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Protocol

EpiDerm™ Phototoxicity Test (EpiDerm H3D PT)

For Use with EPI-200-PHO

Note 1: *This protocol was originally developed by Dr. Manfred Liebsch of ZEBET (Berlin, Germany) under a grant from FFVFF (Zurich, Switzerland). ZEBET (Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments) is a division of The German Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV). The protocol was refined in co-operation with Dr. Frank Gerberick of Procter & Gamble (Cincinnati, USA) and Dr. Uwe Pfannenbecker (Beiersdorf, Hamburg, Germany) and was successfully used in a prevalidation study under a grant from ECVAM (Ispra, Italy), "Evaluation of the Prevalidation Process", subproject: "Prevalidation of the EpiDerm™ Skin Phototoxicity Test" - Phase III (blind trial). The current protocol is based on SOP ZEBET "Phototoxicity protocol For Use with EpiDerm™ Model (EPI-200)" (1997) with minor modifications that reflect long-term experiences gained with this test over the period of almost 20 years (Kandarova and Liebsch, 2017).*

Performing the EpiDerm Phototoxicity Assay (EpiDerm H3D PT) as outlined herein, fulfills the criteria set forth in OECD TG 498.

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1. RATIONALE

Phototoxicity (photo-irritation) is defined as acute 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 substance**.**

The present assay is designed to detect the phototoxic potential of topically applied chemicals and formulations by using a three-dimensional (3D) human epidermis model, EpiDerm™. The EpiDerm human 3-dimensional phototoxicity (H3D PT) assay involves application of test materials to the air exposed surface (stratum corneum), mimicking the *in vivo* situation and allowing for the prediction of the phototoxic potency of test materials applied in usage concentrations. The assay is based on a comparison of the cytotoxicity of a chemical when tested with and without additional exposure to a non-toxic dose of UVA + visible light. Cytotoxicity is determined using the MTT assay which measures the mitochondrial conversion of MTT to formazan (Mosmann 1983), determined one day (21 ± 3 hrs) after chemical treatment and UVA exposure.

2. NEED FOR THE ASSAY

It has been shown in a joint EU/COLIPA validation project (Spielmann et al., 1995; Liebsch et al., 1994), that the phototoxic potential of chemicals can be correctly predicted by using cell culture monolayers in a specially designed cytotoxicity assay, the 3T3-NRU-phototoxicity test. However, since the phototoxic potential of a chemical predicted using a cellular system may not be relevant when topically applied to the skin at low concentrations (e.g. in a formulation), there is a need for adjunct tests, which allow for the assessment of safe usage concentrations on a dose per area basis before testing them in humans. Reconstituted skin models and epidermal models have been shown to be able to predict both, photo-irritancy (Edwards et al., 1994; Rouget et al., 1994; Liebsch et al., 1995**)**, as well as the photoprotective action of sunscreens (Liebsch et al., 1995). In addition, skin models are suitable for testing formulations (e.g. emulsions, suspensions) which the 3T3 test cannot handle. In a testing strategy which is based purely on *in vitro* tests, there is a need to combine the basic 3T3- NRU-PT with other *in vitro* tests, which allow for assessment of safety or phototoxic potency of formulations. The *in vitro* phototoxicity test using the EpiDerm reconstructed epidermis model (EpiDerm H3D PT), which was developed and pre-validated almost 20 years ago, is very suitable for this purpose (Kandarova and Liebsch, 2017). The EpiDerm H3D PT is accepted by ICH as a useful component of the testing strategy for pre-clinical testing of topically applied pharmaceuticals, in ICH guideline S10 (ICH S10 Photosafety Evaluation of Pharmaceuticals) and fulfills the requirements of OECD test guideline, TG 498: In vitro Phototoxicity: Reconstructed Human Epidermis Phototoxicity Test Method (OECD 2021).

3. BASIC PROCEDURE

On the day of receipt, the EpiDerm tissues are removed from shipping package and conditioned by a 1-hour pre-incubation followed by overnight pre-incubation for release of transport stress related compounds. After the overnight pre-incubation, the tissues are transferred to fresh assay medium and topically exposed to 5 concentrations of the test material (dissolved in H_2O or sesame oil, or suspended in sesame oil) using n=2 tissues per concentration (i.e. 1 vehicle control + 5 concentrations = 12 tissues). A second set of 12 tissues is treated identically. The tissues are incubated with test materials overnight (21±3 hrs). On the next day, all tissues are transferred to DPBS, one set of tissues is exposed to UVA (**+UVA part of the test**) for 1 hour (dose = 6 J/cm^2) and the other set is kept in the dark for the same period (**-UVA part of the test**). The tissues are then rinsed with DPBS to remove any residual test material, transferred to new 6 well plates with fresh assay medium, and incubated overnight (21 ± 3 hrs). On the following day, the tissues are transferred to a 24 well plate containing MTT solution and are incubated for 3 hours. After 3 hr, the tissues are transferred to isopropanol for formazan extraction. The optical density is determined at 570 nm using a plate spectrophotometer and cell viability is calculated for each tissue as % of the corresponding vehicle control either irradiated (+UVA) or non-irradiated (- UVA).

3.1 Test System Description

The reconstructed human epidermal model, EpiDerm™ (EPI-200, MatTek, Ashland, MA, USA and Bratislava, Slovakia) consists of normal human-derived epidermal keratinocytes, which have been cultured to form a multilayered highly differentiated model of the human epidermis. The model contains organized basal, spinous, and granular layers, and a multilayered stratum corneum with intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo. A generic description of the general and functional conditions that reconstructed human skin models need to comply with can be found in the new OECD Test Guideline 431 *In vitro* Skin Corrosion: Human Skin Model (OECD 2019) and Test Guideline 439 *In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method (OECD 2020).

The EpiDerm tissues are cultured on specially prepared cell culture inserts (surface area 0.6 cm²) and shipped world-wide as kits, containing 24 tissues shipped on agarose together with the necessary amount of culture media, DPBS, 6-well plates, and 24-well plates (MatTek part #: EPI-200-PHO). In addition, the MTT kit (containing MTT concentrate, diluent, and extractant) is provided by MatTek per request.

3.2 Quality Control of the Test System

The EpiDerm model is manufactured according to defined quality assurance procedures. All biological components of the epidermis and the culture medium are tested by the manufacturer for viral, bacterial, fungal and mycoplasma contamination. MatTek determines the ET-50 value following exposure to Triton X- 100 (1%) for each EpiDerm lot. The ET-50 must fall within a range established based on a historical database of results. Histology can be order (part # EPI-PHO).

3.3 Precautions

The epidermal cells used to produce EpiDerm are isolated from healthy volunteers negative to HIV and Hepatitis. Nevertheless, handling procedures for biological materials should be followed:

a) It is recommended to wear gloves during handling the EpiDerm tissues and kit components.
b) After use, the EpiDerm tissues, test materials, and all culture media should be decontaminated prior to disposal (e.g. using 10% bleach, special containers, or autoclaving).

Note: *Due to long pre-incubation, exposure and post-incubation periods, it is necessary to perform the test under aseptic conditions in a laminar flow hood.*

4. MATERIALS

4.1 Materials and equipment not provided with the EPI-200-PHO kits

4.1.1 Materials

Sterile, blunt-edged forceps	*For transferring tissues*
6-well tissue culture plates (in addition to those provided)	*If, instead of **replacing** media, inserts are transferred to new plates with media*
24-well culture plates (in addition to those provided)	*For UVA irradiation, if not performed in 6-well plates*
96-well plates (flat-bottom)	*For OD reading in plate spectrophotometer*
Sterile disposable pipette tips	*For pipetting test articles onto tissues, different volumes*
Beakers (200 mL)	*For rinsing tissues*
Sterile, capped glass or plastic test tubes	*For preparing the concentration series*
Repeat pipettor (2 mL)	*For adding the extractant solution*

Positive displacement pipettes (20 µL, 50 µL)	*For application of viscous test materials*
Adjustable pipet (100 µL)	*For pipetting the concentration series*
Adjustable pipet (200 µL)	*For pipetting the concentration series*
Adjustable pipet (1000 µL)	*For medium change*
Sterile blotting paper	*For drying the tissues after washing*
Bulb-headed Pasteur pipette	*For spreading on the tissue surface*
Sterile cotton swabs	*For drying the tissue surface*
Parafilm	*For avoiding evaporation during isopropanol extraction*
Sterile wash bottle	*For washing of the tissues*
Mortar and pestle	*For grinding of solids*

