Systemic Phototoxicity Protocol

For Use with EpiDerm™ Model (EPI-200)

This protocol was modified from one originally developed by Dr. Manfred Liebsch of ZEBET (Berlin, Germany) to determine photo-toxicity for topically applied materials. The current protocol models "systemic" exposure of test articles by adding them directly to the culture medium.

CONTENTS:

1 RATIONALE

Phototoxicity (photoirritation) is here 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 a chemical by using a three dimensional human epidermis model*. The test is based upon 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 expressed as reduction of mitochondrial conversion of MTT to formazan [1], determined one day after chemical treatment and UVA exposure.

** The EpiDerm System [2, 3] consists of normal, human-derived epidermal keratinocytes which have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organised basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo. The EpiDerm tissues (surface 0.6 cm²) are cultured on specially prepared cell culture inserts (Millicells®, 10 mm) and shipped world-wide as kits, containing 24 tissues on shipping agarose.*

2 NEED FOR THE ASSAY

Both topically applied and systemically administered medications have the potential to induce photosensitivity. Photosensitivity includes photoallergic reactions, which are immunologically mediated, and phototoxic effects, which can occur following an initial exposure to the drug and sunlight. Phototoxicity is much more common than photoallergy and the majority of medications causing photosensitivity are systemic phototoxicants. All medications must be screened for potential photo-toxicity.

Reconstituted skin models models have shown to be able to predict both, photoirritancy [4, 5, 8], as well as the photoprotective action of sunscreens [5] of topically applied materials. The current protocol attempts to extend the phototoxicity test involving a human skin model may be useful for risk benefit analysis for systemically administered pharmaceuticals.

3 BASIC PROCEDURE

After 24 hour storage at 4ºC to simulate standard overnight shipping, EpiDerm™ tissues are transferred to 6-well plates with pre-warmed assay medium. After 1 hour equilibration of the tissue to standard conditions (37ºC, 5% CO_2) the medium is exchanged with fresh medium. Then, the test material (dissolved in H_20 or DMSO as a 100X concentrate) is added to the culture medium for 2 tissues per test article. A second set of 12 tissues is treated identically. Tissues are incubated for 3 hours. After 3 hours, the tissues are transferred to phosphate buffered saline (PBS). One set of tissues is exposed to 10J/cm² UVA (**+UVA part of the test**) and the other set is kept in the dark for the same period (**-UVA part of the test**). Tissues are then rinsed with PBS, transferred to new 6 well plates with fresh medium and incubated over night. Next day, assay medium is replaced by MTT-medium and tissues are incubated for 3 hours with MTT. Tissues are then rinsed with PBS, and the formazan is extracted with Isopropanol. Optical density is determined at 540/570 nm in a plate spectrophotometer and cell viability is calculated for each tissue as % of the corresponding vehicle control either irradiated or unirradiated.

4 MATERIALS

4.1 Materials, <u>not provided</u> with the Kits:

4.1.1 Laboratory aids

Sterile, blunt-edged forceps	*for transferring inserts*
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	
5 Beakers (50 ml)	
sterile, capped glass or plastic test tubes	*for preparing the concentration series*
Repeat pipetter (2 ml)	*for adding the extractant solution*
Positive displacement pipettes (20 µl, 50µl)	*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 ul)	*for medium change*

4.1.2 Technical Equipment

Autoclave	*for sterilising forceps*
37°C humidified incubator with 5% CO_2,	*for incubating tissues prior to and during assays*
vacuum source/trap	*for aspirating solutions*
laminar flow hood	*for transferring tissues under sterile conditions and for application of test materials.*
37°C water bath	*for warming up Assay Medium, PBS etc.*
96-well Spectrophotometer (Plate-Reader) equipped with filter 570 nm or 540 nm	*for reading optical density at 570 nm,*
Shaker for cell culture plates	*for extraction of formazan*
Laboratory centrifuge 1500 x g	*for centrifugation of MTT medium*

