Appendix D
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1.0 STANDARD OPERATING PROCEDURE (SOP) FOR THE NORMAL HUMAN EPIDERMAL KERATINOCYTE NEUTRAL RED UPTAKE CYTOTOXICITY TEST - A TEST FOR BASAL CYTOTOXICITY

1.1 Background and Introduction

This SOP, based on a NRU assay by Borenfreund and Puerner (1984) using epidermal keratinocytes (Heimann and Rice, 1983), was obtained from the Institute of *In Vitro* Sciences (IIVS). Formulations for the media and solutions correspond to Clonetics® products by BioWhittaker, Inc. For the present purpose of being a recommended standard test for basal cytotoxicity, the protocol from IIVS was embellished by adding details on equipment, media and reagent components, and experimental procedure to make it easier for novice users to follow. For the specific purpose of this guidance document, the RC regression for prediction of acute oral systemic rodent toxicity (Halle, 1998; Spielmann et al., 1999) is included as the prediction model in Section 1.8.

1.2 Rationale

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

Healthy normal human keratinocytes (NHK) cells, when appropriately maintained in culture in a sub-confluent state, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in cell death and/or a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the vital dye, NR, after two days of chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

1.3 Basic Procedure

NHK cells are seeded into 96-well plates and maintained in culture until cells form a 30-50% confluent monolayer. They are then exposed to the test compound over a range of six to eight concentrations. After 48 hours (h) exposure, NRU is determined for each treatment concentration and compared to that determined in control cultures. For each treatment (i.e., concentration of the test chemical) the percent inhibition of growth is calculated. The IC_{50} (a.k.a., NRU_{50}, the concentration producing 50% reduction of NR uptake) is calculated from the concentration-response and expressed as µg/ml or mmol/l.

1.4 Test Limitations

- Volatile chemicals tend to evaporate under the conditions of the test; thus the IC_{50} may be variable, especially when the toxicity of the compound is fairly low. This can be overcome if plates are sealed with CO_2 permeable plastic film, which is impermeable to volatile chemicals.
- Materials that are not readily soluble in serum-free aqueous media may be difficult to test, and their *in vivo* toxicity potentially underestimated.
- Other chemicals that are difficult to test include those that are unstable or explosive in water.
- The *in* vivo toxicity of substances that specifically attack dividing cells may be overestimated.
- It is possible that low cell viability readings may result in those cases where a chemical has a relatively selective effect upon the

lysosomes/endosomes of the cell. An example of this would be chloroquine sulphate, which alters the pH of lysosomes/endosomes, an effect that inhibits NRU.

- Red chemicals absorbing in the range of NR might interfere with the test, provided they are present in sufficient amounts within the cells after washing, and are soluble in the NR solvent.

1.5 Material

1.5.1 *Cell Lines*

NHK cells (e.g., **Clonetics #CC-2507** for cryopreserved cells or **Clonetics #CC-2607** for proliferating cells**,** BioWhittaker, Inc., USA)

1.5.2 *Technical Equipment*

- Incubator: 37° ± 1°C, humidified, 5 ± 1 % CO_2/air
- Laminar flow clean bench (standard: "biological hazard")
- Water bath: 37° ± 1°C
- Inverse phase contrast microscope
- Centrifuge
- Laboratory balance
- 96-Well plate photometer equipped with 540 or 550 nm filter
- Shaker for microtiter plates
- Cell counter or hemocytometer
- Pipetting aid
- Pipettes, 8-channel-pipettes, dilution block
- Cryotubes
- Tissue culture flasks (80 cm^2, 25 cm^2)
- 96-Well tissue culture microtiter plates (e.g., Nunc # 167 008)

Note: Tissue culture flasks and microtiter plates should be prescreened to ensure that they adequately support the growth of NHK.

