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No. 129

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNEP, UNIDO, UNITAR, WHO and OECD

Environment Directorate

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FOREWORD

This document presents the OECD Draft Guidance Document on "*Using Cytotoxicity Tests To Estimate Starting Doses For Acute Oral Systemic Toxicity Tests*". The project for developing this guidance document was proposed by the United States. Comments on a first draft were requested from the Working Group of National Coordinators for the Test Guidelines Programme (WNT) in June 2009. A revised draft was approved at the WNT meeting held on 23-25 March 2010. The Joint Meeting of Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 19 July 2010.

This document is published under the responsibility of the joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

PREAMBLE

1. A number of national and international projects have established a relationship between in vitro cytotoxicity and *in vivo* acute lethality. The Multicentre Evaluation of *In vitro* Cytotoxicity (MEIC) Program, established in 1989 by the Scandinavian Society for Cell Toxicology, investigated the ability of in vitro cytotoxicity test methods to predict acute oral lethality in humans using 50 reference substances. The MEIC program was based on the hypothesis that the basal cytotoxicity detected by in vitro test methods is responsible for a large proportion of *in vivo* toxic effects, and that in vitro cell culture systems could therefore be used to model in vivo acute oral toxicity.

2. Another national initiative, the Registry of Cytotoxicity (RC) database assembled by the German Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) at the Federal Institute for Risk Assessment (BfR) contains rodent acute oral LD50 values and published IC50 values from various *in vitro* cytotoxicity assays for a total of 347 substances. A linear regression-model using the log-transformed IC50 values and log-transformed rodent oral LD50 values was developed for the prediction of acute oral LD50 values from IC50 values.

3. The MEIC and BfR-ZEBET data were also considered at a 1996 workshop, where the use of in vitro cytotoxicity data to determine the starting doses for rodent acute oral toxicity tests and subsequently reduce the number of animals used were discussed as a way to reduce animal use for the classification and labelling of chemicals.

4. The concept of using *in vitro* cytotoxicity data to determine the starting doses for rodent acute oral toxicity tests was further discussed and evaluated at an International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and sponsored by the US National Toxicology Program (NTP), the US National Institute of Environmental Health Sciences (NIEHS) and the US Environmental Protection Agency (EPA) that was held in Arlington, USA in October 2000. The meeting recommended that the method should be further evaluated.

5. The NTP Center for the Evaluation of Alternative Toxicological Methods (NICEATM) collaborated with the European Centre for the Validation of Alternative Methods (ECVAM), a component of the European Commission's Joint Research Centre, to further characterize the usefulness of in vitro cytotoxicity assays as predictors of starting doses for acute oral lethality assays. NICEATM and ECVAM designed a multi-laboratory validation study to evaluate the performance of two standardized in vitro basal cytotoxicity test methods using 72 reference substances with high quality *in vivo* data. Based on the results of the study, ICCVAM and ECVAM recommended that the RC regression model using an IC50 value from an in vitro basal cytotoxicity test could be used to predict an LD50 value for use as a starting dose for the Acute Toxic Class (ATC) method (TG 423) or the Up-and-Down Procedure (UDP) (TG 425) Test Guideline. Simulations for the reference substances showed that using in vitro cytotoxicity assays to estimate an LD50 to use as a starting dose could potentially reduce animal use by up to 28% for acute oral toxicity testing, and as much as 50% for non-classified substances.

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INTRODUCTION

6. The concept of using *in vitro* cytotoxicity data to determine the starting doses for rodent acute oral toxicity tests was discussed and evaluated at an International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity convened in 2000 (ICCVAM, 2001a). The approach involves using an IC_{50} value from an *in vitro* basal cytotoxicity test with the Registry of Cytotoxicity (RC) regression to predict an LD_{50} value for use as a starting dose for the Acute Toxic Class (ATC) method or the Up-and-Down Procedure (UDP) test method (Spielmann et al., 1999). Simulations showed that using *in vitro* cytotoxicity assays to estimate an LD_{50} to use as a starting dose in the UDP could potentially reduce animal use by 25-40% (Spielmann et al., 1999; ICCVAM, 2001a).

7. To investigate the usefulness and limitations of standardized cytotoxicity tests for estimating starting doses for acute oral toxicity tests, the 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] sponsored and organized an international validation study using 72 coded substances tested in three laboratories (ICCVAM, 2006a). BALB/c 3T3 mouse fibroblasts (3T3) and normal human epidermal keratinocytes (NHK) were selected and neutral red uptake (NRU) was used as the cytotoxicity endpoint in the NICEATM-ECVAM validation study. This was consistent with the recommendations included in ICCVAM's initial Guidance Document (ICCVAM, 2001b) for this purpose, which were based on reproducible results for both test methods in earlier validation efforts (ICCVAM 2001b). Based on the results of the NICEATM-ECVAM validation study, these test methods are now recommended for routine consideration before using rats for acute toxicity studies by U.S. regulatory and public health agencies (ICCVAM, 2006c)[1]. When determined to be appropriate and used to estimate starting doses, animal use can be reduced for each study by as much as 50% (ICCVAM, 2006a, b). These recommendations are consistent with the findings of an independent international scientific peer review panel, which concluded that the methods were adequately reliable and reproducible for use in a weight-of-evidence approach for determining starting doses for acute oral toxicity tests (ICCVAM, 2006b). (Definitions used in the context of this Guideline are set out in Annex 1.)

8. A number of large national and international projects established the initial relationship between *in vitro* cytotoxicity and *in vivo* lethality. The Multicentre Evaluation of *In vitro* Cytotoxicity (MEIC) Program, established in 1989 by the Scandinavian Society for Cell Toxicology, investigated the ability of *in vitro* cytotoxicity test methods (using 50 reference substances) to predict acute oral lethality in humans (Bondesson et al., 1989). The MEIC program was based on the hypothesis that the basal cytotoxicity detected by *in vitro* test methods is responsible for a large proportion of *in vivo* toxic effects, and that *in vitro* cell culture systems could therefore be used to model *in vivo* acute oral toxicity. The mechanistic basis of similarities between animal death and cell death is that all cells, regardless of whether they are in animals or *in vitro* cell cultures, have similar cellular mechanisms; for example, energy production and maintenance of cell membrane integrity The ability of the MEIC *in vitro* IC_{50} data to predict human acute oral lethality was assessed using human lethal blood/serum concentrations (LC) compiled from three

[1] See http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_recommend.htm for U.S. agency responses to ICCVAM recommendations.

different data sets: 1) clinically measured acute LC values; 2) acute LC values measured post-mortem; and 3) peak LC values derived from approximate LC_{50} curves over time after exposure. A partial least squares analysis indicated that the IC_{50} data generated from as many as 61 test methods predicted the three sets of LC data well with determination coefficients (R^2) of 0.77, 0.76, and 0.83 (Ekwall et al., 2000).

9. Another national initiative, the RC database assembled by ZEBET (Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch [German Center for Documentation and Evaluation of Alternative Methods to Animal Experiments]), contains rodent acute oral LD_{50} values from the Registry of Toxic Effects for Chemical Substances (RTECS®, Symyx Technologies, Inc. Sunnyvale, California, USA. [http://www.symyx.com/products/databases/bioactivity/rtecs/index.jsp]) and published IC_{50} values from various *in vitro* cytotoxicity assays for 347 substances (Halle, 1998; 2003). Halle (1998, 2003) calculated a linear regression using the log-transformed IC_{50} values (in mM) and log-transformed rodent oral LD_{50} values (in mmol/kg) to develop a model for the prediction of acute oral LD_{50} values from IC_{50} values (R^2=0.45; $p < 0.001$ for slope). The acceptable prediction interval for the LD_{50} was empirically defined by Halle (1998, 2003) as approximately one-half an order of magnitude on either side of the best-fit linear regression (i.e. ± log 5, or ±0.699). This interval was based on eight published linear regressions calculated using *in vitro* mammalian cell cytotoxicity IC_{50} values from various toxic endpoints across approximately eight orders of magnitude and oral LD_{50} values from rat, mouse, or rat and mouse. Seventy-three percent (252/347) of the RC substances fall within the prediction interval.

10. The MEIC and ZEBET data were also considered at a 1996 workshop, where the use of *in vitro* cytotoxicity data to determine the starting doses for rodent acute oral toxicity tests and subsequently reduce the number of animals used were discussed as a way to reduce animal use for the classification and labeling of chemicals (Seibert et al., 1996).

INITIAL CONSIDERATIONS

Background Information

11. The NRU *in vitro* basal cytotoxicity assay procedure is based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye (Borenfreund and Puerner, 1985). 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. Such adverse changes cause cell death and/or inhibition of cell growth, which then decrease the amount of NR retained by the culture. Since the concentration of NR dye desorbed from the cultured cells is directly proportional to the number of living cells, cytotoxicity is expressed as a concentration-dependent reduction of the uptake of NR after chemical exposure. The NRU assay uses a 96-well plate format for the production of replicate measurements at eight test substance concentrations.

12. Data from the *in vitro* tests can be used for estimating the starting dose for acute oral systemic toxicity tests. The *in vivo* starting dose is an estimated LD_{50} value calculated by inserting the *in vitro* IC_{50} value into a regression formula derived from 282 substances for which there are both historical rat oral LD_{50} values and *in vitro* IC_{50} values from the RC (ICCVAM, 2006a). For the 72 chemicals tested in the NICEATM/ECVAM *in vitro* basal cytotoxicity validation study, inter-laboratory reproducibility of the IC_{50}, measured by the average coefficient of variation (CV), was 47% for the 3T3 NRU assay and 28% for the NHK NRU assay. Computer-simulated acute oral systemic toxicity testing of the test substances indicated that the animal savings, which were calculated by comparing the number of animals used with the NRU-determined starting dose to the number of animals used with the default starting dose, were similar using either the 3T3 or the NHK NRU assays to determine starting doses (ICCVAM, 2006a). The NICEATM-ECVAM validation study methods (ICCVAM, 2006a, b, c) demonstrated that the two test methods are useful and reproducible for this purpose. The similarity of animal savings for the 3T3 and NHK NRU tests is due to the general similarity of the IC_{50} values produced (i.e. 85% [61/72] of the

substances tested in the NICEATM-ECVAM validation study had 3T3 and NHK NRU IC_{50} values within one order of magnitude (ICCVAM, 2006a) and the minimization of differences by using a log regression equation to predict LD_{50}.

