DB-ALM Protocol n° 46 : BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test

Basal Cytotoxicity, Eye Irritation

The cytotoxic effect of chemicals upon Balb/c 3T3 cells in culture is measured by cell viability (Neutral Red Uptake, NRU) and total cell protein (Kenacid Blue R dye binding method).

Résumé

Healthy Balb/c 3T3 cells (an established murine fibroblastic cell line), when maintained in culture continuously divide and multiply over time. The basis of this test is that a cytotoxic chemical (regardless of site or mechanism of action) will interfere with this process and, thus, result in a reduction of the growth rate as reflected by cell number. The degree of inhibition of growth, related to the concentration of the test compound, provides an indication of toxicity.
Further details can be found in **Method Summary:** "Neutral Red Uptake (NRU) Assay", available from DB-ALM.

Experimental Description

Endpoint and Endpoint Measurement:

CELL VIABILITY: neutral red uptake measured spectrophotometrically

NEUTRAL RED UPTAKE

PROTEIN CONTENT: the protein content is determined spectrophotometrically using the Kenacid Blue Assay

Endpoint Value:

IC50: the concentration of test substance expressed as mg/ml that induces 50% inhibition of neutral red uptake

Experimental System(s):

3T3 FIBROBLASTS (MOUSE): commercially available cell line derived from BALB/c 3T3 mice

Basic Procedure

Balb/c 3T3 cells are maintained in culture and exposed to test compounds over a range of concentrations. The cultures are visually examined after 24 hours, the highest tolerated dose (HTD) estimated, and the number of viable cells and/or the total cell protein content determined, after 24 hours exposure, by the Neutral Red Uptake and Kenacid Blue methods respectively. The nature of the assays is such that both may be used on the same cultures provided that the Neutral Red Uptake (an indication of the number of viable cells) determination is performed first.

The number of cells in the presence of test chemicals is compared to that observed in control cultures and the percent inhibition of growth calculated. The IC_{50} concentration (i.e. the concentration producing 50% inhibition of growth) is determined and expressed as µg/ml or mmol/l. This value enables a comparison of the relative cytotoxicity of the test compounds to be performed.

Test Compounds and Results Summary

Proposed list of substances to be tested in the FRG Interlaboratory Study for Replacement of the Draize Test

Chemical	CAS Number
2-Propane-1-ol	107-18-6
Acetone	67-64-1
Acetonitrile	75-05-8
Acrylamide	79-06-1
Aniline	62-53-3

Ascorbic acid	50-81-7
Benzalkonium chloride	8001-54-5
Benzoic acid	65-85-0
2-Butoxyethanol	111-76-2
Copper (II) sulphate	7758-98-7
Cyclohexanol	108-93-0
Dimethylsulphoxide (DMSO)	67-68-5
EDTA-Na salt	13235-36-4
Ethanol	64-17-5
Glutamic acid	56-86-0
Isobenzoic furano dione	85-44-9
Nicotinamide	98-92-0
Nitrobenzene	98-95-3
Phenol	108-95-2
Propylene glycol	57-55-6
Pyridine	110-86-1
Salicylic acid	69-72-7
SDS	151-21-3
Tetrachloroethene	127-18-4
Thiourea	62-56-6
Tin (II) chloride	7772-99-8
Toluene	108-88-3

Chemicals (such as alcohols, acids, solvents, and metal salts)

Discussion

Cell culture procedure
The maintenance and culture of a cell line such as Balb/c 3T3 is a relatively simple and inexpensive technique. The application of such cultures to determine cytotoxicity enables the rapid, highly reproducible, testing of many chemicals on a routine basis.

There are certain limitations of the technique, some of which concern the character of the compounds to be tested:
Volatile chemicals tend to evaporate under the conditions of the test, thus the IC_{50} value may be variable, especially when the toxicity of the compound is fairly low. This has been overcome to some extent by adapting the procedure for use in 96-well plates as the smaller surface area of the well in these dishes reduces the extent of evaporation. In addition, plates can be sealed with CO_2 permeable plastic film which is impermeable to volatile chemicals thus decreasing evaporation.