4.1.3 UVA-vis Irradiation equipment

UV-sun simulator type SOL 500 or SOL 3	*Dr. K. Hönle GmbH, Frauenhoferstr. 5, D-82152 Martinsried, Germany* *Contact: Dr. G. Schmid* *☎: +49-89-856 08-0* *Fax: +49-89-856 08-48*
Any appropriate, adjustable and stable tripod	*For the fixation of the SOL 500*
UVA-meter, type No. 37, Dr. Hönle	*For everyday check of calibration*
UVA-meter, type No. 37, Dr. Hönle	*Use only as a reference in case of unexpected readings with the everyday radiometer*
Filter, type H1, Dr. Hönle	*Use to cut-off emitted UVB*

4.1.4 Solutions, Reagents

DMSO (reagent grade) source: Sigma Aldrich	*Vehicle for test materials*
H_2O Aqua Pur (Millipore®), or distilled H_20	*Vehicle for test materials*
PBS with Ca^{++} and Mg^{++}: ~ 500ml per test (e.g. Gibco # 14040)	*For rinsing-off test materials after irradiation*

4.1.5 Computer software

(MS Windows) software for Plate spec-trophotometer	*software must be able to export data*
MS Excel 5.0	*For and data analysis in the Data Spreadsheet*

4.2 Epi-200 Kit Components

*Examine all kit components for integrity. If there is a concern call MatTek Corporation immediately (Mitch Klausner, ☎ +1-508-881-6771, **Fax** +1-508-879-1532).*

1	Sealed 24-well plate	*Contains 24 inserts with tissues on agarose*
2	24-well plates	*Use for MTT assay and formazan extraction*
4	6-well plates	*Use for pre-incubation (and assay)*
1 bottle	Serum-Free Assay Medium	*DMEM-based medium*
1 bottle	PBS Rinse Solution (100 mL)	*Use for rinsing the inserts in MTT assay*
1 vial	1% Triton X-100 Solution (10 mL)	*Skin irritant reference chemical* ***Do not use in present assay***
1	MTT Assay Protocol	*steps are included in the present SOP*

4.3 MTT-100 Assay Kit Components

1 vial, *2 ml*	MTT concentrate	
1 vial, *8 ml*	MTT diluent	*For diluting MTT concentrate*
1 bottle, *60 mL*	Extractant Solution (Isopropanol)	*For extraction of formazan crystals*

5 METHODS

5.1 Expiration and Kit Storage

Epi-200 kits are stored overnight to simulate standard express shipment to testing laboratories on Tuesday. Alternatively, kits may be stored until Wednesday. Storage conditions are given in the following table.

part #	description	conditions	shelf life
EPI-200	EpiDerm™ cultures	refrigerate (4°C)	until Friday
EPI-100	assay medium	refrigerate (4°C)	7 days
MTT-099	MTT diluent	refrigerate (4°C)	7 days
MTT-100	MTT concentrate	freeze (- 20°C)	2 month

All lot numbers of all components are recorded.

5.2 Calibration of the Solar Simulator

Note: *New metal halide burners should be burned for ~100 hrs prior to first use to achieve a stable emittance. According to Dr Hoenle the burner has a shelflife (in which the spectrum is stable) of at least 800 hrs. Recording of lamp usage hours is, therefore, recommended. Extended use is only acceptable if the emitted energy spectrum can be checked.*

1. Mount the SOL 500 / SOL 3 lamp, equipped with a H1-filter, on any appropriate stable tripod allowing fine-adjustment of the exposure distance.
2. Adjust SOL 500 / SOL 3 to a distance of about 60 cm.
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 (type 37, Dr. Hönle), equipped with an UVA-sensor of the same serial number.
4. Adjust distance of SOL 500 / SOL 3 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 difference of **1.5 and 1.9 mW/cm²** can be accepted, if positions of the plates with low and high irradiance are changed after half time of the irradiation (30 minutes) is reached (like chess castling).*

Calibration of the SOL 500 / SOL 3 shall be checked as described above each time before performing a phototoxicity assay. In case measurements with the UV radiometer reveal unexpected results, either the metal halide burner may have reached the end of it's shelflife, or the radiometer is de-calibrated due to various reasons. In this case, a second reference radiometer of the same type and calibration, which is has not been handled every day and kept in the dark shall be used for cross check.

