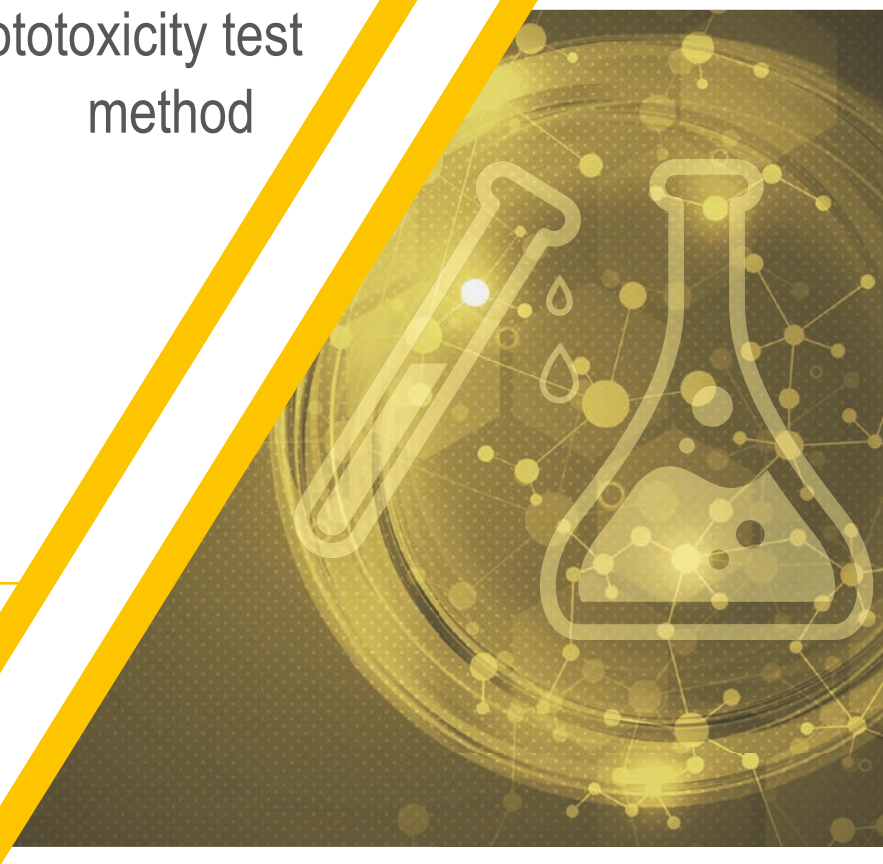
Section 4
Health effects

Test Guideline No. 498

In vitro Phototoxicity: Reconstructed Human Epidermis Phototoxicity test method



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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR TESTING OF CHEMICALS

In vitro Phototoxicity: Reconstructed Human Epidermis Phototoxicity test method

INTRODUCTION

1. Phototoxicity (photoirritation) is defined as an acute toxic response elicited by topically or systemically administered photoreactive chemicals after the exposure of the body to environmental light. Within the context of skin exposures to phototoxic chemicals, phototoxic responses are elicited after an acute exposure of skin to photoactive chemicals and subsequent exposure to light. The test method does not address photosensitizers, which are photoactive chemicals that can induce an immunologically-mediated response in exposed skin.

2. This Test Guideline (TG) addresses the human health endpoint of phototoxicity, specifically as it relates to topical skin exposures to phototoxic chemicals. The *in vitro* reconstructed human epidermis phototoxicity test (RhE PT) is used to identify the phototoxic potential of a test chemical after topical application in reconstructed human epidermis (RhE) tissues in the presence and absence of simulated sunlight (see paragraphs 37-38 for the characterization of simulated sunlight). Phototoxicity potential is evaluated by the relative reduction in viability of cells exposed to the test chemical in the presence as compared to the absence of simulated sunlight. Chemicals identified as positive in this test may be phototoxic *in vivo* following topical application to the skin, eyes, and other external light-exposed epithelia.

3. This TG is based on the *in vitro* test system of the reconstructed human epidermis (RhE), which closely mimics the biochemical and physiological properties of the outermost layers of the human skin, i.e., the epidermis. The RhE test system uses human-derived keratinocytes as a cell source to reconstruct an epidermal model with representative histology and cytoarchitecture. The test method combines the basic test chemical exposure and viability assessment methodologies described in the OECD TG 439 on *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test (1) with the standardized irradiation procedures described in the OECD TG 432 on In Vitro 3T3 NRU Phototoxicity Test (2). Exposure times in this TG are notably longer than those described in TG 439 to ensure sufficient time for chemical ingredients to diffuse into the tissue model nearest the target cells where reactive phototoxic species may be induced at the time of irradiation.

4. An assessment of the general performance was based on an *ad hoc* evaluation of individual literature citations (3)(4)(5)(6)(7)(8)(9)(10) including an initial test method pre-validation reported in 1999 (4) with a sensitivity of 86.7% and specificity of 93.3% (Phase III, set of 10 chemicals tested twice independently in three laboratories). The data were presented to the COLIPA Phototoxicity task-force in 1999 (11). The task-force concluded that 3D RhE skin model could be a useful tool to address bioavailability of a test chemical. When used in combination with the 3T3 NRU PT as a complementary test, it will allow a clear decision in the case of borderline predictions of acute phototoxicity. The 3D RhE skin model test was also regarded as

advantageous in helping to assess the phototoxic potency. A follow-up study on phototoxic potency was supported by ECVAM during 2003-2006 (12).

5. To support the development of the new OECD phototoxicity guideline, the OECD phototoxicity expert group conducted an *ad hoc* evaluation of the pre-validation data, together with other literature, on the ability of the 3D RhE skin models to predict acute phototoxic potential in vitro. From the literature review, a database of over 60 test chemicals evaluated in the EpiDerm™ skin model was derived. The aggregate datasets provided specificity and sensitivity values higher than those obtained in the pre-validation study (4), as previously sensitivity values were compromised by erroneous instructions on the solvent use (10). It should be noted that other studies performed after the initial pre-validation study, whilst using the same SOP, were not conducted as formal validation studies.

6. Definitions used in this TG are provided in ANNEX 1

INITIAL CONSIDERATIONS AND LIMITATIONS

7. Many types of chemicals have been reported to induce phototoxic effects (13)(14)(15)(16). Their common feature is their ability to absorb light energy within the sunlight emission spectrum. Photoreactions require sufficient absorption of light quanta. Thus, before testing is considered, a UV/visible absorption spectrum of the test chemical should be determined according to OECD TG 101 on UV-VIS Absorption Spectra (17). It has been reported that if the molar extinction/absorption coefficient (MEC) is less than 1000 L mol^{-1} cm^{-1}, the chemical is unlikely to be photoreactive (18)(19). Such chemicals may not need additional testing with the *in vitro* RhE PT or any other biological test for adverse photochemical effects (3)(20). In general, this principle applies to all test chemicals, however, more specific guidelines may apply depending on the intended use of the chemical or potential exposure conditions. The RhE PT test can be used as a stand-alone method, and also in a tiered testing strategy for topically applied substances following specific guidelines (such as ICH S10 for pharmaceuticals). The term "test chemical" is used in this TG to refer to what is being tested and is not related to the applicability of the RhE PT to the testing of substances and/or chemical mixtures. Limited information is currently available on the applicability and performance of the RhE PT to mixtures of known composition. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this TG, it should be first considered if the results of such testing will be scientifically meaningful.

