REACTIVE OXYGEN SPECIES (ROS) ASSAY TO EXAMINE PHOTOREACTIVITY OF CHEMICALS

Issued by: ROS assay Validation Management Team
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1. INTRODUCTION

The purpose of this document is to recommend a protocol for assaying the photoreactivity of chemicals based on reactive oxygen species (ROS). Photoreactivity is defined as the property of a chemical to react with another molecule as a consequence of photon absorption. Excitation of molecules by light can lead to generation of ROS such as superoxide anion (SA) and singlet oxygen (SO) through energy transfer mechanisms. The ROS assay does not measure phototoxicity directly, but rather is a physicochemical test that can be applied to that purpose, similar to measurement of UV absorbance.

2. INITIAL CONSIDERATION

Validation studies conducted by JaCVAM showed that the ROS assay has 100% sensitivity for predicting phototoxicants but can result in some false positives [1-3]. Based on the results of the validation studies, conducting this assay would classify a test chemical into one of the following: photoreactive, weakly photoreactive, non-photoreactive, or inconclusive. Photoreactive, weakly photoreactive, or inconclusive results would be a flag for follow-up phototoxicity assessment. Non-photoreactive results indicate a very low probability of phototoxicity, and no further phototoxicity testing would be suggested. In the ROS assay, measurement is first made at a concentration of 200 μM (final concentration). If interference such as precipitation or coloration (exhibiting peak absorbance at 440 or 560 nm) is observed at 200 μM, measurements are made at 20 μM. When precipitation or coloration is found at 20 μM, the substance is considered incompatible with the ROS assay. Since the ROS assay is designed to evaluate directly the photoreactivity of chemicals, it is not suitable for detecting chemicals that induce in vivo phototoxicity by indirect mechanisms such as porphyria and pseudoporphyria.

3. PRINCIPLE OF THE TEST METHOD

Drug-induced photoirritation can be defined as an inflammatory reaction of the skin after topical or systemic administration of pharmaceutical substances. There are several classes of drugs (including antibacterials, thiazide diuretics, non-steroidal anti-inflammatory drugs, quinolones, and tricyclic antidepressants) which are nontoxic by themselves but could become reactive when exposed to environmental light and thereby result in undesired side effects. The primary event in any photosensitization process 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 reaction and SO through type II reaction by photo-excited drug molecules. These appear to be the principal intermediate species in the phototoxic response. From the standpoint of risk assessment, previous research has demonstrated that determination of ROS from pharmaceutical substances irradiated with UVA/B and visible light would be of help in recognizing their phototoxic potential.

In the ROS assay, SO generation was detected by spectrophotometric measurement of *p*-nitrosodimethyl aniline (RNO) bleaching, followed by decreased absorbance of RNO at 440 nm [4]. 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

SA generation was detected by the 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 [5]. Thus, SA can reduce NBT to NBT^+, the formation of which can be monitored spectrophotometrically at 560 nm.

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

4. DESCRIPTION OF THE TEST METHOD

Technical equipment

Solar simulators:

Suntest CP series (Atlas Material Technology, Chicago, IL, USA) or SXL-2500V2 (Seric, Tokyo, Japan) with a fan and UVC cut filter (Spectrum shown in Appendix 1)

UVA detector: e.g. #0037 (Dr. Hönle, München, German) or UD series (Topcon, Tokyo, Japan)

Quartz reaction container (Ozawa Science, Aichi, Japan, Appendix 2) or equivalent

Microplate spectrophotometer, equipped with 440 and 560 nm filters
Microscope
Thermometer
Vortex mixer
Plate shaker
Sonicator
Pipettes
Polypropylene tubes
Plastic 96-well plates (clear, non-treated, flat-bottom)
Plastic and glass laboratory ware

Solar simulator

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 UVC cut filter. Recommended solar simulators and UVA intensity on the plate position measured by UVA detector #0037 (Dr. Hönle) are as follows:

Suntest CPS+ or CPS (Atlas) with UV cut filter (<290 nm),
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 (Appendix 1)

SXL-2500V2 (Seric) with UV cut filter (<300 nm),
3.0 to 5.0 mW/cm^2 for 1 hour,
11 to 18 J/cm^2 of UVA intensity (Appendix 1)

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 temperature. Standard temperature for a solar simulator with temperature control is 25°C. The acceptable temperature range during irradiation is 20° to 29°C. If a solar simulator other than the two recommended models is used, the reference chemical set listed in section 6 is to be tested prior to performing the ROS assay to ensure that measured values of SO and SA are close to those mentioned in section 6.

