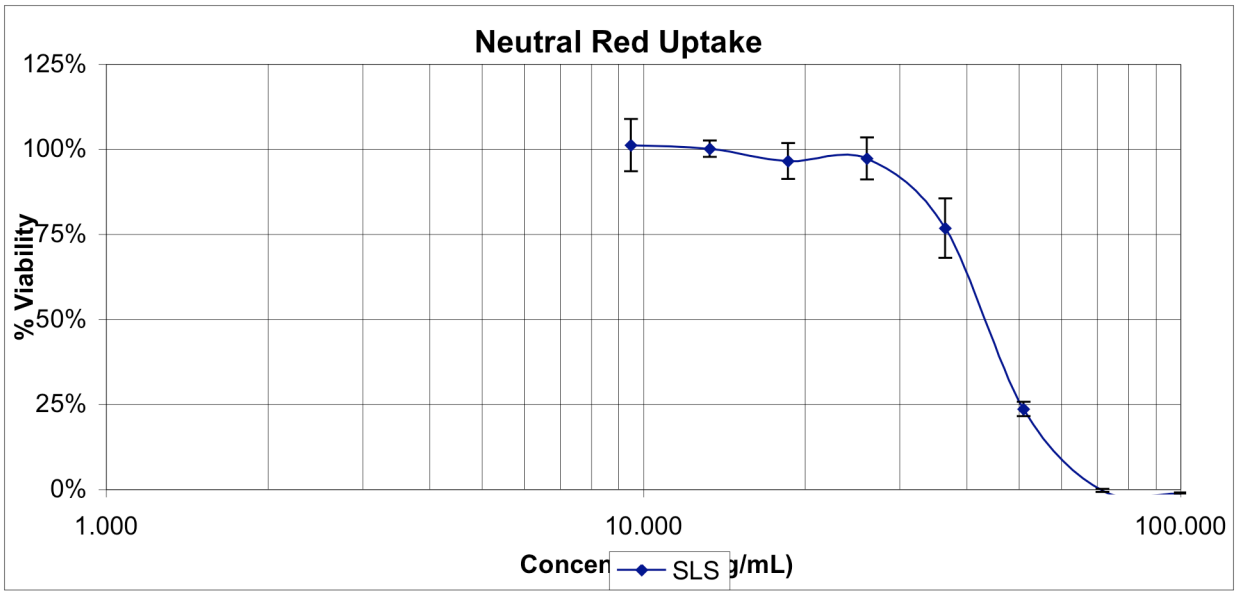
Personal communication, Technical Services, Cambrex Bio Science, Walkersville, MD