8. The reliability and relevance of the *in vitro* RhE PT was evaluated in multiple studies (3)(4)(5)(6)(7)(8)(9)(10). The procedures and prediction model presented in this TG are designed to distinguish between phototoxic and non-phototoxic chemicals. However, specific procedures and prediction models exist in the literature to address phototoxic potency for topically applied chemicals and mixtures. This TG is not designed to predict other adverse effects that may arise from the combined action of a chemical and light (e.g., it does not address photo-genotoxicity, photoallergy, or photocarcinogenicity). Furthermore, the TG has not been designed to address indirect mechanisms of phototoxicity, effects of metabolites of the test chemical, or evaluate the phototoxicity potential of individual chemicals in mixtures.

9. The *in vitro* RhE PT does not need to be performed with a metabolic activation system, although the RhE tissues have limited metabolic activity (21). There is no evidence at this time that any phototoxic compound would be missed in the absence of metabolic activation (22).

10. Test chemicals absorbing light in the same range as MTT formazan (colored chemicals), or test chemicals able to directly reduce the vital dye MTT (to MTT formazan) may interfere with the cell viability measurements if those chemicals persist in or on the test system at the time of the viability assessment, and may need to use adapted controls to correct for the interference (see paragraphs 59-66) in section "*Corrections for MTT-reducing Materials and Colorants*". Alternatively, the HPLC/UPLC spectrophotometry procedure to measure MTT formazan can be used (see paragraphs 65-66).

11. Although most of the studies performed with RhE PT utilized the UVA/visible light part of the solar spectrum, some studies confirm that the RhE tissues can also tolerate UVB exposure (5) (23)(24) under controlled conditions. This is an advantage compared to most of the cell-line based assays (2) that do not tolerate the UVB part of the spectrum well (25).

12. A single testing run should be sufficient for a test chemical when the classification is unequivocal. However, in cases of borderline results, such as non-concordant results from replicate tissues, a second run should be considered, as well as a third one in case of discordant results between the first two runs. In the repeated runs, the concentrations of the test chemical may be adjusted to better capture the range of responses around the borderline or equivocal concentration(s) (see Interpretation of Results and Prediction Model for details).

13. The phototoxicity potential of a test chemical is determined by testing multiple concentrations in RhE tissues in the presence and absence of simulated sunlight. The testing of three to five concentrations in two replicates is generally sufficient to ensure obtaining acceptable test results from at least one concentration of the test chemical to make a valid prediction. Specific criteria for acceptable test results are presented with the prediction model.

PRINCIPLE OF THE TEST

14. Phototoxicity potential in the RhE PT is evaluated by the relative reduction in viability in RhE tissues exposed to the test chemical in the presence as compared to the absence of a non-cytotoxic dose of simulated sunlight.

15. The test chemical is applied topically to a three-dimensional RhE tissue, composed of human-derived epidermal keratinocytes that have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*. Accordingly, RhE tissues are ideally suited for directly modeling exposures of chemicals on native skin *in vivo* and have been validated to predict the skin irritation and corrosion hazards of chemicals and mixtures without the need for test chemical dilution (1)(26).

16. In brief, several concentrations of test chemical prepared in a solvent are applied topically to RhE tissues and incubated at standard culture conditions (37 ± 1 °C, 5 ± 1% CO_2, 90 ± 10% RH) for 18 to 24 hours to allow penetration into the living tissue. In general, three to five concentrations are tested to ensure obtaining results from at least one concentration that meets the criteria for a valid test. A positive control and appropriate solvent controls are also applied topically to RhE tissues and tested in parallel. Half of the tissues in each treatment group are irradiated with 6 J/cm^2 of simulated sunlight (+Irr) while the remaining half are held at room temperature in the dark (−Irr) (see paragraph 37 & 38 for details). After a post-exposure incubation period of 18 to 24 hours, relative viability is determined in both the irradiated (+Irr) and non-irradiated (−Irr) treatment groups by measuring the enzymatic conversion of the vital dye MTT (3-[4,5 dimethylthiazol 2 yl] 2,5-diphenyltetrazolium bromide, thiazolyl blue (CAS number

298-93-1) into a blue formazan salt that is measured photometrically after extraction from the tissues.

17. Phototoxicity potential is determined by comparing the relative reduction in viability in each irradiated treatment group to that of the equivalent non-irradiated treatment group.

18. The experimental design is based on the pre-validation study performed by ZEBET (4)(27) and follow up-studies conducted with this protocol. The follow-up studies suggested some minor modifications that led to better reproducibility and sensitivity of the test. The updated protocol was published in 2017 (10). The procedure described in this TG is based on the updated protocol.

19. This test method can be used as a stand-alone test method to address phototoxicity, especially in cases of limited test material solubility or endpoint-compatibility issues with OECD TG 432 (2) and OECD TG 495 on ROS Assay for Photoreactivity (28). This test method can also be used in a tiered testing strategy in combination with the OECD TG 432 and/or OECD TG 495 (8)(11)(22)(29).

DEMONSTRATION OF PROFICIENCY

20. Prior to the routine use of the test method, laboratories should demonstrate technical proficiency by correctly classifying the six Proficiency Substances listed in **Table 1**. In situations where a listed chemical is unavailable or cannot be used for other justified reasons, another chemical for which adequate *in vivo* and *in vitro* reference data are available may be used (e.g., from the list of reference chemicals (3)(4)(5)(6)(7)(8)(9)(10)) provided that the same selection criteria as described in Table 1 are applied. Using an alternative proficiency substance should be justified.

21. As part of the proficiency testing, if users are naïve to utilizing the RhE model within the testing facility, it is recommended that users verify the barrier properties of the tissues after receipt as specified by the RhE model producer. This is particularly important if tissues are shipped over long distance/time periods. However, once a test method has been successfully established and proficiency in its use has been demonstrated, such verification will not be necessary on a routine basis.