5.3 Quality Controls

5.3.1 UVA Sensitivity of the Epi-200 Tissues

Note: *A UVA sensitivity experiment should be performed once the test is newly set up in a laboratory. If UVA sensitivity of the tissues is within the acceptance range this type of experiment should be repeated in greater intervals (e.g. once every 6 months).*

1. Incubate 24 tissues (37°C, 5 % CO_2) for **at least 1 hr** in 6-well plates with 0.9 ml assay medium /well to allow release of metabolites and debris accumulated during the shipment.
2. Adjust irradiance of the SOL 500 / SOL 3 to **1.7 mW/cm^2** (measure through plate lid !)
3. For UV irradiation, transfer 21 tissues to a 24-well plate filled with 0.3 mL assay medium per well.
4. Prepare a 24 well plate with 0.3 ml assay medium per well and transfer the 3 tissues serving as non-irradiated control. Place this plate in a dark box at room temperature.
5. Start irradiation of the 21 tissues **through the lid of the plate**. Use a fan to prevent H_20 condensation under the lid. Every 30 minutes (= 3 J/cm^2) transfer 3 tissues from the irradiation site to the dark box. The resulting dose series is **3, 6, 9, 12, 15, 18, 21** J/cm^2.
6. Incubate tissues over night (18 - 24 hrs.) at 37°C, 5 % CO_2, 90 % humidity
7. Determine tissue viability according to **5.5**. Compared to the non irradiated tissues (100 % viability) up to 10 J/cm^2 (= 60 minutes) there shall be no reduction of viability exceeding 20%. The historical $ID_{50\ UVA}$ is in the range of ~12 - 18 J/cm^2 (see **ANNEX F**).

5.3.2 Negative Control

The **absolute OD** of the negative control tissues in the MTT-test (see **5.5**) is an indicator of tissue viability obtained in the testing laboratory after shipping procedure and under specific conditions of the assay.

Tissue viability is meeting the acceptance criterion if the mean OD of the two negative control tissues (determined without reference filter) is ***OD ≥ 0.8****.*

5.3.3 Positive Control: Chlorpromazine (CPZ)

A positive control, **Chlorpromazine** (dissolved in H_20) at 2.5 mg%, is included in each phototoxicity test.

5.3.4 Maximum inter tissue viability difference of tissue couples

The new spreadsheet calculates differences in viability between tissue couples that are treated identically. According to the historical data base of ZEBET the mean difference between untreated tissue duplicates is 9% ± 7% (S.D.).

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.*

5.4 Test Sample Preparation and Test Concentrations

According to their solubility, chemicals are applied either as solution in **water**, or as solution in DMSO.

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 in DMSO. Water soluble test materials (category 1-4) shall be tested dissolved in water.

5.4.1 Concentration series

Results from the photo-toxicity test are valid only for the concentrations actually tested. For example, if a material is non-photo-toxic as a certain concentration it is possible that it would be photo-toxic at a higher concentration. Conversely, if a material is photo-toxic at a certain concentration, it is possible that at lower concentrations it is non-photo-toxic. Thus, it is desirable to prepare a concentration series of the test material bracketing the actual end use concentration (in the bloodstream). 10X, 3X, 1X, 0.3X, and 0.1X of the end use concentration are recommendded if sufficient test article is available. Where possible, the highest concentration of a test material should show cytotoxicity in non-irradiated tissues.

5.4.2 Preparation of test article in assay medium

Prepare a 100 X concentrate of the test article in ultrapure H_2O or in DMSO. For each test article concentration to be tested, a minimum of 4 ml of test article/assay medium is required. Add 1 part concentrate to 99 parts assay medium (EPI-100-MM) supplied with the kits (e.g. 0.05 ml concentrate/4.95 ml assay medium).