ANNEX 1
Microsoft EXCEL® Example Spreadsheet Template

Test Facility :	A					Study Number.:	A1					
Chemical Code :	SLS					96-Well Plate ID :	A11					
2nd Chem. Code*:	11					Experiment ID :	XX					
					96-WELL PLATE MAP							
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
C	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
D	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
E	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
F	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
G	Blank	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2	Blank
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
					RAW ABSORBANCE DATA (OD_{550})							
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.044	0.044	0.045	0.045	0.045	0.046	0.051	0.057	0.057	0.043	0.041	0.044
B	0.042	0.456	0.043	0.043	0.130	0.300	0.395	0.414	0.418	0.402	0.401	0.042
C	0.043	0.407	0.042	0.041	0.130	0.294	0.383	0.382	0.413	0.375	0.385	0.044
D	0.043	0.438	0.042	0.043	0.147	0.337	0.409	0.404	0.438	0.436	0.391	0.047
E	0.044	0.448	0.041	0.045	0.132	0.321	0.429	0.414	0.416	0.420	0.441	0.042
F	0.045	0.411	0.040	0.042	0.127	0.375	0.397	0.402	0.422	0.447	0.403	0.043
G	0.041	0.405	0.043	0.040	0.124	0.361	0.444	0.442	0.425	0.448	0.405	0.044
H	0.041	0.041	0.048	0.042	0.042	0.044	0.042	0.042	0.040	0.044	0.041	0.041
Max	0.045	0.456	0.043	0.045	0.147	0.375	0.444	0.442	0.438	0.448	0.441	0.047
Min	0.041	0.405	0.040	0.040	0.124	0.294	0.383	0.382	0.413	0.375	0.385	0.041
Next Max	0.044	0.448	0.042	0.043	0.132	0.361	0.429	0.414	0.425	0.447	0.405	0.044
Next Min	0.042	0.407	0.041	0.041	0.127	0.300	0.395	0.402	0.416	0.402	0.391	0.042
Rmax	-0.250	-0.157	-0.333	-0.400	-0.652	-0.173	-0.246	-0.467	-0.520	-0.014	-0.643	-0.500
Rmin	0.250	0.039	0.333	0.200	0.130	0.074	0.197	0.333	0.120	0.370	0.107	0.167
					CORRECTED ABSORBANCE (Sample OD_{550} - Mean Blank OD_{550})							
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.001	-0.002	0.002	0.002	0.001	0.005	0.008	0.009	-0.001	-0.002	0.001
B	-0.001	0.413	-0.004	-0.001	0.087	0.255	0.349	0.365	0.370	0.359	0.358	-0.001
C	0.000	0.364	-0.005	-0.003	0.087	0.249	0.337	0.333	0.365	0.332	0.342	0.001
D	0.000	0.395	-0.005	-0.001	0.104	0.292	0.363	0.355	0.390	0.393	0.348	0.004
E	0.001	0.405	-0.006	0.002	0.089	0.276	0.383	0.365	0.368	0.377	0.398	-0.001
F	0.002	0.368	-0.007	-0.001	0.084	0.330	0.351	0.353	0.374	0.404	0.360	0.000
G	-0.002	0.362	-0.004	-0.004	0.081	0.316	0.398	0.393	0.377	0.405	0.362	0.001
H	-0.002	-0.002	0.002	-0.001	-0.001	-0.001	-0.005	-0.008	-0.009	0.001	-0.002	-0.002
Mean Blank =	0.043		0.047	0.044	0.044	0.045	0.047	0.050	0.049	0.044		
					RELATIVE VIABILITY (% OF VEHICLE CONTROL)							
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		110.7%	-0.9%	-0.1%	23.2%	68.4%	93.4%	97.7%	99.0%	96.1%	96.0%	
C		97.6%	-1.2%	-0.7%	23.2%	66.7%	90.2%	89.1%	97.7%	88.9%	91.7%	
D		105.9%	-1.2%	-0.1%	27.7%	78.3%	97.2%	95.0%	104.4%	105.2%	93.3%	
E		108.6%	-1.5%	0.4%	23.7%	74.0%	102.5%	97.7%	98.5%	100.9%	106.7%	
F		98.7%	-1.7%	-0.4%	22.4%	88.5%	94.0%	94.5%	100.1%	108.2%	96.5%	
G		97.1%	-0.9%	-0.9%	21.6%	84.7%	106.5%	105.2%	100.9%	108.4%	97.1%	
H												

Test Facility :	A				Study Number.:	A1				
Chemical Code :	SLS				96-Well Plate ID :	A11				
2nd Chem. Code*:	11				Experiment ID :	XX				
	VC1	C1	C2	C3	C4	C5	C6	C7	C8	VC2
Conc. (µg/mL) :	0.0	100	71.4	51.0	36.4	26.0	18.6	13.3	9.49	0.0
Mean Corr. OD :	0.385	-0.005	-0.001	0.088	0.286	0.363	0.360	0.374	0.378	0.361
SD :	0.023	0.001	0.002	0.008	0.033	0.023	0.020	0.009	0.029	0.020
Mean Vehicle Control :	0.373									
Mean Blank :	0.043									
% of Vehicle Control :	103.1%	-1.3%	-0.3%	23.6%	76.8%	97.3%	96.5%	100.1%	101.3%	96.9%
SD :	6.0%	0.3%	0.5%	2.1%	8.7%	6.2%	5.3%	2.4%	7.7%	5.2%
% CV :	5.86%	-25.1%	-150%	9.09%	11.4%	6.33%	5.47%	2.39%	7.59%	5.41%
Mean VC - VC1 (%) :	-3.1%									
Mean VC - VC2 (%) :	3.1%									
Mean Absolute OD :	0.416									
					Visual Observations					
	VC	C1	C2	C3	C4	C5	C6	C7	C8	
ENTER CODES:	1	4	4	3	2	1	1	1	1	
				Interpolated IC_{50} :		4.32E+01	µg/mL			



