Appendix C
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1.0 STANDARD OPERATING PROCEDURE (SOP) FOR THE BALB/C 3T3 NEUTRAL RED UPTAKE CYTOTOXICITY TEST - A TEST FOR BASAL CYTOTOXICITY

1.1 Background and Introduction

The present *in vitro* SOP based on Borenfreund and Puerner (1985) was originally elaborated in 1990 by ZEBET (German National Centre for the Documentation and Evaluation of Alternatives to Animal Experimentation) in co-operation with participants of the German BMFT (Ministry of Research and Technology) sponsored "BGA (Federal Health Institute) eye irritation validation study" (Spielmann et al., 1991). The SOP was used in the second phase of the study, data base development, to assess the cytotoxicity of 150 test chemicals under blind conditions (Spielmann et al., 1996). The test had successfully undergone an interlaboratory assessment phase in which 35 chemicals were tested in 12 laboratories with five independent repeat tests per laboratory (Spielmann et al., 1991). The SOP was submitted in 1992 to INVITTOX, where it is still available as Protocol No. 46 (FRAME, 1992) and published in a methods handbook (Liebsch and Spielmann, 1995).

For the present purpose of being a recommended standard test for basal cytotoxicity, the protocol was refined by adding some paragraphs and appendices, none of which change the original method. The additions are based on experience made with a modification of the test, the 3T3 Neutral Red Uptake Phototoxicity Test (3T3NRU-PT), which has meanwhile gained regulatory acceptance. The additions cover test acceptance criteria and recommendations on the concentration series to be tested. 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 for the specific used described in this document. Two deletions were made with regard to the original SOP. The second endpoint, a cell protein staining with Kenacid Blue (KB), was deleted because it did not contribute to the test. For about 90% of the chemicals tested, the KB_{50} values were close or even identical to the NR_{50} values, but in several cases where necrotic cells were fixed to the plastic material of the plates and then stained with KB, the KB_{50} values led to an under-prediction of cytotoxicity. The second deletion is the microscopic determination of the "highest tolerated dose" (HTD), since this measure turned out to be too subjective and yielded insufficient interlaboratory comparability in the validation study.

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 BALB/c 3T3 cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the vital dye, NR, after one day (= one cell cycle) of chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

1.3 Basic Procedure

BALB/c 3T3 cells are seeded into 96-well plates and maintained in culture for 24 hours (h) (~ 1 doubling period) to form a semi-confluent monolayer (see Section 1.6.1 for more information on cell maintenance and culture procedures). They are then exposed to the test

compound over a range of eight concentrations. After 24 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., 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.
- Other chemicals that are difficult to test include those that are unstable or explosive in water.
- Due to low metabolic capacity of the BALB/c 3T3 cells, the test is likely to underestimate the toxicity of chemicals that require metabolic activation to a toxic intermediary or product.
- The *in vivo* toxicity of substances that specifically attack dividing cells may be overestimated.
- The toxicity of substances that bind to serum proteins may be underestimated. This is overcome to a certain extent by lowering the serum content from 10% to 5% during chemical exposure. Theoretically, serum-free media can be developed for any cell line, but does not yet exist for the BALB/c 3T3 cells.
- 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 sulfate, 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*

BALB/c 3T3 cells, clone 31 (e.g., ECACC # 86110401, European Collection of Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK; CCL-163, American Type Culture Collection [ATCC], Manassas, VA, USA)

1.5.2 *Technical Equipment*

- Incubator: 37ºC, humidified, 7.5 % CO_2/air
- Laminar flow clean bench (standard: "biological hazard")
- Water bath: 37ºC
- Inverse phase contrast microscope
- Laboratory burner
- Centrifuge (optionally: equipped with microtiter plate rotor)
- Laboratory balance
- 96-Well plate photometer equipped with 540 nm filter
- Shaker for microtiter plates
- Cell counter or hemacytometer
- 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)

1.5.3 *Chemicals, Media, and Sera*

- Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine (e.g., ICN-Flow Cat. No. 12-332-54)

- L-Glutamine 200 mM
  (e.g., ICN-Flow # 16-801-49)

- New Born Calf Serum (NBCS)
  (e.g., Biochrom # SO 125)

Note: Due to lot variability of NBCS, first check a lot for growth stimulating properties with 3T3 cells (20-25 hrs doubling time) and then reserve sufficient amount of NBCS.

