Section 4
Health effects

Test Guideline No. 495

Reactive Oxygen Species (ROS) Assay for Photoreactivity

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR TESTING OF CHEMICALS

Ros (Reactive Oxygen Species) Assay For Photoreactivity

INTRODUCTION

1. Phototoxicity is defined as a toxic response is elicited by topically or systemically administered photoreactive chemicals after the exposure of the body to environmental light. Several classes of photoreactive chemicals could cause phototoxic reactions when activated by light at otherwise non-toxic doses. Phototoxicity can be categorized as photoirritation, photoallergy, and photogenotoxicity (1). Photoirritation is characterized as an acute light-induced skin response to a photoreactive chemical. Photoallergy is an immune-mediated reaction in which light may cause a structural change in a drug so that it acts as a hapten, possibly by binding to proteins in the skin (2). Photogenotoxicity is a genotoxic response after exposure to a chemical by two mechanisms: either directly by photoexcitation of DNA or indirectly by excitation of photoreactive chemicals.

2. In 2002, regulatory agencies in the US (US Food and Drug Administration, FDA) and EU (European Medicines Agency, EMA) published guidelines for photosafety assessments of drug candidates (3)(4). In 2004, the Organisation for Economic Co-operation and Development (OECD) adopted Test Guideline 432: *In vitro* 3T3 Neutral Red Uptake (NRU) Phototoxicity Test as a validated methodology for evaluating the phototoxic potential of chemicals (5). The EMA also published a concept paper in 2008 (6), which proposes a testing strategy that merges the testing proposals recommended by FDA and EMA. Considering these documents, the International Council of Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) published ICH S10 guideline, "Photosafety Evaluation of Pharmaceuticals" in 2014 (7).

3. According to above referenced guidelines, chemicals or drug candidates need to be examined for their phototoxic potential. Since light must be absorbed by a compound in order for photochemical reactions to take place (8), the phototoxic potential of chemicals is related to the photochemical properties of compounds, especially light absorption properties within 290–700 nm. The guidelines suggested the need for measurement of the light absorption properties of chemicals as a first round of screening (3)(4). The ICH S10 guideline recommends UV-visible light absorption spectral analysis as a criterion for evaluating the phototoxic potentials of drugs (7); however, UV-visible light absorption of chemicals would not always correlate directly with their phototoxic potential, so a

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combination of UV data (molar extinction coefficient, MEC) with other appropriate screening systems might be advantageous in avoiding false predictions.

4. In addition to light absorption and distribution to light-exposed tissue, the generation of a reactive species from chemicals following absorption of UV-visible light is described as a key determinant of chemicals for causing direct phototoxic reactions in an older guidance document (7)(9). Thus, the Reactive Oxygen Species (ROS) assay (10)(11) has been also included by the ICH S10 guideline as an optional initial *in chemico* screening tool for evaluating the photoreactivity of pharmaceuticals (7).

5. As an alternative method for *in vivo* phototoxicity testing, the OECD TG432 (5) describes an *in vitro* 3T3 NRU Phototoxicity Test and sets specific criteria for evaluating phototoxic hazard. The 3T3 NRU Phototoxicity Test evaluates photo-cytotoxicity by the relative reduction in viability of cells exposed to the chemical in the presence versus absence of light. Chemicals identified by this test are likely to be photoreactive, following systemic application and distribution to the skin, or after topical application. Although most of the photoirritant chemicals were correctly identified by the 3T3 NRU Phototoxicity Test, it provided false predictions for almost half of the chemicals in the photoallergens group. However, the 3T3 NRU Phototoxicity Test was not originally designed for specific prediction of chemical photoallergenicity (2). The 3T3 NRU Phototoxicity Test seems to be less reliable for photoallergenicity prediction. The photochemical assays such as ROS assay and UV/VIS spectral analysis can predict photoallergenic potential of tested chemicals, although there is still a substantial risk of false positive predictions (12).

6. Definitions used are provided in Annex A.

INITIAL CONSIDERATION AND LIMITATIONS

7. Before photosafety assessments are considered, a UV-visible light absorption spectrum of the test chemical should be determined according to OECD Test Guideline 101(13). Based on an analysis of data, the ICH S10 guideline has suggested that no further photosafety testing is needed if the MEC of a chemical is less than 1,000 $L \cdot mol^{-1} \cdot cm^{-1}$ (7). Few phototoxic chemicals showed a MEC less than 1,000 $L \cdot mol^{-1} \cdot cm^{-1}$ and these chemicals may not need to be tested in the ROS assay or any other photosafety assessments (9) (14) (15). Data collected for the limits of photoreactivity are discussed in Henry *et al.* (16) and Bauer *et al.* (17). It should be noted that phototoxicity by indirect mechanisms (e.g., pseudoporphyria or porphyria), although rare, could still occur. For compounds with MEC values of 1000 $L \cdot mol^{-1} \cdot cm^{-1}$ or higher, if the drug developer chooses to conduct a test for photoreactivity a negative result could support a decision that no further photosafety assessment is warranted.