Quartz reaction container

A quartz reaction container is used to avoid loss of UV due to passing through a plastic lid and vaporization of the reaction mixture [6]. A made-to-order container (See Appendix 2.) or its equivalent is recommended. If a different container is used, a lid or seal with high UV transmittance must be used. In this case, a feasibility study using the reference chemicals is to be conducted to determine an appropriate level of exposure to UV and visible light.

Reagents

The following reagents are to be used and stored according to the instructions of manufacturers.

NaH_2PO_4・$2H_2O$ (CAS No. 13472-35-0)

Na_2HPO_4・$12H_2O$ (CAS No. 10039-32-4)

p-Nitrosodimethylaniline (RNO, CAS No. 138-89-6)

Imidazole (CAS No. 288-32-4)

Nitroblue tetrazolium chloride (NBT, CAS No. 298-83-9)

Purified water

Preparation of reagents

All reagents are to be sonicated and used within 1 month after preparation. Representative preparation methods are shown as follows.

20 mM sodium phosphate buffer (NaPB), pH 7.4

Weigh 593 mg of NaH_2PO_4・$2H_2O$ and 5.8 g of Na_2HPO_4・$12H_2O$, 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)

Dissolve 3 mg of RNO in 100 mL of 20 mM NaPB.

Store in a refrigerator and protect from light.

20 mM imidazole

Dissolve 13.6 mg of imidazole in 10 mL of 20 mM NaPB.

Dilute the 2×10^{-2} M imidazole solution 100 times with 20 mM NaPB.

Store in a refrigerator and protect from light.

0.4 mM nitroblue tetrazolium chloride (NBT)

Dissolve 32.7 mg of NBT in 100 mL of 20 mM NaPB.

Store in a refrigerator and protect from light.

Test chemicals

Test chemicals are to be stored appropriately until termination of the study and their stability during the test period is to be confirmed. One concentration level, 200 μM (final concentration), is to be used. A 20-μM concentration can be used if precipitation before light exposure, coloration, or other interference is observed in the reaction mixture at 200 μM.

Preparation of test chemical solutions

The test chemical solutions are to be prepared using a solvent just before use. Each test chemical is to be weighed in a tube, and solvent added to a concentration 10 mM. The tube is to be mixed with

a vortex mixer and sonicated for 5 to 10 minutes under UV-cut illumination or shade. All preparations are to be protected from light. The final concentration in a reaction mixture is to be 200 μM. When precipitation before light exposure, coloration, 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 using the solvent. For chemicals that are not soluble in DMSO, 20 μL of DMSO is to be contained in the reaction mixture.

Positive and negative control chemicals

Quinine hydrochloride (CAS No. 6119-47-7) is to be used at 200 μM (final concentration) as a positive control. Sulisobenzone (CAS No. 4065-45-6) is to be used at 200 μM (final concentration) as a negative control.