6 Experimental Procedure

First day of testing

Note: *It is essential that, before the test is started, tissues are incubated for* ***at least 1 hr*** *in assay medium into which they can release metabolites and debris accumulated during the shipment/overnight storage. This medium has to be replaced before the assay is started. For this important incubation 6-well plates have to be used with 0.9 mL medium per well.*

1. Prewarm assay medium in a 37°C waterbath.

2. Pipet 0.9 ml of assay medium into each well of sterile 6-well plates.

3. Using sterile techniques transfer the inserts (be sure to remove all transport agar) into 6-well plates containing prewarmed assay medium. Any air bubbles trapped underneath the inserts should be released. Incubate for 1-1.5 hr at 37°C, 5% CO_2. *Record the starting and ending incubations times in the lab notebook.*

4. While tissues are in the incubator, prepare the test articles according to **5.4.1**. *Record preparations in the lab notebook.*

5. After 1-1.5 hr incubation, transfer the inserts to a 24-well plates containing 0.3 ml of PBS/well.

6. Per each test concentration, use 4 EpiDerm™ tissues: Two tissues are used in the **(-UVA)** cytotoxicity part and 12 in the **(+UVA)** phototoxicity part of the test. Both parts of the test are dosed identically: Also, apply the vehicle control (VC) to 4 tissues as well. Mark lids and plates to prevent from transposition errors.

7. Once all tissues have been dosed, cover the plates with the lids and incubate for 3 hrs at 37°C, 5% CO_2.

8. Remove 24-well plates from the incubator. Irradiate (**+UVA**)-plates (covered with lids) for **22.5 min** with **7.5 mW/cm^2** (= 10 **J/cm^2**) at room temperature. Ventilate with fan to prevent condensation under the lid. Place (**-UVA**)-plates in the dark at room temperature.

9. After UVA irradiation is completed, use wash bottle with sterile PBS and rinse each insert of the (+UVA) plates and (-UVA) plates. Then transfer all inserts to the new 6-well plates containing 0.9 ml/well of assay medium**.**

10. Incubate (+UVA)- and (UVA) plates over night (18-24 hrs) at 37°C, 5 % CO_2

Second day of testing

1. Prepare MTT medium: thaw MTT concentrate in a water bath and dilute with MTT diluent. Spin down (300×g, 5 min) to remove any precipitate. Prewarm MTT medium (water bath) to 37°C.

2. For *each* test material prepare *one* 24-well plate with 300 µL prewarmed MTT medium per well. Label plates (lid and bottom) and transfer tissue inserts according to the plate design given below. Any air bubbles trapped underneath the inserts should be released.

4. Incubate 24 well plate 3 hours (37°C, 5 % CO_2). *Record start and stop time for MTT incubation in the LAB NOTEBOOK.*

***Note**: Deviations from 3 hour time for MTT incubation will result in different MTT readings. For consistency the 3 hour MTT incubation time should be adhered strictly.*

5. After incubation, remove the cell culture inserts, blot the bottom of the inserts, and transfer the inserts to new 24 well plates.

6. Immerse the inserts in extractant by gently pipetting 2 mL extractant solution (isopropanol) **into** each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.

7. Seal the 24 well plate (e.g. with a zip bag) to inhibit isopropanol evaporation. *Record start time of extraction in the lab notebook.* Extract 2 hrs with shaking (~120 rpm) at room temperature or overnight without shaking.

8. After the formazan extraction period is complete, decant the extract in the insert back into the well from which the insert was taken. Afterwards the insert can be discarded.

9. Transfer 200µL aliquots of the blue formazan extract into a 96-well flat bottom microtiter plate. Prior to the transfer, pipet the extract up and down to homogenize. Read OD in a plate spectrophotometer at **570 nm**.

6.1 Data analysis

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 an EXCEL spreadsheet. The EXCEL spreadsheet is set up so that the viability of each tissue is calculated by dividing by the OD for the tissue by the average OD for the unexposed, non-irradiated negative control tissues. In addition, the difference in viability between the non-irradiated and irradiated tissues for each test article is computed.

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7 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 prediction model.

The prediction model for systemic photo-toxicity is based on the prediction of Liebsch that was developed for topically applied materials.

Liebsch based the topical photo-toxicity prediction model on analysis of historical data of the maximum possible difference in the viability of identically treated EpiDerm™ tissues according to which any difference exceeding 30% has to be regarded significant ($p < 0.001$). Since the UVA irradiation has no cytotoxic effect itself, a phototoxic activity can be predicted if viability of tissues treated with identical test chemical concentrations differs by more than 30% in the irradiated and the non irradiated part of the test.