Other chemicals which are difficult to test include those which are unstable or explosive in water.
Other difficulties are related to the nature of the cell line, i.e. rapidly growing, non-differentiating cells of very low metabolic activity, hence raising problems of direct extrapolation of results to the *in vivo* situation.

The system is likely to underestimate the toxicity of chemicals which require metabolic activation to a toxic intermediary or product. However, metabolic activation is not an essential factor for assessing the irritation potential of chemicals.

Substances which specifically attack dividing cells may appear to be of a much higher order of toxicity than they would be *in vivo*.

The toxicity of substances which bind to serum proteins (i.e. such as those found in newborn calf serum) may be also underestimated.

Several laboratories have shown that the peripheral wells of 96-well plates do not sustain cell growth at the same rate as the inner wells. As a result many laboratories use the peripheral wells as blanks only (containing medium).

Neutral Red uptake assay
Neutral Red is preferentially taken up into the lysosomes/endosomes of the cell. Any chemical having a localised effect upon the lysosomes/endosomes will, therefore, result in an artificially low (or possible high) reflection of cell viability and cell number. This factor does, however, make the system useful to detect those chemicals which selectively affect the lysosomes, especially when it is used in conjunction with other tests capable of determining cell number. An example of this would be chloroquine sulphate which alters the pH of lysosomes/endosomes, an effect which inhibits Neutral Red uptake.

One major drawback of the assay is the precipitation of the Neutral Red dye into readily visible, fine, needle-like crystals. When this occurs it is almost impossible to reverse, thus producing inaccurate readings. To avoid this precipitation, NR medium is incubated overnight and centrifuged before being added to the cells. Additionally, some chemicals induce this precipitation, therefore, a visual inspection stage in the procedure is very important.

Kenacid Blue R dye binding assay
One of the drawbacks of this assay is that the Kenacid Blue dye may, on occasion, precipitate. The likelihood of precipitation increases as the length of handling time increases, therefore 96-well plates should be agitated regularly and inspected visually for uneven blue colour. The process is, however, readily reversed by agitation, so any odd reading should be retested after agitation to obviate the possibility of precipitation.
Another problem is the deposition of a ring of dried protein which may occur around the walls of the well, at the air/medium interface. This arises if the culture medium is not properly removed or through excessive evaporation. Such precipitated protein will give an inaccurate assessment of total cellular protein.
It should be noted that in certain cases care should be taken in the interpretation of results. In the Kenacid Blue assay cells treated with organic acids or alcohols become fixed to the bottom of the plate - including dead cells. As a result, although the protein content determined decreases dose-dependently at lower concentrations, it returns to higher values at higher concentrations of test chemical.
Advantages of this system include:
It can be repeated more than once on the same cells.
Cells can be fixed and the staining performed later.
The cell distribution can easily be seen with the naked eye when stained with the Kenacid Blue before desorbing, thus giving a rapid indication of the success of the assay.
Despite the limitations of the system it provides a simple screen for the rapid assessment of the toxicity of compounds.
The advantages and disadvantages of both the Neutral Red Uptake and the Kenacid Blue protein assays are presented above. A direct comparison of the Kenacid Blue and the Neutral Red methods may also be of value to someone considering the choice of endpoint.
Once initiated the Neutral Red Uptake assay need to be completed, i.e. once the cells have been incubated with the Neutral Red and the dye is taken up into the lysosomes, the process of fixing and destaining has to follow immediately.
With Kenacid Blue the cells can be fixed and the staining/destaining step can be performed later.
The Kenacid Blue test is repeatable more than once on the same cells, i.e. once the cells have been incubated with the Neutral Red and the dye is taken up into the lysosomes, the process of fixing and destaining must follow on immediately.

