Follow-up study on the predictive capacity of the 3T3 Neutral Red Uptake cytotoxicity assay to correctly identify substances not classified for acute oral toxicity under the EU CLP system (LD_{50} > 2 000 mg/kg)

Final Study Report

ANNEXES

Prepared by the European Centre for the Validation of Alternative Methods (ECVAM)

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LIST OF ANNEXES

ANNEX A

Study protocol of HSL

TEST METHOD PROTOCOL
for the BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test

A Test for Basal Cytotoxicity for an *In Vitro* Validation Study
Phase III

November 4, 2003

Prepared by

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

Based on Standard Operating Procedure Recommendations from an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Institute of Environmental Health Sciences (NIEHS)
National Institutes of Health (NIH)
U.S. Public Health Service
Department of Health and Human Services

TEST METHOD PROTOCOL

The BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test
A Test for Basal Cytotoxicity
Phase III

I. PURPOSE

The purpose of this study is to evaluate the cytotoxicity of test chemicals using the BALB/c 3T3 Neutral Red Uptake (NRU) cytotoxicity test. The data will be used to evaluate the intra- and inter-laboratory reproducibility of the assay and effectiveness of the cytotoxicity assay to predict the starting doses for rodent acute oral systemic toxicity assays. This test method protocol outlines the procedures for performing the cytotoxicity test and supports the *in vitro* validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM) and sponsored by NIEHS, U.S. Environmental Protection Agency, and ECVAM. This test method protocol applies to all personnel involved with performing the cytotoxicity assay.

A. BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test

The 3T3 NRU test will be performed to analyze the *in vitro* toxicity of 60 blinded/coded test chemicals. This test will be used to determine IC_{20}, IC_{50}, and IC_{80} values for the predetermined set of test chemicals of varying toxicities.

II. SPONSOR

A. Name:	National Institute of Environmental Health Sciences (NIEHS); The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)	
B. Address:	P.O. Box 12233 Research Triangle Park, NC 27709	
C. Representative:	*Named Representative*	

III. IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

A. Test Chemicals:	*Blinded Chemicals (60)*		
B. Controls:	Positive:	Sodium Lauryl Sulfate
	Vehicle (Negative):	Assay medium (DMEM containing 5% NBCS, 4 mM L-Glutamine, 100 IU/mL Penicillin, 100 µg/mL Streptomycin)
	Solvent:	Assay medium, DMSO, or ethanol directed by the Study Management Team, for preparation of test chemicals

IV. TESTING FACILITY AND KEY PERSONNEL

A. Facility Information

1) Name:
2) Address:
3) Study Director:
4) Laboratory Technician(s):
5) Scientific Advisor:
6) Quality Assurance Director:
7) Safety Manager:
8) Facility Management:

B. Test Schedule

1) Proposed Experimental Initiation Date:
2) Proposed Experimental Completion Date:
3) Proposed Report Date:

V. TEST SYSTEM

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 mammalian 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 NR after chemical exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

VI. DEFINITIONS

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

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log IC50 - X)\text{HillSlope}}}$$

where Y= response, X is the logarithm of dose (or concentration), Bottom is the minimum response, Top is the maximum response, logIC50 is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve.

B. *Documentation*: all methods and procedures will be noted in a Study Workbook; logs will be maintained for general laboratory procedures and equipment (e.g., media preparation, test chemical preparation, incubator function); all optical density data obtained from the

spectrophotometer plate reader will be saved in electronic and paper formats; all calculations of ICx values and other derived data will be in electronic and paper format; all data will be archived.

VII. PROCEDURES

A. Materials

1. Cell Lines

BALB/c 3T3 cells, clone 31
CCL-163, LGC Reference Materials, Customer Service, Queens Road, Teddington, Middlesex, TW110LY, UK
CCL-163, American Type Culture Collection [ATCC], Manassas, VA, USA)

2. Technical Equipment

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

a) Incubator: 37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air
b) Laminar flow clean bench/cabinet (standard: "biological hazard")
c) Water bath: 37ºC ± 1ºC
d) Inverse phase contrast microscope
e) Sterile glass tubes with caps (e.g., 5 mL)
f) Centrifuge (optionally: equipped with microtiter plate rotor)
g) Laboratory balance
h) 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm ± 10 nm filter
i) Shaker for microtiter plates
j) Cell counter or hemocytometer
k) Pipetting aid
l) Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette), dilution block
m) Cryotubes
n) Tissue culture flasks (e.g., 75 - 80 cm^2, 25 cm^2)
o) 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Falcon tissue culture-treated)
p) pH paper (wide and narrow range)
q) Multichannel reagent reservoir
r) Waterbath sonicator
s) Magnetic stirrer
t) Antistatic bar ionizer/antistatic gun (optional for neutralizing static on 96-well plates)
u) Dry heat block (optional)
v) Adhesive film plate sealers (e.g., Excel Scientific SealPlate ,Cat # STR-SEAL-PLT or equivalent)
w) Vortex mixer
x) Filters/filtration devices

[Note: Tissue culture flasks and microtiter plates should be prescreened to ensure that they adequately support the growth of 3T3 cells. Multi-channel repeater pipettes may be used for plating cells in the 96-well plates, dispensing plate rinse solutions, NR medium, and desorb solution. Do not use the repeater pipette for dispensing test chemicals to the cells.]

3. Chemicals, Media, and Sera

a) Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine; should have high glucose [4.5gm/l] (e.g., ICN-Flow Cat. No. 12-332-54)
b) L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49)
c) New Born Calf Serum (NBCS or NCS) (e.g., Biochrom # SO 125)
d) 0.05 % Trypsin/0.02 % EDTA solution (e.g., SIGMA T 3924, ICN-Flow, # 16891-49)
e) Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}(for trypsinization)
f) Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}(CMF-HBSS)
g) Dulbecco's Phosphate Buffered Saline (D-PBS) [formulation containing calcium and magnesium cations; glucose optional] (for rinsing)
h) Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49)
i) Neutral Red (NR) Dye – tissue culture-grade; liquid form (e.g., SIGMA N 2889); powder form (e.g., SIGMA N 4638)
j) Dimethyl sulfoxide (DMSO), U.S.P. analytical grade (Store under nitrogen @ -20ºC)
k) Ethanol (ETOH), U.S.P. analytical grade (100 %, non-denatured for test chemical preparation; 95 % can be used for the desorb solution)
l) Glacial acetic acid, analytical grade
m) Distilled H_2O or any purified water suitable for cell culture and NR desorb solution (sterile)
n) Sterile/non-sterile paper towels (for blotting 96-well plates)

[Note: Due to lot variability of NBCS/NCS, first check a lot for growth stimulating properties with 3T3 cells (approximately 20-24 h doubling time) and then reserve a sufficient amount of NBCS/NCS. May use pre-tested serum lot from Phases Ia, Ib, and II of the validation study if the serum has been stored under appropriate conditions and shelf-life has not expired.]

B. Preparations of Media and Solutions

[Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures will be adequately documented.]

1. Media

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

a) for freezing (Freeze Medium); contains 2X concentration of NBCS/NCS and DMSO of final freezing solution
   40 % NBCS/NCS
   20 % DMSO

b) for routine culture (Routine Culture Medium)
   10 % NBCS/NCS
   4 mM Glutamine

c) for test chemical dilution (Chemical Dilution Medium)

4 mM	Glutamine
200 IU/mL	Penicillin
200 µg/mL	Streptomycin

d) for dilution of NR stock solution (NR Dilution Medium)

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

[Note: The Chemical Dilution Medium with test chemical will dilute the serum concentration of the Routine Culture Medium in the test plate to 5 %. Serum proteins may mask the toxicity of the test substance, but serum cannot be totally excluded because cell growth is markedly reduced in its absence.]

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

2. Neutral Red (NR) Stock Solution

The liquid tissue culture-grade stock NR Solution will be the first choice for performing the assay (e.g., SIGMA #N2889, 3.3 mg/mL). Store liquid tissue culture-grade NR Stock Solution at the storage conditions and shelf-life period recommended by the manufacturer.

If the liquid form is not available, the following formulation can be prepared.

EXAMPLE: 0.25 g NR Dye powder in 100 mL H_2O

The NR Stock Solution (powder in water) should be stored in the dark at room temperature for up to two months.

3. Neutral Red (NR) Medium

EXAMPLE:

0.758 mL (3.3 mg NR dye/mL solution)	NR Stock Solution
99.242 mL	NR Dilution Medium (pre-warmed to 37° C)

The final concentration of the NR Medium is **25 µg NR dye/mL** and aliquots will be prepared on the day of application.

[Note: The NR Medium shall be filtered (e.g., Millipore filtering, 0.2 – 0.45 µm pore size) to reduce NR crystals. Aliquots of the NR Medium should be maintained at 37° C (e.g., in a waterbath) before adding to the cells and used within 30 min of preparation but also used within 15 min after removing from 37° C storage.]

4. Ethanol/Acetic Acid Solution (NR Desorb)

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

C. Methods

1. Cell Maintenance and Culture Procedures

BALB/c 3T3 cells are routinely grown as a monolayer in tissue culture grade flasks (e.g., 75 - 80 cm^2) at 37ºC ± 1ºC, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO_2/air. The cells should be examined on a daily (i.e., on workdays) basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted in a Study Workbook.

2. Receipt of Cryopreserved BALB/c 3T3 Cells

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

3. Thawing Cells

Thaw cells by putting ampules into a water bath at 37°C ± 1ºC. Leave for as brief a time as possible.

a) Resuspend the cells in pre-warmed Routine Culture Medium and transfer into pre-warmed Routine Culture Medium in a tissue-culture flask.

b) Incubate at 37ºC ± 1ºC, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO_2/air.

c) When the cells have attached to the bottom of the flask (within 4 to 24 h), decant the supernatant and replace with fresh pre-warmed (37ºC) medium. Culture as described above.

d) Passage at least two times before using the cells in a cytotoxicity test.

A fresh batch of frozen cells from the stock lot of cells should be thawed out and cultured approximately every two months. This period resembles a sequence of about 18 passages.

4. Routine Culture of BALB/C 3T3 Cells

When cells exceed 50 % confluence (but less than 80 % confluent) they should be removed from the flask by trypsinization:

a) Decant medium, briefly rinse cultures with 5 mL PBS or Hanks' BSS (<u>without Ca^{2+}, Mg^{2+}</u>) per 25 cm^2 flask (15 mL per 75 cm^2 flask). Wash cells by gentle agitation to remove any remaining serum that might inhibit the action of the trypsin.

b) Discard the washing solution. Repeat the rinsing procedure and discard the washing solution.

c) Add 1-2 mL trypsin-EDTA solution per 25 cm^2 to the monolayer for a few seconds (e.g., 15-30 seconds).

d) Remove excess trypsin-EDTA solution and incubate the cells at room temperature.

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

5. Cell Counting

After detaching the cells, add 0.1-0.2 mL of pre-warmed (37ºC) Routine Culture Medium/cm^2 to the flask (e.g., 2.5 mL for a 25 cm^2 flask). Disperse the monolayer by gentle trituration. 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 (e.g., Coulter counter).

6. Subculture of Cells

After determination of cell number, the culture can be sub-cultured into other flasks or seeded into 96-well microtiter plates. BALB/c 3T3 cells are routinely passaged at suggested cell densities as listed in the table (approximate doubling time is 20-24 h). The individual laboratories will need to determine and adjust the final density to achieve appropriate growth.

Table 1. Cell Density Guidelines for Subculturing

Days in Culture	Seeding Density (cells/cm^2)	Total Cells per 25 cm^2 flask	Total Cells per 75 cm^2 flask
2	16800	4.2×10^5	1.26×10^6
3	8400	2.1×10^5	6.3×10^5
4	4200	1.05×10^5	3.15×10^5

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

7. Freezing Cells (procedure required only if current stock of cells is depleted)

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

a) Centrifuge trypsinized cells at approximately 200 x g.

b) Suspend the cells in cold Routine Culture Medium (half the final freezing volume) so a final concentration of $1\text{-}5 \times 10^6$ cells/mL can be attained.

c) Slowly add cold Freeze Medium to the cells so that the solvent will equilibrate across the cell membranes. Bring the cell suspension to the final freezing volume. The final cell suspension will be 10 % DMSO. Aliquot the cell suspension into freezing tubes and fill to 1.8 mL.

d) Place the tubes into an insulated container (e.g., styrofoam trays) and place in a freezer (-70 to -80°C) for 24 h. This gives a freezing rate of approximately 1°C/min. The laboratory needs to ensure that the freezing protocol is applicable to the 3T3 cells and that the cells are viable when removed from cryopreservation.

e) Place the frozen tubes into liquid nitrogen for storage.

8. Preparation of Cells for Assays

a) Cultured cells that are going to be used in seeding the 96-well plates should be fed fresh medium the day before subculturing to the plates. On the day of plate seeding, prepare a cell suspension of 2.0 – 3.0x10^4 cells/mL in Routine Culture Medium. Using a multi-channel pipette, dispense 100 µl Routine Culture Medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate (See **Section VII.F.1**). In the remaining wells, dispense 100 µl of a cell suspension of 2.0 – 3.0x10^4 cells/mL (= 2.0 – 3.0x10^3 cells/well). The seeding density should be noted to ensure that the cells in the control wells are not overgrown after three days (i.e., 24 h incubation in step **b** and 48 h exposure to test chemicals). Prepare one plate per chemical to be tested.

b) Incubate cells for 24 ± 2 h (37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air) so that cells form a less than half (< 50%) confluent monolayer. This incubation period assures cell recovery and adherence and progression to exponential growth phase.

c) Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook.

9. Determination of Doubling Time

a) A cell doubling time procedure was performed on the initial lot of cells that was used in the first cell culture assays of Phase Ia of the Validation Study. The doubling time only needs to be determined in Phase III if there is a change in the lot of cells used. Establish cells in culture and trypsinize cells as per **Section VII.C.4** for subculture. Resuspend cells in NR Dilution Medium (5 % NBCS/NCS). Seed cells at 4200 cells/cm^2.

b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators (37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air).

c) After 4 - 6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue; Nigrosin) if Study Director sees a need. Use appropriate size exclusion limits if using a Coulter counter. Determine the total number of cells and document. Repeat sampling at 24 h, 48 h, 72 h, and 96 h post inoculation. Change culture medium at 72 h or sooner in remaining dishes if indicated by pH drop.

d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase).

D. Preparation of Test Chemicals

The Study Management Team will provide direction on the solvent to be used for each test chemical. [Note: Preparation under red or yellow light is recommended to preserve chemicals that degrade upon exposure to light.]

1. Test Chemicals in Solution

a) Allow test chemicals to equilibrate to room temperature before dissolving and diluting.

b) Prepare test chemical immediately prior to use. Test chemical solutions should not be prepared in bulk for use in subsequent tests. Ideally, the solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate. The SMT may direct the Study Director to store an aliquot (e.g., 1 mL) of the highest 2X stock solution (e.g., low solubility chemicals) in a freezer (e.g., -70°C) for use in future chemical analyses.

c) For chemicals dissolved in DMSO or ethanol, the final DMSO or ethanol concentration for application to the cells must be 0.5 % (v/v) in the vehicle controls and in all of the eight test concentrations.

d) The stock solution for each test chemical should be prepared at the highest concentration found to be soluble in the solubility test conducted per the *Test Method Protocol for Solubility Determination*. Thus, the highest test concentration applied to the cells in each range finding experiment is:
- 0.5 times the highest concentration found to be soluble in the solubility test, if the chemical was soluble in Chemical Dilution Medium, or
- 1/200 the highest concentration found to be soluble in the solubility test if the chemical was soluble in ethanol or DMSO.

e) The seven lower concentrations in the range finding experiment would then be prepared by successive dilutions that decrease by one log unit each. The following example illustrates the preparation of test chemical in solvent and the dilution of dissolved test chemical in Chemical Dilution Medium before application to 3T3 cells.

Example: Preparation of Test Chemical in Solvent Using a Log Dilution Scheme

If DMSO was determined to be the preferred solvent at Tier 2 of the solubility test (i.e., 200,000 μg/mL), dissolve the chemical in DMSO at 200,000 μg/mL for the chemical stock solution.

1) Label eight tubes 1 – 8. Add 0.9 mL solvent (e.g., DMSO) to tubes 2 -- 8.

2) Prepare stock solution of 200,000 μg test chemical/mL solvent in tube # 1.

3) Add 0.1 mL of 200,000 μg/mL dilution from tube #1 to tube #2 to make a 1:10 dilution in solvent (i.e., 20,000 μg/mL).

4) Add 0.1 mL of 20,000 μg/mL dilution from tube #2 to tube #3 to make another 1:10

dilution (i.e., 1:100 dilution from stock solution) in solvent (i.e., 2,000 g/mL)

5) Continuing making serial 1:10 dilutions in the prepared solvent tubes.

6) Since each concentration is 200 fold greater than the concentration to be tested, make a 1:100 dilution by diluting 1 part dissolved chemical in each tube with 99 parts of Chemical Dilution Medium (e.g., 0.1 mL test chemical in DMSO + 9.9 mL Chemical Dilution Medium) to derive the eight 2X concentrations for application to 3T3 cells. Each 2X test chemical concentration will then contain 1 % v/v solvent. The 3T3 cells will have 0.05 mL Routine Culture Medium in the wells prior to application of the test chemical. By adding 0.05 mL of the appropriate 2X test chemical concentration to the appropriate wells, the test chemical will be diluted appropriately (e.g., highest concentration in well will be 1,000 g/mL) in a total of 0.1 mL and the solvent concentration in the wells will be 0.5% v/v.

7) A test article prepared in Chemical Dilution Medium, DMSO, or ethanol may precipitate upon transfer into the Routine Culture Medium. The 2X dosing solutions should be evaluated for precipitates and the results recorded in the workbook. It will be permissible to test all of the dosing solutions in the dose range finding assay and main experiments. However, doses containing test article precipitates should be avoided and generally will not be used in the ICx determinations for the definitive tests. Precipitates in 2X dosing solutions are permissible for range finder tests but not for definitive tests.

Document all test chemical preparations in the Study Workbook.

2. pH of Test Chemical Solutions

Prior to or immediately after application of the test chemical to the 96-well plate, measure the pH of the highest 2X dosing concentration of the test chemical (i.e., C1 in the test plate, see Figure 1) in culture medium. Use pH paper (e.g., pH 0 - 14 to estimate and pH 5 – 10 to determine more precise value; or Study Director's discretion) for measurements. The pH paper should be in contact with the solution for approximately one minute. Document the pH and note the color of the 2X concentration medium (i.e., in the EXCEL template). Medium color for all dosing dilutions should be noted in the workbooks. Do not adjust the pH.

3. Concentrations of Test Chemical

a) Range Finder Experiment

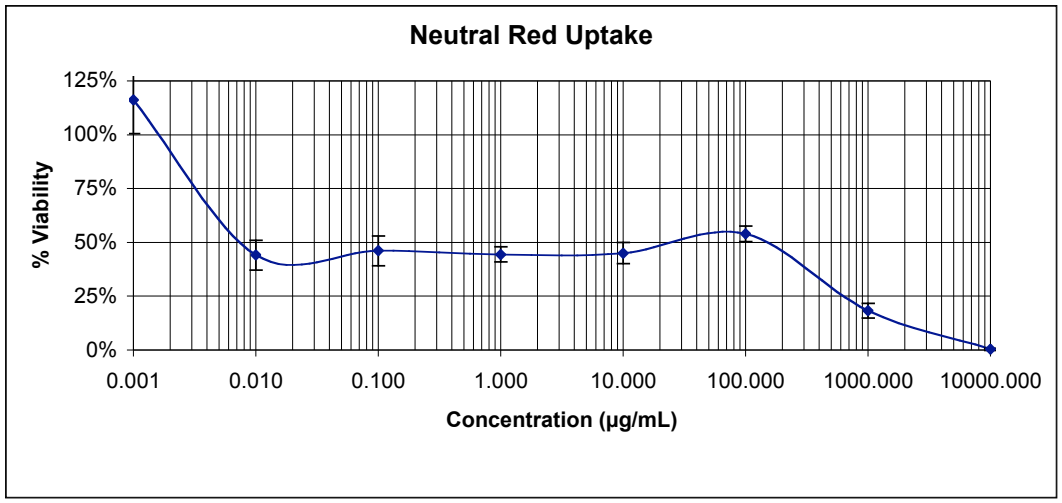
Test eight concentrations of the test chemical by diluting the stock solution with a constant factor covering a large range. The initial dilution series shall be log dilutions (e.g., 1:10, 1:100, 1:1000, etc.).

If a range finder experiment does not generate enough cytotoxicity, then higher doses should be attempted. If cytotoxicity is limited by solubility, then more stringent solubility procedures to increase the stock concentration (to the maximum concentration specified in Section VII.D.3.b.) should be employed. Place the test chemical concentration into an incubator (37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air) and stir or rock for up to 3 hours, if necessary, to facilitate dissolution. For stocks

prepared in medium, vessel caps should be loose to allow for CO_2 exchange. Proceed with dosing solution preparation and dosing.

- If a range finding test produces a biphasic curve, then the doses selected for the subsequent main experiments should cover the most toxic dose-response range (see Example 1 – the most toxic range is 0.001 – 0.1 μg/mL).

Example 1 – Biphasic Curve



b) Main Experiment

[Note: After the range finding assay is completed, the definitive concentration-response experiment shall be performed three times on three different days for each chemical (i.e., one plate per day per chemical.]

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., dilution factor of $\sqrt[6]{10} = 1.47$). Cover the relevant concentration range around the IC_{50} (> 0 % and < 100 % effect) preferably with several points of a graded effect, but with a minimum of two points, one on each side of the estimated IC_{50} value, avoiding too many non-cytotoxic and/or 100 %-cytotoxic concentrations. Experiments revealing less than one cytotoxic concentration on each side of the IC_{50} value shall be repeated, where possible, with a smaller dilution factor (see **Section VII.E.5.a.4**). Each experiment should have at least one cytotoxicity value > 0 % and ≤ 50.0 % viability and at least one cytotoxicity value > 50.0 % and < 100 % viability. A progression factor of 1.21 [$\sqrt[12]{10}$] is regarded the smallest factor achievable and will be the lowest dosing interval required.)

Determine which test chemical concentration is closest to the IC_{50} value (e.g., 50 % cytotoxicity). Use that value as a central concentration and adjust dilutions higher and lower in equal steps for the definitive assay.

Maximum Doses to be Tested in the Main Experiments
If minimal or no cytotoxicity was measured in the dose range finding assay, a maximum dose for the main experiments will be established as follows:

- For test chemicals prepared in Chemical Dilution Medium, the highest test article concentration that may be applied to the cells in the main experiments will be either 100 mg/mL, or the maximum soluble dose. Test chemical will be weighed into a glass tube and the weight will be documented. A volume of Chemical Dilution Medium will be added to the vessel so that the concentration is 200,000 μg/mL (200 mg/mL). The solution is mixed using the mechanical procedures that produced solubility when performing the solubility test specified in *Test Method Protocol for Solubility Determination*. If complete solubility is achieved in medium, then 7 additional serial stock dosing solutions may be prepared from the 200 mg/mL 2X stock. If the test chemical is insoluble in medium at 200 mg/ml, proceed by adding medium, in small incremental amounts, to attempt to dissolve the chemical by using the sequence of mechanical procedures specified in *Test Method Protocol for Solubility Determination*. More stringent solubility procedures may be employed if needed based on results from the range finder experiment (**Section VII.D.3.a.**). The highest soluble stock solution will be used to prepare the 7 additional serial stock dosing solutions.

- For test chemicals prepared in either DMSO or ethanol, the highest test article concentration that may be applied to the cells in the main experiments will be either 2.5 mg/mL, or less, depending upon the maximum solubility in solvent. Weigh the test chemical into a glass tube and document the weight. Add the appropriate solvent (determined from the original solubility test) to the vessel so that the concentration is 500,000 μg/mL (500 mg/mL). Mix the solution using the sequence of mechanical procedures specified in *Test Method Protocol for Solubility Determination*. If complete solubility is achieved in the solvent, then 7 additional serial stock dosing solutions may be prepared from the 500 mg/mL 200X stock. If the test chemical is insoluble in solvent at 500 mg/ml, proceed by adding solvent, in small incremental amounts, to attempt to dissolve the chemical by again using the sequence of mixing procedures. The highest soluble stock solution will be used to prepare the 7 additional serial stock dosing solutions.

- If precipitates are observed in the 2X dilutions, continue with the experiment, make the appropriate observations and documentation, and report data to the SMT.

c) Test Chemical Dilutions

The dosing factor of 3.16 ($= \sqrt[2]{10}$) divides a log into two equidistant steps, a factor of 2.15 ($= \sqrt[3]{10}$) divides a decade into three steps. The factor of 1.47 ($= \sqrt[6]{10}$) divides a log into six equidistant steps, the factor of 1.78 ($\sqrt[4]{10}$) divides a log into four equidistant steps, and the factor of 1.21 ($= \sqrt[12]{10}$) divides the log into 12 steps.

EXAMPLE:

10						31.6						100
10				21.5				46.4				100
10		14.7		21.5		31.6		46.4		68.1		100
10	12.1	14.7	17.8	21.5	26.1	31.6	38.3	46.4	56.2	68.1	82.5	100

The technical production of decimal geometric concentration series is simple. An

example is given for factor 1.47:

Dilute 1 volume of the highest concentration by adding 0.47 volumes of diluent. After equilibration, dilute 1 volume of this solution by adding 0.47 volumes of diluent...(etc.).

E. Test Procedure

1. 96-Well Plate Configuration

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

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb
B	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
C	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
D	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
E	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
F	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
G	VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
H	VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb

VC1 and VC2 = VEHICLE CONTROL
$C_1 – C_8$ = Test Chemicals or PC (SLS) at eight concentrations (C1 = highest, C8 = lowest)
b = BLANKS (Test chemical or PC, but contain **no** cells)
VCb = VEHICLE CONTROL BLANK (contain **no** cells)

2. Application of Test Chemical

a) Two optional methods for rapidly applying the 2X dosing solutions onto the 96-well plates may be utilized.

1) The first method is to add each of the 2X dosing solutions into labeled, sterile reservoirs (e.g., Corning/Costar model 4870 sterile polystyrene 50 mL reagent reservoirs; or Corning/Transtar model 4878 disposable reservoir liners, 8-channel; or other multichannel reservoirs).