8. The reliability and relevance of the ROS assay was recently evaluated in a multi-laboratory validation study using two different solar simulators (18)(19)(20)(21). In both solar simulators, the intra- and inter-day precisions for quinine, a positive control, were found to be above 90%, and the data suggested high inter-laboratory reproducibility (19). In a multi-laboratory validation study, the ROS assay on 2 standards and 42 coded chemicals, including 23 phototoxins and 19 non-phototoxic drugs/chemicals, provided no false negative predictions upon defined criteria as compared with the *in vitro/in vivo* phototoxicity. The, sensitivity, individual specificity, positive and negative predictivities of the ROS assay on the 42 tested chemical were calculated to be 100%, 42–82%, 75–92% and 100%, respectively. The ROS assay was designed for qualitative photoreactivity assessment of chemicals, the principle of which is

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monitoring of type I (an electron or hydrogen transfer, resulting in the formation of free radical species) and type II (an energy transfer from excited triplet photosensitizer to the oxygen) photochemical reactions in test chemicals exposed to simulated sunlight (10), possibly leading to photodegradation and various phototoxic reactions, including photoirritation, photoallergy, and photogenotoxicity. Further, this assay has been optimised for detecting positive test chemicals. Test chemicals found to be negative in the ROS assay are likely to be negative in *in vivo* test systems; however, additional data may be required to determine if chemicals that are photoreactive in the ROS assay are likely to be positive *in vivo*. The test has not been designed to address indirect mechanisms of phototoxicity, such as effects of metabolites of a test chemical.

9. The applicability domain of the ROS assay is currently restricted to only those chemicals that meet the solubility criteria outlined in the protocol (see paragraph 22). Insoluble chemicals in the reaction mixtures are not suitable for testing with the ROS assay using this protocol (DMSO or NaPB solvent) but might be tested in the ROS assay with addition of solubility enhancers in the reaction mixtures (22)(23)(24). However, further characterization and standardization of procedures using these alternative vehicles should be performed by testing proficiency chemicals before incorporation into routine use. In the ROS assay, superoxide anion (SA) can be measured upon the reduction of nitroblue tetrazolium, and the determination of singlet oxygen (SO) can be made on the basis of bleaching of *p*-nitrosodimethylaniline by oxidized imidazole (11). Test chemicals that interfere with these reactions are sometimes best considered outside of the applicability domain of the ROS assay. For example, ascorbic acid and other reducing chemicals reduce the tetrazolium salt to formazan directly (25). Some skin-lightening cosmetics may also have potent reducing properties that interfere with ROS determinations. Ascorbic acid also accelerates the oxidation of imidazole derivatives (26), providing false positive prediction in the ROS assay.

10. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the ROS assay to the testing of mono-constituent chemicals, multi-constituent chemicals and/or mixtures. Based on the data currently available, the ROS assay was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, phototoxic potency (as determined in *in vivo* studies) and physicochemical properties. Limited information is currently available on the applicability of the ROS assay to multi-constituent chemicals/mixtures (27). When considering testing of mixtures, difficult to test chemical (e.g. unstable) or chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically.

PRINCIPLE OF THE TEST

11. Chemical phototoxicity can be caused by topical and systemic application of chemicals in combination with exposure to environmental light. There are several classes of chemicals that are nontoxic by themselves but could become reactive in the skin or eyes when exposed to environmental light and thereby result in toxicity. The primary event in any phototoxic reaction is the absorption of photons of a wavelength that induces excitation of the chromophore. The excitation energy is often transferred to oxygen molecules, followed by generation of ROS, including SA through type I photochemical reactions and SO through type II photochemical reactions by photo-excited molecules. These appear to be the principal intermediate species in many phototoxic responses. Direct reaction of excited chromophores with cellular constituents may also lead to phototoxicity. Therefore, while the ROS assay may not detect all ultimate mechanisms of phototoxicity, the

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determination of ROS generation from chemicals irradiated with simulated sunlight is indicative of phototoxic potential.

12. In the ROS assay, SO generation is detected by spectrophotometric measurement of *p*-nitrosodimethylaniline (RNO) bleaching, followed by decreased absorbance of RNO at 440 nm (28). Although SO does not react chemically with RNO, the RNO bleaching is a consequence of SO capture by the imidazole ring, which results in the formation of a trans-annular peroxide intermediate capable of inducing the bleaching of RNO, as follows:

SO + Imidazole → [Peroxide intermediate] → Oxidized imidazole

[Peroxide intermediate] + RNO → RNO + Products

13. SA generation is detected by observing the reduction of nitroblue tetrazolium (NBT). As indicated below; NBT can be reduced by SA via a one-electron transfer reaction, yielding partially reduced (2 e^-) monoformazan (NBT^+) as a stable intermediate (29). Thus, SA can reduce NBT to NBT^+, the formation of which can be monitored spectrophotometrically at 560 nm.