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TEST CHEMICAL

Test Facility :	A	Study Number.:	A1
Chemical Code :	SLS	96-Well Plate ID :	A11
2nd Chem. Code*:	11	Experiment ID :	XX

* Testing Facility Accession Code, if applicable

PREPARATION OF TEST CHEMICAL

Solvent:	Medium	Dilution factor:	1.4	
Solvent Conc. (%, v/v) in dosing solutions :	N/A	Highest Stock Conc.:	20,000	µg/mL

Aids used to dissolve : ☐ Vortexing ☐ sonication ☐ heating to 37C

pH (highest medium stock or 2X dosing solution) : 8.0

Medium Clarity/Color (highest 2X dosing solution): clear red If ppt, note lowest conc.:

Concentration Series (µg/mL)

C1	C2	C3	C4	C5	C6	C7	C8
100	71.4	51.0	36.4	26.0	18.6	13.3	9.49

Positive Control (SLS) 100 - 9.49 µg/mL

CELL LINE/TYPE

Name:	BALB/c 3T3	Supplier:	ATCC	Lot No.	not provided
Passage No.:	69	Passage No. in Assay:	75	Proliferating/frozen	24-May-02

CELL CULTURE CONDITIONS

Medium:	DMEM	Supplier:		Lot No.:	
Serum:	NCS	Supplier:		Lot No.:	
Serum Conc.:		Growth Medium:	10%	Treatment Medium:	0%

TEST ACCEPTANCE CRITERIA

No. of values >50% and <100%:	3	No. of values >0% and ≤50%:	1	Accept?	YES
VC: % Difference between Col 2 and mean VC.:			-3%	Accept?	YES
PC: Hill Function R^2 Value of SLS:		0.99		Accept?	YES
PC: IC_{50} of SLS:		43.2	µg/mL	Accept?	YES

TIMELINE

Cell Seeding Date	Dose Application Date	OD_{550} Determination Date

TEST RESULTS

VC: Mean Corrected OD_{550}:	0.373			Hill Function R^2 Value:	0.9869
log IC20 :	1.551E+00 µg/mL	log IC50 :	1.635E+00 µg/mL	log IC80 :	1.718E+00 µg/mL
IC20 :	3.56E+01 µg/mL	IC50 :	4.32E+01 µg/mL	IC80 :	5.22E+01 µg/mL

Test Chemical F.W. : 288.4

IC_{20} :	0.12331183 mM	IC_{50} :	0.1496252 mM	IC_{80} :	0.18113599 mM

ANNEX II

TEST METHOD PROCEDURE
Solubility Determination of Test Substances

PROPOSAL

This procedure was designed to identify the solvent that would provide the highest soluble concentration of a test substance so there would be uniform availability of the substance to cells used for *in vitro* basal cytotoxicity testing. The solubility exercises can be performed in a routine and repeatable manner and provide guidelines to effectively prepare test substances for toxicity testing in the NRU test methods.

TEST SYSTEM

The solubility test procedure is based on attempting to dissolve substances in various solvents with increasingly rigorous mechanical techniques. The solvents to be used, in the order of preference, are cell culture medium, DMSO, and ETOH. Determination of whether a substance has dissolved is based entirely on visual observation for the purposes of this protocol. A substance has dissolved if the solution is clear and shows no signs of cloudiness or precipitation.

PROCEDURES

Preparation of the keratinocyte medium and supplements will follow all procedures in the NHK NRU protocol.

Materials – see Section VI.A

Preparations of Media and Solutions – see Section VI.B

All solutions glassware, pipettes, etc., should be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures should be adequately documented.

