

ICCVAM-Recommended Test Method Protocol

Normal Human Keratinocyte NRU Cytotoxicity Test Method

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In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for
Acute Oral Systemic Toxicity Tests”*

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

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APPENDIX C2

**TEST METHOD PROTOCOL FOR THE NHK NRU
CYTOTOXICITY TEST METHOD**

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ICCVAM Recommended Protocol for the Normal Human Epidermal Keratinocyte (NHK) 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 Normal Human Epidermal Keratinocyte (NHK) 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 the 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.

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

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), EC₅₀ 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 EC₅₀ is the equal to the IC₅₀.

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

IC₅₀: 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)

- Keratinocyte assay medium

5.3 Solvent Control

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

6.0 PROCEDURES

6.1 Materials

6.1.1 Cell Line

- Normal Human Epidermal Keratinocytes (NHK) cells. Non-transformed cells; from cryopreserved primary or secondary cells (e.g., Clonetics #CC-2507 or equivalent - Cambrex [Cambrex Bio Science, 8830 Biggs Ford Road, Walkersville, MD].¹

6.1.2 Technical Equipment²

- Incubator: 37 °C ±1 °C, 90% ±10% humidity, 5.0% ±1.0% CO₂/air
- Laminar flow clean bench/cabinet (standard: "biological hazard")
- Waterbath: 37 °C ±1 °C
- 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 cm², 25 cm²)
- 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Corning/COSTAR 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

¹ Keratinocytes should be procured only through commercial sources and not by preparing primary culture from donated tissues.

² Suggested brand names/vendors are listed in parentheses. Equivalents may be used.

Note: Prescreen tissue culture flasks and microtiter plates to ensure that they adequately support the growth of NHK 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.

6.1.3 Chemicals, Media, and Sera

- Keratinocyte Basal Medium without Ca⁺⁺ (e.g., KBM[®], Clonetics CC-3104) that is completed by adding supplements (e.g., KBM[®] SingleQuots[®], Clonetics CC-4131) to achieve the proper concentrations of epidermal growth factor, insulin, hydrocortisone, antimicrobial agents, bovine pituitary extract, and calcium (e.g., Clonetics Calcium SingleQuots[®], 300 mM CaCl₂, Clonetics CC-4202).
- HEPES Buffered Saline Solution (HEPES-BSS) (e.g., Clonetics # CC-5022)
- 0.025% Trypsin/Ethylenediaminetetraacetic acid (EDTA) solution (e.g., Clonetics # CC-5012)
- Trypsin Neutralizing Solution (TNS) (e.g., Clonetics # CC-5002)
- Phosphate Buffered Saline (PBS)
- Dulbecco's Phosphate Buffered Saline (D-PBS) (formulation containing calcium and magnesium cations; glucose optional)
- Neutral Red (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°C)
- 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
- Hanks' Balanced Salt Solution without Ca²⁺ or Mg²⁺ (CMF-HBSS) (e.g., Invitrogen # 14170)
- Distilled H₂O or any purified water suitable for cell culture and NR desorb solution (sterile)
- Sterile/non-sterile paper towels (for blotting 96-well plates)

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

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

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

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

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

KBM[®] SingleQuots[®] contain the following stock concentrations and volumes:

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

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

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

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., 0.33 g NR dye powder in 100 mL H₂O) 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:

1.0 mL (3.3 mg NR dye/mL)	NR Stock Solution
99.0 mL	Routine Culture Medium (pre-warmed to 37 °C)

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

Note: 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% H₂O

6.3 **Methods**

6.3.1 Cell Maintenance and Culture Procedures

- NHK cells are routinely grown as a monolayer in tissue culture grade flasks (e.g., 25 cm²) at 37 °C ±1 °C, 90% ±10% humidity, and 5.0% ±1.0% CO₂/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. Cells should not reach confluence.
- All cell culture studies should follow good cell culture practices (Hartung et al. 2002).

