

DB-ALM Protocol n° 78 : 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay

Phototoxicity

The cytotoxicity of the test compound to 3T3 cells is assessed by Neutral Red Uptake following exposure in the presence or absence of UVA light. (*Validation study protocol*)

Objective & Application

TYPE OF TESTING	: screening, adjunct
LEVEL OF ASSESSMENT	: toxic potential
PURPOSE OF TESTING	: classification and labelling

The test method was granted regulatory approval for phototoxicity testing (Method B.41, EU 2000, 2008; OECD Test Guideline 432, OECD 2004) and it is used to predict acute phototoxicity effects in animals and humans *in vivo* (OECD, 2004 and EU, 2008).

The 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT) is designed to detect the phototoxicity induced by the combined action of a chemical and light by using an *in vitro* cytotoxicity assay with the Balb/c 3T3 mouse fibroblast cell line.

The test identifies compounds that act *in vivo* phototoxic after systemic application, as well as compounds, including UV filter chemicals, that act as photoirritants after topical application and distribution to the skin.

The test is not designed to predict other adverse effects that may arise from combined action of a chemical and light nor allows an assessment of phototoxic potency (EU, 2000 and OECD, 2004).

Résumé

The basis of this test is a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of UVA light. Cytotoxicity is measured as an inhibition of the capacity of the cell cultures to take up a vital dye, neutral red, one day after treatment according to Borenfreund & Pruener (1985).

Within this SOP phototoxicity (photoirritation) is defined as a toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after systemic administration of a chemical (Spielmann *et al.*, 1994a).

Experimental Description

Endpoint and Endpoint Measurement:

CELL VIABILITY: Cell viability, determined as inhibition of the capacity of the cell cultures to take up vital dye, neutral red

NEUTRAL RED UPTAKE

Endpoint Value:

EC₅₀

Experimental System(s):

3T3 FIBROBLASTS: BALB/c 3T3 mouse fibroblast cell line

Basic Procedure

Balb/c 3T3 cells are maintained in culture for 24 hrs for formation of monolayers. Two 96-well plates per test chemical are then preincubated with eight different concentrations of the chemical for 1 hr. One plate is then exposed to a dose of 5 J/cm² UVA (+UV experiment), whereas the other plate is kept in the dark (-UV experiment). The treatment medium is then replaced with culture medium and after 24 hrs cell viability is determined by Neutral Red Uptake for 3 hrs.

Cell viability obtained with each of the eight concentrations of the test chemical is compared with that of untreated controls and the percent inhibition is calculated. For prediction of phototoxic potential the concentration responses obtained in the presence and in the absence of UV irradiation are compared, usually at the EC₅₀ level, i.e. the concentration inhibiting cell viability by 50% of untreated controls.

Data Analysis/Prediction Model

Two versions of a prediction model were applied by the independent statistician. The phototoxicity factor (PTF) version compared two equi-effective concentrations (the EC₅₀ value, defined as the concentration of test chemical, which reduces neutral red uptake by 50%) with and without UV light. However, since no EC₅₀ value was obtained for some chemicals in the absence of UVA, another version was devised, based on the Mean Phototoxic Effect (MPE), whereby all parts of the dose-response curves could be compared.

For further details see section 5 "Prediction Model" of the attached SOP.

Test Compounds and Results Summary

30 chemicals have been tested in the blind trial of the EU/COLIPA international validation study on *in vitro* tests for phototoxic potential (1992-1997). All selected chemicals were taken from those recommended by the ECVAM Workshop on *In vitro* Phototoxicity Testing (Spielmann *et al.*, 1994a). In a subsequent special study of ECVAM (1997) 8 UV filter chemicals were tested, all taken from the 1995 edition of Annex VII of the EU Cosmetics Directive 76/768/EEC (Spielmann *et al.*, 1998a)

Status

During 1992-1997, the 3T3 NRU PT was refined and evaluated in **the EU/COLIPA international validation study on *in vitro* tests for phototoxic potential** (Liebsch *et al.*, 1994 and Spielmann *et al.*, 1994b,c, 1998b)

Based on the successful outcome of the study, at its 9th meeting, the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed the statement that the 3T3 NRU PT was a scientifically validated test and it was ready to be considered for regulatory acceptance (ESAC, 1998a).

In addition, the test has also been evaluated in a subsequent special study on selected UV filter chemicals from Annex VII of EU Directive 76/768/EEC in 1997 (Spielmann *et al.*, 1998a)

ESAC unanimously endorsed also the outcome of the study with UV filter chemicals confirming the validity of the test, which demonstrated to be applicable for testing these type of chemicals for their phototoxic potential (ESAC, 1998b).

After the endorsement of ESAC, as well as by DG III and by DG XI, the 3T3 NRU Phototoxicity granted regulatory acceptance by the European Competent Authorities by including it into "Annex V as method B.41. Phototoxicity" of the "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" (EU, 2000).

Furthermore, in 2004, *In Vitro* 3T3 NRU Phototoxicity Test was adopted as the OECD Test Guideline No 432 (OECD, 2004).

In May 2008, all the test methods of Annex V of the Directive 67/548/EEC have been incorporated into the Annex to the Commission Regulation 440/2008/EC, laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (EU, 2008).

