ECVAM DATABASE SERVICE ON ALTERNATIVE METHODS TO ANIMAL EXPERIMENTATION

INVITTOX Protocol KeratinoSens

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A. Protocol Introduction

KeratinoSens

Induction of antioxidant-response-element dependent luciferase activity in the keratinocyte ARE-reporter cell line KeratinoSens to identify potential skin sensitizing chemicals

OBJECTIVES & APPLICATIONS

Type of Testing:
Screening method to detect electrophilic molecules with skin sensitizing potential providing detailed dose-response data. May be used as stand alone method to identify the majority of sensitizers. Ideally to be used as part of an integrated testing strategy.

Level of Toxicity Assessment:
Serves as a screening method to detect hazard if used as a global method on different chemical classes.
Quantitative data may be used for potency assessment within specific structural classes, esp. using a read across approach with comparison to related chemicals of known sensitization potential.

Purpose of Testing:
If used as stand-alone method, use may be restricted to classification and labelling, relevant to "REGULATION (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures".
If used as part of an integrated testing strategy and combined with read-across, the quantitative results generated should facilitate at least a basic risk assessment and safety prediction, e.g. definition of maximal use levels.

Context of Use:
Method intended to partially replace and reduce the number of tests needed of the currently used local lymph node assay (LLNA) for skin sensitization, the only validated test for classification of substances as skin sensitizers (R43).

Applicability Domain:
Technically, all chemicals soluble in either water or DMSO can be tested in the current protocol. Extremely hydrophobic molecules with a cLogP > 7 cannot be tested due to solubility issues in DMSO and water. Testing in these cases is sometimes possible at a lower maximal concentration. Compounds with a cLogP of up to 5 were successfully tested. No experience so far for molecules between cLogP 5 and 7, but these are rather rare chemicals.
The majority of skin sensitizers appears to be detected based on the current dataset, but few potential skin sensitizers with an exclusive chemical reactivity towards Lysine-residues turn out to be false-negatives. Hence, in an integrated testing strategy, the test should be combined with a reactivity assay which recognizes specific amine-reactive chemicals.
The test can recognize a variety of pro-haptens, but some phenolic pro-haptens presumably requiring an activation step by P450 enzymes are not detected with the test.

BASIS OF THE METHOD

The only feature all skin sensitizers have in common is their intrinsic electrophilicity or their potential to be metabolically transformed to electrophilic chemicals. The signaling pathway with the repressor protein Keap1(Kelch-like ECH-associated protein 1) and the transcription factor Nrf2 (nuclear factor (erythroid-derived 2)-like 2), which binds to the antioxidant / electrophile response element (ARE / EpRE), is known to respond to electrophilic chemicals and it was found to be a valuable cellular endpoint to detect skin sensitizers in vitro [1, 2]. This result was confirmed by independent laboratories [3, 4].

The sensor protein Keap1 contains highly reactive Cys residues. In un-induced conditions, Keap1 is bound to Nrf2, which targets Nrf2 for proteolytic degradation [5]. Covalent modification of crucial Cys residues by small molecules leads to dissociation of Keap1 from the transcriptional regulator Nrf2, which then activates genes (mainly genes coding for phase II detoxifying enzymes) having an antioxidant response element (ARE) in their promoter sequence [5, 6]. Measurement of the induction of this signaling pathway in a reporter cell line provides a high-throughput cell-based *in vitro* test to screen for the skin sensitization potential of novel chemicals. The *in vivo* relevance of this signalling pathway for contact allergy and in particular for the T_H1 response, has been established by Kim et al.[7]. The evidence for the up-regulation of Nrf2-regulated genes by skin sensitizers has recently been reviewed [8].

Many phase II genes contain an ARE-element in their promoter. One particular gene is AKR1C2 coding for an aldo-ketoreductase [9]. This particular gene was identified as one of the target genes up-regulated by contact sensitizers in dendritic cells [10, 11].