$SA + NBT \rightarrow O_2 + NBT^+$

Demonstration of Proficiency

14. Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency, using the proficiency chemicals listed and described in Annex C. The 9 proficiency chemicals (Nos. 1–9) for the two recommended solar simulators (Suntest CPS+ or CPS and SXL-2500V2) or the 17 proficiency chemicals (Nos. 1–17) for a solar simulator other than the two recommended models are to be tested to ensure that measured values of SO and SA on all proficiency chemicals are within the range described in Annex C.

PROCEDURE

Solar simulator

15. Typically calibrated solar simulators are used because photoreactivity in the presence of natural sunlight is of concern, due to the spectral differences of global positioning and the time of day. For other circumstances where photoreactivity in response to artificial light is of interest, other sources of light may be considered. An appropriate solar simulator is to be used for irradiation of UV and visible light. The irradiation power distribution is to be kept as close to that of outdoor daylight as possible by using an appropriate filter to reduce UVC wavelengths. Recommended test conditions are as follows:

Solar simulator with filter to reduce UV wavelengths <290 nm (See Annex B)

- 1.8 to 2.2 mW/cm^2 (e.g. the indicator setting value of 250 W/m^2 for CPS+) for 1 hour,
- 6.5 to 7.9 J/cm^2 of UVA intensity (Annex B).

SXL-2500V2 (Seric) with UV filter (to reduce wavelengths <300 nm)

- 3.0 to 5.0 mW/cm^2 for 1 hour,
- 11 to 18 J/cm^2 of UVA intensity (Annex B).

16. The solar simulator is to be equipped with an appropriate temperature control or fan to stabilize the temperature during irradiation, because ROS production is affected by

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temperature. Standard temperature for a solar simulator with temperature control is 25°C. The acceptable temperature range during irradiation is 20 to 29°C (20)(21).

Quartz reaction container

17. A quartz reaction container is used to avoid loss of UV due to passing through a plastic lid and vaporization of the reaction mixture (20)(21)(30). Specifications for the recommended container are provided in Annex D. If a different container is used, a lid or seal with high UV transmittance should be used. In this case, a feasibility study using the reference chemicals (Nos. 1–17) is to be conducted to determine an appropriate level of exposure to UV and visible light.

Reagents

18. All reagents should be used within 1 month after preparation and should be sonicated immediately prior to use (20)(21). Representative preparation methods are shown as follows:

20 mM sodium phosphate buffer (NaPB), pH 7.4
- Weigh 593 mg of $NaH_2PO_4 \cdot 2H_2O$ (CAS No. 13472-35-0) and 5.8 g of $Na_2HPO_4 \cdot 12H_2O$ (CAS No. 10039-32-4), add 900 mL of purified water, adjust with HCl to a pH of 7.4, dilute with purified water up to 1 L, and mix.
- Store in a refrigerator or at room temperature.

0.2 mM *p*-nitrosodimethylaniline (RNO, CAS No. 138-89-6)
- Dissolve 3 mg of RNO in 100 mL of 20 mM NaPB.
- Store in a refrigerator and protect from light.

0.2 mM imidazole (CAS No. 288-32-4)
- Dissolve 13.6 mg of imidazole in 10 mL of 20 mM NaPB.
- Dilute the 20 mM imidazole solution 100 times with 20 mM NaPB.
- Store in a refrigerator and protect from light.

0.4 mM nitroblue tetrazolium chloride (NBT, CAS No. 298-83-9)
- Dissolve 32.7 mg of NBT in 100 mL of 20 mM NaPB.
- Store in a refrigerator and protect from light.

Solvents

19. Use analytical grade DMSO at first. For chemicals that are not soluble in DMSO, 20 mM NaPB is to be used as a solvent. Some chemicals react with DMSO and test chemical stability in DMSO should be determined. If the test chemical is not soluble or stable in DMSO or NaPB, other solvents may be used. However the test chemical must be demonstrated to be stable in the selected solvent, and SO and SA ranges for proficiency chemicals must fall within the ranges defined in Annex C.

Test chemicals

20. Test chemicals must be prepared fresh, immediately prior to use unless data demonstrate their stability in storage. It is recommended that all chemical handling and the initial treatment of cells be performed under light conditions that would avoid photoactivation or degradation of the test chemical prior to irradiation. Chemicals should be tested at 200 µM (final concentration). A 20-µM concentration can be used if precipitation occurs before light exposure, coloration, or other interference is observed in the reaction mixture at 200 µM. A positive result at 20 µM can be used to indicate

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photoreactivity; however, a negative result at the lower 20 µM concentration is not indicative of absence of photoreactivity. The molecular weight of the test chemical must be available.

