# MB RESEARCH LABORATORIES
# STANDARD PROTOCOL
# 702-03

**1.0 TITLE OF STUDY: 3T3 Neutral Red Uptake Phototoxicity Assay**

**2.0 OBJECTIVE:** The cytotoxicity and phototoxicity of the test compound to 3T3 cells (in the presence or absence of UVA light) is assessed by Neutral Red Uptake. The 3T3 Neutral Red Uptake Phototoxicity Assay (3T3 NRU PT), based on the OECD Guideline for Testing of Chemicals: No. 432, is designed to detect the phototoxicity induced by the combined action of a test article and solar-simulated UVA + visible light in an *in vitro* cytotoxicity assay using the Balb/c 3T3 mouse fibroblast cell line as the test system. The assay identifies aqueous-soluble compounds (or formulations) that have the potential to exhibit *in vivo* phototoxicity after systemic application.

**3.0 TEST ARTICLE:**

3.1: Source: All test articles will be supplied by the Sponsor. Prior to initiation of the study, the Sponsor should provide the Study Director with test article characterization (refer to Section 13 herein).

3.1.1: Absorbance Spectrum: The Sponsor will supply MB Research with the UV-VIS absorbance spectrum of the test article prior to study initiation.

3.2: Label: Each test article will be identified by source, name and/or code number, date of receipt at MB Research, and MB Project Number.

3.3: Test Article Description: The observable physical properties of the test article will be recorded.

3.4: Storage: Refer to Section 13.

3.5: Safety: Based on the information provided by the Sponsor, appropriate routine safety precautions will be exercised in the handling of the test article.

3.6: Vehicle and Controls will be considered 100% active/pure for the purpose of dosage calculations.

3.6.1: Vehicle: The vehicle will be sterile Hanks' Balanced Salt Solution (HBSS). If necessary, a test article can be prepared in a solvent (e.g. Dimethyl sulfoxide (DMSO), or ethanol) at 100-fold the desired final concentration and will be specified in Section 13 of this protocol.

3.6.2: Positive Control: Chlorpromazine (CPZ), source and other I.D. or composition data will be recorded in the raw data and included in the final report.

**4.0 TEST SYSTEM AND JUSTIFICATION:**

4.1: Test System: Cells are available from the American Type Culture Collection (ATCC), or from the European Collection of Cell Cultures (ECACC).

4.2: Justification: Balb/c 3T3 cells are recommended by the test guidelines.

## 5.0 EXPERIMENTAL DESIGN:

5.1: Materials:

- Hanks' Balanced Salt Solution (HBSS) (Sigma, cat.# H8264, or equivalent)
- Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma, cat.# D8662, or equivalent)
- Chlorpromazine (CPZ) (Sigma, cat.# C0982, or equivalent)
- Neutral Red (NR) medium (Sigma, cat.# N2889, or equivalent)
- Solar Simulated Light (SSL) – Honle SOL-500 solar simulator, emitting ultraviolet A (UVA) light, with an H-1 filter to cut off UVB and UVC

5.2: Basis of the Method: The test compares the cytotoxicity of a test article when tested in the presence and in the absence of a non-cytotoxic dose of Solar Simulated light (SSL). Cytotoxicity will be 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)[1].

5.3: Experimental Description:

| | | |
|---|---|---|
| **Endpoint and Detection** | **:** | Cell viability, determined as inhibition of the capacity of the cell cultures to take up the vital dye, Neutral Red |
| **Test Parameter** | **:** | $EC_{50\ No\ SSL}$, $EC_{50+SSL}$ |
| **Test System** | **:** | Balb/c 3T3 mouse fibroblast cell line (clone A31 or equivalent; ATCC CCL-163, or ECACC #86110401) |

Balb/c 3T3 cells will be seeded in microplates and maintained in culture for 24 hours for formation of monolayers. Ideally, the central 60 wells of the microplate will be used. Two 96-well plates per test article will be preincubated with up to eight different concentrations of the test article for one hour. One plate will be exposed to a dose of 5 J/cm² SSL, while the other plate will be kept in the dark (No SSL). The treatment medium will be replaced with culture medium and, after 24 hours, and the cell viability will be determined by Neutral Red Uptake for 3 hours (See Appendix A).

Cell viability obtained with each of the concentrations of the test article will be compared with that of vehicle controls and the percent inhibition (of viability) will be calculated. For prediction of phototoxic potential, the concentration responses obtained in the presence and absence of SSL irradiation will be compared, usually at the $EC_{50}$ level, i.e. the concentration inhibiting cell viability by 50% of vehicle controls.