The cell line KeratinoSens is stably transfected with a modified vector pGL4.17 from Promega Inc A 56-base-pair genetic element containing the ARE sequence from the AKR1C2 gene (shown below) and the SV40 promotor were inserted upstream of the luciferase gene . The resulting vector was transfected into HaCaT keratinocytes and clones with a stable insertion were selected in the presence of Geneticin / G418. The selected clone 8 (termed KeratinoSens) was further propagated as a reporter cell line. Induction of luciferase is the read-out / endpoint evaluated to determine sensitization potential in this test. Luciferase induction directly indicates activation of a gene regulated by the AKR1C2-ARE element. Cytotoxicity is measured in parallel, and if the gene induction is only observed at cytotoxic concentrations, this is indicative of a false-positive gene-induction generated by a skin irritant.

ARE regulatory sequence from the AKR1C2 gene inserted into the novel reporter vector:

5'-TGGTCGCAAGGTGTGCAAGCTGCTGAGTCACCCTGACTGCATCAACCCCAGGAGCT

EXPERIMENTAL DESCRIPTION

Endpoint & Endpoint Detection:
Two endpoints are measured: (i) Luciferase induction after a 48 h treatment with test chemicals and (ii) cytotoxicity as determined with the MTT assay recorded in a parallel plate with the same cell batch and made up with the same dilutions of the test chemicals.

Endpoint Value:
For Luciferase induction the maximal fold-induction over solvent control (I_{max}) and the concentration needed to reach an 1.5 fold induction (EC1.5) are calculated. For cytotoxicity the IC50 value is extrapolated.

Test System(s):
The KeratinoSens cell line is derived from the human keratinocyte culture HaCaT. It contains a stable insertion of a Luciferase gene under the control of the ARE-element of the gene AKR1C2.

Basic Procedure:
Cells are grown for 24 h in 96-well plates. The medium is then replaced with medium containing a final level of 1% of the solvent DMSO containing the test chemical. Each compound is tested at 12 concentrations in the range from 0.98 to 2000 µM. Each test plate contains 7 test chemicals, 6 wells with the solvent control, 1 well with no cells for background value and 5 wells with a dose response of the positive control cinnamic aldehyde. In each repetition, three parallel replicate plates are run with this same set-up, and a forth parallel plate is prepared for cytotoxicity determination. For graphical illustration of the set-up see Annex 1.

DATA ANALYSIS/ PREDICTION MODEL

Chemicals are rated positive in the assay, if (i) the EC1.5 value (concentration for 1.5 fold, statistically significant gene induction) is below 1000 µM, and if (ii) the cellular viability at the EC1.5 determining concentration (i.e. the lowest measured concentration with a gene induction > 1.5) is at > 70%. Compounds that only induce the gene activity at cytotoxic levels are not rated positive, as this is the case for some non-sensitizing skin irritants. With this prediction model an accuracy of 85.1% for a list of 67 chemicals was found, if the test was used as stand alone method. The accuracy was raised to 89% if the test was combined with a peptide reactivity assay.
At the current stage, no global assessment of the use for potency assessment has been made, as for potency assessment (i) either very specific classes of chemicals should be compared to each other with this test or (ii) the quantitative data generated with the test should be used as part of an ITS.

TEST COMPOUNDS & RESULTS SUMMARY

The test was initially used to screen a library of 67 reference compounds. This set of chemicals included (i) the skin sensitizers from the Sens-it-iv list, (ii) the ECVAM/COLIPA list published by Casati *et al.* and (iii) the ICCVAM list of performance standards for alternative endpoints in the LLNA and (iv) further chemicals selected from the ICCVAM database.
Table 1 gives the structural classes tested, and the performance of the assay for these classes:

Table 1. Chemical classes in the 'Silver list' used to evaluate the predictive capacity