21. The test chemical solutions are to be prepared immediately before use in a solvent as described in paragraph 19. Each test chemical is to be weighed in a tube, and solvent added to achieve a 10 mM concentration of the test chemical (20)(21). The tube is to be mixed with a vortex mixer and sonicated for 5 to 10 minutes. All preparations are to be protected from strong UV and intense visible light (e.g. direct overhead light, working near windows exposed to natural light) at all times during preparation. When precipitation before light exposure or other interference is observed in the reaction mixture at 200 µM, a 1-mM solution (20 µM as the final concentration) is to be prepared by dilution of the stock solution of chemicals at 10 mM using DMSO. For chemicals that are not soluble in DMSO, 20 µL of DMSO (2 v/v%) is to be contained in the reaction mixture.

Positive and negative controls

22. Stock solutions of quinine hydrochloride (a positive control, CAS No. 6119-47-7) and sulisobenzone (a negative control, CAS No. 4065-45-6) are to be prepared at 10 mM each in DMSO (final concentration of 200 µM) according to the above procedure, divided into tubes, and stored in a freezer (generally below -20°C) for up to 1 month. The stock solution is to be thawed just before the experiment and used within the day.

Test procedure

23. A typical 96-well plate configuration is as follows, but other configurations are also acceptable:

Figure 1. An example of a typical plate configuration

	1	2	3	4	5	6	7	8	9	10	11	12
A					Singlet oxygen							
B		B	P	N	T1	T2	T3	T4	T5	T6	T7	
C		B	P	N	T1	T2	T3	T4	T5	T6	T7	
D		B	P	N	T1	T2	T3	T4	T5	T6	T7	
E		B	P	N	T1	T2	T3	T4	T5	T6	T7	
F		B	P	N	T1	T2	T3	T4	T5	T6	T7	
G		B	P	N	T1	T2	T3	T4	T5	T6	T7	
H					Superoxide anion							

B: Blank
P: Positive control (Quinine)
N: Negative control (Sulisobenzone)
T1-T7: Test chemical No. 1-7

24. A tube (e.g. 1.5 mL micro tube) and a plastic clear flat bottomed 96-well microplate are to be used. The reaction mixture is to be prepared by vortex mixing and/or sonication under UV-cut illumination or shade. The same volume of DMSO, 20 µL, is to be added in a vehicle control instead of test chemical solution.

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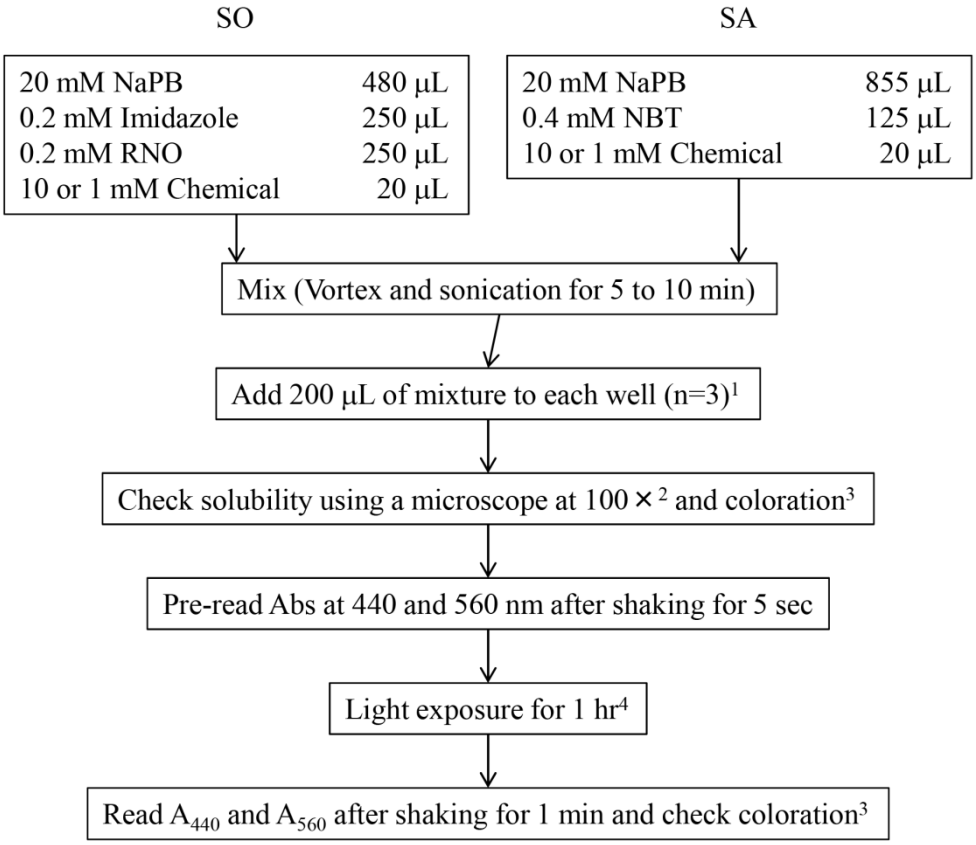
Figure 2. Workflow diagram if the stock solution of the test chemical is prepared in DMSO.