13. Animal savings are highest for chemicals with LD_{50} >5000 mg/kg. An animal savings of up to 50% is possible using the cytotoxicity approach to a starting dose, compared to the number of animals used with the default starting dose in the UDP (OECD, 2008). This may be achieved if the cytotoxicity test is performed first and *in vitro* data predict an LD_{50} > 5000 mg/kg. The UDP would proceed with a starting dose of 5000 mg/kg rather than the default starting dose of 175 mg/kg; thus, three animals would be used instead of six to determine the LD_{50} (ICCVAM, 2009). For chemicals with LD_{50} >5000 mg/kg, average animal use for the UDP was reduced by up to 22% per test and average animal use for the ATC (OECD, 2001a) method was reduced by up to 28% per test. A review of toxicity values in the European Union reveals that the majority of industrial substances tested for regulatory purposes have an LD_{50} of >2000 mg/kg. Eighty-seven percent of the chemicals in the New Chemicals Database, maintained at the Institute for Health and Consumer Protection (IHCP, DG-JRC, Ispra [http://ecb.jrc.it]), have LD_{50} >2000 mg/kg (Bulgheroni et al., 2009). Although animal savings for the Fixed Dose Procedure (FDP; OECD, 2001b) were not evaluated during the NICEATM-ECVAM validation study, the same principles would apply.

Limitations

14. The limitations of the *in vitro* NRU methods are largely due to the differences between whole animal and cell culture systems. Animal and cell culture systems are different with respect to how a substance or toxicant is delivered to the cell and how it is distributed within the cell, metabolized, and excreted. After oral administration, animals must absorb the toxicant from the gastrointestinal tract. The toxicant may or may not be bound to serum proteins, which would reduce its availability to the target organ. The toxicant may be metabolized before, during, and/or after its distribution to the target organs, or the toxicant or its metabolites may be excreted before reaching the target organ. As a consequence, the most critical target organs may not be exposed to the active metabolite, or be exposed for only a limited time or to a relatively small fraction of the administered dose.

15. In contrast, in a cell culture system, the test substance is applied directly to the target cells and the only membranes that must be traversed are those of the target cell and its sub-cellular organelles. Cell culture systems may or may not include serum proteins, which could reduce the availability of toxicant to the target site. 3T3 and NHK cells have little to no capacity to metabolize xenobiotic compounds. Anything excreted from the cell remains in the culture medium and is available to the other cells in the culture. As a result, the cells in culture (as opposed to cells in an animal) may be exposed to a test substance for the entire duration of the test protocol. Animals and cell culture systems may also differ with respect to the target on which a toxicant acts. If a toxicant acts in a specialized organ system *in vivo*, it may not produce a toxic effect by the same mechanism in cultured cells that are derived from a tissue different from the target organ. For example, a substance that affects a neuroreceptor-mediated pathway in animals would not be expected to produce a similar toxicity in 3T3 or NHK cells; if toxicity is seen in these cell cultures, it may be from a different mechanism or in a different concentration relationship than *in vivo*.

PRINCIPLE OF THE TEST METHOD

16. This Guidance Document describes methods to determine the *in vitro* basal cytotoxicity of test substances using NRU assays and the use of the *in vitro* data to determine starting doses for *in vivo* acute oral systemic toxicity tests. The NRU assay is performed in a dose-response format to determine the concentration that reduces NRU by 50% compared to the controls (i.e. the IC_{50}). The IC_{50} value is used in a linear regression equation to estimate the oral LD_{50} value (dose that produces lethality in 50% of the animals tested), which is then used to determine a starting dose for acute oral systemic toxicity testing

using rats for the UDP, the ATC method, or FDP. The use of the NRU test method in a weight-of-evidence approach to determine starting doses for these acute oral systemic toxicity tests might reduce the number of animals required for the tests, and for relatively toxic substances, might reduce the number of animals that die or require humane euthanasia due to severe toxicity. For estimating starting doses, *in vitro* data should be considered along with all other data and information such as quantitative structure-activity relationship (QSAR) predictions, the LD_{50} of related substances, and other existing data to estimate a dose that is likely to be close to the actual LD_{50} value.

17. Standardized test method protocols (Stokes et al., 2008) provide details for performing NRU tests with rodent or human cells. The NRU *in vitro* basal cytotoxicity assay involves exposing cells in culture to a test substance for 48 hours. The test substance is rinsed off the cells and the cells are then incubated with NR dye. The concentration of NR dye eluted from the cells is then quantitated spectrophotometrically. Stokes et al. (2008) describes the methods for testing substances using the immortalized rodent cell line, 3T3, and primary human cells, NHK, in the NRU assay. The results for the two cell types proved to be similar in the validation study; however, the 3T3 NRU assay is more cost and time effective than the NHK NRU assay. Methods for preparation and dilution of substances to be tested in the *in vitro* NRU tests are also described along with a tiered solubility procedure to determine the best solvent for testing the substance of interest. Because the NHK NRU assay requires special attention concerning the cell culture medium, a medium pre-qualification procedure is provided (Annex 2).

DESCRIPTION OF THE TEST METHODS

Testing Formats

Range finder test

18. This is the initial cytotoxicity test performed to determine the starting doses for the main test. The NRU assays test eight concentrations of the test substance or the positive control (PC) by diluting the stock test substance solution in log dilutions to cover a large concentration range (see paragraphs 29-34).

Main test

19. The main test of the cytotoxicity assays is performed to determine the IC_{50} value (i.e. see Annex 3). The concentration closest to the range finder test IC_{50} value serves as the midpoint of the concentrations tested in the main test. Compared to the range finder test, the main test uses a smaller dilution factor for the concentrations tested (see paragraph 35).

Preparations for the 3T3 NRU Assay

Cells

20. The permanent murine fibroblast cell line, BALB/c 3T3 cells, clone 31, should be obtained from well qualified national/international cell culture repositories.

21. All cell stock and cultures used for testing should be certified as free of mycoplasma and bacterial contamination and should be checked routinely (as per specific laboratory protocols and standard operating procedures [SOPs]).

Media and culture conditions

22. Laboratories should follow SOPs in all cell culture aspects. Routine cell passage for the BALB/c 3T3 cells should use a culture medium containing high glucose (4.5 g/L) Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with non-heat-inactivated 10% newborn calf serum[2] (NCS), and 4 mM L-Glutamine. Antibiotics will be used in the culture medium that contains the test substance (see paragraph 46). Proper preparation of the culture medium should include pH adjustment (e.g. with sodium bicarbonate) and proper osmolarity maintenance. Cells should be cultivated at 37°C ±1°C, 90% ±10% humidity, and 5.0% ±1.0% CO_2/air. Cell culture conditions should assure that the cell cycle time is within the historical range of the cell line. The historical cell cycle time (doubling time) for 3T3 cells was approximately 18 hours (average of three laboratories) in the NICEATM-ECVAM validation study (ICCVAM, 2006a [Section 2.3.1.1]).

Preparation of cultures

23. The 3T3 cells from cryogenically-preserved stock should be sub-cultured at least twice before using the cells in the 3T3 NRU assay. Remove cells from flasks through trypsinization when cells reach 50% to 80% confluence. The passages of 3T3 cells from frozen stock should be limited to approximately 18 passages to avoid phenotypic and genotypic changes that may occur as the culture ages.

24. Cells in routine culture medium should be plated into 96-well tissue culture microtiter plates at a density of $2.0 - 3.0 \times 10^3$ cells/100 µL/well. Refer to Annex 4 for recommended 96-well plate template. Cultivate cells for 24 hours ±2 hours to form a less than half (< 50%) confluent monolayer. This incubation period assures adequate cell recovery and adherence to allow for progression to the exponential growth phase.

Preparations for the NHK NRU Assay

Cells

25. Primary, non-transformed normal NHK can be substituted for the BALB/c 3T3 cells for the cytotoxicity assay. The NHK cells should come from cryopreserved primary or secondary pooled neonatal foreskin cells procured only through commercial sources rather than preparing a primary culture from donated tissues.

26. All cell stock and cultures used for testing should be certified as free of mycoplasma and bacterial contamination and should be checked routinely (as per specific laboratory protocols and SOPs).

Media and culture conditions

27. Laboratories should follow SOPs in all cell culture aspects. Routine cell passage for the NHK cells should include a serum-free defined keratinocyte basal culture medium supplemented with 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, and 30 µg/mL bovine pituitary extract (e.g. KBM® [Clonetics CC-3104], KBM® SingleQuots® [Clonetics CC-4131], and Clonetics Calcium SingleQuots® [CC-4202]). Cells should be incubated at 37°C ±1°C, 90% ±10% humidity, and 5.0% ±1.0% CO_2/air. Cell culture conditions should assure that the cell cycle time is within the historical range of the cell type. The historical cell cycle time (doubling time) for NHK cells was approximately 19 hours

[2] Calf serum is also acceptable (ICCVAM, 2006c).

(average of three laboratories) in the NICEATM-ECVAM validation study (ICCVAM, 2006a [Section 2.3.1.2]).

Preparation of cultures

28. Propagate NHK cells (from cryopreserved pool) in 25 cm^2 tissue culture flasks. When cells reach 50% to 80% confluence, remove cells from flasks through trypsinization (quench the trypsinization by adding trypsin neutralizing solution).

29. Prepare a cell suspension of $1.6 – 2.0x10^4$ cells/mL in NHK routine culture medium. Dispense 125 µL of the cell suspension ($2.0 – 2.5x10^3$ cells/well) to the test wells of a 96-well tissue culture microtiter plate. Refer to Annex 4 for recommended 96-well plate template. Dispense 125 µL routine culture medium into the peripheral blank wells.

30. Cultivate cells for 48 – 72 hours (37ºC ±1ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air) so that cells form a >20% confluent monolayer. This incubation period assures adequate cell recovery and adherence to allow for progression to the exponential growth phase.

Preparation of Test Substance

Test substances in solution

31. Equilibrate test substances to room temperature before dissolving and diluting. Prepare the test substance immediately prior to use rather than preparing in bulk for use in subsequent tests. The solutions should be clear and have no noticeable precipitate. Microscopic evaluation of test substance solutions is recommended to assist in the visual determination of solubility of the test substance. Prepare at least 1-2 mL total volume of each stock dilution to ensure an adequate quantity for all of the test wells in a single 96-well plate. Preparation of test substances under red or yellow light is recommended to preserve substances that degrade upon exposure to light (See Annex 6).

32. Culture medium is the preferred solvent for dissolving test substances followed by dimethyl sulfoxide (DMSO) and ethanol (EtOH). See Annex 5 for the solubility protocol and Annex 6 for suitable physicochemical properties of test substances. Preparation of test substances in culture medium will follow solubility steps (tiers) 1, 2, and 3 in Annex 5. For substances dissolved in DMSO or EtOH, the final DMSO or EtOH concentration for application to the cells should be no more than 0.5% (v/v) in the vehicle controls (VCs) and in all of the eight test concentrations. The concentration of DMSO or EtOH should be the minimum concentration needed to dissolve the test substance.