1.5.3 *Chemicals, Media, and Sera*

- Keratinocyte Growth Medium (KGM) complete with epidermal growth factor, insulin, hydrocortisone, antimicrobial agents and supplemented with bovine pituitary extract (e.g., Clonetics # CC-3001)
- HEPES Buffered Saline Solution (HEPES-BSS) (e.g., Clonetics # CC-5022)
- 0.025% Trypsin/EDTA solution (e.g., Clonetics # CC-5012)
- Trypsin Neutralizing Solution (TNS) (e.g., Clonetics # CC-5002)
- Phosphate Buffered Saline (PBS)
- 10% fetal bovine serum (FBS)
- Neutral Red (NR)
- Dimethyl sulfoxide (DMSO), analytical grade
- Ethanol (ETOH), analytical grade
- Glacial acetic acid, analytical grade
- Hanks' Balanced Saline Solution without Ca^{2+} or Mg^{2+} (CMF-HBSS) (e.g., Invitrogen # 14170)
- Formaldehyde
- Calcium chloride
- Distilled H_2O or any purified water suitable for cell culture

1.5.4 *Preparations*

Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, 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).

1.5.4.1 ***Culture and Treatment Medium***

KGM supplemented with**:**

0.1 ng/ml	Human recombinant epidermal growth factor
5 g/ml	Insulin
0.5 g/ml	Hydrocortisone
50 g/ml	Gentamicin
50 ng/ml	Amphotericin B
0.15 mM	Calcium
2 ml	7.5 mg/ml Bovine pituitary extract

Complete media should be kept at 4°C and stored for no longer than two weeks.

1.5.4.2 ***Neutral Red (NR) Stock Solution***

0.4 g	NR Dye
100 ml	H_2O

Make up prior to use and store dark at room temperature for up to two months.

1.5.4.3 ***Neutral Red (NR) Medium***

1 ml	NR Stock Solution
79 ml	KGM

Note: The NR medium should be incubated overnight at 37ºC and centrifuged at 600 x g for 10 min (to remove NR crystals) before adding to the cells. Alternative procedures (e.g., Millipore filtering) can be used as long as they guarantee that NR medium is free of crystals.

1.5.4.4 ***Wash/Fix Solution***

0.5%	Formaldehyde
1.0%	Calcium chloride
98.5%	H_2O

1.5.4.5 ***Ethanol/Acetic Acid Solution (NR Desorb)***

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

Prepare immediately prior to use. Do not store for longer than 1 h.

1.5.4.6 ***Preparation of Test Chemicals***

1. The test article should be dissolved in KGM, deionized distilled water, ETOH, DMSO, acetone, or other appropriate solvent. Other solvents may be used provided they have been tested to be non-cytotoxic at the final concentration used in the test. If the solvent is something other than KGM, a 100X concentrate of each desired final concentration of test article should be prepared. This 100X concentrated dosing solution is then diluted 1:100 directly into sterile pre-warmed (37°C) KGM. This should ensure that the final solvent concentration in culture wells should not exceed 1% (v/v) in the vehicle controls and in all of the eight test concentrations. Check carefully to determine whether the chemical is still dissolved after the transfer from solvent stock solution to medium, and reduce highest test concentration, if necessary. The stability of the test article under the actual experimental conditions should be determined for each experiment.
2. Measure the pH of the highest concentration of the test chemical. If strong acids or bases have changed the pH of the medium, they should be neutralized with 0.1N NaOH or 0.1N HCI. In this case, prepare highest concentration of the chemical in ~ 80% of final volume, measure pH, neutralize, and add KGM to desired final volume.
3. Vortex mixing and/or sonication and/or warming to 37°C may be used, if necessary, to aid solubilization. The concentrations used for relatively insoluble chemicals should range from the soluble to the precipitating dose.

Note: Test chemical must be freshly prepared immediately prior to use. Preparation under red light may be necessary, if rapid photodegradation is likely to occur.

1.6 Methods

A good discussion of the techniques used in the multiple-well plate assays, such as those described in this section, is given by Harbell (2001).

1.6.1 *Cell Maintenance and Culture Procedures*

1.6.1.1 *Receipt of Keratinocytes*

Upon receipt of proliferating keratinocytes, the cultures will be observed microscopically for signs of distress (e.g., floating cells, excessive debris, or lack of mitotic figures). Cultures exhibiting these properties will be discarded. Then perform the following:

- Decontaminate the outside of the culture flasks with 70% ETOH.
- Incubate the cultures at 37º ± 1ºC for a minimum of 60 minutes (min) to allow the temperature of the medium to equilibrate.
- Aseptically remove the medium and replace with fresh KGM warmed to approximately 37º C.
- Unless otherwise specified, the cultures are then incubated at 37º ± 1ºC and 5 ± 1% CO_2 in air.