Determination of Solubility

- Solubility should be determined in a step-wise procedure that involves attempting to dissolve a test substance at a relatively high concentration with the sequence of mechanical procedures specified in **Mechanical Procedures**. **Table 1** and **Figures 1** and **2** illustrate the step-wise procedures. The hierarchy of preference of solvent for dissolving test substances is medium, DMSO, and then ETOH. If the substance does not dissolve in the solvent, the volume of solvent is increased so as to decrease the test substance concentration by a factor of 10, and then the sequence of mechanical procedures are repeated in an attempt to solubilize the substance at the lower concentrations.
- For testing solubility in medium, the starting concentration is 200,000 μg/ml (i.e., 200 mg/mL) in Tier 1, but for DMSO and ETOH the starting concentration is 200,000 μg/ml (i.e., 200 mg/mL) in Tier 3.

Methods

A. Tier 1 begins with testing 200 mg/mL in Routine Culture Medium (see **Table 1**).
   1. Weigh approximately 100 mg (100,000 μg) of the test substance into a glass tube. Document the substance weight.
   2. Add approximately 0.5 mL of medium into the tube so that the concentration is 200,000 μg/ml (200 mg/mL).
   3. Mix the solution as specified in **Mechanical Procedures**. If complete solubility is achieved, then additional solubility procedures are not needed.

B. If the test substance is insoluble in Tier 1 at 200 mg/mL, then proceed to Tier 2.
   1. Weigh approximately 10 mg (10,000 μg) of the test substance into a glass tube. Document the substance weight.
   2. Add approximately 0.5 mL of medium into the tube so that the concentration is 20,000 μg/ml (20 mg/mL).
   3. Mix the solution as specified in **Mechanical Procedures**. If complete solubility is achieved, then additional solubility procedures are not needed.

C. If the test substance is insoluble in Routine Culture Medium, proceed to Tier 3.

   1. Add enough medium, approximately 4.5 mL, to attempt to dissolve the substance at 2 mg/mL by using the sequence of mixing procedures. If the test substance dissolves in medium at 2 mg/mL, no further procedures are necessary.
   2. If the test substance does NOT dissolve in medium, weigh out approximately 100 mg test substance in a second glass tube and add enough DMSO to make the total volume approximately 0.5 mL (for 200 mg/mL) and mix the solution as specified in **Mechanical Procedures**.
   3. If the test substance does not dissolve in DMSO, weigh out approximately 100 mg test substance in another glass tube and add enough ETOH to make the total volume approximately 0.5 mL (for 200 mg/mL) and mix the solution as specified in **Mechanical Procedures**.
   4. If the substance is soluble in either solvent, no additional solubility procedures are needed.

D. If the substance is NOT soluble in Routine Culture Medium, DMSO, or ETOH at Tier 3, then continue to Tier 4 in **Table 1**.
   1. Add enough solvent to increase the volume of the three (or four) Tier 2 solutions by 10 and attempt to solubilize again using the sequence of mixing procedures. If the test substance dissolves, no additional solubility procedures are necessary.
   2. If the test substance does NOT dissolve, continue with Tier 5 and, if necessary, Tier 6 using DMSO and ETOH.
   3. Tier 5 begins by diluting the Tier 4 samples with DMSO or ETOH to bring the total volume to 50 mL. The mixing procedures are again followed to attempt to solubilize the substance.

4. Tier 6 is performed, if necessary, by weighing out another two samples of test substance at ~10 mg each and adding ~50 mL DMSO or ETOH for a 200 μg/mL solution, and following the mixing procedures.

Example

- If complete solubility is not achieved at 20,000 μg/mL in Routine Culture Medium at Tier 2 using the mixing procedures, then the procedure continues to Tier 3 by diluting the solution to 5 mL with medium and mixing again.
- If the substance is not soluble in Routine Culture Medium, two samples of ~ 100 mg test substance are weighed to attempt to solubilize in DMSO and ETOH at 200,000 μg/mL (i.e., 200 mg/mL). Solutions are mixed following the sequence of procedures prescribed in **Mechanical Procedures** in an attempt to dissolve.
- If solubility is not achieved at Tier 3, then the solutions prepared in Tier 3 are diluted by 10 so as to test 200 μg/mL in media, and 20,000 μg/mL in DMSO and ETOH. This advances the procedure to Tier 4. Solutions are again mixed in an attempt to dissolve.
- If solubility is not achieved in Tier 4, the procedure continues to Tier 5, and to Tier 6 if necessary (see **Figures 1** and **2** and **Table 1**).