Last update: December 2008

PROCEDURE DETAILS, 7 September 1998

3T3 Neutral Red Uptake (NRU) Phototoxicity Assay DB-ALM Protocol n° 78

The protocol presents the standard operation procedure used in the EU/COLIPA international validation study on *in vitro* tests for phototoxic potential (1992-1997) (Spielmann *et al.*, 1998b).

During the preparation of the regulatory test guidelines some refinements have been introduced into the test method. Therefore, the proposed update of the SOP has been sent to the person responsible for the method for review and can be provided on request. As soon as new information will become available this version of the protocol will be updated.

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1. Background

The present *in vitro* test protocol was elaborated by the COLIPA Task Force "In vitro Photoirritation" in co-operation with ECVAM and ZEBET within the EU/COLIPA validation project of *in vitro* phototoxicity tests. The basic test design was developed in 1991 at Beiersdorf AG, Hamburg (D). The first SOP was drafted in 1992 by ZEBET, Berlin (D), and after approval by participating laboratories tested in a prevalidation trial within the joint EU/COLIPA initiative. The SOP was refined and approved again in 1994, processed as DB-ALM Protocol No. 78, and tested again in the formal validation study. From experience gained within the validation study the SOP was again refined in 1997. In addition to the test protocol, the present version contains a strategy for the use of solvents, and a suggestion for the maximum concentration of the test chemicals. The SOP was also used in the "ECVAM Special Study on UV filter chemicals (1997)", in which two versions of the prediction model were applied successfully.

2. Materials

2.1. CELL LINE

Balb/c 3T3 cells, clone 31, e.g. ECACC # 86110401
European Collection of Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK

Note: ATCC Balb/c 3T3, Clone 31, 163 CCL were successfully used revealing equivalent results in the EU/COLIPA validation study and ECVAM "Special Study".

Note: Since UV-sensitivity of the cells increases with ageing a sufficient stock of cells should be produced by inoculation and freezing of the cells at a low passage number (<100).

2.2. TECHNICAL EQUIPMENT

- UV-sun simulator, type SOL-500 (Dr. Hönle, D-82152 Planegg)
- UVA-meter(s), type No. 37 (Dr. Hönle, D-82152 Planegg)
- Filter, type H1 (Dr. Hönle, D-82152 Planegg)

- Any appropriate, adjustable and stable fixation of the SOL-500 (e.g. tripod)
- Incubator 37°C, humidified, 7.5 % CO₂/air
- Laminar flow clean bench (standard: "biological hazard")
- Water bath 37°C
- Phase contrast microscope
- Laboratory burner
- Centrifuge (optionally: equipped with microtiter plate rotor)
- Laboratory balance
- 96-well plate photometer equipped with 540 nm filter
- Shaker for microtiter plates
- Cell counter or haemocytometer
- Pipetting aid
- Pipettes, 8-channel-pipettes, dilution block
- Cryotubes
- Tissue culture flasks (80cm²) or petri dishes
- 96-well tissue culture microtiter plates (e.g. Nunc, # 167 008)

2.3. CHEMICALS, MEDIA, SERA

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

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

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

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

Trypsine/EDTA solution (e.g. ICN-Flow, # 16891-49)

Phosphate buffered saline (PBS)
without Ca⁺⁺ and Mg⁺⁺ (for trypsinisation)

Phosphate buffered saline (PBS)
with Ca⁺⁺ and Mg⁺⁺ (chemical treatment medium)

Earle's Balanced Salt Solution (EBSS)
without phenol red (e.g. ICN-Flow, #. 18-002-54)

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

Neutral Red

DMSO (analytical grade)

Ethanol (analytical grade)

Glacial acetic acid (analytical grade)

distilled H₂O or any purified water suitable for cell culture

2.4 PREPARATIONS

Note: All solutions (except Neutral Red stock solution, Neutral Red Medium and Neutral Red Desorb), glassware, etc. shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

2.4.1 Culture Medium

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

A) for Routine Culture
10 % NBCS
4 mM Glutamine
100 IU Penicillin
100 µg/mL Streptomycin

(B) for Freezing
20 % NBCS
7 - 10 % DMSO

(C) for Neutral Red Medium
10% NBCS
4 mM Glutamine
100 IU Penicillin
100 µg/mL Streptomycin

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

2.4.2 Neutral Red (NR) Stock Solution

0.4 g Neutral Red Dye
100 ml H₂O

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

2.4.3 Neutral Red (NR) Medium

1 ml Neutral Red Stock Solution
79 ml DMEM

The NR medium should be incubated overnight at 37°C and centrifuged at 600xg for 10 minutes (to remove NR crystals) before adding to the cells. Alternative procedures (e.g. Millipore filtering) can be used as long as they guarantee that the NR medium is free of any crystals.

2.4.4 Ethanol / Acetic Acid Solution (Neutral Red Desorb)

1 % Glacial Acetic Acid Solution
50 % Ethanol
49 % H₂O

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

2.4.5 Preparation of Test Chemicals

Exposure of the cells with test chemicals and subsequent irradiation is performed in buffered salt solution, since these treatment media are free of proteins and pH indicators. Test chemicals are dissolved either in EBSS or PBS. The highest final concentration of the test chemical shall not exceed 100µg/ml (Spielmann *et al.* 1998).

The solubility of the test chemical is assessed prior to the assay to establish the optimum solvent system, i.e.

whether the stock solution should be made in EBSS, PBS or in organic solvent. For pretesting of solubility, use of the hierarchical procedure shown in **Annex E** is recommended.