Upon receipt of cryopreserved keratinocytes, the cells should be stored in liquid nitrogen.

1.6.1.2 *Thawing Cryopreserved Keratinocytes*

- Thaw cells by putting ampules into a water bath at 37°C for as brief a time as possible. Do not thaw cells at room temperature or by hand. Seed the thawed cells into culture flasks as quickly as possible and with minimal handling.
- Slowly (taking approximately 1-2 min) add 9 ml of KGM to the cells suspended in the cryoprotective solution and transfer 3500 cells/cm^2 into flasks containing routine pre-warmed culture medium.
- Incubate the cultures at 37º ± 1ºC until the cells attach to the flask, at which time the KGM should be removed and replaced with fresh KGM.
- Unless otherwise specified, the cells should be incubated at 37º ± 1ºC and 5 ± 1% CO_2 in air and fed every 2-3 days until they are 50-80% confluent.

1.6.1.3 *Subculturing the Keratinocytes*

- When the keratinocyte culture in a 25 cm^2 flask is 50 to 80% confluent, remove the medium and rinse the culture twice with 5 ml HEPES-BSS. Discard the washing solution.
- 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 min. 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.
- 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 min at approximately 220 x g. Remove the supernatant by aspiration.
- Resuspend the keratinocyte pellet by gentle trituration (to have single cells) in KGM. Count a sample of the cell suspension obtained using a hemacytometer (Trypan Blue exclusion) or cell counter.
- Prepare a suspension of 0.8 to 1.0x10^4 cells/ml in KGM. Transfer the cells into flasks containing pre-warmed growth medium at 3500 cells/cm^2. The keratinocyte cultures

may be sustained through approximately three passages.

For subculturing into 96-well plates, obtain the cell suspension as described above. Add 250 µl cell suspension to the appropriate wells on each 96-well plate. (Note: evaporation of the medium can be a problem; therefore, the edge wells should receive 250 µl PBS. Incubate the cultures in a humidified incubator at $37^o \pm 1^oC$ and $5 \pm 1\%$ CO_2 in air.

1.6.1.4 *Freezing Keratinocytes*

- Harvest the cells as above and resuspend the single cells in cold freezing solution (e.g., 80% growth medium, 10% fetal bovine serum [FBS], 10% DMSO) at $5x10^5$ to $2x10^6$ cells per ml. Aliquot to freezing vials.

- Insulate the vials and place into a -70^oC freezer overnight (12-24 h).

- Place vials into liquid nitrogen for storage.

1.6.2 *Quality Check of Assay (I): Positive Control (PC)*

Of the many chemicals backed by sufficient history or intra- and interlaboratory repeat tests (e.g., those shown in Section 3.2 of the report) **Sodium Lauryl Sulfate (SLS, CAS # 151-21-3)** is one of the most frequently tested, and is therefore recommended as a PC. If a laboratory has not built a historical database on SLS, it is recommended that SLS be tested in a full-scale concentration-response test (at six to eight concentrations), according to Section 1.6.5.2, concurrently with each test article experiment. Once historical data prove reproducibility, the PC might be applied in just one concentration (IC_{50}) on the same plate together with the test chemical (also noted in Section 1.6.6). For the latter procedure, two standard deviations of the IC_{50} for SLS is the acceptance criterion for test sensitivity.

A test meets acceptance criteria, if the IC_{50} for SLS is within 2 standard deviations of the historical mean.

1.6.3 *Quality Check of Assay (II): Vehicle Control (VC)*

The absolute value of optical density (OD_{540} of NRU) obtained in the untreated vehicle control indicates whether the 0.8 to $1x10^4$ cells/ml seeded in each well have grown exponentially with normal doubling time during the three to five days of the assay.