Table 1. Proficiency Substances[1]

	Substance	CAS RN	In vivo[2]	Solvent[3]	Typical phototoxicity ranges [% w/v or % v/v] (references)
			PHOTOTOXIC SUBSTANCES		
1	Chlorpromazine	50-53-3	PT	Water	0.003% – 0.01% (4)
2	Anthracene	120-12-7	PT	EtOH[4]	0.01% – 0.03% (5)(30)
3	Bergamot oil[6]	8007-75-8	PT	Oil[5]	0.0316% – 3.16% (4)(8)
			NON-PHOTOTOXIC SUBSTANCES		
4	Sodium Dodecyl Sulphate	151-21-3	NPT	Water	Non-phototoxic up to highest conc. tested (1%) (4)
5	Octyl salicylate	118-60-5	NPT	Oil[5]	Non-phototoxic up to highest conc. tested (10%) (4)
6	4-Aminobenzoic acid (PABA)	150-13-0	NPT	Oil or EtOH	Non-phototoxic up to highest con. Tested (10%).(27)(30)

Notes: [1] The Proficiency Substances are a subset of the substances used in the pre-validation and follow up studies and the selection is based on the following criteria; (i), the substances are commercially available; (ii), they are representative of the full range of phototoxic effects (from non-phototoxic to strong photoirritants); (iii), they have a well-defined chemical structure; (iv), they are representative of the chemical functionality used in the validation process; (v) they provided reproducible *in vitro* results across multiple testing and multiple laboratories; (vi) they were correctly predicted *in vitro*, and (vii) they are not associated with an extremely toxic profile (e.g., carcinogenic or toxic to the reproductive system) and they are not associated with prohibitive disposal costs, and (viii) results for the selected materials and protocol details are available in the literature.

[2] PT – Phototoxic; NPT – Non-Phototoxic (Note: In vivo classifications were derived from the validation studies of 3T3 NRU PT test (OECD TG 432) and were mostly based on clinical human data as no validated *in vivo* method is available for this endpoint.)

[3] Solvents are suggested, based upon the pre-validation and follow-up study references

[4] EtOH – Ethanol

[5] Oil – Sesame seed oil

[6] Variability in phototoxic response is influenced by the content of impurities, therefore we advise to use a non-purified Bergamot oil, commercially available, specifically for this reason. Non purified Bergamot oils do have significant absorption in UVA and UVB part of the spectra (8).

PROCEDURE

22. The following is a description of the components and procedures of a RhE test method for phototoxicity testing. Standard Operating Procedure (SOP) for the RhE-based tests complying with this TG is available (27). The SOP with minor technical improvements, guidance on solubility, solvents recommendations and with information on the chemicals tested in the EpiDerm protocol has been published in detail in 2017 (10). The SOP for the RhE-based tests complying with this TG should be employed when implementing and using the test method in a laboratory.

General Test System Characterisation

23. Human keratinocytes should be used to reconstruct the epithelium. Multiple layers of viable epithelial cells (basal layer, stratum spinosum, stratum granulosum) should be present under a functional *stratum corneum*. The *stratum corneum* should be multi-layered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic benchmark chemicals (e.g., the surfactants sodium dodecyl sulphate (SDS) and Triton®-X-100 are typically used to test barrier function). The containment properties of the RhE model should prevent the passage of material around the *stratum corneum* to the viable tissue, which would lead to poor modelling of skin exposure. The RhE tissue should be free of contamination by bacteria, viruses, mycoplasma, or fungi.

Functional Conditions

Viability

24. The assay used for quantifying viability is the MTT-assay (31). The viable cells of the RhE tissue can reduce the vital dye MTT into a blue MTT formazan precipitate which is then extracted from the tissue using isopropanol (or a similar solvent). The optical density (OD) of the extraction solvent alone should be sufficiently small, i.e. OD < 0.1. The extracted MTT formazan may be quantified using either a standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure (32). The RhE model developer/supplier should ensure that each batch of the RhE model meets defined quality control criteria for the negative controls. Acceptability ranges (upper and lower limit) for the negative control OD values (in conditions described in paragraph 56) are established by the RhE model developer/suppliers and presented in **Table 2**. The RhE model user should ensure that the results of the solvent (i.e. negative) controls meet the specific test method acceptance criteria. An HPLC/UPLC Spectrophotometry user should use the negative control OD ranges provided in **Table 2** as the acceptance criterion for the solvent (i.e. negative) control.

Table 2. Acceptability ranges for solvent (i.e. negative) control OD values in the MTT assay of the test methods included in this TG

	Lower acceptance limit	Upper acceptance limit
EpiDerm™ (EPI-200)	0.8	2.8

Barrier function

25. The RhE model developer/supplier should ensure that each batch of the RhE model meets defined quality control criteria for barrier function. The barrier function may be assessed either by determination of the concentration at which a benchmark chemical (e.g., sodium dodecyl sulphate (SDS) or Triton®-X-100) reduces the viability of the tissues by 50% (IC_{50}) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50% (ET_{50}) upon application of the benchmark chemical at a specified, fixed concentration. The acceptability ranges for the test methods included in this TG are given in **Table 3**.

Table 3. Barrier Function QC batch release criteria of the RhE models included in this TG

	Lower acceptance limit	**Upper acceptance limit**
EpiDerm™ (EPI-200)	ET_{50} = 4.00 h	ET_{50} = 8.72 h

Morphology

26. Histological examination of the RhE model may be provided by the RhE model developer/supplier demonstrating human epidermis-like structure (including multilayered *stratum corneum*) if this parameter is used in the RhE model developer/supplier's QC release program.

Reproducibility

27. The RhE model developer/supplier should maintain a database of the QC release test results of the viability and barrier function tests to monitor reproducibility over time. It is recommended that the RhE model user maintain a database of the phototoxicity test method positive and solvent (i.e. negative) control results to monitor reproducibility of test method execution over time.

Quality control (QC)

28. The RhE model should only be used if the developer/supplier demonstrates that each batch of the RhE model meets defined production release criteria, among which those for viability (paragraph 24), barrier function (paragraph 25) and morphology (paragraph 26), if applicable, are the most relevant. The relevant QC data should be provided to the test method users, so that they are able to include this information in the test report. Only phototoxicity test results produced with qualified tissues can be accepted for reliable prediction of phototoxicity.

Preparation of Test Chemical and Control Substances

29. Test chemicals must be prepared fresh on the day of testing unless data demonstrate their stability in storage. It is recommended that all chemical handling and the initial treatment of tissues be performed under conditions that would avoid photoactivation or degradation of the test chemical prior to irradiation. The maximum recommended concentration of a test chemical should not exceed 10% since test chemicals may absorb UV and act as a UV filter (10)(27).

30. The testing of three to five concentrations of a test chemical in a solvent is generally sufficient to ensure obtaining acceptable test results from at least one concentration of the test chemical to fulfill requirements to evaluate the test results for phototoxicity potential (see paragraphs 69-71). Ideally, the concentrations of the test chemical should be selected to ensure a cytotoxicity dose response in the absence of irradiation. Guidance for selection of appropriate concentration ranges is given in the SOPs (10)(27).

31. Water soluble test chemicals are prepared in ultra-pure water or if appropriate in buffered salt solutions (e.g., Dulbecco's Phosphate Buffered Saline (DPBS) or Hanks' Balanced Salt Solution (HBSS) without phenol red). The buffer used must be free from protein components and light absorbing components (e.g., pH indicators such as phenol red and vitamins) to avoid interference during irradiation.