With Kenacid Blue the cells can be fixed and the staining/destaining performed later.
Kenacid Blue test can be repeated more than once on the same cells, i.e once the cells are fixed the procedure of dye addition and destaining can be repeated several times. This is obviously out of the question with the Neutral Red assay which is dependent upon live cell uptake of the dye.

Although there is a danger of the Kenacid Blue dye precipitating out it is readily solubilised by agitating the dishes. However, if Neutral Red crystals form they are almost impossible to re-solubilise without removing the stain from the cells as well. This greatly alters the reliability of any readings obtained and has proven to be an occasional occurrence, induced by some chemicals.

Another disadvantage of the Neutral Red assay is the possibility that deceptively low cell viability or cell number readings will result in those cases where a chemical has a relatively selective effect upon the

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

One advantage of Neutral Red assay is that it detects only viable cells. Total protein measurement does not make allowances for necrotic cells which may still be attached to the culture dish and, therefore, may underestimate the toxicity of a compound. It should be noted, however, that the occurrence of adhering dead or dying cells is very rare.

It is possible to perform both the Kenacid Blue and the Neutral Red assays on the same culture, i.e. Neutral Red estimates can be obtained and, as the cells are by then fixed, protein determination can be made using the Kenacid Blue method. Performing both assays would provide a means of checking the sensitivity of the Neutral Red assay, when a chemical is suspected of affecting the lysosomes.

Status

This test, along with several other in vitro systems, was undergoing validation as an alternative test to replace the Draize Rabbit Eye Test, in a national between-laboratory study started in June 1988, by the Federal Health Office (BGA) of the Federal Republic of Germany (FRG).
The aim of this collaborative study was to validate the classification of chemicals, with regard to their irritation potential, using the Neutral Red/Kenacid Blue (NR/KB) cytotoxicity assay and the Hen's Egg Test Chorioallantoic Membrane (HET-CAM) assay according to Lupke. The FRG Public Health Office (BGA) was coordinating the scheme which included 13 toxicology laboratories in the chemical industry, universities, the BGA and other research institutions. 34 substances with a variety of chemical, biochemical, and toxicological characteristics were assessed. The validation was intended to provide comparative data for the development of an alternative routine test scheme, which can be performed under routine laboratory conditions.
The BGA validation project of alternatives for the Draize test consisted of three parts: a preliminary phase, a between-laboratory assessment, and, finally, the development of a database of results. During the preliminary phase the cytotoxicity test and the HET-CAM assay have been established in the different laboratories. The participants have agreed on standard and mandatory protocols and on the choice of chemicals. Two preliminary trials have been performed with 4 test substances.
During the between-laboratory assessment 34 chemical substances of a variety of chemical structure groups have been tested with both alternative techniques in 13 laboratories under conditions defined in the preliminary phase of the study. The main purpose of the study was the comparative and statistical evaluation of all data at the BGA followed by a final scientific validation which could prove of interest to regulatory authorities. This assessment determined both the reproducibility of the results within a given laboratory and of a given test between laboratories. The 3T3-NRU assay had a good between- and within-laboratory reproducibility. The HET-CAM assay identified severe eye irritants (R41) better than the cytotoxicity assays. The authors concluded that the combined use both assays (HET-CAM assay and NRU/KB cytotoxicity test) can be used to classify severe ocular irritants (R41) with sufficient reliability (Spielmann et al., 1996).

Participation in Validation Studies:

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Last update: June 2007

PROCEDURE DETAILS, January 1992

BALB/c 3T3 Neutral Red Uptake (NRU) Cytotoxicity Test
DB-ALM Protocol n° 46

Note: The herewith included details on the procedure have been sent to the person responsible for the method to update or confirm it. As soon as new information will become available this version will be updated.