Sensitizers	**Number of compounds tested**	**Number of correct predictions**
Aldehydes	6	6
Amines	4	3, 1 borderline
Aromatic Esters	2	1
Epoxides	1	1
Haloaromatic Compounds	3	3
Metals	2	2
Michael Acceptors	9	9
Michael Acceptor Aldehydes	3	3
Miscellaneous	5	5
Peroxides	1	0
Phenols	4	2
Thiols	3	2
Non-Sensitizers	**Number of compounds tested**	**Number of correct predictions**
Alcohols	5	5
Aldehydes	1	0
Aromatic Esters [(2)]	4	2
Haloaromatic Compounds	1	1
Miscellaneous	1	1
Organic Acids	7	7
Phenols	1	1
Polysacharides	1	1
Surfactants	2	1

(1) includes propyl paraben, which is a reported human sensitizer

MODIFICATIONS OF THE METHOD

The original SOP was developed and defined based on a series of range finder experiments[1]. This SOP was not further modified, and no modifications appear necessary for the time being. The same SOP as used for the published screening was used in the ring study.

Only two modifications to the prediction model were made based on further screening results:

a) The inclusion of the condition that chemicals are only rated positive if luciferase induction occurs at non-cytotoxic concentrations. This avoids false-positive results for some non-sensitizing irritants

b) Some non-sensitizers occasionally yield false-positive results in some repetitions, if 1.5-fold luciferase induction at a concentration of 2000 µM is rated as positive. Thus, chemicals are rated positive only if the EC1.5 is below 1000 µM in the improved prediction model.

Finally, based on the experience in the ring study, it can be concluded that runs can be accepted if they fulfil one of the two the quantitative acceptance criteria in relation to the positive control cinnamic aldehyde.

DISCUSSION

- Ethical issues: The test is based on a human cell line established 23 years ago. Thus neither human nor animal tissues were required to set-up the test methodology. Fetal calf serum is used in the test, but the level is reduced to 1% in the test plates, thus less then 2 ml of FCS is used to test one compound in the full dose-response and in three repetitions and in triplicate.
- Special equipment: A 96 well luminometer is needed, ideally equipped with an injector to add the luminescene substrate to individual wells immediately before reading the well. The preferred device (GloMaxTM 96 Microplate Luminometer, from Promega) is currently available for less than 12'000 Euro, and thus cost of entry for this technology is low. Some other luminometers can also be used, and four different models have been used in the ring study. Identical results are obtained with Tecan instruments (i.e. the second major supplier of luminometers)
- In principle no hands-on training is needed: All four labs participating in the ring study were able to perform the test just based on the SOP. No visits to the lead lab were organized prior to the ring study. Nevertheless, a short training may accelerate the method transfer in further validation studies. The key limitation for rapid transfer is the availability of a sufficiently sensitive luminometer, paired with the correct test plates fitting the particular device and the correct substrate for the measurement. Based on experience from the ring-trial, a basic initial experiment is now designed to ensure that all these parameters are fulfilled right from the start. This is included as Annex II.
- The protocol can easily be adapted to high throughput testing. A trained experimenter can run (without the use of robotics) at least 42 compounds in one week in triplicate, and thus needs three weeks for the final results in three repetitions on 42 compounds (full dose response at 12 concentrations). By using robotics, the throughput might be significantly enhanced. As the method is based on a very standardized cell biology setup and uses an adherent cell line grown in 96-well plates, it should be possible to run the test on current laboratory robots.
- Cost: To test 1 compound in three repetitions each with three replicates at 12 concentration, the media and FCS cost are: 2.4 €, the luciferase substrate costs are: 72 €, the costs for the Lysis reagent are: 2.5 € the costs for the test plates are: 6.5 €, the costs for other disposables (pipettes, tissue culture dishes, MTT, PBS, DMSO, test tubes) are estimated to be around: 10 €. Thus the cost per compound excluding labor and fixed equipment is: 93.5 €
- Advantages: The reproducibility is very high, the well to well variation of the signal in solvent control wells is very low (around 15%), the test is amendable to high throughput screening, the read-out is simple and does not require many experimental steps (after the washing and lysis step the plates can directly be placed in the luminometer), and thus is much simpler as if the same readout was to be obtained with e.g. RT-PCR. The method is also much cheaper as compared to RT-PCR.
- Limitations: Different substance classes give a different dynamic range, thus the test has a broad applicability domain for the yes/no answer, but limitations in its capacity to predict potency using a global prediction model. The limitations in the applicability domain have been discussed above.