33. Prepare the stock solution for each test substance at the highest concentration found to be soluble in the solubility test (Annex 5). The highest test concentration applied to the cells in a range finding test is as follows:

– 0.5 times the highest concentration found to be soluble in the solubility test, if the substance was soluble in culture medium, or

– 1/200 the highest concentration found to be soluble in the solubility test if the substance was soluble in DMSO or EtOH.

Preparation of test substance dilutions for range finder test

34. This log dilution scheme is appropriate for preparing test substances for the range finder test (see paragraph 13).

35. Dissolve the test substance in DMSO or EtOH at 200 mg/mL to prepare the test substance stock solution (see Figure 1 in Annex 5). Prepare the seven lower concentrations by successive serial dilutions that decrease by one log unit each (e.g. 0.1 mL of solution into 0.9 mL solvent).

36. Each concentration is 200 fold greater than the concentration to be tested. Make a 1:100 dilution by diluting one part dissolved test substance in each tube with 99 parts of medium (e.g. 0.1 mL test substance in DMSO or EtOH + 9.9 mL medium) to derive the eight 2X concentrations for application to the cells. Each 2X test substance concentration will then contain 1% (v/v) solvent.

37. The 3T3 cells will have 50 µL Routine Culture Medium in the wells prior to application of the test substance. Adding 50 µL of any specific 2X test substance concentration to the assigned wells will appropriately dilute the test substance (e.g. highest concentration in well will be 1,000 µg/mL) in 100 µL and the solvent concentration in the wells will be 0.5% (v/v).

38. The NHK cells will have 125 µL of culture medium in the wells prior to application of the test substance. Adding 125 µL of any specific 2X test substance concentration to the assigned wells will appropriately dilute the test substance (e.g. highest concentration in well will be 1,000 µg/mL) in 250 µL and the solvent concentration in the wells will be 0.5% (v/v).

39. A test substance prepared in medium or solvent may precipitate upon transfer into the Routine Culture Medium.

Preparation of test substance dilutions for main test

40. The main test (see paragraph 14) requires a smaller dilution factor than the range finder test. A decimal geometric concentration series of dilutions is recommended and can be used in toxicological tests because such a series has the advantage that independent experiments with wide or narrow dose factors can be easily compared because they share identical concentrations. The dilution 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 (see Table 1). Taking into account pipetting errors, a progression factor of 1.21 is regarded the smallest factor practically achievable. For example, to make dilutions with the dilution factor of 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 diluents, etc. (ICCVAM, 2001b).

Table 1 Maximum Doses for Test Substances Prepared in Routine Culture Medium for the Main Test

Number of Equal Dilutions (Dilution Factor)	Concentration Units[1]
2 (3.16)	10, 31.6, 100
3 (2.15)	10, 21.5, 46.4, 100
4 (1.78)	10, 17.8, 31.7, 56.4, 100
6 (1.47)	10, 14.7, 21.5, 31.6, 46.4, 68.1, 100
12 (1.21)	10, 12.1, 14.7, 17.8, 21.5, 26.1, 31.6, 38.3, 46.4, 56.2, 68.1, 82.5, 100

[1]An example of concentration units is µg/mL.

Preparation of test substances in medium

41. The highest test substance stock concentration in medium for the main tests will be either 100 mg/mL, or the maximum soluble dose divided by 2. If minimal or no cytotoxicity was measured in the range finder test (see paragraph 40), the maximum dose for the main tests is established as follows:

a) Weigh the test substance into a glass tube (glass is preferred but polystyrene may be acceptable) and add routine culture medium to obtain a concentration of 200 mg/mL. If the 200 mg/mL solution used in the range finder test does not produce cytotoxicity, then a stock solution up to 500 mg/mL may be prepared for the main test. Mix the solution using the mixing procedures that produced solubility when performing the solubility test (Annex 5).

b) If complete solubility is achieved in medium, then prepare seven additional serial stock dosing solutions from the 200 mg/mL (or higher concentration) 2X stock.

c) 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 mixing procedures specified in Annex 5. If precipitates are observed in the 2X dilutions, continue with the test and make the appropriate observations and documentation. More stringent solubility procedures may be employed if needed based on results from the range finder test.

d) Use the highest soluble stock solution to prepare the seven additional serial stock dosing solutions.

Maximum doses for test substances prepared in DMSO or EtOH for the main test

42. If the 200 mg/mL solution used in the range finder test does not produce cytotoxicity, then a stock solution up to 500 mg/mL may be prepared for the main test. The maximum concentration for the main test can be determined based on the maximum concentration of DMSO or EtOH that could be added to culture medium without causing cytotoxicity (i.e. 0.5% v/v). The highest test substance concentration that may be applied to the cells in the main tests will be $\leq$ 2.5 mg/mL, depending upon the maximum solubility in solvent.

a) Weigh the test substance into a glass tube and add the appropriate solvent (determined from the original solubility test [Annex 5]) to obtain a concentration of 500 mg/mL. Mix the test substance solution

using the sequence of mixing procedures specified in Annex 5. If complete solubility is achieved in the solvent, then prepare seven additional serial stock dosing solutions from the 500 mg/mL 200X stock.

b) 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.

c) Use the highest soluble stock solution 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.

Test Conditions

Test substance concentrations

Controls

43. *Positive Control (PC)*: Sodium lauryl sulphate (SLS; CASRN 151-21-3)[3]. Prepare a separate 96-well plate of eight PC concentrations so that a complete dose-response curve (Annex 3), rather than a single point estimate, can be obtained. This will assist with troubleshooting the test (Annex 6), if the need arises. Multiple test substance plates can be run with a single PC plate. The PC plate will follow the same schedule and procedures used for the test substance plates.

44. *Vehicle Control (VC)*: The VC consists of routine culture medium when the test substances are dissolved in culture medium. For test substances dissolved in the solvents DMSO or EtOH, the VC consists of routine culture medium with the same amount of solvent (0.5% [v/v]) as is applied to the 96-well test plate.

Test Procedure

Range finder test

45. Test eight concentrations (see paragraph 30) of the test substance by diluting the stock solution using log dilutions (e.g. 1:10, 1:100, 1:1000). If a range finder test does not generate adequate cytotoxicity for the calculation of an IC_{50} value, then higher doses should be attempted. If cytotoxicity is limited by solubility, then more stringent solubility procedures to increase the stock concentration (Annex 5) should be employed.

[3] Other substances can be used as positive controls providing that the cytotoxicity is well characterized and that each test provides an IC_{50} that is consistent with the historical range generated by the laboratory. (See Section 3.1.3 of ICCVAM, 2006c).

Main test

46. Use the range finder IC_{50} value as a central concentration and adjust dilutions higher and lower in equal steps. Alternatively, the test substance concentration closest to the range finder IC_{50} value could be used as the central value.

47. Use a smaller dilution factor for the concentration series of the main test (e.g. dilution factor of $\sqrt[6]{10} = 1.47$) than that used for the range finder test. The slope of the range finder concentration-response can be used to approximate the dilution factor.

48. Cover the relevant concentration range around the IC_{50} (> 0% and < 100% effect), preferably with several points of a graded effect, but with a minimum of two points, one on each side of the IC_{50}, and avoid too many (e.g. > 6) concentrations on either end of the concentration spectrum.

49. Perform a minimum of two main tests for a test substance and average the IC_{50} results.

3T3 NRU Assay

Day 1

50. Prepare a cell suspension and dispense cells to the plate (see paragraph 24).

Day 2

46. Remove Routine Culture Medium from the cells after incubation period by careful inversion of the plate. Gently blot the plate on a sterile paper towel to remove residual culture medium. Add 50 µL of test substance in the test substance dilution medium (DMEM without serum, 4 mM L-Glutamine 200 IU/mL penicillin, 200 µg/mL streptomycin) to the test wells and appropriate blanks. Add 50 µL of test substance dilution medium to the VC wells and appropriate blanks. Refer to Annex 4 for recommended 96-well plate template. Incubate cells for 48 hours ±0.5 hours.

Day 4

Microscopic Procedure

47. After at least 46 hours of treatment, examine each plate with a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record any changes in morphology of the cells due to the cytotoxic effects of the test substance, but do not use these records for any quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells may indicate experimental error and may be cause for rejection of the assay. Perform the NRU assay (see paragraphs 51-56).

NHK NRU Assay

Day 1

48. Prepare a cell suspension and dispense cells to the plate (see paragraphs 23 - 25).

Day 3

49. After the incubation period, do not remove the NHK routine culture medium from the test plate. Add 125 µL of the appropriate concentration of test substance in routine culture medium (see paragraph 33) to the appropriate wells. Incubate cells for 48 hours ±0.5 hours.

Day 5

Microscopic Procedure

50. Microscopic examination of the NHK cells will follow the instructions presented in paragraph 47 for the 3T3 cells.

Neutral Red Uptake Assay

51. For both cell types: After incubation, carefully invert the plate to remove the medium from the wells and rinse the cells carefully with 250 µL/well pre-warmed Dulbecco's Phosphate Buffered Saline (D-PBS). Remove the rinsing solution by inversion of the plate and blot dry on paper towels.

52. *For 3T3 Cells*: Add 250 µL of 25 µg/mL NR dye in DMEM with 5% NCS, 4 mM L-Glutamine, 100 IU/mL Penicillin, and 100 µg/mL Streptomycin to all wells (including the blanks) and incubate at 37ºC ±1ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air for 3.0 hours ±0.1 hr (continue the 3T3 NRU at paragraph 54).

53. *For NHK Cells*: Add 250 µL of 33 µg /mL Neutral Red (NR) dye in NHK routine culture medium to all wells (including the blanks) and incubate at 37ºC ±1ºC, 90% ±10% humidity, 5.0% ±1.0% CO_2/air for 3.0 hours ±0.1 hr (continue the NHK NRU at paragraph 54).

54. After incubation remove the NR medium, and carefully rinse cells with 250 µL/well pre-warmed D-PBS. Remove the solution as above. Add 100 µL NR desorb solution (freshly prepared 49 parts water + 50 parts ethanol + 1 part glacial acetic acid) to all wells (including blanks) to extract the dye.

55. Shake the microtiter plates rapidly on a microtiter plate shaker for 20 – 45 minutes. Protect the plates from light while shaking. Plates should be still for at least five minutes after removal from the plate shaker/mixer. Rupture any bubbles prior to reading the plate.

56. Measure the light absorption (optical density [OD]) within 60 minutes of adding NR desorb solution to each well at 540 nm ±10 nm (OD_{540}) in a microtiter plate reader (spectrophotometer), using the blanks as a reference. Save the data in an appropriate electronic file format for subsequent analysis.

DATA AND REPORTING

Interpretation of Data

57. Use good biological/scientific judgment for determining *unusable* wells (e.g. test wells without cells, wells with contaminated cultures, wells with precipitated test substance) that will be excluded from the data analysis.