4.1.2 Equipment

37±1°C humidified incubator with 5±1% CO_2	*For incubating tissues prior to and during assays*
Laminar flow hood	*For transferring tissues under sterile conditions and for application of test materials.*
37±1°C water bath	*For warming up Assay Medium*
Laboratory balance	*For preparing concentration series*
96-well Spectrophotometer (Plate-Reader) equipped with 570 nm filter	*For reading optical density at 570 nm*
Plate Shaker	*For extraction of formazan*
Vortex	*For keeping test suspensions homogeneous during preparation of the concentration series*

4.1.3 UVA-VIS Irradiation equipment

UV-solar simulator	*For example: type SOL 500 Dr. Hönle GmbH*
Any appropriate, adjustable and stable tripod	*For mounting the SOL 500*
UVA-meter, Dr. Hönle or UVX Radiometer UVP Jena	*For calibration*
Filter, type H1, Dr. Hönle	*Use to cut-off emitted UVB*

4.1.4 Solutions, Reagents

Sesame oil (pharmaceutical grade)	*Solvent for test materials*
Sterile H_2O	*Solvent for test materials*
DPBS without Ca^{++} and Mg^{++}	*For rinsing-off test materials after irradiation*

4.2 Materials need for the assay

EPI-200-PHO kits are shipped from MatTek Corporation (Ashland, MA, USA) or from MatTek In Vitro Life Science Laboratories (Bratislava, Slovakia) on Mondays; kits are shipped on Thursday for shipments to Japan. Upon receipt of the EpiDerm tissues, place the sealed 24-well plates of tissues and the assay medium into the refrigerator (2-8°C). Place the MTT concentrate containing vial in the freezer (-20°C) and the MTT diluent in the refrigerator (2-8°C).

Record lot numbers of all kit components in the Methods Documentation Sheet (MDS) (see ANNEX B).

4.2.1 EPI-200-PHO kit components

Quantity	Item	Use in Assay
1	Sealed 24-well plate	Contains 24 tissues on agarose
2	24-well plates	For MTT viability assay
4	6-well plates	For phototoxicity assay
2 bottles, 50 mL	Assay Medium (EPI-100-ASY)	For phototoxicity assay
1 bottle, 125 mL	DPBS Rinse Solution	Used during irradiation and for rinsing tissues
1 vial, 10 mL	1% Triton X-100 Solution	Skin irritant reference chemical **Do not use in present assay**
1	MK-24-007-0069	EpiDerm Phototoxicity Protocol – hard copy or delivered electronically

4.2.2 MTT-100 Assay Kit Components (ready-to-use kit)

Quantity	Item	Use in Assay
1 vial, 2 mL	MTT concentrate (MTT-100-CON)	Frozen MTT concentrate (5 mg/mL)
1 vial, 8 mL	MTT diluent (MTT-100-DIL)	For diluting MTT concentrate
1 bottle, 60 mL	Extractant Solution (MTT-100-EXT)	For extraction of formazan

4.2.3 Expiration and Kit Storage

Part #	Description	Conditions	Shelf life*
EPI-200-PHO*	EpiDerm cultures	refrigerator (2-8°C)	96 hours
EPI-100-ASY	Assay medium	refrigerator (2-8°C)	14 days
MTT-100-DIL**	MTT diluent	refrigerator (2-8°C)	2 months
MTT-100-CON**	MTT concentrate	freezer (-20 ± 5°C)	2 months
*Refers to storage time @ 2-8°C in unopened package. ** MTT-100 kits must be ordered separately.			

Note: Examine all kit components for integrity. If there is a concern call MatTek immediately.

4.2.4 Contact persons

Yulia Kaluzhny (US)	Silvia Letasiova (EU)
Phone: +1-508-881-6771, ext. 229	Phone: +421-2-3260-7401
Email: *ykaluzhny@mattek.com*	Email: *sletasiova@mattek.com*

4.3 Preparations

4.3.1 MTT Medium (must be prepared fresh on day of use)

If you are using the MTT-100 kit (ready-to-use kit, purchased separately) from MatTek, thaw the MTT concentrate (MTT-100-CON) and dilute with the MTT diluent (MTT-100-DIL). Store the MTT solution in the dark at 4°C for later use on the same day (do not store until next day since MTT will degrade over time).

If you are not using the MTT-100 kit provided by MatTek, prepare a stock solution (5 mg/mL) of MTT in DPBS. Stock solution can be stored frozen (-20 ±5°C) for up to 2 months. Before use, filter the stock solution and dilute the filtrate with assay medium to final concentration (1 mg/mL). Record the preparation in the MDS. Do not store the diluted MTT solution overnight.

Safety precaution: MTT is toxic (Risk phrases: H315, H319, H335, H341). Wear protective gloves while handling MTT solution!

***Note**: MTT is light sensitive. Protect all solutions from light.*

4.3.2 Dulbecco's Phosphate Buffered Saline (DPBS)

Sterile ready-to-use DPBS is recommended. Approximately two liters are sufficient for all rinsing performed with one kit of EpiDerm tissues in this protocol. If DPBS is prepared from powder or 10x concentrated DPBS is used, prepare according to supplier instructions and adjust to pH 7.0 with either NaOH or HCl. Record the pH adjustment in the MDS.

5. METHODS

5.1 Calibration of the Solar Simulator

Before using the irradiation equipment, the solar simulator should be calibrated. New metal halide burners should be burned for ~100 hrs prior to first use to achieve a stable emittance. According to Dr Hönle, supplier of solar simulator type SOL 500, the burner has a useable lifetime (in which the spectrum is stable) of at least 800 hrs. Therefore, recording of lamp usage hours is recommended. Extended use beyond this point is only acceptable if the emitted energy spectrum has been checked.

1. Mount the solar simulator (for example SOL 500), equipped with an H1-filter (UVA only), on any appropriate stable tripod that allows fine-adjustment of the exposure distance.
2. Adjust the solar simulator to a distance of ~60 cm from the tissue surface.
3. Switch the lamp on, wait at least 15 minutes and measure irradiance **through the lid of a cell culture plate** using the calibrated UV radiometer, equipped with an UVA-sensor.
4. Adjust distance of solar simulator to achieve a UVA irradiance of **1.7 mW/cm²** (the resulting dose will be 1 J/cm² per 10 min. exposure time).
5. According to the number of plates to be exposed concurrently, check the exposure area for equal distribution of irradiance: A range of **1.6 - 1.8 mW/cm²** is acceptable. ***Important:** A maximum variation of **1.5 to 1.9 mW/cm²** can be accepted, if the position of the plates with low and high irradiance is changed after half time of the irradiation (30 minutes) is reached.*

Calibration of the solar simulator shall be checked as described above each time before performing a phototoxicity test.

In case measurements with the UV radiometer reveal unexpected results, the metal halide burner may have reached the end of its usable life, or the radiometer is de-calibrated due to various reasons. In the latter case, a second reference radiometer of the same type and calibration should be used for verification.

5.2 Quality Controls

5.2.1 UVA Sensitivity of the EPI-200 Tissues

A UVA sensitivity experiment should be performed once the test is initially set up in a laboratory. If UVA sensitivity of the tissues is within the acceptance range, this type of experiment should be repeated at regular intervals (e.g. once every 6 months).

