DB-ALM Protocol n° 184: LuSens Assay

Skin Sensitisation & Allergic Contact Dermatitis

The LuSens assay uses a luciferase reporter cell line (LuSens cells) based on the activation of the antioxidant response element that can be used to assess the intracellular cysteine reactivity of a substance.

Résumé

Rationale

The LuSens assay is an *in vitro* method for the identification of keratinocyte activating substances using a genetically modified keratinocyte cell-line (LuSens, Ramirez et al., 2014; Bauch et al. 2012). It employs a luciferase reporter gene under the control of the antioxidant response element (ARE) and hence monitors Nrf-2 transcription factor activity. The measured endpoint is the up-regulation of luciferase activity after 48 h of incubation with test substances. This up-regulation is an indicator for the activation of the Keap1/Nrf2/ARE signaling pathway (Ade et al. 2009; Natsch 2012; Natsch and Emter 2008; Vandebriel et al. 2010).
In order to conclude on the keratinocyte activating potential of a test substance, a LuSens experiment comprising at least two, but a maximum of three independent valid repetitions* needs to be carried out. In a valid repetition (i.e. meeting all acceptance criteria), sensitizing potential of the substance is indicated if the luciferase activity equals or exceeds a 1.5 fold induction compared to the vehicle control in 2 (or more than) consecutive non-cytotoxic tested concentrations (viability equal or above 70%), whereby at least three tested concentrations must be non-cytotoxic. The third repetition is only required when the first two repetitions are not concordant (i.e. one repetition is positive and the other is negative, not indicating sensitizing potential). If the first two repetitions of an experiment are either positive or negative, the experiment is completed.

Applicability Domain

As the LuSens assay addresses one key event in the sensitization process, primarily reactivity with cysteine residues of a protein, but subsequently also the activation of the ARE dependent gene expression in keratinocytes, other modes of action serving the key event 2 of the Adverse Outcome Pathway (AOP) of skin sensitization may be missed (OECD, 2012). It is a cell based assay and as such requires the cells to be in an aqueous medium, in principle all water or DMSO soluble test substances can be tested in the LuSens assay. However, the need of an aqueous medium can pose solubility problems for lipophilic substances particularly at higher concentrations. The substances can be solubilized using up to 1% DMSO as a solvent, a concentration not inducing toxic effects in the cells. As a luminescence based assay, any substance that can influence the luciferase protein or quench/increase luminescence may interfere with the assay. However, it is expected that only few substances would do so.

The cells have a relatively low metabolic capacity and correct assessments of pre- and pro-haptens is limited (Fabian et al., 2013), however, several pre-haptens led to positive results in the LuSens assay (i.e. eugenol, isoeugenol, etc.) (Ramirez et al., 2014). Care needs to be taken when handling highly volatile chemicals as they can otherwise contaminate the controls or other substances via the vapor phase or the concentration actually applied can be reduced due to evaporation. Chemicals interacting with MTT may also pose problems, however, this would need to be confirmed (i.e. pigments). To date only some plant extracts and no dyes have been tested and the effect of these types of materials is not known. The method does not (yet) allow the determination of the potency of a sensitizer; hence, predictions are primarily limited to hazard identification, i.e. absence or presence of skin sensitisation potential.

As described by Urbisch and coworkers (Urbisch et al., 2015), the ARE-activation (and thus sensitization) potential of acylating agents is poorly recognized by LuSens, similarly to KeratinoSens™ (Further information regarding KeratinoSens™ can be found in the **DB-ALM dataset**). This effect is most likely related to the fact that the activation of Keap 1 sensor protein is related to cystein reactivity and acylating agents transfer their acyl moiety predominantly to lysine residues (Emter et al., 2013; Aptula et al., 2005). It is expected that ARE-activating assays do not accurately predict the effect of such agents. However, if the LuSens assay is used in a defined approach consisting of multiple tests; some of its limitations may be compensated and correct predictions of the sensitizing potential are still achievable.

* *repetition and test are synonyms in the LuSens protocol*

Experimental Description

Endpoint and Endpoint Measurement

Luciferase activity is an indicator of the activation of a crucial pathway Keap1/Nrf2/ARE that it has been demonstrated in this assay to be essential for the response to skin sensitizers (Natsch and Emter, 2008).