- Test chemicals that are soluble in water up to 100µg/ml should be dissolved in sterile prewarmed (37°C) EBSS or PBS.
- Test chemicals of limited solubility in water (< 0.1mg/ml) should be dissolved in Dimethylsulphoxide (DMSO) at 100-fold the desired final concentration. Ethanol (ETOH) may be considered as the third optional solvent.
The solvent shall be present at a constant volume of 1% (v/v) in the negative controls and in all eight test concentrations, i.e. the test chemical is dissolved in DMSO or ETOH and 1 part of this stock solution is added to 99 parts of sterile prewarmed (37°C) EBSS or PBS.

Measure the pH of the highest concentration of the test chemical. Since strong acids and bases may influence the buffer capacity of EBSS or PBS, they should be neutralised with 0.1n NaOH or 0.1n HCl. In this case, prepare highest concentration of the chemical in ~ 80% of final EBSS/PBS volume, measure pH, neutralise, and add EBSS or PBS to final volume.

Vortex mixing and/or sonication and/or warming to 37°C may be used, if necessary, to aid solubilization. The concentrations used for relatively insoluble chemicals should range from the soluble to the precipitating dose.

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

3. Methods

3.1 CALIBRATION OF THE SOLAR SIMULATOR

The SOL-500 shall be calibrated as described below on each occasion before performing a phototoxicity assay.

1. Mount the SOL-500, equipped with a H1-filter, on any appropriate stable tripod allowing fine-adjustment of the exposure distance.
2. Adjust SOL-500 to a distance of ~ 60cm.
3. Measure irradiance with calibrated UV radiometer (Dr. Hönle #0037) equipped with UVA-sensor of the same serial number.
4. Fine adjust distance of SOL-500 to achieve an UVA irradiance of 1.7mW/cm² (resulting dose: 1J/cm² per 10min. exposure). According to the number of plates to be exposed, check the exposure area for equal distribution of irradiance: A range of 1.6-1.8mW/cm² is acceptable. A maximum difference of 1.5-1.9mW/cm² can be accepted, if the positions of the plates with low and high irradiance are exchanged after half time of the irradiation (25 minutes).

Note: In the EU/COLIPA study the solar simulator SOL-3 (Dr. Hönle) was successfully used instead of the SOL-500. Spectra of SOL-3 and SOL-500 are nearly identical in short wavelengths up to 550 nm. In the upper range of visible light (> 550 nm - 700nm) the SOL-500 irradiance decreases, whereas the irradiance of the SOL 3 remains at the same level.

Note: New metal mercury halide burners should be burned for about 100 hrs prior to first use to achieve a stable spectral emittance.

Note: Make sure, the 9 Volt battery of the UV radiometer has a sufficient capacity, since a weak battery will influence precision of the readings without warning on the display!

Note: In case measurements with the UV radiometer reveal unexpected results, either the burner may have reached the end of its life, or the radiometer is decalibrated due to various reasons. In this case, a second reference radiometer of the same type and calibration, which has not been handled every day and kept in the dark shall be used for cross checking.

3.2 QUALITY CHECK OF CELLS: UVA SENSITIVITY

Note: When the 3T3 NRU PT test is established for the first time, it is most important to check the UV sensitivity of the 3T3 cells. Once the Test is successfully established, it is sufficient to perform the UV

sensitivity check on a regular basis, e.g. once every six months.

Prepare 10 microtiter plates with 1×10^4 cells /100 μ L per well and grow overnight. Next day, replace medium with EBSS or PBS. Keep one plate in the dark at room temperature, and irradiate nine plates with 1.7mW/cm² UVA.

Expose the 1st plate 10min (= 1J/cm²), the 2nd plate 20 min (= 2J/cm²) ...etc. up to the 9th plate. Replace EBSS or PBS with DMEM and incubate all ten plates overnight. Determine cell viability (NRU uptake) according to 3.6 "**Test Procedure**" for each the 9 irradiated plates and compare with viability of the control plate kept in the dark (= 100%). Plot cell viability vs. UVA dose.

The cells meet acceptance criteria if after irradiation with a UVA dose of 5J/cm² (UVA dose used in the phototoxicity test) viability is at least 80% of non irradiated cells. At 9J/cm² the reduction of cell viability should not exceed 50%.

3.3 QUALITY CHECK OF ASSAY (I): POSITIVE CONTROL

Chlorpromazine (CPZ) tested in a full-scale phototoxicity test on two plates according to 3.6 "**Test Procedure**" concurrently with the test chemicals serves as positive reference. Determine NRU in the absence (-UVA) and presence (+UVA) of irradiation (5 J /cm²):

A test meets acceptance criteria, if for CPZ

the EC₅₀ + UVA is within the range of: 0.1-2.0 μ g/ml

the EC₅₀ - UVA is within the range of: 7.0-90.0 μ g/ml

the factor (PIF) between the two EC₅₀ values is at least: 6

3.4 QUALITY CHECK OF ASSAY (II): NEGATIVE CONTROL

The absolute value of optical density (OD540 of NRU) obtained in the untreated negative control indicates whether the 1×10^4 cells seeded per well have grown exponentially with normal doubling time during the two days of the assay:

A test meets acceptance criteria if the mean OD540 of untreated controls is >0.3

To check for systematic errors, untreated negative controls are placed both at the left side (row 2) and the right side (row 11) of the 96-well plate (see ANNEX B):

A test meets acceptance criteria if the left and the right mean negative control does not differ by more than 15% from the mean of all controls (mean \pm 15%).