STATUS

In Development:
The protocol in its current form does not require any further development and has been tested in an extended ring study.
We currently conduct some basic research to evaluate whether in the future additional endpoints may be included, but based on the current knowledge it is sufficient to evaluate the two endpoints cytotoxicity and luciferase induction.

Known Laboratory Use:
The assay has been used in the ring study by four different external laboratories. All these laboratories have a licence to further use the assay for their own internal research, but no results of such additional research has been reported yet.
The method is used routinely in the Givaudan laboratory to screen novel chemicals.

Participation in Evaluation Studies:
A detailed evaluation study was conducted in four external laboratories plus our own laboratory. The study involved two stages:

a) Method transfer stage: Testing of seven chemicals. Participants knew the chemicals, but only knew historical data for three of the chemicals

b) Further evaluation of predictive capacity: Testing of 21 blind coded chemicals.

The chemical selection included all chemicals from the ECVAM/COLIPA list published by Casati *et al.* and the ICCVAM list of performance standards for alternative endpoints in the LLNA. All experiments were run with three repetitions.

The inter-laboratory variability was only slightly higher as compared to the intra-laboratory variability. The details of these results are summarized in separate documents (Attachments 8a, 8b, 10a, 10b, 10c, 10d and 12b to the TST). A summary of the predictive capacity for the 28 chemicals tested is given below in Table 2.

Table 2. The rating of 28 chemicals in five laboratories and Cooper statistics
The data give the number of positive repetitions (significant gene induction above 1.5-fold at a concentration below 1000 µM) of the total number of repetitions done. In red and orange chemicals rated positive, in green chemicals rated negative.

		Positive with EC 1.5 up to 1000 uM					
	Study phase	GIVhist	GIV	Lab1	Lab2	Lab3	Lab4
Sensitizers							
Hexyl cinnamic aldehyde	MT	2 of 2	1 of 3	0 of 3	1 of 3	3 of 3	3 of 3
Citral	MT	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Ethylene glycol dimethacrylate	MT	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
2,4-Dinitrochlorobenzene	MT	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
4-Methylaminophenol sulphate (M	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
(5-chloro)-Methylisothiazolinone	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Phenyl benzoate	BC	1 of 4	0 of 3	0 of 3	0 of 3	0 of 3	1 of 3
Imidazolidinyl urea	BC	3 of 4	2 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Oxazolone	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
4-Phenylenediamine	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Cinnamic aldehyde	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Isoeugenol	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
tetramethylthiuramdisulfide	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
2-Mercaptobenzothiazole	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Eugenol	BC	0 of 4	1 of 3	1 of 3	3 of 3	3 of 3	1 of 3
Cinnamyl alcohol	BC	4 of 4	3 of 3	2 of 3	3 of 3	3 of 3	3 of 3
Glyoxal	BC	4 of 4	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
4-nitrobenzylbromide	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Methyldibromo glutaronitrile	BC	2 of 2	3 of 3	3 of 3	3 of 3	3 of 3	3 of 3
Non-sensitizers							
Isopropanol	BC	0 of 2	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3
Methyl salicylate	MT	0 of 2	0 of 3	1 of 3	0 of 3	1 of 3	1 of 3
Chlorobenzene	MT	0 of 2	0 of 3	0 of 3	0 of 3	0 of 3	1 of 3
Sulfanilamide	MT	0 of 2	0 of 3	0 of 3	0 of 3	1 of 3	0 of 3
Salicylic acid	BC	0 of 2	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3
Sodium lauryl sulfate	BC	**0 of 2**	**3 at cytotc**	**1 at cytotc**	**1 at cytotc**	**1 at cytotc**	3 of 3
Lactic acid	BC	1 of 4	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3
Glycerol	BC	0 of 4	0 of 3	0 of 3	0 of 3	0 of 3	0 of 3
Diethyl phthalate	BC	0 of 2	0 of 3	0 of 3	1 of 3	0 of 3	2 of 3
correct positive		17	16	16	17	18	17
correct negative		9	9	9	9	9	7
false positive		0	0	0	0	0	2
false negative		2	3	3	2	1	2
n		28	28	28	28	28	28
Sensitivity		89.5	84.2	84.2	89.5	94.7	89.5
Specificity		100.0	100.0	100.0	100.0	100.0	77.8
Accuracy		92.9	89.3	89.3	92.9	96.4	85.7

