DB-ALM Protocol n° 155 : KeratinoSens™

Skin Sensitisation & Allergic Contact Dermatitis

The KeratinoSens™ is an *in vitro* test method which quantifies luciferase gene induction as a measure of the activation of the Keap1-Nrf2-antioxidant/electrophile response element (ARE)-dependant pathway in an immortalized adherent cell line derived from HaCaT human keratinocytes transfected with a selectable plasmid. The Keap1-Nrf2-ARE pathway is reported to be a major regulator of cyto-protective responses to electrophile and oxidative stress by controlling the expression of detoxification, antioxidant and stress response enzymes and proteins. The involvement of the Keap1-Nrf2-ARE regulatory pathway in skin sensitisation has been demonstrated in a number of *in vivo* studies (Kim et al., 2008; El Ali et al., 2013; Van der Veen et al., 2013). Therefore, information from the KeratinoSens™ is considered relevant for the assessment of the skin sensitisation potential of chemicals.

Résumé

The purpose of the test is to contribute to the evaluation of the skin sensitization potential of chemicals. Induction of cyto-protective pathways in keratinocytes in response to electrophiles and oxidative stress is addressing the second key event of the skin sensitization Adverse Outcome Pathway (AOP; OECD, 2012).

The role of the Keap1-Nrf2-ARE regulatory pathway in the detection of electrophiles is well-established. Therefore, test methods able to measure the activation of this pathway are considered relevant for the assessment of the skin sensitization potential of chemicals. Further details on the context of use and applicability of the method can be found in the **Method Summary of KeratinoSens** in DB-ALM, in the EURL ECVAM Recommendation (EURL ECVAM, 2014) and in the OECD TG No 442D “KEY EVENT BASED TEST GUIDELINES 442D, In Vitro Skin Sensitisation assays addressing the AOP Key Event on: Keratinocyte activation" (OECD, 2018).

Experimental Description

Endpoint and Endpoint Measurement:

GENE EXPRESSION: The KeratinoSens™ measures luciferase gene induction following 48 hours exposure of the keratinocyte reporter cell line to test chemicals.

Endpoint Value:

Luciferase induction and cytotoxicity are measured as biological response variables. The following endpoint variables are reported based on these measurements: Positive/negative rating according specific prediction model based on the two biological variables.

EC 1.5, EC2 and EC3: Extrapolated concentration of a test compound, needed for a 1.5- / 2- and 3- fold luciferase induction.

IC30 and IC50 values: Concentration to reduce cellular viability by 30 and 50%, respectively.

I_{max}: Maximal fold induction of the luciferase gene over solvent control.

Experimental System(s):

KeratinoSens™: an immortalised adherent cell line derived from HaCaT human keratinocytes transfected with a selectable plasmid. This plasmid contains the luciferase gene under the transcriptional control of the AKR1C2 ARE sequence upstream of the SV40 promoter. The AKR1C2 gene was identified as one of the genes up-regulated by contact sensitisers in dendritic cells (Ryan et al., 2004; Gildea et al., 2006).

Basic Procedure

Luciferase gene induction is measured in the cell lysates by luminescence detection and using a well-established light producing luciferase substrate. The test method is applicable to test pure chemicals soluble in water, cell exposure medium described herein or in DMSO, the solvents prescribed by the protocol (see below). Mixtures can be tested, but there is little information on the predictivity of this and other methods for the assessment of skin sensitisation of mixtures. Predictivity for nanomaterials and medical devices has not been assessed in detail. Currently, until more data are available, it is recommended to only test eluates from nanomaterials and medical devices.

The positive control used is Cinnamic Aldehyde (CAS 14371-10-9) and the negative control is DMSO.

Data Analysis/Prediction Model

Test chemicals are identified as potential skin sensitizers if the I_{max} is statistically significantly higher than 1.5-fold as compared to the basal luciferase activity and the EC1.5 value is below 1000 µM in at least two out of the three repetitions. In addition at the lowest concentration with a gene induction ≥1.5 fold the cellular viability should be above 70% and the dose-response for luciferase induction, should be similar between the repetitions.

Test Compounds and Results Summary

The experimental procedure was developed by Givaudan (Emter et al., 2010). From 2009 to 2010 Givaudan coordinated a validation study on the KeratinoSens™ Test Method, focusing on its transferability and reproducibility (Natsch et al., 2011).

The accuracy of the method to discriminate between sensitising and non-sensitising chemicals was 90% (sensitivity 87%, specificity 100%) for the set of chemicals evaluated in the validation study (n=21). The accuracy of the KeratinoSens ™ as judged versus evidence from the LLNA was found to be of 75% (sensitivity 75%, n=77; specificity 75%, n=104) by EURL ECVAM (EURL ECVAM, 2014). These figures are similar to those published in the scientific literature and based on Givaudan in-house data generated with 145 chemicals (77% accuracy, 79% sensitivity, 72% specificity) (Natsch et al., 2013).

Acceptance Criteria and Proficiency Testing

Acceptance criteria have to be applied to the positive control and to the test chemical's results. At least one of the acceptance criteria for the positive control must be met, otherwise the run is discarded. If only one of the criteria concerning the positive control is fulfilled, it is recommended to carefully check the dose-response of Cinnamic aldehyde in order to decide on the acceptability of the test results.

Discussion

Given the complexity of the biological mechanisms underlying skin sensitisation and the limitations of the currently available non-animal test methods, it is likely that combinations of mechanism-based test methods within Integrated Approaches to Testing and Assessment (IATA) are needed to be able to substitute the regulatory animal tests currently in use to satisfy regulatory requirements for this endpoint. The KeratinoSens™ is considered scientifically valid to be used as part of an IATA, since it was shown to be a reliable method and to be easily transferable to naïve laboratories sufficiently experienced in cell culture techniques (EURL ECVAM, 2014).

Status

Note about the current version:

This version issued March 09 2018 replaces earlier versions. It is aligned with the new OECD guideline version 442D issued in 2018. The technical procedure has not changed, but this version includes a number of technical clarifications and guidance on data evaluation esp. on poorly soluble and cytotoxic compounds. It also includes guidance how to proceed with a version not using animal derived materials.