32. Oil soluble test chemicals are prepared in sesame seed oil or other appropriate oil (e.g., mineral oil that has low UV absorption and is demonstrated to be compatible with the RhE tissues). For test chemicals of limited solubility in water and oils, pure ethanol, or a mixture of acetone:olive oil (4:1 v:v) may be used (10).

33. Other solvents may be considered but should be evaluated prior to use for specific properties including compatibility with the RhE tissues, its ability to react with the test chemical, ability to induce phototoxicity, potential for quenching of the phototoxic effect, radical-scavenging properties and/or chemical stability in the solvent (33). When other solvents are used, it is recommended that a pre-testing with the selected solvent be conducted to ensure solvent stability and compatibility with the test system (see ANNEX 3additional guidance).

34. Vortex mixing, sonication, and/or warming to appropriate temperatures may be used to aid solubilisation, unless the stability of the test chemical is compromised. The procedures used to prepare the test chemical dosing solutions should be documented.

35. Before any testing on the viable reconstructed human tissues is performed, it is recommended to perform the evaluation of the test substance for interference with the measured endpoint (MTT assay). These procedures are described in detail in the SOPs. If potential interference by the test substance on the MTT assay has been determined, the application of adaptive controls is recommended as described in the section "*Corrections for MTT-reducing Materials and Colorants*."

36. A solvent control (used as a negative control) and positive control (PC) should be tested concurrently in each run. The suggested solvent control is either water (solvent for water soluble materials) or sesame seed oil (solvent for oil soluble materials), and/or other solvents used to solubilize the test material. The suggested PC is a solution of chlorpromazine at a final concentration of 0.01% to 0.02% in water (or other aqueous buffered salt solutions such as DPBS or HBSS without phenol red). Additional concentrations can be tested to evaluate dose responses of the chlorpromazine prior to establishing the test to demonstrate proficiency (4)(10)(27).

Irradiation Conditions

37. *Light source:* The choice of an appropriate light source (e.g., a solar simulator) and filters is a crucial factor in phototoxicity testing. Light of the UVA and visible regions is usually associated with phototoxic reactions *in vivo* (15)(28), whereas generally UVB is of less relevance but is highly cytotoxic; the cytotoxicity increases 1000-fold as the wavelength goes from 313 to 280 nm (28). Acceptable light sources emit the entire solar spectrum (290 nm through 700 nm). Adjustment of the spectrum can be performed using filters to attenuate UVB while allowing

transmittance of UVA and visible light (See ANNEX 2:). Furthermore, the wavelengths, irradiance doses employed, and light source equipment (e.g., open or closed system) should not be unduly deleterious to the test system (e.g., from emission of heat/ wavelengths in the infrared region).

38. The simulation of sunlight with solar simulators is considered the preferable artificial light source. The spectral irradiance of the filtered solar simulator should be close to that of outdoor daylight (34). Both xenon arcs and (doped) mercury-metal halide arcs are used as solar simulators (30). The latter have the advantage of emitting less heat and being cheaper, but the match to sunlight is not as good as that provided by xenon arcs. All solar simulators emit significant quantities of UVB and should be suitably filtered to attenuate UVB wavelengths (Annex 2). Because cell culture plastic materials contain UV stabilisers, the transmitted spectrum should be measured through the same type of plate lid as will be used in the assay. Irrespective of measures taken to attenuate parts of the spectrum by filtering or by unavoidable filter effects of the equipment, the spectrum recorded below these filters should not deviate from standardised outdoor daylight (34). External light standard D65, the internationally recognized emission standard for outdoor daylight, is provided in ISO DIS 18909:2006. An example of the spectral irradiance distribution of the filtered solar simulator used in pre-validation and follow-up studies with the EpiDerm™ model is given in (10)(30)(23). See also ANNEX 2: Figure 1.

39. *Dosimetry*: The intensity of light (irradiance) should be regularly checked before each phototoxicity test using a suitable calibrated broadband UVA-meter. Irradiance should be measured through the same type of plate lid as will be used in the assay.

40. An irradiance dose of approximately 6 J/cm^2 (as measured in the UVA range) was determined to be non-cytotoxic in the RhE tissues and sufficiently potent to excite chemicals to elicit phototoxic reactions (4)(10)(30). To achieve 6 J/cm^2 within a time period of 60 minutes, irradiance was adjusted to 1.7 mW/cm^2 of UVA/visible light (see ANNEX 2: Figure 2). Alternate exposure times and/or irradiance values may be used to achieve 6 J/cm^2 using the formula:

$$\text{t (min)} = \frac{\text{irradiation dose (J/cm}^2) \times 1000}{\text{irradiance (mW/cm}^2) \times 60} \qquad (1\,\text{J} = 1\,\text{Wsec})$$

41. The RhE tissue model is tolerant to UVB irradiation (5)(23)(24) and inclusion of UVB irradiation may be appropriate in some cases (e.g., when absorption for the test chemical of interest is exclusively in the UVB wavelength region). The presence of the UVB portion of the spectra should be monitored and reported in the final report along with any changes in irradiance.

42. Similarly, if a different light source is used, the irradiation should be calibrated so that a dose regimen can be selected that is not deleterious to the cells but sufficient to excite standard phototoxins. A functional check should be performed by testing the proficiency chemicals described in Table 1 and also presented in Figure 2.

43. *Radiation sensitivity of the cells:* A UVA-sensitivity experiment should be performed once the test is newly set up in a laboratory. A brief description of the method and expected outcome is given in the SOPs. The viability of the irradiated tissues exposed to 6 J/cm^2 should be ≥ 80% relative to the tissues that were not irradiated.

Test procedure

44. *Tissue conditioning:* Upon receipt of the RhE tissues, examine all kit components for integrity. Under sterile conditions, transfer tissues to 6-well plates containing 0.9 mL medium/well. Place the plates into the incubator at standard culture conditions (37 ± 1 °C, 5 ± 1% CO_2, 90 ± 10% RH) for minimum of 60 minutes. Pre-incubation can be extended overnight, however the medium should be exchanged after the first 60 minutes.

45. After a minimum of 60 minutes pre-incubation, the tissues in the 6-well plates will be removed from the incubator and the medium under the tissues will be exchanged with warmed (37 ºC) fresh assay medium. The tissues may be dosed immediately, or placed back into the incubator until dosing is initiated. For each treatment condition or treatment group, four tissues will be treated such that two tissues are used in the cytotoxicity part of the assay (treated in the absence of irradiation) and two are used in the phototoxicity part of the assay (treated in the presence of irradiation).