6.3.2 Receipt of Cryopreserved Keratinocyte Cells

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

6.3.3 Thawing Cells

- Thaw cells by putting ampules into a waterbath at 37 °C ± 1 °C. Leave for as brief a time as possible. Do not thaw cells at room temperature or by hand. Seed the thawed cells into culture flasks as quickly as possible and with minimal handling.
- Slowly (taking approximately 1-2 minutes) add 9 mL of pre-warmed Routine Culture Medium to the cells suspended in the cryoprotective solution and transfer cells into flasks containing pre-warmed Routine Culture Medium.
- Incubate at 37 °C ±1 °C, 90% ±10% humidity, and 5.0% ±1.0% CO₂/air.
- When the cells have attached to the bottom of the flask (within 4 to 24 hours), the Routine Culture Medium should be removed and replaced with fresh Routine Culture Medium.
- Unless otherwise specified, the cells should be incubated at 37 °C ±1 °C, 90% ±10% humidity, 5.0% ±1.0% CO₂/air and fed every 2-3 days until they exceed 50% confluence (but less than 80% confluent).

6.3.4 Subculture of NHK Cells to 96-Well Plates

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

- When the keratinocyte culture in a 25 cm² flask >50% confluence (but <80% confluent; cell should not be 100% confluent), remove the medium and rinse the culture twice with 5 mL HEPES-BSS. The first rinse may be left on the cells

for up to 5 minutes and the second rinse should remain on the cells for approximately 5 minutes. Discard the washing solutions.

- Add 2 mL trypsin/EDTA solution to each flask and remove after 15 to 30 seconds. Incubate the flask at room temperature for 3 to 7 minutes. When more than 50% of the cells become dislodged, rap the flask sharply against the palm of the hand.
- When most of the cells have become detached from the surface, rinse the flask with 5 mL of room temperature TNS. If more than one flask is subcultured, the same 5 mL of TNS may be used to rinse a total of up to two flasks.
- Then rinse the flask with 5 mL CMF-HBSS and transfer the cell suspension to a centrifuge tube.
- Pellet the cells by centrifugation for 5 minutes at approximately 220 x g. Remove the supernatant by aspiration.
- Resuspend the keratinocyte pellet by gentle trituration (to have single cells) in Routine Culture Medium. It is important to obtain a single cell suspension for exact counting. Count a sample of the cell suspension using a hemocytometer or cell counter.
- Prepare a cell suspension $\sim 1.6 - 2.0 \times 10^4$ cells/mL in Routine Culture Medium. Using a multi-channel pipette, dispense 125 μ L Routine Culture Medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate. In the remaining wells, dispense 125 μ L of the cell suspension ($2 \times 10^3 - 2.5 \times 10^3$ cells/well). Prepare one plate per substance to be tested (see **Figure C2-1**).
- Incubate cells ($37^\circ\text{C} \pm 1^\circ\text{C}$, 90% $\pm 10\%$ humidity, and 5% $\pm 1\%$ CO_2/air) so that cells form a 20+% monolayer ($\sim 48-72$ hours). This incubation period assures cell recovery and adherence and progression to exponential growth phase.
- Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the study workbook.

Figure C2-1 96-Well Plate Configuration for Positive Control (PC) and Test Substance Assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	C _{1b}	C _{2b}	C _{3b}	C _{4b}	C _{5b}	C _{6b}	C _{7b}	C _{8b}	VCb	VCb
B	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb
C	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb
D	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb
E	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb
F	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb
G	VCb	VC1	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC2	VCb
H	VCb	VCb	C _{1b}	C _{2b}	C _{3b}	C _{4b}	C _{5b}	C _{6b}	C _{7b}	C _{8b}	VCb	VCb

VC1 and VC2 = Vehicle control

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

C_xb = Blanks (Test substance or PC, but contain **no** cells)

VCb = VC blank (contain **no** cells)

6.3.5 Determination of Doubling Time

- Establish cells in culture and trypsinize cells as per **Section 6.3.4** for subculture. Resuspend cells in appropriate culture medium. Use **Table C2-1** to determine seeding densities.
- 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 °C ±1 °C, 90% ±10% humidity, 5.0% ±1.0% CO₂/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. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin). Determine the total number of cells and document.
- Repeat sampling at 24 hours, 48 hours, 72 hours, and 96 hours post inoculation. Change culture medium at 72 hours or sooner in remaining dishes if indicated by pH drop (i.e., pH <7).
- Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. The doubling time will be in the log (exponential) phase of the growth curve. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase).