MECHANICAL PROCEDURES

A. The following hierarchy of mixing procedures will be followed to dissolve the test substance:
   1. Add test substance to solvent as in Tier 1 of **Table 1**. (Test substance and solvent should be at room temperature.)
   2. Gently mix at room temperature. Vortex the tube (1 –2 minutes).
   3. If test substance has not dissolved, use waterbath sonication for up to 5 minutes.
   4. If test substance is not dissolved after sonication, then warm solution to 37°C for 5 - 60 minutes. This can be performed by warming tubes in a 37°C waterbath or in a CO_2 incubator at 37°C. The solution may be stirred during warming (stirring in a CO_2 incubator will help maintain proper pH).
   5. Proceed to Tier 2 (and Tiers 3-6, if necessary of **Table 1** and repeat procedures 2-4).

B. The preference of solvent for dissolving test substances is Routine Culture Medium, DMSO, and then ETOH. Thus, if all solvents for a particular tier are tested simultaneously and a test substance dissolves in more than one solvent, then the choice of solvent follows this hierarchy. For example, if, at any tier, a substance were soluble in Routine Culture Medium and DMSO, the choice of solvent would be medium. If the substance were insoluble in medium, but soluble in DMSO and ETOH, the choice of solvent would be DMSO for both assays.

Table 1 Determination of Solubility in Routine Culture Medium, DMSO, or ETOH

Tier	1	2	3	4	5	6
Total Volume Routine Culture Medium	0.5 mL	0.5 mL	5 mL	50 mL		
Concentration of Test Substance Tier 1: Add ~ 100 mg to a tube. Add enough medium to equal Tier 1 volume. If insoluble, go to Tier 2. Tier 2: Add ~10 mg to another tube. Add enough medium to equal the first volume. Dilute to subsequent volumes if necessary.	200,000 µg/mL (200 mg/mL)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	200 µg/mL (0.20 mg/mL)		
Total Volume DMSO/			0.5 mL	5 mL	50 mL	
Concentration of Test Substance (Add ~100 mg to a large tube. Add enough DMSO or ETOH to equal the first volume. Dilute with subsequent volumes if necessary.)			200,000 µg/mL (200 mg/mL)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	
Total Volume DMSO/ETOH						50 mL
Concentration of Test Substance (Add ~10 mg to a large tube. Add enough DMSO or ETOH to equal 50 mL.)						200 µg/mL (0.2 mg/mL)
EQUIVALENT CONCENTRATION ON CELLS	100,000 µg/mL (100 mg/mL)	10,000 µg/mL (10 mg/mL)	1000 µg/mL (1 mg/mL)	100 µg/mL (0.1 mg/mL)	10 µg/mL (0.01 mg/mL)	1 µg/mL (0.001 mg/mL)

[NOTE: The amounts of test substance weighed and Routine Culture Medium added may be modified from the amounts given above, provided that the targeted concentrations specified for each tier are tested.]

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TIER 1

STEP 1:	200 mg/mL test substance (TS) in 0.5 mL Routine Culture Medium • if TS soluble in medium, then **STOP**. • if TS insoluble in medium, then go to STEP 2.

TIER 2

STEP 2:	20 mg/mL TS in 0.5 mL Routine Culture Medium • if TS soluble, then **STOP**. • if TS insoluble, then go to STEP 3.

TIER 3

STEP 3:	200 mg/mL TS in DMSO
	• if TS soluble, then **STOP**.
	• if TS insoluble, test at 200 mg/mL in ETOH.
	  • if TS soluble, then **STOP.**
	  • If TS insoluble, go to STEP 4.