Known Laboratory Use:

Givaudan (Natsch et al., 2013)

BASF (Bauch et al., 2012)

Institute of In Vitro Science (IIVS) (Gan et al., 2013)

Dow Chemicals (Settivari et al., 2012)

Transfer to > 50 academic and commercial labs has been completed.

Participation in Validation Studies:

The KeratinoSens™ has undergone a validation study (Natsch et al. 2013) followed by an independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) and finalised with EURL ECVAM recommendation (EURL ECVAM, 2014).

Regulatory Acceptance:

The test method was adopted **as OECD Test Guideline No 442D** (OECD, 2015). A revised version has been adopted in June 2018 (OECD, 2018). TG 442D provides an *in vitro* procedure proposed for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (UN, 2017).

Proprietary and/or Confidentiality Issues

The KeratinoSens™is a trade mark of the test method developer (Givaudan SA, Switzerland). The KeratinoSens™ cell line is made available to third parties at no cost.

Abbreviations and Definitions

AKR1C2: Aldo-keto reductase family 1, member C2

AOP: Adverse Outcome Pathway

ARE : antioxidant response element

DMSO: Dimethyl sulfoxide

EC 1.5/2/3: Extrapolated concentration for a 1.5/2/3 fold luciferase induction

IC 30/50: Concentration for reduction of cellular viability by 30% or 50% as determined with the MTT assay

FBS: Foetal bovine serum

IATA: Integrative Approaches to Testing and Assessment

I_{max}: Maximal induction of luciferase activity over solvent control over the complete dose-response range measured

Keap1 : Kelch-like ECH-associated protein 1

MTT : Thiazolyl Blue Tetrazolium bromide

Nrf2 : nuclear factor (erythroid-derived 2)-like 2

PBS: Phosphate buffered saline

SOP: Standard Operating procedure

SV40: Simian virus 40

Last update: 23 July 2018

PROCEDURE DETAILS, 9 March 2018

KeratinoSens™
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The protocol is based on the Standard Operating Procedure (SOP) used in the Givaudan-coordinated validation study on the KeratinoSens™ Method. Study templates and an example of data analysis are available from the DB-ALM website. Go to the section related to the Protocol No. 155 and select **Downloads**.

Contact Details

Dr. Andreas Natsch
Givaudan
Ueberlandstrasse 138
Duebendorf CH-8600
Switzerland
email: andreas.natsch@givaudan.com

Dr. Roger Emter
Givaudan
Ueberlandstrasse 138
Duebendorf CH-8600
Switzerland
email: roger.emter@givaudan.com

Materials and Preparations

Cell or Test System

The transgenic cell line KeratinoSens with a stable insertion of the Luciferase construct is supplied by Givaudan on dry ice. Upon receipt, it should be propagated to passage 2 - 4 and multiple vials of the resulting cell population should be stored in liquid nitrogen as a homogeneous stock. Cells from this stock are then used for routine testing. The cells propagated from this original stock can then be kept in culture for a maximum of 25 passages.

Equipment

Fixed Equipment

- Sterile hood for cell culture work
- CO_2 incubator
- Multi-channel pipettes for volumes between 10 µl and 200 µl
- 96 well plate Luminometer with an injector (single injector sufficient, no need for double luciferase measurement)
- Models which have been used successfully with this protocol:
    - GloMaxTM 96 Microplate Luminometer (Promega)
    - Infinity F500 (Tecan)
    - Infinity M200 (Tecan)
    - FLUOstar OPTIMA (BMG Labtech)
    - Orion II/MPL4 microplate luminometer; without injector (Berthold)
- 96 well plate absorbance reader (equipped for reading at 600 nm) for MTT measurement

Media, Reagents, Sera, others

Below are listed the reagents used for the routine testing. For most cell-culture products, alternative products from other manufacturers will work equally.

	Product	Company	Catalog Number
Medium	D-MEM (Dulbecco's Modified Eagle Medium), liquid with GlutaMAX™ I, 1000 mg/L D-Glucose, Sodium Pyruvate	Gibco	21885-025
Serum	Foetal bovine serum (FBS) Origin: South America An alternative source of the serum can be used with the standard supplier for each Laboratory Heat inactivated by heating up to 56°C and keeping at 56°C for 30 min.	AMIMED	2-01F10-I
Alternative for FBS: Human Serum	pooled human male AB plasma	Sigma-Aldrich UK	H4522

	Product	Company	Catalog Number
Phosphate buffered saline	DPBS	Gibco	14190
Trypsin	0.05% Trypsin-EDTA	Gibco	25300
Alternative to Trypsin	Trypzean ™	Sigma-Aldrich	T3499
G-418	Geneticin (G418)	Gibco	10131-027
EDTA	Ethylenediamin-tetra-acetic acid trinatrium salt	FLUKA	03710
Solvent	DMSO	Sigma	41650
Lysis buffer	Passive Lysis Buffer, 5x	Promega	E1941
Luciferase substrate	Luciferase Assay System 10-Pack	Promega	E1501
MTT	Thiazolyl Blue Tetrazolium bromide	Fluka	88415
Positive control	Cinnamic aldehyde, MW 132.16, CAS-Nr. **14371-10-9** , > 99%	Aldrich	239968
White 96 well culture plates	Lia-Plate, white, Tissue culture (TC), 96 well, flat bottom, with lid, sterile	Greiner Bio-One	655 083
Transparent 96 well culture plates	Tissue culture (TC) test plate, 96 well, flat bottom	Orange Scientific	5530100
Addhesive foils to cover plates during 2 day incubation period	Sealing tape SI	Nunc	0236366
Culture plates	Culture Dishes 100 x 20 mm	Milian	TP-93100
CryoTubes	CryoTube 1,8 ml SI	Nunc	368632

Attention points:

For the ring study each laboratory used their own FBS supplier and this did not affect the results.