1. Transfer EpiDerm tissues from the shipping agar into the 6-well plates containing 0,9 mL assay medium per well and pre-incubate them in the humidified incubator for 1 hour at 37±1°C, 90 ± 10% rH, 5 ±1 % CO_2 to allow release of metabolites accumulated during the storage on agar. After 1 hour, change medium and further pre-incubate overnight at 37±1°C, 90 ± 10% rH, 5 ±1 % CO_2.
2. Adjust irradiance of the SOL 500 to 1.7 mW/cm^2 (measure through the plate lid).
3. For UV irradiation, transfer 21 tissues to 6-well plates filled with 0.9 mL of DPBS per well.
4. Transfer 3 tissues serving as non-irradiated control to 6-well plate filled with 0,9 mL of DPBS per well. Cover this plate using aluminium foil or place it into the dark at room temperature until irradiation of the UVA+ part of the experiment is completed.
5. Start irradiation of the 21 tissues through the lid of the plate. Every 30 minutes (= 3 J/cm^2) transfer 3 tissues from the irradiation site to the dark box. The resulting dose series will be 3, 6, 9, 12, 15, 18, 21 J/cm^2.
6. After irradiation of the tissues is over, transfer then to new 6-well plates prefilled with 0,9 mL of fresh assay medium and incubate tissues for 21±3 hours at 37±1°C, 90 ± 10% rH, 5 ±1 % CO_2.
7. Determine tissue viability using MTT assay and calculate tissue viability of the irradiated tissues compared to the non-irradiated tissues (100 % viability). For doses ≤ 6 J/cm^2 (= 60 minutes), the tissue viability should not decrease by >20%. The historical ID50 UVA is in the range of ~12 - 18 J/cm^2 (see ANNEX F).

5.2.2 Assay Acceptance Criterion 1: Negative Control

The **absolute OD** of the negative control tissues in the MTT test is an indicator of tissue viability obtained in the testing laboratory after shipping and under the specific conditions of the assay. Tissue viability fulfills the acceptance criterion if the mean OD of the negative control tissues (determined without reference filter) is ≥ **0.8 and ≤ 2.8**.

A solvent control (used as a negative control) must be tested concurrently in each run.

5.2.3 Assay Acceptance Criterion 2: Positive Control: Chlorpromazine (CPZ)

It is not necessary to include a positive control into each phototoxicity test since this reduces the number of concentrations of the test chemical. When the assay is newly established, perform a full experiment with five concentrations of **Chlorpromazine (CPZ)** (dissolved in H_2O) ranging from 0.001% to 0.1%. Repeat this test on a regular basis (~ every 6 months). **A dose dependent reduction of cell viability occurring only in the UVA-irradiated tissues should be observed between 0.00316% and 0.0316%** (see **ANNEX D**).

5.2.4 Assay Acceptance Criterion 3: Maximum viability difference of tissue duplicates

The difference in viability between tissue duplicates that are treated identically must be ≤ **30%.** According to the historical data base of ZEBET, the mean difference between untreated tissue duplicates is 9% ± 7% (S.D.).

Note: A difference **> 30%** *(i.e. exceeding the 99% confidence interval) between two tissues treated identically should be regarded as a rejection criterion, and re-testing of the chemical is recommended if the resulting viability is near to the classification cut-off.*

5.3 Test Sample Preparation and Test Concentrations

According to their solubility, chemicals are applied either as a solution in **water**, or as solution or suspension in **sesame oil**.

DESCRIPTIVE TERM	RANGE OF SOLUBILITY	% (w/v)	CATEGORY
very soluble	> 1000 mg/mL	>100.00	1
freely soluble	> 100 mg/mL - 1000 mg/mL	>10.00	2
soluble	> 30 mg/mL - 100 mg/mL	>3.00	3
sparingly soluble	> 10 mg/mL - 30 mg/mL	>1.00	4
slightly soluble	> 1 mg/mL - 10 mg/mL	>0.10	5
very slightly soluble	> 0.1 mg/mL - 1 mg/mL	>0.01	6
practically insoluble	0.1 mg/mL and lower	<0.01	7

As a basic recommendation, poorly water soluble test materials (category 5-7) should be tested dissolved or suspended in sesame oil. Water soluble test materials (category 1-4) should be tested dissolved in water. If suspensions are tested, use appropriate techniques for preparing e.g. a homogenizer, vortex or sonicator.

5.3.1 Concentration series

Prepare five concentrations of the test material. Where possible, the highest concentration of a test material should show cytotoxicity in non-irradiated tissues. Since many test chemicals are likely to absorb UV they can act as UV-filter. Therefore, the highest test concentration should **not exceed 10%**.

If there is no information on skin toxicity of the test material available, start with the following concentration series:

vehicle	% (w/v)	% (w/v)	% (w/v)	% (w/v)	% (w/v)
oil	10	3.16	1	0.316	0.1
water	1	0.316	0.1	0.0316	0.01

Note: *This series works well for most oil soluble test materials. Materials dissolved in water may permeate through the stratum corneum more quickly. If a water-soluble test material is highly cytotoxic, the concentration series may have to be shifted to a lower range in a second experiment.*

5.3.2 Application of test samples

Solutions in **H_2O**: Apply **50 µL** on the top of the tissues and gently spread with bulb headed Pasteur pipette.

Solutions in **sesame oil**: Apply **25 µL** on the top of tissues and gently spread with bulb headed Pasteur pipette. Preferably use a positive displacement pipette.

Solutions in **ethanol**: If a material is poorly soluble in water and in sesame oil, another solvent that can be used is ethanol (purity ≥ 99.8 %). Apply **25 µL** of the diluted test article on the top of the tissues.

5.4 Experimental Procedure (see also ANNEX A)

DAY 0 – Day prior to dosing

1. Upon receipt of the shipment, examine all kit components for integrity. If there is a concern, contact MatTek Corporation or MatTek IVLSL immediately.
2. Record all information about the supplied materials into the MDS.
3. Store the DPBS at room temperature and the vial containing the MTT concentrate in the freezer (-20±5°C).
4. Let the assay medium reach room temperature (20-25°C). Do not pre-heat to 37°C.
5. Pipette 0.9 mL of the assay medium into each well of sterile 6-well plates (for 24 tissues prepare 4 sterile 6-well plates).
6. Under sterile conditions, open the plastic bag containing the 24-well plate with the tissues. Open the plate and remove the sterile gauze. Carefully (using sterile forceps) take out each insert containing the EpiDerm tissue. Remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on sterile filter paper or gauze, and place the tissues in an empty, sterile 24-well plate.
7. Perform visual inspection of the inserts within 5 min. Record any tissue defects and excess moisture on the surface. Do not use tissues with defects or tissues with excessive moisture on the surface.
8. Dry the surface of the tissues with a sterile cotton tip swab and transfer tissues to 6-well plates pre-filled with 0.9 mL medium. Place the plates containing the tissues into the incubator (37±1°C, 5±1% CO_2, 90% ± 10% RH). Equilibrate the tissues in the incubator for 60 ± 5 min.
9. At the end of the first (60 minute) pre-equilibration period, exchange the medium in the 6-well plates. Further, pre-equilibrate the tissues (37±1°C, 5±1% CO_2, 90% ± 10% RH) overnight for 18 ± 3 hr.
10. Place the rest of the assay medium into the refrigerator (5±3°C).
11. Prepare and sterilize all devices which will be used in the assay and prepare sufficient amount of rinsing DPBS for the next day (approximately 2 L per 24 inserts).

DAY 1 – Exposure

1. Place all devices, solutions, and chemicals necessary for the test into the sterile hood.
2. For each test material, prepare a series of five concentrations in the vehicle in which the test material has the best solubility, see **5.3 and 5.3.1**.
3. Pre-warm the assay medium in a 37°C water bath.
4. Exchange the medium in all 6-well plates with 0.9 mL per well of fresh, prewarmed assay medium.
5. For each test material, use one EpiDerm™ kit (EPI-200-PHO) (24 tissues): Twelve tissues are used for the **(-UVA)** cytotoxicity part and 12 for the **(+UVA)** phototoxicity part of the test. Both parts of the test are dosed identically: Apply the vehicle control (VC) plus 5 concentrations of the test chemical (C1 -C5), each onto duplicate tissues according to **5.4.2**. The tissues are dosed with **50 µL** of solutions in water or **25 µL** of solutions in sesame oil or ethanol using standard or positive displacement pipette.

Mark the lids and the plates to prevent transposition errors.

+ UVA Plate 1

VC	C 1	C 2
VC	C 1	C 2

+ UVA Plate 2

C 3	C 4	C 5
C 3	C 4	C 5

- UVA Plate 1

VC	C 1	C 2
VC	C 1	C 2

- UVA Plate 2

C 3	C 4	C 5
C 3	C 4	C 5

***Note**: Covering the plate with self-adhesive plastic film to prevent from evaporation of test articles into adjacent wells in general is not necessary in this test. To be on the safe side, position the lowest concentration (C1) beside the vehicle control (VC).*

6. Once all tissues have been dosed, cover the plates with the lids and incubate for 21 ± 3 hr at 37±1°C, 5±1% CO_2, 90% ± 10% RH.