Table C2-1 Guidelines for Establishing Cell Cultures¹

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

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

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 II** 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 II**. 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 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 for Range Finding Tests Solvent Using a Log Dilution Scheme

If DMSO is determined to be the preferred solvent at Tier 3 of the solubility test (i.e., 200,000 µg/mL), dissolve the substance in DMSO at 200,000 µg/mL for the chemical stock solution. 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 substance in each tube with 99 parts of culture medium (e.g., 0.1 mL of test substance in DMSO + 9.9 mL culture medium) to derive the eight 2X concentrations for application to NHK cells. Each 2X test substance concentration will then contain 1% (v/v) solvent.
 - The NHK cells will have 0.125 mL of culture medium in the wells prior to application of the test substance. By adding 0.125 mL of the appropriate 2X test substance concentration to the appropriate wells, the test substance will be diluted appropriately (e.g., highest concentration in well will be 1,000 µg/mL) in a total of 0.250 mL and the solvent concentration in the wells will be 0.5% (v/v).
- A test substance prepared in DMSO or ETOH may precipitate upon transfer into the Routine Culture Medium. The 2X dosing solutions should be evaluated for precipitates and the results recorded in the study workbook. It is permissible to test all of the dosing solutions in the dose range finding assay 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.

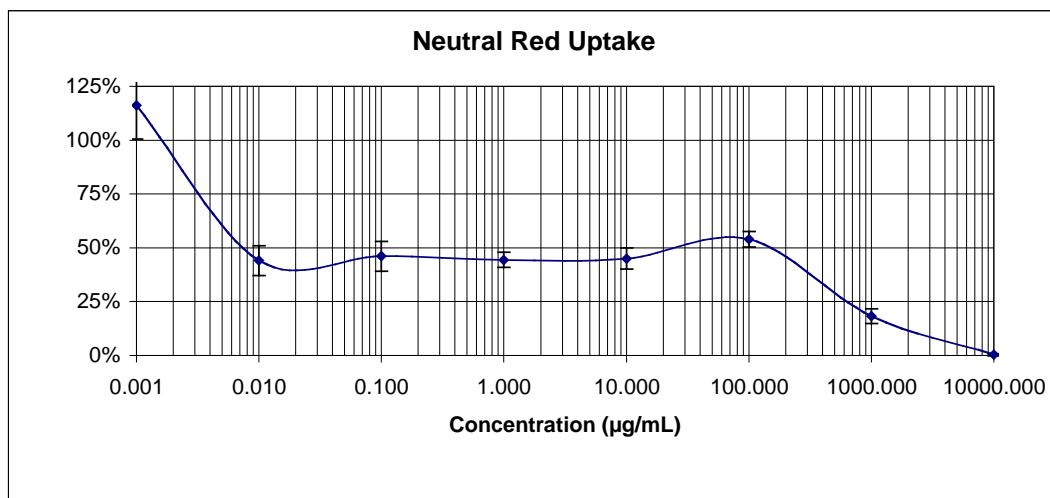
6.4.2 pH of Test Substance Solutions

- 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 C2-1**) 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 III** 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 °C ±1 °C, 90% ±10% humidity, 5.0% ±1.0% CO₂/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 CO₂ 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 C2-1** – the most toxic range is 0.001 – 0.1 µg/mL) that reduces viability to 50%.

Example C2-1 Biphasic Curve



- Main Test (Definitive Test)
 - 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 $\sqrt[6]{10} = 1.47$).
 - Cover the relevant concentration range around the IC₅₀ (>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 IC₅₀ value, avoiding too many non-cytotoxic and/or 100%-cytotoxic concentrations.

- Determine which test substance concentration is closest to the IC₅₀ value. Use that value as a central concentration and adjust dilutions higher and lower in equal steps for the definitive assay.
- The 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 Routine Culture 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 Routine Culture Medium will be added to the vessel so that the concentration is 200,000 µg/mL (200 mg/mL).
- The solution is mixed using the mechanical procedures that produced solubility when performing the solubility test (See **ANNEX II**).
- 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 II**.
- 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 II**.
- If complete solubility is achieved in the solvent, then seven additional serial stock dosing solutions may be prepared from the 500 mg/mL 200X stock.
- If the test substance is insoluble in solvent at 500 mg/mL, proceed by adding solvent, in small incremental amounts, to attempt to dissolve the substance by again using the sequence of mixing procedures. The highest soluble stock solution will be used to prepare the seven additional serial stock dosing solutions.

