ICCVAM-Recommended Test Method Protocol

BALB/c 3T3 NRU Cytotoxicity Test Method

Originally published as Appendix C1 of "ICCVAM Test Method Evaluation Report: In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests"

NIH Publication No. 07-4519 – Published 2006

Available at: http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_tmer.htm

This page intentionally left blank

ICCVAM Recommended Protocol for the BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test - A Test for Basal Cytotoxicity

1.0 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 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 joint independent *in vitro* validation study organized by 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).

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

2.0 TEST SYSTEM

The NRU cytotoxicity assay procedure is 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 diffuses through the plasma membrane and concentrates in lysosomes where it electrostatically binds to the anionic lysosomal matrix. Toxicants can alter the cell surface or the lysosomal membrane to cause lysosomal fragility and other adverse changes that gradually become irreversible. Thus, cell death and/or inhibition of cell growth decreases the amount of neutral red retained by the culture. Healthy proliferating mammalian cells, when properly 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 NR after chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

3.0 KEY PERSONNEL

3.1 Laboratory

- Study Director (only recommended if testing is performed in accordance with Good Laboratory Practice [GLP] guidelines)
- Laboratory Technician(s)

3.2 Testing Facility

- Scientific Advisor
- Quality Assurance Director (only necessary if testing is performed under GLP)
- Safety Manager
- Facility Management

4.0 DEFINITIONS

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

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC50 - \log X)HillSlope}}$$

where Y=response (i.e., % viability), X is the substance concentration producing the response, Bottom is the minimum response (0% viability, maximum toxicity), Top is the maximum response (maximum viability), EC_{50} is the substance concentration at the response midway between Top and Bottom, and HillSlope describes the slope of the curve. When Top=100% viability and Bottom=0% viability, the EC_{50} is the equal to the IC_{50}.

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 (OD) 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

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

5.0 IDENTIFICATION OF CONTROL SUBSTANCES

5.1 Positive Control (PC)

- Sodium lauryl sulfate (SLS)

5.2 Vehicle Control (VC)

- Assay medium (Dulbecco’s Modification of Eagle’s Medium [DMEM] containing 5% New Born Calf Serum (NCS), 4 mM L-Glutamine, 100 IU/mL Penicillin, 100 µg/mL Streptomycin)

5.3 Solvent Control

- VC 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.

6.0 PROCEDURES

6.1 Materials

6.1.1 Cell Line

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

6.1.2 Technical Equipment[1]

- Incubator: 37 ºC ±1 ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air
- Laminar flow clean bench/cabinet (standard: "biological hazard")
- Waterbath: 37 ºC ±1 ºC
- Inverse phase contrast microscope
- Sterile glass tubes with caps (e.g., 5 mL)
- Centrifuge
- Laboratory balance
- 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm ±10 nm filter with maximum absorbance of 3
- Shaker for microtiter plates
- Cell counter or hemocytometer
- Pipetting aid
- Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette), dilution block
- Cryotubes
- Tissue culture flasks (e.g., 75 - 80 cm^2, 25 cm^2)
- 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Falcon tissue culture-treated)
- pH paper (wide and narrow range)
- Multichannel reagent reservoir
- Waterbath sonicator
- Magnetic stirrer
- Antistatic bar ionizer/antistatic gun (optional: to neutralize 96-well plate static)
- Dry heat block (optional)
- Adhesive film plate sealers (e.g., Excel Scientific SealPlate™, Cat # STR-SEAL-PLT or equivalent)
- Vortex mixer
- Filters/filtration devices

Note: Prescreen tissue culture flasks and microtiter plates to ensure that they adequately support the growth of 3T3 cells. Use multi-channel repeater pipettes 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.

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

6.1.3 Chemicals, Media, and Sera

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

6.2 Preparation 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.

6.2.1 Media

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

- **Freeze Medium:** contains 2X concentration of NCS and DMSO of final freezing solution
  - 40% NCS
  - 20% DMSO
- **Routine Culture Medium**
  - 10% NCS
  - 4 mM L-Glutamine

- **Chemical Dilution Medium**[2]
  - 4 mM L-Glutamine
  - 200 IU/mL Penicillin
  - 200 µg/mL Streptomycin
- **NR Dilution Medium**
  - 5% NCS
  - 4 mM Glutamine
  - 100 IU/mL Penicillin
  - 100 µg/mL Streptomycin

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

6.2.2 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.