2) The second method utilizes a “dummy” plate (i.e., an empty sterile 96-well plate) prepared to hold the dosing solutions immediately prior to treatment of the test plate

(with cells). The test chemical and control dosing solutions should be dispensed into the dummy plate in the same pattern/order as will be applied to the plate containing cells. More volume than needed for the test plate (i.e. greater than 50 µl/well) should be in the wells of the dummy plate.

At the time of treatment initiation, a multi-channel micropipettor is used to transfer the 2X dosing solutions, from the reservoirs or dummy plate, to the appropriate wells on the treatment plate (as described in step c. below). These methods will ensure that the dosing solutions can be transferred rapidly to the appropriate wells of the test plate to initiate treatment times and to minimize the range of treatment initiation times across a large number of treatment plates, and to prevent "out of order" dosing. Do not use a multichannel repeater pipette for dispensing test chemical to the plates.

b) After 24 h ± 2 h incubation of the cells, remove Routine Culture Medium from the cells by careful inversion of the plate (i.e., "dump") over an appropriate receptacle. Gently blot the plate on a sterile paper towel so that the monolayer is minimally disrupted. Do not use automatic plate washers for this procedure nor vacuum aspiration.

c) Immediately add 50 µL of fresh pre-warmed Routine Culture Medium to all of the wells, including the blanks. Fifty microliters (50 µL) of dosing solution will be rapidly transferred from the 8-channel reservoir (or dummy plate) to the appropriate wells of the test plate using a single delivery multi-channel pipettor. For example, the VC may be transferred first (into columns 1, 2, 11, and 12), followed by the test article dosing solutions from lowest to highest dose, so that the same pipette tips on the multi-channel pipettor can be used for the whole plate. [The Vehicle Control blank (VCb) wells (column 1, column 12, wells A2, A11, H2, H11) will receive the Vehicle Control dosing solutions (which should include any solvents used). Blanks for wells A3 – A10 and H3 – H10 shall receive the appropriate test chemical solutions for each concentration (e.g., wells A3 and H3 receive C_1 solution).

d) Incubate cells for 48 h ± 0.5 h (37ºC ± 1ºC, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO_2/air).

e) **Positive Control**: For each set of test chemical plates used in an assay, a separate plate of positive control concentrations will be set up following the concentration range established in the development of the positive control database in Phase I of the Validation Study. If multiple sets of test chemical plates are set up, then clearly designate the positive control plates for each set; each set will be an individual entity. The Study Director will decide how many test chemical plates will be run with a positive control plate. The mean IC_{50} ± two and a half standard deviations (SD) for the SLS acceptable tests from Phases Ia, Ib, and II (after the removal of outliers) are the values that will be used as an acceptance criterion for test sensitivity for the 3T3 NRU assay. This plate will follow the same schedule and procedures as used for the test chemical plates (including appropriate chemical concentrations in the appropriate wells and meeting test acceptance criteria – see **sections VII.E.1, E.2, and E.5**).

3. Microscopic Evaluation

After at least 46 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 any changes in morphology of the cells due to the cytotoxic effects of the test

chemical, 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. Use the following Visual Observations Codes in the description of cell culture conditions. Numerical scoring of the cells (see **Section VII.E.3**) should be determined and documented in the Study Workbook and in the appropriate section of Addendum II of the EXCEL study template.

Visual Observations Codes

Note Code	Note Text
1	Normal Cell Morphology
2	Low Level of Cell Toxicity
3	Moderate Level of Cell Toxicity
4	High level of Cell Toxicity
1P	Normal Cell Morphology with Precipitate
2P	Low Level of Cell Toxicity with Precipitate
3P	Moderate Level of Cell Toxicity with Precipitate
4P	High level of Cell Toxicity with Precipitate
5P	Unable to View Cells Due to Precipitate

4. Measurement of NRU

a) Carefully remove (i.e., "dump") the medium with test chemical and rinse the cells very carefully with 250 µL pre-warmed D-PBS. Remove the rinsing solution by dumping and remove excess by gently blotting on paper towels. Add 250 µL NR medium (to all wells including the blanks) and incubate (37ºC ± 1ºC, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO_2/air) for 3±0.1 h. Observe the cells briefly during the NR incubation (e.g., between 2 and 3 h – Study Director's discretion) for NR crystal formation. Record observations in the Study Workbook. Study Director can decide to reject the experiment if excessive NR crystallization has occurred.

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

c) Decant and blot D-PBS from the plate.

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

e) Shake microtiter plate rapidly on a microtiter plate shaker for 20 – 45 min to extract NR from the cells and form a homogeneous solution. Plates should be protected from light by using a cover during shaking.

f) Plates should be still for at least five minutes after removal from the plate shaker (or orbital mixer). If any bubbles are observed, assure that they have been ruptured prior to reading the plate. Measure the absorption (within 60 minutes of adding NR Desorb solution) of the resulting colored solution at 540 nm ± 10 nm in a microtiter plate reader (spectrophotometer), using the blanks as a reference. [Note: Phases Ia and Ib data show the mean OD value for the plate blanks to be 0.057 ± 0.043 for 3T3 cells (± 2.5 standard

deviations; data from 3 labs; N = 189). Use this range as a **guide** for assessment of the blank values.] Save raw data in the Excel format as provided by the SMT.

5. Quality Check of 3T3 NRU Assay

a) Test Acceptance Criteria

All acceptance criteria (i.e., criteria 1, 2, and 3) must be met for a test to be acceptable.

1) The PC (SLS) IC_{50} must be within ± two and a half (2.5) standard deviations of the historical mean established by the Test Facility (as per **VII.E.2.e**), and must meet criteria 2 and 3, and must have an r^2 (coefficient of determination) value calculated for the Hill model fit (i.e., from PRISM® software) ≥ 0.85.

2) The left and right mean of the VCs do not differ by more than 15% from the mean of all VCs.

3) At least one calculated cytotoxicity value > 0 % and ≤ 50.0 % viability and at least one calculated cytotoxicity value > 50.0 % and < 100 % viability must be present.

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

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

[Note: A corrected mean $OD_{540 \pm 10nm}$ of 0.103 - 0.813 for the VCs is a target range but will not be a test acceptance criterion. Range determined from Phase Ib VC OD values from 3 laboratories (mean ± 2.5 standard deviations, N = 98).]

b) Checks for Systematic Cell Seeding Errors

To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row 2) and the right side (row 11 for the test plates) of the 96-well plate. Aberrations in the cell monolayer for the VCs may reflect a volatile and toxic test article present in the assay. If volatility is suspected, then proceed to **Section VII.E.6**.

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.

6. Volatility of Test Chemicals

Highly volatile test chemicals may generate vapors from the treatment medium during the test chemical treatment incubation period. These vapors may become resorbed into the treatment medium in adjacent wells, such that culture wells nearest the highest doses may become contaminated by exposure to resorbed test article vapors. If the test chemical is particularly

toxic at the doses tested, the cross contamination may be evident as a significant reduction in viability in the vehicle control cultures (i.e., VC1) adjacent to the highest test chemical doses.

If potential test article volatility is suspected (e.g., for low density liquids) or if the initial range finder test (non-sealed plate) results show evidence of toxic effects in the control cultures (i.e., > 15 % difference in viability between VC1 [column 2] and VC2 [column 11]), then seal the subsequent test plates by the following procedure.

a) Plate Sealer Method

1) Plates and chemicals will be prepared as usual according to **Sections VII.D and VII.E**.
2) Immediately after the 96-well culture plate has been treated with the suspected volatile chemical (**Section VII.E.2.b**), apply the adhesive plate sealer (e.g., using a hand, microplate roller, etc.) directly over the culture wells. Assure that the sealer adheres to each culture well (well tops should be dry). Place the 96-well plate cover over the sealed plate and incubate the plate under specified conditions (**Section VII.E.2.b**). [Note: Do not jam the plate lid over the film to avoid deforming the sealer and causing the sealer to detach from culture wells. Loose fit of the plate lid is acceptable.]
3) At the end of the treatment period, the plate sealer should be carefully removed to avoid spillage. Continue with the NRU assay as per **Section VII.E.4**.

F. Data Analysis

The Study Director will use good biological/scientific judgment for determining "unusable" wells that will be excluded from the data analysis and provide explanations for the removal of any data from the analysis.

A calculation of cell viability expressed as NRU is made for each concentration of the test chemical by using the mean NRU of the six replicate values (minimum of four acceptable replicate well) per test concentration (blanks will be subtracted). This value is compared with the mean NRU of all VC values. Relative cell viability is then expressed as percent of untreated VC. If achievable, the eight concentrations of each chemical tested will span the range of no effect up to total inhibition of cell viability. Data from the microtiter plate reader shall be transferred to the Excel® spreadsheet template provided by the SMT. The template will automatically determine cell viability, IC_{50} values by linear interpolation, and perform statistical analyses (including statistical identification of outliers). The template will also calculate the concentrations associated with 20 %, 50 %, and 80 % viability using the Hill slope and EC_{50} (i.e., IC_{50}) from the Hill function analysis.

The Hill function analysis shall be performed using statistical software (e.g., GraphPad PRISM® 3.0) and a template specified by the SMT to calculate IC_{20}, IC_{50}, and IC_{80} values (and the associated confidence limits) for each test chemical.

The Testing Facility shall report data using at least three (3) significant figures and shall forward the results from each assay to the SMT through the designated contacts in electronic format and hard copy upon completion of testing. The SMT will be directly responsible for the statistical analyses of the Validation Study data.

VIII. REFERENCES

Hackenberg, U. and H. Bartling. 1959. Messen und Rechnen im pharmakologischen Laboratorium mit einem speziellen Zahlensystem (WL24-System). Arch. Exp. Pathol. Pharmakol. 235: 437-463.

Spielmann, H., S. Gerner, S. Kalweit, R. Moog, T. Wirnserberger, K. Krauser, R. Kreiling, H. Kreuzer, N.P. Luepke, H.G. Miltenburger, N. Müller, P. Murmann, W. Pape, B. Siegmund, J. Spengler, W. Steiling, and F.J. Wiebel. 1991. Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany. Toxicol. *In Vitro* 5: 539-542.

Test Method Protocol for Solubility Determination. In Vitro Cytotoxicity Validation Study. Phase III. August 29, 2003. Prepared by The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).

IX. APPROVAL

_____ _____

SPONSOR REPRESENTATIVE **DATE**
(Print or type name)

_____ _____

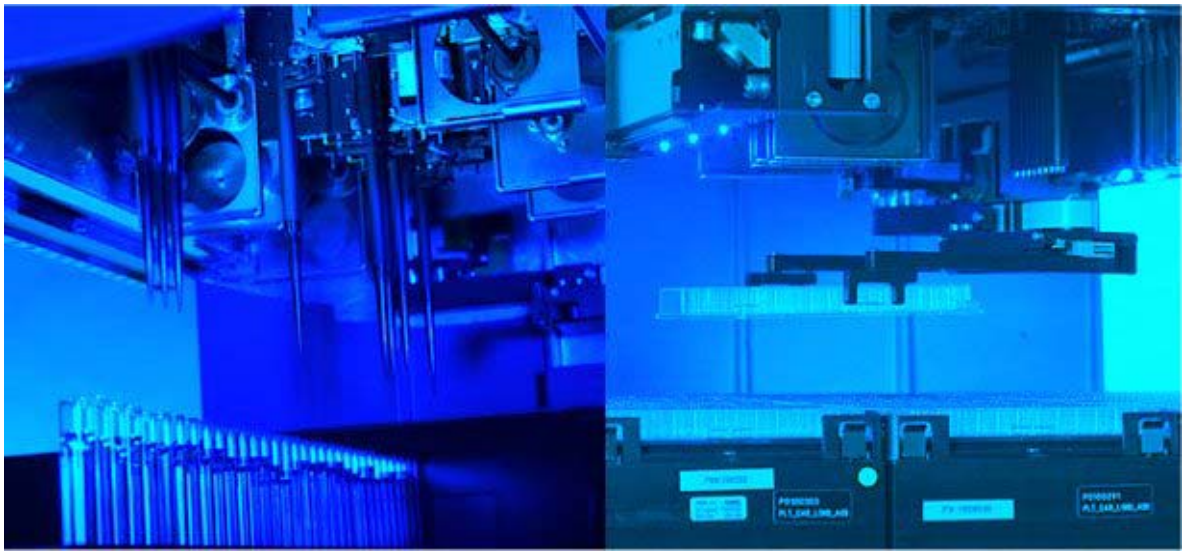
Test Facility STUDY DIRECTOR **DATE**
(Print or type name)

ANNEX B

Study protocol of JRC

Automated 3T3/NRU standard operating protocol for acute cytotoxicity testing of chemicals

Mounir Bouhifd, Maurice Whelan



2006

InViTech (IHCP Action 4224, AL M. Whelan)
Deliverable No. D2.1

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

Institute for Health and Consumer Protection

Content:

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Abbreviations

CDM	Chemical Dilution Medium
CT	Chemical Tube
DF	Dilution Factor
DP	Dilution Plate
DPBS	Dulbecco's Phosphate Buffered Saline
DTV	Dilution Transfer Volume
ES	Experiment Supervisor
GUI	Graphical User Interface
HW	Header Worksheet
IWL	Input Work List
NRD	Neutral Red Desorb
NRM	Neutral Red Medium
NRSS	Neutral Red Stock Solution
NRU	Neutral Red Uptake
OT	Osmometer Tube
OWL	Output Work List
PP	Physiochemical Plate
PTP	Pilot Test Platform
RCM	Routine Culture Medium
RF	Range Finding
RTP	Routine Test Platform
SOP	Standard Operating Protocol
TP	Test Plate (seeded)
VC	Vehicle Control
VCb	Vehicle Control Blanks

Introduction

In the last decade, automated systems are increasingly used for the discovery and development of new drugs in order to reduce cost and time. The driving force is to shorten the pipeline of drug development. This change of pace in the drug discovery process has been stimulated by advances in cell and molecular biology allowing now the testing of thousands of substances on numerous cellular therapeutic targets.

A similar approach can be taken to study chemical substances for which in vivo data are available. A number of in vitro assays for cytotoxicity and target organ toxicities developed in the IHCP laboratories have the potential to be adapted to microtiter plate formats and redesigned for automated standard operating procedures. Automated cell-based screening systems of different scales and dimensions are commercially available and can be used for this purpose after adaptation. The automated test facility of the IHCP consists of the Pilot Test Platform (PTP) and the Routine Test Platform (RTP). Both platforms (P/RTP) are supported by Cell Culturing Labs to provide seeded plates and Chemical Labs where stock solutions of test chemicals are prepared, analysed and stored.

This will provide a new approach to assess the properties of in vitro systems and their inter-relationships as well as (combined) predictive values. The goal will be to run different in vitro cell and tissue-based assays using different endpoint detection systems and to measure a multitude of mechanisms as part of an integrated testing strategy for determining acute systemic toxic effects including target organ toxicities.

The SOP on which this automated process in based was devised to support the in vitro validation study organised by NICEATM and ECVAM (sponsored by NIEHS, the U.S. Environmental Protection Agency and ECVAM) in which the 3T3/NRU test is being used to analyse the in vitro cytotoxicity of 60 blinded/coded test chemicals of varying toxicities, to determine their IC_{20}, IC_{50}, and IC_{80} values.

The Neutral Red Uptake (NRU) cytotoxicity assay is a cell survival/viability chemosensitivity test 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 and to detect a reduction of growth rate.

The purpose of this document is to provide a detailed technical description of the automated SOP for acute cytotoxicity assessment of chemicals based on 3T3/NRU assay and its implementation in our institute. First, the main components of the automated in-vitro testing platform will be described, then the automation of the assay will be detailed and finally a technical description of the execution of an automated run will be given.

Part I: The automated *in-vitro* testing platform

There are a wide variety of options available for automated in-vitro testing platforms. The solution adopted in our institute corresponds to precise and specific needs detailed in the technical annex of the call of tenders for the purchase of the Automated cell-based system.

The automated test facility of the IHCP consists of the Pilot Test Platform (PTP) and the Routine Test Platform (RTP). Both platforms (P/RTP) are supported by Cell Culturing Labs to provide seeded plates and Chemical Labs where stock solutions of test chemicals are prepared, analysed and stored. The test platforms are used to carry out both range finding (RF) and definitive (Main) concentration-response experiments.

The Pilot Test Platform (PTP) and the Routine Test Platform (RTP) are flexible, modular, specialised systems for the full automation of a wide variety of cell based assays. Both systems include components for the automation of all liquid handling and transportation procedures manufactured by Hamilton Company, together with third party. The entire solution is driven and managed by the Hamilton software. All automation tasks are governed by a Plate Scheduler Software, in order to optimize the incubation timing and the utilization of the resources available in the platform (reader, washer, etc).

The following table summarize all instrumentation included in the PTP and RTP systems, with a short description of the purpose of each instrument. In the next paragraph we'll analyze in detail the feature of each device.

Instrument	Short Description	System
Microlab STAR	Liquid handling workstation	PTP and RTP
Microlab SWAP 1400	Plate handling robotic arm	PTP and RTP
EasyPEEL	Device to remove sealing pads	PTP and RTP
Cytomat 2C15	Incubator, 42 microplate capacity	PTP
Cytomat 6000	Incubator, 189 microplate capacity	RTP
ELX405R	Microplate washer	PTP and RTP
ALPS 300	Plate Sealer	PTP and RTP
FluoStar OPTIMA	Plate Reader	PTP and RTP
Variomag Teleshake 70	Temperature controlled plate shaker	PTP and RTP
RoboPH	8 channel ph meters	PTP and RTP
3900 Osmometer	Osmometer	PTP and RTP
3932-D1	Class II enclosure	PTP and RTP
Vector Scheduler Plus	Scheduling software	PTP and RTP

Table 1: Summary of the main components of the automated platforms

Each single device is integrated in a robotic environment, the following drawing shows how the layout looks like.

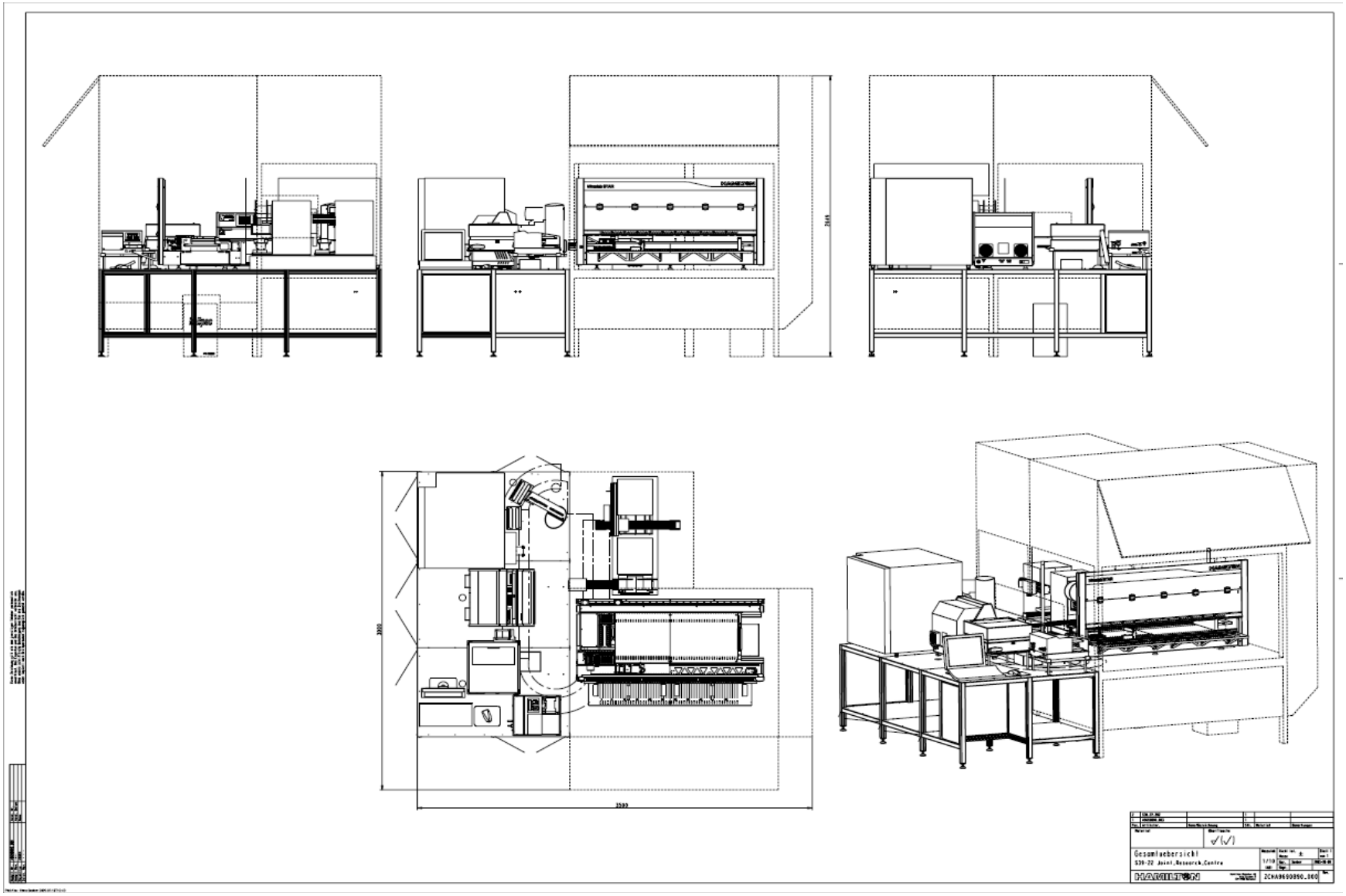

Figure 1: Layout of the automated platform

1. PTP and RTP technical description: detailed profile of all components

Taking into account the table presented above, we'll now describe in detail the features of each component of the robotic system.

1.1. MICROLAB STAR

Figure 2: Microlab Star Liquid handling station

The MICROLAB STAR is an advanced liquid handling system and is the core of our platform. Both platforms are equipped with the following components:

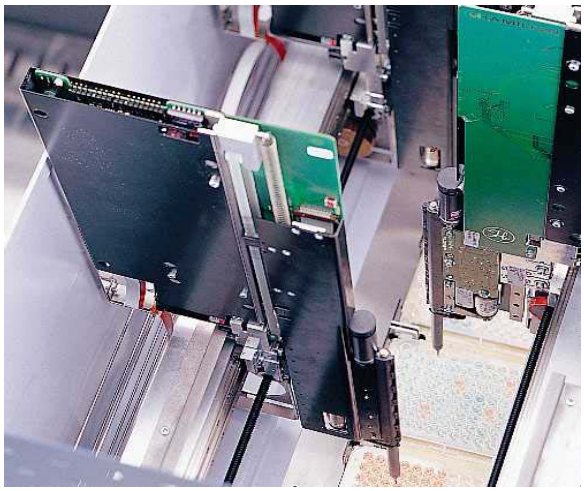

Figure 3: Pippeting channels

8 independent channels, asymmetrically spreadable ; the air displacement technology allows to keep sterility in the liquid handling path (no system liquid, no tubing, no valves = no contamination) and to achieve the highest performance for precision and accuracy, even for sub-microliter volumes.

Figure 4: Pippeting needles

8 independent syringes, volume 1000 ml (volume range from 0,3ml to 1000ml) : even if it is possible to configure the system with higher volume syringes, the volume mentioned was chosen in order to improve precision down to 1ml and therefore approach the test chemical dilutions in the best possible conditions. As it was described in the publications and in the technical annex, the dilution of the test chemicals is a fundamental part of the protocol and, in some cases, it would be necessary to dispense small amounts of product (for example 1ml). Since dispensing this volume with an high volume syringe (for example 5000ml or 10000ml) would necessary become imprecise, it was decided to use the best performing syringe and take advantage of the total independent spreading of the channels to dispense high diluent volumes (> 1000ml). In fact, more channels can aspirate in the same reagent vessel and dispense in the same tube simply adjusting the spacing to the destination tube geometry. It is also important to point out that the imprecision on multiple dispensing of the same high volume (for example, dispensing 5 times 1000ml) is much less than the imprecision of dispensing 1ml with an high volume syringe

Figure 5: Needles washing

Active wash station: thanks to the CO-RE technology, the system can aspirate and dispense indifferently using fixed or disposable tips, it become therefore necessary an efficient tool to wash fixed tips. Hamilton offers an advanced active wash station that can use two different washing solutions : one scenario could be that one solution is an aggressive sterilizing detergent and the second one is just sterile distilled water, in order to remove all carryover and keep sterility

CO-RE Grip: gripper module to move plates and objects in the deck space (for example: load/unload shakers)

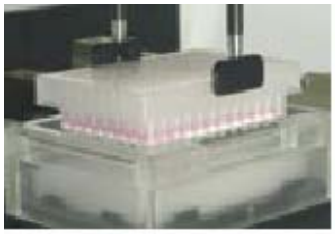

Figure 6: Core Grip plate transport

Monitored Air Displacement (MAD) this technique detects if the volume defined in the protocol was aspirated regularly or some errors occurred (tip blocked or bubbles or foaming), it is therefore very important for the general reliability of the protocol and it becomes mandatory in case the process takes several days of unattended operations, like the application we're automating with the PTP and RTP systems

Liquid Level Detector (LLD): A liquid level detector based on a dual technology capacity and pressure is used:. It is precise and reliable, false triggers or bubble/foam errors never occur, generating a total confidence of the operator in the system.

Standard carriers to hold plates, tips and reagents were added to the MICROLAB STAR deck. In addition, several customized accessories have been developed specifically for this application, as described in the following list :

Temperature controlled shakers: the need of shaking microplates in a temperature control environment was taken into account, together with the throughput requirements. As suggested in the technical annex, an estimated time of 45 minutes was considered in the design of the proper shaker.