Up-regulation of the luciferase activity after 48 h treatment. This up-regulation is triggered by the activation of ARE promoter sequence genetically introduced into keratinocytes. The activation of ARE indicates the activation of the Keap1/Nrf2/ARE signaling pathway.

Viability measured by MTT.

Experimental/Test System

Transgenic keratinocyte reporter cell line which expresses luciferase under the regulation of the rat Antioxidant Response Element (ARE). The ARE promoter belongs to the NADPH:quinone oxidoreductase 1 gene from rat.

Basic Procedure

The LuSens assay, described below, consists of two phases: a **cytotoxicity range finder experiment** and the **main experiment** . The cytotoxicity range finder is used to select the adequate concentration range that will be tested in the main experiment. In the main experiment is evaluated the expression of luciferase induced by the test item at non-cytotoxic concentrations.

a. Cytotoxicity range finder experiment

Cells are suspended in 9 mL of assay media (DMEM with 10% FBS Superior, Biochrom) per T75 flask and subsequently quantified with a Casy cell counting system (Roche, Germany). For analysis of cell viability, cells are seeded into clear flat bottom 96 well plates (TPP, Switzerland; $1x10^4$ in 120 µL per well).

Test substances are dissolved in DMSO in a series of 1:2 dilutions starting at 200 mM (100x stock solution). Substances are further diluted (1:25) in medium to obtain 4x stock solution. Final DMSO concentrations in the assay do not exceed 1%. Treatment is performed by applying 50 µL of the test substance to each well (final volume: 200 µL) for 48 h. Each substance is tested at twelve concentrations in triplicate.

Assessment of cell viability is performed using the MTT assay as mentioned above. From the range finding experiments, the concentration in which cell viability corresponds to no less than 75% (CV75) is calculated. The highest tested concentration in the main experiment is then 1.2x CV75 or an 1.2-fold of 1.2x CV75 (e.g. 1.2^2x CV75, 1.2^3x CV75 etc.). In cases where no cytotoxicity is observed 2000 µM (or 2000 µg/mL when no molecular weight is available) is the maximum concentration assessed in the main experiment.

b. Main experiment for luciferase expression and cell viability

For analysis of luciferase expression, cells are seeded into white flat bottom 96 well plates (Perkin Elmer; $1x10^4$ in 120 µL per well). Test substances are dissolved in DMSO (100x stock solution) at concentrations according to the preliminary cytotoxicity data. Substances are further diluted (1:25) in medium to obtain 4x stock solution. Final DMSO concentration on the cells does not exceed 1%. The highest tested concentration is 1.2x CV75 or an 1.2-fold of 1.2x CV75 (e.g. 1.2^2x CV75, 1.2^3x CV75 etc.). In cases where no cytotoxicity is observed 2000 µM (or 2000 µg/mL when no molecular weight is available) is the maximum concentration assessed in the main experiment.

Treatment is performed by applying 50 µL of the test substance dilution to each well (final volume: 200 µL) for 48 h. Each substance is tested in at least six concentrations in triplicate. If the classification in both tests differs, a third test is conducted. After treatment, cell culture media is removed and cells are washed twice with 300 µL PBS (with Ca^{2+}/Mg^{2+}). After washing, 200 µL of Steady-Glo® working solution (one part of PBS(Ca^{2+}/Mg^{2+} free) and one part of Steady-Glo®Mix reagent (Promega, Germany)) are added to each well. Plates are gently shaken in the dark for at least 5 min and luminescence then measured using a luminometer (e.g. Perkin Elmer “Victor 3” 1420 Multilabel counter).

For analysis of cell viability, cells are seeded in clear flat bottom 96 well plates (TPP, Switzerland; 1x10 ^{4} in 120 µL per well). Test substances are dissolved in DMSO (100x stock solution). Substances are further diluted (1:25) in medium to obtain 4x stock solution. Final DMSO concentration on the cells does not exceed 1%. Treatment is performed by applying 50 µL of the test substance dilution to each well (final volume: 200 µL) for 48 h. Each substance is tested in six concentrations (each concentration in triplicate). In addition, the assay is performed in at least 2 independent experiments.