5.4: Neutral Red (NR) Medium: NR solution will be prepared to yield a 50 µg/ml working solution. The NR medium should be allowed to stand at room temperature, or incubated overnight at 37ºC ± 1ºC, and centrifuged at approximately 600G for 10 minutes (to remove NR crystals) before adding to the cells. Alternatively, 0.2 – 10 µm filtering can be used.

5.5: Neutral Red Extractant:

1) 1.0% Glacial Acetic Acid Solution, 50% Ethanol, 49% $H_2O$
   or
2) 95% Ethanol, 5% $H_2O$

The Neutral Red Extractant is best prepared immediately prior to use.

5.6: Preparation of Test Articles: Exposure of the cells with test articles and subsequent irradiation will be performed in buffered salt solution, since these treatment media are free of proteins and pH indicators. Test articles will be dissolved in either HBSS or DPBS. The highest final concentration of the test article shall not exceed 1000 µg/ml (Spielmann et al., 1998)[2]

The solubility of the test article will be assessed prior to the assay to establish the optimum solvent system, i.e. whether the stock solution should be made in HBSS, DPBS or in organic solvent. For pretesting of solubility, use of the hierarchical procedure shown in Appendix B is recommended.

- Test articles that are soluble in water up to 1000 µg/ml should be dissolved in sterile prewarmed (37ºC ± 1ºC) HBSS or DPBS.

- Test articles of limited solubility in water (< 1000 µg/ml) should be dissolved in Dimethylsulfoxide (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 vehicle controls and in all eight test concentrations, i.e. the test article will be dissolved in a solvent (vehicle) consisting of 1 part DMSO or EtOH added to 99 parts of sterile prewarmed (37ºC ± 1ºC) HBSS or DPBS.

The pH of the highest concentration of the test article will be measured. Since strong acids and bases may influence the buffer capacity of HBSS or DPBS, they should be neutralized with 0.1N NaOH or 0.1N HCl. In this case, the highest concentration of the test article in approximately 80% of final HBSS/DPBS volume will be prepared, the pH will be measured, neutralize, and HBSS or DPBS will be added to the final volume. Vortex mixing and/or sonication and/or warming to 37ºC ± 1ºC may be used, if necessary, to aid solubilization. The concentrations used for relatively insoluble test articles should range from the soluble to the precipitating dose. If a precipitate is present, the concentration will be centrifuged at 100-200G for 5 min and the supernatant will be used for dosing.

The test article must be freshly prepared immediately prior to use. Preparation under red or yellow light may be necessary if rapid photodegradation is known or likely to occur.

5.7: Selection of Concentrations:

5.7.1: Range Finder Screen: Eight concentrations of the test article will be tested, with both + SSL and No SSL. Concentrations will be prepared by diluting the stock Test Article solution with a constant factor (e.g. $^{2}\sqrt{10} = 3.16$, see Appendix C) with HBSS/DPBS, covering a large range starting at 1000 µg/ml, or 0.1%, e.g.:

1000 ⇒ 316 ⇒ 100 ⇒ 31.6 ⇒ 10 ⇒ 3.16 ⇒ 1.0 ⇒ 0.316 µg/ml

5.7.2: Definitive Test: Depending on the slope of the concentration-response curve estimated from the range finder, the dilution factor in the concentration series of the definitive test should be smaller (e.g. $^{6}\sqrt{10} = 1.47$, see Appendix C) than that in the range finder screen and centered on the two $EC_{50}$'s, the $EC_{50+SSL}$ and the $EC_{50\ No\ SSL}$. Each relevant concentration range will be targeted to include at least three cytotoxic concentrations between 10% and 90%. If the chosen concentration range does not accomplish this, the test will be considered a screen and the definitive test will be repeated.

5.8: Irradiation: The No SSL plate will be kept in the dark at room temperature. The +SSL plate will be irradiated with approximately 1.7 mW/cm² (= 5 J/cm²) of UVA from a SOL 500 Solar Simulator. The UVA dose will be monitored with a calibrated UVA radiometer. Temperature will be recorded using a calibrated digital thermometer.

## 6.0: TEST PROCEDURE

6.1: Pre-test: The Balb/c 3T3 cell line will be plated and grown to 60-80% confluence (approximately 37ºC ± 1ºC, 5% ± 1% $CO_2$) in growth medium, such as Dulbecco's Modified Eagle's Medium (DMEM) containing 0.1% Gentamicin and 10% fetal bovine serum (DMEM-10-FBS), to obtain a sufficient quantity of cells for the experiment.