Preparation of positive and negative control chemical solutions

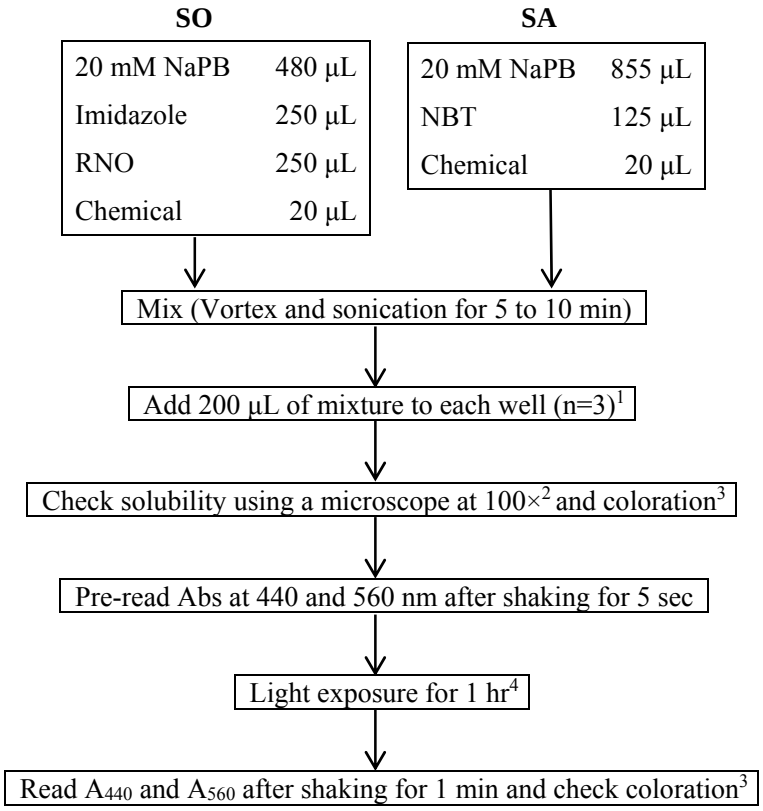
Stock solutions of quinine and sulisobenzone are to be prepared at 10 mM each in DMSO (final concentration of 200 μM) according to the above procedure, divided into tubes, and stored in a freezer (generally below -20˚C) for up to 1 month. The stock solution is to be thawed just before the experiment and used within the day.

Solvent

Use analytical grade DMSO at first. For chemicals that are not soluble in DMSO, 20 mM of NaPB is to be used. When a test chemical is insoluble in either DMSO or 20 mM NaPB, try bovine serum albumin (BSA) or other solvent [7-8]. Prior to use of BSA or other solvent, however, perform a feasibility study (see Section 6) using the reference chemicals to determine appropriate test conditions. The results of ROS assays using BSA or other solvents, however, are not suitable for regulatory purposes until these solvents have been properly evaluated.

Test procedure

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 blank instead of test chemical solution.



Notes

1) Avoid using peripheral wells. More than one test chemical can be tested on a plate. A typical 96-well plate configuration is as follows:

	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

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.

5. DATA AND REPORTING

Data analysis

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: Blank before light exposure (mean)

b: Blank 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: Blank before light exposure (mean)

b: Blank after exposure (mean)

Criteria for data acceptance

The following criteria are to be satisfied in each experiment.

No precipitation of test chemical in the reaction mixture before light exposure.

No coloration of test chemical in the reaction mixture before or after light exposure.

No technical problems, including prescribed temperature range, when collecting data set.

Raw OD value: 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 value at 200 μM (mean of 3 wells)

SO: 319 to 583

SA: 193 to 385

Negative control value at 200 μM (mean of 3 wells)

SO: -9 to 11

SA: -20 to 2

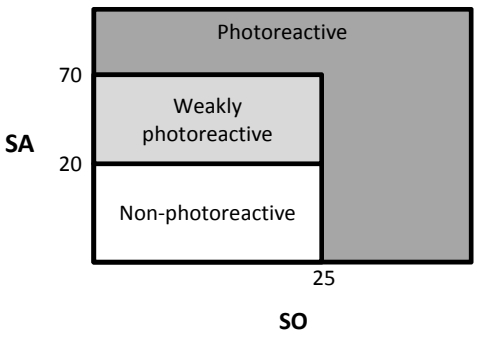
Criteria for judgment

Each test chemical is to be judged as follows:

Judgment[1, 2]	**Concentration[3]**	**SO (mean of 3 wells)**		**SA (mean of 3 wells)**
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
Non-photoreactive	200 μM	<25	and	<20
Inconclusive	The results do not meet the above-mentioned criterion.			

Notes

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. Twenty μM can be used for judgment when precipitation or coloration is observed at 200 μM. For regulatory purposes, the stability of the test chemical in the reaction mixture both before and after light exposure is to be confirmed when results at 20 μM are used for judgment as a non-photoreactive chemical for which no further phototoxicity testing is necessary.
4. Interference such as precipitation or coloration.