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Cell line

Balb/c 3T3 cells, catalogue number 03-405-83 - Flow, Meckenheim, FRG

Equipment

Incubator - 37.5^{0}C, humidified, 7.5% CO_2/air
Clean bench
Water bath, 37.5^{0}C
Phase contrast microscope
Laboratory burner
Centrifuge with microtiter rotor
Laboratory balance
Osmometer
Immuno reader
Shaker for microtiter plates
Cell counter or haemocytometer
Pipetting aid
Pipettes, 8-channel-pipettes, dilution block
Cryotubes
80cm^2 tissue culture flasks or petri dishes
96-well tissue culture microtiter plates
Permeable plastic film - Greiner, No 676001
Computer with evaluation program "Symphony" (adapted for four types of readers which were in use in the participating laboratories)

Materials

1 x DMEM fluid medium without L-glutamine - Flow, Meckenheim, West Germany
Glutamine, 200mM
Newborn calf serum (NBCS) - Biochrom, Berlin

Trypsin/EDTA solution - Flow
Special salt solution - Flow
Penicillin/streptomycin solution - Flow
Neutral Red
Kenacid Blue
Ethanol
Glacial acetic acid
Double distilled water
Dimethylsulphoxide (DMSO)
Phosphate buffered saline with calcium and magnesium
(PBS-with Ca^{2+} and Mg^{2+})
Phosphate buffered saline without calcium and magnesium
(PBS-without Ca^{2+} and Mg^{2+})
Potassium acetate

Make up the following:

Culture medium
DMEM (buffered with sodium bicarbonate) supplemented with:
(Final concentrations of the compounds, in medium, are quoted)

(a) for routine culture
10% Newborn calf serum
4mM Glutamine
100IU Penicillin/100µg/ml Streptomycin.

(b) for treatment
5% Newborn calf serum
4mM Glutamine
100IU Penicillin/100µg/ml Streptomycin.

(c) for freezing
Growth medium with 20% NBCS and 7-10% DMSO

Complete medium should be kept at 4^0C and stored for no longer than two weeks.

Neutral Red stock solution
0.4g neutral red dye
100ml PBS-with Ca^{2+} and Mg^{2+}

Make up prior to use and store for up to two months.

Neutral Red (NR) medium
1ml of Neutral Red stock solution
79ml assay medium.

Make up prior to use.
The NR medium should be incubated overnight at 37^0C and centrifuged at 600 x g. for 10 minutes (to remove NR crystals) before adding to cells.

Ethanol/acetic acid solution (Neutral Red Desorb)
1% glacial acetic acid solution
50% ethanol
49% double distilled water
Prepare immediately prior to use. Do not store for longer than 1 hour.

Stock Kenacid Blue stock solution
0.4g Kenacid Blue stain
250ml glacial acetic acid
630ml double distilled water.
Make up prior to use.

Kenacid Blue solution

88ml Kenacid Blue stock solution
12ml glacial acetic acid
Prepare immediately prior to use.

Kenacid Blue washing solution
10% ethanol
5% glacial acetic acid
85% double distilled water
Prepare immediately prior to use.

Kenacid Blue desorbing solution
98.15g potassium acetate (1M)
700ml ethanol
300ml double distilled water

Trypsin/EDTA solution
Make up a 0.05% trypsin/0.02% EDTA in a salt solution.

Test Compounds
Dissolve in sterile treatment medium, ethanol, methanol or dimethyl sulphoxide (DMSO), as appropriate, at 100-fold the desired final concentration in the case of solvents. The final solvent concentration should be kept at a constant level of 1% in culture medium.
Volatile chemicals should be tested in microtitre plates sealed by a plastic film permeable to CO_2.
Insoluble and viscous substances are very difficult to test. In such cases the supernatant of a saturated solution is used as the highest concentration in the test.

All solutions, glassware, etc., are sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

METHOD

A) **Cell Maintenance And Culture Procedures**
Balb/c 3T3 cells are routinely grown as a monolayer in 80cm^2 tissue culture grade flasks, at 37.5^{0}C in a humidified atmosphere of 7.5% CO_2.
The cells should be examined, on a daily basis, under a phase contrast microscope, and any changes in morphology or their adhesive properties, noted.