6.2: 1st day:

Cells will be harvested by trypsinization and prepared as a cell suspension of $1x10^5$/ml in appropriate culture medium. Using a multi-channel pipette, 100 µl of culture medium only (no cells) will be dispensed into the peripheral wells of a 96-well tissue culture microtiter plate (i.e. blanks). 100 µl of a cell suspension of $1x10^5$ cells/ml (= $1x10^4$ cells/well) will be dispensed into the remaining central 60 wells. Two plates will be prepared for each test article, one to be used for determination of cytotoxicity (No SSL), and the other to be used for determination of phototoxicity (+SSL).

Cells will be incubated for approximately 24 hours (5.0% ± 1.0% $CO_2$, 37ºC ± 1ºC) until they form a half-confluent monolayer. This incubation period allows for cell recovery and adherence.

6.3: 2nd day:

After incubation, culture medium will be decanted from the cells and the cells will be washed carefully with 150 µl HBSS/DPBS. If necessary, excess rinse buffer will be decanted and blotted. 100 µl of vehicle containing the appropriate concentration of test article will be added. The cells will be incubated for 1 hour (5.0% ± 1.0% $CO_2$, 37º ± 1ºC).

To perform the +SSL part of the assay, the cells will be irradiated at room temperature for 50 minutes through the lid of the 96-well plate with 1.7 mW/cm² (= 5 J/cm²). If necessary, the area will be ventilated with a fan to prevent $H_2O$ condensation under the lid. Duplicate plates (No SSL) will be kept at room temperature in a dark box for approximately 50 minutes (= UVA exposure time).

After SSL irradiation, the test solution will be decanted and the cells will be washed once with 150 µl HBSS/DPBS. The HBSS/DPBS will be replaced with culture medium and the plates will be incubated at 37º ± 1ºC overnight (18-24 hours).

6.4: 3rd day:

6.4.1: Microscopic Evaluation: The cells will be examined under a phase contrast microscope. Changes in cell morphology due to cytotoxic effects of the test article will be recorded. This check will be performed to exclude experimental errors, not for evaluation of cytotoxicity or phototoxicity.

6.4.2: Measurement of Neutral Red Uptake (NRU): The uptake of the vital dye Neutral Red into the lysosomes/endosomes and vacuoles of living cells will be used as a quantitative indication of cell number and viability.

6.4.2.1: Culture medium will be removed and 100 µl Neutral Red (NR) medium will be added. The cells will be incubated at 37ºC ± 1ºC, in a humidified atmosphere of 5.0% ± 1% $CO_2$, for three hours.

6.4.2.2: After incubation, the NR medium will be removed and the cells will be washed with 150 µl of HBSS/DPBS.

6.4.2.3: The HBSS/DPBS will be completely decanted and blotted. (Optionally, the reversed plate will be centrifuged.)

6.4.2.4: Exactly 150 µl of NR Extractant solution will be added.

6.4.2.5: The microtiter plate will be shaken, either rapidly on a microtiter plate shaker for 10 minutes or slowly for up to 2 hours on a platform shaker, until NR has been extracted from the cells and formed a homogeneous solution.

6.4.2.6: The absorption of the resulting colored solution will be measured at 540 nm (without a reference filter) in a microtiter plate reader, using the mean of the outer wells (blanks) as a reference. The readout will be immediately printed or the file will be saved in Standard ASCII format.

6.5: Quality Checks of Assay:

6.5.1: Positive Control: Chlorpromazine (CPZ) will be the positive reference. CPZ will be tested in a full-scale phototoxicity test on two plates, concurrently with the test articles. NRU will be determined in the absence (No SSL) and presence (+SSL) of irradiation (5 J /cm²):

A CPZ test will meet acceptance criteria if:

- the $EC_{50}$ +SSL is within the range of: 0.1-2.0 µg/ml
- the $EC_{50}$ No SSL is within the range of: 7.0-90.0 µg/ml
- the Photo-Irritancy Factor (PIF) between the two $EC_{50}$ values is at least: 6

6.5.2: Vehicle (Negative) Control: The optical density ($OD_{540}$) will be obtained in the vehicle control, which indicates whether the 1 X $10^4$ (10,000) cells seeded per well have proliferated normally time during the two days of the assay. A test will meet acceptance criteria if the mean $OD_{540}$ of vehicle controls is greater than or equal to 0.4 AU.