DAY 2 – Irradiation

1. Remove the 6-well plates from the incubator.
2. Transfer the tissues into new 6-well plates pre-filled with 0.9 mL of DPBS.
3. Irradiate the **+ UVA**-plates (covered with lids!) for 60 min with 1.7 **mW/cm²** of UVA (= **6 J/cm²**, measured through the lid) at room temperature.
4. Place the **– UVA**-plates in a dark box or cover them with aluminum foil at room temperature.
5. While the tissues being irradiated, prepare new 6-well plates pre-filled with 0.9 mL of fresh assay medium per well.
6. After the UVA irradiation is complete, wash all tissues (+ UVA and - UVA) using a wash bottle filled with sterile DPBS, dry the tissues using sterile blotting paper, and transfer them in 6-well plates prepared in step 5.
7. Incubate the +UVA and -UVA plates for 21 ± 3 hr at 37±1°C, 5±1% CO_2, 90% ± 10% RH.

DAY 3 – MTT Viability Test

MTT Test

1. Prior to the MTT assay, label a sufficient number of 24-well plates. Use the following design:

VC	C 1	C 2	C 3	C 4	C 5	+UVA
VC	C 1	C 2	C 3	C 4	C 5	+UVA
VC	C 1	C 2	C 3	C 4	C 5	- UVA
VC	C 1	C 2	C 3	C 4	C 5	- UVA

2. Prepare the MTT solution (1 mg/mL) from frozen concentrate according to Section 4.3.1 and pipette 0.3mL of MTT solution in each well of a 24-well plate.
3. Remove the inserts from the 6-well plates, blot the bottom of the inserts, and transfer them into the 24- well plates, pre-filled with 0.3 mL of MTT solution. Place the plates in the incubator (37±1°C, 5± 1% CO_2, 90% ±10% RH), record the start time of MTT incubation in the **MDS** and incubate for **3 hours ± 5 min**.

Note: *The 3 hours +/- 5 min MTT incubation time must be strictly adhered to. Deviations from the 3 hour time for MTT incubation will result in different MTT readings.*

4. After the incubation is complete, transfer the tissues to a new 24-well plate pre-filled with 2.0 mL of extractant solution (isopropanol) per well. The level will rise above the upper edge of the insert, thus completely submerging the tissue.
5. Seal the 24-well plates (e.g. with Parafilm or place into a sealable plastic bag) to inhibit extractant evaporation. Record start time of extraction in the MDS and extract formazan for at least 2 hours at room temperature with gentle shaking on a plate shaker (~ 120 rpm).
6. As an alternative, overnight extraction is also possible. Seal the plates as described above and extract in refrigerator at +5 ±3°C in the dark, without shaking. Before using the extracts, shake for at least 15 min on a plate shaker.
7. After the extraction period is complete, pierce the inserts with an injection needle or bulb-headed Pasteur pipette and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded. Before transferring the extract to 96 well plates pipette up and down 3x until the extractant solution is homogenous.
8. For each tissue, transfer 3 × 200 µL aliquots***** of the blue formazan solution into a 96-well flat bottom microtiter plate. For the 96 well plate, use **exactly** the well design given below since this configuration is used in the EXCEL data spreadsheet. Read the OD in a plate spectrophotometer at **570 nm**, **without** a **reference filter**. Alternatively, ODs can be read at **540 nm**. Isopropanol is used as a blank.

***** ***Note:*** *In contrast to other spectrophotometers, in plate readers pipetting errors influence the OD. Therefore, 3 formazan aliquots shall be taken from each tissue extract. In the data sheet these 3 aliquots will be automatically reduced to one value by calculating the mean of the three aliquots. Thus, for calculations from each single tissue only one single mean OD-value is used.*

 # Note: Readings are performed without a reference filter, since the "classical" reference filter often used in the MTT test (630 nm) is still within the absorption curve of formazan. Since filters may have a ± tolerance, in some cases the reference filter reduces the dynamics of the signal (OD) up to 40%.

Fixed 96 well-plate design (for OD reading in plate photometer, 3 aliquots per tissue)

VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	*+UVA*
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	*-UVA*
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
Blank	**Blank**											
tissue 1	*tissue 2*	*tissue 1*	*tissue 2*	*tissue 1*	*tissue 2*	*tissue 1*	*tissue 2*	*tissue 1*	*tissue 2*	*tissue 1*	*tissue 2*	

5.5 Documentation

5.5.1 Method Documentation Sheet, MDS

The MDS allows users to ensure the correct set up, calibration and function of the equipment used in each assay as well as correct weights, applications etc. The MDS is designed as a paper document "in the spirit of GLP". For each kit, make a hardcopy of the MDS, fill in and sign the requested information, starting the day prior to testing and ending after the test has been completed.

Note: If several tests are performed per week, pipette verification (weighing H_2O on a balance) is only necessary once at the beginning of each week. Nevertheless, if adjustable pipettes are used, the correct adjustment shall be checked and recorded in the MDS before each test.

5.5.2 Data Spreadsheet

The MS EXCEL workbook EpiDermPHO.xls is available from MatTek. Data files of optical densities (ODs) generated by the microplate reader are copied from the reader software to the Windows Clipboard and then pasted into the first map of the EXCEL spreadsheet in the fixed 96-well format given above (*Note: Only 74 wells of the 96 wells are used!*).

6. PREDICTION MODEL

The rules used to transform quantitative or qualitative data of a toxicological test into a prediction of a toxic potential or potency are called the prediction model. The prediction model is based on analysis of historical data of the difference in the viability between test material exposed, UVA irradiated and non-irradiated EpiDerm tissues. Since the UVA irradiation (6 J/cm^2) has no cytotoxic effect, a phototoxic test material can be identified if viability of tissues treated with identical test chemical concentrations differs ≥ 30% in the irradiated versus the non-irradiated tissues.

For each concentration of a test chemical, the mean OD of the n=2 tissues treated with this concentration is determined and expressed as relative percentage viability of the vehicle control. Identical calculations are performed for the (+UVA) part of the test.

A chemical is predicted to have **a phototoxic potential** if one or more test concentrations of the (+UVA) part of the experiment reveal a decrease in viability of **≥ 30%** when compared with identical concentrations of the (-UVA) part of the experiment.

Prediction of phototoxicity is supported if, in addition, the (+UVA) induced reduction in tissue viability shows a dose response relationship.

***Note**: It is a quite common observation for certain phototoxins, that a severe effect may be reduced again at higher doses. This is due to the UV absorbing properties of the test chemical which can act as UV filter if an excessive dose is applied so that some of the test article remains on the stratum corneum.*

7. REFERENCES

Edwards SM, Donally TA, Sayre RM, Rheins LA, Spielmann H, Liebsch M (**1994**). Quantitative *in vitro* assessment of phototoxicity using a human skin model; Skin². *Photodermatol. Photoimmunol. Photomed **10**: 111-117.*

ICH HARMONISED TRIPARTITE GUIDELINE. Photosafety Evaluation of Pharmaceuticals – S10. November 2013. https://database.ich.org/sites/default/files/S10_Guideline.pdf.

Kandarova H and Liebsch M (2017). The EpiDerm™ Phototoxicity Test (EpiDerm™ H3D-PT). in Alternatives for Dermal Toxicity Testing. Eskes C, van Vliet E, Maibach HI (eds.). Springer International Publishing AG 2017.

Klausner M, Kubilus J, Ricker HA, Neal PJ (**1995**). UVB irradiation of an organotypic skin model, EpiDerm™, results in significant release of cytokines. *The Toxicologist, 15 (1)., Soc. of Toxicology.*

Liebsch M, Spielmann H, Balls M, Brand M, Doring B, Dupuis J, Holzhutter HG, Klecak G, L'Eplattenier H, Lovell WW, Maurer T, Moldenhauer F, Moore L, Pape WJW, Pfannenbecker U, Potthast J, De Silva O, Steiling W, Willshaw A (**1994**): 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. **Eds**. A. Rougier, A. Goldberg, H. Maibach; *Mary Ann Liebert Publ., New York; pp. 243 – 254.*

Liebsch M, Doring B, Donelly TA, Logemann P, Rheins LA, Spielmann H (**1995**). Application of the human dermal model Skin^2 ZK 1350 to phototoxicity and skin corrosivity testing. *Toxic. in Vitro Vol. 9, No. 4, 557 – 562.*

Mosmann T (**1983**). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays *J. Immun. Meth. **65**: 55-63.*

OECD (**2021**). OECD guideline for the testing of chemicals No 498: In vitro Phototoxicity: Reconstructed Human Epidermis Phototoxicity Test Method. Organisation for Economic Cooperation and Development, Paris, France.