58. After subtraction of the blank OD_{540} value, calculate the cell viability for each test well as percent of the mean VC OD_{540} value. Cell viability can be calculated using a spreadsheet template (e.g. Microsoft Excel®). Ideally, the eight concentrations of each substance tested will span the range of no effect up to total inhibition of cell viability.

59. Perform a Hill function analysis of the replicate cell viability data for each concentration using statistical software (*e.g.* GraphPad PRISM® http://www.graphpad.com/prism/Prism.htm) to calculate the IC_{50} for each test substance. The Hill function is recommended because all the dose-response information, rather than a few points around the IC_{50}, is used. The Hill function also provides the slope of the dose-response curve (see Annex 1 and Annex 6, paragraph 5).

Quality and Quantity of Data

Test acceptance criteria

60. The mean corrected absorbance of the left (VC1) and the mean corrected absorbance of the right (VC2) columns of VCs (refer to Annex 4 for the recommended 96-well plate template) do not differ by more than 15% from the mean corrected absorbance of all VCs.

61. At least one calculated cytotoxicity value > 0% and ≤ 50% viability and at least one calculated cytotoxicity value > 50% and < 100% viability should be present. Exception: If a test has only one point between 0 and 100% **and** the smallest practical dilution factor (i.e. 1.21) was used **and** all other test acceptance criteria were met, then the test is acceptable.

Additional test acceptance criteria for the PC

62. The PC fitted dose-response curve should have an R^2 (coefficient of determination) ≥ 0.85 for the Hill model fit.

63. The PC IC_{50} value should be within ±2.5 standard deviations (SD) of the historical mean established by the laboratory. A minimum of ten cytotoxicity tests of the positive control should be performed to develop the initial historical database (ICCVAM, 2006c).

Evaluation of Results

Anticipated results

64. For either NRU test, blank OD_{540} values should be approximately 0.05 (ICCVAM, 2006a). The corrected OD_{540} for the VCs can be expected to average 0.476 ±0.117 (SD) for the 3T3 NRU and 0.685 ±0.175 (SD) for the NHK NRU (ICCVAM, 2006a). IC_{50} values for the positive control, SLS, should be 41.5 ±4.8 (SD) µg/mL (n = 233) for the 3T3 NRU assay and 3.11 ±0.72 µg/mL (n = 114) for the NHK NRU assay. Annex 3 shows a typical dose-response curve for SLS in the 3T3 NRU assay. IC_{50} results for the test substances in the NICEATM/ECVAM *in vitro* basal cytotoxicity validation study ranged from 0.005 to 38,878 µg/mL (1.1×10^{-5} to 422 mM) for the 3T3 NRU test method and 0.00005 to 49,800 µg/mL (6.4×10^{-8} to 49,800 mM) for the NHK NRU test method (ICCVAM, 2006a).

Application of Results

Determination of the starting doses for acute oral systemic toxicity tests (see Annex 7)

65. The IC_{50}-LD_{50} regressions using IC_{50} values from the 3T3 NRU or the NHK NRU with those from the RC using the 47 chemicals that were common to the RC and the NICEATM-ECVAM validation study showed that neither regression was significantly different from the 47 chemical RC regression (p=0.642 for the 3T3 NRU regression and p=0.759 for the NHK NRU regression). Thus, either 3T3 NRU IC_{50} or NHK NRU IC_{50} can be used. Use the IC_{50} value in mM in the following regression formula to estimate the log LD_{50} in mmol/kg:

$$\log LD_{50} \text{ (mmol/kg)} = 0.439 \log IC_{50} \text{ (mM)} + 0.621 \text{ (ICCVAM, 2006a).}$$

Convert the log LD_{50} to LD_{50} and then convert to mg/kg units by multiplying by the molecular weight of the test substance.

66. The starting dose for the UDP is the next dose lower than the estimated LD_{50} in the default dose progression. The default dose progression for the UDP is 1.75, 5.5, 17.5, 55, 175, 550, and 2000 mg/kg

using a limit test of 2000 mg/kg or 1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000 mg/kg using a limit test of 5000 mg/kg (OECD, 2008).

67. The starting dose for the ATC method and the sighting study for the FDP is the next dose lower than the estimated LD_{50} in the default dose progression. The default dose progression for the ATC method and the FDP is 5, 50, 300, or 2000 mg/kg for the 2000 mg/kg limit test or 5, 50, 300, 2000, or 5000 mg/kg for the 5000 mg/kg limit test (OECD, 2001a, b).

68. For substances with no molecular weight, IC_{50} values in μg/mL can be used in the following regression formula to estimate the LD_{50} in mg/kg:

$$\log LD_{50}\ (mg/kg) = 0.372 \log IC_{50}\ (\mu g/mL) + 2.024 \text{ (ICCVAM, 2006a)}$$

Test Report

69. The test report should contain the following test and test substance information:

Test and Control Substances

- chemical/substance name(s), synonyms, CASRN, formula weight, if known
- purity and composition of the substance or preparation (in percentage[s] by weight)
- physicochemical properties (e.g. physical state, volatility, pH, stability, chemical class, water solubility)
- solubilisation of the test/control substances (e.g. vortexing, sonication, warming, grinding) prior to testing, if applicable.

Solvent

- solvent name
- justification for choice of solvent
- solubility of the test substance in the solvent
- percentage of solvent in treatment medium and vehicle controls

Cells

- cell type used and source of cells
- absence of mycoplasma or bacterial contamination
- cell passage number

Test Conditions (1); experimental information

- experiment start and completion dates
- details of test procedures used
- description of modifications made to the test procedure
- reference to historical data of the test model (e.g. solvent and PCs)
- description of the evaluation criteria used

Test Conditions (2); cell culture information

- lot numbers and product manufacturers for reagents, serum, medium, supplements, culture-ware, etc.)
- composition of culture medium used for routine cell culture and test substance application

Test Conditions (3); incubation before and after treatment

- incubation conditions (i.e. 37°C ±1°C, 90% ±10% humidity, and 5.0% ±1% CO_2/air)
- duration of incubation (pre-treatment; post-treatment)

Test Conditions (4); treatment with test substance

- rational for selection of concentrations of the test substance
- solubility of the test substance and rationale of the highest test concentration
- composition of the treatment medium

- duration of the test substance treatment

Test Conditions (5); NR viability test

- composition of NR treatment medium
- duration of NR incubation
- incubation conditions (i.e. 37°C ±1°C, 90% ±10% humidity, and 5.0% ±1.0% CO_2/air)
- NR extraction conditions (extractant; duration)
- wavelength used for spectrophotometric reading of NR optical density

Information Concerning the Sponsor and the Test Facility

- name and address of the sponsor, test facilities, study director, and participating laboratory technicians
- justification of the test method and specific protocol used

Test Method Integrity

- the procedure used to ensure the integrity (i.e. accuracy and reliability) of the test method over time (e.g. use of the PC data)

Criteria for an Acceptable Test

- acceptable VC differences between each column of wells and the mean of both columns
- acceptable concurrent PC ranges based on historical data (include the summary historical data)
- number of toxic points on either side of the IC_{50} (i.e. number of points > 0 and ≤ 50% viability and > 50 and < 100% viability)

Results

- tabulation of data from individual test samples (e.g. IC_{50} values for the reference substance and the PC, absolute and derived OD_{540} readings, reported in tabular form, including data from replicate repeat experiments as appropriate, and the means and standard deviations for each experiment)

Description of Other Effects Observed

- cell morphology, precipitate, NR crystals, etc.

Discussion of the Results

Conclusions

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ANNEX 1

DEFINITIONS

Coefficient of determination: In linear regression, it denotes the proportion of the variance in Y and X that is shared. Its value ranges between zero and one and it is commonly called "R^2." For example, $R^2 = 0.45$, indicates that 45% of the variance in Y can be explained by the variation in X and that 45% of the variance in X can be explained by the variation in Y.

Coefficient of variation: A statistical representation of the precision of a test. It is expressed as a percentage and is calculated as follows: (standard deviation/mean) × 100%

Confluence: A state in which cells in culture encounter other cells in the same culture to form a complete sheet of cells (monolayer). Confluence is determined as a percentage of cell coverage of the tissue culture vessel growth surface (e.g. cell monolayer is 80% confluent).

Cytotoxicity: The adverse effects resulting from interference with structures and/or processes essential for cell survival, proliferation, and/or function. For most chemicals/substances, toxicity is a consequence of non-specific alterations in "basal cell functions" (i.e. via mitochondria, plasma membrane integrity, etc.), which may then lead to effects on organ-specific functions and/or death of the organism. These effects may involve the integrity of membranes and the cytoskeleton, cellular metabolism, the synthesis and degradation or release of cellular constituents or products, ion regulation, and cell division.

Hill function: The IC_{50} values are determined from the concentration-response using a Hill function which is a four-parameter logistic mathematical model relating the concentration of the test substance to the response (typically following a sigmoidal shape):

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

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), EC_{50} is the substance concentration at the response midway between Top and Bottom, and HillSlope describes the slope of the curve. When Top=100% viability and Bottom=0% viability, the EC_{50} is the equal to the IC_{50}.

Hill function (rearranged): Some unusual dose-responses do not fit the Hill function well. To obtain a better model fit, the Bottom parameter can be estimated without constraints (i.e. Bottom not necessarily any particular value). However, when Bottom≠0, the EC_{50} reported by the Hill function is not the same as the IC_{50} since the Hill function defines EC_{50} as the point midway between Top and Bottom. Thus, the Hill function calculation using the Prism® software was rearranged to calculate the concentration corresponding to the IC_{50} as follows:

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

where IC_{50} is the concentration producing 50% toxicity, EC_{50} is the concentration producing a response midway between the Top and Bottom responses; Top is the maximum response (maximum survival),

Bottom is the minimum response (0% viability, maximum toxicity), Y=50 (i.e. 50% response), 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}.

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

LD_{50}: The calculated value of the oral dose that produces lethality in 50% of test animals (rats and mice). The LD_{50} values serve as reference values for the *in vitro* tests.

Neutral red uptake (NRU): Concentration of neutral red dye in the lysosomes of living cells. Altering the cell surface or the lysosomal membrane by a toxicological agent causes lysosomal fragility and other adverse changes that gradually become irreversible. The NRU test method makes it possible to distinguish between viable, damaged, or dead cells because these changes result in decreased uptake and binding of NR measurable by optical density absorption readings in a spectrophotometer.

Optical density (OD_{540}): The absorption (i.e. OD_{540} measurement) of the resulting coloured solution (colorimetric endpoint) in the NRU assay measured at 540 nm ±10 nm in a spectrophotometric microtiter plate reader using blanks as a reference.

RC regression (Halle, 1999, 2003): $\log (LD_{50}) = 0.435 \log (IC_{50}) + 0.625$; for estimating an LD_{50} value in mmol/kg (body weight) from an IC_{50} value in mM. Developed using the 347 IC_{50} and oral LD_{50} (282 rat and 65 mouse) values from the RC.