Lysis buffer is the only complex reagent which is specific to the indicated supplier and where no alternative products were tested yet, and which contains a proprietary composition known only to Promega.

For the luciferase substrate, the Promega quality should be used for licence reasons (See **Annex 4**). Ideally the luminometer is equipped with an injector, and then a flash substrate is used (substrate giving only short but intense light production). If no injector is available, a Glow-substrate (yielding long-time steady light emission at low intensity) has also successfully been used, but it can generate issues with sensitivity or with a gradient over the plate if long integration times are needed.

It is important that the test plates for the luminescence reading exactly fit the geometry of the reader: If the height of the plates is not sufficient, there can be a well-to-well interference by light emitted in one well influencing the results in the adjacent well. This may especially be the case if a Glow-substrate is used.

Note: Three factors are crucial for luminescence readings:
(i) The choice of a sensitive luminometer,
(ii) of a plate format with sufficient height to avoid light cross-contamination
(iii) a substrate with sufficient light output to ensure sufficient sensitivity and low variability.

Annex 2 describes a basic experimental setup, which should be performed as a first experiment, in order to validate that these three points are met.

Preparations

Media and Endpoint Assay Solutions

Maintenance medium

The maintenance medium for the KeratinoSens™ cell line is prepared by supplementing 500 ml D-MEM with 50 ml FBS (final FBS concentration: 9.1 %) and 5.5 ml G418 Gibco (final concentration 500 µg/ml). The medium is stored at 4°C and used within 28 days. Instead of FBS, alternatively human serum may be used.

Medium for freezing the cells

D-MEM containing 20% FBS and 10% DMSO. Instead of FBS, alternatively human serum may be used.

Exposure medium

Supplementing 495 ml D-MEM with 5 ml FBS (final FBS concentration: 1%). No G418 is added. The medium is stored at 4°C and used within 28 days. Instead of FBS, alternatively human serum may be used.

EDTA solution 10%, pH 8

10 g EDTA is dissolved in 100 ml H_2O and NaOH is added to bring the solution to pH8, sterilized by filtration.

Solubilisation trial

Chemicals which are known to have a low water solubility, are subjected to a solubilisation trial. The test chemical can be either dissolved directly at a concentration of 1 - 2 mM in exposure medium containing 1% DMSO. Alternatively, the chemical is dissolved at 200 mM in DMSO and, the DMSO solution is diluted 100-fold in the exposure medium.
This solution can be further serially diluted, and the different dilutions are allowed to settle for at least 2 h in transparent tubes. By visual observation, it is determined whether the test chemical did fully dissolve at a given concentration or at least forms a stable dispersion without apparent phase separation or precipitation. This observation is relevant when evaluating the final result. Chemicals of poor solubility (i.e. phase separation / precipitation at high concentration) are tested at lower maximal concentrations than 2 mM.

When testing mixtures, it should be verified that all constituents are dissolved in the test medium or at least form a stable dispersion (e.g. by visual inspection of the mixture dissolved at the maximal final test concentration in the test medium, showing that no undissolved residues remain and that no precipitates or phase separation form if the solution is left to settle for 2 hours).

Test Compound solutions and positive control solution

All chemicals are dissolved to a final concentration of 200 mM in DMSO. To this end 20 – 40 mg of chemicals are weighted into pre-tared glass vials. A volume of DMSO calculated according to the following formula is added:

$$V = 5 \times \frac{(p \div 100) \times w}{MW} - \frac{w}{1000}$$ Where:

V is the volume of DMSO in ml to be added
p is the purity of the chemical in %
MW is the molecular weight of the chemical in g / mol
w is the exact weight of the chemical added to the vial in mg

All DMSO solutions can be considered self-sterilizing, and no sterile filtration is applied to any DMSO solution.

Chemicals not soluble in DMSO are dissolved and diluted in exposure media and the solutions are sterilized by filtration through a 0.2 µM filter. Final DMSO concentration is always adjusted to 1% in all exposure solutions.

Chemicals which have no defined molecular weight (such as small polymers) are tested considering a *pro forma* molecular weight of 200, or, in other words, the stock solution is prepared to a concentration of 40 mg / ml or 4 % (w/v).

Positive Control(s)

Cinnamic aldehyde is dissolved to a final concentration of 200 mM in DMSO as described above. This solution is further diluted to a final concentration of 6.4 mM by adding 32 µl of the 200 mM solution to 968 µl of DMSO.

Negative Control(s)

There is no negative control chemical tested in each run. As control the DMSO solvent control is used, and each test plate contains six wells with the DMSO control, as indicated below:

Preparation of the 100 × DMSO Master plate

Based on the 200mM stock DMSO solutions of the test chemicals, the 100 × DMSO master plate is prepared. It contains seven different test chemicals in rows A – G and a control row in row H. For the test chemical rows, 100 µl of DMSO is pipetted into column 1 to 11. For each test chemical then 200 µl of the 200mM stock solution is added to column 12. Serial dilutions are then prepared by transferring 100 µl from column 12 to column 11, mixing by repeated pipetting (at least 3 times) in column 11 and then transferring again 100 µl to column 10 and so forth.

The control row contains 100 µl DMSO only in column 1 – 6 and column 12. To column 7 – 10 100 µl of DMSO are added and to column 11 200 µl of the 6.4 mM stock solution of cinnamic aldehyde is added. Serial dilutions of the cinnamic aldehyde solution starting from column 11 and ending in column 7 are then made as described above for the test compound dilutions.