46. *Dose Application*: The RhE tissues are treated topically. For solutions in water or aqueous buffer, 50 µL of dosing solutions are applied topically on the RhE tissue and gently spread, if necessary, with a sterile bulb-headed Pasteur pipette (a Pasteur pipette which has been flame-melted to create a small round bulb at one end), or similar device. For solutions in oil, 25 µL of dosing solutions are applied topically on the RhE tissue and gently spread, if necessary, with a sterile bulb-headed Pasteur pipette. If the spreading is not sufficient, consider applying a sterile nylon mesh (circular shape) topically on the tissue as an additional spreading tool (which acts by capillary action to cover the tissue surface). For solvents that may be irritating to skin, the dosing volume should be limited to avoid solvent cytotoxicity. For example, dosing solutions in ethanol, or acetone – olive oil mixture (4:1) should not exceed 20 to 25 µL, since higher volumes may lead to cytotoxicity.

47. Once dosed, tissues are placed back into the incubator and incubated overnight (18 to 24 hours) at standard culture conditions.

48. On the following day, transfer the tissues into new 6-well plates pre-filled with 0.9 mL of buffered solution (e.g., DPBS or HBSS without phenol red) or 24-well plates pre-filled with 0.3 mL of buffered solution. The use of a phenol-red-free buffered salt solution is recommended since irradiation in cell culture medium may lead to increased variability and production of cytotoxic photo-products (35).

49. *Irradiation*: Irradiate the +Irr plates (covered with lids) for 60 minutes with 1.7 mW/cm^2 (or equivalent) at room temperature to achieve 6 J/cm^2 of simulated sunlight. If the light source generates excess heat and induces condensation under the plate lids, ventilate the plates with a fan. Place the −Irr plates in the dark (e.g., in a box) at room temperature, preferably in the same exposure room as for the tissues being irradiated. If a mesh was used during dosing, the mesh should be carefully removed (for example using fine forceps) prior to the irradiation/dark exposures. Prepare new 6-well plates containing 0.9 mL of warmed (37 ºC) fresh assay medium per well.

50. After the irradiation is completed, use a wash bottle with sterile CMF-DPBS and rinse each tissue. About 20 washes are needed to effectively remove the materials from the tissue surface. The procedures used to remove the dosing dilutions should be documented and presented in the final report. Transfer all washed inserts to the new plates containing fresh media. The surface of each tissue should be carefully dried using a sterile cotton tipped swab.

51. In cases where the test material characteristics may impede or block the irradiation (e.g., dark colored or opaque materials), the dosing dilutions should be removed prior to the irradiation

or dark exposure conditions. Under the test conditions, test chemical that permeates into the RhE tissue during the 18 to 24-hour exposure period is bioavailable within the tissue at the time of irradiation. The 18 to 24 hour exposure time prior to irradiation is notably longer than the exposure times applied in TG 439 for Skin Irritation Test (1) and accordingly ensures sufficient time for test chemical to diffuse into the tissue model nearest the target cells where reactive phototoxic species may be induced at the time of irradiation. Sterile cotton swabs soaked in a rinse medium (e.g., DPBS without Ca^{++} & Mg^{++} (CMF-DPBS)) may be used to remove the test chemical prior to the UVA/visible light or dark exposure conditions. Justification for removing the test material doses prior to irradiation should be presented in the study report.

52. Incubate the tissues overnight (18 to 24 hours) at standard culture conditions.

53. *MTT Viability Assay*: A 1 mg/mL MTT solution will be prepared, warmed at 37 ºC, and 300 µL pipetted into the appropriate wells of a labeled 24-well plate. After the 18 to 24-hour incubation, the tissue inserts are removed from the 6-well plates, the bottom of the inserts blotted on sterile gauze or paper towels, and transferred into the appropriate wells of the labeled 24-well MTT plates. The 24-well plates are incubated at standard culture conditions for 3 hours.

54. After the MTT incubation, the inserts are removed from the 6-well plates, the bottom of the inserts blotted on sterile gauze or paper towels, and transferred into the appropriate wells of new labeled 24-well plates. The tissues are extracted in 2 mL of isopropanol (extraction solution). The 24-well plates will be sealed (e.g., with Parafilm) and the formazan extracted for at least 2 hours at room temperature with gentle shaking on a plate shaker. Alternatively, overnight extraction is also possible. The plates are sealed as described above and extracted at room temperature in the dark, without shaking. Before sampling the extracts, shake for at least 15 minutes on a plate shaker.

55. After the extraction is completed, the tissue inserts may either be lifted out of the well and the extraction solution decanted into the well from which the insert was taken, or the tissues may be pierced (e.g., with a 20 gauge injection needle) and the extraction allowed to drain into the well from which the insert was taken (the insert can be discarded). The extract will be mixed by pipetting “up and down” at least 3 times until the extraction solution is homogenous. For each tissue, 200 µL aliquots of the extraction solution are pipetted into a labeled 96-well flat bottom microtiter plate. Finally, 200 µL aliquots of isopropanol will be added to the wells designated for the blanks.

56. The optical density (OD) of the 96-well plate will be determined using a microtiter-plate spectrophotometer using a wavelength between 540 and 595 nm, preferably at 570 nm (with a filter band pass of maximum ± 30 nm). No reference filter reading is required. Alternatively, the absorbance of the formazan extraction samples can be determined using an HPLC/UPLC-spectrophotometry procedure (32).

Cell Viability Calculations

57. *Viability Calculation.* The OD values obtained with each test chemical can be used to calculate the percentage of viability relative to the solvent (i.e. negative) control, which is set to 100%. In case HPLC/UPLC-spectrophotometry is used, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent solvent (i.e. negative) control.

58. The relative viability (or % of Control) of each of the test chemical or positive control-treated tissues (+Irr) will be calculated relative to the mean of the appropriate solvent (i.e., negative) control-treated tissues (+Irr). Similarly, the relative viability (or % of Control) of the test

article or positive control-treated tissues (−Irr) will be calculated relative to the mean of the appropriate solvent (i.e. negative) control-treated tissues (−Irr). The individual % of Control values are averaged to calculate the mean % of Control (viability) per concentration for each of the +Irr and −Irr exposures. The following equation will be used:

$$\%\ of\ Control = \frac{Corrected\ OD\ of\ each\ Test\ Chemical\ or\ Positive\ Control\ Treated}{Corrected\ OD\ of\ Negative/Solvent\ Control} \times 100$$

Corrections for MTT-reducing Materials and Colorants

59. Optical properties of the test chemical or its chemical action on MTT (e.g., chemicals may prevent or reverse the colour generation as well as cause it) may interfere with the assay leading to a false estimate of viability. This may occur when a specific test chemical is not completely removed from the tissue by rinsing or when it penetrates the epidermis. If a test chemical acts directly on the MTT (e.g., MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls should be used to detect and correct for test chemical interference with the viability measurement. Detailed description of how to correct direct MTT reduction and interferences by colouring agents is available in the SOPs for the OECD Validated test methods on skin and eye irritation and corrosion (5)(25).