3.5 CONCENTRATIONS OF TEST CHEMICAL

3.5.1. Range finder Experiment

Test +UVA and -UVA eight concentrations of the test chemical by diluting the stock solution with a constant factor (e.g. $2\sqrt[10]{10} = 3.16$, see ANNEX D) with EBSS/PBS, covering a large range, e.g.:

1 » 3.16 » 10 » 31.6 » 100 » 316 » 1.000 » 3.160 μ g/ml

3.5.2 Main Experiment

Depending on the slope of the concentration- response curve estimated from the range finder, the dilution factor in the concentration series of the main experiment should be smaller (e.g. $6\sqrt[10]{10} = 1.47$): Try to cover the relevant concentration range, avoiding too many non-cytotoxic and/or 100%-cytotoxic concentrations.

Experiments revealing less than three cytotoxic concentrations (between 10% and 90% effect) shall be repeated - where possible - with a smaller dilution factor.

3.6. TEST PROCEDURE (SEE ANNEX A)

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

1. Prepare a cell suspension of 1×10^5 /ml in culture medium. Using a multi-channel pipette, dispense 100 μ L culture medium only into the peripheral wells of a 96-well tissue culture microtiter plate (=blanks). In the remaining wells, dispense 100 μ L of a cell suspension of 1×10^5 cells/ml (= 1×10^4 cells/well). Per one test chemical, prepare two plates each: one for determination of **cytotoxicity (-UVA)**, and the other for determination of **phototoxicity (+UVA)**.
2. Incubate cells for **24 h** (7.5% CO₂, 37°C) until they form a half-confluent monolayer. This incubation period allows for cell recovery and adherence

2nd day

1. After incubation, decant culture medium from the cells and wash twice with 150 μ L EBSS/PBS. Add 100 μ L of EBSS/PBS containing the appropriate concentration of test chemical. Incubate cells for 1 h (7.5% CO₂, 37°C).
2. To perform the **+UVA** part of the assay, irradiate the cells at room temperature for 50min through the lid of the 96-well plate with 1.7mW/cm² (= 5 J/cm²). Ventilate with a fan to prevent H₂O condensation under the lid. Keep duplicate plates **(-UVA)** at room temperature in a dark box for 50min (= UVA exposure time).
3. Decant test solution and wash twice with 150 μ L EBSS/PBS. Replace EBSS/PBS by culture medium and incubate at 37°C overnight (18-22 h).

3rd day

A) Microscopic Evaluation

Examine cells under a phase contrast microscope. Record changes in morphology of the cells due to cytotoxic effects of the test chemical. This check is performed to exclude experimental errors. Records are not used for evaluation of cytotoxicity or phototoxicity.

B) Measurement of Neutral Red Uptake (NRU)

This method is based upon that of Ellen Borenfreund (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.

1. Wash the cells with 150 μ L prewarmed EBSS/PBS. Remove the washing solution by gentle tapping. Add 100 μ L Neutral Red (NR) medium and incubate at 37°C, in a humidified atmosphere of 7.5% CO₂, for 3 hrs.
2. After incubation, remove the NR medium, and wash cells with 150 μ L EBSS/PBS.
3. Decant and blot EBSS/PBS totally. (Optionally: centrifuge reversed plate.)
4. Add exactly 150 μ L NR Desorb (ethanol/acetic acid) solution.
5. Shake microtiter plate rapidly on a microtiter plate shaker for 10min until NR has been extracted from the cells and formed a homogeneous solution.
6. Measure the absorption of the resulting coloured solution at 540nm in a microtiter plate reader, using the blanks as a reference. Save file in Standard ASCII format.

4. Data Analysis

To apply the rules for predicting phototoxic potential (see section 5 "**Prediction Model**") it is necessary to analyse the concentration-cytotoxicity response curves concurrently obtained in the presence (+UV) and in the absence (-UV) of irradiation. Two versions of the PM exist, one based on comparison of single concentrations (PIF, see 5.1) and one based on comparison of two curves (MPE, see 5.2). To apply the PIF model, any appropriate procedure can be used to calculate the EC₅₀, i.e. the concentration inhibiting cell viability by 50%. For application of the MPE model, a special software program is needed.

4.1 DETERMINATION OF EC50 FOR PIF (SEE SECTION 5.1)

Where possible, the concentration of a test chemical reflecting a 50% inhibition of cell viability (EC_{50}) is determined. This can be done either

- by applying any appropriate non-linear regression procedure (preferably a Hill function or logistic regression) to the concentration-response data. Before using the EC_{50} for further calculations, the quality of the fit should be appropriately checked.

or

- by applying a simple graphical fitting method. In this case it is recommended to use probability paper with "x=log" and "y=probit" scales as in most cases the concentration response function will become almost linear after this transformation.

4.2 COMPARISON OF CONCENTRATION RESPONSE CURVES FOR MPE (SEE SECTION 5.2)

To apply the refined MPE prediction model a special software has to be used. This software uses the algorithms published by Holzhütter (1997). The software is presently available for MS Windows 3.x ("PHOTO16") and will be available later for Windows 95 ("PHOTO32").