OECD (**2020**). OECD guideline for the testing of chemicals No 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method. Organisation for Economic Cooperation and Development, Paris, France.

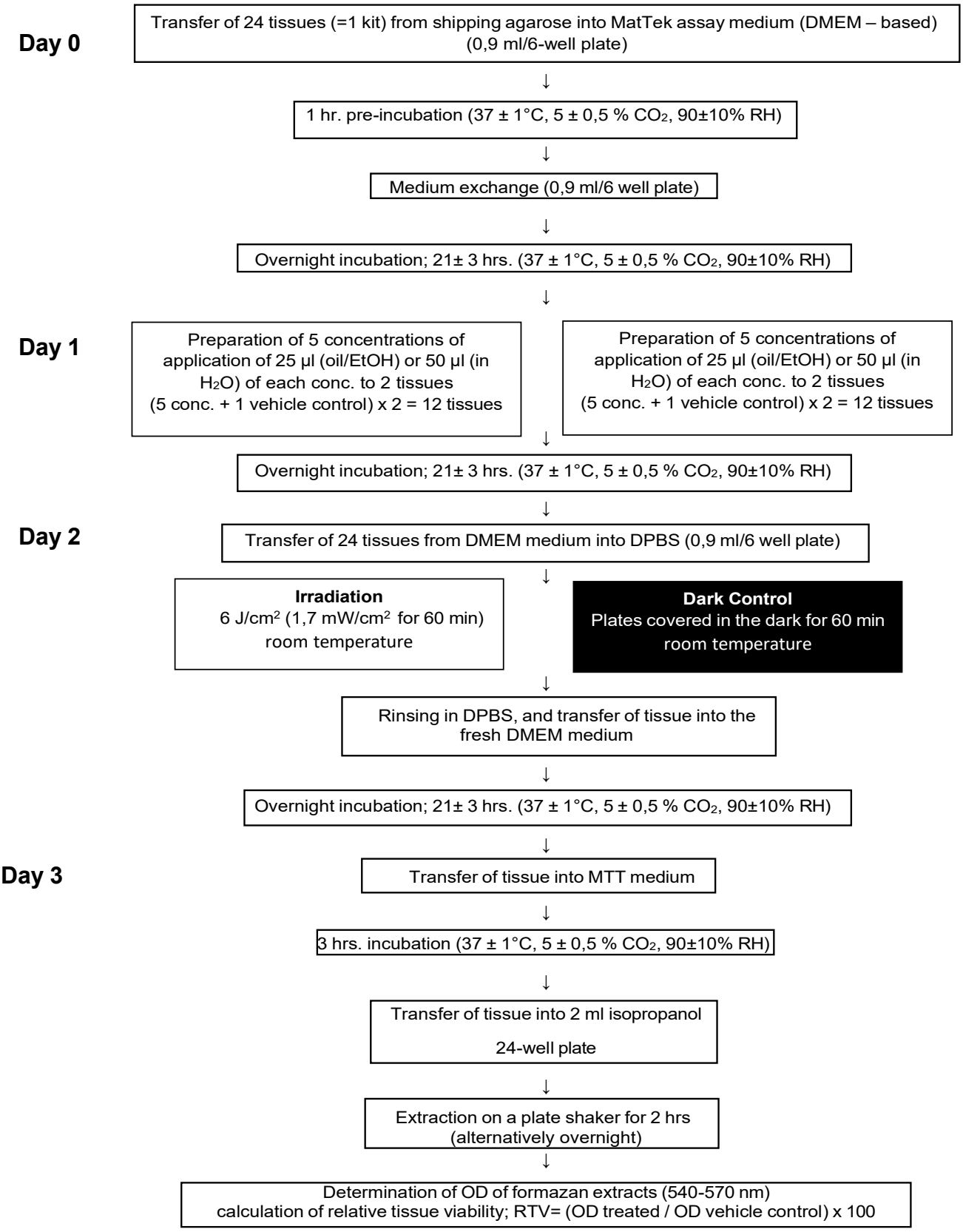
OECD (**2019**). OECD guideline for the testing of chemicals No 431: In vitro skin corrosion: reconstructed human epidermis (RHE) test method. Organisation for Economic Cooperation and Development, Paris, France.

Perkins MA, Osborne R, Johnson GR (**1996**). Development of an *in vitro* Method for Skin Corrosion Testing. *Fundamental and Applied Toxicology **31**: 9-18.*

Rouget R, Cohen C, Rougier A (**1994**). A reconstituted human Epidermis to assess cutaneous irritation, photo-irritation and photoprotection *in vitro*. **In:** Alternative Methods in Toxicology, Vol. 10: *In vitro* Skin Toxicology - Irritation, Phototoxicity, Sensitization. **Eds**. A. Rougier, A. Goldberg, H. Maibach; *Mary Ann Liebert Publ., New York; pp. 141 - 149* Eskes C, van Vliet E, Maibach HI (eds.). Springer International Publishing AG 2017.

Spielmann H, Liebsch M, Pape WJW, Balls M, Dupius J, Klecak G, Lovell WW, Maurer T, De Silva O, Steiling W (**1995**). The EEC COLIPA in vitro photoirritancy program: results of the first stage of validation. **In:** Irritant Dermatitis: New clinical and experimental aspects. **Eds**. P. Elsner and H.I. Maibach *T. Karger Publ., Basel; pp. 256-264.*

ANNEX A: PROCEDURE OUTLINE



ANNEX B: METHODS DOCUMENTATION SHEET (MDS)

Assay:……

Date: …….

Test material:

Corresponding XLS Data File Name: ……

PERFORMED BY: .....SIGNATURES: .....

Time Protocol

Receipt of EpiDerm™ tissues (date, day, hour):

…….

ID:

Experimental schedule

Procedure	**Date (dd-mm-yy)**	**Set 1**		**Set 2**		**Remark**
		start	stop	start	stop	
Pre-incubation 1 (60 ± 5 min)						
Pre-incubation 2 (18 ± 3hrs)						
Exposure (21 ± 3 hrs)						
Irradiation (60 min.)						
Post-incubation (21 ± 3 hrs)						
MTT test (3 hrs ± 5 min)						
Extraction (minimum 2hrs)						
Measurement						

Devices Verification

Incubator verification

Incubator #	CO_2 < 5±1 % >	Temperature < 37 ± 1°C >	Check water in reservoir (✓)

ID/ Date:

Refrigerator verification

Refrigerator #	Temperature < 5°C ± 3°C >

ID/ Date:

Water bath verification

Water bath #	Temperature < 37 ± 1°C >

ID/ Date:

In case that your devices are controlled by central computer, fill in the following table instead of fields above:

Name of the device	device #	reference

ID/ Date:

Pipette verification (triplicate weightings)

Pipette 3 x H_2O into a small baker on a laboratory scale and record readings in g. Perform pipette verification only once per week and refer to it in all assays of this week. If adjustable pipettes are used, check adjustment daily.

	0.9 mL	2 mL	300 µL	200 µL	25 µL	30 µL
	.....H_2O weight in g.....					
1.						
2.						
3.						
Mean						
SD						

ID/ Date:

Kit Components *(**MTT-100 Kit Must Order Separately)*

EpiDerm™ (EPI-200-SIT) Lot no.:	Production date:
Assay medium (EPI-100-ASY) Lot no.:	Expiration date:
MTT concentrate (**MTT-100-CON); 2 mL Lot no.:	Expiration date:
MTT diluent (**MTT-100-DIL); 8 mL Lot no.:	Expiration date:
MTT extractant (**MTT-100-EXT), 60 mL Lot no.:	Expiration date:
DPBS (TC-PBS); 125 mL Lot no:	Expiration date:
1% Triton X-100	Expiration date:
Position of ice-packs: (direct contact of the ice-packs with the skin must be avoided)	
Other remarks	

ID/ Date:

Visual Quality Control of the tissues

Use scores: 1- very good, 2-good, 3- acceptable, 4- not acceptable

APPERANCE	**KIT 1**	**KIT 2**
MACRO.		
No of excluded tissues with: - edge defects - air bubbles - extensive moisture on the surface		

Specific observations:

Solutions

Note: In case you are preparing your own MTT stock solution and/or DPBS fill in the following forms:

MTT Stock Solution Preparation: 5 mg/mL :

- MTT batch N° : …….....
- Weight : …….....
- DPBS batch N°: ……...
- DPBS Volume added: …….
- Preparation date: ……...
- Expiration date : …….....
- Stocking place : Refrigerator N°…….....