For each concentration of a test chemical, the mean OD of the tissue couple treated with this concentration is determined and expressed as relative percentage viability of the untreated vehicle controls. Identical calculations are performed for the (+UVA) part of the test and the (-UV) 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 **exceeding 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.

8 REFERENCES

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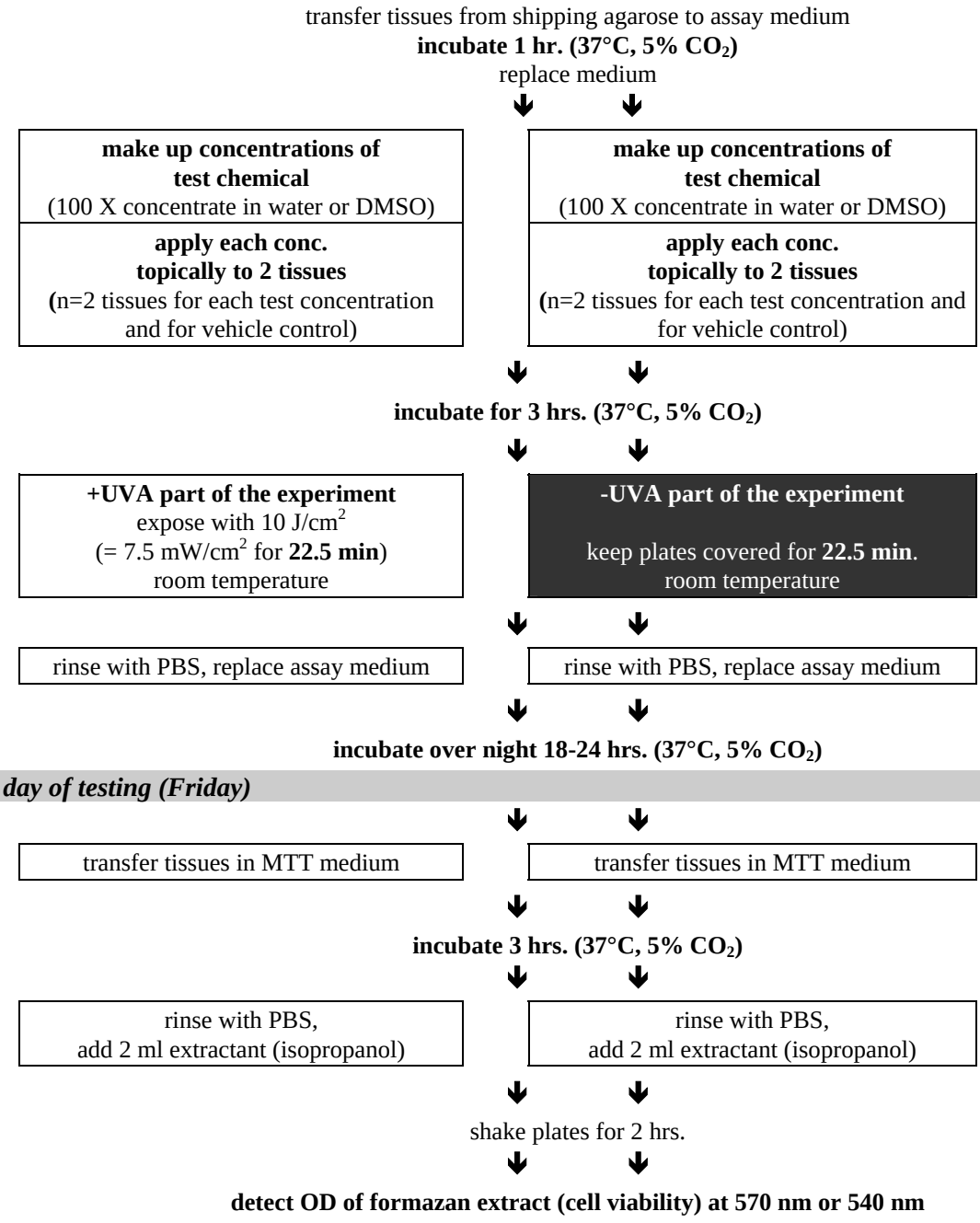
9 ANNEX A: Procedure Steps