If precipitates are observed in the 2X dilutions, continue with the test 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 C2-2 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 NHK NRU assay for test substances will use the 96-well plate configuration shown in **Figure C2-1**.

6.5.2 Application of Test Substance

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 125 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 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 48 - 72 hours (i.e., after cells attain 20+ % confluency [see **Section 6.3.4**]) incubation of the cells, add 125 μ L of the appropriate concentration of test substance, the PC, or the VC (see **Figure C2-1** for the plate configuration) directly to the test wells. Do not remove Routine Culture Medium for re-feeding the cells.
 - The dosing solutions will be rapidly transferred from the 8-channel reservoir (or dummy plate) to the test plate using a single delivery multi-channel pipettor. For example, the VC may be transferred first (into columns 1, 2, 11, and 12), followed by the test substance dosing solutions from lowest to highest dose, so that the same pipette tips on the multi-channel pipettor can be used for the whole plate. The 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 solution for each concentration (e.g., wells A3 and H3 receive C₁ solution).
 - Incubate cells for 48 hours \pm 0.5 hours (37 °C \pm 1 °C, 90% \pm 10% humidity, and 5.0% \pm 1.0% CO₂/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 proposed so that a complete dose response curve, rather than a single point estimate, can be obtained. This will assist with troubleshooting, 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*³ in medium should be identified and noted.

- Use the following Visual Observations Codes (**Table C2-2**) 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.

Table C2-2 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 Routine Culture Medium with test substance 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 $^{\circ}$ C \pm 1 $^{\circ}$ C, 90% \pm 10% humidity, and 5.0% \pm 1.0% CO₂/air) for 3 hours \pm 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 μ L pre-warmed D-PBS.
- Decant and blot D-PBS from the plate.
- Add exactly 100 μ L NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.

³ 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.

- 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 \pm 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 0.043 - 0.059 for the VC blanks is a target range of ODs but not a test acceptance criterion (range = mean OD \pm 2.5 standard deviations; mean = 0.054; SD = 0.003; N = 114).⁴

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 NHK NRU Assay

6.5.5.1 *Test Acceptance Criteria for PC*

- All acceptance criteria must be met by the PC for a test to be acceptable.
 - The PC (SLS) IC₅₀ must be within \pm 2.5 standard deviations (SD) of the historical mean established by the Test Facility and must have an R² (coefficient of determination) \geq 0.85 (calculated for the Hill model fit using PRISM[®] software). The NICEATM/ECVAM validation study generated the following PC data (ICCVAM 2006):
 - IC₅₀ mean = 3.11 μ g/mL; SD = 0.72 (n = 114)
 - Range for IC₅₀ mean \pm 2.5 SD = 1.31 μ g/mL – 4.91 μ g/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 \leq 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 \leq 50% viability and at least one calculated cytotoxicity value $>$ 50% and $<$ 100% viability must be present.

⁴ Data from NICEATM/ECVAM validation study (ICCVAM 2006).

Note: A corrected mean $OD_{540 \pm 10nm}$ of 0.205 – 1.645 for the VCs is a target range of ODs but not a test acceptance criterion (range = mean OD ± 2.5 standard deviations; mean = 0.685; SD = 0.175; N = 114).⁵

Exception

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

Stopping Rule for Insoluble Substances

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

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 may also 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).

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

- 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**.

6.6 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 IC₅₀ values by linear interpolation, and perform statistical analyses (including statistical identification of outliers) (see **ANNEX III** for an example spreadsheet template).
- A Hill function analysis should be performed using statistical software (e.g., GraphPad PRISM[®]) and a template to calculate IC₂₀, IC₅₀, and IC₈₀ 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 IC₅₀ 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 EC₅₀ reported by the Hill function is not the same as the IC₅₀ since the Hill function defines EC₅₀ 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 IC₅₀ as follows.

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

where IC₅₀ is the concentration producing 50% toxicity, EC₅₀ 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 REFERENCE MATERIALS

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ANNEX I

TEST METHOD PROCEDURE

Prequalification of Normal Human Epidermal Keratinocyte Growth Medium

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

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

1.0 TEST SYSTEM

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

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

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

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

2.0 PROCEDURES

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

2.1 Materials

See **Section 6.1** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method.