Concentrations are chosen according to preliminary MTT cytotoxicity assays. Assessment of viable cells is also performed by MTT cytotoxicity assay. In parallel to the test substances, a positive control (EGDMA, 120 µM or a different concentration inducing luciferase expression above 2.5 fold) is also tested in all cases and in most cases a negative control is also included (DL- Lactic acid (LA), 5000 µM).

Discussion

The LuSens assay has been tested systematically, by using the procedure described hereinafter, with a set of 74 test substances with and without sensitizer potential in humans and in the local lymph node assay (LLNA) (the list of test compounds can be found in **Annex 1**). It is a robust assay for identification of skin sensitizers, presenting a reproducibility over 90% and a predictivity of 83% when compared to human data (Ramirez et al., 2014). When comparing to the KeratinoSens™ assay, for a set of 69 test substances that have been tested in both assays, it was calculated an overall interchangeability of 88% (Urbisch et al., 2015). Most importantly, the integration of LuSens in the “2 out or 3 approach” including the direct peptide reactivity assay (DPRA) and the human cell line activation test resulted in similar accuracies towards prediction of sensitization when compared to LLNA or human data (Bauch et al.; 2012, Ramirez et al., 2014 and Urbisch et al., 2015, see also **Annex 5**).

Status

In Development

The development of this assay has been completed.

Known Laboratory Use

As part of a defined approaches for identification of skin sensitizers. Please see **Annex 5**: The LuSens Assay as a suitable and valid replacement of the KeratinoSens™ assay in the “2 out of 3” Approach.

Participation in Validation Studies

BASF has organized and completed an inter-laboratory validation study in which the method was transferred to 4 laboratories (see below) and the predictive capacity was also evaluated in each laboratory (Ramirez et al., 2016).

a. Burleson Research Technologies Inc, 120 First Flight Lane, Morrisville, NC, USA.
b. DSM Nutritional Products Ltd, Wurmisweg 576, CH-4303 Kaiseraugst, Switzerland.
c. Institute for In Vitro Sciences, 30 West Watkins Mill Road, Suite 100, MD 20878, USA.
d. The Procter & Gamble Company, 8700 Mason Montgomery Road, Mason, OH 45040, USA.

An independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) regarding the BASF-coordinated Performance Standards-based validation of the LuSens method for skin sensitisation testing has been published in July 2016 (ESAC, 2016).

Regulatory Acceptance

The test method was adopted as **OECD Test Guideline 442D** in June 2018. An updated version of the TG has been issued (OECD, 2018).

Proprietary and/or Confidentiality Issues

Not applicable

Health and Safety Issues

General Precautions

The use of LuSens cells does not represent any kind of risk for human health or the environment; they are to be used in a BS1 laboratory.

MSDS Information

Please refer to MSDS of substances used in the assay.

Abbreviations and Definitions

AOP: Adverse Outcome Pathway

ARE: antioxidant response element

CV75: concentration leading to viability of 75% compared to solvent control

Cys: cysteine

DMSO: Dimethylsulfoxide

DPRA: direct peptide reactivity assay

EGDMA: ethylene glycol dimethacrylate

FCS: Fetal calf serum

FN: false negative

FP: false positive

Keap1: Kelch like ECH-associated protein 1

LA: DL-Lactic acid

LLNA: Local lymph node assay

MTT: Methyltetrazolium bromide

µL: microliter

µM: micromolar

Nrf2: Nuclear factor erythroid 2-related factor 2

PBS: phosphate buffered saline

RN: right negative

RP: right positive

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LuSens Assay
DB-ALM Protocol n° 184

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Materials and Preparations

TEST SYSTEM

Keratinocyte cell line genetically modified to express luciferase under the expression of ARE of the NADPH:quinone oxidoreductase 1 gene. The cells can be obtained from acCELLerate GmbH (Germany, **https://www.accellerate.me/product/instacell-skin-sensitization-assay-kits.html**).

EQUIPMENT

Fixed Equipment

Sterile cell culture bank (Herasafe KS 18, Thermo Electron Corporation), incubator for cell culture (Heraeus, 37°C), water bath (Julabo, 37°C), centrifuge for plates, 50 mL and 10 mL tubes (TPP), luminometer (i.e. GloMax, Promega) and CASY cell counter (OMNI Life Science (OLS)).