DPBS Solution Preparation:

- Preparation date: …….
  - pH adjustment (to 7.0)…….…….....
  - Type of sterilisation.....
- Preparation date: ……..…….....
- Expiration date : ……..…….....

Preparation of test chemical and application

Test article: _____	Vehicle used (H_2O / oil):	
Homogenisation technique: _____	Solution / suspension:_____	Conc. (%) _____
Application volume (µL):		
Stock preparation:		
1. dilution:		
2. dilution:		
3. dilution:		
4. dilution:		
5. dilution:		

SPECTROPHOTOMETRIC MEASUREMENT

Plate Configuration for Reading (for transfer to Spreadsheet EpiDermPHO.xls):
Record the positions of substances on 96-well plate.

VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	*+UVA*
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	*-UVA*
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
VC	VC	C1	C1	C2	C2	C3	C3	C4	C4	C5	C5	
Blank	**Blank**											
tissue 1	*tissue 2*	*tissue 1*	*tissue 2*	*tissue 1*	*tissue 2*	*tissue 1*	*tissue 2*	*tissue 1*	*tissue 2*	*tissue 1*	*tissue 2*	

Note: **Turn on the reader 10 min before reading plate.**

Check plate photometer filter
Tick correct (✓) filter setting

Reading Filter: 570 nm	
No Reference Filter	

ID/ Date:

Archival

Raw data saved in/as:

Spreadsheet saved in/as:

MDS saved in/as:

ANNEX C: EXCEL SPREADSHEET

EpiDerm Phototoxicity Test

Chemical:	Acridine
Tissue-lot no.:	1701 C
Exp. no.:	6
Solvent:	water
Date:	Jun-11-2021
Irradiation:	6 J/cm^2
Application time:	21 hours
Prediction:	Phototoxin

Summary

TA (%)	-UVA	+UVA	Δ Viability
control	100.0	100.0	0.0
0.010	102.2	87.6	14.6
0.032	107.0	6.7	100.3
0.100	99.8	10.7	89.1
0.316	21.1	6.9	14.2
1.000	8.8	7.2	1.6

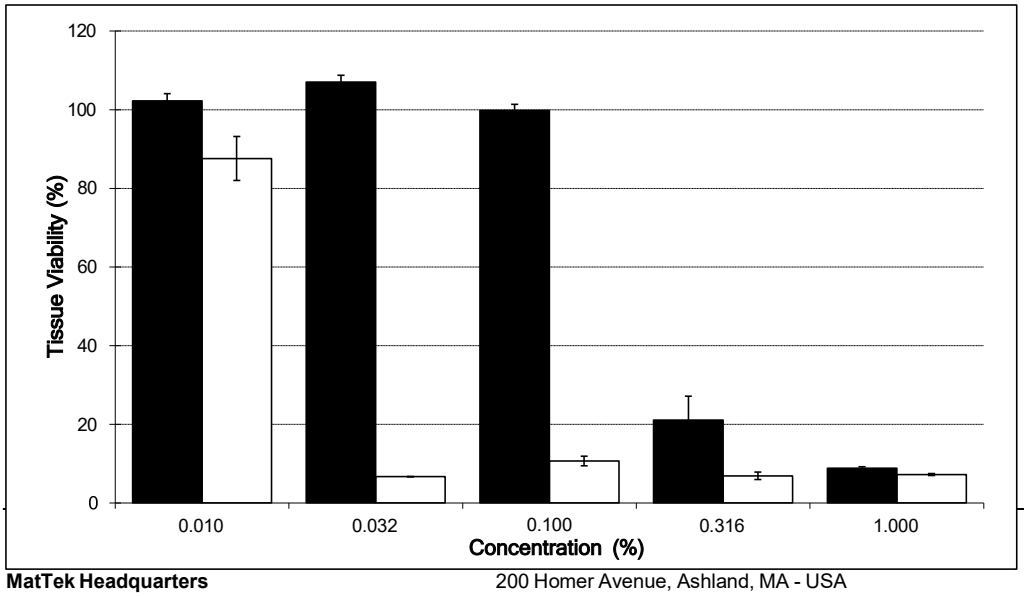
Raw data:

w/o UVA

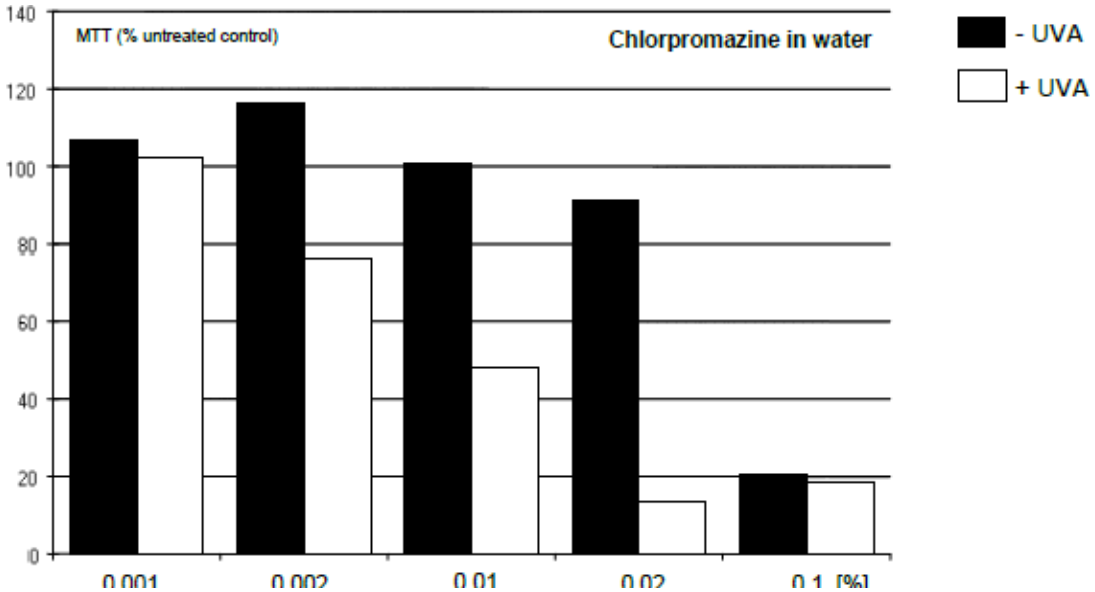
TA (%)	Tissue 1	Tissue 2	Tissue 3	mean	Mean OD	D OD tissue	tissue viability	Mean Viability	Difference D tissue
control	0.798	0.827	0.825	0.817	0.848	0.377	96.3	100.0	7.466
	0.895	0.878	0.867	0.880			103.7		
0.010	0.859	0.850	0.845	0.851	0.867	0.410	100.4	102.2	3.694
	0.887	0.892	0.869	0.883			104.0		
0.032	0.921	0.926	0.921	0.923	0.908	0.476	108.8	107.0	3.497
	0.900	0.893	0.886	0.893			105.3		
0.100	0.837	0.840	0.823	0.833	0.847	0.404	98.2	99.8	3.104
	0.858	0.868	0.853	0.860			101.3		
0.316	0.246	0.224	0.220	0.230	0.179	0.166	27.1	21.1	12.102
	0.132	0.127	0.123	0.127			15.0		
1.000	0.084	0.076	0.074	0.078	0.075	0.042	9.2	8.8	0.747
	0.079	0.072	0.064	0.072			8.4		