Figure 7: temperature-controlled shaker (PTP: 3 positions – RTP: 8 positions)

Considering a throughput of minimum 100 microplates per run, as estimated in the RTP system, shaking 45 minutes each plate means a total shaking time of 75 hours (single position shaker). The total protocol timing was then evaluated and simulated in the Vector Scheduler Plus software, and it was determined the minimum number of shaking positions needed in order to avoid that this step would become the bottleneck of the entire protocol. All these calculations came to the conclusion that 8 positions are needed in the RTP system and 3 positions in the PTP system : even if this configuration heavily impacts the total price, we believe it is absolutely necessary for the proper execution of the protocol. If the shaking time was lower than 45 minutes, the number of positions could be reduced accordingly to the new time.
Once identified the specifications as mentioned above, the proper shaker was chosen from the market offer, again taking into account the significant shaking time during the run and therefore the need to have a professional, reliable unit.
The Variomag Teleshake S70 from Inheco was selected because of the quality and the positive feedback from previous installations. A total of 8 (RTP) or 3 (PTP) positions were integrated in the STAR deck, as it'll be shown in the deck layout presentation.

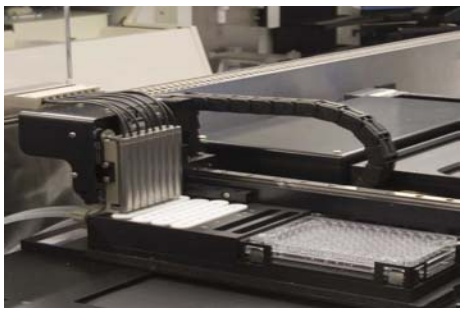

Figure 8: pH module

RoboPH device: as it was mentioned in the Hamilton Company profile, one of the three business units at Hamilton is developing and manufacturing electrochemical sensors. Merging the know-how of this business unit together with the Robotics one, our R&D team was able to design and manufacture an innovative ph meters fully integrated in the Microlab STAR.

Basically, it incorporates 8 pH sensors mounted in a head, the entire assembly can be gripped and moved in the deck thanks to the CO-RE technology. RoboPH is able to monitor ph in microplate wells (8 wells per measurement) and in any kind of other container (for example the test chemicals one) , since the 8 sensors are also spreadable.
After each measuring cycle, an efficient washing procedure is applied to the sensors, removing completely all carryover elements. This special washing station was derived by the fixed tips one described above, and it is located in the STAR deck, just like the RoboPH parking position : when the

system is executing other tasks, this device is dropped in a dedicated position near the wash station. RoboPH is auto-calibrating and it takes approx. 5 minutes to measure one 96 well plate, including the washing cycle. At the end, all measurements are stored in an Excel files together with the plate/sample identification.

CO-RE Technology: this technology enables to pipette with 8 teflon coated needles and disposable tips. Because of the aggressive nature of some test chemicals and the overall need to maintain sterility, CO-RE technology enables a good compromise, since the user can decide if some samples will be dispensed with the fixed and others with the disposable tips. In addition, CO-RE allows to use the Gripper tool to move plates and the RoboPH device

Fixed Tip Size	Volume	Trueness	Precision
10µl	1µl	5%	7%
10µl	5µl	2.5%	1.6%
10µl	10µl	1.5%	1%
300µl	10µl	5%	4%
300µl	50µl	2.0%	2%
300µl	200µl	1.5%	1.0%
1000µl	20µl	6.0%	6.0%
1000µl	100µl	3.0%	2.5%
1000µl	1000µl	2.0%	1.0%

Table 2: MICROLAB STAR performance for both disposable and fixed tips

1.2. MICROLAB SWAP

The Microlab SWAP is a plate-handling device and represents the element of connection between all the components of the robotic station.
The version chosen in this configuration is the SWAP 1400 : thanks to its wide X range (1400mm) can easily reach all the peripheral devices needed to run the application.
It is important to mention that it has a "smart hand" able to detect if the microplate was picked up or not, and it allows parallel operations, which means it can operate while the STAR or the other devices are running separate protocols, optimizing throughput and resources.

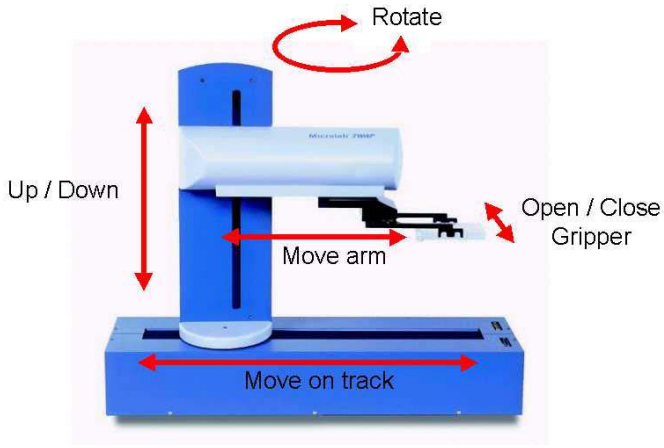

Figure 9: MICROLAB SWAP plate handling device (with 5 degrees of freedom)

1.3. Third party instrumentation

KENDRO CYTOMAT INCUBATOR

Figure 10: Cytomat 6000 (left) and Cytomat 2C15 (right)

Kendro Cytomat series are the most known incubators for robotic environments, with hundreds of installations worldwide.

Two different models were selected for the PTP and RTP units, both models have CO2, humidity and temperature control, the only difference is the capacity in terms of number of plates :

- Cytomat 2C15 : this model offers a capacity of 42 microplates, therefore it was selected for the PTP system. Internally 2 stainless steel stackers manage the space, each one holding 21 microplates. A linear robotic arm handles all plate movements to and from the external door, eventually with bar code reader identifications.

- Cytomat 6000 : the capacity is 189 microplates, in this case the number of stacker is 9, each one holding 21 microplates. The internal space looks like a carousel, the robotic arm is in fact circular and not linear.

Microplates are loaded and unloaded with the lid or eventually sealed, liquid handling operations are performed in the Microlab STAR which is also managing the de-lidding or pad removing of each plate.
The incubator is fully controlled by the software: all the operations are driven by the scheduler, just like the monitoring of the main incubator parameters.

WASHER BIOTEK ELX405R

Figure 11: Automatic plate washer

This plate washer was selected thanks to its performances in handling cell culture and cell based assays. It has a manifold with 96 dual needles, all parameters related to buffer dispense and waste removal are fully programmable (pressure, vacuum, arm speed), therefore the optimization of extremely delicate assays is straight forward. Regarding cell based assay, it is important to mention that the manifold incorporates a special needle which is dispensing to the side of the well and not to the bottom, preserving the cell layer adherent to the well bottom. The model we selected incorporates a valve to switch between several buffers and reagents (for example D-PBS, NR medium, etc.), washing solutions can also be wormed as requested by the protocol.

HJ-BIOANALYTIK Sealer and Peeler

As specified in the technical annex, sealing and peeling of plates should be possible on-line and with no manual intervention. The solution proposed is based on the two devices RoboSeal and RoboPeel from HJ-BIOANALYTIK. These devices can handle a wide selection of foil materials compatible with cell assays (porous membranes).

The sealer is fully controlled by the software, it is physically installed under the hood because it is requested to seal cell plates just after the addition of the diluted test chemicals, therefore under sterile conditions

Figure 12: Sealing and peeling devices

Optical Reader BMG FLUOSTAR

Figure 13: Optical reader

The FLUOstar OPTIMA is a fully automated microplate based multi-detection reader designed for life science laboratories in academia and industry. The FLUOstar incorporates four different measurement principles:

- Fluorescence Intensity - including FRET and multilable applications
- Time-Resolved Fluorescence - including DELFIA™
- High-Performance Luminescence (flash and glow) - dedicated luminescence circuit
- Absorbance - UV/Vis with 1 cm path length correction

The reader is fully controlled by the software, the readout are stored in the computer hard disk for data titration or transfer to external LIMS.

OSMOMETER

The measurement of osmometry in a robotic environment requires a small amount of sample to be aliquoted into tubing or vials, and the integration of a multi sample osmometer in the robotic system. We evaluated this option and decide to integrate a freezing point osmometer from Advanced Instrument, model 3900.

The system can run up to 30 samples without any user intervention, at the end, if needed, more samples can be aliquoted and run.

Figure 14: Osmometer

CLASS II Laminar Flow Hood

The nature of the assay requires a high quality enclosure to ensure sterility, cell protection and eventually operator protection from test chemicals aerosol.
Clean Air is the most referenced one in case of high quality solutions, therefore an enclosure from this company was selected. The cabinet is compliant to all kind of certifications and regulations, the classification is Class II biohazard safety cabinet.
The cabinet is designed to fit both the Microlab STAR (with all the accessories mentioned above) and the plate sealer, which are the two components that require the maximum sterility.

1.4. SOFTWARE: VECTOR SCHEDULER PLUS

The software in charge of managing all the components described so far represents one of the key elements of the entire automated solution.
The basic software requirements are:

- incorporate all drivers of instruments needed in the robotic system, for full remote control of each device
- schedule plate tasks in order to avoid that more than one plate require access to the same device at the same time
- allow the user to change most of the parameters for any possible need in terms of protocol modification or new protocol setup

Basically, this software incorporates a large list of drivers, including all the devices mentioned in this system, and allows the operator to create easy to complex tasks simply generating a single plate linear process where each step is programmed in a graphical interface (for example, load the serial dilution protocol in the STAR, add diluted samples to cell plates, incubate "x" hours, unload the plate and send it to the washer, etc).

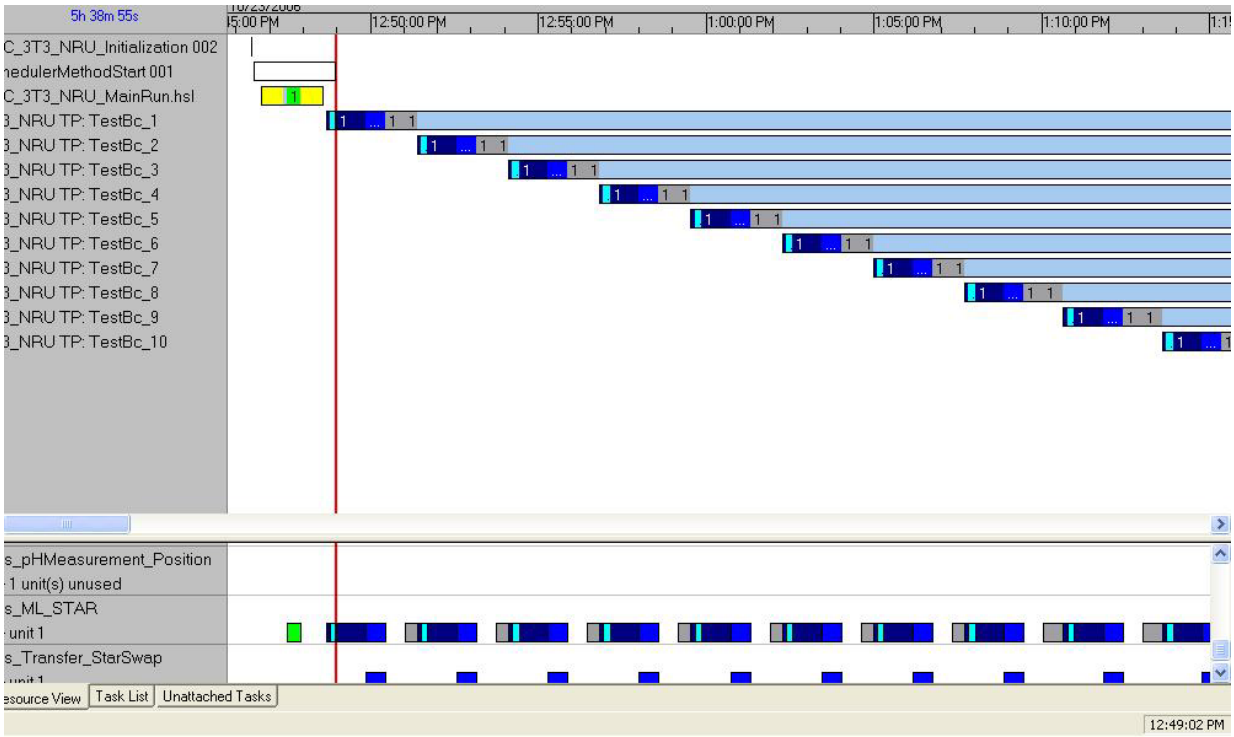

Figure 15: Vector Scheduler Plus Software.

Each line represents a single microplate cycle, it is delayed from the previous one to avoid that more plates need to use the same device at the same time.

Figure 16: Photograph of the automated platform (PTP)

Part II: The automated assay

2. Process description

The automated process is illustrated in Figure 17. It can be divided in two stages associated with pre- and post-incubation (i.e. of TP with test chemical), denoted Stage I and Stage II, respectively. The automated run starts once the operator has placed the TP rack in the incubator, and loaded the CT carrier, empty DP/PP plates and the relevant media on the P/RTP. During Stage I the processing of each TP is carried out serially. Thus once a DP is prepared for a test chemical, a TP is retrieved from the incubator, treated with the test chemical and then returned to the incubator. The processing steps are then repeated for the next chemical. In stage II, the processing of plates is done in a staggered-parallel fashion, due to the availability of more than one shaking station. The details of each step are now described.

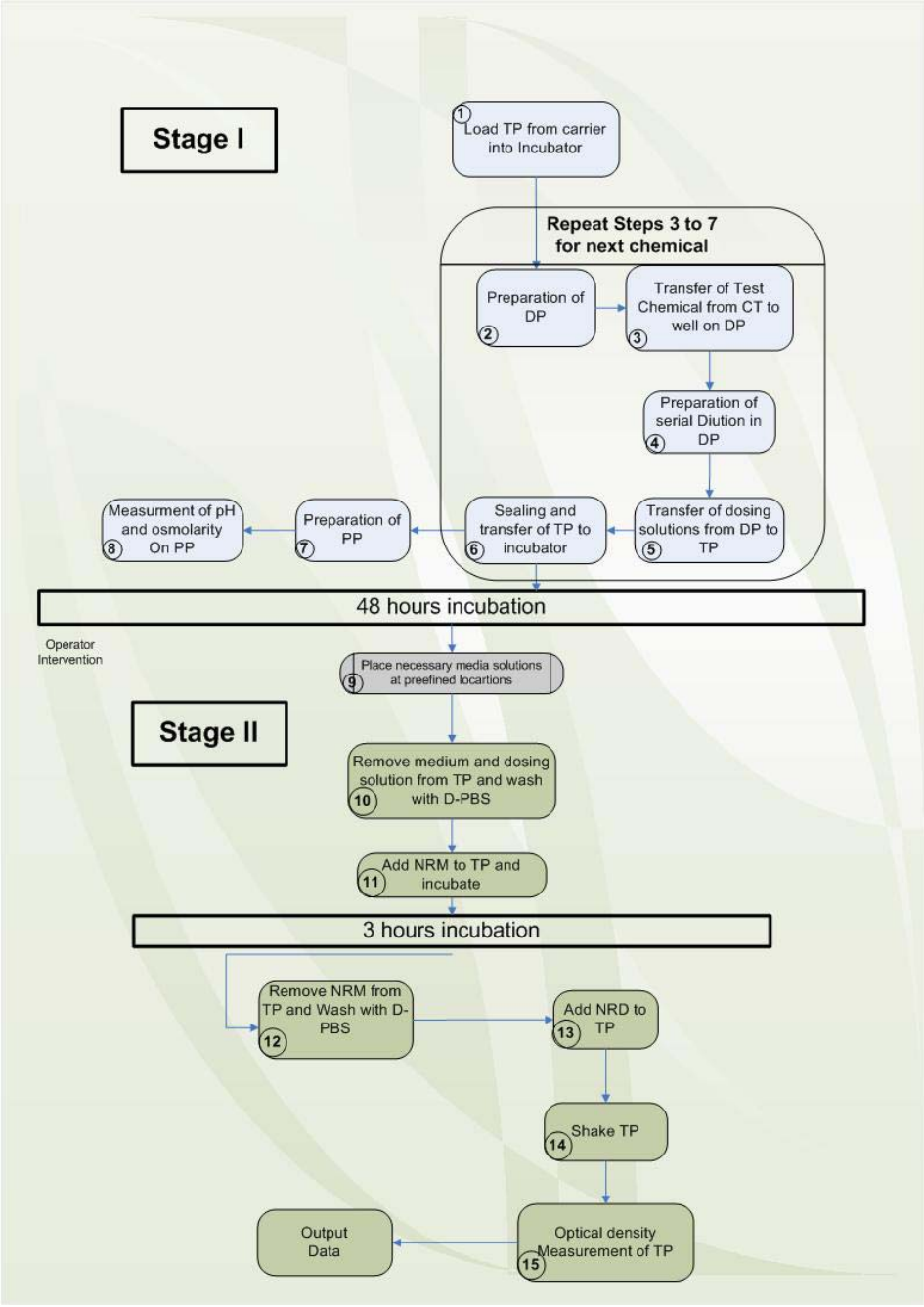

Figure 17: Process flowchart of automated 3T3/NRU assay.

2.1. Preparation for an automated run

The seeding of the test plates (TP) is carried out 24 hours prior to an experiment/run. Each TP used is labelled with a unique barcode ID, generated specifically for a particular run. All wells on the TP, except those along the plate periphery, are seeded. Freshly seeded TPs are placed in an incubator in the Cell Culture Lab and incubated for 24 hours to allow the cells to recover and proliferate. Cell counting, plate seeding and quality inspection are carried out manually in the Cell Culture Lab. After the 24 hour incubation period, the medium is replaced with 50 µl of fresh pre-warmed routine culture medium (RCM), added to all wells on the TP. TPs are then loaded into a rack which is inserted directly in the incubator on the P/RTP. When an automated run commences, the system (incubator function) makes a scan of all TPs within the incubator to register their location and barcode label.

Test chemical stock solutions are prepared immediately prior to the start of an automated run. Each stock solution (minimum vol. 2.5 ml) is contained with a sealed chemical tube (CT), labelled with a barcode, located on a tube carrier. The position of tubes within the carrier is not important since when an automated run commences, the system detects the presence of tubes within the carrier and registers their position and barcode label. The Input Work List (IWL) contains operational parameters (e.g. dilution factor, sealing requirement) associated with a particular chemical/tube, identified by the barcode.

A number of deck positions on the P/RTP are reserved for empty sterile 96 deep-well plates to be used as serial dilution plates (DP) and for the physiochemical properties plate (PP). A plate carrier may also be used to increase the number of empty plates available if required. The treatment of each TP requires the use of one DP. Only one PP is used for the whole run since each test chemical is contained within one well on the PP. Thus the total number of empty sterile 96-well plates to be made available equals the number of TPs plus one.

At the beginning of an automated run, only two media are placed on the P/RTP, namely, the Chemical Dilution Medium (CDM) used in preparing the DPs, and the Routine Culture Medium (RCM) used for preparing the PP. After treatment of the cells with the test chemical and subsequent incubation (i.e. ~48 hours later), the operator must place Neutral Red Stock Solution (NRSS), Neutral Red Medium (NRM) and Neutral Red Desorb (NRD) on the P/RTP for the endpoint assay. Neutral Red Medium (NRM) must be kept at 37 °C while on the TP and used preferable within 30 minutes after preparation.

2.2. Stage I

Step 1: Detect test plates (TP) and chemical tubes (CT)
The set of TPs (seeded 24 hours earlier and with fresh RCM) located in the incubator on the P/RTP are automatically scanned/detected and their barcodes are registered. The system also detects the presence of CTs in the tube carrier and registers their barcodes. Checks are then performed to i) confirm that the number of TPs is the same as indicated in the IWL and ii) that the CT-barcodes read during the scan of the tube carrier are the same as those listed in the IWL. If the plate-tube association flag (see section 5.1) is true, then the system checks to see if the TP-barcodes scanned in the incubator correspond to those listed in the IWL.

<start loop for processing of each test chemical sequentially>

Step 2: Preparation of DP
An empty sterile 96 deep-well plate is retrieved and placed at a predefined position, to become the DP for the test chemical currently being processed.
990 µl of CDM is pipetted in Column 3 (corresponding to concentration C_1).
A fixed volume FV (specified in the IWL) of CDM is pipetted into every well in columns 4 to 10 on the DP (corresponding to concentrations C_2 to C_8 of dosing solution).
[use new tips, one set is sufficient for all of step 7]

Step 3: Transfer of test chemical from CT to well A-1 on DP.
200 µl of the test chemical (from the stock solution at highest concentration of) is pipetted from its sealed CT into the H-1 well of the DP.

Step 4: Preparation of serial dilutions in DP
First, 10 µl of test chemical from well H-1 of the DP are dispensed in well A-3 of the DP. This operation is repeated 7 more times to load the remaining wells in that column (i.e. wells B-3 to H-3). Thus column 3 contains the test chemical at the maximum concentration (2X), denoted C_1. Then a certain volume of test chemical, denoted Dilution Transfer Volume (DTV), specified in the IWL, is then transferred from well B-1 on the DP to all the wells on column 4 (concentration C_2). The serial dilution processes then continues by transferring the fixed volume DTV, from column 4 to 5, 5 to 6, and so on.

[use new tips, one set is sufficient for all of step 7]

Step 5: Transfer of dosing solutions from DP to TP
Retrieve a seeded TP from the incubator. In a column by column fashion, pipette 50 µl of dosing solution from each well on the DP, into the appropriate well on the TP. The Vehicle Controls (VC), containing only CDM, should be transferred first of all (i.e. column 1,2, 11 and 12), followed by the dosing solutions containing the test chemical, starting from the lowest concentration (C_8 column 10) and working towards the highest concentration (C_1 in column 3). The final configuration of the TP is illustrated is Fig. 3. (For the first pass, the dosing solutions in DP01, produced using the test chemical from CT01, are used to treat TP01, for the second pass, the dosing solutions in DP02, produced using the test chemical from CT02, are used to treat TP02, and so on).

Step 6: Sealing and transfer of TP to incubator
The treated TP is first sealed, if specified in the IWL, and then transferred to the on-line incubator where it should remain for 48 h ± 0.5 h (37°C ± 1°C, 90% ± 5% humidity, 5% ± 1% CO_2/air).

[Repeat steps 2 to 7 for next chemical]

	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

Figure 18: Configuration of the 96-well test plate (TP).

VC1/VC2	Vehicle Controls (contain only cells in medium)
C1 – C8	Cells with dosing solutions of test chemicals (C1 = highest conc., C8 = lowest)
Cxb	Chemical Blanks (contain test chemical and medium)
VCb	Vehicle Control Blanks (contains only medium)

Step 7: Preparation of PP
Once all the TPs specified in the IWL are treated, the additional DP placed in position 24 is retrieved and placed in a specific deck position to prepare the PP. First, 990 µl are pipetted to wells A1, A2, …. The number of the wells that will be pipetted correspond to the number of the chemicals in the experiment. Then 10 µl of chemical is added to wells A1, A2, ….

Step 10: Measurement of pH and Osmolarity Sealing and transfer of PP to incubator
The PP, containing 250 µl of the highest dose concentration (C_1) for each test chemical (one chemical per well), is transferred to the pH station and a sub-process is started to measure the pH and Osmolarity of each sample (minimum sample volume is 200 µl for the osmometer).

[exchange/dump disposable tips]

<repeat steps 3-7 for processing of next test chemical>

2.3. Stage II

Operator intervention: The operator must place Neutral Red Stock Solution (NRSS), Neutral Red Medium (NRM) and Neutral Red Desorb (NRD) on the P/RTP for the endpoint assay. Warm D-PDS should also be available to the plat washer.

<start of loop for processing of each TP to determine NRU>
[exchange/dump disposable tips]

Step 11: Remove medium and dosing solution from TP and Wash TP with D-PBS
A TP is retrieved from the incubator, 48 hours after the incubation commenced. The TP is placed on the washing station. If a seal is present it is removed beforehand. The washer removes and dumps the medium plus dosing solution (100 µl in total) by rinsing in warm D-PBS (one rinse with 250 µl D_PBS is used in manual SOP). After washing, any residual D-PBS in the wells should have been removed.

Step 12: Add NRM to TP and incubate
Add 250 µl of NRM (pre-warmed to 37°C) to all the wells on the TP and return it to the incubator. Incubate for 3 hours ± 0.1 hour (37°C ± 1°C, 90% ± 5% humidity, 5% ± 1% CO_2/air).

Step 13: Remove NRM from TP and Wash TP with D-PBS
A TP is retrieved from the incubator and placed on the washing station 3 hours after the incubation with NR commenced. The NR-medium plus dosing solution (250 µl in total) is then removed and dumped during washing with warm D-PBS (one rinse with 250 µl D_PBS is used in manual SOP). After washing, any residual D-PBS should not be present.

Step 14: Add NRD to TP
Add 100 µl of NRD to all the wells on the TP.

Step 15: Shake TP
Transfer the TP to a free shaking station and shake for 45 minutes. Plates should be protected from ambient light during this operation. After shaking, allow TP to settle for 5 minutes.

Step 16: Optical density measurement of TP
Transfer the TP to the optical reader. Measure the optical density at 540 nm ± 10 nm. Once the optical measurement is completed, transfer the TP to a plate carrier, for subsequent disposal.

<repeat steps 9-16 for processing of all the TP contained in the incubator>
Note. The processing of TPs from Step 9 to 11 is carried out serially. After the 3 hour incubation, however, TPs are processed with Steps 12 to 16 in a staggered-parallel fashion. The delay introduced between each plate is a function of the number of shaking stations available (e.g. 45 minutes shaking, with 3 shakers, gives a 15 minutes delay between plates).

3. Process interfaces

3.1. Input Work List and GUI

The interaction with the process will be carried out through two specific interfaces, namely an Excel file entitled the Input Work List (IWL), and a software graphical user interface (GUI). The basic idea behind this strategy is to provide a reliable and secure information platform that ensures traceability and minimum operator intervention. The Experiment Supervisor (ES) is responsible to define the IWL which contains all the operational parameters needed to run an experiment and also all the relevant information for complete traceability of results. Once prepared, the IWL is write-protected and passed to the operator. The operator interacts with the GUI and will indicate the IWL corresponding to the run. He will then be asked to check/confirm the run parameters (number of test plates, tubes, empty plates, media etc…) before starting the run.

Input Work List

The Input Work List (IWL) is prepared by the ES. It is an Excel spreadsheet file and is *unique* to each run. The first worksheet of the excel file (called Header Worksheet, HW) will contain a header with general experimental descriptors (e.g. Experiment ID, SOP ID, number of TPs …) and also the details concerning the particular cell culture used in the experiment. For this protocol, only one cell culture type is used (i.e. 3T3 cells). However, since provision is made for CT-TP association (flag in the IWL), the protocol could also be run in the future using different cell types seeded in different plates. In this case, the IWL should provide all the relevant information for all cell types present and define which cells and CT is associated with each TP.

