

**TEST METHOD PROTOCOL
for the BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test**

**A Test for Basal Cytotoxicity for an *In Vitro* Validation Study
Phase III**

November 4, 2003

Prepared by

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

**Based on Standard Operating Procedure Recommendations from an
International Workshop Organized by the Interagency Coordinating Committee
on the Validation of Alternative Methods (ICCVAM)**

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

TEST METHOD PROTOCOL

The BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test A Test for Basal Cytotoxicity Phase III

I. PURPOSE

The purpose of this study is to evaluate the cytotoxicity of test chemicals using the BALB/c 3T3 Neutral Red Uptake (NRU) cytotoxicity test. The data will be used to evaluate the intra- and inter-laboratory reproducibility of the assay and 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 cytotoxicity test and supports the *in vitro* validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM) and sponsored by NIEHS, U.S. Environmental Protection Agency, and ECVAM. This test method protocol applies to all personnel involved with performing the cytotoxicity assay.

A. BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test

The 3T3 NRU test will be performed to analyze the *in vitro* toxicity of 60 blinded/coded test chemicals. This test will be used to determine IC₂₀, IC₅₀, and IC₈₀ values for the predetermined set of test chemicals of varying toxicities.

II. SPONSOR

- A. Name: National Institute of Environmental Health Sciences (NIEHS); The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
- B. Address: P.O. Box 12233
Research Triangle Park, NC 27709
- C. Representative: *Named Representative*

III. IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

- A. Test Chemicals: *Blinded Chemicals (60)*
- B. Controls:
- | | |
|---------------------|---|
| Positive: | Sodium Lauryl Sulfate |
| Vehicle (Negative): | Assay medium (DMEM containing 5% NBCS, |
| | 4 mM L-Glutamine, 100 IU/mL Penicillin, 100 µg/mL Streptomycin) |
| Solvent: | Assay medium, DMSO, or ethanol directed by the Study Management Team, for preparation of test chemicals |

IV. TESTING FACILITY AND KEY PERSONNEL

A. Facility Information

- 1) Name:
- 2) Address:
- 3) Study Director:
- 4) Laboratory Technician(s):
- 5) Scientific Advisor:
- 6) Quality Assurance Director:
- 7) Safety Manager:
- 8) Facility Management:

B. Test Schedule

- 1) Proposed Experimental Initiation Date:
- 2) Proposed Experimental Completion Date:
- 3) Proposed Report Date:

V. 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.

VI. DEFINITIONS

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

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{IC}_{50} - X) \text{HillSlope}}}$$

where Y= response, X is the logarithm of dose (or concentration), Bottom is the minimum response, Top is the maximum response, logIC₅₀ is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve.

- 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 chemical preparation, incubator function); all optical density data obtained from the spectrophotometer plate reader will be saved in electronic and paper formats; all calculations of IC_x values and other derived data will be in electronic and paper format; all data will be archived.

VII. PROCEDURES

A. Materials

1. Cell Lines

BALB/c 3T3 cells, clone 31

CCL-163, LGC Reference Materials, Customer Service, Queens Road, Teddington, Middlesex, TW110LY, UK

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.]

- a) Incubator: 37°C ± 1°C, 90 % ± 5 % humidity, 5.0 % ± 1 % CO₂/air
- b) Laminar flow clean bench/cabinet (standard: "biological hazard")
- c) Water bath: 37°C ± 1°C
- d) Inverse phase contrast microscope
- e) Sterile glass tubes with caps (e.g., 5 mL)
- f) Centrifuge (optionally: equipped with microtiter plate rotor)
- g) Laboratory balance
- h) 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm ± 10 nm filter
- i) Shaker for microtiter plates
- j) Cell counter or hemocytometer
- k) Pipetting aid
- l) Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette), dilution block
- m) Cryotubes
- n) Tissue culture flasks (e.g., 75 - 80 cm², 25 cm²)
- o) 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Falcon tissue culture-treated)
- p) pH paper (wide and narrow range)
- q) Multichannel reagent reservoir
- r) Waterbath sonicator
- s) Magnetic stirrer
- t) Antistatic bar ionizer/antistatic gun (optional for neutralizing static on 96-well plates)
- u) Dry heat block (optional)
- v) Adhesive film plate sealers (e.g., Excel Scientific SealPlate™, Cat # STR-SEAL-PLT or equivalent)
- w) Vortex mixer
- x) 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 chemicals 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 (NBCS or 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) Dimethyl sulfoxide (DMSO), U.S.P. analytical grade (Store under nitrogen @ -20°C)
- k) Ethanol (ETOH), U.S.P. analytical grade (100 %, non-denatured for test chemical 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 NBCS/NCS, first check a lot for growth stimulating properties with 3T3 cells (approximately 20-24 h doubling time) and then reserve a sufficient amount of NBCS/NCS. May use pre-tested serum lot from Phases Ia, Ib, and II of the validation study if the serum has been stored under appropriate conditions and shelf-life has not expired.]