Routine culture of Balb/c 3T3 cells
When the cells approach confluence they should be removed from the flask by trypsinisation:

Decant the medium and rinse the cultures with PBS-without Ca^{2+} and Mg^{2+} e.g. 5ml is used for a 25cm^2 flask.
Wash cells by gentle agitation to remove any remaining serum which might otherwise inhibit the action of the trypsin.
Discard the washing solution.
Add 1-2ml trypsin-EDTA solution to the monolayer.
Incubate for ~1 minute, at 37.5^{0}C.
Remove excess Trypsin-EDTA solution and incubate the cells at 37.5^{0}C.
After 2-3 minutes, lightly tap the flask to detach the cells into a single cell suspension.

Cell counting
After detaching the cells, add 0.1-0.2ml of routine culture medium/cm2 flask i.e. 2.5ml for a 25cm^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 haemocytometer or cell counter.

Subculture
After determination of cell number, the culture can be subcultured into another flask or seeded into a 96-well microtiter plate.
Balb/c 3T3 cells are routinely passaged at a cell density of 1 x 10^5 cells/ml in 80 cm^2 flasks, every 3-4 days (average doubling time, 20-24 hours).

N.B. It is important that cells are in an exponential growth phase when they are taken for the cytotoxicity assay.

Freezing
Stocks of Balb/c 3T3 cells can be stored in sterile, freezing tubes in liquid nitrogen. Dimethyl sulphoxide is used as a cryoprotective agent.
Centrifuge trypsinised cells at 200 x g.
Suspend the cells in routine culture medium, containing 20% NBCS, at a concentration of 1-5 x 10^6 cells/ml.
Aliquot 120-180µl of cooled DMSO into freezing tubes and fill to 1.8ml with the cell suspension.
Place the tubes into a freezer at -80^{0}C for 24 hours. This gives a freezing rate of 1^{0}C/minute.
Place the frozen tubes into liquid nitrogen for storage.

Thawing
Thaw cells by putting ampoules into a water bath at 37.5^{0}C. Leave for as brief a time as possible.
Resuspend the cells and transfer into routine culture medium. Incubate at 37.5^{0}C in a humidified 7.5% CO_2 atmosphere.
When the cells have attached to the bottom of the flask (this may take up to ~4 hours), decant the supernatant and replace with fresh medium.
Culture as described above.
Passage two to three times before using the cells in a cytotoxicity test.
A fresh batch of frozen cells should be thawed out approximately every two months.

B) **Determination of Cytotoxicity**

Preparation of cell cultures
Prepare a cell suspension of 10 x 10^4 cells/ml in routine culture medium.
Using a multi-channel pipette, dispense medium only into the peripheral wells of a 96-well tissue culture microtiter plate. In the remaining wells *dispense* 100µl volumes of cell suspension (in routine culture medium) at a cell concentration of 1 x 10^4 cells/well.
Incubate the cells for 24 hours in a humidified atmosphere with 7.5% CO2 at 37.5^{0}C, until they form a half confluent monolayer. This incubation period allows for cell recovery and adherence.

Treatment
Make up a range of doses of test substance by diluting with treatment medium. The first run for each chemical should have concentrations covering a large range, e.g. 0.01-10/100 mg/l. In subsequent runs the concentration range should be narrower, e.g. 0.5-5 mg/l. The final concentrations are reached with a constant dilution factor

($\sqrt[8]{10}$).
Eight concentrations, with six replicates, should be run for each chemical on two separate occasions, excluding the initial rangefinder.

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

After 24 hours incubation, aspirate the medium from the cells.
Add 100µl of treatment medium containing the appropriate

concentration of test chemical.
Incubate at 37.5^{0}C for 24 hours.