The schematic setup of the 100 × DMSO master plate is shown below, concentrations are given in mM:

	1	2	3	4	5	6	7	8	9	10	11	12
A	comp.1 0.098	comp.1 0.195	comp.1 0.39	comp.1 0.78	comp.1 1.56	comp.1 3.125	comp.1 6.25	comp.1 12.5	comp.1 25	comp.1 50	comp.1 100	comp.1 200
B	comp.2 0.098	comp.2 0.195	comp.2 0.39	comp.2 0.78	comp.2 1.56	comp.2 3.125	comp.2 6.25	comp.2 12.5	comp.2 25	comp.2 50	comp.2 100	comp.2 200
C	comp.3 0.098	comp.3 0.195	comp.3 0.39	comp.3 0.78	comp.3 1.56	comp.3 3.125	comp.3 6.25	comp.3 12.5	comp.3 25	comp.3 50	comp.3 100	comp.3 200
D	comp.4 0.098	comp.4 0.195	comp.4 0.39	comp.4 0.78	comp.4 1.56	comp.4 3.125	comp.4 6.25	comp.4 12.5	comp.4 25	comp.4 50	comp.4 100	comp.4 200
E	comp.5 0.098	comp.5 0.195	comp.5 0.39	comp.5 0.78	comp.5 1.56	comp.5 3.125	comp.5 6.25	comp.5 12.5	comp.5 25	comp.5 50	comp.5 100	comp.5 200
F	comp.6 0.098	comp.6 0.195	comp.6 0.39	comp.6 0.78	comp.6 1.56	comp.6 3.125	comp.6 6.25	comp.6 12.5	comp.6 25	comp.6 50	comp.6 100	comp.6 200
G	comp.7 0.098	comp.7 0.195	comp.7 0.39	comp.7 0.78	comp.7 1.56	comp.7 3.125	comp.7 6.25	comp.7 12.5	comp.7 25	comp.7 50	comp.7 100	comp.7 200
H	blank solvent	blank solvent	blank solvent	blank solvent	blank solvent	blank solvent	0.4 mM cinn. ald.	0.8 mM cinn. ald.	1.6 mM cinn. ald.	3.2 mM cinn. ald.	6.4 mM cinn. ald.	no cells blank

For test chemicals not soluble in DMSO, all the dilutions can be made in test medium.

The DMSO level in all the wells of the final test solution must in these cases also be adjusted to 1% as for the other compounds. This is detailed below, in the “ **Test Material Exposure Procedures** ” section.

Method

Test System Procurement

Givaudan will share the recombinant cell line KeratinoSens with third laboratories under a material transfer agreement. Previously, a transfer fee was requested for assay implementation. In order to make all assays under OECD guideline 442D, version 2018, equally accessible, Givaudan decided to waive this fee effective February 1th, 2018. Only shipping costs have to be covered by recipient after this date. Stocks of the cells can then be prepared by the test lab based on the culture received from Givaudan (For information, see **Contact Person** section).

The luciferase gene luc2 in the KeratinoSens cell line is patent-protected by Promega Corp. It can be used by any laboratory for research use and testing for the sensitisation potential of chemicals with the proviso that the substrate used for the assay is purchased from Promega (see **Annex 4**).

Routine Culture Procedure

Thawing: Upon receipt, the frozen cells should be transferred to a liquid nitrogen tank for prolonged storage. To thaw the cells, they should be warmed in a 37ºC water bath. The cells are then resuspended in 10 ml maintenance medium and pelleted by centrifugation at 125 g for 5 min to get rid of the DMSO used for freezing. The cell pellet is then resuspended in 10 ml of maintenance medium with 9.1% FBS without G418. Cells are plated in a 10 cm tissue culture dish. G418-containing medium is only added in the next passage.

Maintenance: Cells are maintained in Dulbecco's modified Eagle's medium containing Glutamax (Gibco/Invitrogen) supplemented with 9.1 % bovine fetal serum human serum and 500 µg/ml G418 at 37ºC in the presence of 5% CO_2. 80-90% confluent cells are washed twice with DPBS containing 0.05% EDTA, then Trypsin-EDTA (1 ml / plate) is added and plates are put back into the 37°C incubator. Alternatively, Trypzean may be used. After cells have detached (usually after 5 – 10 min), they are resuspended in 10 ml maintenance medium and split at a ratio of 1: 3 - 1:12 in fresh medium and grown to 80-90% confluency. With a split ratio of 1:3, cells need 2 days to reach confluency again, in a ratio of 1:6, cells need 3 days (normally done for the weekend) and in a ratio of 1:12, 4 days.

Antibiotics against microbial contaminations are not used in the standard cultivation of these cells, nor are they used when cells are seeded for testing.

Routinely, 100 mm culture dishes are used. However, cells may also be grown in T75 flasks.

Freezing: For the preparation of frozen stocks, the cells are harvested as described above, pelleted by centrifugation (125 g for 5 min), and resuspended in growth medium containing 20% FBS or human serum and 10% DMSO at a density of 3 - 4 × 10^6 cells per ml. The cells are aliquoted into CryoTubes and frozen in a -80ºC freezer using a Freezing Container. After 24 h, they are then transferred to liquid nitrogen.

Cell seeding for testing:

- Cells are split on Friday afternoon in a split ratio of 1:6 and 1:12 and grown for 3 – 4 days in 10 cm culture dishes.
- On Monday morning the media is replaced with fresh medium.
- The cells from the 1:6 split are then used to prepare assay plates on Monday afternoon, whereas the cells from the 1:12 split are used on Tuesday afternoon to prepare additional assay plates.
- At the stage of preparing assay plates, cells should be 80- 90 % confluent, but should never be grown to full confluency.
- The cells are washed twice with PBS containing 0.05% EDTA, harvested as described above, re-suspended in DMEM with 9.1% FBS without G418 and adjusted to a density of 80'000 cells / ml.
- The cells are then distributed to the 96-well plates, 125 µl (containing 10'000 cells) per well. It is very important to avoid sedimentation of the cells during this step and to assure that the same cell number is distributed to all wells. If this is not carefully assured, this step may give the highest well-to-well variability in the assay. Cell counting is routinely being done using a counting chamber. CASY Counting system and a Coulter Counter (Model ZM) were also successfully used by other laboratories in the interlaboratory study.
- At least four parallel plates are prepared for each batch of seven test chemicals: Three white 96 well plates (assay plates for Luciferase activity) and at least one transparent 96 well plate (cell viability assay plate for MTT assay); one plate is required by the protocol, two parallel plates can be used to further reduce data variability).
- After seeding the cells into the 96 wells plates, leave the plates in the sterile hood for 30 min in order that cells can attach evenly distributed. Else cells may settle at one side of the well due to medium movement when placing plates in the incubator.