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) for freezing (Freeze Medium); contains 2X concentration of NBCS/NCS and DMSO of final freezing solution

40 %	NBCS/NCS
20 %	DMSO
- b) for routine culture (Routine Culture Medium)

10 %	NBCS/NCS
4 mM	Glutamine
- c) for test chemical dilution (Chemical Dilution Medium)

4 mM	Glutamine
200 IU/mL	Penicillin
200 µg/mL	Streptomycin
- d) for dilution of NR stock solution (NR Dilution Medium)

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

[Note: The Chemical Dilution Medium with test chemical 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 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.

If the liquid form is not available, the following formulation can be prepared.

EXAMPLE: 0.25 g NR Dye powder in 100 mL H₂O

The NR Stock Solution (powder in water) 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 solution)	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 30 min of preparation but also used within 15 min after removing from 37° C storage.]

4. Ethanol/Acetic Acid Solution (NR Desorb)

1 %	Glacial acetic acid solution
50 %	Ethanol
49 %	H ₂ O

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 cm²) at 37°C \pm 1°C, 90 % \pm 5 % humidity, and 5.0 % \pm 1 % CO₂/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 water bath at 37°C \pm 1°C. Leave for as brief a time as possible.

- Resuspend the cells in pre-warmed Routine Culture Medium and transfer into pre-warmed Routine Culture Medium in a tissue-culture flask.
- Incubate at 37°C \pm 1°C, 90 % \pm 5 % humidity, and 5.0 % \pm 1 % CO₂/air.
- When the cells have attached to the bottom of the flask (within 4 to 24 h), decant the supernatant and replace with fresh pre-warmed (37°C) medium. Culture as described above.
- 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:

- a) Decant medium, briefly rinse cultures with 5 mL PBS or Hanks' BSS (without Ca^{2+} , Mg^{2+}) per 25 cm² flask (15 mL per 75 cm² flask). Wash cells by gentle agitation to remove any remaining serum that might inhibit the action of the trypsin.
- b) Discard the washing solution. Repeat the rinsing procedure and discard the washing solution.
- c) Add 1-2 mL trypsin-EDTA solution per 25 cm² to the monolayer for a few seconds (e.g., 15-30 seconds).
- d) Remove excess trypsin-EDTA solution and incubate the cells at room temperature.
- e) After 2-3 minutes (min), 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 (37°C) Routine Culture Medium/cm² to the flask (e.g., 2.5 mL for a 25 cm² flask). Disperse the monolayer by gentle trituration. It is important 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. BALB/c 3T3 cells are routinely passaged at suggested cell densities as listed in the table (approximate doubling time is 20-24 h). The individual laboratories will need to 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.

- a) Centrifuge trypsinized cells at approximately 200 x g.
- b) Suspend the cells in cold Routine Culture Medium (half the final freezing volume) so a final concentration of $1-5 \times 10^6$ cells/mL can be attained.
- c) 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.
- d) Place the tubes into an insulated container (e.g., styrofoam trays) and place in a freezer (-70 to -80°C) for 24 h. This gives a freezing rate of approximately 1°C/min. 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.
- e) Place the frozen tubes into liquid nitrogen for storage.

8. Preparation of Cells for Assays

- a) Cultured cells that are going to 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 $2.0 - 3.0 \times 10^4$ 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 VII.F.1**). In the remaining wells, dispense 100 µl of a cell suspension of $2.0 - 3.0 \times 10^4$ cells/mL ($= 2.0 - 3.0 \times 10^3$ 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 h incubation in step **b** and 48 h exposure to test chemicals). Prepare one plate per chemical to be tested.
- b) Incubate cells for 24 ± 2 h ($37^\circ\text{C} \pm 1^\circ\text{C}$, 90 % \pm 5 % humidity, 5.0 % \pm 1 % CO₂/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.
- c) 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) A cell doubling time procedure was performed on the initial lot of cells that was used in the first cell culture assays of Phase Ia of the Validation Study. The doubling time only needs to be determined in Phase III if there is a change in the lot of cells used. Establish cells in culture and trypsinize cells as per **Section VII.C.4** for subculture. Resuspend cells in NR Dilution Medium (5 % NBCS/NCS). Seed cells at 4200 cells/cm².