6.2.3 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: Filter the NR Medium (e.g., Millipore filtering, 0.2 – 0.45 µm pore size) to reduce NR crystals. Maintain aliquots of the NR Medium at 37 °C (e.g., in a waterbath) before adding to the cells and use within 60 minutes of preparation and within 15 minutes after removing from 37 °C storage. Examine the solution for crystals prior to use.

6.2.4 ETOH/Acetic Acid Solution (NR Desorb)

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

[2] 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.

6.3 Methods

6.3.1 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 37 ºC ±1 ºC, 90% ±10% humidity, and 5.0% ±1.0% CO_2/air.
- Examine the cells on a daily (i.e., on workdays) basis under a phase contrast microscope, and note any changes in morphology or their adhesive properties in a study workbook.
- All cell culture studies should follow good cell culture practices (Hartung et al. 2002).

6.3.2 Receipt of Cryopreserved BALB/c 3T3 Cells

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

6.3.3 Thawing Cells

Thaw a fresh batch of frozen cells from the stock lot of cells and culture approximately every two months. This period resembles a sequence of about 18 passages.

- Thaw cells by putting ampules into a waterbath at 37 °C ±1 ºC. 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 ºC ±1 ºC, 90% ±10% humidity, and 5.0% ±1.0% CO_2/air.
- 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 (37 ºC) medium. Culture as described above.
- Passage at least two times before using the cells in a cytotoxicity test.

6.3.4 Routine Culture of BALB/C 3T3 Cells

Remove cells from the flask by trypsinization when they exceed 50% confluence (but less than 80% confluent):

- Decant medium, briefly rinse cultures with 5 mL PBS or HBSS (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.
- Discard the washing solution. Repeat the rinsing procedure and discard the washing solution.
- Add 1-2 mL trypsin-EDTA solution per 25 cm^2 to the monolayer for a few seconds (e.g., 15-30 seconds).
- Remove excess trypsin-EDTA solution and incubate the cells at room temperature.
- After 2-3 minutes, lightly tap the flask to detach the cells into a single cell suspension.

6.3.5 Cell Counting

- After the cells are detached, add 0.1-0.2 mL of pre-warmed (37 ºC) 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).

6.3.6 Subculture of Cells

BALB/c 3T3 cells are routinely sub-cultured into other flasks or seeded into 96-well microtiter plates (see **Figure C1-1** for 96-well test plate configuration) and passaged at suggested cell densities as listed in **Table C1-1** (approximate doubling time is 20-24 hours). Laboratories must determine and adjust the final density to achieve appropriate growth.

Figure C1-1 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 controls
C_1 – C_8 = Test Substances or PC (SLS) at eight concentrations (C_1 = highest, C_8 = lowest)
C_xb = Blanks (Test substance or PC, but contain **no** cells)
VCb = VC blanks (contain **no** cells)

Table C1-1 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.

6.3.7 Freezing Cells

(Procedure required only if current stock of cells is depleted.)
Store stocks of BALB/c 3T3 cells in sterile freezing tubes in a liquid nitrogen freezer. DMSO is used as a cryoprotective agent.

- Centrifuge trypsinized cells at approximately 200 x *g*.
- 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.
- 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.
- 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/minute). 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.
- Place the frozen tubes into liquid nitrogen for storage.

6.3.8 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.[3]
  - Prepare a cell suspension of 2.0 – 3.0x10^4 cells/mL in Routine Culture Medium on the day of plate seeding.
  - Use a multi-channel pipette to dispense 100 µL Routine Culture Medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate (See **Figure C1-1**).
  - Dispense 100 µL of a cell suspension of 2.0 – 3.0x10^4 cells/mL (= 2.0 – 3.0x10^3 cells/well) in the remaining wells.
- Incubate cells for 24 hours ±2 hours (37 ºC ±1 ºC, 90% ±10% humidity, 5.0% ±1.0% 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.

[3]Note the seeding density to ensure that the cells in the control wells are not overgrown after three days (i.e., 24 hour incubation and 48 hour exposure to test substances). Prepare one plate per substance to be tested.

- 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.

6.3.9 Determination of Doubling Time

- Establish cells in culture and trypsinize cells as per **Section 6.3.4** for subculture. Resuspend cells in NR Dilution Medium (5% NCS). Seed cells at 4200 cells/cm^2.
- Seed five sets of cell culture vessels in triplicate (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 ºC ±1 ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air).
- 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 and document. Study Director may determine cell viability by dye exclusion (e.g., Trypan Blue; Nigrosin). Use appropriate size exclusion limits if using a Coulter counter.
- Repeat sampling at 24-, 48-, 72-, and 96-hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop.
- 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).