RC rat-only millimole regression: $\log (LD_{50}) = 0.439 \log (IC_{50}) + 0.621$; for estimating an LD_{50} value in mmol/kg (body weight) from an IC_{50} value in mM; developed from the IC_{50} values (in mM) and acute oral LD_{50} values (in mmol/kg) for the 282 substances with rat LD_{50} values in the RC database (Halle 1998, 2003).

RC rat-only weight regression: $\log (LD_{50}) = 0.372 \log (IC_{50}) + 2.024$; for estimating an LD_{50} value in mg/kg (body weight) from an IC_{50} value in µg/mL; developed from the IC_{50} values (in µg/mL) and acute oral LD_{50} values (in mg/kg) for the 282 substances with rat LD_{50} values in the RC database (Halle 1998, 2003).

Solubility: The amount of a test substance that can be dissolved (or thoroughly mixed with) culture medium or solvent. The solubility protocol was based on a U.S. EPA guideline (EPA, 1996) that involves testing for solubility in a particular solvent, beginning at a relatively high concentration and proceeding to successively lower concentrations by adding more solvent as necessary for dissolution. Testing stops when, upon visual observation, the procedure produces a clear solution with no cloudiness or precipitate.

Volatility: Ability of a test chemical/substance to evaporate. A general indicator of excessive volatility in the NRU test methods is the percent difference in the mean OD_{540} values for the two VC columns on the test plate (i.e. excessive volatility contaminates the VC column adjacent to the highest test substance concentration). If the difference is greater than 15%, then excessive chemical/substance volatility can be suspected, especially if the VC adjacent to the highest test concentration had a significantly reduced OD_{540} value. Excessive volatility may be an issue for compounds with a specific gravity of less than 1.

Weight-of-evidence: A weight-of-evidence approach is the use of the strengths and weaknesses of a collection of information as the basis for a conclusion that may not be evident from the individual data. For estimating starting doses, in vitro data should be considered along with all other data and information such as quantitative structure-activity relationship (QSAR) predictions, the LD_{50} of related substances,, and other existing data, to estimate a dose that is likely to be close to the actual LD_{50} value.

ANNEX 2

PREQUALIFICATION OF NORMAL HUMAN EPIDERMAL KERATINOCYTE (NHK) GROWTH MEDIUM

1. Keratinocyte Basal Medium and the medium supplements supplied by a manufacturer for use with NHK cells should be prequalified to demonstrate their ability to perform adequately in the NHK NRU assay. The quality control (QC) test data should be obtained from the manufacturer for each potential lot of medium and supplements.

Test System

2. The NHK NRU assay is performed to analyze NHK growth characteristics and the *in vitro* toxicity of SLS, as measured by the IC_{50}, for each NHK medium/supplement combination being tested. Test every combination of medium/supplements expected to be used in subsequent NHK NRU tests.

3. Establish NHK cultures using each medium/supplement combination to be tested, and subculture the cells 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/supplement (if available) for which performance has been previously established.

Test Methods

4. Establish NHK cultures with cryopreserved cells seeded into individual 25 cm^2 tissue culture flasks using a proven medium/supplement combination (i.e. the control medium) and each test medium/supplement combination.

5. Suspend freshly thawed cells initially into 9 mL of control medium and then add the cell suspension to 25 cm^2 culture flasks containing pre-warmed control or test medium. Use cell seeding densities in flasks (1 flask/density/medium) of 1 x 10^4, 5 x 10^3, and 2.5 x 10^3 cells.

6. Subculture the cells on three different days into 96-well plates for three subsequent NRU tests (three test plates total [one plate per day] for each medium/supplement combination and each control).

7. Sub-culturing the cells and application of the SLS will follow the procedures in paragraph 25 of the Guidance Document in reference to appropriate cell confluency. Cell numbers should be recorded for each flask prior to sub-culturing to the 96-well plates. Doubling time may be measured as an additional quality assurance check.

Test Procedure

8. Preparation of SLS should follow the main test procedures for testing compounds in keratinocyte routine culture medium. Cells cultured in control medium and in each test medium/supplement combination should be tested in parallel for their sensitivity to SLS.

9. SLS concentrations should be the same or similar to those used previously with control medium/supplements. The SLS concentration range used in an *in vitro* validation study was 0.6 μg/mL – 20.0 μg/mL (ICCVAM, 2006a).

Microscopic Evaluation

10. Changes in morphology of the cells due to cytotoxic effects of the SLS (prior to measurement of NRU) should be recorded. In addition to the general microscopic evaluation of the cell cultures, the following specific observations should be made:

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])

Data Analysis and Test Evaluation

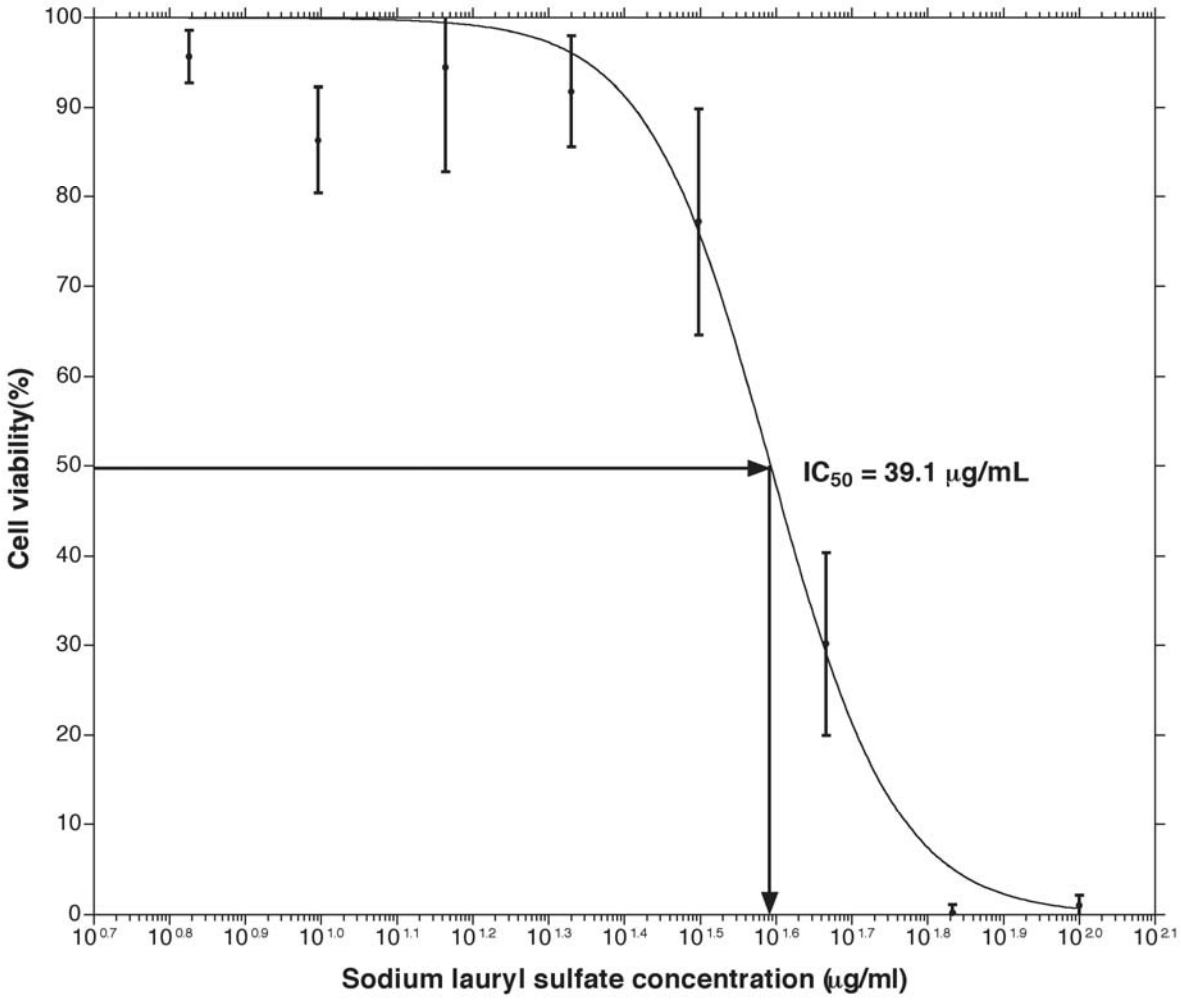
11. See Test Acceptance Criteria (paragraphs 60-63) to determine acceptability of a test plate. Other criteria that should be considered include the following:

- mean corrected OD_{540} of the VCs. *Note: The target range for corrected mean OD_{540} = 0.248 - 1.123 for the VCs (range = mean OD_{540} ±2.5 standard deviations; mean = 0.685; SD = 0.175; N = 114 [ICCVAM, 2006a]).*
- cell morphology and confluence of the VCs at the end of the 48-hour treatment.
- doubling time for NHK cells.

12. 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.

ANNEX 3

TYPICAL DOSE-RESPONSE FOR SODIUM LAURYL SULFATE (SLS) IN THE NEUTRAL RED UPTAKE TEST USING BALB/C 3T3 MOUSE FIBROBLASTS



The points and error bars show the means and standard deviations, respectively, for the percent cell viability response of the six replicate wells at each of the eight concentrations: 6.8, 10, 14.7, 21.5, 31.6, 46.4, 68.1, and 100 μg/mL. The curved line shows the fit of the concentration-response to the Hill function.

ANNEX 4

96-WELL PLATE TEMPLATE

	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

96-Well plate configuration for positive control (PC) and test substance assays.

Rows A through H show the locations of the eight rows of the 96-well plate, while the columns numbered 1 through 12 show the locations of the 12 columns of the 96-well plate.

VC1 and VC2 are the left (VC1) and right (VC2) vehicle control wells, which contain cells, routine culture medium and solvent (if used). VCb wells are VC blanks that contain routine culture medium and solvent [if used], but not cells.

C_1 – C_8 are the eight test substance or PC (sodium lauryl sulfate [SLS]) concentrations. C_1 is the highest concentration and C_8 is the lowest. Each concentration tested has six replicate wells. C_xb are blank wells that contain test substance or PC, but not cells.

ANNEX 5

SOLUBILITY PROTOCOL

SOLUBILITY DETERMINATION OF TEST SUBSTANCES

1. **This protocol identifies the solvent that provides the highest soluble concentration of a test substance for uniform availability of the substance to cells in *in vitro* basal cytotoxicity testing.**

2. The solubility test procedure is based on attempting to dissolve a test substance in various solvents with increasingly rigorous mixing techniques. The solvents to be used, in the order of preference, are cell culture medium, DMSO, and EtOH. Determination of whether a test substance has dissolved can be based on visual observation using a microscope. A test substance has dissolved if the solution is clear and shows no signs of cloudiness or precipitation (see paragraph 26 in the main body of the Guidance Document).