NOTE: The procedure describes an individual experimental setup. Two repetitions of the full experiment are needed to derive a prediction if concordant results are obtained, while three repetitions are needed if the first two calls are not-concordant.

NOTE: FBS-free culture is possible using human serum instead of FBS. The following procedures should be followed if human serum is used:

Cells that have previously been cultured in FBS, should be weaned into human serum over at least 3 passages. Provided that the cells are showing healthy morphology and comparable growth rates with those in FBS, a cell bank should then be created for future use.
KeratinoSens cell line, when cultured in human serum, should be cultured up to a maximum passage number of 22 for optimal performance.
When a new batch of human serum is used, an internal validation of the serum batch including cell morphology, growth rates and I_{max} / EC1.5 values with at least the positive control, and preferably representative reference chemicals (at least one sensitiser and one non sensitiser) should be conducted, with subsequent reservation of successfully performing serum batches for long term use.

Test Material Exposure Procedures

- After seeding, the cells are grown for 24 h in the 96-wells microtiter plates in presence of 9.1 % FBS or human serum without G418.
- The medium is then removed by aspiration and replaced with 150 µl DMEM-medium containing 1% FBS but without G418.
- The 100 x DMSO master plate (prepared as described above) is replicated into a fresh plate (see "**Master plate with medium**" in **Annex 1**) (10 µl solution per well) and the DMSO solution is diluted 25-fold by adding 240 µl of DMEM-medium containing 1% FBS or human serum.
- For chemicals dissolved in cell culture medium, 10 µl per well of the stock solution, 10 µl per well of DMSO and 230 µl of DMEM-medium containing 1% FBS or human serum are mixed to adjust to the same DMSO level.
- This resulting 4 × master plate with medium is then distributed to the replicate assay plates: 50 µl each to three white assay plates for luciferase activity and 50 µl to one cell viability assay plate (see **Annex 1**).
- All the plates are then covered with a foil (Sealing tape SI, Nunc) to avoid evaporation of volatile compounds and to avoid cross-contamination between wells by volatile compounds.
- The plates are then incubated for an additional 48 hours in the CO_2 incubator.

Note: For chemicals not soluble in DMSO two options are available:

a. Dissolve test chemical at 200 mM in test medium. Prepare Master plate as done for a DMSO master plate. Then dilute into Master plate with medium as described above.
b. Dissolve test chemical directly at 8 mM in test medium containing 4 mM DMSO and directly dilute this solution in test medium with 4 % DMSO to obtain the Master plate with medium.

With all these options a Master plate with medium is obtained with 4 mM DMSO and maximal 8 mM test of chemicals. Other options (different volume for addition to the cells, different concentrations) are compatible with this test protocol and the guideline, as long as final level of DMSO in the exposure medium is 1%.

Endpoint Measurement

Luciferase Activity

- After the incubation time, the supernatant is aspirated from the white assay plates and discarded.
- The cells are washed once with DPBS.
- To each well, 20 µl of passive lysis buffer is added (at this stage, the formation of foam should be avoided by careful pipetting) and the cells are incubated for 20 min at RT (Note: Between processing of successive assay plates, the time should be equal or greater than the cycle time for the luminometer to read one plate in order to ensure constant lysis time for each plate).
- The plates with the cell lysate are then placed in the luminometer for reading: The luminometer is programmed to:

  i. add 50 µl of the luciferase substrate to each well,
  ii. to then wait for 1 second and
  iii. then to integrate the luciferase activity for 2 seconds. Thus the cycle time to read one plate is 10 min.

Alternative settings may be needed depending on the model of luminometer used.

MTT assay

- An MTT solution is prepared in DPBS (5mg/ml in), 2.7 ml of this solution is added to 20 ml cell culture medium containing 1% FBS (or human serum).
- For the cell viability assay plate, the medium is replaced with 200 µl of this fresh medium containing MTT.
- The plates are covered with a sealing tape and returned to the incubator.
- After 4 hours incubation, the medium is removed and 200 µl of a 10% SDS solution is added to each well.
- **Note:** Alternatively (for experiments finishing on Friday), the plates are frozen, thawed on the following Monday, SDS is added and plates are read on Tuesday.
- The plate is covered with a sealing tape and placed protected from light in the incubator. After overnight incubation to dissolve the cells, the plate is rocked on an orbital shaker for 10 min. Then, the absorption at 600nm is determined for each well. Alternatively (for experiments finishing on Friday), the plates are left in the incubator protected from light over the weekend and read on the following Monday.

Alternatively, after the MTT containing medium is removed, cells can be solubilised by the addition of 50µl isopropanol. After shaking for 30 minutes, the absorption is measured at 570 nm with a spectrophotometer.

Note: Alternatively, viability can also be assessed with the PrestoBlue assay, see **Annex 3** and the publication (Emter and Natsch, 2015).

Acceptance Criteria

A) Cinnamic aldehyde as positive control must be positive, thus the gene induction by this control must be statistically significant above the threshold of 1.5 in at least one dose.

B) The I_{max} and the EC 1.5 for cinnamic aldehyde is calculated. The targets are: (i) Average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8, and (ii) the EC 1.5 value should be between 7 µM and 30 µM. At least one of these criteria must be met, otherwise the run is discarded. If only one criteria is fulfilled, it is recommended to carefully check the dose-response of cinnamic aldehyde in order to decide on acceptability.

C) For acceptance of the test for a given master plate in a given repetition, the average variability in the 3 × 6 solvent control wells for each master plate/repetition should be below 20%. The variability is calculated as 100 × [standard deviation (18 DMSO wells) / average (18 DMSO wells)]. If the variability is higher, results are discarded.

NOTE: Potential outlier removal. There are 6 solvent control wells per well, 18 in total for the triplicate plates. The data for maximal one well per plate can be removed as outlier in case one well is > 25 % lower or higher than the average of the other 5 wells. This may occasionally happen for the well H1 at the corner of the plate.