To check for systematic errors, Vehicle Controls (VC) will be placed at both the left side (row 2) and the right side (row 11) of the center 60 wells of the 96-well plate. A test will meet acceptance criteria if the mean of the six left ($VC_L$) wells and the mean of the six right ($VC_R$) wells vehicle control do not differ by more than 15% from the mean of all twelve VC wells (Global Mean = $VC_{12}$). Thus, VC must be ≤ 0.15. See formula below:

$$\frac{VC_{12} - VC_R}{VC_{12}} \quad \textbf{and} \quad \frac{VC_{12} - VC_L}{VC_{12}} \quad \textbf{must} \leq 0.15$$

The irradiated negative control wells (mean of 12 wells) must show a viability of at least 80% when compared to non-irradiated negative control wells (mean of 12 wells).

6.5.3: Radiation Sensitivity: The irradiated negative control wells (mean of 12 wells) must show a viability of at least 80% when compared to non-irradiated negative control wells (mean of 12 wells).

## 7.0 DATA ANALYSIS:

7.1: Viability: The mean absorbance value for each dose group will be calculated and expressed as percent viability for each sample using the following formula:

$$\% \text{ viability} = 100 \times (OD_{sample} / OD_{negative\ control})$$

The data will be presented as a graph of percent viability (linear y-axis) vs dose (log x-axis).

7.2: The concentration-cytotoxicity response curves concurrently obtained in the presence (+SSL) and absence (No SSL) of irradiation will be analyzed to apply the rules for predicting phototoxic potential (see “Prediction Model" section, Appendix D)

7.3: Photo-Irritancy Factor (PIF) Model: The analysis for the 3T3 NRU Phototoxicity Assay will utilize the PIF model using an Excel spreadsheet.

7.3.1: PIF Calculation: To apply the PIF, any appropriate procedure can be used to calculate the $EC_{50}$ ($EC_{50}$ = the concentration inhibiting all viability by 50%). A compound is a phototoxin if PIF is ≥ 5. See example below:

$$PIF = \frac{EC_{50}\ (-UV)}{EC_{50}\ (+UV)} \geq 5$$

7.3.2: $EC_{50}$ Calculation: $EC_{50}$ will be calculated by a method of logarithmic extrapolation, where x = log (concentration) will be plotted vs. y = % viability. A logarithmic regression (such as in MS Excel) will be used to obtain an equation that allows interpolation of the $EC_{50}$. The points chosen for the regression will be the test article concentrations yielding values just below and above 50% viability.

7.4: Mean Photo Effect (MPE) Prediction Model: If results from the PIF model are indeterminate, Phototoxicity will be determined by the MPE using the 3T3 NRU Phototox Prediction Software developed by ZEBET (see Appendix D). The MPE model will only be offered to the Sponsor if the PIF results are indeterminate in two attempts of the definitive test. The Phototox software cannot be fully validated at this time due to lack of controls to ensure compliance with 21 CFR Part 11. Because OECD recommends the use of the Phototox software, a GLP deviation will be placed in the report to reflect that the Phototox software does not comply, but installation, operational and performance qualification testing has been conducted to ensure that the software has been installed correctly and performs as intended with the integrated system.

**8.0 TEST DURATION:** The duration of the 3T3 Neutral Red Uptake Phototoxicity Test Assay is approximately four days.

## 9.0 REFERENCES:

1. E. Borenfreund and J. A. Puerner. Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol Lett* 24:119-124, 1985. (Abstract)

2. H. Spielmann, M. Balls, J. Dupuis, W. J. Pape, O. de Silva, H. G. Holzhütter, F. Gerberick, M. Liebsch, W. W. Lovell, and U. Pfannenbecker. A Study On UV Filter Chemicals From Annex VII Of European *Directive 76/768/EEC,* In The *In Vitro* 3T3 NRU Phototoxicity Test. *ATLA* 26:679-708, 1998. (Abstract)

3. U. Hackenberg and H. Bartling. Measurement & calculation in pharmacological laboratories with a special numbering system (WL24-system). *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* 235:437-463, 1959. (Abstract)

4. G. Archer, M. Balls, L. H. Bruner, R. D. Curren, J. H. Fentem, H. G. Holzhütter, M. Liebsch, D. P. Lovell, and J. A. Southee. The Validation Of Toxicological Prediction Models. *ATLA* 25:505-516, 1997. (Abstract)

5. H. Spielmann, M. Balls, M. D. Brand, B. Doring, H. G. Holzhütter, S. Kalweit, G. Klecak, H. L'Eplattenier, M. Liebsch, W. W. Lovell, T. Maurer, F. Moldenhauer, L. Moore, W. J. Pape, U. Pfannenbecker, J. M. Potthast, O. de Silva, W. Steiling, and A. Willshaw. EEC/COLIPA project on *in vitro* phototoxicity testing: First results obtained with a Balb/c 3T3 cell phototoxicity assay . *Toxicol In Vitro* 8:793-796, 1994. (Abstract)