3. The solubility test procedure is a step-wise tiered procedure to determine the appropriate solvent for use in the test methods. Each tier involves attempting to dissolve the test substance in one or more solvents at test substance concentrations that will yield the same concentration (when dissolved in any solvent) on the cells (with 0.5% [v/v] DMSO or EtOH for those substances not soluble in medium). If the test 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 mixing procedures are repeated in an attempt to solubilise the substance at the lower concentration. 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 the culture medium, DMSO, and EtOH hierarchy. If, at any tier, a substance were soluble in 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.

Determination of Solubility Using the Step-Wise (Tiered) Procedure

4. *Tier 1*: Weigh 100 mg of the test substance into a glass tube. Add approximately 0.5 mL of medium into the tube to get 200 mg/mL. Mix the solution. If complete solubility is achieved, then additional solubility procedures are not needed.

5. *Tier 2*: If the test substance is insoluble in Tier 1 at 200 mg/mL, then proceed to Tier 2. Weigh 10 mg of the test substance into a glass tube. Add approximately 0.5 mL of medium to get 20 mg/mL. Mix the solution. If complete solubility is achieved, then additional solubility procedures are not needed.

6. *Tier 3*: If the test substance is insoluble in Tier 2 at 20 mg/mL, 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 100 mg test substance in a second glass tube and add approximately 0.5 mL DMSO to get 200 mg/mL and mix the solution. If the test substance does not dissolve in DMSO, weigh 100 mg test substance in another glass tube and add approximately 0.5 mL EtOH to get 200 mg/mL and mix the solution. If the substance is soluble in either solvent, no additional solubility procedures are needed.

7. *Tier 4*: If the substance is insoluble in Test Substance Dilution Medium, DMSO, or EtOH at Tier 3, then continue to Tier 4. Add enough solvent to increase the volume of the three (or four) Tier 2 solutions by 10 and attempt to solubilise 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.

8. *Tier 5*: Dilute the Tier 4 samples with DMSO or EtOH to bring the total volume to 50 mL and attempt to solubilise again using the sequence of mixing procedures.

9. *Tier 6*: Weigh two samples of test substance at 10 mg each, add approximately 50 mL DMSO or EtOH for a 200 μg/mL solution, and following the mixing procedures.

Mixing Procedures

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

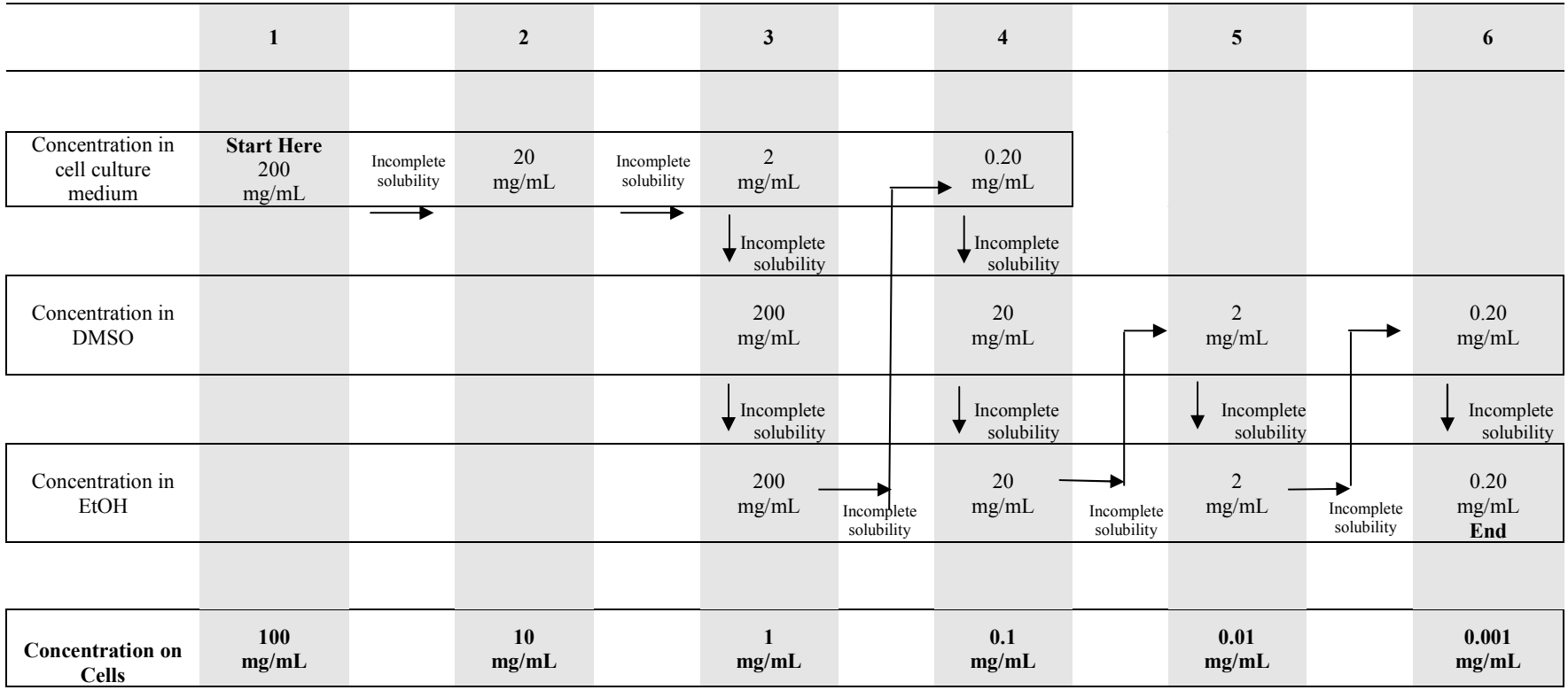
a) Gently mix at room temperature by vortexing for 1 – 2 minutes.

b) If test substance has not dissolved, use water-bath sonication for up to 5 minutes.

c) If test substance is not dissolved after sonication, then warm solution to 37°C for 5 - 60 minutes in a water-bath or in a CO_2 incubator. The solution may be stirred during warming (stirring in a CO_2 incubator will help maintain proper pH).

d) Proceed to Tier 2 (and Tiers 3-6, if necessary and repeat mixing procedures a - b).

Figure 1. Flow Chart for Determination of Test Substance Solubility in Medium, Dimethyl Sulfoxide (DMSO), or Ethanol (EtOH).



Testing starts with 200 mg/mL cell culture medium and proceeds to 0.2 mg/mL in EtOH if the test substance is not completely soluble. Mixing procedures are applied at each concentration step to enhance dissolution. Testing stops at any step during which the test substance achieves solubilit

ANNEX 6

TROUBLESHOOTING

1. The success of a NRU test outcome depends upon achieving adequate cell growth, sufficient cytotoxicity for the calculation of an IC_{50} value, the absence of NR crystals, and a good fit of the concentration-response data to the Hill function. Cells should be in the exponential phase of growth during chemical/substance exposure. Control OD_{540} values should typically be at least 0.3, although lower OD_{540} measurements can be justified if the cells look healthy and the response to SLS is adequate. If neither of these conditions is met, suspect mycoplasma (or other; e.g. bacterial, fungal) contamination, inadequate environmental conditions (temperature, CO_2, humidity), cell culture medium, or cell culture medium components (i.e. serum for the 3T3 or growth factors for the NHK). Although 100% confluence at the end of the exposure period is satisfactory for the 3T3 cells, it is undesirable for the NHK cells. Confluent NHK cells produce growth factors that inhibit growth and promote differentiation.

2. Solubility is often the limiting factor in achieving sufficient cytotoxicity for the calculation of an IC_{50} value, especially for relatively nontoxic test substances. Insoluble substances may produce a precipitate or a film in the stock solution or in the cell culture wells. Solvents other than those recommended in this protocol may be used if the concentration used does not produce cytotoxicity. Additional procedures such as stirring or heating for longer periods may also increase test substance solubility. Users should be aware that inadequate toxicity upon exposure to volatile substances might, in fact, be an artefact of the "airborne" substance escaping the wells. A reduction in the viability of the VC cultures adjacent to the highest concentration of a test substance may suggest that this substance has volatilized (see VC1 wells in the recommended 96-well palate template in Annex 4). However, adequate cytotoxicity for some volatile agents is achievable with the use of plastic film sealers to retain the vapours and minimize contamination of neighbouring VC wells.

3. Insoluble substances or those unstable in aqueous environments are not compatible with the test systems. Volatile substances may yield acceptable results if CO_2 permeable plastic film is used to seal the test plates. Testing for corrosive substances is unnecessary since there is no regulatory requirement for acute oral systemic toxicity testing for known corrosives. The 3T3 NRU test method may underestimate the toxicity of substances that are highly bound to serum proteins because the culture medium contains 5% serum during substance exposure. The toxicity of substances that specifically affect lysosomes may be overestimated because they may affect NRU binding, and therefore, retention, in the cell. Red substances (and other coloured substances) that absorb light in the optical density range of NR may interfere with the test if they remain inside the cell in sufficient amounts after washing and are soluble in the NR solvent.

4. NR dye crystals interfere with OD_{540} measurements. Blank OD_{540} values may increase from the typical 0.05 to approximately 0.10 or higher. Preparation and maintenance of the NR dye solution is a key factor in minimizing crystal formation. Therefore, the NR dye solution should be made fresh, filtered, and maintained at 37ºC prior to application to the cells.

5. The calculation of an appropriate IC_{50} value depends upon the fit of the concentration-response data to the Hill function. Toxicants that are specific for acting at a single phase of the cell cycle may yield

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

a concentration-response in which percent viability oscillates greatly around 50% with the increasing log doses of the range finder test. In these situations, the main test should focus on the lowest concentrations that produce 50% reduction in viability. Concentration-responses, for which the percent viability plateaus with increasing concentration, rather than decreasing to 0%, tend to fit the Hill function poorly (i.e. $R^2 < 0.9$). The fit is generally improved by allowing the Hill function to fit the Bottom parameter of the Hill function rather than by constraining it to 0% viability. Then, however, the EC_{50} of the standard Hill function will not be equivalent to the concentration that reduces viability by 50%. The Hill function calculation should be rearranged to calculate the IC_{50} as follows:

where IC_{50} is the concentration producing 50% toxicity, EC_{50} is the concentration producing a response midway between the Top and Bottom responses; Top is the maximum percent viability, Bottom is the minimum viability (maximum toxicity), Y=50 (i.e. 50% response), 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}.