A test meets acceptance criteria if the mean OD_{540} of VCs is ≥ 0.3

To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row **2**) and the right side (row **11**) of the 96-well plate (see Appendix E):

A test meets acceptance criteria if the left and the right mean of the VCs do not differ by more than 15% from the mean of all VCs.

Checks for cell seeding errors may also be performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent. Microscopic evaluation obviates the need for two rows of VCs.

1.6.4 *Quality Check of Concentration-Response*

If possible, the test article concentrations for the definitive assay will be chosen such that at least six treatments will be available for the determination of the IC_{50}. Ideally, two concentrations should result in expected survivals lower than 50%, one concentration should result in an expected survival of approximately 50% and two concentrations should result in expected survivals greater than 50%.

1.6.5 *Concentrations of Test Chemical*

1.6.5.1 *Range Finder Experiment*

In this preliminary NR bioassay, six to eight decreasing concentrations of the test material are selected based upon the available information for the test material. The test article should be dissolved in KGM, water, DMSO, acetone,

ETOH, or other appropriate solvent. The maximum solvent concentration (other than water or KGM) should be 1%. One way to determine concentrations of the chemical to be tested is to dilute the stock solution several times by a constant factor (e.g., $\sqrt[2]{10} = 3.16$, see Appendix F), covering a large range, e.g.:

1 ⇒3.16⇒10 ⇒31.6 ⇒100 ⇒316⇒ 1000 ⇒ 3160 µg/ml

1.6.5.2 *Main Experiment*

Depending on the slope of the concentration-response curve estimated from the range finder, the dilution/progression factor in the concentration series of the main experiment should be smaller (e.g., $\sqrt[6]{10} = 1.47$) to avoid too many non-cytotoxic and/or 100%-cytotoxic concentrations. Experiments revealing less than three cytotoxic concentrations in the relevant range shall be repeated, **where possible**, with a smaller dilution factor. (Taking into account pipetting errors, a progression factor of 1.21 is regarded the smallest factor achievable.)

1.6.6 *Test Procedure*

See Table D.1 for a flowchart of the test procedure. Appendix G contains a template recommended for documenting the relevant data.

1st day *after growing up the cells from frozen stock:*

1. Prepare a suspension of 0.8-1x10^4 cells/ml in KGM. Using a multi-channel pipette, dispense 250 µl cell suspension to the appropriate wells on each 96-well tissue culture microtiter plate. [Note: evaporation of the medium can be a problem; therefore, the edge wells should receive 250 µl PBS for blanks.] Prepare one plate per chemical to be tested and one plate for the PC.

[Note: Individual plates for the PC are for establishing an historical database. Once an IC_{50} mean has been determined, only that PC concentration need be included in the test material plate.]

2. Incubate cells (37 ± 1°C and 5 ± 1% CO_2) until a 30-50% confluent monolayer is produced (~24-72 h). This incubation period assures cell recovery and adherence and progression to the exponential growth phase.

3. 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 systematic cell seeding errors.

2nd day:

1. After 24-72 h incubation, remove culture medium from the cells by inverting the uncovered 96-well plates over a liquid discard container and then gently blotting the plates several times on sterile paper towels.

2. Immediately add 125 µl fresh KGM to each well. Add 125 µl of the test article dilutions, positive control dilutions and solvent control dilution to the appropriate wells. Wells designated as blanks receive 125 µl KGM.

3. Incubate cells for 48 h (37 ± 1°C and 5 ± 1% CO_2).

3rd day:

A) Microscopic Evaluation

After 48 h treatment, examine each plate under a phase contrast microscope to identify test chemical precipitate, systematic cell seeding errors and growth characteristics of control and treated cells. Record changes in morphology of the cells due to cytotoxic effects of the test chemical, but do not use these records for the calculation of any quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells may indicate experimental error and may be cause for rejection of the assay.

B) Measurement of NRU

This method is based upon that of Ellen Borenfreund (Borenfreund and Puerner, 1985). The uptake of the NR into the lysosomes/endosomes and vacuoles of living cells

is used as a quantitative indication of cell number and viability.

1. Remove the treatment medium and add 250 µl NR medium to each well, except for blanks, which receive 250 µl KGM. Incubate at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 for 3 h.