- Trypsin/EDTA solution
  (e.g., ICN-Flo, # 16891-49)

- Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}(for trypsinization)

- PBS with Ca^{2+} and Mg^{2+}(for rinsing)

- Penicillin/streptomycin solution
  (e.g. ICN-Flow # 16-700-49)

- Neutral Red (NR)

- Dimethyl sulfoxide (DMSO), analytical grade

- Ethanol (ETOH), analytical grade

- Glacial acetic acid, analytical grade

- 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 ***Media***

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

(A) for Freezing

20 %	NBCS
7 - 10 %	DMSO

(B) for Routine Culture

10 %	NBCS
4 mM	Glutamine
100 IU	Penicillin
100 µg/ml	Streptomycin

(C) for Treatment with Test Chemicals

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

Note: The serum concentration of treatment medium is reduced to 5%, since serum proteins may mask the toxicity of the test substance. Serum cannot be totally excluded because cell growth is markedly reduced in its absence.

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	DMEM

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 ***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 1h.

1.5.4.5 ***Preparation of Test Chemicals***

1. Depending on the solubility, dissolve test chemical either in sterile treatment medium, or ETOH, or DMSO, as appropriate - at 100-fold the desired final concentration in the case of solvents. Other solvents may be used provided they have been tested to be non-cytotoxic at the final concentration used in the test. The final solvent concentration should be kept at a constant level of 1-2 % (v/v) in the vehicle controls and in all of the eight test concentrations. This means, the test chemical is dissolved in the solvent first, and then 1-2 part(s) of this stock solution is added to 98-99 parts of sterile pre-warmed (37°C) medium. Check carefully to determine whether the chemical is still dissolved after the transfer from solvent stock solution to medium, and reduce the highest test concentration, if necessary.
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 HCl. In this case, prepare highest concentration of the chemical in ~ 80% of final volume, measure pH, neutralize, and add medium 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

1.6.1 ***Cell Maintenance and Culture Procedures***

BALB/c 3T3 cells are routinely grown as a monolayer in 80 cm^2 tissue culture grade flasks, at 37°C in a humidified atmosphere of 7.5 % CO_2. The cells should be examined on a daily basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted. Cells should be checked regularly for the absence of mycoplasma contamination and only used if none is found.

1.6.1.1 ***Routine Culture of BALB/C 3T3 Cells***

· When cells approach confluence they should be removed from the flask by trypsinization:

— Decant medium, rinse cultures with ~5 ml PBS (without Ca^{2+}, Mg^{2+}) per 25 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.

— Add 1-2 ml trypsin-EDTA solution to the monolayer for a few seconds.

— Remove excess trypsin-EDTA solution and incubate the cells at 37°C.

— After 2-3 minutes (min), lightly tap the flask to detach the cells into a single cell suspension.

1.6.1.2 ***Cell Counting***

After detaching the cells, add 0.1-0.2 ml of routine culture medium/cm flask, i.e., 2.5 ml for a 25 cm^2 flask. Disperse the monolayer by gentle trituration. It is important to obtain a single cell suspension for exact counting. Count a sample of the cell suspension obtained using a hemocytometer or cell counter.

1.6.1.3 Subculture

After determination of cell number, the culture can be sub-cultured into another flask or seeded

into a 96-well microtiter plate. BALB/c 3T3 cells are routinely passaged at a cell density of ~ 1 x 10^6 cells in 80 cm^2 flasks every 3-4 days (average doubling time is 20-24 h).

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

1.6.1.4 ***Freezing***

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

- Centrifuge trypsinized cells at 200 x g.
- Suspend the cells in routine culture medium, containing 20 % NBCS, at a concentration of 1-5x10^6 cells/ml.
- Aliquot 120-180 µl of cooled DMSO into freezing tubes and fill to 1.8 ml with the cell suspension.
- Place the tubes into a freezer at -80°C for 24 h. This gives a freezing rate of 1°C/min.
- Place the frozen tubes into liquid nitrogen for storage.

1.6.1.5 ***Thawing***

Thaw cells by putting ampules into a water bath at 37°C. Leave for as brief a time as possible.

- Resuspend the cells and transfer into routine culture medium.
- Incubate at 37°C in a humidified 7.5 % CO_2 atmosphere.
- When the cells have attached to the bottom of the flask (this may take up to 4 h), decant the supernatant and replace with fresh medium. Culture as described above.
- Passage two to three times before using the cells in a cytotoxicity test.

A fresh batch of frozen cells should be thawed out approximately every two months. This period resembles a sequence of about 18 passages.