Data quality

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

Test report

The test report must include the following information:

Test chemical

- Name and lot No.
- Physical nature and purity
- Storage condition
- Stability during the test period
- UV/vis absorption spectrum, maximum molar extinction coefficient at 290 to 700 nm, and/or photostability, if known
- Preparation of test chemical solution
- Final concentrations tested

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

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

Archives

The study report and all raw data is to be retained according to the SOP in the testing facility.

6. REFERENCE CHEMICALS FOR THE FEASIBILITY STUDY

To perform a ROS assay, it is necessary first to ensure irradiation conditions that satisfy the recommended criteria using the positive and negative controls, after which reference chemicals are to be tested in a feasibility study. The reference chemical set, as selected from the validation studies, and acceptable value ranges are shown in Table 1 and Table 2. It is important that the values for SO and SA obtained in the feasibility study be similar to the following values.

Table 1 Standard chemical set for laboratories to demonstrate proficiency using solar simulators of Suntest CPS/CPS+ (Atlas) or SXL-2500V2 (Seric) and the acceptable range

No.	Chemical	CAS No.	SO	SA	Solvent	Concentration
11	Doxycycline hydrochloride	10592-13-9	115 to 429	230 to 468	DMSO	200 μM
12	Norfloxacin	70458-96-7	131 to 271	57 to 161	DMSO	200 μM
13	8-Methoxy psoralen	298-81-7	31 to 137	0 to 126	DMSO	200 μM
14	Fenofibrate	49562-28-9	77 to 203	-31 to 11	DMSO	20 μM
15	p-Aminobenzoic acid	150-13-0	-8 to 12	-11 to 7	DMSO	200 μM
16	Benzocaine	94-09-7	-7 to 9	-7 to 17	DMSO	200 μM
17	Erythromycin	114-07-8	-15 to 11	-9 to 21	DMSO	200 μM
18	Octyl salicylate	118-60-5	-5 to 11	-8 to 20	DMSO	20 μM
19	L-Histidine	71-00-1	-8 to 12	8 to 120	NaPB	200 μM

The values were calculated as mean +/- 1.96 SD from the validation data.

Note: Underline parts were corrected because of errors in writing.

Table 2 Recommended chemical set for the other solar simulators and the acceptable range

No.	Chemical	CAS No.	SO	SA	Solvent	Concentration
21	Acridine	260-94-6	182 to 328	121 to 243	DMSO	200 µM
22	Chlorpromazine hydrochloride	69-09-0	-56 to 70	66 to 106	DMSO	200 µM
23	Diclofenac	15307-79-6	34 to 416	47 to 437	DMSO	200 µM
24	Doxycycline hydrochloride	10592-13-9	115 to 429	230 to 468	DMSO	200 µM
25	Furosemide	54-31-9	31 to 225	-7 to 109	DMSO	200 µM
26	Ketoprofen	22071-15-4	120 to 346	77 to 151	DMSO	200 µM
27	8-Methoxy psoralen	298-81-7	31 to 137	0 to 126	DMSO	200 µM
28	Nalidixic acid	389-08-2	54 to 246	88 to 470	DMSO	200 µM
29	Norfloxacin	70458-96-7	131 to 271	57 to 161	DMSO	200 µM
30	Omeprazole	73590-58-6	-221 to 103	30 to 216	DMSO	200 µM
31	Promethazine hydrochloride	58-33-3	20 to 168	-3 to 77	DMSO	200 µM
32	Fenofibrate	49562-28-9	77 to 203	-31 to 11	DMSO	20 µM
33	p-Aminobenzoic acid	150-13-0	-8 to 12	-11 to 7	DMSO	200 µM
34	Benzocaine	94-09-7	-7 to 9	-7 to 17	DMSO	200 µM
35	Erythromycin	114-07-8	-15 to 11	-9 to 21	DMSO	200 µM
36	Octyl salicylate	118-60-5	-5 to 11	-8 to 20	DMSO	20 µM
37	L-Histidine	71-00-1	-8 to 12	8 to 120	NaPB	200 µM

The values were calculated as mean +/- 1.96 SD from the validation data.

7. GLOSSARY

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

3T3 NRU-PT: *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 $L\ mol^{-1}\ cm^{-1}$).

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

Phototoxicity: An acute light-induced tissue response to a photoreactive chemical.