6. H. Spielmann, M. Liebsch, B. Doring, and F. Moldenhauer. First Results of an EC/COLIPA Validation Project of *in vitro* Phototoxicity Testing Methods. *ALTEX* 19:22-33, 1994. (Abstract)

7. H. Spielmann, M. Balls, J. Dupuis, W. J. Pape, G. Pechovitch, O. de Silva, H. G. Holzhütter, R. H. Clothier, P Desolle, F. Gerberick, M. Liebsch, W. W. Lovell, T. Maurer, U. Pfannenbecker, J. M. Potthast, M Csato, D Sladowski, W. Steiling, and P Brantom. The International EU/COLIPA In Vitro Phototoxicity Validation Study: Results of Phase II (Blind Trial). Part 1: The 3T3 NRU Phototoxicity Test. *Toxicol In Vitro* 12:305-327, 1998.

8. M. Liebsch, H. Spielmann, M. Balls, M. D. Brand, B. Doering, J. Dupuis, H. G. Holzhütter, G. Klecak, H. L'Eplattenier, W. W. Lovell, T. Maurer, F. Moldenhauer, L. Moore, W. J. Pape, U. Pfannenbecker, J. M. Potthast, O. de Silva, W. Steiling, and A. Willshaw. First Results of the EC/Colipa Validation Project "In Vitro Phototoxicity testing". In: *Alternative Methods in Toxicology, Vol.10: In Vitro Skin Toxicology – Irritation, Phototoxicity, Sensitization.*, edited by A. Rougier, H. I. Maibach, and A. M. Goldberg, New York:Mary Ann Liebert Publ., 1994, p. 243-254.

9. H. G. Holzhütter. A general measure of *in vitro* phototoxicity derived from pairs of dose-response curves and its use for predicting the *in vivo* phototoxicity of chemicals. *ATLA* 25:445-462, 1997. (Abstract)

10. H. G. Holzhütter and J. Quedenau Mathematical modeling of cellular responses to external signals. J. Biol. Systems, 3: 127-138 (1995)

11. Anonymous. Manual for 3T3 NRU Phototox Software, Version 2.0. BfR Zebet, 2002.

## 10.0 AMENDMENT TO THE PROTOCOL:

Any amendment to or deviation from this protocol will be fully documented in the study file, including the reason for the change, authority for change and the date.

## 11.0 RECORDS TO BE MAINTAINED:

11.1: Collection of Data: All data generated during the conduct of this study will be recorded in ink on data collection sheets. All entries will be dated, initialed and verified by another person.

11.2: Final Report: The final report may include, but not limited to, a description of the methods and experimental design, results, discussion, conclusion, data table and the Quality Assurance statement. The content of the final report will meet the requirements of the applicable Good Laboratory Requirements.

11.3: Retention of Data: All data generated during the conduct of this study will be archived at MB Research for at least 10 years from the date of the final report. The Sponsor will be contacted in writing to determine final disposition of the records. If the Sponsor fails to respond within 90 days, the archived items will be properly discarded.

11.3.1: Raw Data will be filed at MB Research by project number.

11.3.2: Final Report will be filed at MB Research by sponsor name and MB project number.

11.3.3: Test Article: Refer to Section 13 for test article disposition. If this study exceeds 28 days, it is recommended that the Sponsor archive a sample of the test article to meet the requirements for retention samples as defined in 40 CFR 160.195(c) and (h) and 40 CFR 792.195(c) and (h).

11.3.4: Test Article Mixtures: These are not routinely retained. However, upon written request from the sponsor, and at additional cost, an aliquot of the test article mixture will be retained and forwarded to the Sponsor.

## 12.0 GOOD LABORATORY PRACTICES:

This study will be conducted in accordance with the Good Laboratory Practices of the EPA, 40 CFR 160 and 792, FDA 21 CFR 58, and the OECD, Principles on Good Laboratory Practice, Revised 1997.

12.1: Protocol: MB Research will have on file a copy of this protocol, signed and dated by both the responsible MB Study Director and the sponsor's authorized representative.

12.2: Quality Assurance: The Quality Assurance Unit will inspect at least one critical phase of this study, audit the raw data and the report in accordance with the Standard Operating Procedures of MB and the applicable regulatory requirements.