60. To identify direct MTT reducers, each test chemical, at the highest test concentration, should be added to freshly prepared MTT solution. If the MTT mixture containing the test chemical turns blue/purple, the test chemical is presumed to directly reduce MTT and a further functional check on non-viable RhE tissues should be performed, independently of using the standard absorbance (OD) measurement or an HPLC/UPLC-spectrophotometry procedure. This additional functional check employs killed tissues that possess only residual metabolic activity but absorb the test chemical in a similar way as viable tissues. Each MTT reducing test chemical is applied on at least two killed tissue replicates (e.g., one tissue to be irradiated and one tissue exposed under the dark conditions) at the highest test concentration, which undergo the entire testing procedure to generate a non-specific MTT reduction (NSMTT).

61. A single NSMTT control is sufficient per test chemical regardless of the number of independent tests/runs performed. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the MTT reducer minus the percent non-specific MTT reduction obtained with the killed tissues exposed to the same MTT reducer, calculated relative to the solvent (i.e. negative) control run concurrently to the test being corrected (%NSMTT).

62. To identify potential interference by coloured test chemicals or test chemicals that become coloured when in contact with water or isopropanol, and to determine the need for additional controls, analysis of the test chemical in water (environment during exposure) and/or isopropanol (extracting solution) should be performed. If the test chemical in water and/or isopropanol absorbs light in the range of 570 ± 30 nm, additional colorant controls should be used. Alternatively, an HPLC/UPLC-spectrophotometry procedure should be used in which case these controls are not required.

63. When performing the standard absorbance (OD) measurement, each interfering coloured test chemical is applied on at least two viable tissues (e.g., one tissue to be irradiated and one tissue exposed under the dark conditions) at the highest test concentration, which undergoes the entire testing procedure but is incubated with medium instead of MTT solution during the MTT incubation step to generate a non-specific colour (NSCliving) control. The NSCliving control

needs to be performed concurrently to the testing of the coloured test chemical and in case of multiple testing, an independent NSCliving control needs to be conducted with each test performed (in each run) due to the inherent biological variability of living tissues. The true tissue viability is then calculated as the percent tissue viability obtained with living tissues exposed to the interfering test chemical and incubated with MTT solution minus the percent non-specific colour obtained with living tissues exposed to the interfering test chemical and incubated with medium without MTT, run concurrently to the test being corrected (%NSCliving).

64. It is important to note that non-specific MTT reduction and non-specific colour interferences may increase the readouts of the tissue extract above the linearity range of the spectrophotometer. On this basis, each laboratory should determine the linearity range of their spectrophotometer with MTT formazan (CAS # 57360-69-7) from a commercial source before initiating the testing of test chemicals for regulatory purposes. The standard absorbance (OD) measurement using a spectrophotometer is appropriate to assess direct MTT-reducers and colour interfering test chemicals when the ODs of the tissue extracts obtained with the test chemical without any correction for direct MTT reduction and/or colour interference are within the linear range of the spectrophotometer.

65. For coloured test chemicals which are not compatible with the standard absorbance (OD) measurement due to strong interference with the MTT assay, the alternative HPLC/UPLC-spectrophotometry procedure to measure MTT formazan may be employed. The HPLC/UPLC-spectrophotometry system allows for the separation of the MTT formazan from the test chemical before its quantification (32). For this reason, NSCliving or NSCkilled controls are never required when using HPLC/UPLC spectrophotometry, independently of the chemical being tested. NSMTT controls should nevertheless be used if the test chemical is suspected to directly reduce MTT or has a colour that impedes the assessment of the capacity to directly reduce MTT. When using HPLC/UPLC-spectrophotometry to measure MTT formazan, the percent tissue viability is calculated as percent MTT formazan peak area obtained with living tissues exposed to the test chemical relative to the MTT formazan peak obtained with the concurrent solvent (i.e. negative) control. For test chemicals able to directly reduce MTT, true tissue viability is calculated as the percent tissue viability obtained with living tissues exposed to the test chemical minus %NSMTT. Finally, it should be noted that direct MTT-reducers that may also be colour interfering, which are retained in the tissues after treatment and reduce MTT so strongly that they lead to ODs (using standard OD measurement) or peak areas (using UPLC/HPLC-spectrophotometry) of the tested tissue extracts that fall outside of the linearity range of the spectrophotometer cannot be assessed, although these are expected to occur in only very rare situations.

66. HPLC/UPLC-spectrophotometry may be used also with all types of test chemicals (coloured, non-coloured, MTT-reducers and non-MTT reducers) for measurement of MTT formazan. Due to the diversity of HPLC/UPLC-spectrophotometry systems, qualification of the HPLC/UPLC-spectrophotometry system should be demonstrated before its use to quantify MTT formazan from tissue extracts by meeting the acceptance criteria for a set of standard qualification parameters based on those described in the U.S. Food and Drug Administration guidance for industry on bio-analytical method validation (36).

Criteria for a Valid Test

67. The following acceptance criteria should be met for a valid test run:

- The difference in the relative viability values between the two replicate tissues treated with the solvent (i.e. negative) or positive controls should not exceed 20 %.

- The viability of the solvent (i.e. negative) controls tested in the absence of irradiation should fall within the acceptance range presented in **Table 2.**
- The viability of the solvent (i.e. negative) controls tested in the presence of irradiation should result in a viability of ≥80% when compared to the solvent (i.e. negative) controls tested in the absence of irradiation; this control demonstrates absence of excessive radiation sensitivity of the cells, as described in paragraph 43.
- The positive control should result in a positive prediction.

68. The following criteria should be met for each of the test substance treatment groups to be evaluable for phototoxicity potential:

- The viability of the test substance-treated tissues in the absence of irradiation should be sufficiently high (for example, >35% viability) to ensure ability to make both phototoxic and not phototoxic predictions at the maximum recommended concentration of 10% (100 mg/mL), or when the maximum concentrations are limited by cytotoxicity, at the highest tolerated dose(s).

Interpretation of Results and Prediction Model

69. A chemical is predicted to be **phototoxic** (or to have phototoxicity potential) if the relative viability values for one or more test concentrations treated in the presence of irradiation result in a decrease in viability ≥ 30% when compared to the relative viability values for the same concentrations treated in the absence of irradiation.

70. A chemical is predicted to be **not phototoxic** (or to not have phototoxicity potential) if none of the relative viability values for the test concentrations treated in the presence of irradiation result in a decrease in viability ≥ 30% when compared to the relative viability values for the same concentrations treated in the absence of irradiation.

71. If none of the test concentrations result in a phototoxic prediction and at least one of the concentrations falls within 5% of the cutoff value, and/or non-concordant results from replicate tissues are obtained, a second run should be considered, as well as a third one in case of discordant predictions between the first two runs. In this case, it is recomended to consider a concentration range that is closer to the concentration in which the potentially phototoxic outcome was observed.

DATA AND REPORTING:

Data

72. *Quality and quantity of data.* Appropriate concentrations which capture the concentration-responses in the presence and absence of irradiation should be selected to allow meaningful analysis of the data. Equivocal, borderline, or unclear results should be clarified by further testing. In such cases, modification of experimental conditions (e.g., concentrations tested) should be considered.