Data files of optical densities (OD540) generated by the microplate reader are imported as ASCII files into NRU-PIT2, which saves data in a standard format and fits the concentration response curves with the procedure FitGraph, specially developed for the proper handling of non-monotonous curves (Holzhütter & Quedenau 1995).

In addition, the program compares corresponding (+UV) and (-UV) couples of experiments, predicts the phototoxic potential (both according to the PIF model and the MPE model) and calculates a toxicity probability which takes into account whether predictions based on results near the classification cut-off are influenced by the intra-assay and inter-assay variability.

5. Prediction Model

Note: The mathematical rules for the prediction of *in vivo* toxicity potential from *in vitro* test data is called Prediction Model (PM, for details see Archer et al., 1997). In the present test prediction of phototoxic potential is based on comparison of the two concentration response curves concurrently obtained in the presence (+UVA) and absence (-UVA) of UVA irradiation. This can be achieved either by comparison of two equally effective inhibition concentrations (original PM, see 5.1), or, by comparison of the +UV and -UV concentration response curves on a grid of concentrations from the concentration range shared by both curves (refined PM, see 5.2).

5.1 ORIGINAL VERSION BASED ON THE PHOTO-IRRITANCY FACTOR (PIF)

The PM was developed from interlaboratory data obtained in a EU/COLIPA prevalidation study (Spielmann et al., 1994a,b) and applied in a formal validation study (Spielmann et al., 1997).

It is based on comparison of two equally effective cytotoxic chemical concentrations (EC_{50} values) obtained in concurrently performed experiments in the presence (+UV) and absence (-UV) of UVA irradiation, which are used to calculate a photo-irritancy factor (PIF):

$$(1) \quad PIF = \frac{EC_{50}(-UV)}{EC_{50}(+UV)}$$

Discriminant analysis of the PIFs obtained in the prevalidation study revealed a cut-off value of $PIF = 5$ for phototoxic potential (e.g. Liebsch et al., 1994). The resulting classification rule is:

$$(2) \quad > PIF = \frac{C_{\max}(-UV)}{EC_{50}(+UV)}$$

If **PIF < 5**: no phototoxic potential predicted. If **PIF > 5**: phototoxic potential predicted

The PIF can be calculated only if the concentration-response curves obtained in the presence and the absence of UV-light drop down below 50% of the controls, because only in these cases two EC₅₀ values (-UVA and +UVA) can be determined.

Therefore, the prediction model takes into account two additional classification rules:

If a chemical is only cytotoxic +UV and is not cytotoxic when tested -UV, the PIF cannot be calculated, although this is a result indicating phototoxic potential. In these cases, a > PIF can be calculated if the (-UV) cytotoxicity test is performed up to the highest test concentration (C_{max}) and this value is used for calculation of the > PIF:

$$(2) \quad > \text{PIF} = \frac{C_{\max}(-UV)}{EC_{50}(+UV)}$$

Since the ">PIF" is not an exact numerical value, no biostatistical procedure could be applied to determine the optimum cut-off. Consequently, the classification rule has to be:

If only a "> PIF" can be obtained, then any value >1 predicts phototoxic potential.

If both, EC₅₀ (-UV) and EC₅₀ (+UV) cannot be calculated due to the fact that a chemical does not show any cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In these cases a formal "PIF = *1" is used to characterise the result:

$$(3) \quad \text{PIF} = *1 = \frac{C_{\max}(-UV)}{C_{\max}(+UV)}$$

A "PIF = *1" predicts no phototoxic potential

Note: In cases (2) and (3) the absolute concentrations achieved should be taken into consideration.

5.2 REFINED VERSION OF THE PREDICTION MODEL BASED ON THE MEAN PHOTO EFFECT (MPE)

A major limitation of the PIF prediction model is the fact that the PIF is based on the comparison of two equally-effective concentrations (EC₅₀) in the dark and light experiments, which cannot be determined in every case.

To overcome this limitation, a novel measure for the phototoxic potential of chemicals, the mean photo effect (MPE), has recently been proposed (Holzhütter, 1997). It is based on a comparison of the +UV and -UV concentration response curves on a grid of concentrations i ($i=1, \dots, N$) chosen from the common concentration range of the (-UV) and (+UV) experiments. The photo effect (PE _{i}) at concentration c_i is computed as a product of the concentration effect (CE _{i}) and the response effect (RE _{i}). The mean photo effect (MPE) is obtained by averaging across all PE _{i} values.

Analogous to PIF, the MPE can be used in a prediction model for the phototoxic potential of chemicals by comparing it with a critical cut-off value, MPE_c. The cut-off value MPE_c = 0.1 was derived from a first application of the MPE-based prediction model to data obtained in phase II of the EU/COLIPA study in a test carried out by the FRAME/University of Nottingham laboratory according to the same test design, but with primary human keratinocytes instead of 3T3 cells (Holzhütter, 1997).