6.4 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 I** of this protocol.

6.4.1 Test Substances in Solution

- Equilibrate test substances to room temperature before dissolving and diluting.
- 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 quantitative chemical 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 VCs and in all of the eight test concentrations. The concentration of DMSO or ETOH

should be the lowest possible concentration needed to dissolve the test substance.

- 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 I**. Thus, the highest test concentration applied to the cells in each range finding test is:
  - 0.5 times the highest concentration found to be soluble in the solubility test, if the substance was soluble in Chemical Dilution Medium, or
  - 1/200 the highest concentration found to be soluble in the solubility test if the substance was soluble in ETOH or DMSO.

Example: Preparation of Test Substance in Solvent for Range Finding Tests 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. The seven lower concentrations in the range finding test are prepared by successive dilutions that decrease by one log unit each.

- Label eight tubes 1 – 8. Add 0.9 mL solvent (e.g., DMSO) to tubes 2 - 8.
- Prepare stock solution of 200,000 μg test substance/mL solvent in tube # 1.
- 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).
- 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). Continue making serial 1:10 dilutions in the prepared solvent tubes.
- 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).
- 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 finder tests and main tests. 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.

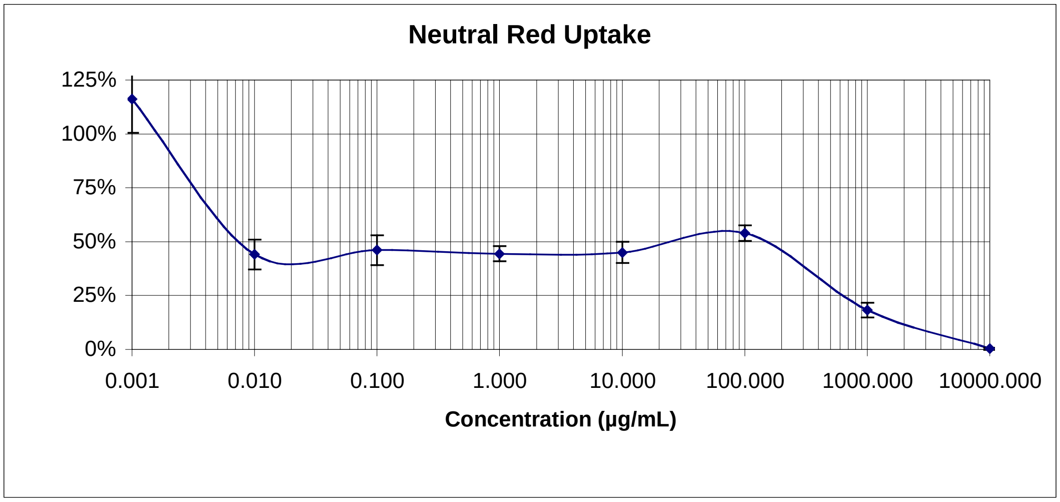
6.4.2 pH of Test Substance Solutions

- 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 C1-1**) 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 II** for an example template). Medium color for all dosing dilutions should be noted in the study workbook. Do not adjust the pH.

6.4.3 Concentrations of Test Substance

- Range Finder Test
  - 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 test 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 6.4.4**) should be employed.
  - Place the test substance concentration into an incubator (37 ºC ± 1 ºC, 90% ±10% humidity, 5.0% ±1.0% 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 finder test produces a biphasic curve, then the doses selected for the subsequent main tests should cover the most toxic dose-response range (see **Example C1-1** – the most toxic range is 0.001 – 0.1 μg/mL) that reduces viability to 50%.

Example C1-1 Biphasic Curve



- Main Test (Definitive Test)
    - o Depending on the slope of the concentration-response curve estimated from the range finder test, the dilution/progression factor in the concentration series of the main test should be smaller (e.g., dilution factor of $\sqrt[6]{10} = 1.47$).
    - o 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.
    - o 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.
    - o The minimum number of definitive tests that should be performed for a test substance is two.

6.4.4 Maximum Doses to be Tested in the Main Test

If minimal or no cytotoxicity was measured in the dose range finder test, a maximum dose for the main tests will be established as follows:

6.4.4.1 *For test substances prepared in Chemical Dilution Medium*

- The highest test substance concentration that may be applied to the cells in the main tests 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 I**).