[1] Avoid using peripheral wells. More than one test chemical can be tested on a plate.

[2] Some chemicals might precipitate in the reaction mixture. It is therefore important to check solubility prior to irradiation. Solubility of each reaction mixture in its well is to be observed with a microscope prior to irradiation. Test chemical concentrations are to be selected so as to avoid precipitation or cloudy solutions.

[3] The reaction mixture is to be checked for coloration with the naked eye.

[4] The 96-well plate is to be placed in the quartz reaction container. A quartz cover is to be set on the plate and fastened with bolts. Ensure that temperature and other ambient conditions are stable when using the solar simulator. Measure UVA intensity and temperature at the plate position using a UVA detector and thermometer both before and after irradiation.

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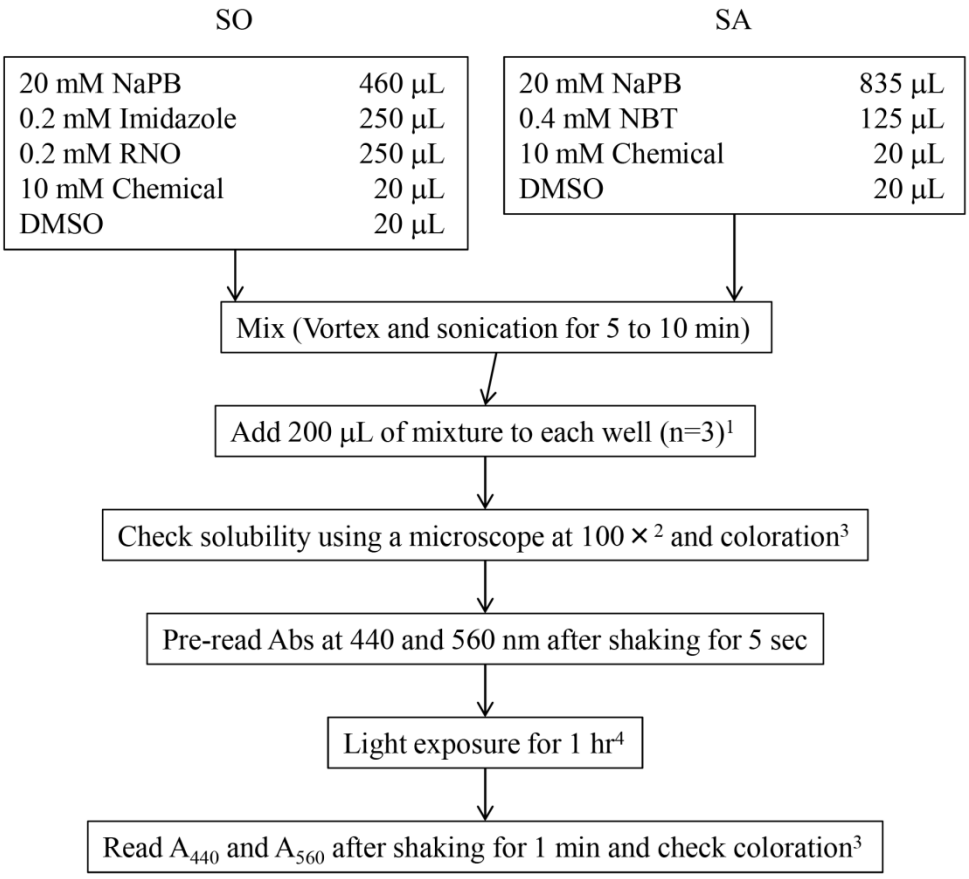
Figure 3. Workflow diagram if the stock solution of the test chemical is prepared in 20 mM NaPB.



[1] Avoid using peripheral wells. More than one test chemical can be tested on a plate.

[2] Some chemicals might precipitate in the reaction mixture. It is therefore important to check solubility prior to irradiation. Solubility of each reaction mixture in its well is to be observed with a microscope prior to irradiation. Test chemical concentrations are to be selected so as to avoid precipitation or cloudy solutions.

[3] The reaction mixture is to be checked for coloration with the naked eye.

[4] The 96-well plate is to be placed in the quartz reaction container. A quartz cover is to be set on the plate and fastened with bolts. Ensure that temperature and other ambient conditions are stable when using the solar simulator. Measure UVA intensity and temperature at the plate position using a UVA detector and thermometer both before and after irradiation.