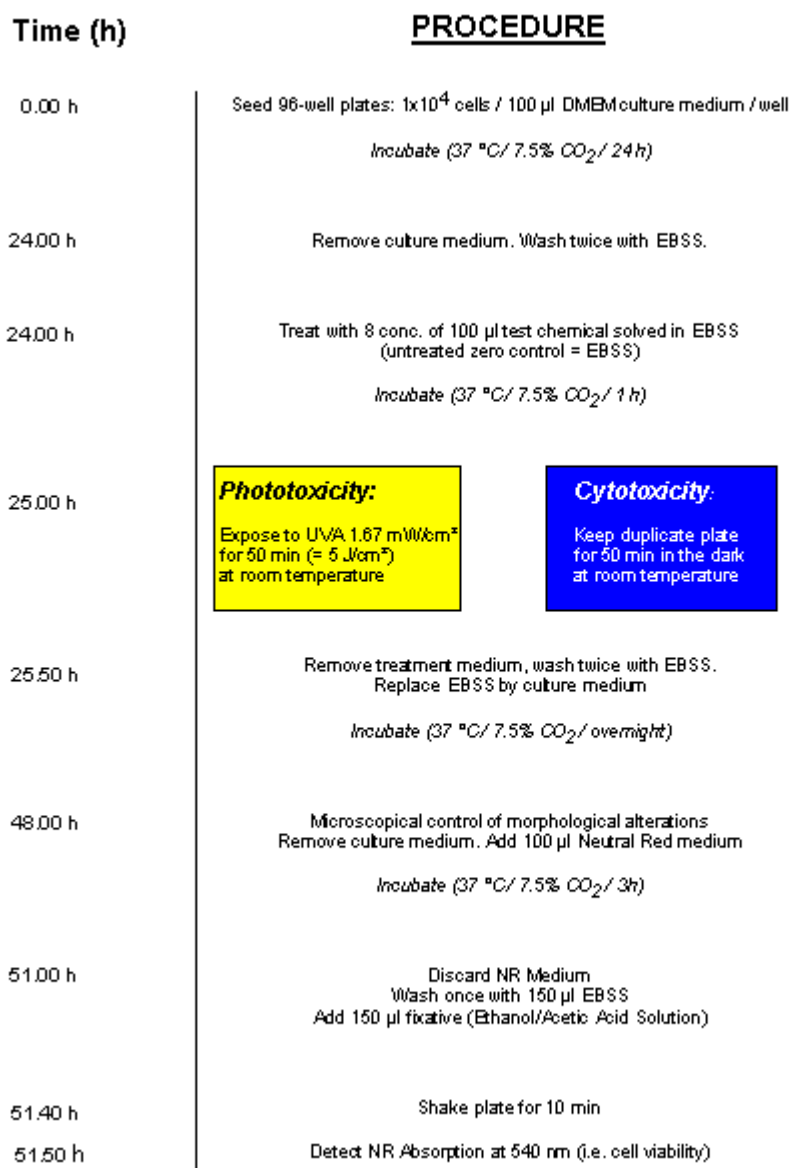
MPE

prediction of *in vivo* phototoxic potential

< 0.1	non-phototoxic
≥ 0.1	phototoxic

Note: In contrast to the PIF, the MPE cannot be easily calculated by everyone without using a special software. The development of a special program "PHOTO16" (for MS Windows 3.11) or "PHOTO32" (for Windows 95) by Dr. Holzhütter was funded by ECVAM and ZEBET and is, therefore, freeware available from ZEBET.

ANNEX A NRU PT Test: Flow Chart



ANNEX B 96-well plate configuration

Note: The plate configuration shown below is a recommendation. In case the software NRU-PIT2 is used for analysing the data the configuration has to be exactly as shown below.

Note: Since evaporation may take place in the peripheral wells, it is recommended to use these wells for blanks only, which correct possible adsorption of Neutral Red to the plastic.

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	B	b	b	b	b	b	b	b	b
B	b	UC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	UC	b
C	b	UC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	UC	b
D	b	UC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	UC	b
E	b	UC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	UC	b
F	b	UC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	UC	b
G	b	UC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	UC	b
H	b	b	b	B	b	b	b	b	b	b	b	b

- UC = UNTREATED CONTROL
(mean viability set to 100%)
- C1 - C8 = TEST CHEMICAL at eight concentrations
(C1 = lowest, C8 = highest)
- b = BLANKS
(containing no cells, but treated with NR medium
and with NR Desorb solution)

ANNEX C Cell Maintenance and Culture Procedures

Balb/c 3T3 cells are routinely grown as a monolayer in 80cm² tissue culture grade flasks, at 37°C in a humidified atmosphere of 7.5 % CO₂. 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 medium and rinse cultures with PBS minus Ca⁺⁺ / Mg⁺⁺ (~5 ml is used for a 25 cm² 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 for a few seconds.
- Remove excess Trypsin-EDTA solution and incubate the cells at 37 °C.
- After 2 - 3 min, 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/cm flask i.e. 2.5 ml for a 25 cm² 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 $\sim 1 \times 10^6$ cells in 80cm² flasks, every 3 - 4 days (average doubling time, 20-24 h).

Note: It is important that cells are in 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 $1-5 \times 10^6$ cells/ml.
- Aliquot 120-180 μ l of cooled DMSO into freezing tubes and fill to 1.8ml with the cell suspension.
- Place the tubes into a freezer at -80°C for 24h. This gives a freezing rate of 1°C/min.
- Place the frozen tubes into liquid nitrogen for storage.

Thawing

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

- Resuspend the cells and transfer into routine culture medium.
- Incubate at 37°C in a humidified 7.5% CO₂ atmosphere.
- When the cells have attached to the bottom of the flask (this may take up to 4 hrs), 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.