- 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 mechanical procedures specified in **ANNEX I**.
- More stringent solubility procedures may be employed if needed based on results from the range finder test (**Section 6.4.3**). The highest soluble stock solution will be used to prepare the seven additional serial stock dosing solutions.

6.4.4.2 *For test substances prepared in either DMSO or ETOH*

- The highest test substance concentration that may be applied to the cells in the main tests 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 I**.
- 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.

6.4.4.3 *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, 1.78 (= $\sqrt[4]{10}$) into four steps, 1.47 (= $\sqrt[6]{10}$) into six steps, and 1.21 (= $\sqrt[12]{10}$) into 12 steps.

Example C1-2 Example of Decimal Geometric Concentration Series for Factor 1.47

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.).

6.5 Test Procedure

6.5.1 96-Well Plate Configuration

The 3T3 NRU assay for test substances will use the 96-well plate configuration as shown in **Figure C1-1**.

6.5.2 Application of Test Substance and Positive Control

6.5.2.1 *Application of Test Substance*

- Two optional methods for rapidly applying the 2X dosing solutions onto the 96-well plates may be utilized.
  - 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).
  - 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, use a multi-channel micropipettor 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.
- 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.
- 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 VC blank (VCb) wells (column 1, column 12, wells A2, A11, H2, H11) will receive the VC 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).
- Incubate cells for 48 hours ±0.5 hours (37 ºC ±1 ºC, 90% ±10% humidity, and 5.0% ±1.0% CO_2/air).

6.5.2.2 *Application of Positive Control (PC)*

- For each set of test substance plates used in an assay, prepare a separate plate of PC concentrations. A separate plate for the PCs is used so that a complete dose response curve, rather than a single point estimate, can be obtained. This will assist with troubleshooting the experiment, if the need arises.
- If multiple sets of test substance plates are set up, clearly designate the PC 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 PC 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 6.5.1**, **6.5.2**, **and 6.5.5**).

6.5.3 Microscopic Evaluation

- After at least 46 hours of treatment, examine each plate under an inverse 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. *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. Substances that may etch the plastic or *film out*[4] in medium should be identified and noted.
- Use the following Visual Observations Codes (**Table C1-2**) in the description of cell culture conditions. Numerical scoring of the cells should be determined and documented in the study workbook and in the appropriate section of the Microsoft Excel® template.

[4] Film out indicates that a substance comes out of solution and forms a layer over the medium and the well. If a precipitate forms or if a substance films out, then the concentration to which the cells are exposed may not be the same as the concentration placed into the test well.

Table C1-2 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

6.5.4 Measurement of NRU

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 (37 ºC ±1 ºC, 90% ±10% humidity, and 5.0% ±1.0% CO_2/air) for 3.0 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.

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

Decant and blot D-PBS from the plate.

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

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.

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.

Save raw data in the Microsoft Excel® template.

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 (SD); mean = 0.048; SD = 0.007; N = 233)[5].

Note: The range of linearity of the microplate reader should be confirmed, as per in-house standard operating procedures. Additionally, all equipment should be calibrated according to manufacturer's instructions.

6.5.5 Test Acceptance Criteria for the 3T3 NRU Assay

6.5.5.1 *Test Acceptance Criteria for PC*

- All acceptance criteria must be met for a PC test to be acceptable.
  - The PC (SLS) IC_{50} must be within ± 2.5 standard deviations (SD) of the historical mean established by the Test Facility and must have an R^2 (coefficient of determination) ≥0.85 (calculated for the Hill model fit using PRISM® software). The NICEATM/ECVAM validation study generated the following PC data (ICCVAM 2006):
    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
  - 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.

6.5.5.2 *Test Acceptance Criteria for Test Substances*

- All acceptance criteria must be met by the test substances for a test to be acceptable.
  - 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.

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)[8].

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.

[5] Data from NICEATM/ECVAM validation study (ICCVAM 2006).

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).

6.5.3.3 *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 6.5.6**.
- Checks for cell seeding errors also may be performed by examining each plate under an inverse phase contrast microscope to assure that cell quantity is consistent.

6.5.6 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.

- Plates and substances will be prepared as usual according to **Sections 6.4** and **6.5**.
- Immediately after the 96-well culture plate has been treated with the suspected volatile substance (**Section 6.5.2**), 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 6.5.2**). 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.
- 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 6.5.4**.