73. For each run, data from individual replicate tissues (e.g., OD values and calculated percentage cell viability data for each test chemical, including classification) should be reported, including data from repeat experiments, as appropriate. In addition, Viability means ± Difference

between the duplicate tissues for each run should be reported. Observed interactions with MTT reagent and coloured test chemicals should be reported for each tested chemical.

Test Report

74. The test report should include the following information:

Test Chemical and Control Substances:

- Mono-constituent substance: chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.;
- Multi-constituent substance, UVCB and mixture: characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents;
- Physical appearance, water solubility, and any additional relevant physicochemical properties;
- Source, lot number if available;
- Preparation of the test chemical/control substance prior to testing, if applicable (e.g., warming, grinding);
- Stability of the test chemical, expiration date, or date for re-analysis if known;
- Storage conditions;
- Solvent (justification for the choice of solvent; solubility of the test chemical in solvent)

RhE model and protocol used (and rationale for the choice, if applicable):

- RhE model used (including batch number);
- Complete supporting information for the specific RhE model used including its performance. This should be provided as a Certificate of Analysis or QC release report by the tissue developer/supplier and may include, but is not limited to;
    i) Viability;
    ii) Barrier function;
    iii) Morphology;
    iv) Quality controls (QC) of the model

- Reference to historical data of the model. This should include, but is not limited to acceptability of the QC data with reference to historical batch data;
- Statement of proficiency in performing the test method by testing of the proficiency substances.

Test Conditions:

- Calibration information for measuring device (e.g., spectrophotometer), wavelength and band pass (if applicable) used for quantifying MTT formazan, and linearity range of measuring device; Description of the method used to quantify MTT formazan;
- Description of the qualification of the HPLC/UPLC-spectrophotometry system, if applicable;
- Light source – irradiation conditions:
  - rationale for selection of the light source used;

- manufacturer and type of light source and radiometer;
- full spectral irradiance characteristics of the light source;
- transmission and absorption characteristics of the filter(s) used;
- characteristics of the radiometer and details on its calibration;
- distance of the light source from the test system;
- UVA irradiance at this distance, expressed in mW/cm^2;
- duration of the irradiation exposure;
- UVA dose (irradiance x time), expressed in J/cm^2;
- temperature of cell cultures during irradiation and cell cultures concurrently kept in the dark.

Test Procedure:

- Details of the test procedure used (including washing procedures used after exposure period);
- Doses of test chemical and control substances used;
- Rationale for selection of concentrations of the test chemical used in the presence and in the absence of irradiation;
- Type and composition of solvent/vehicle;
- Duration and temperature of exposure and post-exposure incubation period;
- Indication of controls used for direct MTT-reducers and/or colouring test chemicals, if applicable;
- Number of tissue replicates used per test chemical and controls (PC, solvent (i.e. negative) control, and NSMTT, and NSCliving, if applicable);
- Description of decision criteria/prediction model applied based on the RhE model used;
- Description of any modifications to the test procedure (including washing procedures).

Run and Test Acceptance Criteria:

- Acceptance criteria for variability between tissue replicates for positive and solvent (i.e. negative) controls;
- Acceptance criteria for solvent (i.e. negative) control OD values;
- Acceptance criteria for the viability of the solvent (i.e. negative) controls in the presence of irradiation relative to those in the absence of irradiation;
- Acceptance criteria for the positive control.

Results:

- Tabulation of data for individual test chemical for each run and each replicate measurement including OD or MTT formazan peak area, percent tissue viability, mean percent tissue viability and difference between tissues;
- If applicable, results of controls used for direct MTT-reducers and/or colouring test chemicals including OD or MTT formazan peak area, %NSMTT, %NSCliving, final corrected relative viability;
- Results obtained with the test chemical(s) and control substances in relation to the defined run and test acceptance criteria;
- Description of other effects observed;
- The derived classification with reference to the prediction model/decision criteria used

Discussion of the results.

Conclusions.

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ANNEX 1: Definitions

Mixture: A mixture or a solution composed of two or more chemicals in which they do not react (14).

Irradiance: the intensity of ultraviolet (UV) or visible light incident on a surface, measured in W/ m^2 or mW/ cm^2.

Dose of light: the quantity (**=** intensity x time) of ultraviolet (UV) or visible radiation incident on a surface, expressed in Joules **(=** W x s) per surface area, e.g., J/ m^2 or J/ cm^2.

UV light wavebands: the designations recommended by the CIE (Commission Internationale de L'Eclairage) are: **UVA** (315-400nm) **UVB** (280-315nm) and **UVC** (100-280nm). Other designations are also used; the division between UVB and UVA is often placed at 320nm, and the UVA may be divided into UV-A1 and UV-A2 with a division made at about 340nm.

Relative tissue viability: tissue viability expressed in relation to solvent (i.e. negative) controls which have been taken through the whole test procedure (either +Irr or -Irr) but not treated with test chemical.

MEC (Molar Extinction/absorption Coefficient): a constant for any given molecule under a specific set of conditions (e.g., solvent, temperature, and wavelength) and reflects the efficiency with which a molecule can absorb a photon (typically expressed as $L \cdot mol^{-1} \cdot cm^{-1}$).

Phototoxicity: 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.

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method (20).

Cell viability: Parameter measuring total activity of a cell population e.g., as ability of cellular mitochondrial dehydrogenases to reduce the vital dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue), which depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

Chemical: means a substance or a mixture.
Concordance: This is a measure of test method performance for test methods that give a categorical result, and is one aspect of relevance. The term is sometimes used interchangeably with accuracy, and is defined as the proportion of all chemicals tested that are correctly classified as positive or solvent (i.e. negative). Concordance is highly dependent on the prevalence of positives in the types of test chemical being examined (20).

HPLC: High Performance Liquid Chromatography.

IATA: Integrated Approach on Testing and Assessment

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue tetrazolium bromide.

Negative Control: see Solvent Control

NSCkilled: Non-Specific Colour in killed tissues.

NSCliving: Non-Specific Colour in living tissues.

NSMTT: Non-Specific MTT reduction.

Performance standards (PS): Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of Reference Chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of Reference Chemicals (20).

Positive Control (PC): a replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (20).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility (20).

Replacement test: A test which is designed to substitute for a test that is in routine use and accepted for hazard identification and/or risk assessment, and which has been determined to provide equivalent or improved protection of human or animal health or the environment, as applicable, compared to the accepted test, for all possible testing situations and chemicals (20).

Run: A run consists of one or more test chemicals tested concurrently with a solvent (i.e. negative) control and with a PC.

Sensitivity: The proportion of all positive/active test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (20).

Solvent Control: A replicate containing all components of a test system except for the test chemical, but including the solvent that is used. It is used to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent, and in this Test Method is used as a negative control in the data analyses. This sample is processed with test chemical-treated samples and other control samples.

Specificity: The proportion of all negative/inactive test chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (20).