The IWL will contain a number of additional worksheets, one worksheet for each TP in the run. The worksheets will be named Plate 1, Plate 2, and so on. Each worksheet will contain the parameters that described the chemical tested on that TP, plus any additional information considered relevant for traceability purposes. If by any chance a particular chemical is tested on more than one TP (e.g. multiple control plates), then the ES should copy the information on the chemical from one plate-worksheet to another.

The input file will be prepared in advance (e.g. days before the run) by the ES. Once the input file is completed, it is write-protected and stored in a dedicated folder on the server. The storage folder name will be the experiment ID. This folder will host all files generated during the experiment. The IWL filename will be the experiment ID concatenated with the string "_IWL". An example layout for the IWL is shown in Fig. 4.

	A	B	C	D	E	F	G	H	I	J	K
1											
2	Chemical name					Tube ID				CT_10	
3	Cass No.			cas01		Plate ID				TP_11	
4	Solvent					Dilution factor					
5	Solvent Conc.					Highest stock Conc. (M)					
6	Aids used to dissolve					Plate sealing				0	
7	pH (highest conc. tested):			LEAVE EMPTY		Medium Clarity/Color (highest conc.					
8											
9	Final Volume (FV)			60		Dilution Transfer Volume (DTV)				286	
10											
11				C1	C2	C3	C4	C5	C6	C7	C8
12	Concentration Series (M)										
13											

	A	B	C	D	E	F	G	H	I	J	K
1	Operator ID		Experiment ID								
2	Start Date		Start Time								
3	SOP cell procedure		SOP endpoint ID								
4											
5	Number of test plates	4	Number of chemical tubes		4						
6											
7	Experiment Description										
8	Notes										
9											
10	Cell culture type			Cells Supplier		Cells Lot no.					
11	Original Passage no			Cell passage no. in assay							
12	Proliferating/frozen					Cell seeding date					
13											
14	Medium Type		Medium Supplier			Medium Lot no.					
15											
16	Serum Type 1		Serum Supplier 1			Serum 1 Lot no.					
17	Serum 1 Conc. Growth Med.				Serum 1 Conc. Treatment Med.						
18											
19	Serum Type 2			Serum Supplier 2			Serum 2 Lot no.				
20	Serum 2 Conc. Growth				Serum 2 Conc. Treatment Med.						
21	Serum 2 Conc. Growth				Serum 2 Conc. Treatment Med.						
22											
23	Plate/tube association	1									
24											
25	**Step Selection:**										
26	Preparation of PP	1									
27	pH measurement	1									
28	Osmolarity measuremer	1									
29	Note: The "pH measurement" step will only start, if the "Preparation of PP" step is selected too.										
30											
31											

Figure 19: Example of the layout of the Input Work List (IWL).

Operator interaction via GUI

At the beginning and during the run, the operator will interact with the system via a Graphical User Interface (GUI). The operator name (same as login username), start-date and start-time will be taken directly from the computer OS. When a run is launched, the operator will be requested to point to an IWL file corresponding to the experiment to be run. Based on the information read by the system from the IWL, the operator will be asked to confirm the number of TPs and CTs associated with the run. Once this step is passed, the system should commence with recognition and barcode scanning of all TPs and CTs present. When this is finished, and the correct numbers of TPs and CTs have been identified, the operator should be presented with a list (CAS No.) of the chemicals that will be tested in the experiment. If TP-CT association is flagged true, then the corresponding plate label/barcode

should be also presented in the list. This list should be accepted by the operator before the process continues. Finally, the operator will be asked to confirm the presence of the necessary media and labware on the deck before starting the run.

3.2. Output Work List

The Output Work List (OWL) is also an Excel file and is generated automatically by the system. The base file is generated by the system by simply making a copy of the IWL, retaining the filename but changing '_IWL' to '_OWL'. The file will be stored in the same folder as the IWL. The complete OWL is then produced by writing the output data (e.g. results, system parameters) to it. The Header Worksheet in the IWL contains some fields which are left blank by the Experiment Supervisor but which are then filled during the writing of the OWL. At the moment, these particular fields are related to the operator ID and the start date and time of the experiment.

The results (measurements) acquired for each plate should be written to pre-defined cells in the relevant plate-worksheet. In addition to the main results from the plate-reader, traceability data related to each TP will be extracted from the system and written to the OWL. These parameters are as follows:

- Concentration (test chemical) application date and time
- Endpoint determination date and time
- pH measurement for concentration C_1 (from PP)
- Osmolarity measurement for concentration C_1 (from PP)
- Absorbance data for concentration C_1 (from PP)
- Other key time points (to be defined)

An example of the layout for the plate-worksheet of the OWL is given in Fig. 5.

1													
2	Chemical name				Tube ID				CT_10				
3	Cass No.		cas01		Plate ID				TP_11				
4	Solvent				Dilution factor								
5	Solvent Conc.				Highest stock Conc. (M)								
6	Aids used to dissolve				Plate sealing				0				
7	pH (highest conc. tested):		5.083634377		Medium Clarity/Color (highest conc. tested):								
8													
9	Final Volume (FV)		60		Dilution Transfer Volume (DTV)				286				
10													
11			C1	C2	C3	C4	C5	C6	C7	C8			
12	Concentration Series (M)		100.00	82.64	68.30	56.45	46.65	38.55	31.86	26.33			
13													
14													
15													
16					RAW DATA O.D. Measurement								
17		1	2	3	4	5	6	7	8	9	10	11	12
18	A	0.305	0.297	0.282	0.298	0.279	0.315	0.328	0.291	0.314	0.327	0.328	0.330
19	B	0.320	0.806	0.314	0.291	0.322	0.334	0.478	0.581	0.672	0.660	0.736	0.318
20	C	0.303	0.827	0.315	0.320	0.305	0.330	0.434	0.595	0.678	0.761	0.768	0.323
21	D	0.302	0.833	0.306	0.310	0.295	0.331	0.484	0.644	0.744	0.793	0.801	0.294
22	E	0.329	0.809	0.303	0.322	0.330	0.325	0.482	0.682	0.706	0.779	0.854	0.337
23	F	0.294	0.865	0.339	0.317	0.326	0.345	0.481	0.620	0.647	0.689	0.776	0.320
24	G	0.302	0.711	0.312	0.291	0.314	0.344	0.427	0.603	0.623	0.691	0.671	0.312
25	H	0.291	0.305	0.296	0.300	0.295	0.305	0.302	0.291	0.311	0.323	0.322	0.319
26													
27		pH (highes conc. tested):			7.0836344								
28													
29		Conc. application date:			2006-09-26 - 10:22:11								
30													
31		Endpoint determination date:			2006-09-28 - 14:18:30								
32													
33		Osmolarity measurement (mOs			334								
34													

Figure 20: Layout of the Output Worklist

Part III: The automated run

The 3T3/NRU automated process has been described in an internal document [1]. The process was divided in two stages associated with pre- and post-incubation (i.e. of TP with test chemical), denoted Stage I and Stage II, respectively. In this document, we describe the general preparation tasks that have to be performed before starting a run.
First, the handling of the different washing bottles is described then the sequence of the positioning of the different labware on the deck is detailed. The instrumentation initialisation (and calibration when needed) is given. Finally, the software run interface is shown.
These preparation tasks are given for stage I and II.

4. Stage I

4.1. Preparation of wash bottles

All the wash bottles are labelled. This task is valid for Stage I & stage II:

A- Washing station (MicroLabStar needles wash)
1- Fill the 2 bottles labelled *Fluid 1* and *Fluid 2* with Filtered water
2- Check the bottle labelled *Waste* is empty

B- pH washer
1- Fill the 2 bottles labelled pH washer with deionised water
2- Check the bottle labelled *pH Waste* is empty

C- Active waste (MicroLabStar) positioned in the pH washing station
1- Empty waste bottle

D- Washer Biotek:
1- Fill the bottle labelled *"A"Buffer* with pre-warmed D-PBS
2- Empty the bottles labelled *Waste* and *Waste Overflow*.

4.2. Preparation of the MicroLabStar deck

For convenience, the sequence and positioning and of the labware is shown on a layout representing the deck configuration used

A- Lid park position

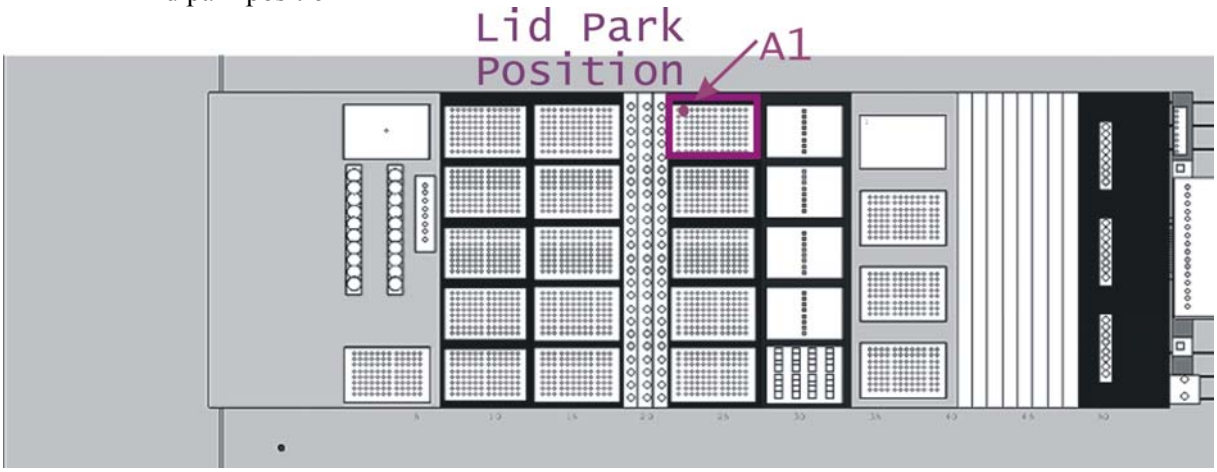

Figure 21: lid park position on the deck

[1] Automated process for 3T3/NRU cytotoxicity assay prepared on 2004-11-04 and revised on 2005-07-12

Place 1 empty de-lidded 96-well.
Use same 96-well plate than Test Plate TP
This position serves as a park position for lids.
Place 1 empty de-lidded 96-well.
Use same 96-well plate than Test Plate TP
The plate is oriented in order to have the well A1 at the top left (forward – left on the deck). This orientation is valid for all plates positioned on the deck.

B- PP Plate

Leave empty.
During the run, the PP position is used for various tasks and should be left empty. When needed, the system will automatically load a 96 well plate to prepare the PP plate. (Now, we are using the lid plate as the PP plate).
The position corresponds to the pH measurement station

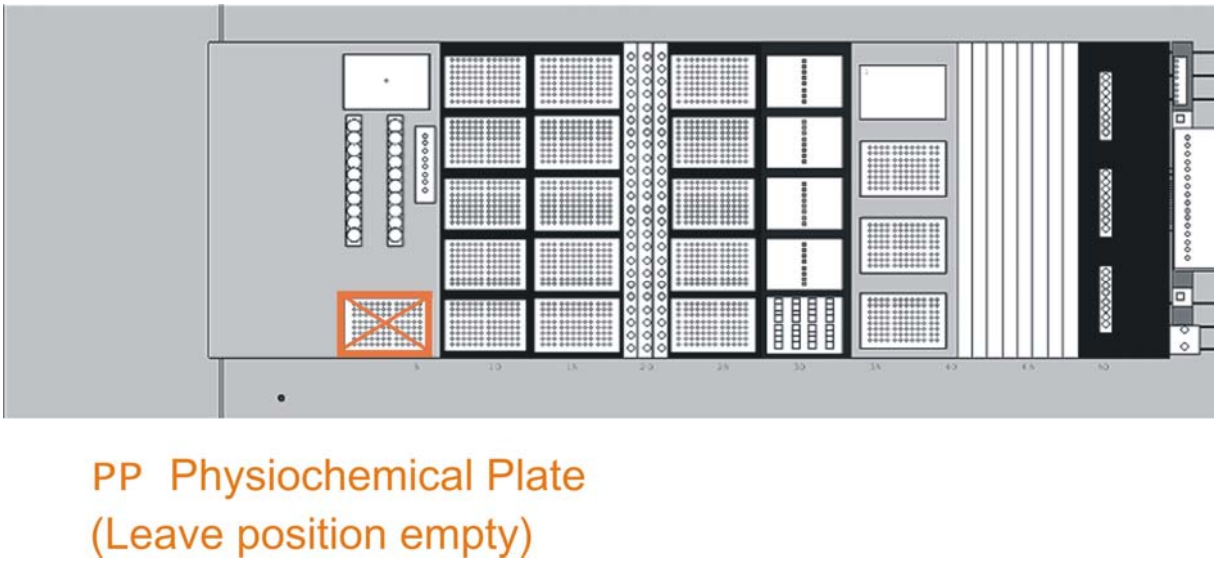

Figure 22: physiochemical plate position

C- Tips

In stage I, only standard volume tips (300µl) are used.
Position1: 8-tips take. 8 tips are picked up at a time. Position is tracked with global variable. Count Valid from run-to-run.
Position 2: 1-tip take. Individual tips are picked up. Position is tracked with global variable. Count valid from run-to-run.
Position 3 to 7: Each position is a stack of 4 sets of tips.

Positions 1 and 2 are tracked with global variable. The count is valid from run-to-run.
During the run , when Positions 1 and 2 are empty, they are loaded automatically sequentially from stacks 3 to 7.
Positions 3 to 7 are tracked with local variable. The count is not valid from run-to-run. Positions should be filled before any new run. These positions should not be refilled at stage II or if a second batch is scheduled during the same run.

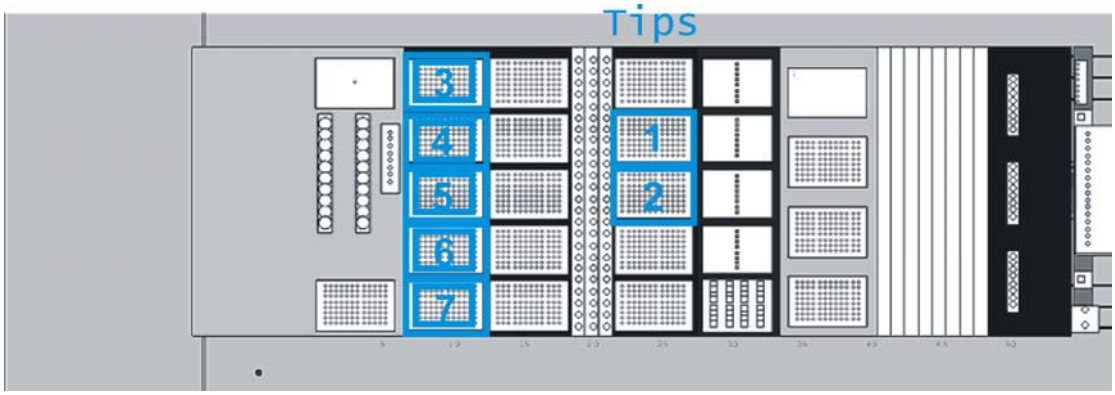

Figure 23: Tips racks position

D- Dilution plates

Positions 1 to 3: One DP
Position 4 to 9: 3 DP at each position.
Position 10: 1 DP. This DP will be used to prepare the diluted chemicals for the physiochemical measurements.
Do not place more than 3 DP in positions 4 to 10.
Same orientation of plates: A1 to top left.

Figure 24: dilution plates position

E- Transfer position
Leave position empty. It serves as a transfer position form ML-Swap to ML-Star.

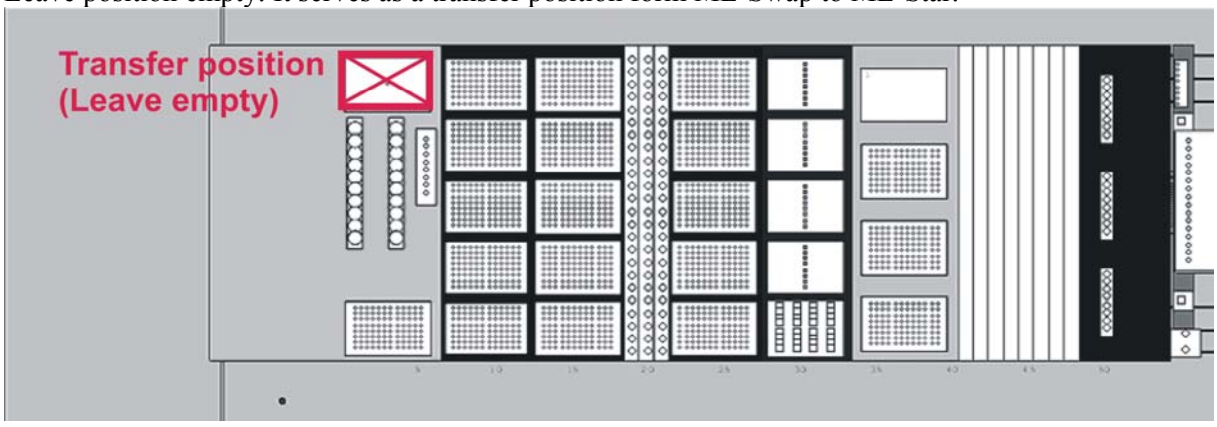

Figure 25: Plate transfer position

F- RCM container

Fill the liquid container with sufficient Culture routine medium

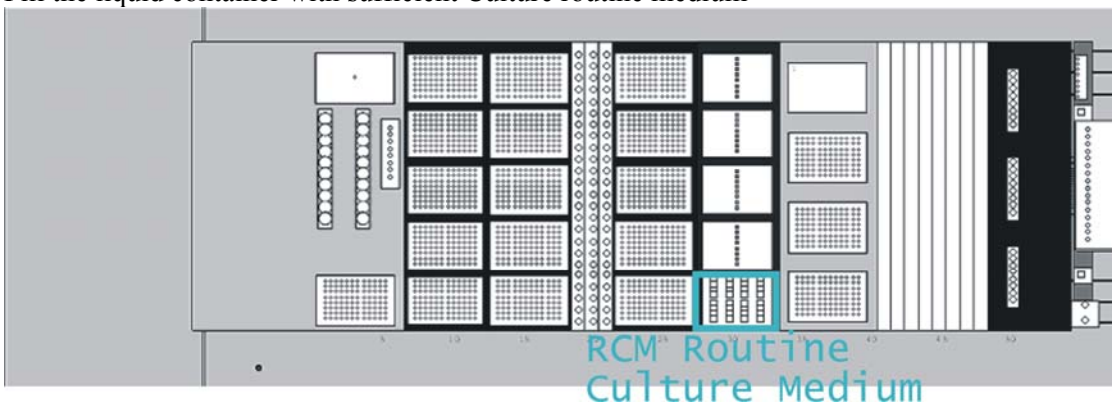

Figure 26: Routine culture media containers position

G- CDM container

Fill the liquid container with sufficient Chemical Dilution medium

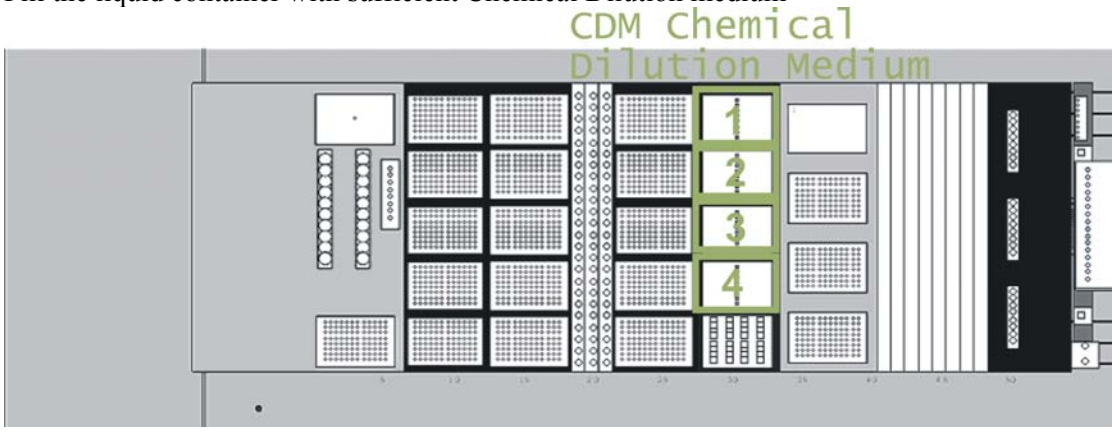

Figure 27: Chemical dilution media containers position

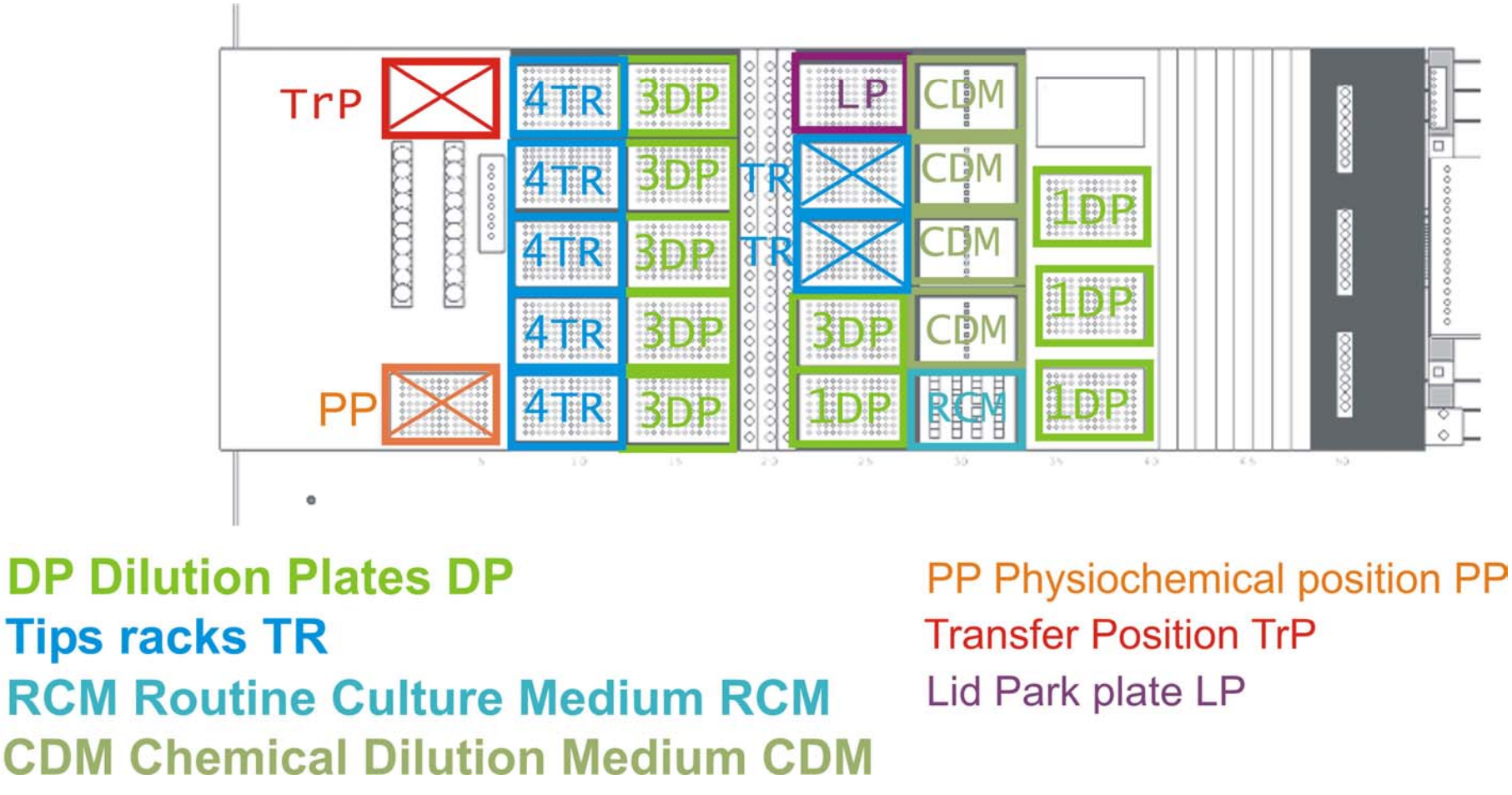

Figure 28: Layout summary for Stage I.

4.3. Instrumentation preparation

A- ML-Star
Switch ON if needed
Be sure that Swap is at safe position

B- Optical reader
Switch ON if needed

C- Washer
Switch ON if needed

D- Osmometer
Switch ON if needed. At start-up, the instrument goes in a calibration process.
Perform calibration tasks:

Load the osmo-tubes rack.

D- Sealer/Peeler:
Switch ON if needed. Check foil on place.