After treating the cells for 24 hours, examine the cells under a phase contrast microscope. Record any changes in growth behaviour or morphology of the cells due to the cytotoxic effect of the test chemical. Estimate and record, microscopically, the Highest Tolerated Dose (HTD) i.e. the test substance concentration which causes the minimum morphological defects to the cells.

C) **Determination of Cell Growth Inhibition**

(i) ***Neutral Red measurement***
This method is based upon that of Borenfreund and Puerner, 1985. The uptake of the vital dye Neutral Red into the lysosomes/endosomes and vacuoles of living cells is used as a quantitative indication of cell number and viability.

At the end of the 24 hours treatment period.
Wash the cells with 100µl prewarmed PBS-with Ca^{2+} and Mg^{2+}. Remove the washing solution by gentle tapping.
Add 100µl NR medium and incubate at 37.5^{0}C, in a humidified atmosphere of 7.5% CO_2, for 3 hours.
After 3 hours incubation, remove the NR medium, and wash cells with 100µl PBS-with Ca^{2+} and Mg^{2+}.
Add exactly 150µl NR desorb (ethanol/acetic acid) solution.
Shake microtiter plate rapidly on a microtiter plate shaker for 10 minutes until NR has been extracted from the cells and formed a homogeneous solution.
Measure the absorbance of the resulting coloured solution at 540nm (reference filter 380nm) on a microtiter plate reader, using the blank as a reference.

(ii) ***Kenacid Blue measurement***
This method is based on that of Knox et al. (1986). The measurement of total cell protein provides a quantitative indication of cell number in a culture.

After assessing Neutral Red uptake. Remove the NR desorb solution.
Wash the cells once with 100µl NR desorb (ethanol/acetic acid) solution.
Add 100µl KB solution. Make up on day of use.
Shake plate for 10 minutes.
Remove unbound stain by washing twice with 150µl Kenacid Blue washing solution.
Shake plates for a further 10 minutes.
Centrifuge the plate at 600 x g. for 5 minutes to remove all the remaining washing solution.
Add 150µl Kenacid Blue desorb solution and rapidly shake plates for 10 minutes, until KB has been extracted from the cells and formed a homogeneous solution.
Measure the absorbance of the resulting coloured solution at 570nm (reference filter 380nm) on a microtiter plate reader, using the blank as a reference.

Results
An estimation of the number of viable cells (determined by the Neutral Red Uptake method) or total cell protein (measured using the Kenacid Blue method) is made on each culture dish as outlined above. The results obtained under test conditions are compared to the appropriate control and converted to a percentage value. The eight concentrations of each compound tested should span the range of, no effect up to 95-100% inhibition of cell growth.

A Hill function (% inhibition being a function of the concentration) is

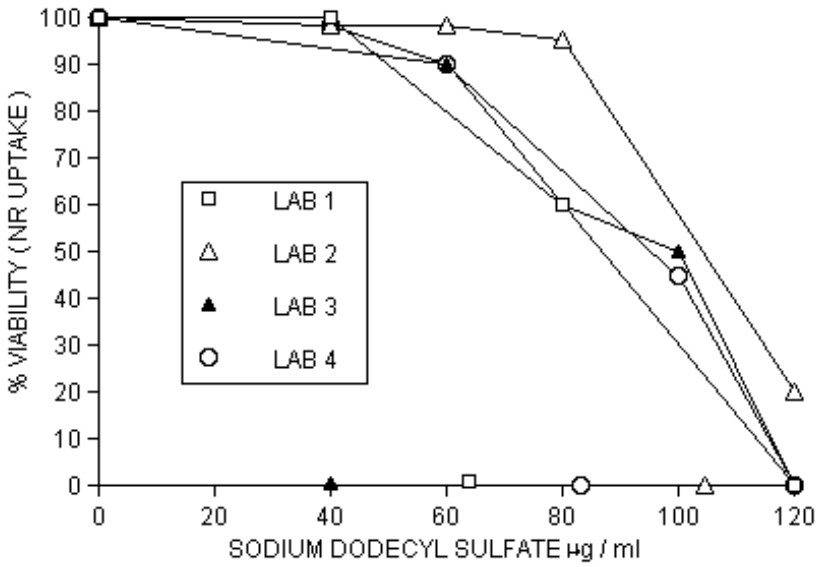
fitted to the results of the eight concentrations using the method of least squares. Hill functions are sigmoidal in shape and represent a good model for many dose response curves. IC 50-values together with the corresponding confidential intervals for the 200 chemicals of data base development will be calculated from the Hill function
The computer programme 'symphony' was adapted to the needs of the interlaboratory study by a software specialist. The programme has been modified to pick up data from four different types of Elisa readers. The average optical densities are 0.6 for NR and 1.0 for KB.