ANNEX D Decimal Geometric Concentration Series

In general dose-response relationships are non linear, but can be linearised to some extent by logarithmic transformation of the x-axis. Usually this has to be done when EC₅₀ values are calculated either by regression analysis or by graphical estimation.

If dose series (in cell culture: concentration series!) are done with arithmetic steps, transformation of the x-axis will result in an unequal distribution of measuring points. Therefore, the use of geometric concentration series (= constant dilution factor) is recommended. The most simple geometric series are dual geometric series, like e.g. factor 2. These series have the disadvantage of permanently changing chains within the series (2, 4, 8, 16, 32, 64, 128, 256...).

The decimal geometric series, first described by Hackenberg & Bartling (1959) for the use in toxicological and pharmacological studies has the advantage, that independent experiments with wide and with narrow dose factors can be easily compared, and, furthermore under certain circumstances can even be merged together:

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 dose factor of **3.16** ($= 2\sqrt{10}$) divides a decade into 2 equal chains, the dose factor of **2.15** ($= 3\sqrt{10}$) divides a decade into 3 equal chains, the dose factor of **1.47** ($= 6\sqrt{10}$) divides a decade into 6 equal chains, and the dose factor of **1.21** ($= 12\sqrt{10}$) divides the decade into 12 equal chains.

Therefore, for reasons of an easier biometrical evaluation of the data it is recommended to use decimal geometric concentration series rather than dual geometric series.

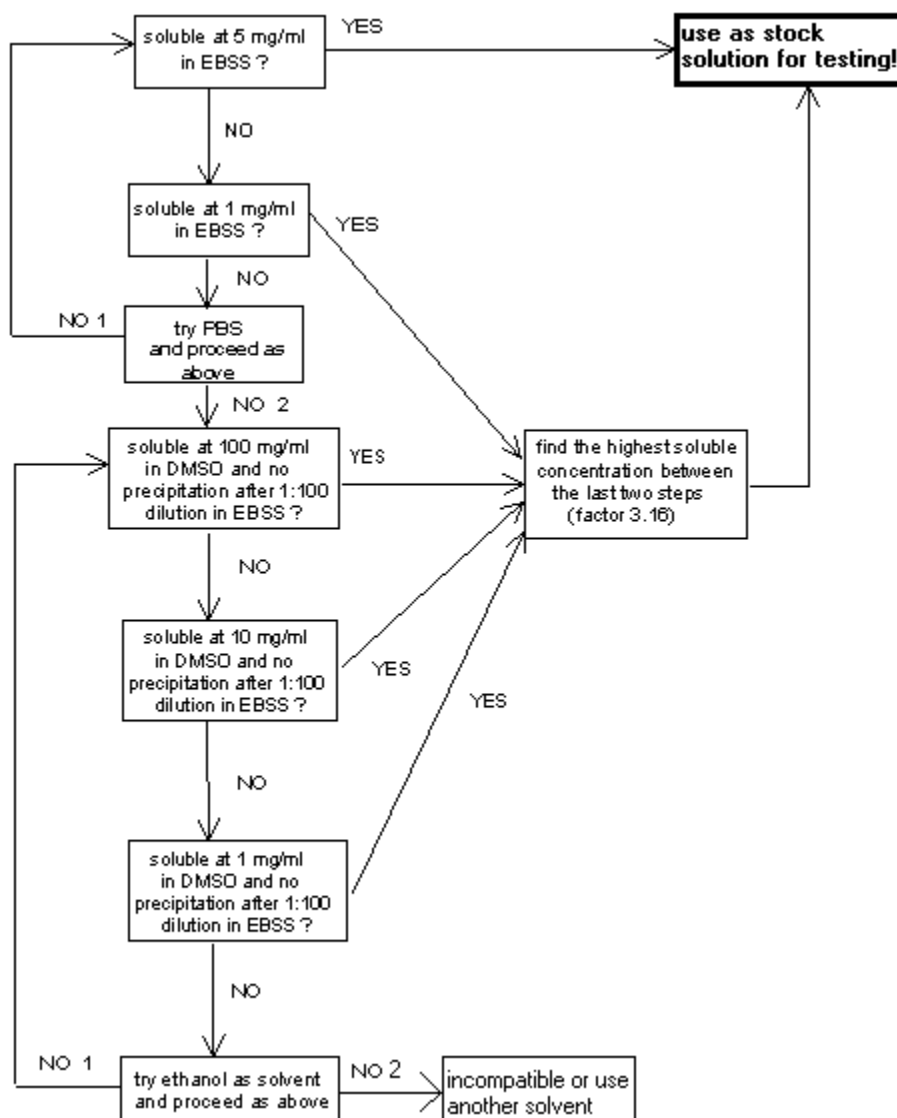
The technical production of decimal geometric concentration series is very easy. An example is given for 1.47:

Dilute 1 volume of highest dose by adding 0.47 volumes of diluent. After equilibration dilute 1 volume of this solution by adding 0.47 volumes of diluent...(and so on).