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DATA AND REPORTING

Data analysis

25. Data from three wells for each chemical concentration is used to calculate mean and standard deviation.

SO

Decrease of $A_{440} \times 1000 = [A_{440}(-) - A_{440}(+) - (a - b)] \times 1000$
$A_{440}(-)$: Absorbance before light exposure at 440 nm
$A_{440}(+)$: Absorbance after light exposure at 440 nm
a: Vehicle control before light exposure (mean)
b: Vehicle control after exposure (mean)

SA

Increase of $A_{560} \times 1000 = [A_{560}(+) - A_{560}(-) - (b - a)] \times 1000$
$A_{560}(-)$: Absorbance before light exposure at 560 nm
$A_{560}(+)$: Absorbance after light exposure at 560 nm
a: Vehicle control before light exposure (mean)
b: Vehicle control after exposure (mean)

Criteria for data acceptance

26. The following criteria are to be satisfied in each experiment.

- No precipitation of test chemical in the reaction mixture before light exposure.
- No color interference by test chemical in the reaction mixture before or after light exposure.
- No technical problems, including temperature range (20–29°C), when collecting data set.
- The ranges of raw A_{440} and A_{560} values: 0.02 to 1.5.
- Historical positive and negative control values are to be developed by each laboratory based on a mean +/-2 SD. The following range was defined based on the 95% confidence interval (mean +/- 1.96SD) obtained from the validation data. When a solar simulator other than a recommended model is used, establish modified criteria based on 95% confidence interval.
-

  Positive control (quinine hydrochloride) value at 200 µM (mean of 3 wells)
  SO: 319 to 583
  SA: 193 to 385
  Negative control (sulisobenzone) value at 200 µM (mean of 3 wells)
  SO: -9 to 11
  SA: -20 to 2
- Laboratories should demonstrate technical proficiency, as described in Annex C, prior to routine use of the test method described in this Test Guideline.

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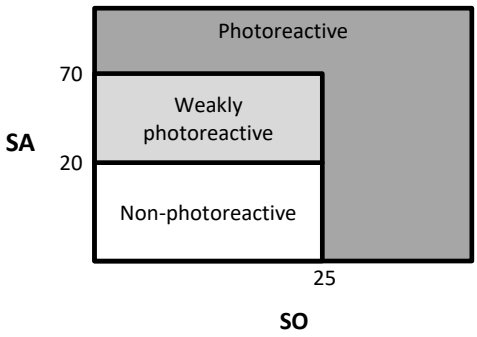
Criteria for judgment

27. Each test chemical is to be judged as follows:

ROS assay prediction model

Judgment[1, 2]	Concentration[3]	SO (mean of 3 wells)[6]		SA (mean of 3 wells)[6]
Photoreactive	200 µM	≥25	and	≥70
		<25 and/or I[4]	and	≥70
		≥25	and	<70 and/or I[4]
Weakly photoreactive	200 µM	<25	and	≥20, <70
Photoreactive	20 µM	≥25	and	≥20
Non-photoreactive	200 µM	<25	and	<20
Inconclusive	The results do not meet any of the above-mentioned criteria.[5]			



[1] A single experiment is sufficient for judging results, because the ROS assay shows good intra- and inter-laboratory reproducibility in the validation studies.

[2] If precipitation, coloration, or other interference is observed at both 20 and 200 µM, the chemical is considered incompatible with the ROS assay and judged as inconclusive.

[3] 20 µM can be used for judgment when precipitation or coloration is observed at 200 µM. A positive results at 20 µM can be used to indicate photoreactivity; however, a negative result at the lower 20 µM concentration is not indicative of absence of photoreactivity.

[4] Interference such as precipitation or coloration.

[5] Positive prediction can be made on the basis of SO only, SA only, or both; however, both SO and SA values should be obtained for reliable negative prediction.

[6] Classification criteria defined in published manuscripts. (11)(20)(21)

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Data quality

28. Studies for regulatory purposes are to be conducted to the highest of quality standards, with data collection records readily available, in compliance with GLP regulations whenever possible, and all documents checked by the Quality Assurance Unit of the laboratory.

Test report

29. The test report should include the following information:

Test chemical:

- identification data, common generic names and IUPAC and CAS number, if known;
- physical nature and purity;
- physicochemical properties relevant to conduct of the study;
- UV/vis absorption spectrum;
- stability and photostability, if known.

Control chemicals:

- name, manufacturer, and lot No.;
- physical nature and purity;
- storage condition;
- preparation of control chemical solutions;
- final concentrations tested.

Solvent:

- name, manufacturer, and lot No.;
- justification for choice of solvent;
- solubility of the test chemical in solvent.

Irradiation condition:

- manufacturer and type of the solar simulator used;
- rationale for selection of the solar simulator used;
- UVA detector used;
- UVA irradiance, expressed in mW/cm^2
- UVA dose, expressed in J/cm^2;
- temperature before and after irradiation.

ROS assay procedure.

Acceptance and decision criteria.

Results.

Discussion.

Conclusions.

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Annex A. Definitions

3T3 NRU Phototoxicity Test: *In vitro* 3T3 neutral red uptake phototoxicity test.

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

Dose of light: The quantity [= intensity × time (seconds)] of UV or visible light incident on a surface, expressed in J/m^2 or J/cm^2.