6.4 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 OD of the six replicate values (minimum of four acceptable replicate wells) per test concentration (blanks will be subtracted). This value is compared with the mean OD of all VC wells. Relative cell viability is then expressed as percent of mean VC OD. 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 II** for an example spreadsheet template).
- A Hill function analysis should be performed using statistical software (e.g., GraphPad PRISM®) and a template to calculate IC_{20}, IC_{50}, and IC_{80} values (and the associated confidence limits) for each test substance. The Hill function is recommended because all the dose-response information, rather than a few points around the IC_{50}, are used. Additionally, the slope of the curve can be assessed using the Hill function.
- 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, the Bottom parameter can be estimated without constraints. However, when Bottom≠0, the EC_{50} reported by the Hill function is not the same as the IC_{50} since the Hill function defines EC_{50} as the point midway between Top and Bottom. The Hill function calculation using the Prism® software should be rearranged to calculate the concentration corresponding to the IC_{50} as follows.

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

where IC_{50} is the concentration producing 50% toxicity, EC_{50} is the concentration producing a response midway between the Top and Bottom responses; Top is the maximum response (100% viability, maximum survival), Bottom is the minimum response (0% viability, maximum toxicity), Y=50 (i.e., 50% viability), and HillSlope describes the slope of the response. The X from the standard Hill function equation is replaced, in the rearranged Hill function equation, by the IC_{50}.

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

7.0 BACKGROUND MATERIALS

Borenfreund E, Puerner JA. 1985. Toxicity determination *in vitro* by morphological alterations and neutral red absorption. Toxicol Lett 24:119-124.

ICCVAM. 2006. Background Review Document – *In Vitro* Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity. NIH Publication No. 07-4518. Research Triangle Park, NC:National Institute for Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/invitro.htm [accessed 21 November 2006].

Riddell RJ, Panacer DS, Wilde SM, Clothier RH, Balls M. 1986. The importance of exposure period and cell type in *in vitro* cytotoxicity tests. Altern Lab Anim 14:86-92.

U. S. Environmental Protection Agency. 1996. Product Properties Test Guidelines. OPPTS 803.7840. Water Solubility: Column Elution Method; Shake Flask Method. EPA712-C-96-041. Washington, DC: U.S. Environmental Protection Agency.

8.0 CITED REFERENCES

Hartung T, Balls M, Bardouille C, Blanck O, Coecke S, Gstrauthaler G, et al. 2002. Good cell culture practice: ECVAM Good Cell Culture Practice Task Force report 1. Altern Lab Anim 30:407-414. Available: http://ecvam.jrc.it/publication/index5007.html [accessed 01 November 2006].

ANNEX I

TEST METHOD PROCEDURE
Solubility Determination of Test Substances

1.0 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 Neutral Red Uptake (NRU) test methods. All individuals involved in solubility assessments should be trained to understand solvent and solubility issues.

2.0 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 can be based on visual observation using a microscope. A test substance has dissolved if the solution is clear and shows no signs of cloudiness or precipitation.

3.0 PROCEDURES

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

3.1 Materials

See **Section 6.1** of Test Method Protocol for the BALB/c 3T3 NRU Cytotoxicity Test Method Protocol.

3.2 Preparation of Media and Solutions

See **Section 6.2** of Test Method Protocol for the BALB/c 3T3 NRU Cytotoxicity Test Method. 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.

3.3 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 **Annex I, Section 3.5**. **Table C1-3** and **Figures C1-2** and **C1-3** 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.

Table C1-3 Determination of Solubility in Chemical Dilution Medium, DMSO, or ETOH

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)

Abbreviations: DMSO=Dimethyl sulfoxide; ETOH=Ethanol.
Note: The amounts of test substance weighed and Chemical Dilution Medium added may be modified from the amounts given above, provided that the targeted concentrations specified for each tier are tested.

Figure C1-2 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 • 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**

Abbreviations: DMSO=Dimethyl sulfoxide; ETOH=Ethanol.

Figure C1-3 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

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

3.4 Methods

3.4.1 <u>Tier 1</u>

- Tier 1 begins with testing 200 mg/mL in Chemical Dilution Medium (see **Table C1-3**).
  - Weigh approximately 100 mg (100,000 µg) of the test substance into a glass tube. Document the test substance weight.
  - Add approximately 0.5 mL of medium into the tube so that the concentration is 200,000 µg/mL (200 mg/mL).
  - Mix the solution as specified in **Annex I, Section 3.5**. If complete solubility is achieved, then additional solubility procedures are not needed.