2.2 Preparation of Media and Solutions

See **Section 6.2** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method.

2.3 Methods

See **Section 6.3** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method.

- NHK cultures should be established with cryopreserved cells seeded into individual tissue culture 25 cm² flasks using a proven medium/supplement combination (i.e., the control medium) and each test medium/supplement combination.
- Suspend freshly thawed cells initially into 9 mL of control medium and then add the cell suspension to 25 cm² culture flasks containing pre-warmed control or test medium. Cell seeding densities (1 flask/density/medium) of 1 x 10⁴, 5 x 10³, and 2.5 x 10³ are recommended.
- The cells should be subcultured on three different days into 96-well plates (see **Table C2-3**) for three subsequent NRU tests (three test plates total [one plate per day] for each medium/supplement combination and each control).

Table C2-3 Subculture Protocol

Flask	Subculture: 1 Test Plate and 1 Control Plate	Application of SLS
#1 (1 x 10 ⁴ cells/mL)	Day A	Day X
#2 (5 x 10 ³ cells/mL)	Day B	Day Y
#3 (2.5 x 10 ³ cells/mL)	Day C	Day Z

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

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

2.4 Doubling Time

See **Section 6.3.5** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method. A doubling time experiment may be considered as an additional quality assurance check.

2.5 Preparation of SLS

See **Section 6.4.1** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method.

- Preparation of SLS concentrations/dilutions should follow the main test (definitive assay) procedures specifically for testing compounds in Routine Culture Medium as outlined in **Section 6.4.3** of the Test Method Protocol for the NHK NRU Cytotoxicity Test Method.
- The concentrations/dilutions should be the same or similar to those used previously with control medium/supplements.
- SLS concentration ranges used by three laboratories in the NICEATM/ECVAM validation study were 20.0 µg/mL – 1.4 µg/mL and 10.0 µg/mL – 0.6 µg/mL (ICCVAM 2006).

2.6 Test Procedure

See **Sections 6.5.1, 6.5.2, and 6.5.4** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method.

- The C₁ test concentration will be the highest SLS concentration and C₈ the lowest concentration.
- Cells cultured in control medium and in each test medium/supplement combination should be tested in parallel for their sensitivity to SLS (see **Annex I, Section 2.3**).
- Each of the three test plates of the new medium/supplement combinations is considered a replicate test plate.

2.7 Microscopic Evaluation

See **Section 6.5.3** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method.

Changes in morphology of the cells due to cytotoxic effects of the SLS (prior to measurement of NRU) should be recorded as per procedures outlined in **Section 6.5.3** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method. In addition to the general microscopic evaluation of the cell cultures, the Study Director should make the following specific observations:

- *General culture observations*
 - Rate of proliferation (e.g., rapid, fair, slow)
 - Percent confluence (e.g., daily estimate)
 - Number of mitotic figures (e.g., average per field)
 - Contamination (present/not present)
- *Cell morphology observations*
 - Overall appearance (e.g., good, fair, poor)
 - Colony formation (e.g., tight/defined, fair, loose/migrating)
 - Distribution (e.g., even/uneven)
 - Abnormal cells (e.g., enlarged, vacuolated, necrotic, spotted, blebby - [average per field])

2.8 Data Analysis and Test Evaluation

See **Section 6.6** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method. Test Acceptance Criteria in **Section 6.5.5** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method should be used to determine acceptability of a test plate. Other criteria that should be considered by the Study Director includes the following:

- Mean corrected OD₅₄₀₋₅₅₀ of the VCs. Note: The target range for corrected mean OD_{540 ±10nm} = 0.248 - 1.123 for the VCs, but it is not a test acceptance criterion (range = mean OD ±2.5 standard deviations; mean = 0.685; SD = 0.175; N = 114).⁶
- Cell morphology and confluence of the VCs at the end of the 48 hour treatment
- Doubling time

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

⁶ Data from NICEATM/ECVAM validation study (ICCVAM 2006).

ANNEX II

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 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 or using a microscope. A test substance has dissolved if the solution is clear and shows no signs of cloudiness or precipitation.

3.0 PROCEDURES

3.1 Materials

See **Section 6.1** of Test Method Protocol for the NHK NRU Cytotoxicity Test Method.