G- general:
Ensure pH station filled with: Kcl, pH4, pH7 and pH10 calibration solutions.
Check core-grip tweezers on place.
Ensure sealer foil sufficient for run.
Check bio-hazard bin on place

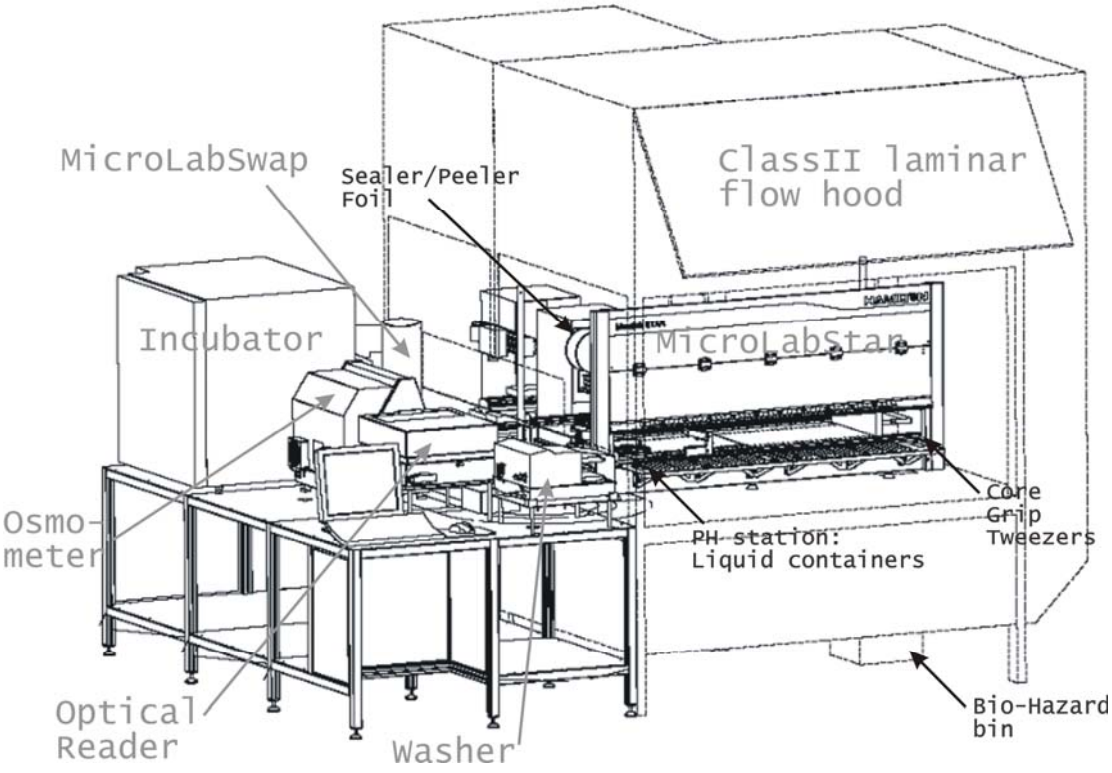

Figure 29: Automated platform drawing

4.4. Run Start

The run starts after:

1- putting the chemical tubes CT rack in the deck. This is an auto-load position, i.e. the rack is positioned in the rails (outside the ML Star) in order to allow a bar-code reading. The CT rack could also be loaded in the ML-Star deck, it will be then automatically moved to the rails, bar-code read and repositioned ion the deck. The bar codes labels should be carefully placed in the tubes to allow the reading. There is no particular sequence of loading the tubes in the first rack. If more than 32 chemicals are used, the first rack must be completely filled before using the second rack.

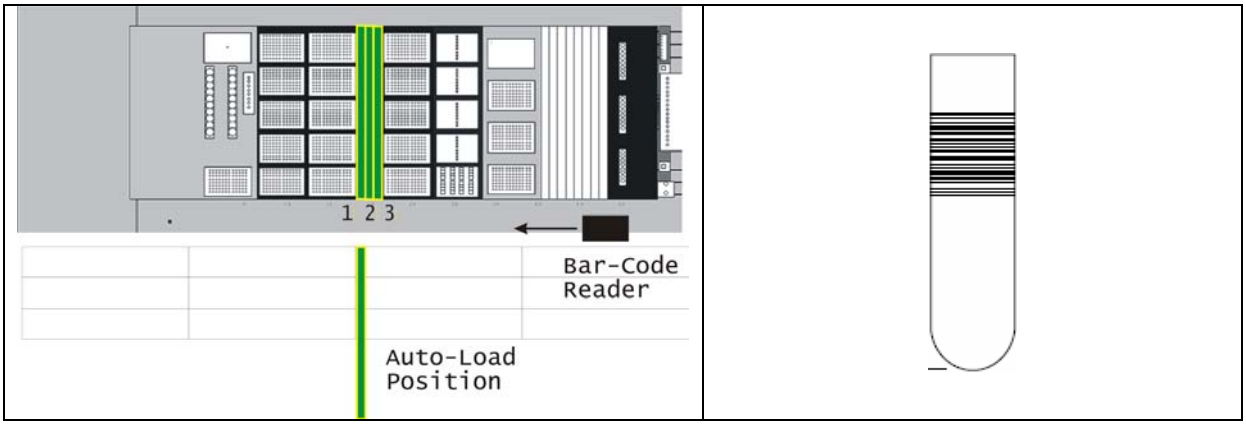

Figure 30: Sealed chemical tubes auto-load

2- Loading the Test plates TP in the incubator. The bar-code reader scans all positions in the incubator, the positioning of the plates is thus random. The TP label is positioned in the back of the rack to allow reading (the bar code reader is positioned behind the load racks)

The CT label must be glued within a range of between 20mm to 100mm from the bottom of the tube. The label must fit tightly at an angle of approximately 90° to the tube.

The TP barcode must fit on side A of the plate.
The barcode must be positioned in the middle of the plate.
The barcode label must be centered and parallel to the edge of the plate.

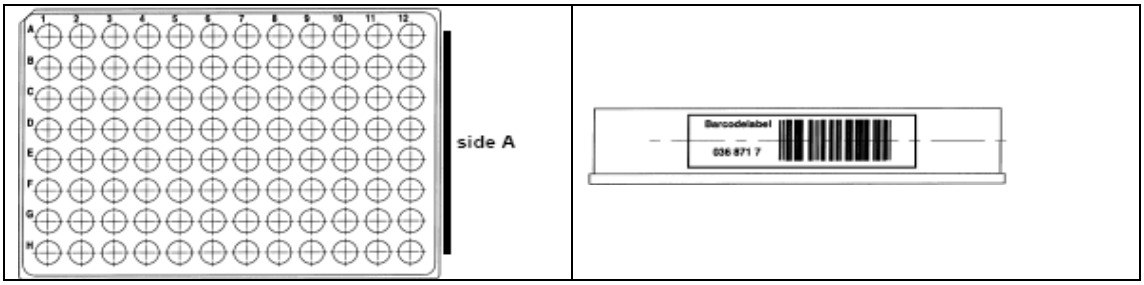

Figure 31: Plate bar-coding

5. Stage II

Once the treatment in stage I is finished, the plates are placed in the incubator.

A message is displayed on the screen to notify the operator to load labware needed for stageII

Concerning the Wash bottles, the instrumentation, No particular tasks to perform. Only the deck configuration changes and new labware has to be loaded

5.1. Preparation of the MicroLabStar deck

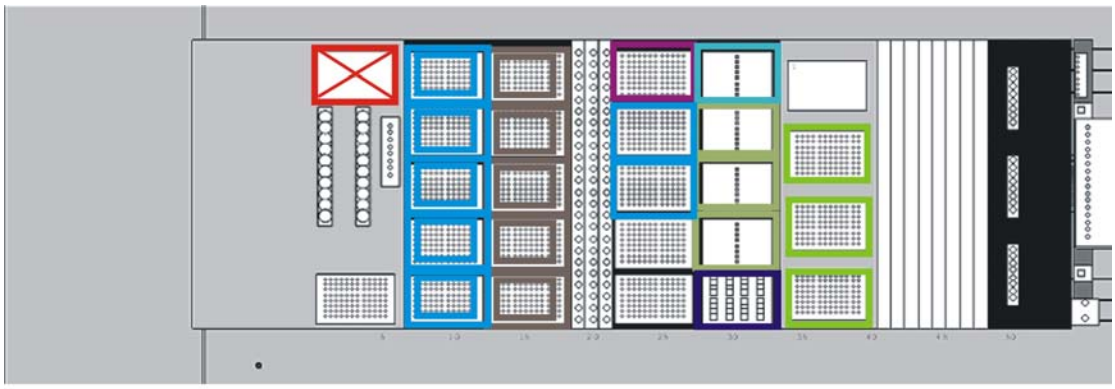

Figure 32: Layout summary for Stage II.

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ANNEX C

Study protocol of IIVS

NEUTRAL RED UPTAKE BIOASSAY IN BALB/c 3T3 MOUSE FIBROBLASTS – A SCREEN FOR LOW TOXICITY

1.0 PURPOSE

The purpose of this study is to evaluate the potential cytotoxicity of test articles as measured by a reduction of neutral red uptake (NRU) in cultures of BALB/c 3T3 fibroblasts, and is designed as a screen to discriminate between highly toxic materials and those of minimal toxicity (as defined by a predicted rodent oral LD_{50} > 2000 mg/kg). This protocol is based upon modifications of the procedures described in the protocol used in the validation of the Neutral Red Uptake in BALB/c 3T3 cytotoxicity test supported by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM).

2.0 SPONSOR

2.1	Name:	Institute for In Vitro Sciences, Inc.
2.2	Address:	30 W. Watkins Mill Rd. Gaithersburg, MD 20878
2.3	Representative:	Hans A. Raabe, M.S.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Articles:	See Protocol Attachment 1	
3.2	Controls:	Positive: Negative:	sodium lauryl sulfate (SLS) vehicle control

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Institute for In Vitro Sciences, Inc.
4.2	Address:	30 W. Watkins Mill Road, Suite 100 Gaithersburg, MD 20878
4.3	Study Director:	Gregory Moyer, M.B.A.

5.0 TEST SCHEDULE

5.1	Proposed Experimental Initiation Date:	March 24, 2008
5.2	Proposed Experimental Completion Date:	September 24, 2008
5.3	Proposed Report Date:	November 19, 2008

6.0 TEST SYSTEM

BALB/c 3T3 mouse fibroblasts obtained from American Type Culture Collection [ATCC], (Manassas, VA, USA) will be used in this study. The BALB/c 3T3 mouse fibroblasts have been selected as the target cell because of wide availability, rapid growth in culture and ready response to toxic assault.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

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 mammalian 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 NR after chemical exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

The experimental design of this study consists of a solubility or miscibility test to select (or confirm) a suitable solvent for the test article, the determination of the pH of the neat test article (if possible), the determination of the pH at the highest concentration of test article in the culture medium, and at least two definitive neutral red bioassays.

The neutral red bioassay is evaluated on the basis of the uptake of the dye, neutral red, by the BALB/c 3T3 fibroblasts. The amount of neutral red is measured spectrophotometrically. Data are presented in the form of relative survival (relative neutral red uptake) versus test article concentration.

The methods for the neutral red bioassay are modifications of the procedures used by Borenfreund and Puerner (1984).

NOTE: since the goal of this protocol is to discriminate between highly toxic materials and those of minimal toxicity (as defined by a predicted rodent oral LD_{50} > 2000 mg/kg), the assay will utilize *in vitro* doses expected to be predictive of this range. Accordingly, the range of dilutions to be tested will typically be 20,000 µg/mL to 80 µg/mL (unless test chemical solubility limits the maximum doses).

7.1 Cell Type

BALB/c 3T3 Fibroblasts, clone 31, CCl-163, from American Type Tissue Collection (ATCC), Manassas, VA, USA.

7.2 Technical Equipment

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

7.2.1 Incubator: 37ºC ± 1ºC, 90 % ± 10 % humidity, 5 % ± 1 % CO_2/air (standard culture conditions)
7.2.2 Laminar flow clean bench (standard: "biological hazard")
7.2.3 Water bath: 37ºC ± 1ºC
7.2.4 Inverse phase contrast microscope
7.2.5 Sterile glass tubes with caps (e.g., 5 mL)
7.2.6 Centrifuge
7.2.7 Laboratory balance
7.2.8 96-well plate spectrophotometer equipped with 550 nm filter
7.2.9 Shaker for microtiter plates
7.2.10 Cell counter or hemacytometer
7.2.11 Pipetting aid
7.2.12 Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette)
7.2.13 Tissue culture flasks (75 - 80 cm^2, 25 cm^2)
7.2.14 96-well flat bottom tissue culture microtiter plates (e.g., Falcon tissue culture-treated)
7.2.15 pH paper (wide and narrow range)
7.2.16 Multichannel reagent reservoir
7.2.17 Waterbath sonicator
7.2.18 Magnetic stirrer
7.2.19 Antistatic bar ionizer/antistatic gun (optional for neutralizing static on 96-well plates)
7.2.20 Adhesive film plate sealers (e.g., Excel Scientific SealPlate™,Cat # STR-SEAL-PLT or equivalent)
7.2.21 Vortex mixer
7.2.22 Filters/filtration devices

7.3 Chemicals, Media, and Sera

7.3.1 Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine; should have high glucose [4.5 g/L] (e.g., ICN-Flow Cat. No. 12-332-54)
7.3.2 L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49)
7.3.3 New Born Calf Serum (NBCS or NCS) (e.g., Biochrom # SO 125)
7.3.4 0.05 % Trypsin/0.02 % EDTA solution (e.g., SIGMA T 3924, ICN-Flow, # 16891-49)
7.3.5 Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}(for trypsinization)
7.3.6 Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}(CMF-HBSS)
7.3.7 Dulbecco's Phosphate Buffered Saline (D-PBS) [formulation containing calcium and magnesium cations; glucose optional] (for rinsing)
7.3.8 Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49)
7.3.9 Neutral Red (NR) Dye – tissue culture-grade; liquid form (e.g., SIGMA N 2889)
7.3.10 Dimethyl sulfoxide (DMSO), U.S.P. analytical grade (Store under nitrogen @ -20ºC)
7.3.11 Ethanol (ETOH), U.S.P. analytical grade (100 %, non-denatured for test chemical preparation; 95 % can be used for the desorb solution)
7.3.12 Glacial acetic acid, analytical grade
7.3.13 Distilled H_2O or any purified water suitable for cell culture and NR desorb solution (sterile)
7.3.14 Sterile, paper towels (for blotting 96-well plates)

7.4 Preparations of Media and Solutions

[Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures will be adequately documented.]

7.4.1 Media

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

7.4.1.1 Routine Culture (Routine Culture Medium)
10 % NBCS/NCS
4 mM Glutamine

7.4.1.2 Test chemical dilution (Chemical Dilution Medium)
4 mM Glutamine

200 IU/mL Penicillin
200 µg/mL Streptomycin

7.4.1.3 Dilution of NR stock solution (NR Dilution Medium)
5 % NBCS/NCS
4 mM Glutamine
100 IU/mL Penicillin
100 µg/mL Streptomycin

[Note: The Chemical Dilution Medium with test chemical will dilute the serum concentration of the Routine Culture Medium in the test plate to 5 %. The final medium formulation applied to the cultures is defined as Assay Medium. Serum proteins may mask the toxicity of the test substance, but serum cannot be totally excluded because cell growth is markedly reduced in its absence.]

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

7.4.2 Neutral Red (NR) Stock Solution

The liquid tissue culture-grade stock NR Solution will be the first choice for performing the assay (e.g., SIGMA #N2889, 3.3 mg/mL). Store liquid tissue culture-grade NR Stock Solution at the storage conditions and shelf-life period recommended by the manufacturer.

7.4.3 Neutral Red (NR) Medium

Example:

0.758 mL (3.3 mg NR dye/mL solution) NR Stock Solution
99 mL 99.242 mL NR Dilution Medium (pre-warmed to 37ºC)

The final concentration of the NR Medium is **25 µg NR dye/mL** and aliquots will be prepared on the day of application.

[Note: The NR Medium shall be filtered (e.g., Millipore filtering, 0.2 – 0.45 µm pore size) used to reduce NR crystals. Aliquots of the NR Medium should be maintained at 37° C (e.g., in a waterbath) before adding to the cells and used within 30 min of preparation but also used within 15 min after removing from 37°C storage.]

7.4.4 Ethanol/Acetic Acid Solution (NR Desorb)

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

7.5 Cell Culture Methods

7.5.1 Cell Maintenance and Culture Procedures

BALB/c 3T3 cells are routinely grown as a monolayer in tissue culture grade flasks (e.g., 75 - 80 cm^2) at 37ºC ± 1ºC, 90 % ± 10 % humidity, and 5.0 % ± 1 % CO_2/air (standard culture conditions). The cells should be examined on a daily (i.e., on workdays) basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted in a Study Workbook.

7.5.2 Receipt of Cryopreserved BALB/c 3T3 cells

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

7.5.3 Thawing Cells

Thaw cells by putting ampules into a water bath at 37°C ± 1ºC. Leave for as brief a time as possible. Resuspend the cells in pre-warmed Routine Culture Medium and transfer into a tissue-culture flask. Incubate at standard culture conditions. When the cells have attached to the bottom of the flask (within 4 to 24 hours), remove the supernatant and replace with fresh pre-warmed (37ºC) medium. Culture as described above. Passage at least two times before using the cells in a cytotoxicity test.

A fresh batch of frozen cells from the stock lot of cells should be thawed out and cultured approximately every two months. This period resembles a sequence of about 18 passages.

7.5.4 Routine Culture of BALB/c 3T3 Cells

When the cultures reach 50 to 80 % confluence, they should be removed from the flask by trypsinization. Remove the medium, and briefly rinse cultures with 5 mL PBS or Hanks' BSS (without Ca^{2+}, Mg^{2+}) per 25 cm^2 flask (15 mL per 75 cm^2 flask). Wash cells by gentle agitation to remove any remaining serum that might inhibit the action of the trypsin. Discard the washing solution. Repeat the rinsing procedure and discard the washing solution. Add 1-2 mL trypsin-EDTA solution per 25 cm^2 to the monolayer for a few seconds (e.g., 15-30 seconds). Remove excess

trypsin-EDTA solution and incubate the cells at room temperature. After 2-3 minutes (min), lightly tap the flask to detach the cells into a single cell suspension. When more than 50% of the cells become dislodged, the flask will be rapped sharply against the palm of the hand. When most of the cells have become detached from the surface, approximately 5-10 mL of Routine Culture Medium is added to each 75 cm^2 flask to obtain a single cell suspension. 3T3 mouse fibroblasts are routinely passaged every 2-4 days (average doubling time, 20-24 hr) to maintain a cell density less than 80% of confluence.

7.5.5 Subculture of BALB/c 3T3 Cells to 96-Well Plates

Cultured cells that are going to be used in seeding the 96-well plates should be fed fresh medium the day before subculturing to the plates. When cultures are between 50 and 80% confluence, a cell suspension will be obtained from the culturing procedure. A hemacytometer and/or Coulter Counter is used to determine cell concentration. The cells are diluted to a concentration of $2.0 – 3.0x10^4$ cells/mL in Routine Culture Medium. Using a multi-channel pipette, dispense 100 µL Routine Culture Medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate. In the remaining wells, dispense 100 µL of a cell suspension of $2.0 – 3.0x10^4$ cells/mL (= $2.0 – 3.0x10^3$ cells/well).

Incubate cells for 24 ± 2 hr at standard culture conditions so that cells form a less than half (< 50%) confluent monolayer.

On the day of dosing, examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate.

7.6 Test Chemical Preparation

Preparation under red or yellow light is recommended to preserve chemicals that degrade upon exposure to light. Test chemicals will be allowed to equilibrate to room temperature before dissolving and diluting. Test chemicals will be prepared immediately prior to use. Ideally, the solutions must not be cloudy nor have noticeable precipitate.

A solubility or miscibility test will be conducted to select (or confirm) a suitable solvent for the test article and to determine the solubility at the highest stock concentration to be used in the assay. The methods for determining solubility will be modifications of those presented in *Test Method Protocol for Solubility Determination* (NICEATM, 2003). Based upon the selection of the solvent, the following sections provide guidance on the highest concentration to be prepared for the subsequent assays.

7.6.1 For chemicals dissolved in Chemical Dilution Medium, a dilution series of eight 2X stock dosing solutions will be prepared for application to the BALB/c 3T3 cells. The stock solution for each test chemical should be prepared at the highest soluble concentration up to 40 mg/mL (40,000 µg/mL) in chemical dilution medium. Therefore, the highest dose tested on the cells would be 20,000 µg/mL. The seven lower concentrations would then be prepared by serial dilution using a uniform dilution factor of 2.2 (resulting in dose intervals of approximately 1/3 log each).

7.6.2 For chemicals dissolved in DMSO or ethanol, the final DMSO or ethanol concentration for application to the cells must be 0.5 % (v/v) in the vehicle controls and in all of the eight test concentrations. The stock solution for each test chemical should be prepared at the highest soluble concentration up to 500 mg/mL (500,000 µg/mL) in DMSO or ethanol. The seven lower concentrations would then be prepared by serial dilution using a uniform dilution factor of 2.2 (resulting in dose intervals of approximately 1/3 log each).

Since each stock concentration in solvent is 200 fold greater than the concentration to be tested, a 1:100 dilution will be made by diluting 1 part dissolved chemical in each tube with 99 parts of Chemical Dilution Medium to derive the eight 2X concentrations for application to the BALB/c 3T3 cells. Each 2X test chemical concentration will then contain 1 % (v/v) solvent. The BALB/c 3T3 cells will have 0.05 mL of Routine Culture Medium in the wells prior to application of the test chemical. By adding 0.05 mL of the appropriate 2X test chemical concentration to the appropriate wells, the test chemical will be diluted appropriately in a total of 0.1 mL and the solvent concentration in the wells will be 0.5% (v/v). Therefore, the highest possible dose exposed to the cells would be 2.5 mg/mL (2500 µg/mL) when starting with a 500 mg/mL DMSO stock.

A test article prepared in DMSO or ethanol may precipitate upon transfer into the Chemical Dilution Medium. The 2X dosing solutions should be evaluated for precipitates and the results will be recorded in the workbook. It will be permissible to test all of the dosing solutions, however, doses containing test article precipitates should be noted.

7.6.3 pH of Test Chemical Solutions

Prior to or immediately after application of the test chemical to the 96-well plate, measure the pH of the highest 2X dosing concentration of the test chemical in Chemical Dilution Medium. Use pH paper to determine the pH. The pH paper should be in contact with the solution for approximately one minute. Document the pH and note the medium color

of the 2X dosing solutions. Medium color for all dosing dilutions should be noted in the workbooks. Do not adjust the pH.

7.7 Test Procedure

7.7.1 96-Well Plate Configuration

The BALB/c 3T3 NRU assay for test chemicals will use the 96-well plate configuration shown in Figure 1.

Figure 1. 96-Well Plate Configuration for Test Chemical Assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	T_1C_8b	T_1C_7b	T_1C_6b	T_1C_5b	T_1C_4b	T_1C_3b	T_1C_2b	T_1C_1b	VCb	VCb
B	VCb	VC_1	T_1C_8	T_1C_7	T_1C_6	T_1C_5	T_1C_4	T_1C_3	T_1C_2	T_1C_1	VC_2	VCb
C	VCb	VC_1	T_1C_8	T_1C_7	T_1C_6	T_1C_5	T_1C_4	T_1C_3	T_1C_2	T_1C_1	VC_2	VCb
D	VCb	VC_1	T_1C_8	T_1C_7	T_1C_6	T_1C_5	T_1C_4	T_1C_3	T_1C_2	T_1C_1	VC_2	VCb
E	VCb	VC_1	T_2C_8	T_2C_7	T_2C_6	T_2C_5	T_2C_4	T_2C_3	T_2C_2	T_2C_1	VC_2	VCb
F	VCb	VC_1	T_2C_8	T_2C_7	T_2C_6	T_2C_5	T_2C_4	T_2C_3	T_2C_2	T_2C_1	VC_2	VCb
G	VCb	VC_1	T_2C_8	T_2C_7	T_2C_6	T_2C_5	T_2C_4	T_2C_3	T_2C_2	T_2C_1	VC_2	VCb
H	VCb	VCb	T_2C_8b	T_2C_7b	T_2C_6b	T_2C_5b	T_2C_4b	T_2C_3b	T_2C_2b	T_2C_1b	VCb	VCb

VC_1 and VC_2 = VEHICLE CONTROL
$T_\#C_1 – T_\#C_8$ = TEST CHEMICAL 1 or 2 at eight concentrations (C1 = highest, C8 = lowest)
$T_\#C_\#b$ = BLANKS (Test chemical 1 or 2, but contain no cells)
VCb = VEHICLE CONTROL BLANK (contain no cells)

7.7.2 Application of Test Chemical

After at least 22 hr incubation of the cells, remove Routine Culture Medium from the cells. Gently blot the plate on a sterile, paper towel so that the monolayer is minimally disrupted. Immediately add 50 µL of fresh pre-warmed Routine Culture Medium to all of the wells, including the blanks. The plates will be returned to the incubator under standard culture conditions until dosing solutions are ready to be dosed.

Each of the 2X dosing solutions will be transferred into labeled, sterile 8-channel reservoirs. After 24 ± 2hr incubation of the cells, after

refeeding the cultures, add 50 µL of the appropriate concentration of test chemical, the PC, or the VC directly to the test wells. The dosing solutions will be rapidly transferred from the 8-channel reservoir to the test plate using a single delivery multi-channel pipettor. For example, the VC may be transferred first (into columns 1, 2, 11, and 12), followed by the test article dosing solutions for the first test article, so that the same pipette tips on the multi-channel pipettor can be used. After changing pipette tips, the dosing solutions for the second test article will be transferred to the test plate in the same manner. The Vehicle Control blank (VCb) wells (column 1, column 12, wells A2, A11, H2, H11) will receive the Vehicle Control dosing solutions (which should include any solvents used). Blanks for wells A3 – A10 and H3 – H10 shall receive the appropriate test chemical dosing solution. Incubate cells for 48 ± 0.5 hr at standard culture conditions.

Positive Control: For each set of test chemical plates used in an assay, a separate plate of positive control (SLS) concentrations will be tested. Eight doses of SLS ranging from 100 µg/mL to 9.49 µg/mL will be tested. The positive control dosing solutions will be prepared in Chemical Dilution Medium, and will be applied as described above, however, each dosing solution will be tested in 6 replicate wells per dose.

Figure 2. 96-Well Plate Configuration for the Positive Control

	1	2	3	4	5	6	7	8	9	10	11	12
A	VCb	VCb	C_8b	C_7b	C_6b	C_5b	C_4b	C_3b	C_2b	C_1b	VCb	VCb
B	VCb	VC_1	C_8	C_7	C_6	C_5	C_4	C_3	C_2	C_1	VC_2	VCb
C	VCb	VC_1	C_8	C_7	C_6	C_5	C_4	C_3	C_2	C_1	VC_2	VCb
D	VCb	VC_1	C_8	C_7	C_6	C_5	C_4	C_3	C_2	C_1	VC_2	VCb
E	VCb	VC_1	C_8	C_7	C_6	C_5	C_4	C_3	C_2	C_1	VC_2	VCb
F	VCb	VC_1	C_8	C_7	C_6	C_5	C_4	C_3	C_2	C_1	VC_2	VCb
G	VCb	VC_1	C_8	C_7	C_6	C_5	C_4	C_3	C_2	C_1	VC_2	VCb
H	VCb	VCb	C_8b	C_7b	C_6b	C_5b	C_4b	C_3b	C_2b	C_1b	VCb	VCb

VC_1 and VC_2 = VEHICLE CONTROL
C_1 –C_8 = POSITIVE CONTROL (SLS) at eight concentrations (C1 = highest, C8 = lowest)
$C_\#b$ = BLANKS (Positive Control, but contain no cells)
VCb = VEHICLE CONTROL BLANK (contain no cells)

7.7.3 Microscopic Evaluation

After at least 46 hours of treatment, examine each plate under 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 chemical.