These acceptance criteria are automatically calculated in the Summary sheet of the Excel file, and results should appear as shown in the example below:

	Criteria		Quality control: Variability blank	
EC 1.5	EC 1.5	Ind. 64 uM	% standard deviation blanks	
12.93	TRUE	TRUE	15.16659	ACCEPTED

The results for these controls are always reported along with the test results.

Data Analysis

For each set of seven chemicals, a copy of the standard file **'KeratinoSens_Evaluation-Sheet_April_09_2013_locked'** is made. Alternatively, the file 'KeratinoSens_Evaluation-Sheet_April_09_2013_formated Graphs' can be used. This file allows viewing of the calculations, and eventual outlier removal if needed'. In case adapted concentration ranges are used, the evaluation sheet 'KeratinoSens_Evaluation-Sheet_Oct_21_2014_different dilution series' can be used.

The fields which need to be filled in are marked yellow. On the 'Summary sheet', the compound identifiers and the plate identifier are inserted. On the sheet 'rep1', the plate readout of the triplicate analysis can directly be inserted in the yellow areas. The second and third repetitions are added to sheet 'rep2' and 'rep3'. The cytotoxicity results are pasted into the sheets 'Cytotoxicity (1) – (3)'.

This file then automatically calculates the gene induction and the wells with statistically significant induction over a given threshold (default value set to 1.5 = 50% enhanced gene activity). Furthermore the maximal induction (I_{max}) and the EC value (concentration for induction above threshold), both with linear and log-linear extrapolation, are calculated similar to the LLNA. The results from the different repetitions are then summarized in the 'Summary sheet'. This sheet also generates for each chemical a plot summarizing the gene induction and cytotoxicity dose-response in all repetitions.

The data are also automatically plotted in the graphs on the different repetition sheets. The automatically calculated I_{max} and the EC values should visually be checked with the help of this graphs, as uneven dose-response curves or large variation may lead to wrong extrapolations which may need to be corrected manually.

Note: Especially in the very rare cases with a statistically non-significant induction ≥ 1.5-fold which is followed by a higher concentration with a statistically significant induction, the automatically calculated value may in some cases be wrong. In such cases a warning (**'Check EC1.5!'**) appears in the summary sheet in the cells S15 – U21. Such a statistically non-significant induction may occur in cases with a very steep dose response, which may lead to differing fold-induction values between replicates which are not normally distributed, and thus the t-test may not be statistically significant even if all three replicates are clearly above the threshold of 1.5.

If a clear dose-response for induction is apparent from the plot, the four parameters needed for the extrapolation of EC1.5 values (concentration and fold-induction below the threshold of 1.5 as well as concentration and fold-induction above the threshold) may then be manually calculated for the respective chemical at the respective repetition. However, these runs are only considered as valid and positive if the fold induction at any (higher) concentration is statistically significant and above the threshold of 1.5 or if at the first concentration above the EC1.5 value the three replicate wells are all clearly above 1.5 (e.g. a case with three replicates giving 2-, 5- and 8- fold induction): this would not pass t-test but is a clear and reproducible, but not normally distributed induction, which may occur at a very steep dose response. This is rated as a positive outcome.

Manual calculation of EC1.5 values:

$$EC1.5 = (C_b - C_a) \times \left(\frac{1.5 - I_a}{I_b - I_a}\right) + C_a$$

Where:
C_a is the concentration above 1.5 fold induction
C_b is the concentration below 1.5 fold induction
I_a is the fold-induction above 1.5 fold induction
I_b is the fold-induction below 1.5 fold induction

In the (very rare) cases of biphasic dose-response curves which do cross the threshold of 1.5 twice, the EC1.5 value is also not correctly calculated. These cases are easily spotted by inspection of the dose-response-plot.

Note: The current prediction model rates any chemical with significant gene induction above 1.5 positive and thus likely to be a sensitizer. Other EC value can automatically be calculated by modifying the threshold in the 'summary sheet', thus EC2 and EC3 values can easily be calculated by just changing this single figure.

Note: For chemicals, which generate a 1.5-fold or higher induction already at the lowest test dose of 0.98 µM, the EC1.5 value cannot be calculated automatically, for these chemicals the EC1.5 value of <0.98 is manually set based on visual inspection of the dose-response curve.

Prediction Model

Chemicals are rated positive if the following conditions are met:

- The I_{max} is ≥ 1.5-fold of the basal luciferase activity and the EC1.5 value is below 1000 µM in all three repetitions or in at least 2 repetitions.
  If an EC1.5 value is calculated automatically in the summary sheet, this already indicates that the gene induction is statistically significant at the corresponding concentration according to a T-test.
- At the lowest concentration with a gene induction ≥ 1.5 fold (i.e. at the EC 1.5 determining value), the cellular viability is above 70%. If this is not the case, a warning ('cytotox') appears in the summary sheet, cells O15 – Q22.
- There is an apparent overall dose-response for luciferase induction, which is similar between the repetitions.

These parameters are automatically calculated and these automatic calculations are correct in the vast majority of the cases. Nevertheless, a careful inspection of the dose-response curves for both endpoints, both in the individual repetitions and in the summary file is recommended for quality control. In particular, uneven dose response curves can lead to wrong extrapolations in few cases, and these are detected by visual inspection.

Note: In rare cases, chemicals, which induce the gene activity very close to the cytotoxic levels, are positive in some repetitions at non-cytotoxic levels, and in other repetitions only at cytotoxic levels. Examples of such molecules are ethyl-hexyl-acrylate or hexyl-cinnamic aldehyde. Such molecules may be retested with an adapted concentration range and more narrow dose-response analysis with dilution of 1.3333-fold between wells instead of two-fold dilutions to decide if induction is at cytotoxic levels or not. An example of such an analysis is described in Emter at al. (2010) for SDS. To perform experiments with such adapted concentration ranges or to test chemicals with no defined molecular weight, an alternative evaluation sheet is available: 'KeratinoSens_Evaluation-Sheet_Oct_21_2014_different dilution series.'