3.2 Preparation of Media and Solutions

See **Section 6.2** of Test Method Protocol for the NHK 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 II, Section 3.5. Table C2-4** and **Figures C2-2** and **C2-3** 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 C2-4 Determination of Solubility in Routine Culture Medium, DMSO, or ETOH

Tier	1	2	3	4	5	6
Total Volume Routine Culture Medium	0.5 mL	0.5 mL	5 mL	50 mL		
Concentration of Test Substance Tier 1: Add ~ 100 mg to a tube. Add enough medium to equal Tier 1 volume. If insoluble, go to Tier 2. Tier 2: Add ~10 mg to another tube. Add enough medium to equal the first volume. Dilute to subsequent volumes if necessary.	200,000 µg/mL (200 mg/mL)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	200 µg/mL (0.20 mg/mL)		
Total Volume DMSO/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 Routine Culture Medium added may be modified from the amounts given above, provided that the targeted concentrations specified for each tier are tested.

Figure C2-2 Solubility Step-Wise (Tiered) Procedure

TIER 1

STEP 1:	200 mg/mL test substance (TS) in 0.5 mL Routine Culture Medium <ul style="list-style-type: none"> • if TS soluble in medium, then STOP. • if TS insoluble in medium, then go to STEP 2.
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TIER 2

STEP 2:	20 mg/mL TS in 0.5 mL Routine Culture Medium <ul style="list-style-type: none"> • if TS soluble, then STOP. • if TS insoluble, then go to STEP 3.
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TIER 3

STEP 3:	200 mg/mL TS in DMSO <ul style="list-style-type: none"> • if TS soluble, then STOP. • if TS insoluble, test at 200 mg/mL in ETOH. <ul style="list-style-type: none"> – if TS soluble, then STOP. – If TS insoluble, go to STEP 4.
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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) <ul style="list-style-type: none"> • 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). <ul style="list-style-type: none"> – 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). <ul style="list-style-type: none"> ▪ if TS soluble, then STOP. ▪ if TS insoluble, then go to STEP 5.
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TIER 5

STEP 5:	2 mg/mL TS in DMSO – increase volume from STEP 4 by 10 (i.e., to 50 mL) <ul style="list-style-type: none"> • 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). <ul style="list-style-type: none"> – if TS soluble, then STOP. – if TS insoluble, then go to STEP 6.
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TIER 6

STEP 6:	0.2 mg/mL TS in 50 mL DMSO <ul style="list-style-type: none"> • if TS soluble, then STOP. • if TS insoluble, test at 0.2 mg/mL in 50 mL ETOH <ul style="list-style-type: none"> – STOP
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Abbreviations: DMSO=Dimethyl sulfoxide; ETOH=Ethanol.

Figure C2-3 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				
Concentration in DMSO					200 mg/mL		20 mg/mL		2 mg/mL		0.2 mg/mL
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

* NHK medium - Keratinocyte Growth Medium (e.g., KGM[®] from Cambrex) for normal human epidermal keratinocytes.

3.4 Methods

3.4.1 Tier 1

- Tier 1 begins with testing 200 mg/mL in Routine Culture Medium (see **Table C2-4**).
 - 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 II, Section 3.5**. If complete solubility is achieved, then additional solubility procedures are not needed.

3.4.2 Tier 2

- 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 II, Section 3.5**. If complete solubility is achieved, then additional solubility procedures are not needed.

3.4.3 Tier 3

- If the test substance is insoluble in Routine Culture 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 II, 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 II, Section 3.5**.
 - If the substance is soluble in either solvent, no additional solubility procedures are needed.

3.4.4 Tier 4

- If the substance is not soluble in Routine Culture Medium, DMSO, or ETOH at Tier 3, then continue to Tier 4 in **Table C2-4**.
 - 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.
 - 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 Routine Culture Medium at Tier 2 using the mixing procedures, then the procedure continues to Tier 3 by diluting the solution to 5 mL with medium and mixing again.
- If the substance is not soluble in Routine Culture Medium, two samples of ~ 100 mg test substance are weighed to attempt to solubilize in DMSO and ETOH at 200,000 µg/mL (i.e., 200 mg/mL). Solutions are mixed following the sequence of procedures prescribed in **Annex II, 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 so as to test 200 µg/mL in media, and 20,000 µg/mL in DMSO and ETOH. This advances the procedure to Tier 4. Solutions are again mixed in an attempt to dissolve.
- If solubility is not achieved in Tier 4, the procedure continues to Tier 5, and to Tier 6 if necessary (see **Figures C2-2 and C2-3** and **Table C2-4**).