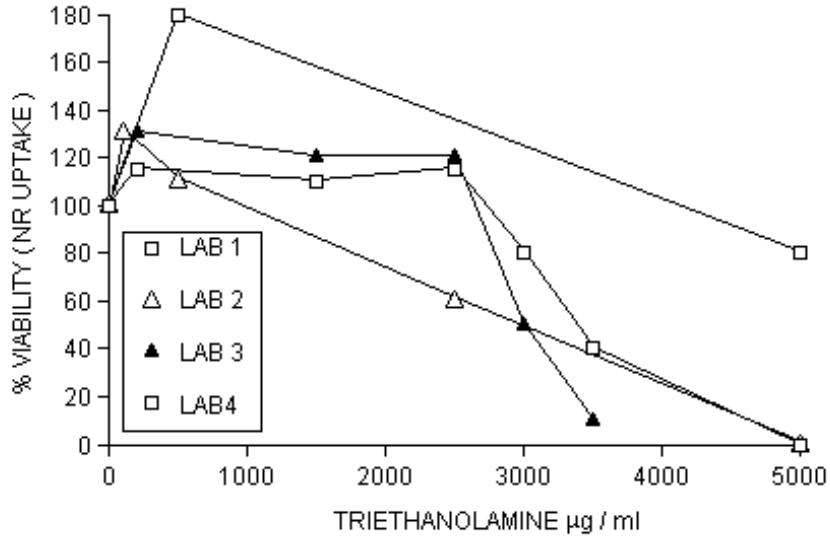
Preliminary results of the interlaboratory study are published in:

Spielmann et al. (1991) . Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany. ***Toxic. in Vitro***, ***5 No.5/6***, 539-542.

Experimental Data

Interlaboratory comparison of the Neutral Red cytotxicity test





Appendix: Scheme Of Neutral Red And Kenacid Blue Tests

Time (h)	Procedure

0.0 h	Seed 96-well plates with 1 x 10^4 cells in 100µl growth medium and incubate at 37.5^{0}C for 24 hours
24.0 h	Remove medium Add 100µl test solution or medium (zero control) with 5%NBCS and incubate at 37.5^{0}C for 24 hours
48.0 h	Detection of HTD value Remove test solution Wash once with 100µl PBS Add 100µl Neutral Red stock solution and incubate at 37.50C for 3 hours
51.0 h	Discard NR medium Wash once with 100µl PBS Centrifuge plate for 5 minutes at 600 g Add 150µl fixative (ethanol/acetic acid solution) Shake plate for 10 minutes
51.5 h	Detection of NR absorbance at 540nm i.e. cell viability
52.0 h	Discard fixative Wash once with 100µl fixative Add 100µl Kenacid Blue solution Shake plate for 10 minutes
52.5 h	Remove surplus Kenacid Blue by washing twice with 150µl Kenacid Blue washing solution Shake plate for 10 minutes
53.0 h	Discard Kenacid Blue washing solution Centrifuge plate for 5 minutes at 600g Add 150µl measuring solution Shake plate for 10 minutes
53.5 h	Detection of Kenacid Blue at 570 nm i.e. cellular protein Calculation of results IC_{50} values

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