3.4.2 <u>Tier 2</u>

- If the test substance is insoluble in Tier 1 at 200 mg/mL, then proceed to Tier 2.
  - Weigh approximately 10 mg (10,000 µg) of the test substance into a glass tube. Document the substance weight.
  - Add approximately 0.5 mL of medium into the tube so that the concentration is 20,000 µg/mL (20 mg/mL).
  - Mix the solution as specified in **Annex I, Section 3.5**. If complete solubility is achieved, then additional solubility procedures are not needed.

3.4.3 <u>Tier 3</u>

- If the test substance is insoluble in Chemical Dilution Medium, proceed to Tier 3.
  - 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.
  - 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 **Annex I, Section 3.5**.
  - 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 **Annex I, Section 3.5**.
  - If the substance is soluble in either solvent, no additional solubility procedures are needed.

3.4.4 <u>Tier 4</u>

- If the substance is insoluble in Chemical Dilution Medium, DMSO, or ETOH at Tier 3, then continue to Tier 4 in **Table C1-3**.

- o 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.
- o If the test substance does not dissolve, continue with Tier 5 and, if necessary, Tier 6 using DMSO and ETOH.

3.4.5 Tier 5

- 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.

3.4.6 Tier 6

- 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 **Annex I, Section 3.5** in an attempt to dissolve.
- If solubility is not achieved at Tier 3, then the solutions prepared in Tier 3 are diluted by 10 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 C1-2** and **C1-3** and **Table C1-3**).

3.5 Mechanical Procedures

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

- Add test substance to solvent as in Tier 1 of **Table C1-3**. (Test substance and solvent should be at room temperature.)
- Gently mix at room temperature. Vortex the tube (1 –2 minutes).
- If test substance has not dissolved, use waterbath sonication for up to 5 minutes.
- 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).

- Proceed to Tier 2 (and Tiers 3-6, if necessary of **Table C1-3** and repeat procedures 2-4).

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.

[This Page Intentionally Left Blank]

ANNEX II

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})

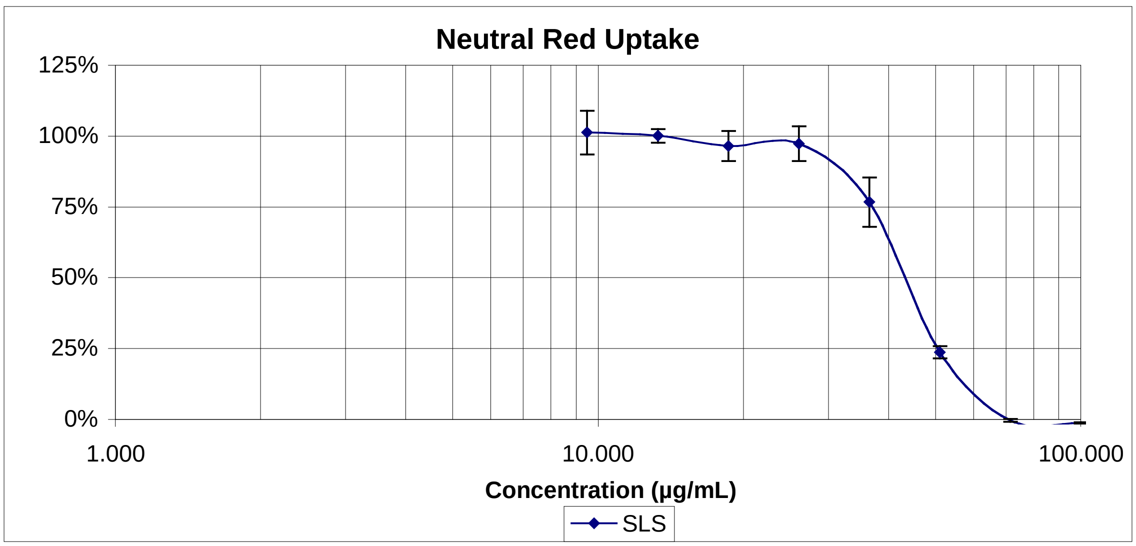
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.001	0.001	-0.002	0.002	0.002	0.001	0.005	0.008	0.009	-0.001	-0.002	0.001
B	-0.001	0.413	-0.004	-0.001	0.087	0.255	0.349	0.365	0.370	0.359	0.358	-0.001
C	0.000	0.364	-0.005	-0.003	0.087	0.249	0.337	0.333	0.365	0.332	0.342	0.001
D	0.000	0.395	-0.005	-0.001	0.104	0.292	0.363	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**			



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

[This Page Intentionally Left Blank]