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 8 concentrations), according to Section 1.6.5.2, concurrently with each 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. For the latter procedure, the 95% confidence interval (CI) of the IC_{50} of SLS has to be established and defined as an acceptance criterion for test sensitivity in the SOP.

The historical mean IC_{50} of SLS (Spielmann et. al., 1991) is **0.093 mg/ml**.

The 95% CI is **0.070 - 0.116 mg/ml.**

A test meets acceptance criteria, if the IC_{50} for SLS is within the 95% CI

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 1×10^4 cells seeded per well have grown exponentially with normal doubling time during the two 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*

The IC_{50} derived from the concentration-response should be backed by at least two, or if possible, three responses between 10 and 90% inhibition of NRU. If this is not the case, and the concentration progression factor can be easily reduced, reject the experiment and repeat it with a smaller progression factor.

1.6.5 *Concentrations of Test Chemical*

1.6.5.1 ***Range Finder Experiment***

Test eight concentrations of the test chemical by diluting the stock solution with 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., $\sqrt[6]{10} = 1.47$). Try to cover the relevant concentration range (between 10% and 90% effect) with at least three points of a graded effect, avoiding 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 C.1 for a flow chart of the test procedure. Appendix G contains a recommended template for documenting the relevant data generated by the BALB/c 3T3 NRU assay.

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

1. Prepare a cell suspension of $1x10^5$ cells/ml in culture medium. Using a multi-channel pipette, dispense 100 µl culture medium only into the peripheral wells of a 96-well tissue culture microtiter plate (= blanks). In the remaining wells, dispense 100 µl of a cell suspension of $1x10^5$ cells/ml (= $1x10^4$ cells/well). 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, one need only include that PC concentration in the test material plate.]

2. Incubate cells for 24 h (7.5% CO_2, 37ºC) so that cells form a half-confluent monolayer. This incubation period assures cell recovery and adherence and progression to 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 experimental errors.

2nd day

1. After 24 h incubation, aspirate culture medium from the cells.

2. Per well, add 100 µl of treatment medium containing either the appropriate concentration of test chemical, or the PC, or nothing but vehicle (VC).

3. Incubate cells for 24 h (7.5% CO_2, 37ºC).

3rd day

A) Microscopic Evaluation

After 24 h treatment, examine each plate under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record changes in morphology of the cells due to cytotoxic effects of the test chemical, but do not use these records for the calculation of HTD or any other 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 NR into the lysosomes/endosomes and vacuoles of living cells is used as a quantitative indication of cell number and viability.

1. Wash the cells with 150 µl pre-warmed PBS. Remove the washing solution by gentle tapping. Add 100 µl NR medium and incubate at 37°C in a humidified atmosphere of 7.5 % $C0_2$ for 3 h.

2. After incubation, remove the NR medium, and wash cells with 150 µl PBS.

3. Decant and blot PBS totally. (Optionally: centrifuge the reversed plate.)

4. Add exactly 150 µl NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.

5. Shake microtiter plate rapidly on a microtiter plate shaker for 10 min until NR has been extracted from the cells and formed a homogeneous solution.

6. Measure the absorption of the resulting colored solution at 540 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 C.1. 3T3 NRU Cytotoxicity Test: Flow Chart

TIME (h)	PROCEDURE
00:00	Seed 96-well plates: $1x10^4$ cells / 100 µl DMEM culture medium / well
	Incubate (37°C / 7.5% CO_2 / 22-24 h)
	↓
23:00	Remove culture medium
	↓
24:00	Treat with 8 concentrations of test chemical in treatment medium (100 µl) (untreated vehicle control = treatment medium)
	Incubate (37°C / 7.5% CO_2 / 24 h)
	↓
48:00	Microscopic evaluation of morphological alterations Remove treatment medium wash once with 150 µl PBS Add 100 µl NR medium
	Incubate (37°C / 7.5% CO_2 / 3 h)
	↓
51:00	Discard NR Medium Wash once with 150 µl PBS Add 150 µl NR desorbing fixative (ETOH/Acetic acid solution)
51:40	↓
51:50	Shake plate for 10 min
	Detect NR Absorption at 540 nm (i.e., cell viability)

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 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 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 (i.e., the IC_{50}) is determined from the concentration-response. This can be done either by applying:

- 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. 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. An example is PHOTO-32, which uses a nonmonotonous curve fitting algorithm (Holzhütter and Quedenau, 1995) and addresses the influence of variability on the IC_{50} by bootstrapping procedures performed on concentration replicates (Holzhütter, 1997).

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:

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

1.9 References

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