6. The prediction of the rat oral LD_{50} values and the determination of starting doses for acute oral systemic toxicity tests by the *in vitro* NRU methods is expected to be poor for substances with mechanisms of toxicity that are not active in the 3T3 or NHK cells. Such toxic mechanisms include specific, receptor-mediated actions on the central nervous system or the heart (ICCVAM, 2006a).

7. The *in vitro* NRU test methods can be applied to a wide range of substances as long as they can be dissolved in the cell culture medium or in a nontoxic solvent (at the concentration used), and do not react with the culture medium. Although these test methods may to be applicable to mixtures, none were evaluated in this validation study. The toxicity of substances that act by mechanisms not expected to be active in 3T3 or NHK cells (e.g. those that are specifically neurotoxic or cardiotoxic) will likely be under-predicted by these test methods. Therefore, until more appropriate cell lines are developed, the results from basal cytotoxicity testing with such substances may not be relevant for predicting certain *in vivo* effects.

ANNEX 7

EXAMPLES FOR ESTIMATION OF STARTING DOSES FOR ACUTE ORAL SYSTEMIC TOXICITY TESTS

(see **Determination of the Starting Doses for Acute Oral Systemic Toxicity Tests** – paragraphs 65 - 68)

EXAMPLE FOR mM IC_{50} VALUE

(See Figure 1 for graphical representation)

1,1,1-Trichloroethane (MW 133.4)

3T3 NRU IC_{50} = 153.3 mM

log LD_{50} (mmol/kg) = 0.439 log IC_{50} (mM) + 0.621

(RC Rat-Only Millimole Regression [ICCVAM, 2006a]).

log LD_{50} (mmol/kg) = (0.439 x 0.2.186 mM) + 0.621

log LD_{50} (mmol/kg) = 1.580

LD_{50} = 38.019 mmol/kg

Estimated LD_{50} = 38.019 mmol/kg x 133.4 mg/mmol

Estimated LD_{50} = 5072 mg/kg

UDP Starting Dose

Default doses: 1.75, 5.5, 17.5, 55, 175, 550, and 2000 mg/kg (limit test of 2000 mg/kg)
1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000 mg/kg (limit test of 5000 mg/kg)

Estimated LD_{50} = 5072 mg/kg; Starting dose = 5000 mg/kg, one default dose below the estimated LD_{50}.

ATC Starting Dose

Default doses: 5, 50, 300, and 2000 mg/kg (limit test of 2000 mg/kg)
5, 50, 300, 2000, and 5000 mg/kg (limit test of 5000 mg/kg)

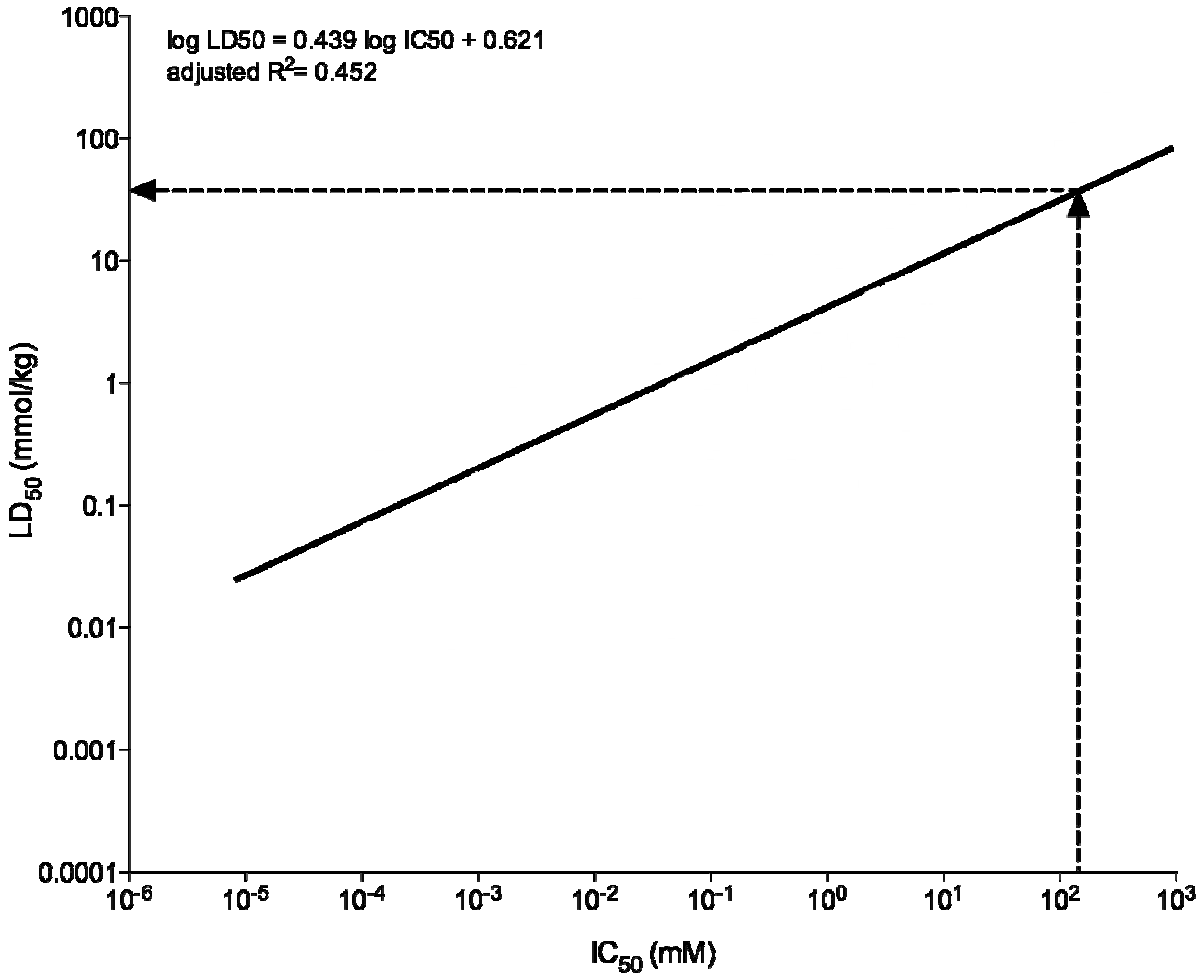
Estimated LD_{50} = 5072 mg/kg; Starting dose = 5000 mg/kg, one default dose below the estimated LD_{50}.

FDP Starting Dose

Default doses: 5, 50, 300, and 2000 mg/kg (limit test of 2000 mg/kg)
5, 50, 300, 2000, and 5000 mg/kg (limit test of 5000 mg/kg)

Estimated LD_{50} = 5072 mg/kg; Starting dose for sighting study = 5000 mg/kg, one default dose below the estimated LD_{50}.

Figure 1 RC Rat Only Millimole Regression – Correlation of IC_{50} to Estimated LD_{50}



Dashed lines show correlation of the IC_{50} value to the LD_{50} value on the regression line

EXAMPLE FOR μg/mL IC_{50} VALUE

(See Figure 2 for graphical representation)

1,1,1-Trichloroethane (MW 133.4)

3T3 NRU IC_{50} = 20453 μg/mL

log LD_{50} (mg/kg) = 0.372 log IC_{50} (μg/mL) + 2.024

(*RC Rat-Only Weight Regression [ICCVAM, 2006a]).*

ICCVAM, 2006a)

log LD_{50} (mg/kg) = (0.372 x 4.311) + 2.024

log LD_{50} (mg/kg) = 3.628

LD_{50} = 4246 mg/kg

UDP Starting Dose

Default doses: 1.75, 5.5, 17.5, 55, 175, 550, and 2000 mg/kg (limit test of 2000 mg/kg)
1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000 mg/kg (limit test of 5000 mg/kg)

Estimated LD_{50} = 4246 mg/kg; Starting dose = 2000 mg/kg, one default dose below the estimating LD_{50} for limit test of 2000 mg/kg; *Starting dose = 1750 mg/kg, one default dose below the estimated LD_{50} for* limit test of 5000 mg/kg.

ATC Starting Dose

Default doses: 5, 50, 300, or 2000 mg/kg (limit test of 2000 mg/kg)
5, 50, 300, 2000, or 5000 mg/kg (limit test of 5000 mg/kg)

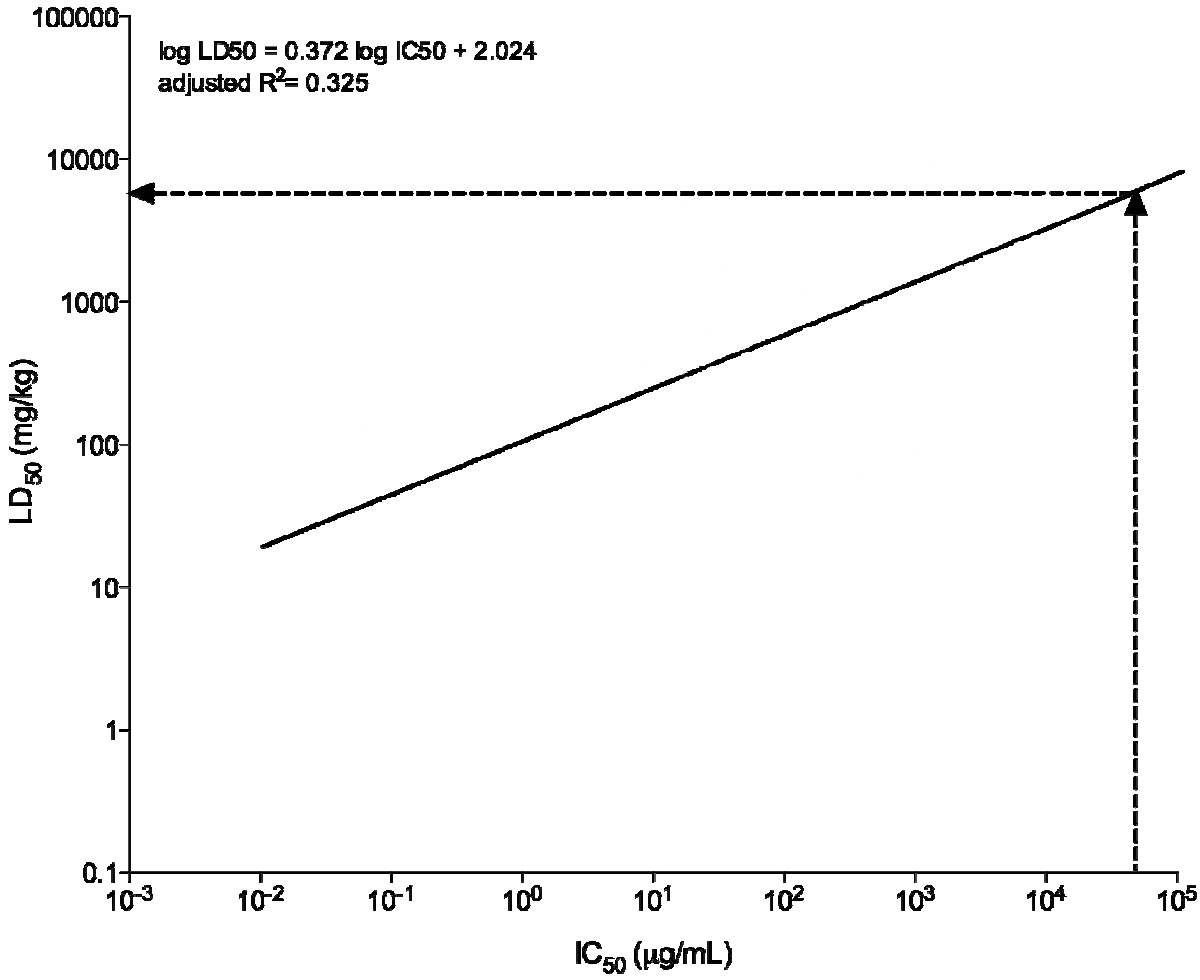
Estimated LD_{50} = 4246 mg/kg; Starting dose = 2000 mg/kg, one default dose below the estimated LD_{50} for limit test of 2000 or 5000 mg/kg.