(*) Note: induction at cytotox concentrations for SDS was not considered positive in Cooper stats
If 2 of 3 reps are positive and overall dose response is given in all reps, compound is considered positive
If only one rep is positive and dose response is not evident compound is considered negative

MT: Method transfer / phase I data (7 chemicals)
BC: Blind study / phase II data (21 chemicals)

NB: Where possible please complete all fields using non-numerical bullet-points and format lists in chronological or alphabetical order as appropriate.

Participation in Validation Studies:
No formal validation study has been conducted so far, yet the parameters of the evaluation study reported above may fulfil the key criteria of pre-validation studies

- number of test chemicals (28)
- number of repetitions (3)
- number of labs (5)
- the use of blind coded chemicals

Regulatory Acceptance:
Not applicable

PROPRIETARY &/OR CONFIDENTIALITY ISSUES

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 with the proviso that the substrate used for the assay is purchased from Promega (see Annex 3). If the assay is used to offer commercial service, a licence fee needs to be paid to Promega, but this is not prohibitive as it is the current business model of Promega.
A patent from the year 2001 (EP1130086 A1) on the general use of reporter cell lines derived from HaCaT for the use of toxicity screening has been abandoned, and thus does not pose any limitations.
Givaudan has decided to follow a no-patent strategy for new developments in alternative assays, and thus has not filed any patent applications on either KeratinoSens nor on the general principle of using Nrf2-regulated genes as screening targets in order to not hamper validation and regulatory acceptance.
Givaudan will share the recombinant cell line KeratinoSens with third laboratories under a material transfer agreement. No licence fee will be reimbursed for research and validation studies. A licence fee may be asked for commercial testing.

ABBREVIATIONS & DEFINITIONS

ARE :	antioxidant response element
Keap1 :	Kelch-like ECH-associated protein 1
Nrf2 :	nuclear factor (erythroid-derived 2)-like 2
AKR1C2:	Aldo-keto reductase family 1, member C2
DMSO:	Dimethyl sulfoxide
FCS:	Foetal calf serum
PBS:	Phosphate buffered saline
MTT :	Thiazolyl Blue Tetrazolium bromide
EC 1.5:	Extrapolated concentration for a 1.5 fold of luciferase induction
I_{max}:	Maximal induction of luciferase activity over solvent control over the complete dose-response range measured
EC50:	Concentration for reduction of cellular viability by 50% as determined with the MTT assay

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B. Technical Description

Procedure Details, **Latest Version:** SOP_KeratinoSens Version 1.3.

KeratinoSens assay

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HEALTH & SAFETY ISSUES

The reagents used do not require specific safety measure. The laboratory needs to follow good cell culture practice. The key health issue comes from the testing of potential skin sensitizers, and thus any contact of the test compounds with the skin should be avoided.

MATERIALS AND PREPARATIONS

CELL /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 passage number of 25.

EQUIPMENT

Fixed Equipment:

- Sterile hood for cell culture work
- CO_2 incubator
- 8 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), preferred model is the GloMaxTM 96 Microplate Luminometer (Promega)

- Other models which have been used successfully:
  - 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

Consumables, 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.

- For the ring study each laboratory used their own FCS 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 3). 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 and (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.

	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 calf serum Origin: South America An alternative source of the serum can be used with the standard supplier for each Laboratory	AMIMED	2-01F10-I

	Product	Company	Catalog Number
Phosphate buffered saline	DPBS	Gibco	14190
Trypsin	0.05% Trypsin-EDTA	Gibco	25300
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. 104-55-2, > 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

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 FCS (final FCS concentration: 9.1 %) and 5.5 ml Geneticin Gibco (final concentration 500 µg/ml). The medium is stored at 4°C and used within 28 days.