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 C2-4**. (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 CO₂ incubator at 37 °C. The solution may be stirred during warming (stirring in a CO₂ incubator will help maintain proper pH).

- Proceed to Tier 2 (and Tiers 3-6, if necessary of **Table C2-4** and repeat procedures 2-4).

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

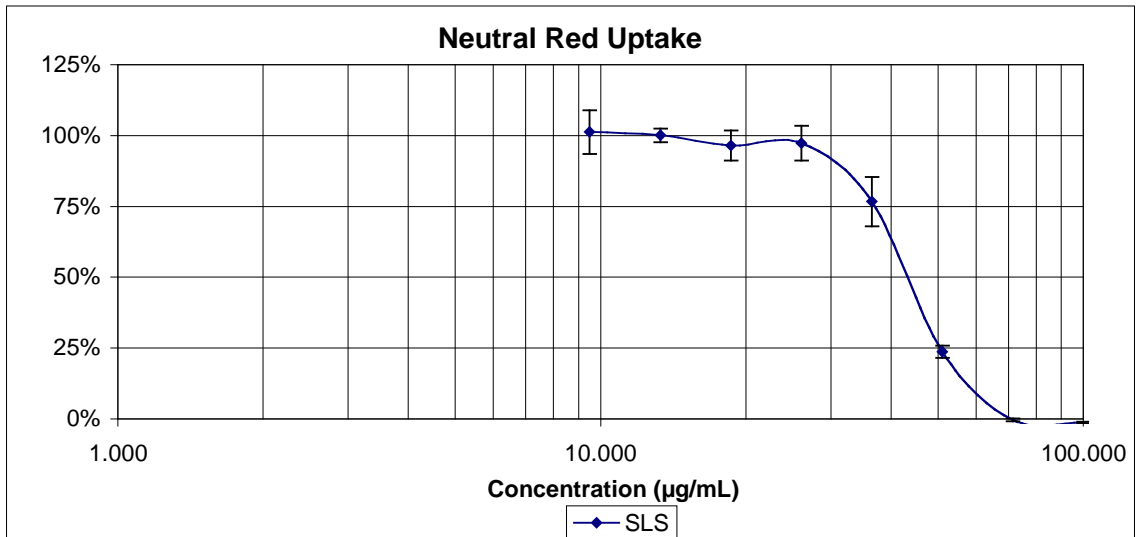
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ANNEX III

Microsoft EXCEL® Example Spreadsheet Template

Test Facility : A												
Chemical Code : SLS												
2nd Chem. Code* : 11												
	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													
ENTER CODES:		VC	C1	C2	C3	C4	C5	C6	C7	C8			
		1	4	4	3	2	1	1	1	1			
Interpolated IC₅₀ :						4.32E+01		µg/mL					



TEST CHEMICAL																									
Test Facility : A			Study Number.: A1																						
Chemical Code : SLS			96-Well Plate ID : A11																						
2 nd 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 : <input type="checkbox"/> Vortexing <input type="checkbox"/> sonication <input type="checkbox"/> 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)																									
<table border="1"> <thead> <tr> <th>C1</th> <th>C2</th> <th>C3</th> <th>C4</th> <th>C5</th> <th>C6</th> <th>C7</th> <th>C8</th> </tr> </thead> <tbody> <tr> <td>100</td> <td>71.4</td> <td>51.0</td> <td>36.4</td> <td>26.0</td> <td>18.6</td> <td>13.3</td> <td>9.49</td> </tr> </tbody> </table>										C1	C2	C3	C4	C5	C6	C7	C8	100	71.4	51.0	36.4	26.0	18.6	13.3	9.49
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 IC ₂₀ :		1.551E+00 µg/mL		log IC ₅₀ :		1.635E+00 µg/mL		log IC ₈₀ :		1.718E+00 µg/mL															
IC ₂₀ :		3.56E+01 µg/mL		IC ₅₀ :		4.32E+01 µg/mL		IC ₈₀ :		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																			