UVA: Ultraviolet light A (wavelengths between 320 and 400 nm).

UVB: Ultraviolet light B (wavelengths between 290 and 320 nm).

UVC: Ultraviolet light C (wavelengths between 190 and 290 nm).

8. REFFERENCES

[1] ROS assay validation management team. Validation report for the international validation study on ROS (Reactive Oxygen Species) assay as a test evaluating phototoxic potential of chemicals (Atlas Suntest version), 2013.

[2] ROS assay validation management team. Validation report for the international validation study on ROS (Reactive Oxygen Species) assay as a test evaluating phototoxic potential of chemicals (Seric version), 2013.

[3] Onoue S, Hosoi K, Wakuri S, Iwase Y, Yamamoto T, Matsuoka N, Nakamura K, Toda T, Takagi H, Osaki N, Matsumoto Y, Kawakami S, Seto Y, Kato M, Yamada S, Ohno Y, Kojima H. Establishment and intra-/inter-laboratory validation of a standard protocol of reactive oxygen species assay for chemical photosafety evaluation. J Appl Toxicol, Article first published online, 13 June, 2012 (DOI: 10.1002/jat.2776).

[4] I. Kraljić, S. El Mohsni. A new method for the detection of singlet oxygen in aqueous solutions. Photochem Photobiol, 28, 577-81, 1978.

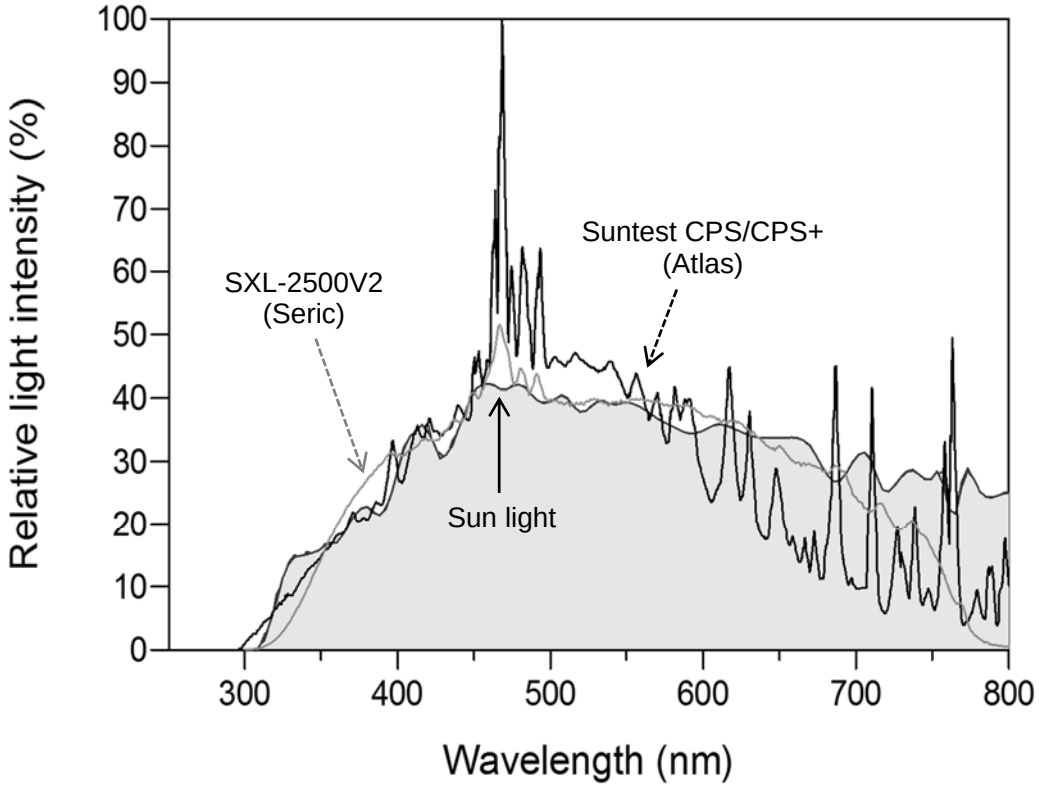
[5] Pathak MA, Joshi PC. Production of active oxygen species ($^1O_2$ and $O_2^{-\cdot}$) by psoralens and ultraviolet radiation (320-400 nm). Biochim Biophys Acta, 798, 115-26, 1984.