Consumables

Substance/ Solution	Company	Catalog No.
D-MEM	PAN	P04-04510
FBS Superior (or equivalent)	Biochrom	S 0613/5
Penicillin/Streptomycin	Biochrom	A 2213
Puromycin dihydrochloride	Sigma	P9620-10mL
SDS	Sigma	L3771-100G
Dimethyl sulfoxide (DMSO)	Fisher Chemical	D/4121/PB15
Acetic acid 100%	Merck	1.00063.1011
Thiazolyl Blue Tetrazolium Bromide (MTT)	Sigma	M5655-1G
PBS (without Ca2+/Mg2+)	PAN	P04-36500
PBS (with Ca2+/Mg2+)	PAN	P04-35500
Steady-Glo Luciferase Assay System Alternative: ONE-Glo Luciferase Assay System	Promega Promega	E2520 E6120
Trypsin/EDTA	Biochrom	L2143
Ethylendiamine-tetra- acetic acid trinatrium salt (EDTA)	Sigma	03710-250g
White 96 well culture plates, with white bottom	PerkinElmer	6005680
Transparent 96 well culture plates	TPP	92696
Sealing tape	Nunc / Thermo	236366
Culture flask (T75)	TPP	90076
Cryo tubes	VWR (Brand)	479-1203

MEDIA, REAGENTS, SERA, OTHERS

Preparations

Media and Endpoint Assay Solutions

Medium No.1 (growth media)

Substance / Solution	Quantity	Catalog No.
D-MEM	500 mL	PAN / P04-04510
FBS Superior*	50 mL	Biochrom S 0613/5
Penicillin/Streptomycin	5 mL	Biochrom A 2213
Puromycin dihydrochloride	25 µL	Sigma P9620-10 mL

Medium No.2

Substance / Solution	Quantity	Catalog No.
D-MEM	500 mL	PAN / P04-04510
FBS Superior *	50 mL	Biochrom S 0613/5

Medium No.3

Substance / Solution	Quantity	Catalog No.
D-MEM	500 mL	PAN / P04-04510
FBS Superior*	5 mL	Biochrom S 0613/5

All media can be use and stored for a maximum of 4 weeks at 4°C.

Lysis buffer

Substance / Solution	Quantity	Catalog No.
SDS	10 g	Sigma L3771-100G
Dimethyl sulfoxide (DMSO)	99.6 mL	Fisher Chemical D/4121/PB15
Acetic acid 100%	0.4 mL	Merck 1.00063.1011

→ Stable at room temperature for 6 months

MTT solution

Substance / Solution	Quantity	Catalog No.
Thiazolyl Blue Tetrazolium Bromide (MTT)	5 mg/mL	Sigma M5655-1G
PBS without Mg2+/Ca2+		PAN / P04-36500

→ Sterile filtered

→ Stable at 4°C for 3 months

* only EU-certified; if this serum is not available in your country, please try the serum that you regularly use for other mammalian cell culture systems

Steady-Glo® Luciferase Assay System, Promega

Substance/ Solution	Quantity	Catalog No.
Steady-Glo Luciferase Assay System	100 mL	Promega E2520

→ Mixed, stable at -20°C for 2 weeks

Freezing medium

Substance/ Solution	Quantity
Growth media (Medium No.1)	18 mL
DMSO	2 mL

→ The freezing media will be prepared always fresh.

All media can be use and stored for a maximum of 4 weeks at 4°C.

Test Compounds

The **list of test compounds** is available as **Annex 1** in the **Downloads** section of this **DB-ALM Protocol No. 184**.

Positive Control(s)

Ethylene glycol dimethacrylate (EGDMA), 120 µM

Negative Control(s)

DL-Lactic acid, 5000 µM

Method

The method consists of two phases, the range finder experiment and the main experiment.

A training video on the LuSens assay can be found under following link:

https://doi.org/10.5281/zenodo.5878268

The following **time schedule** is an example for maintenance of the culture during propagation phase, set up for the cytotoxicity range finder test and main test.