TIER 4

STEP 4:	0.2 mg/mL TS in medium (one or both) – increase volume from STEP 2 by 10 (i.e., to 50 mL)
	• if TS soluble in both media, then **STOP**.
	• if TS insoluble in one medium, test at 20 mg/mL in DMSO – increase volume from STEP 3 by 10 (i.e., to 5 mL).
	  • if TS soluble, then **STOP.**
	  • if TS insoluble, test at 20 mg/mL in ETOH – increase volume from STEP 3 by 10 (i.e., to 5 mL).
	  • if TS soluble, then **STOP.**
	  • if TS insoluble, then go to STEP 5.

TIER 5

STEP 5:	2 mg/mL TS in DMSO – increase volume from STEP 4 by 10 (i.e., to 50 mL)
	• if TS soluble, then **STOP.**
	• if TS insoluble, test at 2 mg/mL in ETOH – increase volume from STEP 4 by 10 (i.e., to 50 mL).
	  • if TS soluble, then **STOP.**
	  • if TS insoluble, then go to STEP 6.

TIER 6

STEP 6:	0.2 mg/mL TS in 50 mL DMSO • if TS soluble, then **STOP.** • if TS insoluble, test at 0.2 mg/mL in 50 mL ETOH • **STOP**

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Figure 2 Solubility Flow Chart

Tier	1		2		3		4		5		6
Concentration in Medium	**Start Here** 200 mg/mL	Incomplete solubility →	20 mg/mL	Incomplete solubility →	2 mg/mL		0.20 mg/mL				
					↓ Incomplete solubility		↓ Incomplete solubility				
Concentration in DMSO					200 mg/mL		20 mg/mL		2 mg/mL		0.2 mg/mL
					↓ Incomplete solubility		↓ Incomplete solubility		↓ Incomplete solubility		↓ Incomplete solubility
Concentration in ETOH					200 mg/mL	→ Incomplete solubility	20 mg/mL	→ Incomplete solubility	2 mg/mL	→ Incomplete solubility	0.2 mg/mL **End**
Concentration on Cells	100 mg/mL		10 mg/mL		1 mg/mL		0.1 mg/mL		0.01 mg/mL		0.001 mg/mL

Notes: NHK medium - Keratinocyte Growth Medium (e.g., KGM® from Cambrex) for normal human keratinocytes.

ANNEX III

TEST METHOD PROCEDURE
Prequalification of Normal Human Epidermal Keratinocyte Growth Medium

This annex provides the guidelines and testing requirements for prequalifying manufacturer lots of Keratinocyte Basal Medium and the medium supplements for use with the Test Method Protocol for the NHK Neutral Red Uptake (NRU) Cytotoxicity Test. The medium and supplements should be tested so as to demonstrate their ability to perform adequately in the recommended assay.

The Testing Facility should request the quality control (QC) test data from the manufacturer for each potential lot of medium and supplements. Based upon the QC test data, purchase and test the one or two most current lots of medium and supplements that appear to have the potential to support NHK cultures according to the requirements of the aforementioned protocol.

TEST SYSTEM
The NHK NRU test is performed to analyze NHK growth characteristics and the *in vitro* toxicity of sodium lauryl sulfate (SLS), as measured by the IC_{50}, with each NHK medium/supplements being tested.

Every combination of medium/supplements expected to be used should be tested. Potential medium testing/supplement combinations are:

- One lot of medium/one lot of supplements: Test the lot of medium using the lot of supplements.
- Two or more lots of medium/one lot of supplements: Test each lot of medium using the one lot of supplements.
- One lot of medium/two or more lots of supplements: Test the lot of medium using each lot of supplements.

NHK cultures should be established using each medium/supplement combination to be tested, and should be subcultured on three different days into 96-well plates (1 plate per day) for three subsequent SLS cytotoxicity tests using each test medium/supplement combination along with a control medium (if available) for which performance has been previously established.

PROCEDURES
Prequalification of the keratinocyte medium and supplements will follow all procedures in the NHK NRU protocol.