- 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 ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm 5\%$ humidity, $5.0\% \pm 1\%$ 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) if Study Director sees a need. Use appropriate size exclusion limits if using a Coulter counter. Determine the total number of cells and document. Repeat sampling at 24 h, 48 h, 72 h, and 96 h post inoculation. Change culture medium at 72 h 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 Chemicals

The Study Management Team will provide direction on the solvent to be used for each test chemical. [Note: Preparation under red or yellow light is recommended to preserve chemicals that degrade upon exposure to light.]

1. Test Chemicals in Solution

- a) Allow test chemicals to equilibrate to room temperature before dissolving and diluting.
- b) Prepare test chemical immediately prior to use. Test chemical solutions should not be prepared 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 SMT may direct the Study Director to store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility chemicals) in a freezer (e.g., -70°C) for use in future chemical analyses.
- c) For chemicals dissolved in DMSO or ethanol, the final DMSO or ethanol 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 chemical should be prepared at the highest concentration found to be soluble in the solubility test conducted per the *Test Method Protocol for Solubility Determination*. 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 chemical was soluble in Chemical Dilution Medium, or

- 1/200 the highest concentration found to be soluble in the solubility test if the chemical was soluble in ethanol or DMSO.
- e) The seven lower concentrations in the range finding experiment would then be prepared by successive dilutions that decrease by one log unit each. The following example illustrates the preparation of test chemical in solvent and the dilution of dissolved test chemical in Chemical Dilution Medium before application to 3T3 cells.

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

If DMSO was determined to be the preferred solvent at Tier 2 of the solubility test (i.e., 200,000 µg/mL), dissolve the chemical 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 chemical/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 chemical in each tube with 99 parts of Chemical Dilution Medium (e.g., 0.1 mL test chemical in DMSO + 9.9 mL Chemical Dilution Medium) to derive the eight 2X concentrations for application to 3T3 cells. Each 2X test chemical 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 chemical. By adding 0.05 mL of the appropriate 2X test chemical concentration to the appropriate wells, the test chemical 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 article prepared in Chemical Dilution Medium, DMSO, or ethanol may precipitate upon transfer into the Routine Culture Medium. The 2X dosing solutions should be evaluated for precipitates and the results recorded in the 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 article precipitates should be avoided and generally will not be used in the IC_x determinations for the definitive tests. Precipitates in 2X dosing solutions are permissible for range finder tests but not for definitive tests.

Document all test chemical preparations in the Study Workbook.

2. pH of Test Chemical Solutions

Prior to or immediately after application of the test chemical to the 96-well plate, measure the pH of the highest 2X dosing concentration of the test chemical (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 EXCEL® template). Medium color for all dosing dilutions should be noted in the workbooks. Do not adjust the pH.

3. Concentrations of Test Chemical

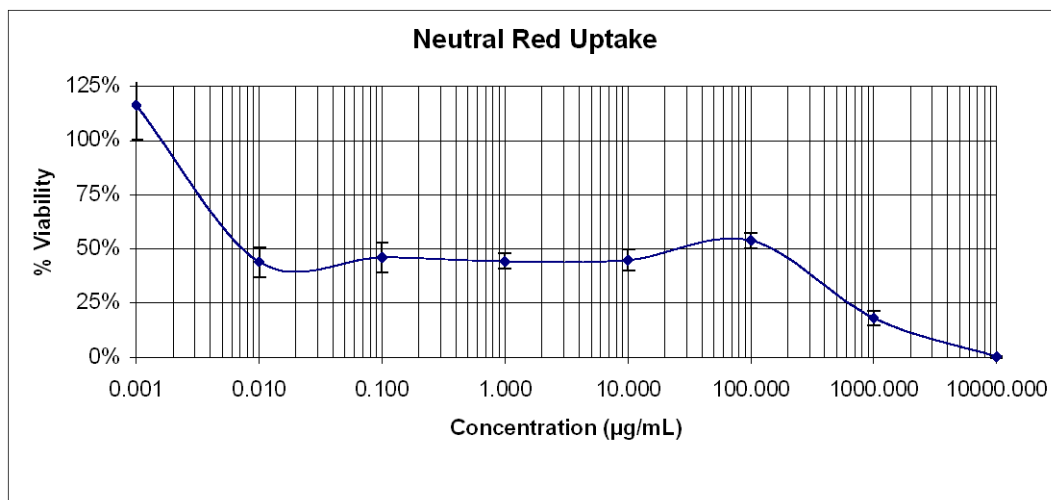
a) Range Finder Experiment

Test eight concentrations of the test chemical by diluting the stock solution with a constant factor covering a large range. The initial dilution series shall be 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 VII.D.3.b.) should be employed. Place the test chemical concentration into an incubator ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm 5\%$ humidity, $5.0\% \pm 1\%$ 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 $\mu\text{g}/\text{mL}$).