MEC: Molar Extinction Coefficient (also called molar absorptivity) is 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 $L \cdot mol^{-1} \cdot cm^{-1}$).

Photoreactivity: The property of chemicals that react with another molecule as a consequence of absorption of photons.

Phototoxicity: Toxic responses that can be elicited after the 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.

ROS: Reactive Oxygen Species, including superoxide anion (SA) and singlet oxygen (SO).

SA: Superoxide anion is one of radical species, generated from photo-irradiated chemicals through type I photochemical reaction.

SO: Singlet oxygen is one of radical species, generated from photo-irradiated chemicals through type II photochemical reaction.

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

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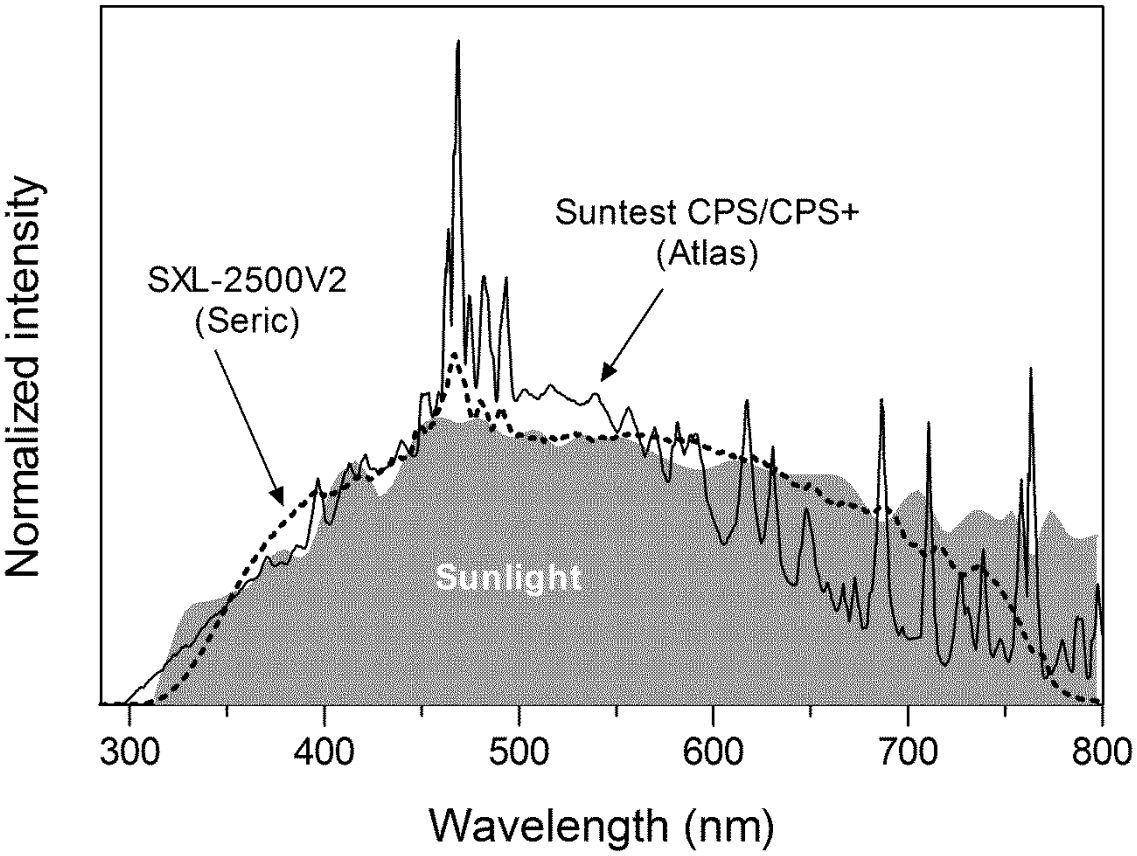
Annex B. Spectrum of solar stimulators used in the validation studies.



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Annex C. Proficiency Chemicals

Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected ROS prediction for proficiency chemicals recommended in the Table. For Suntest CPS/CPS+ (Atlas) or SXL-2500V2 (Seric) solar simulators, nine chemicals (Nos. 1–9) are to be tested. For other solar simulators, all 17 chemicals (Nos. 1–17) are to be tested. These proficiency chemicals were selected to represent the range of responses for phototoxic potential. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the ROS assay are available, and that they were used in the JaCVAM-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study (20)(21).

Table A C.1. Table of proficiency chemicals.

The expected ROS prediction for proficiency chemicals and the acceptable range..