7.7.4 Measurement of NRU

Carefully remove (i.e., "dump") the medium (with test chemical) and rinse the cells very carefully with 250 µL pre-warmed D-PBS. Remove the rinsing solution by dumping and remove excess by gently blotting on sterile, paper towels. Add 250 µL NR medium (to all wells including the blanks) and incubate at standard culture conditions for 3±0.1 hr. Observe the cells briefly during the NR incubation (e.g., between 2 and 3 hr) for NR crystal formation. Record observations in the Study Workbook.

After incubation, remove the NR medium, and carefully rinse cells with 250 µL pre-warmed D-PBS. Decant and blot D-PBS from the plate. Add 100 µL NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.

Shake microtiter plate rapidly on a microtiter plate shaker for 20 – 45 min to extract NR from the cells and form a homogeneous solution. Plates should be protected from light by using a cover during shaking.

Measure the absorption (within 60 minutes of adding NR Desorb solution) of the resulting colored solution at 550 nm in a microtiter plate reader.

7.7.5 Volatility of Test Chemicals

Highly volatile test chemicals may generate vapors from the treatment media during the test chemical treatment incubation period. These vapors may become resorbed into the treatment medium in adjacent wells, such that culture wells nearest the highest doses may become contaminated by exposure to resorbed test article vapors. If the test chemical is particularly toxic at the doses tested, the cross contamination may be evident as a significant reduction in viability in the vehicle control cultures (i.e., VC2) adjacent to the highest test chemical doses.

If potential test article volatility is suspected (e.g., for low density liquids) or if the results from the initial trial conducted without a plate sealer shows evidence of toxic effects in the control cultures (i.e., > 15 % difference in viability between VC1 [column 2] and VC2 [column 11]), then seal the subsequent test plates by the following procedure.

Plate Sealer Method

Plates and chemicals will be prepared as usual as described above. Immediately after the 96-well culture plate has been treated with the suspected volatile chemical, apply the adhesive plate sealer directly over the culture wells. Assure that the sealer adheres to each culture well. Place the 96-well plate cover loosely over the sealed plate and incubate the plate under standard culture conditions. [Note: Do not jam the plate lid over the film to avoid deforming the sealer and causing the sealer to detach from culture wells. A loose fit of the plate lid is acceptable.]

At the end of the treatment period, the plate sealer should be carefully removed to avoid spillage. Continue with the NRU assay as per **§ 7.7.3**.

7.8 Data Analysis

The mean OD_{550} value for the vehicle control-treated blank wells (VCb) will be calculated. Similarly, the mean OD_{550} values for each of the test article-treated blank wells (C_nb) will be calculated for each dose group. The corrected OD_{550} values for each vehicle control (VC) culture well will be calculated by subtracting the mean VCb OD_{550} value from the individual VC OD_{550} values. Similarly, the corrected OD_{550} values for each of the culture wells treated with each test article dilution or positive control dilution will be determined.

The mean corrected OD_{550} value for the VC will be calculated. The % of Vehicle Control (relative viability) will be calculated for each individual culture well, by dividing the corrected mean OD_{550} value from each well by the mean corrected OD_{550} value of the VC. A mean % of VC value for each dose group will be calculated. A plot of the % of VC versus the concentration of the test article or positive control, will establish a dose response curve for the test article and positive control. Wherever possible, an NRU_{50} value will be interpolated from the plot. In cases where the % of VC is less than 50% throughout the tested dose range, then the NRU_{50} value will be presented as less than the lowest dose tested. In cases where the % of VC is greater than 50% throughout the tested dose range, then the NRU_{50} value will be presented as greater than the highest dose tested.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

Both acceptance criteria must be met for a test to be acceptable.

1) The neutral red bioassay will be accepted if the positive control compound causes an NRU_{50} that falls within 2 standard deviations of the historical mean.

2) For each test plate, the left and the right mean of the VCs should not differ by more than 15 % from the mean of all VCs.

9.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. A summary will be presented for each treatment group. The report will also include a discussion of results. A copy of the protocol used for the study and any significant deviation(s) from the protocol will appear as a part of the final report.

10.0 RECORDS AND ARCHIVES

A separate working notebook will be used to record the materials and procedures used to perform this study. Upon completion of the final report, all raw data, reports and specimens will be retained in the archives for a period of either a) 5 years, b) the length of time specified in the contract terms and conditions, or c) as long as the quality of the preparation affords evaluation, whichever is applicable.

11.0 REFERENCES

Hackenberg, U. and H. Bartling. 1959. Messen und Rechnen im pharmakologischen Laboratorium mit einem speziellen Zahlensystem (WL24-System). Arch. Exp. Pathol. Pharmakol. 235: 437-463.

Spielmann, H., S. Gerner, S. Kalweit, R. Moog, T. Wirnserberger, K. Krauser, R. Kreiling, H. Kreuzer, N.P. Luepke, H.G. Miltenburger, N. Müller, P. Murmann, W. Pape, B. Siegmund, J. Spengler, W. Steiling, and F.J. Wiebel. 1991. Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany. Toxicol. *In Vitro* 5: 539-542.

Test Method Protocol for Solubility Determination. In Vitro Cytotoxicity Validation Study. Phase III. August 29, 2003. Prepared by The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).

12.0 APPROVAL

_____ _____
SPONSOR REPRESENTATIVE DATE

Hans A Raabe, M.S.
(Print or Type Name)

_____ _____
IIVS STUDY DIRECTOR DATE

PROTOCOL ATTACHMENT 1

IIVS Test Article Code	Sponsor Designate
08AA25	C740
08AA26	C493
08AA27	C688
08AA28	C565
08AA29	C161
08AA30	C342
08AA31	C605
08AA32	C183
08AA33	C526
08AA34	C496
08AA35	C814
08AA36	C949
08AA37	C974
08AA38	C529
08AA39	C691
08AA40	C518
08AA41	C902
08AA42	C973
08AA43	C147
08AA44	C368
08AA45	C616
08AA46	C330
08AA47	C404
08AA48	C446
08AA49	C371
08AA50	C549
08AA51	C170
08AA52	C889
08AA53	C642
08AA54	C558
08AA55	C154
08AA56	C662
08AA57	C398
08AA58	C726
08AA59	C233
08AA60	C701
08AA61	C598
08AA62	C309
08AA63	C817
08AA64	C696

IIVS Test Article Code	Sponsor Designate
08AA65	C934
08AA66	C323
08AA67	C865
08AA68	C606
08AA69	C959
08AA70	C270
08AA71	C238
08AA72	C708
08AA73	C151
08AA74	C580
08AA75	C223
08AA76	C366
08AA77	C856
08AA78	C638
08AA79	C400
08AA80	C247

REGULATORY REQUIREMENTS:

Will this study be conducted according to **GLPs** ? [ ] **YES** or [**X**] **NO**
If **YES**, please indicate which agency(ies) guidelines are to be followed:
[ ] FDA; [ ] EPA TSCA; [ ] EPA FIFRA; [ ] OECD; [ ] Other

Will this study be submitted to a regulatory agency ? [ ] **YES** or [**X**] **NO**
If **YES**, please indicate to which agency(ies) the study will be submitted:
[ ] FDA; [ ] EPA TSCA; [ ] EPA FIFRA; [ ] Other

ANNEX D

Codes of the test chemicals

D. CODES OF THE TEST CHEMICALS

HSL	JRC	IIVS	Chemical	Chemical number
B135	A403	C740	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate	1.
B569	A355	C493	1,2,4-Trichlorobenzene	2.
B280	A288	C688	1,2-Benzenedicarboxylic acid	3.
B505	A367	C565	1,2-Dichlorobenzene	4.
B467	A746	C161	1-Naphthylamine	5.
B948	A300	C342	1-Phenyl-3-pyrazolidone	6.
B952	A149	C605	2-(2-Butoxyethoxy)ethanol	7.
B929	A567	C183	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	8.
B985	A125	C526	2,4,6-Tris(dimethylaminomethyl)phenol	9.
B143	A773	C496	2,6-Diethylaniline	10.
B427	A572	C814	2-Butoxyethyl acetate	11.
B375	A636	C949	2-Chloro-4-nitroaniline	12.
B299	A818	C974	2-Ethylhexyl acrylate	13.
B520	A717	C529	2-Phenoxyethanol	14.
B134	A204	C691	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone	15.
B267	A137	C518	Acetophenone	16.
B261	A779	C902	Aconitine	17.
B813	A782	C973	Ammonium chloride	18.
B543	A768	C147	Barium chloride	19.
B844	A836	C368	Benzaldehyde	20.
B608	A682	C616	Benzyl benzoate	21.
B822	A346	C371	Brucine	22.
B673	A658	C330	Caprylic acid	23.
B920	A799	C404	Copper sulphate	24.
B875	A699	C446	Diallyl phthalate	25.
B159	A189	C549	Diepoxide 126	26.
B833	A716	C170	Di-"isodecyl" phthalate	27.
B447	A266	C889	Diisopropanolamine	28.
B732	A495	C642	Dimethyldioctadecylammonium chloride	29.
B855	A341	C558	Edetic acid	30.
B236	A255	C154	Ethoxyquin	31.
B827	A139	C662	Ethyl acetoacetate	32.
B416	A201	C398	Ethyl chloroacetate	33.
B684	A815	C726	Glycerol triacetate	34.
B908	A752	C233	Maleic acid	35.
B877	A893	C701	Malononitrile	36.
B866	A144	C598	Methenamine	37.
B739	A294	C309	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine	38.
B326	A379	C817	Octyl 3,4,5-trihydroxybenzoate	39.
B707	A168	C696	*P*-benzoquinone	40.
B241	A538	C934	Phthalic anhydride	41.
B824	A298	C323	Potassium sulfate	42.
B756	A399	C865	Resorcinol	43.
B583	A274	C606	Sodium cyanate	44.

HSL	JRC	IIVS	Chemical	Chemical number
B680	A269	C959	Sodium salt of chloroacetic acid	45.
B914	A308	C270	Sorbitan monolaurate	46.
B997	A359	C238	Tetramethylthiuram monosulphide	47.
B122	A724	C708	Triethanolamine	48.
B718	A485	C151	Triethylene glycol dimethacrylate	49.
B111	A961	C580	Tripotassium citrate	50.
B121	A568	C223	Tris(nonylphenyl) phosphite	51.
B164	A883	C366	Trizinc bis(orthophosphate)	52.
B123	A197	C856	Tween 20	53.
B828	A982	C638	Urea	54.
B625	A800	C400	Zinc distearate	55.
B321	A461	C247	Zinc oxide	56.

ANNEX E

Additional information
(GHS classification system for acute oral toxicity)

TABLE OF CONTENTS

E. ADDITIONAL INFORMATION (GHS CLASSIFICATION SYSTEM FOR ACUTE ORAL TOXICITY)

In this Annex we present the additional analyses performed to determine the predictive capacity of the 3T3 NRU test method protocols for estimating the additional hazard classification categories used by U.S. Federal agencies for acute oral toxicity, specifically, the GHS category 5 (2 000 $\leq$ LD_{50} $\leq$ 5 000 mg/kg) and GHS unclassified (LD_{50} > 5 000 mg/kg).

The evaluation of concordance, over-prediction, and under-prediction of these GHS toxicity categories was done using both NICEATM/ECVAM regressions models (i.e., weight and molar units). Moreover, the results of applying the optimum IC_{50} cut-off value, established from a training set (RC data set) for distinguishing between classified and unclassified test chemicals using the LD_{50} of 5 000 mg/kg as cut-off value is also calculated in this Annex.

E.1 REGRESSION ANALYSES

This section summarises the GHS predicted acute oral toxicity categories from the *in vivo* 3T3 NRU test method in each laboratory using the millimole regression and weight regression analysis compared to the *in vivo* GHS acute oral toxicity categories.

E.1.1 Millimole regression

E.1.1.1 HSL

When the prediction of the GHS acute oral toxicity categories was assessed in HSL only results from 40 test chemicals were included in the analysis, since for ten test chemicals IC_{50} right censored values were obtained that resulted in estimated LD_{50} values that did not allow to assign the test chemical to the corresponding GHS categories (e.g. LD_{50} > 2 000 mg/kg b.w.). Six test chemicals were not tested in HSL (see Section 6.2 of the final study report and Table E-18).

When the millimole regression was used, the overall GHS acute oral category prediction accuracy was 35% (14/40) (Table E-1). The acute oral toxicity was over-

predicted for 62.5% (25/40) and under-predicted for 2.5% (1/40) of the test chemicals. The three test chemicals belonging to the GHS categories 1 ($LD_{50} \leq 5$) and 2 ($5 < LD_{50} \leq 50$) were not tested in HSL.

The predictions of the remaining GHS acute oral categories were as follows:

- three (75%) of the four test chemicals in the $50 < LD_{50} \leq 300$ mg/kg category (GHS category 3);
- nine (69%) of 13 test chemicals in the $300 < LD_{50} \leq 2\,000$ mg/kg category (GHS category 4);
- one (12.5%) of 8 test chemicals in the $2\,000 < LD_{50} \leq 5\,000$ mg/kg category (GHS category 5;
- one (7%) of 15 test chemicals with $LD_{50} > 5\,000$ mg/kg (GHS unclassified).

Table E-2 shows the results of concordance, over-prediction and under-prediction of three toxicity categories ($LD_{50} \leq 2\,000$ mg/kg, $2\,000 < LD_{50} \leq 5\,000$ mg/kg GHS category 5, $LD_{50} > 5\,000$ mg/kg GHS unclassified) in HSL. There were no under-predicted test chemicals. The toxicity was over-predicted for 87.5% (7/8) of the test chemicals in GHS category 5 ($2\,000 < LD_{50} \leq 5\,000$ mg/kg) and by 93% (14/15) of the GHS unclassified ($LD_{50} > 5\,000$ mg/kg) test chemicals. The overall accuracy for correctly predicting the three toxicity categories using the millimole regression was 48% (19/40).

Table E-1. Prediction of GHS Acute Oral Toxicity Categories in HSL by the 3T3 NRU test method and the millimole regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)						Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 5$	0	0	0	0	0	0	0	0%	0%	0%
$5 < LD_{50} \leq 50$	0	0	0	0	0	0	0	0%	0%	0%
$50 < LD_{50} \leq 300$	0	0	3	1	0	0	4	75%	0%	25%
$300 < LD_{50} \leq 2\,000$	0	1	3	9	0	0	13	69%	31%	0%
$2\,000 < LD_{50} \leq 5\,000$	0	1	0	6	1	0	8	12.5%	87.5%	0%
$LD_{50} > 5\,000$	0	0	0	8	6	1	15	7%	93%	0%
Total	0	2	6	24	7	1	40	35%	62.5%	2.5%
Category Over-predicted	0%	100%	50%	58%	86%	0%				
Category Under-predicted	0%	0%	0%	4%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded cells indicate correct predictions.

Table E-2. Prediction of three acute oral toxicity categories in HSL by the 3T3 NRU test method and the millimole regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)			Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 2\,000$	17	0	0	17	100%	0%	0%
$2\,000 < LD_{50} \leq 5\,000$	7	1	0	8	12.5%	87.5%	0%
$LD_{50} > 5\,000$	8	6	1	15	7%	93%	0%
Total	32	7	1	40	47.5%	52.5%	0%
Category Over-predicted	47%	86%	0%				
Category Under-predicted	0%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded cells indicate correct predictions.

E.1.1.2 JRC

When the prediction of the GHS toxicity categories was assessed in JRC only results from 46 test chemicals were included in the analysis, since for seven test chemicals IC_{50} right censored values were obtained that resulted in estimated LD_{50} values that did not allow assignment of the test chemical to the corresponding GHS category (i.e. LD_{50} > 2 000 mg/kg). In addition, three test chemicals were not tested at JRC (see Section 6.2 of the final study report and Table E-18).

When the millimole regression was used (Table E-3), the total accuracy of GHS acute oral toxicity category prediction was 33% (15/46). The rate for acute oral toxicity under-prediction was 11% (5/46) and for over-prediction was 57% (26/46).

For this analysis, the predictions of each GHS classification category were as follows:

- the one test chemical with $LD_{50} \leq 5$ mg/kg (GHS category 1) was not predicted (0%);
- the two test chemicals in the $5 < LD_{50} \leq 50$ mg/kg category (GHS category 2) were not predicted (0%);
- three (60%) of the five test chemicals in the $50 < LD_{50} \leq 300$ mg/kg category (GHS category 3);
- ten (71%) of 14 test chemicals in the $300 < LD_{50} \leq 2\,000$ mg/kg category (GHS category 4);
- two (25%) of 8 test chemicals in the $2\,000 < LD_{50} \leq 5\,000$ mg/kg category (GHS category 5);
- none (0%) of the 16 test chemicals with $LD_{50} > 5\,000$ mg/kg (GHS unclassified).

Table E-4 shows the results of concordance, over-prediction and under-prediction of three toxicity categories ($LD_{50} \leq 2\,000$ mg/kg, $2\,000 < LD_{50} \leq 5\,000$ mg/kg GHS category 5, $LD_{50} > 5\,000$ mg/kg GHS unclassified) in JRC. None of the test chemicals was under-predicted. Over-prediction was observed in GHS category 5 ($2\,000 < LD_{50} \leq 5\,000$ mg/kg) for 75% (6/8) of the test chemicals and for 100% of the test chemicals in GHS unclassified ($LD_{50} > 5\,000$ mg/kg). The overall accuracy for correctly predicting the three toxicity categories using the millimole regression was 52% (24/46).

Table E-3. Prediction of GHS Acute Oral Toxicity Categories in JRC by the 3T3 NRU test method and the millimole regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)						Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 5$	0	0	0	1	0	0	1	0%	0%	100%
$5 < LD_{50} \leq 50$	0	0	1	1	0	0	2	0%	0%	100%
$50 < LD_{50} \leq 300$	0	0	3	2	0	0	5	60%	0%	40%
$300 < LD_{50} \leq 2\,000$	0	0	4	10	0	0	14	71%	29%	0%
$2\,000 < LD_{50} \leq 5\,000$	0	1	0	5	2	0	8	25%	75%	0%
$LD_{50} > 5\,000$	0	0	0	9	7	0	16	0%	100%	0%
Total	0	1	8	28	9	0	46	33%	57%	11%
Category Over-predicted	0%	100%	50%	50%	78%	0%				
Category Under-predicted	0%	0%	13%	14%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded cells indicate correct predictions.

Table E-4. Prediction of three acute oral toxicity categories in JRC by the 3T3 NRU test method and the millimole regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)			Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq$ 2 000	2 000 < $LD_{50} \leq$ 5 000	LD_{50} > 5 000				
$LD_{50} \leq$ 2 000	22	0	0	22	100%	0%	0%
2 000 < $LD_{50} \leq$ 5 000	6	2	0	8	25%	75%	0%
LD_{50} > 5 000	9	7	0	16	0%	100%	0%
Total	37	9	0	46	52%	48%	0%
Category Over-predicted	41%	78%	0%				
Category Under-predicted	0%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded cells indicate correct predictions.

E.1.1.3 IIVS

When the prediction of the GHS toxicity categories was assessed in IIVS, only 37 test chemicals were included in the analysis since nine test chemicals had right censored IC_{50} values and 10 had left censored IC_{50} values that could not be assigned to one GHS toxicity category (see Table E-18). The reason why IIVS obtained a higher number of IC_{50} censored values compared to the other two laboratories is due to the protocol used, designed around the 2 000 mg/kg b.w. cut-off value according to the original aim of the validation study (see Sections 1.4 and 5.6 of the final study report). The two test chemicals in GHS category 2 were excluded from the analysis due to censored IC_{50} values.

When the millimole regression was used (Table E-5), the overall accuracy for correctly predicting GHS acute oral toxicity classification category was 35% (13/37); oral toxicity was under-predicted for 5% (2/37) of the test chemicals, and over-predicted for 59% (22/37) of the test chemicals.

For this analysis, the predictions of the remaining GHS classification category were as follows:

- the one test chemical with $LD_{50} \leq 5$ mg/kg category (GHS category 1) was not predicted (0%);
- two (67%) of the three test chemicals in the $50 < LD_{50} \leq 300$ mg/kg category (GHS category 3);
- ten (91%) of 11 test chemicals in the $300 < LD_{50} \leq 2\,000$ mg/kg category (GHS category 4);
- one (14%) of seven test chemicals in the $2\,000 < LD_{50} \leq 5\,000$ mg/kg category (GHS category 5);
- none (0%) of the 15 test chemicals with $LD_{50} > 5\,000$ mg/kg (GHS unclassified).

Table E-6 shows the results of concordance, over-prediction and under-prediction of three toxicity categories ($LD_{50} \leq 2\,000$ mg/kg, $2\,000 < LD_{50} \leq 5\,000$ mg/kg GHS category 5, $LD_{50} > 5\,000$ mg/kg GHS unclassified) in IIVS. Only 9 test chemicals were excluded from the analysis since the right censored IC_{50} values resulted in estimated LD_{50} values that could not be assigned to any of the three toxicity

categories. All left censored IC_{50} values were included in the analysis. As for HSL and JRC, no under-predictions of toxicity were obtained. Oral toxicity was over-predicted for 88% (7/8) and 100% (16/16) of the test chemicals in GHS category 5 (2 000 < LD_{50} ≤ 5 000) and GHS unclassified (LD_{50} > 5 000), respectively. The overall accuracy for correctly predicting the three acute oral toxicity categories using the millimole regression was 51% (24/47).

Table E-5. Prediction of GHS Acute Oral Toxicity Categories in IIVS by the 3T3 NRU test method and the millimole regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)						Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 5$	0	0	0	1	0	0	1	0%	0%	100%
$5 < LD_{50} \leq 50$	0	0	0	0	0	0	0	0%	0%	0%
$50 < LD_{50} \leq 300$	0	0	2	1	0	0	3	67%	0%	33%
$300 < LD_{50} \leq 2\,000$	0	0	1	10	0	0	11	91%	9%	0%
$2\,000 < LD_{50} \leq 5\,000$	0	0	0	6	1	0	7	14%	86%	0%
$LD_{50} > 5\,000$	0	0	0	10	5	0	15	0%	100%	0%
Total	0	0	3	28	6	0	37	35%	59%	5%
Category Over-predicted	0%	0%	33%	57%	83%	0%				
Category Under-predicted	0%	0%	0%	7%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded cells indicate correct predictions.

Table E-6. Prediction of three acute oral toxicity categories in IIVS by the 3T3 NRU test method and the millimole regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)			Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq$ 2 000	2 000 < $LD_{50} \leq$ 5 000	LD_{50} > 5 000				
$LD_{50} \leq$ 2 000	23	0	0	23	100%	0%	0%
2 000 < $LD_{50} \leq$ 5 000	7	1	0	8	12.5%	87.5%	0%
LD_{50} > 5 000	11	5	0	16	0%	100%	0%
Total	41	6	0	47	51%	49%	0%
Category Over-predicted	44%	83%	0%				
Category Under-predicted	0%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded indicate correct predictions.

E.1.2 Weight regression

E.1.2.1 HSL

When the prediction of the GHS acute oral toxicity categories was assessed in HSL, results from only 40 test chemicals were included in the analysis (Section E.1.1.1 and Table E-18). With the weight regression model (Table E-7) the overall accuracy was 38% (15/40); toxicity was over-predicted for 60% (24/40) of the test chemicals and under-predicted for 2.5% (1/40) of the test chemicals. The test chemicals belonging to the GHS categories 1 ($LD_{50} \leq 5$) and 2 ($5 < LD_{50} \leq 50$) were not tested in HSL.

For the remaining GHS acute oral toxicity categories the predictions were the following:

- three (75%) of the four test chemicals in the $50 < LD_{50} \leq 300$ mg/kg category (GHS category 3);
- ten (77%) of 13 test chemicals in the $300 < LD_{50} \leq 2\,000$ mg/kg category (GHS category 4);
- one (12.5%) of eight test chemicals in the $2\,000 < LD_{50} \leq 5\,000$ mg/kg category (GHS category 5);
- one (7%) of the 15 test chemicals with $LD_{50} > 5\,000$ mg/kg (GHS unclassified).

Table E-8 shows the results of concordance, over-prediction and under-prediction of three toxicity categories ($LD_{50} \leq 2\,000$ mg/kg, $2\,000 < LD_{50} \leq 5\,000$ mg/kg GHS category 5, $LD_{50} > 5\,000$ mg/kg GHS unclassified) in HSL. None were under-predicted. The toxicity was over-predicted for 87.5% (7/8) and for 93% (14/15) of the test chemicals in GHS category 5 ($2\,000 < LD_{50} \leq 5\,000$ mg/kg) and GHS unclassified ($LD_{50} > 5\,000$ mg/kg), respectively. The overall accuracy for correctly predicting the three toxicity categories using the weight regression was 48% (19/40).

Table E-7. Prediction of GHS Acute Oral Toxicity Categories in HSL by the 3T3 NRU test method and the weight regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)						Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 5$	0	0	0	0	0	0	0	0%	0%	0%
$5 < LD_{50} \leq 50$	0	0	0	0	0	0	0	0%	0%	0%
$50 < LD_{50} \leq 300$	0	0	3	1	0	0	4	75%	0%	25%
$300 < LD_{50} \leq 2\,000$	0	0	3	10	0	0	13	77%	23%	0%
$2\,000 < LD_{50} \leq 5\,000$	0	1	0	6	1	0	8	12.5%	87.5%	0%
$LD_{50} > 5\,000$	0	0	1	11	2	1	15	7%	93%	0%
Total	0	1	7	28	3	1	40	37.5%	60%	2.5%
Category Over-predicted	0%	100%	57%	61%	67%	0%				
Category Under-predicted	0%	0%	0%	4%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded cells indicate correct predictions.