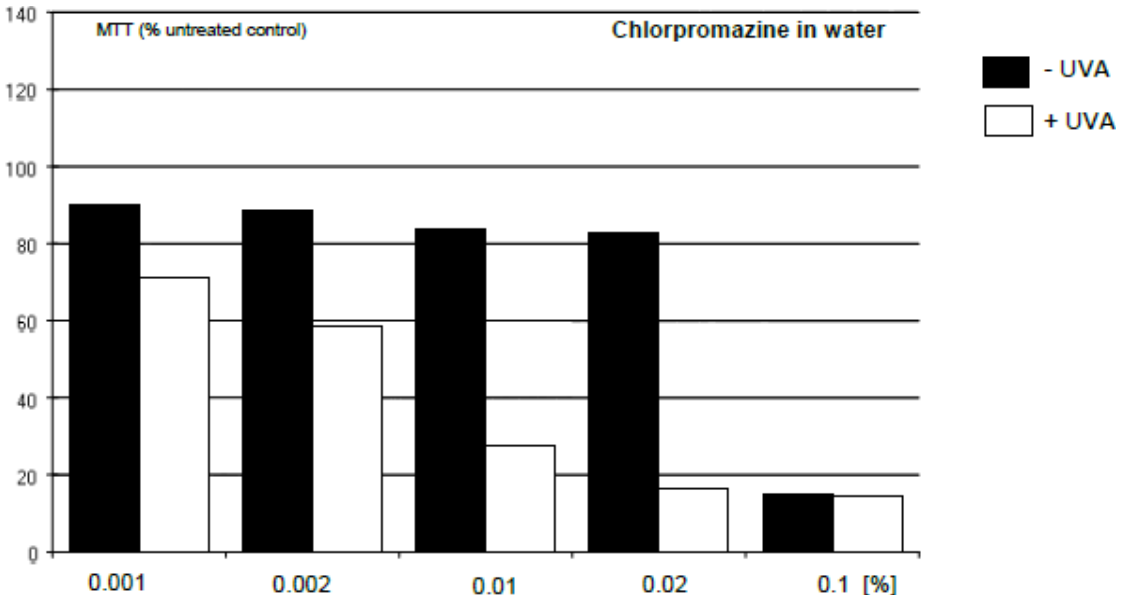
with UVA

TA (%)	Tissue 1	Tissue 2	Tissue 3	mean	Mean OD	D OD tissue	tissue viability	Mean Viability	Difference D tissue
	0.973	1.036	1.061	1.023	0.949	0.586	107.8	100.0	15.6
	0.872	0.870	0.883	0.875			92.2		
0.010	0.777	0.778	0.779	0.778	0.831	0.336	82.0	87.6	11.2
	0.883	0.884	0.885	0.884			93.1		
0.0316	0.065	0.062	0.062	0.063	0.064	0.031	6.6	6.7	0.1
	0.065	0.064	0.063	0.064			6.7		
0.100	0.111	0.112	0.115	0.113	0.101	0.068	11.9	10.7	2.4
	0.093	0.087	0.089	0.090			9.4		
0.316	0.076	0.073	0.074	0.074	0.065	0.046	7.8	6.9	1.9
	0.057	0.056	0.056	0.056			5.9		
1.000	0.071	0.070	0.071	0.071	0.068	0.038	7.4	7.2	0.5
	0.067	0.066	0.065	0.066			7.0		