No.	Chemical[1]	CAS No.	SO [2]	SA [2]	Solvent	Concentration
1	*p*-Aminobenzoic acid	150-13-0	-8 to 12	-11 to 7	DMSO	200 µM
2	Benzocaine	94-09-7	-7 to 9	-7 to 17	DMSO	200 µM
3	Doxycycline hydrochloride	10592-13-9	115 to 429	230 to 468	DMSO	200 µM
4	Erythromycin	114-07-8	-15 to 11	-9 to 21	DMSO	200 µM
5	Fenofibrate	49562-28-9	77 to 203	-31 to 11	DMSO	20 µM
6	L-Histidine	71-00-1	-8 to 12	8 to 120	NaPB	200 µM
7	Norfloxacin	70458-96-7	131 to 271	57 to 161	DMSO	200 µM
8	8-Methoxy psoralen	298-81-7	31 to 137	0 to 126	DMSO	200 µM
9	Octyl salicylate	118-60-5	-5 to 11	-8 to 20	DMSO	20 µM
10	Acridine	260-94-6	182 to 328	121 to 243	DMSO	200 µM
11	Chlorpromazine hydrochloride	69-09-0	-56 to 70	66 to 106	DMSO	200 µM
12	Diclofenac	15307-79-6	34 to 416	47 to 437	DMSO	200 µM
13	Furosemide	54-31-9	31 to 225	-7 to 109	DMSO	200 µM
14	Ketoprofen	22071-15-4	120 to 346	77 to 151	DMSO	200 µM
15	Nalidixic acid	389-08-2	54 to 246	88 to 470	DMSO	200 µM
16	Omeprazole	73590-58-6	-221 to 103	30 to 216	DMSO	200 µM
17	Promethazine hydrochloride	58-33-3	20 to 168	-3 to 77	DMSO	200 µM

[1]All chemicals are solid
[2]The values were calculated as means +/- 1.96 SD from the validation data..

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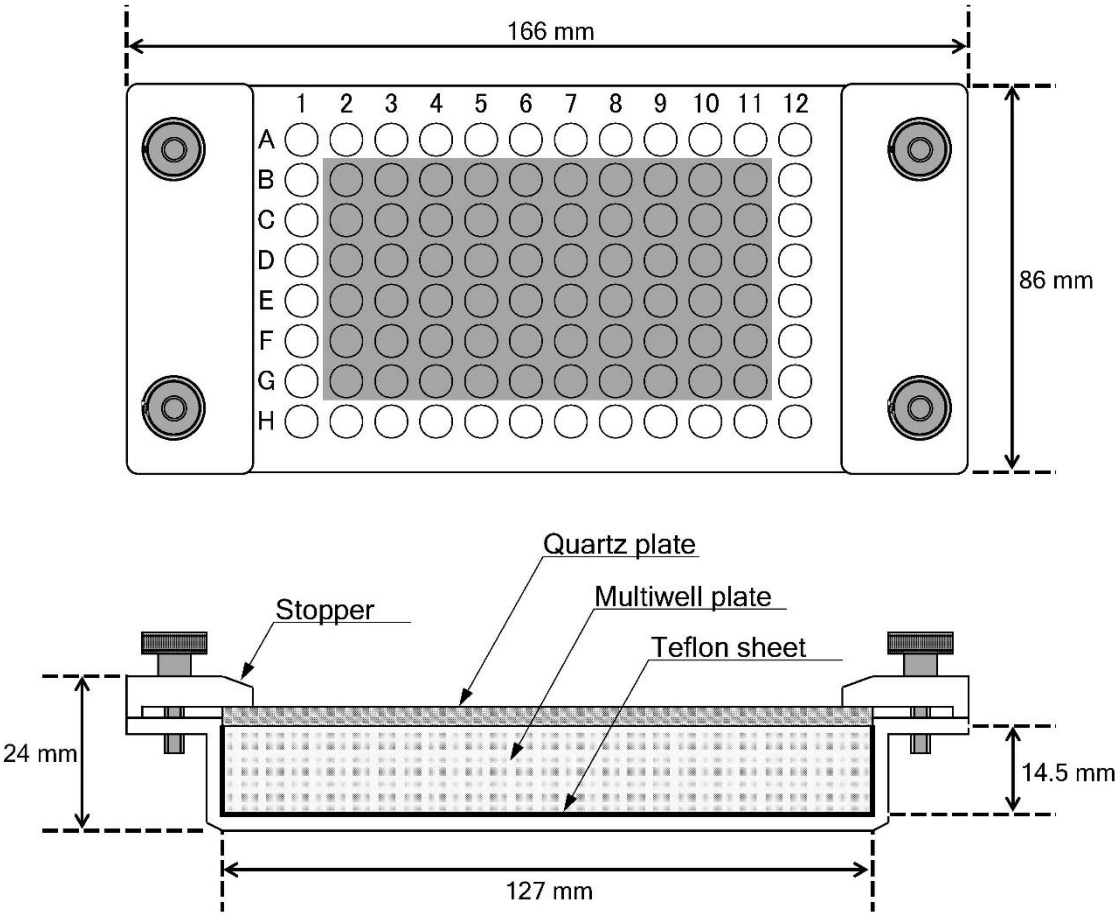
Annex D. Quartz reaction container used in the validation studies.



Recommended thickness of quartz plate: ca. 3 mm.

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