Medium for freezing the cells
D-MEM containing 20% FCS and 10% DMSO.

Medium for exposure to chemicals
Supplementing 495 ml D-MEM with 5 ml FCS (final FCS concentration:1%) No Geneticin is added. The medium is stored at 4°C and used within 28 days.

EDTA solution 10%, pH 8
10 g EDTA is dissolved in 100 ml H2O and NaOH is added to bring the solution to pH8, sterilized by filtration.

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 sterile water and the solutions are sterilized by filtration through a 0.2µM filter.
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 %.

Positive Control Solution(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 Solution(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 these DMSO solutions 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 are made in water.

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.

METHOD

TEST SYSTEM PROCUREMENT:

Stocks of the cells can be prepared by the test lab based on the culture received from Givaudan.

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% FCS without Geneticin. Cells are plated in a 10 cm tissue culture dish. Geneticin-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 % fetal calf serum and 500 µg/ml Geneticin 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. After cells have detached (usually after 5 – 10 min), they are resuspended in 10 medium and split at a ratio of 1: 4 – 1: 16 in fresh medium and grown to 80-90% confluency. With a split ratio of 1:4, cells need 2 days to reach confluency again, in a ratio of 1:8, cells need 3 days (normally done for the weekend) and in a ratio of 1:16 4 -5 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% FCS 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 1:8 or 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 1:8 / 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% FCS without G-418 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.
- Four parallel plates are prepared for each batch of seven test chemicals: Three white 96 well plates and one transparent 96 well plate.

TEST MATERIAL EXPOSURE PROCEDURES:

- After seeding, the cells are grown for 24 h in the 96-wells microtiter plates in presence of 9.1 % FCS without G-418 prior to compound addition.
- The medium is then removed by aspiration and replaced with 150 µl DMEM-medium containing 1% FCS but without Geneticin.
- The 100 × DMSO master plate (prepared as described above) is replicated into a fresh plate (10 µl solution per well) and the DMSO solution is diluted 25-fold by adding 240 µl of DMEM-medium containing 1% FCS.
- For chemicals dissolved in water, 10 µl per well of the stock solution, 10 µl per well of DMSO and 230 µl of DMEM-medium containing 1% FCS 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 and 50 µl to one cytotoxicity plate (see Annex I).
- 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.

ENDPOINT MEASUREMENT(S):

- 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 setting may be needed depending on the model of luminometer used.

- For the cell viability assay plate, the medium is replaced with 200 µl fresh medium containing 1% FCS.
- 27 µl of a MTT solution (5mg/ml in DPBS) is then directly added to each well of the transparent 96-well plate.
- 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.
- The plate is covered with a sealing tape and placed protected from light in the incubator. After overnight incubation to dissolve the cells, 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.

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 7.5 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%. If the variability is higher results are discarded.

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

	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 'Attachment1b_SOP_calculation.xls' is made. 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 above 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 entered in Row 44 – 50 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.

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:

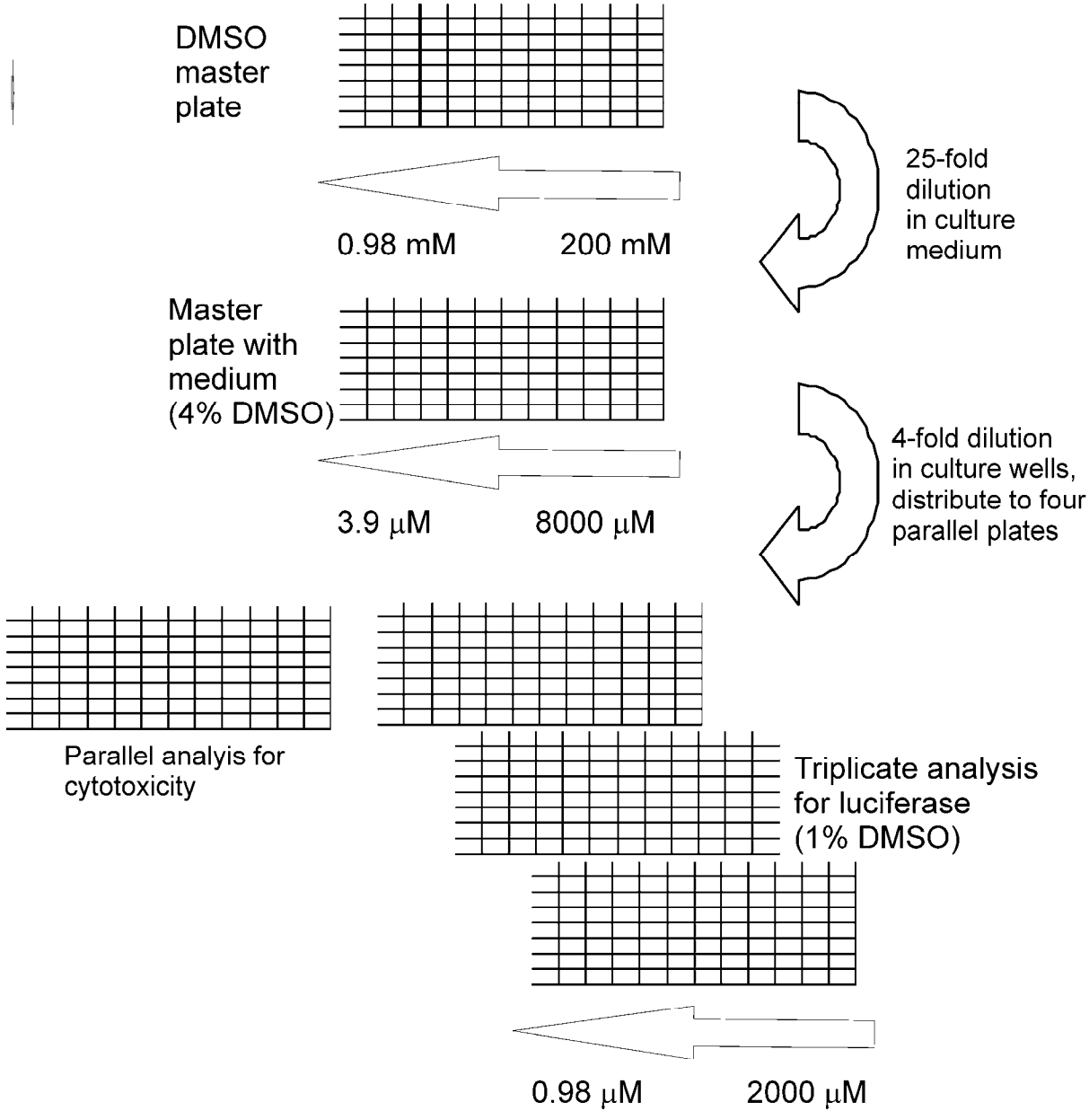
- The I_{max} is > 1.5-fold gene induction and the EC1.5 value is below 1000 µM in all three repetitions or in at least 2 repetitions.
  If an EC1.5 value is calculated automatically in the summary sheet, this already indicates that the gene induction is statistically significant at the corresponding concentration according to a T-test.
- If the I_{max} is exactly equal to 1.5, the chemical is still rated negative and no EC1.5 value is calculated by the evaluation sheet.
- At the lowest concentration with a gene induction above 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 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.

ANNEXES

ANNEX 1. EXPERIMENTAL SETUP, PREPARATION OF THE MASTER PLATE AND DILUTIONS.



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NB: Where possible please complete all fields using non-numerical bullet-points and format lists in chronological or alphabetical order as appropriate.

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 1.3) (-> 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 = Ethyleneglycoldimethacrylate, CAS 97-90-5, a strongly inducing compound

CA = Cinnamic aldehyde, positive reference, CAS 104-55-2

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 PROMEGA LICENCING CONDICTIONS FOR THE LUCIFERASE GENE

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