Example 1 – Biphasic Curve



b) Main Experiment

[Note: After the range finding assay is completed, the definitive concentration-response experiment shall be performed three times on three different days for each chemical (i.e., one plate per day per chemical).]

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. Experiments revealing less than one cytotoxic concentration on each side of the IC_{50} value shall be repeated, where possible, with a smaller dilution factor (see **Section VII.E.5.a.4**). Each experiment should have at least one cytotoxicity value > 0 % and ≤ 50.0 % viability and at least one cytotoxicity value > 50.0 % and < 100 % viability. A progression factor of 1.21 [$\sqrt[12]{10}$] is regarded the smallest factor achievable and will be the lowest dosing interval required.)

Determine which test chemical concentration is closest to the IC_{50} value (e.g., 50 % cytotoxicity). 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 chemicals prepared in Chemical Dilution Medium, the highest test article concentration that may be applied to the cells in the main experiments will be either 100 mg/mL, or the maximum soluble dose. Test chemical 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 $\mu\text{g/mL}$ (200 mg/mL). The solution is mixed using the mechanical procedures that produced solubility when performing the solubility test specified in *Test Method Protocol for Solubility Determination*. 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 chemical is insoluble in medium at 200 mg/mL, proceed by adding medium, in small incremental amounts, to attempt to dissolve the chemical by using the sequence of mechanical procedures specified in *Test Method Protocol for Solubility Determination*. More stringent solubility procedures may be employed if needed based on results from the range finder experiment (**Section VII.D.3.a.**). The highest soluble stock solution will be used to prepare the 7 additional serial stock dosing solutions.
- For test chemicals prepared in either DMSO or ethanol, the highest test article concentration that may be applied to the cells in the main experiments will be either 2.5 mg/mL, or less, depending upon the maximum solubility in solvent. Weigh the test chemical 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 *Test Method Protocol for Solubility Determination*. If complete solubility is achieved in the solvent, then 7 additional serial stock dosing solutions may be prepared from the 500 mg/mL 200X stock. If the test chemical is insoluble in solvent at 500 mg/ml, proceed by adding solvent, in small incremental amounts, to attempt to dissolve the chemical by again using the sequence of mixing procedures. The highest soluble stock solution will be used to prepare the 7 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 Chemical Dilutions

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

The technical production of decimal geometric concentration series is simple. An example is given 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 chemicals will use the 96-well plate configuration as shown in **Figure 1**.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	C ₁ b	C ₂ b	C ₃ b	C ₄ b	C ₅ b	C ₆ b	C ₇ b	C ₈ b	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 ₁ b	C ₂ b	C ₃ b	C ₄ b	C ₅ b	C ₆ b	C ₇ b	C ₈ b	VCb	VCb

VC1 and VC2 = VEHICLE CONTROL

C₁ – C₈ = Test Chemicals or PC (SLS) at eight concentrations
(C₁ = highest, C₈ = lowest)

b = BLANKS (Test chemical or PC, but contain **no** cells)

VCb = VEHICLE CONTROL BLANK (contain **no** cells)

2. Application of Test Chemical

- a) Two optional methods for rapidly applying the 2X dosing solutions onto the 96-well plates may be utilized.
 - 1) The first method is to 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) The second method utilizes 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 chemical 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 chemical to the plates.

- b) After $24 \text{ h} \pm 2 \text{ h}$ 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.
- c) Immediately add $50 \mu\text{L}$ of fresh pre-warmed Routine Culture Medium to all of the wells, including the blanks. Fifty microliters ($50 \mu\text{L}$) 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 article 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 chemical solutions for each concentration (e.g., wells A3 and H3 receive C_1 solution).
- d) Incubate cells for $48 \text{ h} \pm 0.5 \text{ h}$ ($37^\circ\text{C} \pm 1^\circ\text{C}$, $90 \% \pm 5 \%$ humidity, and $5.0 \% \pm 1 \% \text{ CO}_2/\text{air}$).
- e) **Positive Control:** For each set of test chemical plates used in an assay, a separate plate of positive control concentrations will be set up following the concentration range established in the development of the positive control database in Phase I of the Validation Study. If multiple sets of test chemical 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 chemical plates will be run with a positive control plate. The mean $\text{IC}_{50} \pm$ two and a half standard deviations (SD) for the SLS acceptable tests from Phases Ia, Ib, and II (after the removal of outliers) are the values that will be used as an acceptance criterion for test sensitivity for the 3T3 NRU assay. This plate will follow the same schedule and procedures as used for the test chemical plates (including appropriate chemical concentrations in the appropriate wells and meeting test acceptance criteria – see **sections VII.E.1, E.2, and E.5**).