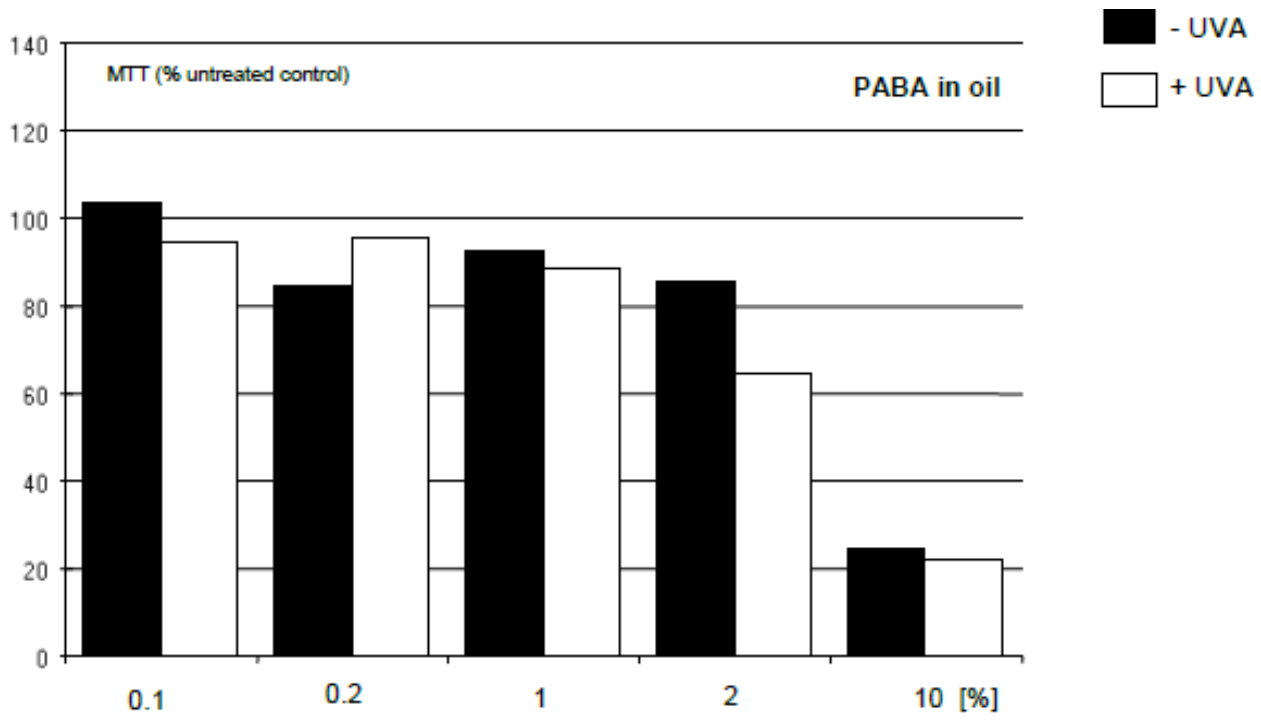


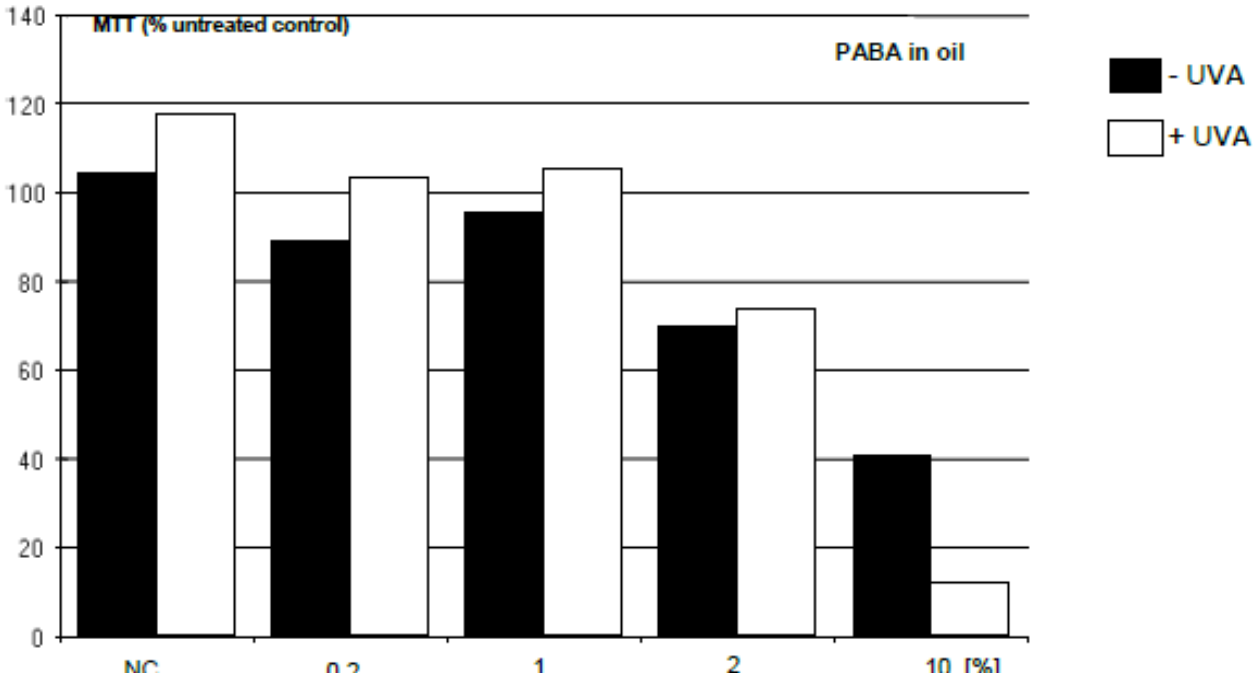
ANNEX D: POSITIVE REFERENCE DATA



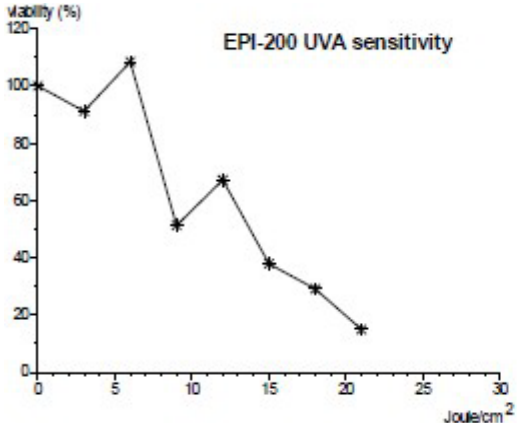


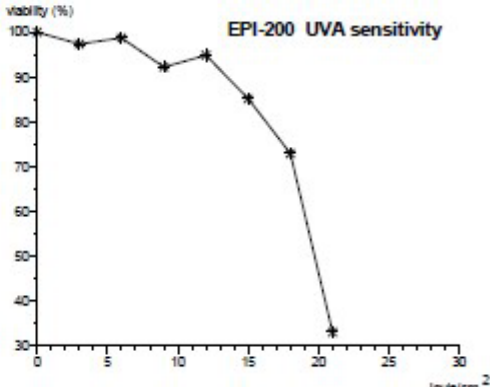
ANNEX E: NEGATIVE REFERENCE DATA

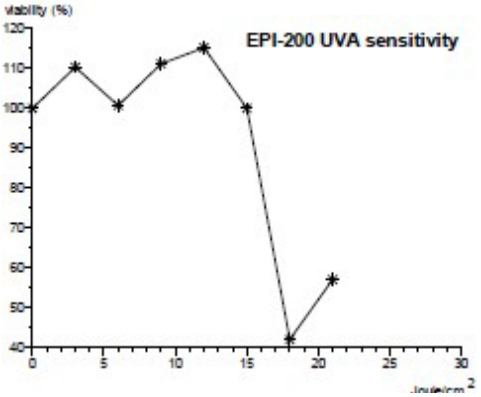


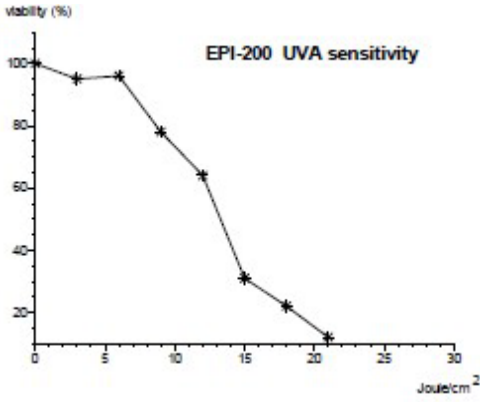


ANNEX F: EPI-200 UVA-SENSITIVITY

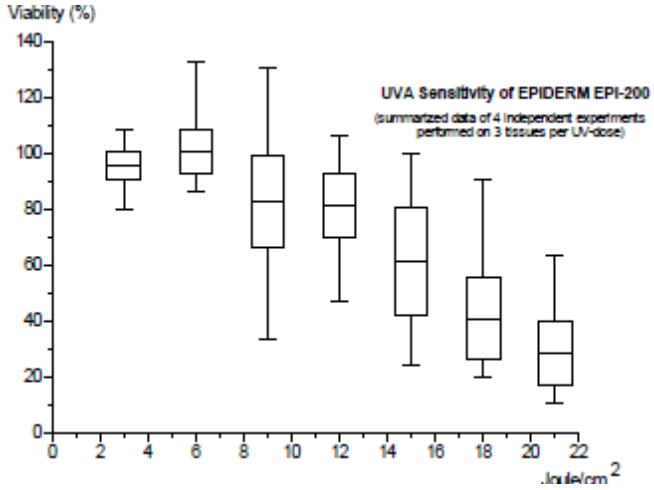








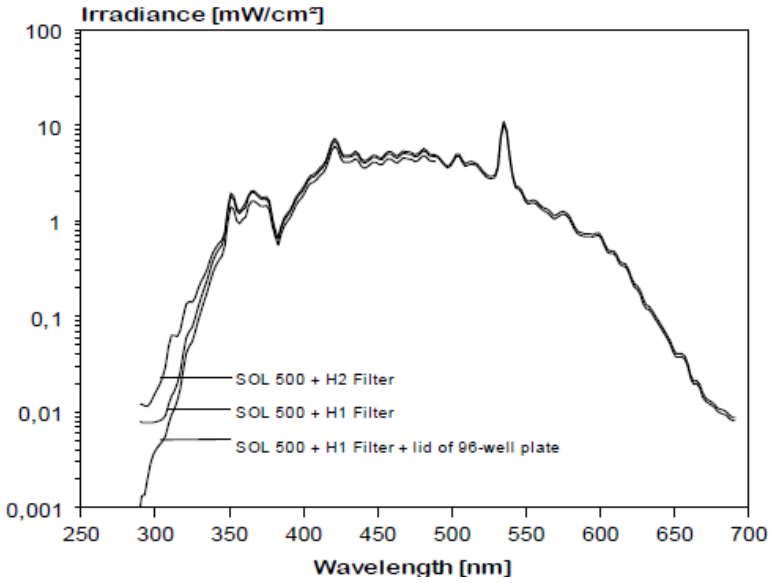
The figures show four independent UV sensitivity experiments performed according to **5.3.1.** The dose of 6 J/cm² used in the EpiDerm™ Phototoxicity Test is not cytotoxic in any of the experiments. In addition, the dose of 6 J/cm² is comparable to doses used in animal tests and has proven to be sufficient to activate phototoxins.



ANNEX G: IRRADIANCE SPECTRUM OF THE SUN SIMULATOR

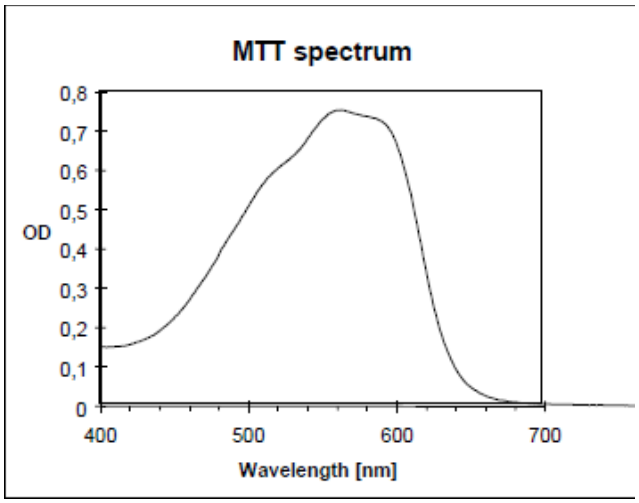
Note: The irradiance spectra of the SOL 3 and SOL 500 are nearly identical up to a wavelength of ~ 550 nm. In the longer wavelength range of visible light (> 550 nm - 700 nm) the SOL 500 irradiance decreases, whereas the irradiance of the SOL 3 remains at the same level. In the EU/COLIPA validation study this difference proved to irrelevant.

(spectrum kindly provided by Beiersdorf AG)



ADDENDUM (13 November 1997)

The spectrum (provided by Beiersdorf AG) shows the FORMAZAN absorption. It explains why the reference filter of 630 nm has been omitted. If the filter is not precise (e.g. 620 nm), the dynamics of the reading can be reduced by ~40%!


MTT spectrum
OD
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
400
500
600
700
Wavelength [nm]