Materials – see Section VI.A

Preparations of Media and Solutions – see Section VI.B

Methods – see Section VI.C

- NHK cultures should be established with cryopreserved cells seeded into individual tissue culture 25 cm^2 flasks using a proven medium/supplement combination (i.e., the "control" medium) and each test medium/supplement combination.
- Suspend freshly thawed cells initially into 9 mL of control medium and then add the cell suspension to 25 cm^2 culture flasks containing pre-warmed control or test medium. Cell seeding densities (1 flask/density/medium) of 1 x 10^4, 5 x 10^3, and 2.5 x 10^3 are recommended.
- The cells should be subcultured on three different days into 96-well plates for three subsequent NRU tests (three test plates total [one plate per day] for each medium/supplement combination and each control).

Flask	**lture: 1 Test Plate and 1 Control Plate**	**Application of SLS**
10^4 cells/mL)	Day A	Day X
10^3 cells/mL)	Day B	Day Y
x 10^3 cells/mL)	Day C	Day Z

- Subculturing the cells and application of the SLS will follow procedures in the protocol in reference to appropriate cell confluency. Cell numbers should be recorded for each flask prior to subculturing to the 96-well plates.

[Note: Use of a control medium assumes that the Testing Facility has recent experience with a medium/supplement combination proven to support adequate NHK growth and provide adequate sensitivity to SLS. It is not absolutely necessary to use a control medium.]

Doubling Time – see Section VI.C.5
A doubling time experiment may be considered as an additional quality assurance check.

Preparation of SLS – see Sections VI.D.1.a, b, and d
Preparation of SLS concentrations/dilutions should follow the main experiment (definitive assay) procedures specifically for testing compounds in Routine Culture Medium as outlined in **Section VI.D.3.b**. The concentrations/dilutions should be the same or similar to those used previously with control medium/supplements. SLS concentration ranges used by three laboratories in the NICEATM/ECVAM validation study were 20.0 μg/mL – 1.4 μg/mL and 10.0 μg/mL – 0.6 μg/mL.

Test Procedure – see Sections VI.E.1, E.2, and E.4
The C_1 test concentration will be the highest SLS concentration and C_8 the lowest concentration. Cells cultured in control medium and in each test medium/supplement combination should be tested in parallel for their sensitivity to SLS (see **Methods**). Each of the three test plates of the new medium/supplement combinations is considered a replicate test plate.

Microscopic Evaluation – see Section VI.E.3
Changes in morphology of the cells due to cytotoxic effects of the SLS (prior to measurement of NRU) should be recorded as per procedures outlined in **Section VI.E.3**.

In addition to the general microscopic evaluation of the cell cultures, the Study Director should make the following specific observations:

General culture observations
- rate of proliferation (e.g., rapid, fair, slow)
- percent confluence (e.g., daily estimate);
- number of mitotic figures (e.g., average per field);
- contamination (present/not present)

Cell morphology observations
- overall appearance (e.g., good, fair, poor)
- colony formation (e.g., tight/defined, fair, loose/migrating)
- distribution (e.g., even/uneven)
- abnormal cells (e.g., enlarged, vacuolated, necrotic, spotted, blebby - [average per field])

Data Analysis and Test Evaluation see Sections VI.E.5 and VI.F

Test Acceptance Criteria in section VI.E.5 will be used to determine acceptability of a test plate. Other criteria that should be considered by the Study Director includes the following:

- Mean corrected $OD_{540\text{-}550}$ of the VCs [Note: The target range for corrected mean $OD_{540 \pm 10nm}$ = 0.248 - 1.123 for the VCs, but it is not a test acceptance criterion (range = mean OD ± 2.5 standard deviations; mean = 0.685; SD = 0.175; N = 114).]
- Cell morphology and confluence of the VCs at the end of the 48 hour treatment
- Doubling time

The Study Director should utilize all observed growth characteristics and test results in addition to comparison of results to the media manufacturer's QC data to determine whether the medium/supplements combinations perform adequately. The Testing Facility should request that the manufacturer reserve a portion of an acceptable lot based on estimates of media need.

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