FDP Starting Dose

Default doses: 5, 50, 300, and 2000 mg/kg (limit test of 2000 mg/kg)
5, 50, 300, 2000, and 5000 mg/kg (limit test of 5000 mg/kg)

Estimated LD_{50} = 4246 mg/kg; Starting dose for sighting study = 2000 mg/kg, one default dose below the estimated LD_{50} for limit test of 2000 or 5000 mg/kg.

Figure 2 RC Rat Only Weight Regression – Correlation of IC_{50} to Estimated LD_{50}



Dashed lines show correlation of the IC_{50} value to the LD_{50} value on the regression line

ANNEX 8

In Vitro and *In Vivo* Data from the NICEATM-ECVAM *In Vitro* Basal Cytotoxicity Validation Study (ICCVAM, 2006a)

Chemical Tested	CASRN	3T3 NRU IC_{50} (µg/mL)[1]	NHK NRU IC_{50} (µg/mL)[1]	Reference Acute Oral LD_{50} (mg/kg)[2, 3]	Chemical Class[4]
1,1,1-Trichloroethane	71-55-6	17248	8122[5]	12078	Organic compound; Halogenated hydrocarbon
2-Propanol	67-63-0	3618	5364	5105	Organic compound; Alcohol
5-Aminosalicylic acid	89-57-6	1667	46.7	3429	Organic compound; Carboxylic acid; Phenol
Acetaminophen	103-90-2	47.7	518	2163	Organic compound; Amide
Acetonitrile	75-05-8	7951	9528	3598	Organic compound; Nitrile
Acetylsalicylic acid	50-78-2	676	605	1506	Organic compound; Carboxylic acid; Phenol
Aminopterin	54-62-6	0.006	669	7	Organic compound; Heterocyclic compound
Amitriptyline HCl	549-18-8	7.05	8.96	348	Organic compound; Polycyclic compound
Arsenic trioxide	1327-53-3	1.96	5.26	25	Inorganic compound; Arsenical
Atropine sulfate	5908-99-6	76	81.8	819	Organic compound; Heterocyclic compound
Boric acid	10043-35-3	1850	421	3426	Inorganic compound; Boron compound; Acids
Busulfan	55-98-1	77.7	260	12	Organic compound; Alcohol; Acyclic hydrocarbon; Sulfur compound
Cadmium II chloride	10108-64-2	0.518	1.84	135	Inorganic compound; Cadmium compound
Caffeine	58-08-2	153	638	310	Organic compound; Heterocyclic compound
Carbamazepine	298-46-4	103	83.2	2805	Organic compound; Heterocyclic compound
Carbon tetrachloride	56-23-5	NA	NA	3783	Organic compound; Halogenated hydrocarbon
Chloral hydrate	302-17-0	183	133	638	Organic compound; Alcohol
Chloramphenicol	56-75-7	128	348	3491	Organic compound; Alcohol; Cyclic hydrocarbon; Nitro compound
Citric acid	77-92-9	796	400	5929	Organic compound; Carboxylic acid
Colchicine	64-86-8	0.034	0.007	15 (mouse)	Organic compound; Polycyclic compound
Cupric sulfate pentahydrate	7758-99-8	42.1	197	474	Inorganic compound; Sulfur compound; Metal
Cycloheximide	66-81-9	0.187	0.073	2	Organic compound; Heterocyclic compound
Dibutyl phthalate	84-74-2	49.7	28.7	8892	Organic compound; Carboxylic acid
Dichlorvos	62-73-7	17.7	10.7	59	Organic compound; Organophosphorous compound
Diethyl phthalate	84-66-2	107	120	9311	Organic compound; Carboxylic acid
Digoxin	20830-75-5	466	0.001	28	Organic compound; Polycyclic compound; Carbohydrate
Dimethylformamide	68-12-2	5224	7760	5309	Organic compound; Amide; Carboxylic acid

Chemical Tested	CASRN	3T3 NRU IC_{50} (μg/mL)[1]	NHK NRU IC_{50} (μg/mL)[1]	Reference Acute Oral LD_{50} (mg/kg)[2, 3]	Chemical Class[4]
Diquat dibromide monohydrate	6385-62-2	8.04	4.48	160	Organic compound; Heterocyclic compound
Disulfoton	298-04-4	133	270	5	Organic compound; Organophosphorous compound; Sulphur compound
Endosulfan	115-29-7	6.35	2.13	28	Organic compound; Heterocyclic Compound; Sulphur compound
Epinephrine bitartrate	51-42-3	59	87.4	4 (mouse)	Organic compound; Alcohol; Amine
Ethanol	64-17-5	6523	10018	11324	Organic compound; Alcohol
Ethylene glycol	107-21-1	24317	41852	7161	Organic compound; Alcohol
Fenpropathrin	39515-41-8	24.2	2.43	76	Organic compound; Nitrile; Ester; Ether
Gibberellic acid	77-06-5	7810[5]	2856	6040	Organic compound; Polycyclic compound
Glutethimide	77-21-4	174	174	600	Organic compound; Heterocyclic compound
Glycerol	56-81-5	24655	24730	19770	Organic compound; Alcohol
Haloperidol	52-86-8	6.13	3.36	330	Organic compound; Ketone
Hexachlorophene	70-30-4	4.19	0.029	82	Organic compound; Cyclic hydrocarbon; Phenol
Lactic acid	50-21-5	3044	1304	3639	Organic compound; Carboxylic acid
Lindane	58-89-9	108	18.7	100	Organic compound; Halogenated hydrocarbon
Lithium I carbonate	554-13-2	562[5]	468	590	Inorganic compound; Lithium compound; Alkylies; Carbon compound
Meprobamate	57-53-4	519	357	1387	Organic compound; Carboxylic acid
Mercury II chloride	7487-94-7	4.12	5.8	40	Inorganic compound; Mercury compound; Chlorine compound
Methanol	67-56-1	NA	1529[6]	8710	Organic compound; Alcohol
Nicotine	54-11-5	361	107	70	Organic compound; Heterocyclic compound
Paraquat	1910-42-5	20.1	61.6	93	Organic compound; Heterocyclic compound
Parathion	56-38-2	37.4	30.3	6	Organic compound; Organo-phosphorous compound; Sulphur compound
Phenobarbital	50-06-6	573	448	224	Organic compound; Heterocyclic compound
Phenol	108-95-2	66.3	75	548	Organic compound; Phenol
Phenylthiourea	103-85-5	79	336	3	Organic compound; Sulphur compound; Urea
Physostigmine	57-47-6	25.8	88.5	5	Organic compound; Carboxylic acid; Heterocyclic compound
Potassium cyanide	151-50-8	34.6	29	7	Inorganic compound; Potassium compound; Nitrogen compound
Potassium I chloride	7447-40-7	3551	2237	2799	Inorganic compound; Potassium compound; Chlorine compound
Procainamide HCl	51-06-9	441	1741	1950	Organic compound; Carboxylic acid; Amide
Propranolol	3506-09-0	13.9	35.3	466	Organic compound; Alcohol; Amine; Polycyclic compound
Propylparaben	94-13-3	26.1	16.6	6332 (mouse)	Organic compound; Carboxylic acid; Phenol
Sodium arsenite	7784-46-5	0.759	0.477	44	Inorganic compound; Arsenical; Sodium compound

Chemical Tested	CASRN	3T3 NRU IC_{50} (μg/mL)[1]	NHK NRU IC_{50} (μg/mL)[1]	Reference Acute Oral LD_{50} (mg/kg)[2, 3]	Chemical Class[4]
Sodium chloride	7647-14-5	4730	1997	4046	Inorganic compound; Sodium compound; Chlorine compound
Sodium dichromate dihydrate	7789-12-0	0.587	0.721	51	Inorganic compound; Sodium compound; Chromium compound
Sodium fluoride	7681-49-4	78	49.8	127	Inorganic compound; Sodium compound; Fluorine compound
Sodium hypochlorite	7681-52-9	1103	1502	10328	Inorganic compound; Sodium compound; Oxygen compound; Chlorine compound
Sodium oxalate	62-76-0	37.7	337	633	Organic compound; Carboxylic acid; Sodium compound
Sodium selenate	13413-01-0	29	10.2	3	Inorganic compound; Sodium compound; Selenium compound
Strychnine	57-24-9	158	62.5	6	Organic compound; Heterocyclic compound
Thallium I sulfate	7446-18-6	5.74	0.152	25	Inorganic compound; Metal; Sulphur compound
Trichloroacetic acid	76-03-9	902	413	5229	Organic compound; Carboxylic acid
Triethylenemelamine	51-18-3	0.272	1.85	4	Organic compound; Heterocyclic compound
Triphenyltin hydroxide	76-87-9	0.017	0.01	329	Organic compound; Organo-metallic compound
Valproic acid	99-66-1	916	512	995	Organic compound; Carboxylic acid; Lipids
Verapamil HCl	152-11-4	34.9	66.5	111	Organic compound; Amine
Xylene	1330-20-7	721[5]	466[5]	4667	Organic compound; Cyclic hydrocarbon

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; CASRN=Chemical Abstract Service Registry Number; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; NA=Not available.

[1]Geometric mean IC_{50} of the laboratory geometric mean values (three laboratories unless otherwise noted).

[2]Based on a geometric mean of acceptable LD_{50} values from adult laboratory rats unless otherwise specified.

[3]Values rounded to the nearest whole number.

[4]Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, developed by the National Library of Medicine (http://www.nlm.nih.gov/mesh/meshhome.html).

[5]Data available from only one laboratory.

[6]Data available from only two laboratories.