## 13.0 SPONSOR REQUEST:

13.1: The sponsor requests that this protocol be implemented:

☐ As written *(or)* ☐ Modifications (per attached)

13.2: Will report be submitted to a regulatory agency? ☐ No ☐ Yes _____(agency)

13.3: Test Article: will be identified in the report and supporting documentation exactly as indicated below:

_____

Lot/Batch #: _____ pH (if available): _____

Physical Description: _____ Special Handling Precautions: _____

Storage Requirements: ☐ Room Temperature ☐ Refrigerated (2-8ºC) ☐ Other: _____

13.3.1: UV/Visible Absorbance Spectrum: supplied to MB? (Refer to section 3.1.1) ☐ Yes ☐ No

13.3.2: Solvent necessary to prepare at 100X stock concentration (if insoluble in HBSS): _____

13.3.3: Range Finder: ☐ Conduct As written ☐ Do Not Conduct

13.3.4: Characterization of the test article is required in support of data submissions and should include identity, strength, purity, composition, stability and uniformity. This data must be reviewed by the Study Director prior to study initiation and included in the final report. (EPA 40 CFR 160.105 and 792.105; FDA 21 CFR 58.105, OECD The Principles of Good Laboratory Practices, sect. 6.2). This information is:

☐ provided *(or)* ☐ not available

13.3.5: Material Safety Data Sheet: ☐ Yes ☐ No

13.3.6: DOT Hazardous Material: ☐ No ☐ Yes (indicate DOT shipping Name) _____

EPA Hazardous Waste: ☐ No ☐ Yes (indicate EPA Waste Number) _____

13.3.7: Disposition of Test Article at Study Termination: (Call for costs)

☐ UPS / Ambient temperature *(no charge)* ☐ Express carrier / Ambient temperature

☐ Overnight carrier / ☐ Dry Ice or ☐ Ice Packs ☐ Discard (proper disposal): _____

13.4: Authorization Statement: This protocol is authorized for implementation at MB Research.

BY: _____ FOR: _____
(signature) (date) (company)

_____ _____
(type/print name) (address)

_____ _____
(title) (city) (st) (zip)

_____ _____
(email) (phone) (fax)

Additional Sponsor representative: _____

**14.0 MB RESEARCH ACKNOWLEDGMENT**: Request for implementation of this protocol and receipt of the test article is acknowledged by MB Research.

14.1: Test Article Identity: _____

_____

_____

14.1.1: Date Received: _____

14.1.2: Physical Description: _____

_____

14.1.3: Test Article Characterization:

☐ Not supplied by Sponsor, or

☐ Received and Reviewed by Study Director

14.2: MB Project Number assigned to this study: _____

14.3: Supplier: The tissue equivalent supplier is: _____

14.4: Proposed Study Dates:

14.4.1: Experimental Start Date: _____

14.4.2: Experimental Term Date: _____

14.4.3: Study Completion Date (Submission of Report): Approximately 4 weeks following Experimental Term Date.