Reference:

Hackenberg, U. & Bartling, H. (1959)
Arch. exp. Pathol. Pharmacol., **235**: 427-463

ANNEX E Strategy for the Use of Solvents



ANNEX F Technical Info on Irradiation Equipment

Reasons for selection of the sun simulator

For phototoxicity testing artificial sources of UVR (solar simulators) shall be used. Xenon arc sources filtered with a SCHOTT WG 320 filter are often recommended and widely used in biological determination of sun protection factors (SPFs) of sunscreens.

These sources are expensive. In contrast to testing of sunscreens with high SPFs, in phototoxicity testing the high irradiance of xenon arc lamps is not needed. In addition, they emit too much heat. Therefore, the best choice in this area is a *doped mercury metal halide source*.

Description of the SOL-500 and Filters

In the EU/COLIPA project laboratories used an identical solar simulator **SOL-500** (Dr. Hönle) and identical UVA radiometer for calibration. The light emission of solar simulators **SOL-500**, SOL 1200 and SOL 2000 is produced by gas discharge of metal halide amongst other things (=doping). The irradiance of the source in the UV/visible range of 280-780nm is higher than found in nature. Thus, SOLs are mostly used for accelerated material ageing tests.

For the 3T3 NRU PT Test, a spectrum almost devoid of UVB (< 320nm) was achieved by filtering with ~50% transmission at a wavelength of 335nm (filter: H1). For the Photo RBC Test UVB was partly included by filtering with the H2 filter. For the EpiDerm™ phototoxicity test H1 or H2 can be used equivalently.

H1 and H2 filter characteristics compared to SCHOTT WG 320 are given in the following **Table**:

Wavelength (nm)	H1 Transmiss. (%)	H2 Transmiss. (%)	WG320 Transmiss. (%)
300	0	0	0
310	2.2	12	9
320	17.3	40	45
330	44.8	70	73
340	67.8	84	85
350	80.3	88	88
360	86.0	89	89
370	87.8	90	90

To avoid bacterial contamination during irradiation, in the 3T3 NRU PT Test (and EpiDerm™ PT Test) irradiation is performed through the polystyrene lid of the of the culture plates, which has an additional filter effect. This effect is shown in **Figure 1**.

Calibration

Calibration of the **SOL-500** shall be checked each time before performing a phototoxicity test. In case of unexpected readings, either the metal halide burner may have reached the end of it's life (usually > 3.000 hrs), or the radiometer is de-calibrated due to various reasons. In this case, a second reference radiometer of identical type and calibration, which has not been handled every day shall be used for cross check.

1. Mount **SOL-500**, equipped with a H1-filter, on any appropriate stable tripod allowing fine-adjustment of the exposure distance.
2. Adjust **SOL-500** to a distance of ~ 60 cm.
3. Switch **SOL-500** on, wait 15min. and measure irradiance through lid of a cell culture plate using UV radiometer (type 37), equipped with an UVA-sensor of the same serial number.

4. Fine adjust distance of **SOL-500** to achieve a UVA irradiance of $1.7\text{mW}/\text{cm}^2$ (The resulting dose will be $1\text{J}/\text{cm}^2$ per 10min. exposure time). According to the number of plates to be concurrently exposed, check the exposure area for equal distribution of irradiance: A range of $1.6\text{-}1.8\text{mW}/\text{cm}^2$ is acceptable. A maximum difference of 1.5 and $1.9\text{mW}/\text{cm}^2$ can be accepted, if positions of two plates with low and high irradiance are changed after half time of the irradiation is reached.

Filtered spectrum of **SOL-500**

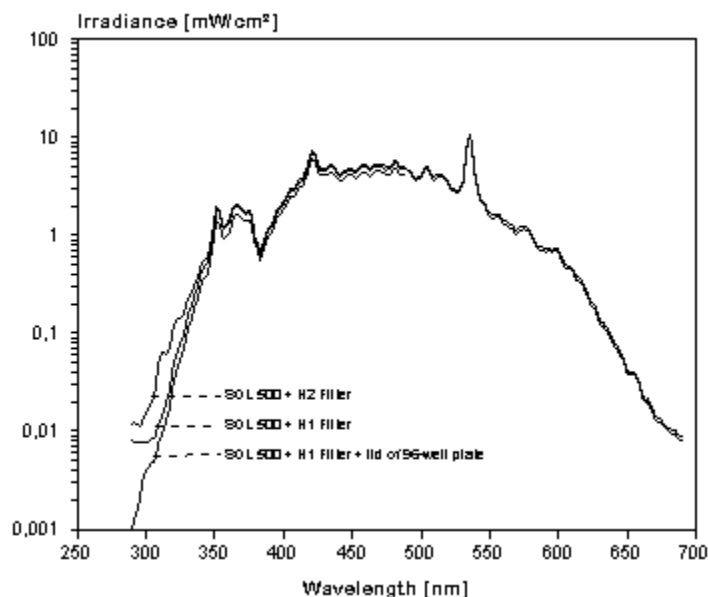


Figure 1 (spectrum kindly provided by Beiersdorf AG)

Purchase information

Dr. K. Hönle GmbH, tel. +49-89-85 608-0
 Fraunhoferstraße 5, fax +49-89-85 608-48
 D-82152 Planegg, Germany
 contact: Dr. G. Schmid / H. J. Herrmann

SOL-500 # 5468
 H1 filter + frame # 4730
 Power unit # 0298 *
 UVA radiometer # 0037

* **please Note:** This # only holds for 230 V~ / 50 Hz
 on request other units can be purchased.

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