Table E-8. Prediction of three acute oral toxicity categories in HSL by the 3T3 NRU test method and the weight regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)			Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 2\,000$	17	0	0	17	100%	0%	0%
$2\,000 < LD_{50} \leq 5\,000$	7	1	0	8	12.5%	87.5%	0%
$LD_{50} > 5\,000$	12	2	1	15	7%	93%	0%
Total	36	3	1	40	47.5%	52.5%	0%
Category Over-predicted	53%	67%	0%				
Category Under-predicted	0%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded cells indicate correct predictions.

E.1.2.2 JRC

When the prediction of the GHS toxicity categories was assessed in JRC, only results from 46 test chemicals were included in the analysis (Section E.1.1.2 and Table E-18). With the weight regression (Table E-9) the overall accuracy for correctly predicting GHS acute oral toxicity classification category was 30% (14/46); oral toxicity was over-predicted for 57% (26/46) of the test chemicals, and under-predicted for 13% (6/46) of the test chemicals.

For this analysis, the predictions of each GHS classification category were as follows:

- the one test chemical with $LD_{50} \leq 5$ mg/kg category (GHS category 1) was not predicted (0%);
- the two test chemicals in the $5 < LD_{50} \leq 50$ mg/kg category (GHS category 2) were not predicted (0%);
- two (40%) of the five test chemicals in the $50 < LD_{50} \leq 300$ mg/kg category (GHS category 3);
- ten (71%) of 14 test chemicals in the $300 < LD_{50} \leq 2\,000$ mg/kg category (GHS category 4);
- two (25%) of 8 test chemicals in the $2\,000 < LD_{50} \leq 5\,000$ mg/kg category (GHS category 5);
- none (0%) of the 16 test chemicals with $LD_{50} > 5\,000$ mg/kg (GHS unclassified).

Table E-10 shows the results of concordance, over-prediction and under-prediction of three toxicity categories ($LD_{50} \leq 2\,000$ mg/kg, $2\,000 < LD_{50} \leq 5\,000$ mg/kg GHS category 5, $LD_{50} > 5\,000$ mg/kg GHS unclassified) in JRC. None of the test chemicals was under-predicted. The toxicity was over-predicted for 75% (6/8) and 100% (16/16) of the test chemicals in the GHS category 5 ($2\,000 < LD_{50} \leq 5\,000$) and GHS unclassified ($LD_{50} > 5\,000$), respectively. The overall accuracy for correctly predicting the three toxicity categories using the weight regression was 52% (24/46).

Table E-9. Prediction of GHS Acute Oral Toxicity Categories in JRC by the 3T3 NRU test method and the weight regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)						Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 5$	0	0	0	1	0	0	1	0%	0%	100%
$5 < LD_{50} \leq 50$	0	0	0	2	0	0	2	0%	0%	100%
$50 < LD_{50} \leq 300$	0	0	2	3	0	0	5	40%	0%	60%
$300 < LD_{50} \leq 2\,000$	0	0	4	10	0	0	14	71%	29%	0%
$2\,000 < LD_{50} \leq 5\,000$	0	0	1	5	2	0	8	25%	75%	0%
$LD_{50} > 5\,000$	0	0	1	13	2	0	16	0%	100%	0%
Total	0	0	8	34	4	0	46	30%	57%	13%
Category Over-predicted	0	0	75%	53%	50%	0				
Category Under-predicted	0	0	0%	18%	0%	0				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded cells indicate correct predictions.

Table E-10. Prediction of three acute oral toxicity categories in JRC by the 3T3 NRU test method and the weight regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)			Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 2\,000$	22	0	0	22	100%	0%	0%
$2\,000 < LD_{50} \leq 5\,000$	6	2	0	8	25%	75%	0%
$LD_{50} > 5\,000$	14	2	0	16	0%	100%	0%
Total	42	4	0	46	52%	48%	0%
Category Over-predicted	48%	50%	0%				
Category Under-predicted	0%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded indicate correct predictions.

E.1.2.3 IIVS

When the prediction of the GHS toxicity categories was assessed in IIVS, only 37 test chemicals were included in the analysis (Section E.1.1.3 and Table E-18). With the weight regression (Table E-11), the overall accuracy for correctly predicting GHS acute oral toxicity classification category was 30% (11/37); oral toxicity was over-predicted for 59% (22/37) of the test chemicals, and under-predicted for 11% (4/37) of the test chemicals.

For this analysis, the predictions of the remaining GHS classification category were as follows:

- the one test chemical with $LD_{50} \leq 5$ mg/kg category (GHS category 1) was not predicted (0%);
- none (0%) of the three test chemicals in the $50 < LD_{50} \leq 300$ mg/kg category (GHS category 3);
- ten (91%) of 11 test chemicals in the $300 < LD_{50} \leq 2\,000$ mg/kg category (GHS category 4);
- one (14%) of seven test chemicals in the $2\,000 < LD_{50} \leq 5\,000$ mg/kg category (GHS category 5);
- none (0%) of the 15 test chemicals with $LD_{50} > 5\,000$ mg/kg (GHS unclassified).

Table E-12 shows the results of concordance, over-prediction and under-prediction of three toxicity categories ($LD_{50} \leq 2\,000$ mg/kg, $2\,000 < LD_{50} \leq 5\,000$ mg/kg GHS category 5, $LD_{50} > 5\,000$ mg/kg GHS unclassified) in IIVS. The 10 test chemicals with left censored IC_{50} values were included in this analysis as the estimated LD_{50} values were < 2 000 mg/kg. As for HSL and JRC, no under-predictions of toxicity were obtained. The toxicity was over-predicted for 88% of the test chemicals in GHS category 5 ($2\,000 < LD_{50} \leq 5\,000$) (7/8) and for 100% of the chemicals in GHS unclassified ($LD_{50} > 5\,000$) (16/16). The overall accuracy for correctly predicting the three toxicity categories using the weight regression was 51% (24/47).

Table E-11. Prediction of GHS Acute Oral Toxicity Categories in IIVS by the 3T3 NRU test method and the weight regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)						Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq 5$	$5 < LD_{50} \leq 50$	$50 < LD_{50} \leq 300$	$300 < LD_{50} \leq 2\,000$	$2\,000 < LD_{50} \leq 5\,000$	$LD_{50} > 5\,000$				
$LD_{50} \leq 5$	0	0	0	1	0	0	1	0%	0%	100%
$5 < LD_{50} \leq 50$	0	0	0	0	0	0	0	0%	0%	0%
$50 < LD_{50} \leq 300$	0	0	0	3	0	0	3	0%	0%	100%
$300 < LD_{50} \leq 2\,000$	0	0	1	10	0	0	11	91%	9%	0%
$2\,000 < LD_{50} \leq 5\,000$	0	0	0	6	1	0	7	14%	86%	0%
$LD_{50} > 5\,000$	0	0	1	12	2	0	15	0%	100%	0%
Total	0	0	2	32	3	0	37	30%	59%	11%
Category Over-predicted	0%	0%	100%	56%	67%	0%				
Category Under-predicted	0%	0%	0%	12.5%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded indicate correct predictions.

Table E-12. Prediction of three acute oral toxicity categories in IIVS by the 3T3 NRU test method and the weight regression

Reference Rodent Oral LD_{50} (mg/kg)[1]	IC_{50}-Predicted GHS Category (mg/kg)			Total	Accuracy	Toxicity Over-predicted	Toxicity Under-predicted
	$LD_{50} \leq$ 2 000	2 000 < $LD_{50} \leq$ 5 000	LD_{50} > 5 000				
$LD_{50} \leq$ 2 000	23	0	0	23	100%	0%	0%
2 000 < $LD_{50} \leq$ 5 000	7	1	0	8	12.5%	87.5%	0%
LD_{50} > 5 000	14	2	0	16	0%	100%	0%
Total	44	3	0	47	51%	49%	0%
Category Over-predicted	48%	67%	0%				
Category Under-predicted	0%	0%	0%				

[1] = Reference rat oral LD_{50} values from Table 4 of the final study report. Shaded indicate correct predictions.

E.2 RECEIVER OPERATING CHARACTERISTIC (ROC) CURVE ANALYSIS USING THE LD_{50} CUT-OFF VALUE OF 5 000 MG/KG B.W.

The ROC analysis was performed to identify the optimum IC_{50} to distinguish between classified and unclassified chemicals according to the GHS classification system and, therefore, applying the LD_{50} cut-off value of 5 000 mg/kg b.w. The curve and coordinates applied to this LD_{50} cut-off value are shown in Figure E-1 and Table E-13, respectively.

As described in Section 10.3 of the final study report, a training set was used to generate the prediction model to be used with the data set generated in this validation study.

An IC_{50} cut-off value of 1 154 μg/ml for the toxicity predictions (classified if the IC_{50} ≤ 1 154 μg/ml and unclassified if the IC_{50} > 1 154 μg/ml) was chosen due to its high sensitivity. Using this IC_{50} value, the predictions of the two categories, classified and unclassified, in the three laboratories are shown in Tables E-14 to E-16.

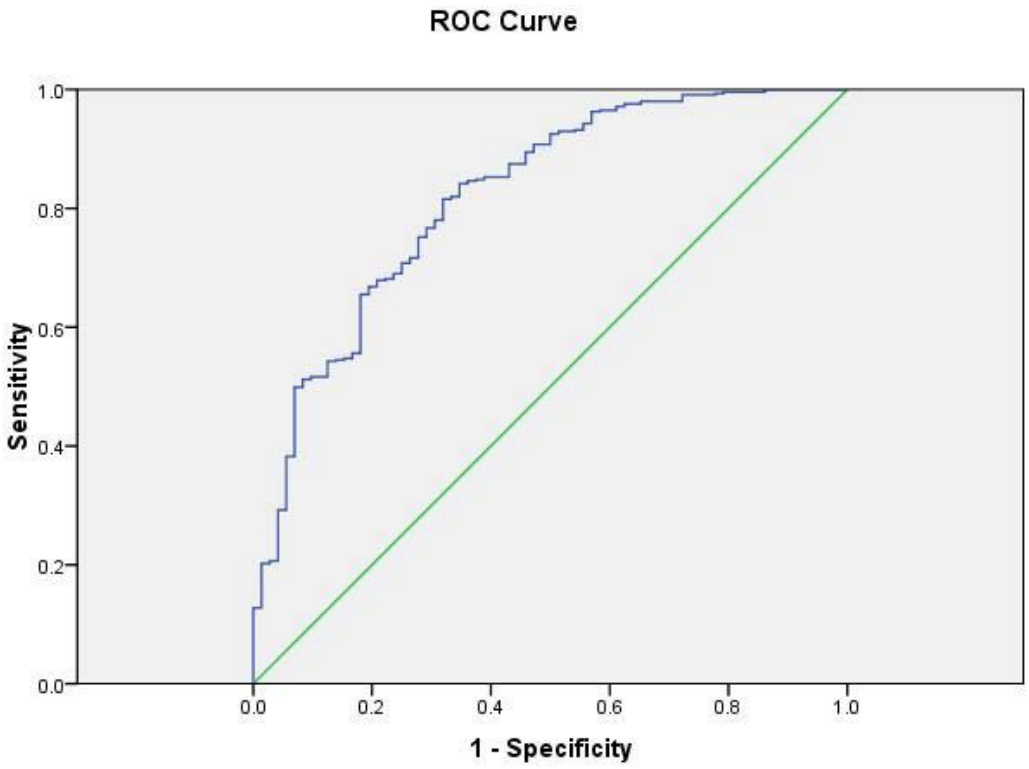

Figure E-1. ROC Plot of the 3T3 NRU test method to discriminate between classified ($LD_{50} \leq 5\,000$ mg/kg b.w.) and unclassified ($LD_{50} > 5\,000$ mg/kg b.w.) test chemicals based on the RC data set.

Table E-13. Criterion values and Coordinates of the ROC Curve			
Positive if Less Than or Equal To[1]	Sensitivity	1 - Specificity	Specificity
98.186605	.552	.167	.833
98.990643	.554	.167	.833
99.219745	.556	.167	.833
99.410858	.556	.181	.819
99.750755	.558	.181	.819
100.268710	.560	.181	.819
100.622400	.563	.181	.819
100.801818	.565	.181	.819
101.111231	.567	.181	.819
103.573031	.569	.181	.819
106.384914	.571	.181	.819
107.435457	.574	.181	.819
108.008878	.576	.181	.819
109.018340	.578	.181	.819
110.832022	.580	.181	.819
111.910629	.582	.181	.819
113.289963	.585	.181	.819
115.328611	.587	.181	.819
116.345391	.589	.181	.819
116.909193	.591	.181	.819
118.356568	.593	.181	.819
119.568800	.596	.181	.819
120.614892	.598	.181	.819
122.070331	.600	.181	.819
124.489706	.602	.181	.819
127.224080	.604	.181	.819
128.117714	.607	.181	.819
128.453640	.609	.181	.819
131.104834	.611	.181	.819
136.684293	.613	.181	.819
141.181661	.615	.181	.819
144.533634	.618	.181	.819
146.588332	.620	.181	.819
147.061477	.622	.181	.819
148.155517	.624	.181	.819
148.978988	.626	.181	.819
150.156619	.629	.181	.819
158.683509	.631	.181	.819
167.491700	.633	.181	.819
169.981089	.635	.181	.819
173.845655	.637	.181	.819
178.223091	.640	.181	.819
180.816506	.642	.181	.819
182.695181	.644	.181	.819
184.582195	.646	.181	.819

Table E-13. Criterion values and Coordinates of the ROC Curve			
Positive if Less Than or Equal To[1]	Sensitivity	1 - Specificity	Specificity
186.363066	.648	.181	.819
188.021450	.651	.181	.819
190.293261	.653	.181	.819
[a]193.352849	**.655**	**.181**	**.819**
195.261577	.655	.194	.806
196.966910	.657	.194	.806
199.562938	.659	.194	.806
203.462623	.662	.194	.806
206.644170	.664	.194	.806
208.500268	.666	.194	.806
210.288959	.668	.194	.806
211.201703	.668	.208	.792
213.034410	.670	.208	.792
215.320020	.673	.208	.792
216.473059	.675	.208	.792
218.119310	.677	.208	.792
223.493456	.679	.208	.792
227.912054	.679	.222	.778
235.188756	.681	.222	.778
243.552842	.681	.236	.764
247.540458	.684	.236	.764
252.825733	.686	.236	.764
257.160928	.688	.236	.764
263.592599	.690	.236	.764
270.127478	.690	.250	.750
277.603899	.692	.250	.750
284.243150	.695	.250	.750
285.980103	.697	.250	.750
288.665175	.699	.250	.750
290.776433	.701	.250	.750
293.113464	.703	.250	.750
296.093679	.705	.250	.750
297.161871	.708	.250	.750
304.996499	.708	.264	.736
313.188970	.710	.264	.736
316.142588	.712	.264	.736
322.578254	.714	.264	.736
327.798113	.716	.264	.736
329.636132	.716	.278	.722
332.832773	.719	.278	.722
[b]336.977883	**.721**	**.278**	**.722**
340.979435	.723	.278	.722
343.596754	.725	.278	.722
345.884229	.727	.278	.722
352.844348	.730	.278	.722

Table E-13. Criterion values and Coordinates of the ROC Curve			
Positive if Less Than or Equal To[1]	Sensitivity	1 - Specificity	Specificity
357.950639	.732	.278	.722
359.320420	.734	.278	.722
372.964042	.736	.278	.722
391.913697	.738	.278	.722
398.731140	.741	.278	.722
400.973338	.743	.278	.722
408.406123	.745	.278	.722
415.331188	.747	.278	.722
417.844940	.749	.278	.722
420.326537	.752	.278	.722
426.233025	.752	.292	.708
434.441088	.754	.292	.708
445.471160	.756	.292	.708
457.375856	.758	.292	.708
463.856968	.760	.292	.708
466.626176	.763	.292	.708
475.067164	.765	.292	.708
483.698990	.767	.292	.708
485.625682	.767	.306	.694
488.112730	.769	.306	.694
493.191475	.771	.306	.694
504.857536	.774	.306	.694
525.491029	.776	.306	.694
540.401262	.778	.306	.694
545.167869	.780	.306	.694
552.853458	.780	.319	.681
559.101397	.782	.319	.681
582.795651	.785	.319	.681
615.217473	.787	.319	.681
626.671774	.789	.319	.681
639.750189	.791	.319	.681
662.032668	.793	.319	.681
681.416834	.796	.319	.681
691.394922	.798	.319	.681
696.050975	.800	.319	.681
702.936998	.802	.319	.681
716.669735	.804	.319	.681
728.133672	.807	.319	.681
737.119570	.809	.319	.681
757.845550	.811	.319	.681
772.961109	.813	.319	.681
775.496482	.815	.319	.681
776.332524	.815	.333	.667
779.026786	.818	.333	.667
788.321423	.820	.333	.667

Table E-13. Criterion values and Coordinates of the ROC Curve			
Positive if Less Than or Equal To[1]	Sensitivity	1 - Specificity	Specificity
796.495383	.820	.347	.653
814.744484	.822	.347	.653
839.1091040	.824	.347	.653
850.493326	.826	.347	.653
862.152726	.829	.347	.653
875.380186	.831	.347	.653
883.463490	.833	.347	.653
901.077550	.835	.347	.653
919.143668	.837	.347	.653
925.858425	.840	.347	.653
931.044063	.842	.347	.653
948.435747	.842	.361	.639
969.438496	.844	.361	.639
981.180090	.846	.361	.639
988.580119	.846	.375	.625
1036.902943	.848	.375	.625
1101.397757	.848	.389	.611
1129.478105	.851	.389	.611
[c]1153.990500	**.853**	**.389**	**.611**
1171.658122	.853	.403	.597
1201.427547	.853	.417	.583
1234.203881	.853	.431	.569
1244.011341	.855	.431	.569
1251.069657	.857	.431	.569
1256.774477	.859	.431	.569
1267.962492	.862	.431	.569
1293.200967	.864	.431	.569
1309.406462	.866	.431	.569
1317.527255	.868	.431	.569
1331.755001	.870	.431	.569
1354.770386	.873	.431	.569
1370.745919	.875	.431	.569
1376.344103	.875	.444	.556

1 = Test result variable(s): IC_{50} (µg/ml). Numbers in bold indicated the three selected thresholds: a = maximises specificity, b = best balance between sensitivity and specificity, c = maximises sensitivity

E.2.1 HSL

Six test chemicals were not tested in HSL (see Section 6.2 of the final study report and Table E-18) and six test chemicals were excluded from the analysis since the IC_{50} right censored values were smaller than the IC_{50} cut-off value chosen (1 154 µg/ml).

Table E-14. Prediction of acute oral toxicity (classified/unclassified) by applying an IC_{50} cut-off value of 1 154 µg/ml in HSL

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified	Unclassified	Total
Toxicity according to the IC_{50} 1 154 µg/ml cut-off value	Classified	24	9	33
	Unclassified	5	6	11
	Total	29	15	44

* = Reference oral LD_{50} value in mg/kg b.w. from Table 4 of the final study report

Sensitivity: 24/29*100 = 82.8%
Specificity: 6/15*100 = 40%
PPV: 24/33*100 = 73.7%
NPV: 6/11*100 = 54.5%
Accuracy: (24+6)/44*100 = 68.2%
FP: 9
FN: 5

The toxicity was under-predicted for 1,2-Dichlorobenzene, 2,6-Diethylaniline, Benzaldehyde, Benzyl benzoate and Glycerol triacetate.

E.2.2 JRC

Three test chemicals were not tested in JRC and another three test chemicals were excluded from the analysis since the IC_{50} right censored values were smaller than the IC_{50} cut-off value chosen (1 154 µg/ml) (Section 6.2 of the final study report and Table E-18).

Table E-15. Prediction of acute oral toxicity (classified/unclassified) by applying an IC_{50} cut-off value of 1 154 µg/ml in JRC

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified	Unclassified	Total
Toxicity according to the IC_{50} 1 154 µg/ml cut-off value	Classified	25	10	35
	Unclassified	8	7	15
	Total	33	17	50

* = Reference oral LD_{50} value in mg/kg b.w. from Table 4 of the final study report

Sensitivity: 25/33*100 = 75.8%
Specificity: 7/17*100 = 41.2%
PPV: 25/35*100 = 71.4%
NPV: 7/15*100 = 46.7%
Accuracy: (25+7)/50*100 = 64%
FP: 10
FN: 8

The toxicity was under-predicted for 1,2,4-Trichlorobenzene, 2,6-Diethylaniline, 2-Butoxyethyl acetate, Acetophenone, Barium chloride, Benzyl benzoate, Ethyl acetoacetate and Glycerol triacetate.

E.2.3 IIVS

Three test chemicals were chemicals were excluded from the analysis since the IC_{50} right censored values were smaller than the IC_{50} cut-off value chosen (1 154 µg/ml) (Section 6.2 of the final study report and Table E-18).

Table E-16. Prediction of acute oral toxicity (classified/unclassified) by applying an IC_{50} cut-off value of 1 154 µg/ml in IIVS

		Reference *in vivo* oral LD_{50} (mg/kg)*		
		Classified	Unclassified	Total
Toxicity according to the IC_{50} 1 154 µg/ml cut-off value	Classified	30	11	41
	Unclassified	4	8	12
	Total	34	19	53

* = Reference oral LD_{50} value in mg/kg b.w. from Table 4 of the final study report

Sensitivity: 30/34*100 = 88.2%
Specificity: 8/19*100 = 42.1%
PPV: 30/41*100 = 73.2%
NPV: 8/12*100 = 66.7%
Accuracy: (30+8)/53*100 = 71.7%
FP: 11
FN: 4

The toxicity was under-predicted for 1,2-Benzenedicarboxylic acid, 1,2-Dichlorobenzene, 2-Butoxyethyl acetate and Glycerol triacetate.

Table E-17 shows the distribution of false negatives (under-predicted toxicity) and false positives (over-predicted toxicity) found in each laboratory when the LD_{50} cut-off value of 5 000 mg/kg was used for unclassified chemicals.

Table E-17. Over and under-prediction of toxicity generated in each laboratory by applying an IC_{50} value of 1 154 µg/ml to discriminate between classified and unclassified chemicals (LD_{50} > 5 000 mg/kg b.w.)

Chem nr	Chemical	Over-predicted			Under-predicted		
		HSL	JRC	IIVS	HSL	JRC	IIVS
2.	1,2,4-Trichlorobenzene					X	
3.	1,2-Benzenedicarboxylic acid						X
4.	1,2-Dichlorobenzene				X		X
8.	2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol	X	X	X			
10.	2,6-Diethylaniline				X	X	
11.	2-Butoxyethyl acetate[a]					X	X
12.	2-Chloro-4-nitroaniline	X	X	X			
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone[a]	X	X				
16.	Acetophenone[a]					X	
19.	Barium chloride					X	
20.	Benzaldehyde[a]				X		
21.	Benzyl benzoate[a]				X	X	
23.	Caprylic acid[a]	X	X	X			
28.	Diisopropanolamine	X	X	X			
29.	Dimethyldioctadecylammoium chloride[a]	X	X	X			
32.	Ethyl acetoacetate[a]					X	
34.	Glycerol triacetate[a]				X	X	X
37.	Methenamine[a]	X	X	X			
46.	Sorbitan monolaurate[a]		X	X			
49.	Triethylene glycol dimethacrylate[a]	X	X	X			
52.	Trizinc bis(orthophosphate)			X			
53.	Tween 20[a]	X	X	X			
56.	Zinc oxide[a]			X			

[a] use as cosmetic ingredient according to the EC CosIng database (see Tables 2 and 3 of the final study report)

Of the falsely negative predicted test chemicals, Benzyl benzoate and 1,2,4-Trichlorobenzene were found by JRC to react with the plastic of the tubes and all three laboratories reported volatility for Acetophenone. Benzaldehyde is sensitive to air, light and moisture and, therefore, required storage under nitrogen. 1,2,-

Dichlorobenzene was found to form precipitates in IIVS. 2,6-Diethylaniline needed to be handled and stored under nitrogen. Furthermore, it was found to be volatile by both HSL and IIVS. Glycerol triacetate is a viscous test chemical and problems could have been encountered during preparation of solutions. JRC and HSL reported volatility for 2-Butoxyethyl acetate and HSL also reported precipitates. In addition, this chemical requires minimal exposure to air. Barium chloride is hygroscopic and both JRC and IIVS have reported precipitates. Ethyl acetoacetate was found to be volatile in all three laboratories. 1,2-Benzenecarboxylic acid has a high log Kow value (9,37) indicating its possible unsuitability for *in vitro* assays.

Previously Strickland et al., using the Registry of Cytotoxicity data set, derived the optimum IC_{50} values to determine the rates for correct hazard prediction and under- and over-prediction of hazard (Poster presentation: J Strickland, M Paris, D Allen, R Tice, W Stokes. Using In Vitro Cytotoxicity Data to Determine When Rat Acute Oral Toxicity Testing Should Start with the Limit Test). These values were considered by the authors as the optimum IC_{50} values (0% over-prediction) to predict that chemicals should be tested at the limit dose in acute oral systemic toxicity tests because animal deaths are not expected at this level. The IC_{50} values established were:

- IC_{50} = 6 569 µg/ml to predict the limit test of 2 000 mg/kg b.w.
- IC_{50} = 19 133 µg/ml to predict the limit test of 5 000 mg/kg b.w.

When these IC_{50} cut-off values were applied to the data set obtained in this validation study, three out of 25 chemicals were correctly predicted as unclassified (LD_{50} > 2 000 mg/kg b.w.): Di-"isodecyl" phthalate (HSL), Potassium sulphate (HSL, JRC), and Urea (HSL, JRC, IIVS), with the IC_{50} = 6 569 µg/ml cut-off value. Applying the IC_{50} = 19 133 µg/ml cut-off value, the correct prediction of unclassified chemicals (LD_{50} > 5 000 mg/kg b.w.) was obtained only in HSL for two chemicals (Di-"isodecyl" phthalate and Urea).