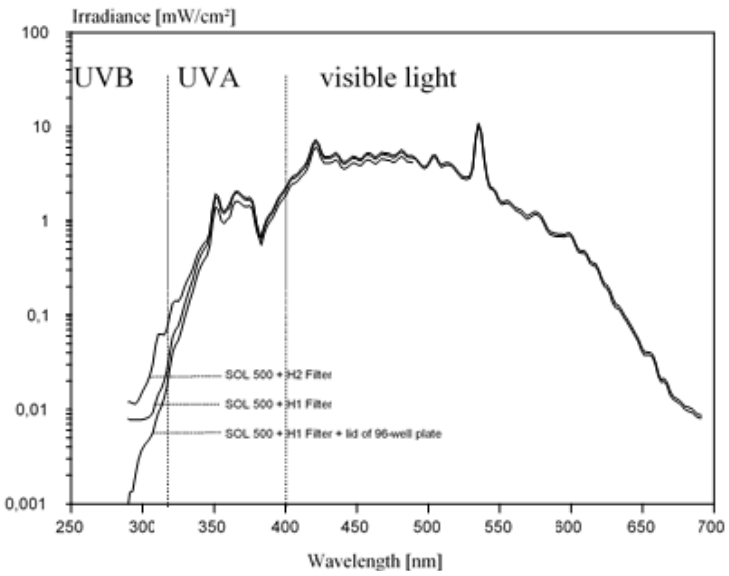
Test chemical: is the term “test chemical” is used to refer to what is being tested.

UPLC: Ultra-High Performance Liquid Chromatography.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials

ANNEX 2: An example of spectral power distribution of the light source and the irradiation sensitivity of RhE

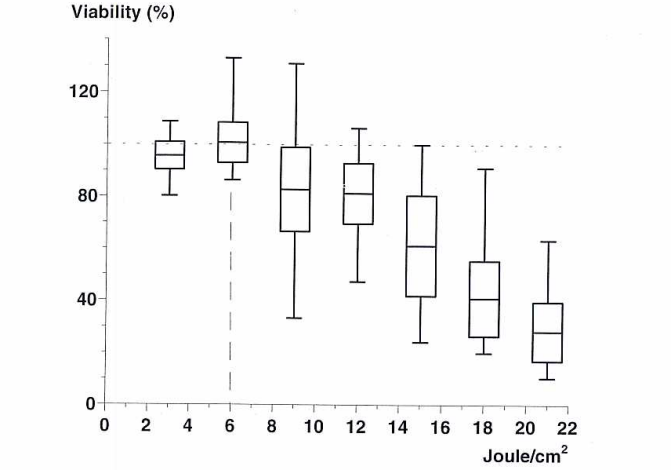
Figure 1. Spectral power distribution of a filtered solar simulator.



Source: Spielmann, H. *et al* (1998) (37)

Figure 1 gives an example of an acceptable spectral power distribution of a filtered solar simulator. It is from the doped metal halide source used in the validation trial of the 3T3 NRU PT as well as pre-validation of the EpiDerm Phototoxicity test and in most of the follow-up studies. The effect of two different filters and the additional filtering effect of the lid of a 96-well cell culture plate are shown. The H2 filter was only used with test systems that can tolerate a higher amount of UVB (skin model test and red blood cell photohemolysis test). In the 3T3 NRU-PT the H1 filter was used. The figure shows that additional filtering effect of the plate lid is mainly observed in the UVB range, still leaving enough UVB in the irradiation spectrum to excite chemicals typically absorbing in the UVB range, like Amiodarone.

Figure 2. Irradiation sensitivity of RhE (as measured in the UVA range)



Source: Liebsch *et al* (1998) (38)

This figure presented in Liebsch *et al* (1998) (38) shows the responses of tissues exposed to increasing concentrations of UVA irradiation relative to non-irradiated tissues. Relative viability was determined using the MTT conversion assay. Each box represents the mean of 12 tissues evaluated over four independent experiments. The tissues tolerated a dose of 6 J/cm^2 without excess cytotoxic effects. The dose is 1.7 mW/cm^2 (of the UVA), and with an irradiation time of 10 min is resulting to in 1J.

ANNEX 3: Considerations in the selection of test chemical solvents

Solvents / vehicles:

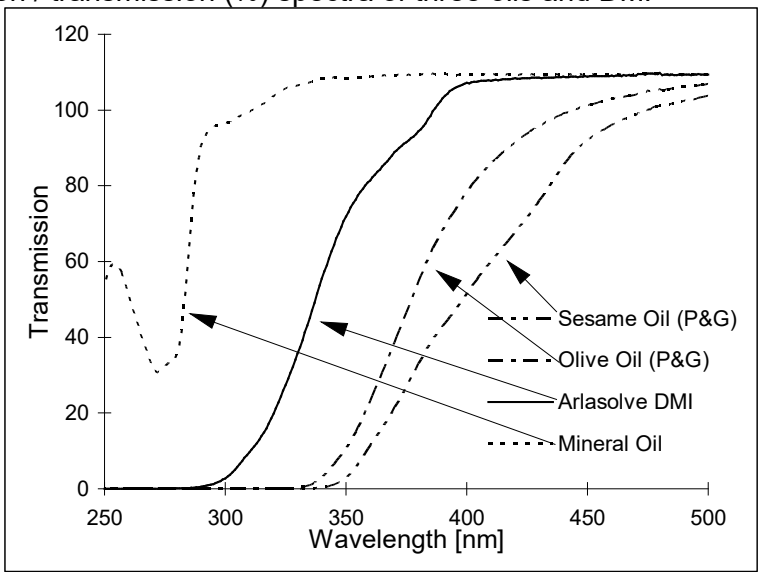
During the development and pre-validation study (3)(38), sesame seed oil was chosen as a solvent and vehicle for chemicals which could not be sufficiently dissolved in water. Several other solvents were investigated by the other laboratories participating in the pre-validation (Figure 3), but the sesame seed oil was chosen for the final experiments. In addition to oily solvents, ethanol and a mixture of acetone:olive oil were suggested for materials that could not be readily solubilised in water or oil (5)(30).

It is of importance to select a solvent that will sufficiently transmit the full spectrum of the simulated sunlight (i.e., the solvent should not show appreciable absorption within the simulated sunlight spectrum). Furthermore, the recommended dosing volume of 50 µL should not be exceeded, since excessive volumes of solvent/vehicle on the tissue surface may create a photo-protective layer.

Furthermore, the biological response of the 3D tissues to the alternative solvent/vehicle should be evaluated. The alternative solvent/vehicle should not cause decrease of tissue viability below 70% of water treated tissues.

The photopotency (i.e. the phototoxic strength) of chemicals may be modulated by the solvent/vehicle as demonstrated in experiments obtained for Chlopromazine in oily and aqueous solutions (3) or with Anthracene tested in oily and ethanolic solutions (3)(30).

Figure 3. Absorption / transmission (%) spectra of three oils and DMI



Source: Liebsch, M., (1998) (38)