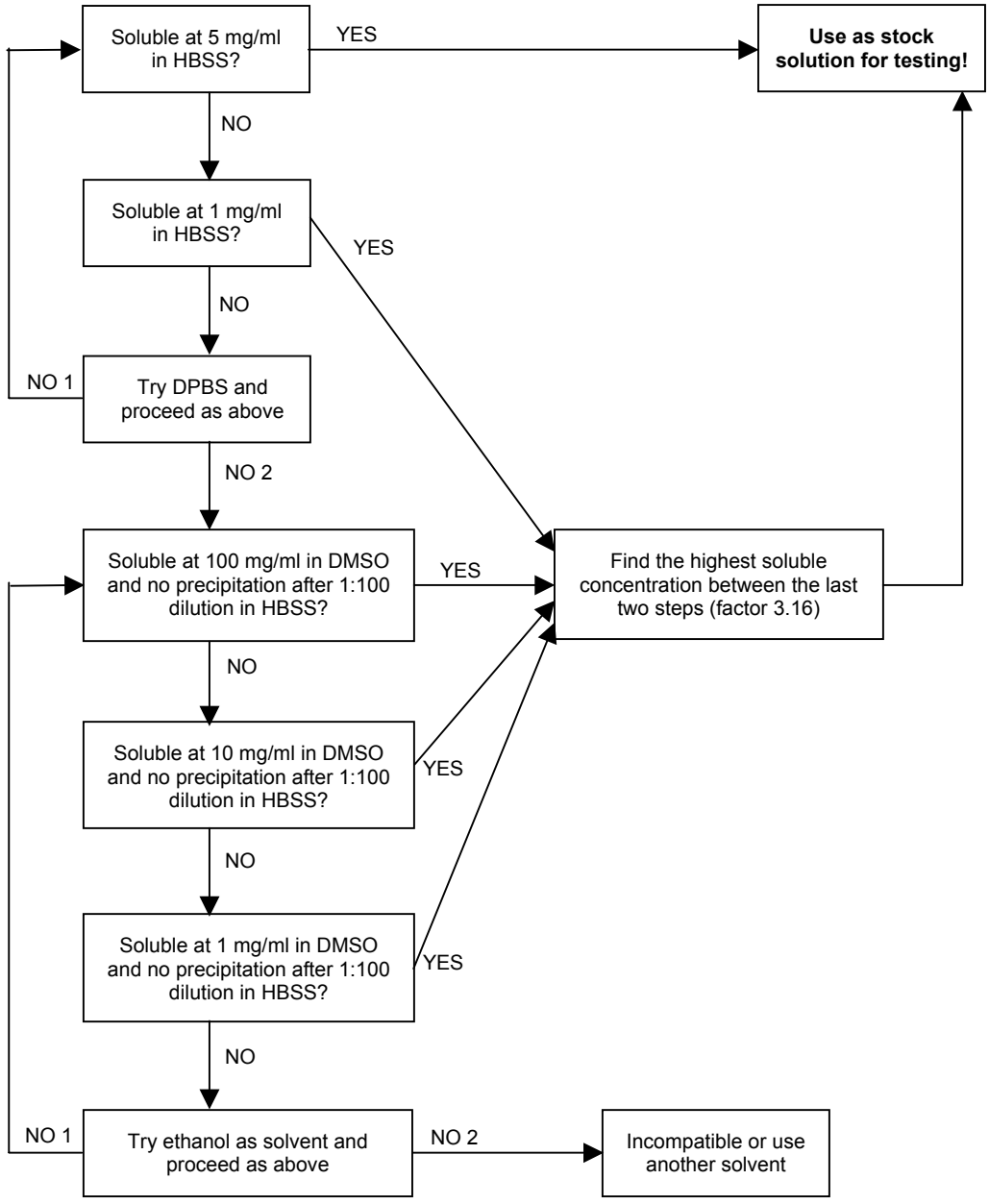
14.5: Approval: This protocol is approved for implementation at MB Research by the below named MB Study Director.

BY: _____
Study Director Date
Testing Facility: MB Research Laboratories
1765 Wentz Road, P. O. Box 178
Spinnerstown, PA 18968

## APPENDIX A: NRU PT TEST: FLOW CHART

| Time (h) | PROCEDURE |
|---|---|
| 0.00 h | Seed 96-well plates: $1x10^4$ cells / 100 µl DMEM culture medium / well<br>*Incubate (37°±1°C / 5.0%±1% $CO_2$ / 24 h)* |
| 24.00 h | Remove culture medium. Wash once with HBSS. |
| 24.00 h | Treat with 8 conc. Of 100 µl test article solved in HBSS<br>(vehicle zero control = HBSS)<br>*Incubate (37°±1°C / 5.0%±1% $CO_2$ / 1 h)* |
| 25.00 h | **Phototoxicity:** Expose to UVA 1.67 mW/cm$^2$ for 50 min (= 5 J/cm$^2$) at room temp.<br>**Cytotoxicity:** Keep duplicate plate for 50 min. in the dark at room temperature |
| 25.50 h | Remove treatment medium, wash with HBSS.<br>Replace HBSS by culture medium<br>*Incubate (37°±1°C / 5.0%±1% $CO_2$ / overnight)* |
| 48.00 h | Microscopical control of morphological alterations<br>Remove culture medium. Add 100 µl Neutral Red medium.<br>*Incubate (37°±1°C / 5.0%±1% $CO_2$ / 3 h)* |
| 51.00 h | Discard NR Medium<br>Wash once with 150 µl HBSS<br>Add 150 µl fixative (Ethanol/Acetic Acid Solution) |
| 51.40 h | Shake plate for 10 min. |
| 51.50 h | Detect NR Absorption at 540 nm (i.e. cell viability) |