Table E-18. Test chemicals excluded from regression and ROC analysis

Chem nr	Chemical	Millimole and weight regression analyses			ROC analysis		
		HSL	JRC	IIVS	HSL	JRC	IIVS
1.	(4-Ammonio-*m*-tolyl)ethyl(2-hydroxyethyl)ammonium sulphate			X[c]			
2.	1,2,4-Trichlorobenzene		X				
3.	1,2-Benzenedicarboxylic acid	X	X	X	X	X	
4.	1,2-Dichlorobenzene	X	X	X		X	
5.	1-Naphthylamine			X[c]			
9.	2,4,6-Tris(dimethylaminomethyl)phenol	X			X		
10.	2,6-Diethylaniline	X	X				
11.	2-Butoxyethyl acetate[a]			X			
13.	2-Ethylhexyl acrylate	X	X	X	X		
15.	4'-Tert-butyl-2',6'-dimethyl-3',5'-dinitroacetophenone[a]			X			X
17.	Aconitine	X[b]		X	X[b]		X
19.	Barium chloride	X			X		
20.	Benzaldehyde[a]	X					
21.	Benzyl benzoate[a]	X	X				
22.	Brucine	X[b]			X[b]		
24.	Copper sulphate[a]			X[c]			
27.	Di-"isodecyl" phthalate			X			
29.	Dimethyldioctadecylammonium chloride[a]						
36.	Malononitrile	X[b]		X[c]	X[b]		
38.	*N*-isopropyl-*N*'-phenyl-*p*-phenylenediamine			X[c]			
39.	Octyl 3,4,5-trihydroxybenzoate			X[c]			
40.	*P*-benzoquinone			X[c]			
41.	Phthalic anhydride			X			X
43.	Resorcinol[a]			X[c]			
46.	Sorbitan monolaurate[a]	X			X		
47.	Tetramethylthiuram monosulphide			X[c]			
51.	Tris(nonylphenyl) phosphite[a]	X	X	X	X	X	
52.	Trizinc bis(orthophosphate)	X[b]	X[b]		X[b]	X[b]	
55.	Zinc distreate[a]	X[b]	X[b]		X[b]	X[b]	
56.	Zinc oxide[a]	X[b]	X[b]	X[c]	X[b]	X[b]	

Millimole and weight regression analyses were used to evaluate concordance, under- and over-prediction of the GHS acute oral toxicity categories.

ROC (Receiver Operating Characteristic) analysis was used to identify the optimum IC_{50} cut-off value for distinguishing between classified and unclassified chemicals applying the LD_{50} cut-off value of 5 000 mg/kg b.w.

[a] use as cosmetic ingredient according to the EC CosIng database (see Tables 2 and 3 of the final study report)

[b] not tested

[c] left censored (included in the analyses performed for three acute oral toxicity categories ($LD_{50} \leq 2\,000$ mg/kg, 2 000 $< LD_{50} \leq 5\,000$ mg/kg GHS category 5, $LD_{50} > 5\,000$ mg/kg GHS unclassified)

E.3 SUMMARY

In this Annex we described the predictive capacity of the 3T3 NRU test method to identify unclassified test chemicals using as cut-off value an LD_{50} value of 5 000 mg/kg b.w., according to the GHS classification system. For this purpose, regression analyses (both millimole and weight) and ROC analysis were used.

In addition, we also showed the performance of the 3T3 NRU test method to predict the GHS acute oral toxicity categories. The distribution of test chemicals among the different GHS toxicity categories was unbalanced in comparison to the NICEATM/ECVAM validation study. This was due to the fact that the test chemicals were selected taking into account the original aim of this follow up validation study [i.e. to assess the predictive capacity of the 3T3 NRU cytotoxicity test to determine if the test chemical correctly falls into one of the two categories, unclassified (LD_{50} > 2 000 mg/kg b.w.), or classified ($LD_{50} \leq$ 2 000 mg/kg b.w.) according to the EU CLP classification system].

The GHS category predictions showed that, similarly to the NICEATM/ECVAM validation study, the 3T3 NRU test method predicted better the GHS acute oral toxicity categories 3 and 4, both with millimole and weight regression analysis (Tables E-1, E-3, E-5, E-7, E-9 and E-11).

With the millimole regression, for the three acute oral toxicity categories ($LD_{50} \leq$ 2 000 mg/kg, 2 000 < $LD_{50} \leq$ 5 000 mg/kg and LD_{50} > 5 000 mg/kg), there were no under-predictions in any of the laboratories. Only one chemical (Di-"isodecyl" phthalate) was correctly predicted as GHS unclassified (LD_{50} > 5 000 mg/kg b.w.) in HSL, whereas in JRC and IIVS none of the unclassified chemicals were correctly predicted. GHS category 5 (2 000 < $LD_{50} \leq$ 5 000 mg/kg) was over-predicted in all three laboratories (87.5% in HSL, 75% in JRC, and 87.5% in IIVS).

With the weight regression, for the three acute oral toxicity categories ($LD_{50} \leq$ 2 000 mg/kg, 2 000 < $LD_{50} \leq$ 5 000 mg/kg and LD_{50} > 5 000 mg/kg), there were no under-predictions in any of the laboratories. Similarly to the millimole regression analysis, in HSL only one chemical (Di-"isodecyl" phthalate) was correctly predicted as GHS

unclassified (LD_{50} > 5 000 mg/kg b.w.) whereas in JRC and IIVS none of the GHS unclassified chemicals were correctly predicted. GHS category 5 (2 000 < LD_{50} ≤ 5 000 mg/kg) was over-predicted in all three laboratories (87.5% in HSL, 75% in JRC, and 87.5% in IIVS).

Overall, the analyses showed that when the LD_{50} limit dose of 5 000 mg/kg b.w. was used, the specificity of the 3T3 NRU test method was poor (7% in HSL, 0% in JRC, and 0% in IIVS) with both millimole and weight regression. In addition, IIVS protocol was particularly designed around the 2 000 mg/kg b.w. cut-off values and, therefore, it was not expected to provide good results with the 5 000 mg/kg b.w. cut-off value.

When the optimum IC_{50} value of 1 154 µg/ml (identified with the RC data set using the ROC analysis) was applied to the data set from the validation study, the sensitivity of the 3T3 NRU test method ranged from 76% to 88% and specificity from 40% to 42% (depending on the laboratory). Overall, 11 test chemicals were under-predicted, of which six are used as cosmetic ingredients according to the EU CosIng database (see Tables 2 and 3 of the final study report and Table E-17). The negative predictive value ranged from 47% to 67%, depending on the laboratory.

In contrast to the regression analysis, when the IC_{50} value of 1 154 µg/ml was applied, the performance of the 3T3 NRU test method to identify unclassified chemicals (LD_{50} > 5 000 mg/kg b.w.) was better. However, the analysis resulted in higher numbers of under-predicted chemicals, 4-8 false negatives, depending on the laboratory (Table E-17). Assuming that the prevalence of positive (LD_{50} ≤ 5 000 mg/kg b.w.) and negative (LD_{50} > 5 000 mg/kg b.w.) chemicals in the EU NCD was 83% and 17%, respectively (Bulgheroni et al 2009), the proportion of unclassified chemicals identified by the 3T3 NRU test method was low (7-7.5%). Therefore, the use of 3T3 NRU test method to identify unclassified chemicals according to the GHS classification scheme would result in a reduction of animal toxicity testing up to 7.5% if the method is used as the first step in a tiered approach.

ANNEX F

Solubility protocol

TEST METHOD PROTOCOL
for Solubility Determination

In Vitro Cytotoxicity Validation Study
Phase III

September 24, 2003

Prepared by

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

Based on Standard Operating Procedure Recommendations from an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Institute of Environmental Health Sciences (NIEHS)
National Institutes of Health (NIH)
U.S. Public Health Service
Department of Health and Human Services

TEST METHOD PROTOCOL

Solubility Determination
Phase III

I. PURPOSE

The purpose of this study is to evaluate the cytotoxicity of test chemicals using the BALB/c 3T3 Neutral Red Uptake (NRU) and normal human keratinocyte (NHK) cytotoxicity tests. The data will be used to evaluate the intra- and inter-laboratory reproducibility of the assay and effectiveness of the cytotoxicity assay to predict the starting doses for rodent acute oral systemic toxicity assays. This test method protocol outlines the procedures for performing solubility determinations for the *in vitro* validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM) and sponsored by NIEHS, U.S. Environmental Protection Agency, and ECVAM. This test method protocol applies to all personnel involved with performing the solubility testing.

A. Solubility Test

The solubility tests will be performed to determine the best solvent to use for each of the 60 blinded/coded test chemicals to be tested in the 3T3 and NHK NRU cytotoxicity tests

II. SPONSOR

A. Name: National Institute of Environmental Health Sciences (NIEHS); The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

B. Address: P.O. Box 12233
Research Triangle Park, NC 27709

C. Representative: *Named Representative*

III. IDENTIFICATION OF TEST SUBSTANCES AND SOLVENTS

A. Test Chemicals: *60 Coded Chemicals (60)*

B. Solvents: Chemical Dilution Medium for 3T3 assay (See **Section VII.B.1**)
Treatment Medium for NHK assay (See **Section VII.B.2**)

IV. TESTING FACILITY AND KEY PERSONNEL

A. Facility Information

1) Name:
2) Address:
3) Study Director:
4) Laboratory Technician(s):
5) Scientific Advisor:
6) Quality Assurance Director:
7) Safety Manager:

8) Facility Management:

B. Test Schedule

1) Proposed Experimental Initiation Date:
2) Proposed Experimental Completion Date:
3) Proposed Report Date:

V. TEST SYSTEM

The solubility test procedure is based on attempting to dissolve chemicals in various solvents with a increasingly rigorous mechanical techniques. The solvents to be used, in the order of preference, are cell culture media, DMSO, and ethanol. Solubility shall be determined in a step-wise procedure that involves attempting to dissolve a test chemical in the solvents (in the order of preference) at relatively high concentrations using the sequence of mechanical procedures (**Section VII.C.2.a**). If the chemical does not dissolve, the volume of solvent is increased so as to decrease the concentration by a factor of 10, and then the sequence of mechanical procedures are repeated in an attempt to solubilize the chemical at the lower concentrations.

Determination of whether a chemical has dissolved is based entirely on visual observation. A chemical has dissolved if the solution is clear and shows no signs of cloudiness or precipitation.

VI. DEFINITIONS

A. *Soluble:* Chemical exists in a clear solution without visible cloudiness or precipitate.

B. *Documentation*: all methods and procedures will be noted in a Study Workbook; logs will be maintained for general laboratory procedures and equipment (e.g., media preparation, solubility testing, laboratory balance calibration); solubility reports will be in electronic and paper format; all data will be archived.

VII. PROCEDURES

A. Materials

1. Technical Equipment

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

a) Water bath: 37ºC ± 1ºC
b) Glass tubes with caps (e.g., 5 mL)
c) Laboratory balance
d) Pipetting aid
e) Pipettes, pipettors (multi-channel and single channel; multichannel repeater pipette), dilution block
f) Waterbath sonicator
g) Dry heat block (optional)

2. Chemicals, Media, and Sera

a) Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine; should have high glucose [4.5gm/l] (e.g., ICN-Flow Cat. No. 12-332-54)
b) L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49)
c) Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49)
d) Dimethyl sulfoxide (DMSO), U.S.P. analytical grade (Store under nitrogen @ -20ºC)
e) Ethanol (ETOH), U.S.P. analytical grade (100 %, non-denatured for test chemical preparation; 95 % can be used for the desorb solution)
f) Keratinocyte Basal Medium without Ca^{++} (KBM™, Clonetics CC-3104) that is completed by adding the KBM™ SingleQuots™ (Clonetics CC-4131) to achieve the proper concentrations of epidermal growth factor, insulin, hydrocortisone, antimicrobial agents, bovine pituitary extract, and calcium (e.g., Clonetics Calcium SingleQuots™, 300 mM $CaCl_2$, Clonetics # CC-4202).

B. Preparations of Media and Solutions

[Note: All solutions glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures will be adequately documented. Completed media formulations should be kept at approximately 2-8° C and stored for no longer than two weeks.]

1. 3T3 Chemical Dilution Medium

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

4 mM	Glutamine
200 IU/mL	Penicillin
200 µg/mL	Streptomycin

2. NHK Treatment Medium

KBM™ (Clonetics CC-3104) supplemented with KBM™ SingleQuots™ (Clonetics CC-4131) and Clonetics Calcium SingleQuots™ (CC-4202) to make 500 mL medium. Final concentration of supplements in medium are:

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

NOTE:
KBM™ SingleQuots™ contain the following stock concentrations and volumes:

0.1 ng/mL	hEGF	0.5 mL
5.0 mg/mL	Insulin	0.5 mL
0.5 mg/mL	Hydrocortisone	0.5 mL
30 mg/mL	Gentamicin, 15 ug/mL Amphotericin-B	0.5 mL

7.5 mg/mL	Bovine Pituitary Extract (BPE)	2.0 mL

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

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

C. Determination of Solubility

The preference of solvent for dissolving test chemicals is medium, DMSO, and then ethanol. Solubility shall be determined in a step-wise procedure that involves attempting to dissolve a test chemical at a relatively high concentration with the sequence of mechanical procedures specified in **Section VII.C.2.a.** If the chemical does not dissolve, the volume of solvent is increased so as to decrease the concentration by a factor of 10, and then the sequence of mechanical procedures in **Section VII.C.2.a** are repeated in an attempt to solubilize the chemical at the lower concentrations. For testing solubility in medium, the starting concentration is 20,000 µg/ml (i.e., 20 mg/mL) in Tier 1, but for DMSO and ethanol the starting concentration is 200,000 µg/ml (i.e., 200 mg/mL) in Tier 2. Weighing out chemical for each solvent (i.e., medium, DMSO, ethanol) can be done all at once, if convenient, but solubility testing (at each tier that calls for more than one solvent) is designed to be sequential - medium, then DMSO, then ethanol – in accordance with the solvent hierarchy (see **Figure 1**). This allows for testing to stop, rather than continue testing with less preferred solvents, if the test chemical dissolves in a more preferred solvent. For example, if a chemical is soluble in medium at a particular tier, testing may stop. Likewise, if a chemical is soluble in DMSO at any tier, testing need not continue with ethanol. However, since the issue of primary importance is testing the solvents and concentrations of test chemical required by any one tier, sequential testing of solvents may be abandoned if the lab can test more efficiently in another way.

1. Method

a) Tier 1 begins with testing 20 mg/mL each in Chemical Dilution Medium and Treatment Medium (see **Table 1**). For each medium, weigh approximately 10 mg (10,000 µg) of the test chemical into glass tubes. Document the chemical weight. Add approximately 0.5 mL of each medium into its respective tube so that the concentration is 20,000 µg/ml (20 mg/mL). Mix the solution as specified in **Section VII.C.2.a.** If complete solubility is achieved in each medium, then additional solubility procedures are not needed.

b) If the test chemical is insoluble in either Chemical Dilution Medium or Treatment Medium, proceed to Tier 2 by adding enough medium, approximately 4.5 mL, to attempt to dissolve the chemical at 2 mg/mL by using the sequence of mixing procedures specified in **Section VII.C.2.a**. If the test chemical dissolves in medium at 2 mg/mL, no further procedures are necessary. If the test chemical does NOT dissolve in one medium or the other (if both are tested in this tier), weigh out approximately 100 mg test chemical in a second glass tube and add enough DMSO to make the total volume approximately 0.5 mL (for 200 mg/mL) and attempt to dissolve the chemical as specified in **Section VII.C.2.a**. If the test chemical does not dissolve in DMSO, weigh out approximately 100 mg test chemical in another glass tube and add enough ethanol to make the total volume approximately 0.5 mL (for 200 mg/mL) and attempt to dissolve the chemical as specified in **Section VII.C.2.a**. If the chemical is soluble in either solvent, no additional solubility procedures are needed.

c) If the chemical is NOT soluble in one or both media, DMSO, or ethanol at Tier 2, then continue to Tier 3 in Table 1 by adding enough solvent to increase the volume of the three (or four) Tier 2 solutions by 10 and attempt to solubilize again using the sequence of mixing procedures in **Section VII.C.2.a.** If the test chemical dissolves, no additional solubility procedures are necessary. If the test chemical does NOT dissolve, continue with Tier 4 and, if necessary, Tier 5 using DMSO and ethanol. Tier 4 begins by diluting the Tier 3 samples with DMSO or ethanol to bring the total volume to 50 mL. The mixing procedures in **Section VII.C.2.a** are again followed to attempt to solubilize the chemical. Tier 5 is performed, if necessary, by weighing out another two samples of test chemical at ~10 mg each and adding ~50 mL DMSO or ethanol for a 200 μg/mL solution, and following the mixing procedures in **Section VII.C.2.a**.

Example: If complete solubility is not achieved at 20,000 μg/mL in either Chemical Dilution Medium or Treatment Medium at Tier 1 using the mixing procedures specified in **Section VII.C.2.a,** then the procedure continues to Tier 2 by diluting the solution to 5 mL (with either of the appropriate media) and mixing again as specified in **Section VII.C.2.a.** If the chemical is not soluble in Chemical Dilution Medium or Treatment Medium, two samples of ~ 100 mg test chemical are weighed to attempt to solubilize in DMSO and ethanol at 200,000 μg/mL (i.e., 200 mg/mL). Solutions are mixed following the sequence of procedures prescribed in **Section VII.C.2.a** in an attempt to dissolve. If solubility is not achieved at Tier 2, then the solutions (Chemical Dilution Medium and/or Treatment Medium, DMSO, and ethanol) prepared in Tier 2 are diluted by 10 so as to test 200 μg/mL in media, and 20,000 μg/mL in DMSO and ethanol. This advances the procedure to Tier 3. Solutions are again mixed as prescribed in **Section VII.C.2.a** in an attempt to dissolve. If solubility is not achieved in Tier 3, the procedure continues to Tier 4, and to 5 if necessary (see **Figure 1** and **Table 1**).

Table 1. Determination of Solubility in Chemical Dilution Medium, Treatment Medium, DMSO, or Ethanol

TIER	**1**	**2**	**3**	**4**	**5**
Total Volume Chemical Dilution Medium/Treatment Medium	0.5 mL	5 mL	50 mL		
Concentration of Test Chemical (Add ~10 mg to a tube. Add enough medium to equal the first volume. Dilute to subsequent volumes if necessary.)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	200 µg/mL (0.20 mg/mL)		
Total Volume DMSO/Ethanol		0.5 mL	5 mL	50 mL	
Concentration of Test Chemical (Add ~100 mg to a large tube. Add enough DMSO or ethanol to equal the first volume. Dilute with subsequent volumes if necessary.)		200,000 µg/mL (200 mg/mL)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	
Total Volume DMSO/Ethanol					50 mL
Concentration of Test Chemical (Add ~10 mg to a large tube. Add enough DMSO or ethanol to equal 50 mL.)					200 µg/mL (0.2 mg/mL)
Equivalent Concentration on Cells	10,000 µg/mL (10 mg/mL)	1000 µg/mL (1 mg/mL)	100 µg/mL (0.1 mg/mL)	10 µg/mL (0.01 mg/mL)	1 µg/mL (0.001 mg/mL)

[NOTE: The amounts of test chemical weighed and Chemical Dilution Medium and Treatment Medium added may be modified from the amounts given above, provided that the targeted concentrations specified for each tier are tested.]

Figure 1. Solubility Flow Chart

TIER 1

STEP 1:	20 mg/mL test chemical (TC) in 0.5 mL Chemical Dilution Medium and Treatment Medium: • if TC soluble in both media, then **STOP**. • if TC insoluble in one medium, then go to STEP 2.

TIER 2

STEP 2:	2 mg/mL TC in medium (one or both) – increase volume from STEP 1 by 10 (i.e., to 5 mL) • if TC soluble, then **STOP**. • if TC insoluble in one medium, then go to STEP 3.

STEP 3:	200 mg/mL TC in DMSO
	• if TC soluble, then **STOP**.
	• if TC insoluble, test at 200 mg/mL in ETOH.
	  • if TC soluble, then **STOP.**
	  • If TC insoluble, go to STEP 4.

TIER 3

STEP 4:	0.2 mg/mL TC in medium (one or both) – increase volume from STEP 2 by 10 (i.e., to 50 mL)
	• if TC soluble in both media, then **STOP**.
	• if TC insoluble in one medium, test at 20 mg/mL in DMSO – increase volume from STEP 3 by 10 (i.e., to 5 mL).
	  • if TC soluble, then **STOP.**
	  • if TC insoluble, test at 20 mg/mL in ETOH – increase volume from STEP 3 by 10 (i.e., to 5 mL).
	  • if TC soluble, then **STOP.**
	  • if TC insoluble, then go to STEP 5.

TIER 4

STEP 5:	2 mg/mL TC in DMSO – increase volume from STEP 4 by 10 (i.e., to 50 mL)
	• if TC soluble, then **STOP.**
	• if TC insoluble, test at 2 mg/mL in ETOH – increase volume from STEP 4 by 10 (i.e., to 50 mL).
	  • if TC soluble, then **STOP.**
	  • if TC insoluble, then go to STEP 6.

TIER 5

STEP 6:	0.2 mg/mL TC in 50 mL DMSO • if TC soluble, then **STOP.** • if TC insoluble, test at 0.2 mg/mL in 50 mL ETOH • **STOP**

2. Mechanical Procedures

a) The following hierarchy of mixing procedures will be followed to dissolve the test chemical:

1) Add test chemical to solvent as in Tier 1 of **Table 1**. (Test chemical and solvent should be at room temperature.)

2) Gently mix at room temperature. Vortex the tube (1 –2 minutes).

3) If test chemical hasn't dissolved, use waterbath sonication for up to 5 minutes.

4) If test chemical is not dissolved after sonication, then warm solution to 37°C for 5 - 60 min. This can be performed by warming tubes in a 37°C water bath or in a CO_2 incubator at 37°C. The solution may be stirred during warming (stirring in a CO_2 incubator will help maintain proper pH).

5) Proceed to Tier 2 (and Tiers 3-5, if necessary of Table 1 and repeat procedures 2-4).

b) The preference of solvent for dissolving test chemicals is Chemical Dilution Medium or Treatment Medium, DMSO, and then ethanol. Thus, if all solvents for a particular tier are tested simultaneously and a test chemical dissolves in more than one solvent, then the choice of solvent follows this hierarchy. For example, if, at any tier, a chemical is soluble in Chemical Dilution Medium and DMSO, but not in Treatment Medium or ethanol, the choice of solvent would be medium for the 3T3 assay and DMSO for the NHK assay. If the chemical were insoluble in both media, but soluble in DMSO and ethanol, the choice of solvent would be DMSO for both assays.

After the lab has determined the preferred solvent for the test chemical and before proceeding to the cytotoxicity testing, the Study Director will submit the solubility test results (laboratory worksheets are preferable), and discuss the solvent selection with the Study Management Team (SMT) of the validation study. The SMT will provide direction on the solvent to be used in each assay for each chemical prior to cytotoxicity testing. If the laboratory has attempted all solubility testing without success, then the SMT will provide additional guidance for achieving test chemical solubility. The SMT anticipates that all validation study test chemicals will be tested in the NRU assays.

The Testing Facility shall forward the results from the solubility tests assay to the SMT through the designated contacts in electronic format and hard copy upon completion of testing. The SMT will be directly responsible for the statistical analyses of the Validation Study data.

VIII. REFERENCES

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

IX. APPROVAL

_____	_____
SPONSOR REPRESENTATIVE	**DATE**
(Print or type name)	
_____	_____
Test Facility STUDY DIRECTOR	**DATE**
(Print or type name)	

ANNEX G

Number of chemicals tested and/or excluded from analysis in each laboratory

Table G-1. Final number of chemicals tested and number of chemicals with censored IC_{50} values obtained in each laboratory

	HSL	JRC	IIVS
Nr. chemicals tested	**50** • 3 chemicals insoluble • 1 chemical impossible to remove from vial • 2 chemicals identified as highly toxic	**53** • 3 chemicals insoluble	**56**
Nr. chemicals for which only RF was performed	11 (not enough cytotoxicity in RF)	6 (not enough cytotoxicity in RF)	0
Nr. chemicals for which both RF and DF were performed	39	47	56 (only DF)*
Nr. chemicals with right censored IC_{50} values	11	8	11
Nr. chemicals with left censored IC_{50} values	0	0	13

* = In the abbreviated protocol used by IIVS the concentration range was predefined and no range finding experiments were performed (see Section 5.6 and Table 4).
RF= range finding experiments; DF = definitive or main experiments

Table G-2. Number of chemicals excluded from the analysis of within-laboratory variability, between-laboratory variability and predictive capacity in each laboratory

	HSL			JRC			IIVS		
Millimole regression analysis	**WLV** 16	**BLV*** 12	**PC** 12	**WLV** 9	**BLV*** 5	**PC** 5	**WLV**: 3	**BLV*** 2	**PC** 2
• [a]**Due to IC_{50} censored values excluded**	6	6	6	2	2	2	2	2	2
• **Due to less than two IC_{50} values accepted and/or available**	4			4			1		
• **Not tested**	6	6	6	3	3	3	0	0	0
Weight regression analysis	**WLV** 17	**BLV*** 16	**PC** 16	**WLV** 10	**BLV*** 10	**PC** 10	**WLV** 10	**BLV*** 9	**PC** 9
• [a]**Due to IC_{50} censored values excluded**	10	10	10	7	7	7	9	9	9
• **Due to less than 2 IC_{50} values accepted and/or available**	1						1		
• **Not tested**	6	6	6	3	3	3	0	0	0
Applying an optimum IC_{50} value (ROC analysis)	**WLV**	**BLV**	**PC**	**WLV**	**BLV**	**PC**	**WLV**	**BLV**	**PC**
• [b]**Due to IC_{50} censored values excluded**	NA	NA	3	NA	NA	1	NA	NA	3
• **Not tested**			6			3			0

WLV = within-laboratory variability; BLV = between-laboratory variability; PC = predictive capacity; NA = not applicable; ROC = receiver operating characteristic.

* = For between laboratory variability, 15 (millimole regression) and 21 (weight regression) test chemicals were excluded from the overall evaluation due to right IC_{50} censored values that resulted in an estimated LD_{50} value smaller than 2 000 mg/kg b.w. (9 chemicals with millimole regression and 15 chemicals with weight regression analyses) or not tested (6 chemicals).

a = right IC_{50} censored values excluded since the estimated LD_{50} were ≤ 2 000 mg/kg b.w.; b= right IC_{50} censored values excluded since they were smaller than the cut-off value of 485.6 µg/ml.