Note: Also very rarely, a chemical may be extremely cytotoxic. Cells should remain > 70% viable at least at two consecutive test concentrations. If this is not the case, chemicals should be retested at lower concentrations than the standard dose-range (i.e. minimal concentration < 0.98 µM).

Compounds that only induce the gene activity at cytotoxic levels are not rated positive, as is the case for some non-sensitizing skin irritants.

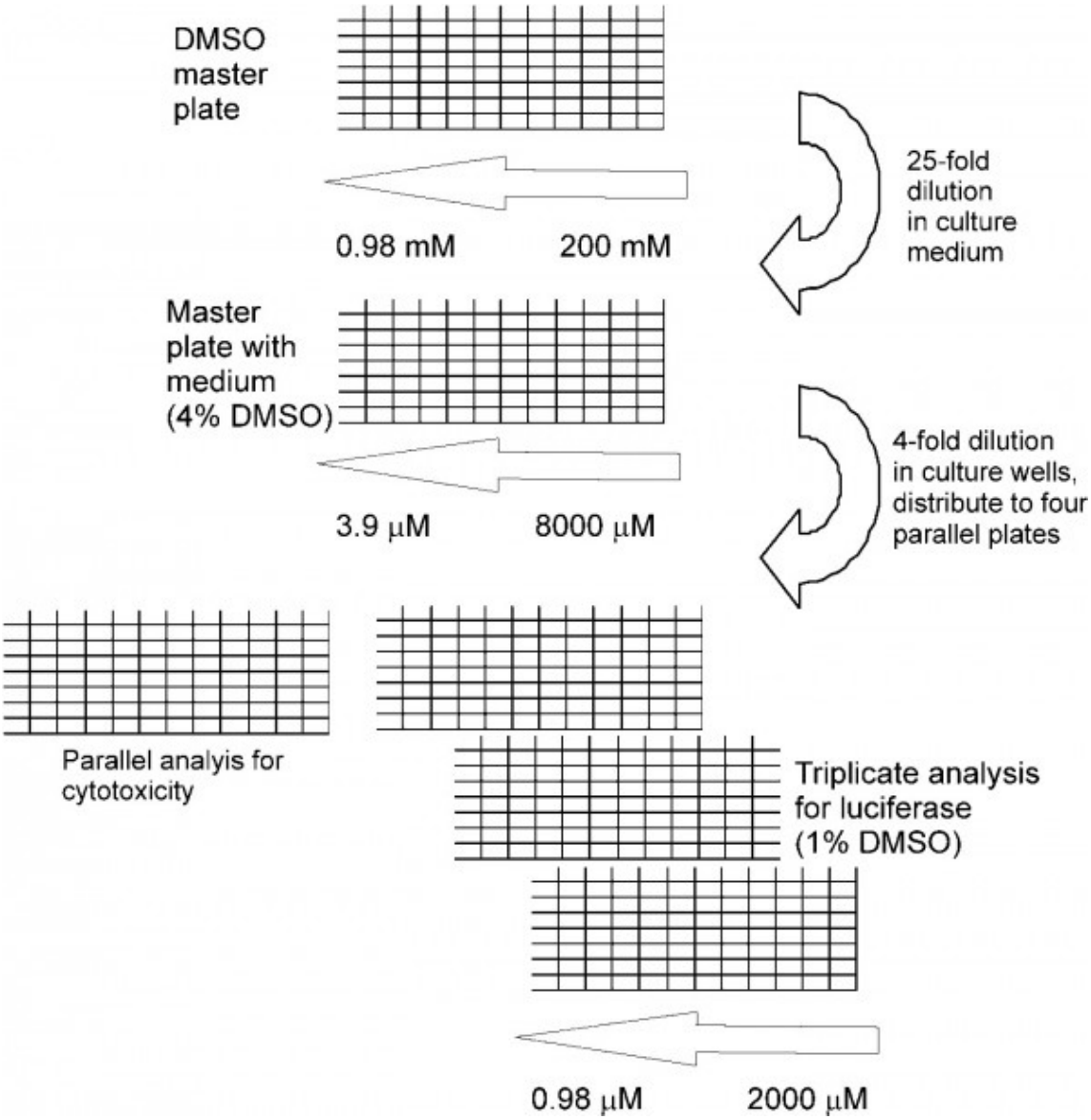
Prediction model for chemicals that are not soluble (or do not form a stable dispersion) at 1000µM:

a. If the test chemical induces luciferase at a lower, non-cytotoxic concentration, where it is still soluble, this positive result can be accepted to rate the chemical as **positive**.
b. If the test chemical does induce cytotoxicity (viability < 70%) at the maximal soluble concentration, but does not induce luciferase up to the maximal soluble concentration, this result can be accepted to rate a chemical as **negative**.
c. If a chemical does not lead to cytotoxicity or luciferase induction at a maximal tested soluble concentration, which is < 1000 µM, this chemical is rated as **inconclusive**.

Note on mixtures and multiconstituent substances:
When testing multi-constituent substances or mixtures, consideration should be given to possible interference of cytotoxic constituents with the observed responses (i.e. the presence of a high content of non-sensitising cytotoxic constituents may mask the response of weakly sensitising components or sensitising components present at low concentration). Thus, it might be scientifically justified to test either single main constituents or several fractions of the mixture to conclude on the sensitisation risk of the complex mixture.

Annexes

Annex 1. Experimental setup, preparation of the master plate and dilutions.



Annex 2. Basic experiment for transferability to ensure optimal luminescence measurements in the KeratinoSens assay

Three parameters are critical to facilitate reliable results:

a) Sufficient sensitivity giving a stable background in control wells

b) No gradient over the plate due to long reading times

c) No light contamination in adjacent wells from strongly active wells

As a first experiment for method transfer, the set-up of the plate below needs therefore to be tested (triplicate analysis according to the SOP).