3. Microscopic Evaluation

After at least 46 h 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 chemical, 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 VII.E.3**) should be determined and documented in the Study Workbook and in the appropriate section of Addendum II of the EXCEL® study 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 chemical and rinse the cells very carefully with 250 μ L pre-warmed D-PBS. Remove the rinsing solution by dumping and remove excess by gently blotting on paper towels. Add 250 μ L NR medium (to all wells including the blanks) and incubate ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm 5\%$ humidity, and $5.0\% \pm 1\%$ CO_2/air) for 3 ± 0.1 h. Observe the cells briefly during the NR incubation (e.g., between 2 and 3 h – 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 μ L pre-warmed D-PBS.
- c) Decant and blot D-PBS from the plate.
- d) Add exactly 100 μ L NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.
- e) Shake microtiter plate rapidly on a microtiter plate shaker for 20 – 45 min 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 $540 \text{ nm} \pm 10 \text{ nm}$ in a microtiter plate reader (spectrophotometer), using the blanks as a reference. [Note: Phases Ia and Ib data show the mean OD value for the plate blanks to be 0.057 ± 0.043 for 3T3 cells (± 2.5 standard deviations; data from 3 labs; $N = 189$). Use this range as a **guide** for assessment of the blank values.] Save raw data in the Excel format as provided by the SMT.

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 \pm two and a half (2.5) standard deviations of the historical mean established by the Test Facility (as per **VII.E.2.e**), 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 .
- 2) The left and 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 $\leq 50.0\%$ viability and at least one calculated cytotoxicity value $> 50.0\%$ 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 Chemicals: 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 chemical.

[Note: A corrected mean $OD_{540 \pm 10nm}$ of 0.103 - 0.813 for the VCs is a target range but will not be a test acceptance criterion. Range determined from Phase Ib VC OD values from 3 laboratories (mean \pm 2.5 standard deviations, N = 98).]

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 article present in the assay. If volatility is suspected, then proceed to **Section VII.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. Volatility of Test Chemicals

Highly volatile test chemicals may generate vapors from the treatment medium during the test chemical 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 to resorbed test article vapors. If the test chemical is particularly toxic at the doses tested, the cross contamination may be evident as a

significant reduction in viability in the vehicle control cultures (i.e., VC1) adjacent to the highest test chemical doses.

If potential test article 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

- 1) Plates and chemicals will be prepared as usual according to **Sections VII.D and VII.E**.
- 2) Immediately after the 96-well culture plate has been treated with the suspected volatile chemical (**Section VII.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 VII.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.]
- 3) 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 VII.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 chemical 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 chemical tested will span the range of no effect up to total inhibition of cell viability. Data from the microtiter plate reader shall be transferred to the Excel® spreadsheet template provided by the SMT. The template will automatically determine cell viability, IC₅₀ values by linear interpolation, and perform statistical analyses (including statistical identification of outliers). The template will also calculate the concentrations associated with 20 %, 50 %, and 80 % viability using the Hill slope and EC₅₀ (i.e., IC₅₀) from the Hill function analysis.

The Hill function analysis shall be performed using statistical software (e.g., GraphPad PRISM® 3.0) and a template specified by the SMT to calculate IC₂₀, IC₅₀, and IC₈₀ values (and the associated confidence limits) for each test chemical.

The Testing Facility shall report data using at least three (3) significant figures and shall forward the results from each assay to the SMT through the designated contacts in electronic format and hard copy upon completion of testing. The SMT will be directly responsible for the statistical analyses of the Validation Study data.

VIII. REFERENCES

Hackenberg, U. and H. Bartling. 1959. Messen und Rechnen im pharmakologischen Laboratorium mit einem speziellen Zahlensystem (WL24-System). Arch. Exp. Pathol. Pharmacol. 235: 437-463.

Spielmann, H., S. Gerner, S. Kalweit, R. Moog, T. Wirnserberger, K. Krauser, R. Kreiling, H. Kreuzer, N.P. Luepke, H.G. Miltenburger, N. Müller, P. Murmann, W. Pape, B. Siegmund, J. Spengler, W. Steiling, and F.J. Wiebel. 1991. Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany. Toxicol. *In Vitro* 5: 539-542.

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IX. APPROVAL

SPONSOR REPRESENTATIVE
(Print or type name)

DATE

Test Facility STUDY DIRECTOR
(Print or type name)

DATE