## APPENDIX B: STRATEGY FOR THE USE OF SOLVENTS



## APPENDIX C: DECIMAL GEOMETRIC CONCENTRATION SERIES

In general, dose-response relationships are nonlinear, but can be linearized to some extent by logarithmic transformation of the x-axis. Usually this has to be done when $EC_{50}$ 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 simplest geometric series are dual geometric series, e.g. factor 2. These series have the disadvantage of permanently changing chains within the series (2, 4, 8, 16, 32, 64, 128, 256, etc.). The decimal geometric series, first described by Hackenberg & Bartling (1959)[3] for the use in toxicological and pharmacological studies, has the advantage that independent experiments with wide and narrow dose factors can be easily compared, and 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** (= $\sqrt[2]{10}$) divides a decade into 2 equal chains, the dose factor of **2.15** (=$\sqrt[3]{10}$) divides a decade into 3 equal chains, the dose factor of **1.47** (= $\sqrt[6]{10}$) divides a decade into 6 equal chains, and the dose factor of **1.21** (=$\sqrt[12]{10}$) 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 one volume of highest dose by adding 0.47 volumes of diluent. After equilibration, dilute one volume of this solution by adding 0.47 volumes of diluent...(and so on). Thus, assuming an initial concentration of 1000 µg/ml, and using the dose factor of 1.47 (= $\sqrt[6]{10}$), the resulting concentrations would be 1000, 681, 464, 316, 215, 147, 100 and 68.1 µg/ml.*

## APPENDIX D: PREDICTION MODEL

The mathematical rule for the prediction of *in vivo* toxicity potential from *in vitro* test data is called the Prediction Model (PM, for details see Archer et al., 1997)[4]. At present, test prediction of phototoxic potential is based on comparison of the two concentration-response curves concurrently obtained in the presence (+SSL) and absence (No SSL) of SSL irradiation. This can be achieved either by comparison of two equally effective inhibition concentrations by comparison of the +SSL and No SSL concentration response curves on a grid of concentrations from the concentration range shared by both curves (refined PM, see MEAN PHOTO EFFECT Section).

### 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*., 1994)[5,6] and applied in a formal validation study (Spielmann *et al*., 1998)[7]. It is based on comparison of two equally effective cytotoxic Test Article concentrations ($EC_{50}$ values) obtained in concurrently performed experiments in the presence (+SSL) and absence (No SSL) of SSL irradiation, which are used to calculate a photo-irritancy factor (PIF):

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)[8]. The resulting classification rule is:

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

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 Solar Simulated light drop down below 50% of the controls, because only in these cases two $EC_{50}$ values (No SSL and +SSL) can be determined. Therefore, the prediction model takes into account two additional classification rules:

If a test article is only cytotoxic +SSL and is not cytotoxic when tested No SSL, the PIF cannot be calculated, although this is a result indicating phototoxic potential. In these cases, a > PIF can be calculated if the (No SSL) cytotoxicity test is performed up to the highest test concentration ($C_{max}$) and this value is used for calculation of the > PIF:

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

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 $EC_{50}$ (No SSL) and $EC_{50}$ (+SSL) cannot be calculated because a test article does not show any cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In this cases a formal "PIF = *1" is used to characterize the result:

**(3)**
$$\mathbf{PIF = *1 = \frac{C_{max}\ (-\ UV)}{EC_{50}\ (+\ UV)}}$$

A "PIF = *1" predicts no phototoxic potential

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

## APPENDIX D: PREDICTION MODEL (cont'd)

### 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 ($EC_{50}$) 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 test articles, the mean photo effect (MPE), has recently been proposed (Holzhütter, 1997)[9]. It is based on a comparison of the +SSL and No SSL concentration response curves on a grid of concentrations i (i=1,...,N) chosen from the common concentration range of the (No SSL) and (+SSL) experiments. The photo effect (PEi) at concentration (ci) is computed as a product of the concentration effect (CEi) and the response effect (REi). The mean photo effect (MPE) is obtained by averaging across all PEi values.

To apply the refined Mean Photo Effect (MPE) prediction model, special software has to be used. This software uses the algorithms published by Holzhütter (1997)[9]. The software is presently available for MS Windows.

Data files of optical densities ($OD_{540}$) generated by the microplate reader are printed out and data is recorded and entered into MS Excel or equivalent program (which fits the concentration response curves with the procedure FitGraph, specially developed for the proper handling of non-monotonous curves (Holzhütter & Quedenau 1995)[10].

The program compares corresponding (+SSL) and (No SSL) 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.

Analogous to PIF, the MPE can be used in a prediction model for the phototoxic potential of test articles by comparing it with a critical cut-off value, MPEc. The cut-off value MPEc = 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)[9].

| MPE | Prediction of *in vivo* phototoxic potential |
|---|---|
| < 0.1 | Non-phototoxic |
| >0.1 and <0.15 | Probably Phototoxic |
| ≥ 0.15 | Phototoxic |

**Note:** In contrast to the PIF, the MPE cannot be easily calculated by everyone without using special software. A special program, "3T3 NRU Phototox, v. 2.0", was obtained from ZEBET)[11].