An analysis then needs to be made to ensure:

a) Clear dose response in row D, with the I_{max} > 20-fold above background, in most cases I_{max} values between 100 and 300 are reached

b) No dose-response in row C and E (no induction value above **1.5** ; ideally not above 1.3) (-> i.e. **no light contamination** esp. next to strongly active wells in the EGDMA row)

c) No statistically significant difference between the rows A, B, C, E, F and G. (i.e. **no gradient** over plate)

d) Variability in any of the rows A, B, C, E, F and G and in the DMSO wells in row H below 20% (i.e. **stable background**)

EGDMA = Ethylene glycol dimethacrylate, CAS: 97-90-5, a strongly inducing compound

CA = Cinnamic aldehyde, positive reference, CAS: 14371-10-9

Plate setup of first training experiment

DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
EGDMA 0.98	EGDMA 1.95	EGDMA 3.9	EGDMA 7.8	EGDMA 15.6	EGDMA 31.25	EGDMA 62.5	EGDMA 125	EGDMA 250	EGDMA 500	EGDMA 1000	EGDMA 2000
DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	CA 4	CA 8	CA 16	CA 32	CA 64	Blank

Annex 3 Alternative viability assessment using the Presto blue® reagent

Note that this Protocol amendment does currently not form part of the protocol validated in the multilaboratory trial or the OECD guideline. However, technical equivalence and equivalent predictivity was shown by intralaboratory comparative data (Emter and Natsch, 2015). This is the preferred method in rapid screenings on multiple chemicals, but may not be considered equivalent when submitting data for regulatory purposes, as it was not included in the ring trial.

The KeratinoSens™ assay includes a cell viability assessment, which serves two purposes:
it forms part of the prediction model to exclude false-positive irritants and cytotoxicity provides some information on sensitiser potency of chemicals which can feed into a multivariate potency model.
In the standard protocol, Nrf2-dependent luciferase induction and the MTT-viability assay are performed in parallel plates. Resazurin-based viability assays do not require cell lysis and are compatible with luciferase measurements in the same cells.
The PrestoBlue® assay can therefore be run in the same assay plates prior to luciferase measurements. Thus, both endpoints then come from the same cells. Equivalent results in terms of predictivity can be achieved with this assay, but with an increased statistical power and lower workload.

In the standard validated KeratinoSens™ assay, triplicate plates are run in each repetition for luciferase induction, while at least one single parallel plate is used for the MTT assay (EURL ECVAM, 2014). Since these four plates are prepared from the same cell suspension and the solutions of test chemicals are prepared in one master plate and distributed to the four assay plates, homogeneous treatment of the four plates used for both endpoints is guaranteed, yet still results do not come from the same cells. With the MTT-assay, this parallel treatment is required since MTT-reduction needs incubation of the dye with living cells over several hours followed by solubilisation of the cells to measure the intracellular precipitate of the reduced dye. Resazurin-based assays on the other hand do not require cell-lysis and are known to be compatible with the luciferase assay as the resazurin dyes can penetrate viable cells, get reduced and fluorescent and can then be detected in the culture supernatant. Following a resazurin-based assay, cells remain viable and can be washed, subjected to cell-lysis and luciferase readings.
The PrestoBlue® assay is a recently developed modified Alamar-blue assay, which can be performed within only 30 min incubation. Based on this rapid response, it is an ideal test to be performed prior to luciferase readings. The short incubation time may ensure that the expression status of the cells / luciferase content is not affected. The sequential nature of this approach makes it easily amenable to automation and robotics and it is recommended when screening large number of chemicals.

Protocol: KeratinoSens™ assay with PrestoBlue®

- Cells were grown for 24 h in 96-well white plates with a transparent bottom.
- The medium is then replaced by fresh medium containing the test substance and a final level of 1% of the solvent DMSO. Each chemical was tested at 12 concentrations of a two-fold dilution series according to this general protocol. Cells are incubated for 48 h with the test substances.
  (→ Up to here no protocol change with exception that transparent plates are used)
- The medium is aspirated and 100 µl of PrestoBlue® reagent (Invitrogen, Zug, Switzerland) diluted 10-fold in DMEM without phenol red is added to each well.
- Plates are incubated for 30 min at 37°C and 5% CO2.
- The fluorescence at 560 excitation and 590 nm emission is determined.
- Cells are rinsed with 125 µl PBS.
- Cells are lysed with 20 µl Passive Lysis Buffer (Promega Duebendorf) at room temperature for 30 min according to the SOP.
- Finally, luciferase activity is read in a Promega Glomax luminometer with automatic injection of 50 µl of the luciferase substrate to each well and integration of the luciferase activity for 2 s.
- All chemicals are tested in two - three independent repetitions. In this modification, both the luciferase and PrestoBlue® endpoints are determined in triplicate in each repetition.
- The same evaluation sheet can be used to evaluate data (it contains space for the three replicate cytotoxicity plates). The fluorescence data can directly be pasted as done for the MTT data.

Annex 4. Promega licensing conditions for the Luciferase gene

BY USE OF THIS PRODUCT, RESEARCHER AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE STATEMENT

Researchers may use this product for research use and *in vitro* testing of chemicals to predict their skin sensitization potential only, no commercial use is allowed. Commercial Use means any and all uses of this product by a party for: (1) product manufacture; and (2) resale of the product for any use. Researchers shall have no right to modify or otherwise create variations of the nucleotide sequence of the luciferase gene. No other use or transfer of this product is authorized without the prior express written consent of Promega. In addition, Researchers must either: (1) use luminescent assay reagents purchased from Promega Corporation for all determinations of luminescence activity of this product; or (2) contact Promega to obtain a license for use of the product. With respect to any uses outside this label license, including any diagnostic, therapeutic or prophylactic uses, please contact Promega for supply and licensing information. NEITHER PROMEGA NOR GIVAUDAN MAKE ANY REPRESENTATIONS OR WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING FOR MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE WITH REGARDS TO THE PRODUCT. The terms of this agreement shall be governed under the laws of the State of Wisconsin, USA. The above license relates to Promega patents and/or patent applications on improvements to the luciferase gene.

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