2. After incubation, decant the NR medium, and add 250 µl Wash/Fix solution to each well.

3. After 2 min, decant and add 100 µl NR Desorb solution to all wells, including blanks.

4. Shake microtiter plate rapidly on a microtiter plate shaker for a minimum of 20 min at room temperature.

5. Measure the absorption of the resulting colored solution at **540-550 nm** in a microtiter plate reader, using the blanks as a reference. Save raw data in a file format (e.g., ASCII, TXT, XLS) appropriate for further analysis of the concentration-response and calculation of IC_{50}.

Table D.1. NHK NRU Cytotoxicity Test: Flow Chart

ASSAY PHASE	PROCEDURE
CELL GROWTH (24:00-72:00 h) [30–50% monolayer confluency]	• Seed 96-well plates: 2.0 to 2.5 x 10^3 cells/250 µl KGM culture medium/well • Incubate (37° ± 1°C, 5 ± 1% CO_2, 24-72 h)
TEST MATERIAL TREATMENT (48:00 h)	• Remove culture medium/add fresh KGM culture medium (125 µl/well) • Treat cells with 6-8 concentrations of test material in treatment medium (125 µl/well) [test material is 2X concentration before adding to wells] for 48 h treatment
PRELIMINARY NEUTRAL RED BIOASSAY (3:00 hours)	• Microscopic evaluation of morphological alterations • Remove treatment medium and add 250 µl/well NR medium • Incubate (37° ± 1°C, 5 ± 1% CO_2, 3 h)
NEUTRAL RED BIOASSAY (0:20 hours)	• Discard NR medium • Add 250 µl/well Wash/Fix solution for 2 min • Remove Wash/Fix solution • Add 100 µl/well NR Desorb (ETOH/acetic acid solution) • Shake plate for 20 min • Detect NR absorption at $OD_{540\text{-}550}$

1.7 Data Analysis

A calculation of cell viability expressed as NRU is made for each concentration of the test chemical by using the mean NRU of the six to eight replicate values per test concentration. This value is compared with the mean NRU of all VC values (provided VCs have met the VC acceptance criteria). Relative cell viability is then expressed as percent of untreated VC. If achievable, the six to eight concentrations of each compound tested should span the range of no effect up to total

inhibition of cell viability.

Where possible, the concentration of a test chemical reflecting a 50% inhibition of cell viability (IC_{50}) is determined from the concentration-response. This can be done either by applying the following:

- a manual graphical fitting method. The use of probability paper with "x = log" and "y = probit" scales is recommended because in most cases the concentration-response function will become almost linear in the relevant range. Alternatively, semi-log paper could also be used for this technique.

  or

- any appropriate non-linear regression procedure (preferably a Hill function* or a logistic regression) to the concentration-response data. Before using the IC_{50} for further calculations, the quality of the fit should be appropriately checked. (* = Hill functions are monotonous and sigmoidal in shape and represent an acceptable model for many dose response curves.)

More sophisticated programs specially developed for concentration-response analysis from 96-well plates can also be used.

Before using the IC_{50} information in any subsequent estimation of rodent LD_{50}, be sure that the IC_{50} data are expressed as mmol/l since the prediction model described in this guidance document is based on the relationship between the LD_{50} (in mmol/kg) and the IC_{50} (in mmol/l).

1.8 Prediction Model

In general, basal cytotoxicity is highly valuable information *per se*, which can be used in combination with other information, e.g., bioavailability, for many purposes in the process of safety or risk evaluation. For the purpose of this document, basal cytotoxicity is to be used to predict starting doses for *in vivo* acute oral LD_{50} values in rodents. After testing the reference chemicals recommended in Section 3.2 of this guidance document and qualifying the test as described in Section 3.1 (see Section 3.3 of the report for examples with two different cell types), best estimates of starting doses for *in vivo* acute oral toxicity tests are predicted according to the following prediction model:

$$\log (LD_{50}\ [\text{mmol/kg}]) = 0.435 \times \log (IC_{50}\ [\text{mmol/l}]) + 0.625$$

1.9 References

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