Please note that every time a new cell cryovial is thawed, the passage number will be set to zero, even though we are aware that they have been cultured until p3 and to p4 for master bank and working bank respectively.

p=cell passage number

In grey: optional test starting date

Working day	Activity
Day 1 (Monday)	Thaw LuSens cryovial (p0)
Day 2 (Tuesday)	Replace Medium No.1
Day 4 (Thursday)	Split cells: p0 → p1
Day 8 (Monday)	Split cells: p1 → p2
Day 11 (Thursday)	1.- For stocks' preparation, freeze cells from p2 2.- For propagation, split cells: p2 → p3
Day 15 (Monday)	Split cells: p3 → p4
Day 18 (Thursday) or Day 19 (Friday)	1.- For stocks' preparation, freeze cells from p4 2.- For propagation, split cells: p4→ p5 3.- For cytotoxicity range finder experiment, start a pre-culture
Day 22 (Monday)	1.- Split cells: p5 → p6 2.- Start cytotoxicity range finder test
Day 23 (Tuesday)	Treat according to concentration-range test
Day 25 (Thursday)	Conduct cytotoxicity range finder test measurement
Day 26 (Friday)	1.- Split cells: p6 → p7 2.- Pre-culture for main test 1(Friday-Monday) 3.- Pre-culture for main test 2 (Friday-Tuesday)
Day 29 (Monday)	1.- Split cells: p7 → p8 2.- Start main test 1 (Preparation of plates for treatment, p7)
Day 30 (Tuesday)	1.- Treatment: main test 1 2.- Start main test 2 (Preparation of plates for treatment, p7)
Day 31 (Wednesday)	Treatment: main test 2
Day 32 (Thursday)	Measurements main test 1 - Luciferase assay - Viability assay (MTT)
Day 33 (Friday)	1.- Split cells, p8 → p9 2.- Measurements main test 2 - Luciferase assay - Viability assay (MTT) 3.- Pre-culture for main test 3 4.- Pre-culture for main test 4
Day 36 (Monday)	1.- Split cells, p9 → p10 2.- Start main test 3, p9 ….and so on up to passage no.15 for main test

ROUTINE CULTURE PROCEDURE

1. Cell propagation and preparation of stocks

1.1. Thawing cryopreserved LuSens cells

a. Cryovials are placed in a 37°C water bath.

b. As soon as the ice has melted, cells are gently resuspended in 10 mL Medium No.1 (without Puromycin).

c. Cells (~1,5 - 2 x 10^6 cells) are seeded into T75 culture flasks containing 10 mL of Medium No.1 (without Puromycin).

d. Medium change is performed in the following 24 h of cell thawing. For this purpose, cell culture media is replaced by 20 mL Medium No.1 (**with Puromycin**), no washing step is needed.

1.2. Maintenance of the culture during propagation phase and pre-culturing

a. Cells are maintained in T75 flasks with 20 mL Medium No.1 at 37°C in a humidified atmosphere containing 5% CO_2 to a confluence of 80-90%.

1.3. Propagation

a. Cells are washed twice with 10 mL PBS containing 0.05% EDTA.

b. PBS is aspirated and cells are trypsinized by adding 1 mL Trypsin/EDTA and incubating the cells at 37°C until cells detach (6-7 minutes)

c. After cells have detached, they are resuspended in 9 mL Medium No.1 per T75 flask.

   - For culture Monday-Friday or Friday-Tuesday: 0.4 x10^6 cells are seeded per T75 culture flask and incubated until the next cell passage.
   - For culture Monday-Thursday or Friday-Monday: 0.68 x10^6 cells are seeded per T75 culture flask and incubated until the next cell passage.

1.4. Cryopreservation

a. Cells are harvested as previously described (**1.3.a**) and centrifuged at 380x g for 5 min.

b. The cell pellet is resuspended in freezing media at a density of 3-5 x 10^6 cells per cryovial (1.8 mL).

c. The 1.8 mL aliquot is then transferred into corresponding cryovials.

d. Cryovials are frozen at -80°C using a freezing container for 24 h. Then vials are transferred to liquid nitrogen (store at liquid phase) for longer storage.

Important:

Viability should be confirmed by microscopic analysis every time the cells are brought into culture. The performance of every new cell batch should be evaluated by performing validity runs (1-2) and comparing the results to previous data (i.e. historical data, when available).
A validity run consists of a LuSens assay performed without test substance, but with all controls (blank medium, vehicle control, EGDMA 120 µM and DL-Lactic acid 5000 µM using the **plate layout of main test 3.2** as shown in **Annex 3**).