[6] Onoue S, Igarashi N, Yamada S, Tsuda Y. High-throughput reactive oxygen species (ROS) assay: an enabling technology for screening the phototoxic potential of pharmaceutical substances. J Pharm Biomed Anal, 46, 187-93, 2008.

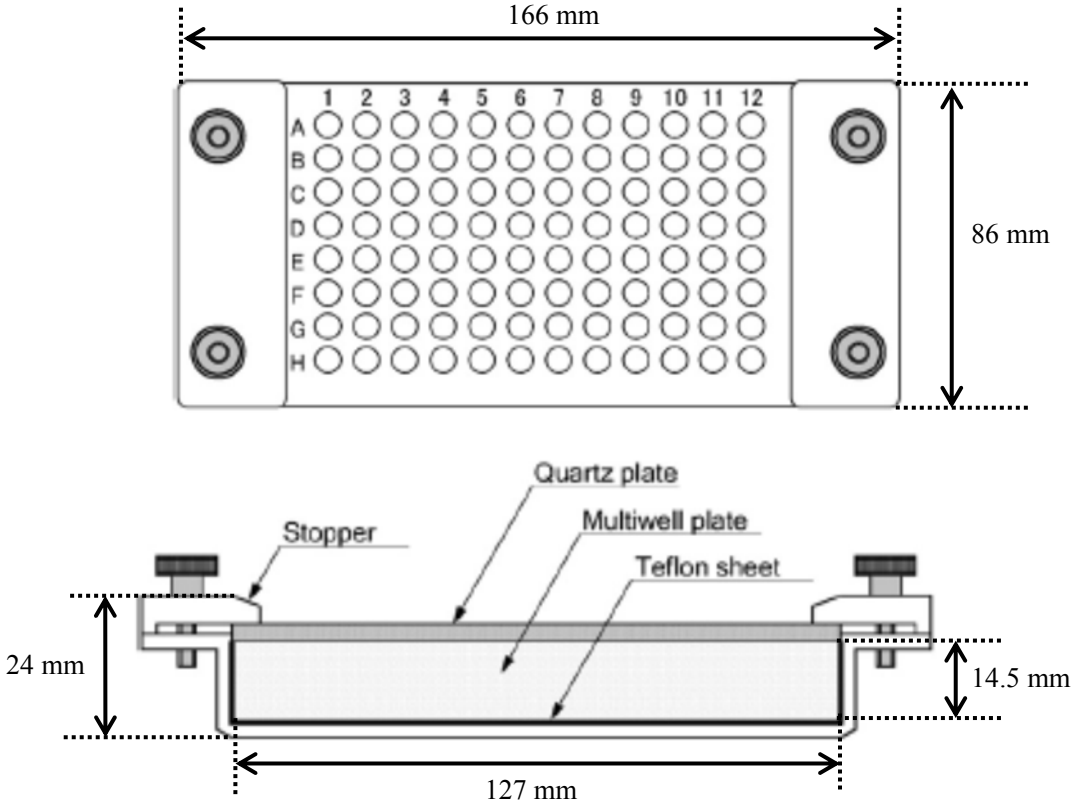
[7] Onoue S, Yamauchi Y, Kojima T, Igarashi N, Tsuda Y. Analytical studies on photochemical behavior of phototoxic substances; effect of detergent additives on singlet oxygen generation. Pharm Res, 25, 861-868, 2008.

[8] Onoue S, Kato M, Yamada S. Development of an albuminous reactive oxygen species assay for photosafety evaluation under experimental biomimetic conditions. J Appl Toxicol, Article first published online, 28 Jan, 2013 (DOI: 10.1002/jat.2846).

Appendix 1 Spectrum of solar simulators used in the validation studies



Appendix 2 Quartz reaction container used in the validation studies



Appendix 3 Amendment

Version 3.2

Page 10: The SA values of benzocaine and erythromycin were corrected in Table 1 because of errors in writing.

	Incorrect	Correct
Benzocaine	7 to 17	-7 to 17
Erythromycin	9 to 21	-9 to 21