First day of testing (Tuesday or Wednesday)

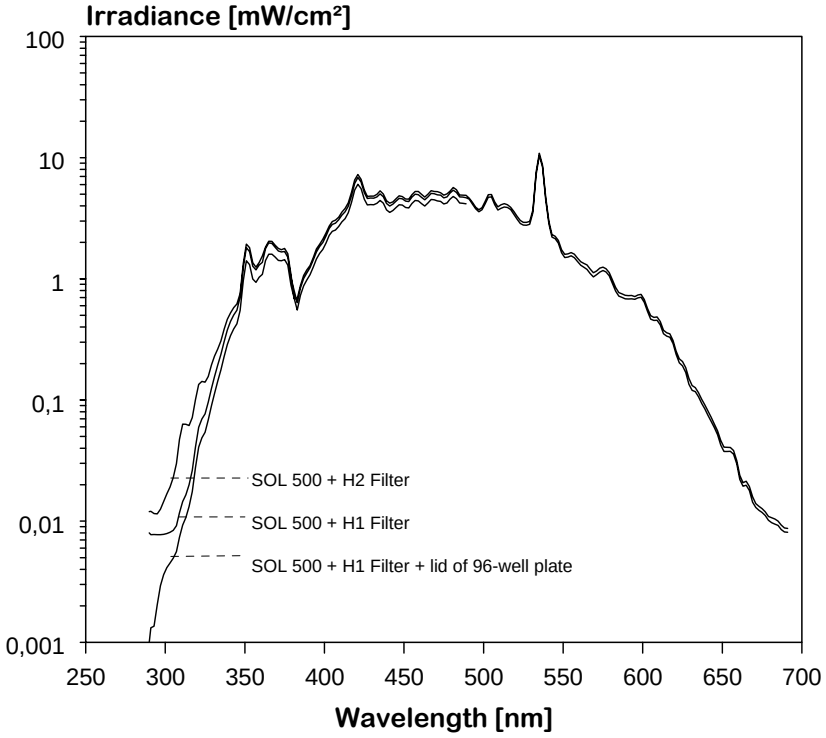


Second day of testing (Friday)

10 ANNEX B: Irradiance spectrum of the sun simulator

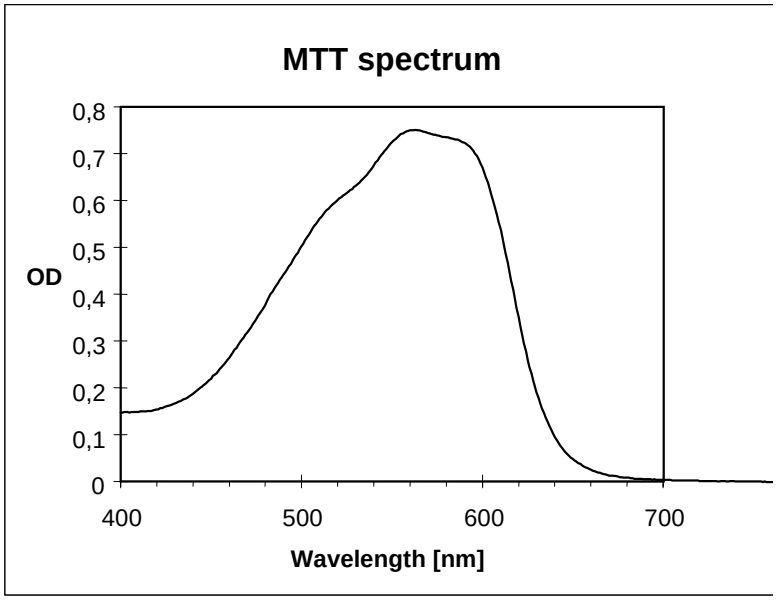
Note: *The irradinace spectra of the SOL 3 and SOL 500 are nearly identical up to a wavelength of ~ 550 nm. In the longer wavelength range of visible light (> 550 nm - 700 nm) the SOL 500 irradiance decreases, whereas the irradiance of the SOL 3 remains at the same level. In the EU/COLIPA validation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