TEST MATERIAL EXPOSURE PROCEDURES

2. Assay

2.1. Cytotoxicity range finder test

2.1.1. Pre-culture (Day 18 or Day 19)

a. Cells are used for pre-culture at p4 → p5 on Thursday or Friday.
   - For pre-culture on Thursday seed T75 flask with 0.4x10 ^{6} cells (assay start on Monday).
   - For pre-culture on Friday seed T75 flask with 0.68x10 ^{6} cells (assay start on Monday) or flask with 0.4x10 ^{6} cells (assay start on Tuesday).
   -

2.1.2. Preparation of plates for treatment (Day 22)

a. At the time of splitting, cells should be 80-90% confluent.

b. Cells are washed twice with 10 mL PBS containing 0.05% EDTA and trypsinized aspreviously described (by adding 1 mL Trypsin/EDTA to the flask and by incubating the cells at 37°C for 6-7 min or until cells have detached).

c. Cells are then resuspended in 9 mL Medium No.2 per T75 flask.

d. Cells are quantified and the cell suspension is adjusted to 83 000 cells per 1 mL.

e. 120 µL of cell suspension (10 000 cells) are seeded as follows:
   - For the testing of one test substance, cells are seeded in a flat bottom 96 well plate (see **plate layout 3.1** in **Annex 3**).

Important: no cells should be seeded into well "H12"

   - Before and during the seeding procedure, cells should be gently but thoroughly mixed to avoid sedimentation and to ensure equal distribution of cells per well.

f. Cells are incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 .

Important: for optimal cell culture conditions please bear in mind that the cell culture plates inside the incubator should be placed away from the ventilator.

2.1.3. Preparation of test and control substances for treatment (Day 23)

a. Test substances are dissolved to prepare stock solution concentrations of up to 200 mM in DMSO. Positive and negative controls are dissolved to prepare a stock solution of 12 mM EGDMA (positive control) and 500 mM DL-Lactic acid (negative control) in DMSO. The DMSO concentration in the test is adjusted to 1%.

Test substances, **positive and negative control substances** have to be **prepared always fresh** as described below and in **Annex 2** and **Annex 3**.

b. Preparation of test substances:

1. From the 200 mM stock solution, a 100x master plate is prepared in DMSO, as described in the **Annex 2** (**2.1** and **2.2**).
2. The 100x DMSO master plate is then used to prepare a 4x master plate in Medium No.3 as described in the **Annex 2** (**2.1** and **2.3**).

3. Finally the following test concentrations are used for the cytotoxicity range finder test: 0.976, 1.953, 3.906, 7.812, 15.625, 31.25, 62.5, 125, 250, 500, 1000 and 2000 µM (see **plate layout 3.1** in **Annex 3**).

c. Preparation of positive and negative controls:

4. Positive control (4x EGDMA): From the 12 mM EGDMA stock solution it is prepared a 1:25 dilution with Medium No.3 (e.g. 8.4 mL Medium No.3 + 350 µL EGDMA stock solution).
5. Negative control (4x DL-Lactic acid): From the 500 mM DL-Lactic acid stock solution it is prepared a 1:25 dilution with Medium No.3 (e.g. 8.4 mL Medium No.3 + 350 µL DL-Lactic acid stock solution).
6. Vehicle control (4x DMSO): DMSO is 1:25 diluted with Medium No.3 (e.g. 8.4 mL Medium No.3 + 350 µL DMSO).

2.1.4. Treatment (Day 23)

a. Aspirate cell culture media from the wells.

b. Add 150 µL Medium No.3 to each well.

c. Add 50 µL (dilution 1:4) of the 4x master plate to A-C, 1-12 (see **Annex 3** (**3.1**)).

d. Add 50 µL (dilution 1:4) of 4x DMSO to G, 1-12

e. Add 50 µL (dilution 1:4) of the Medium No.3 to H, 1-6

f. Add 50 µL (dilution 1:4) of 4x DL-Lactic Acid to H, 7-9

g. Add 50 µL (dilution 1:4) of 4x EGDMA to H, 10-11

h. Seal the plates with adhesive foil to avoid evaporation of volatile compounds and to avoid cross contamination between wells.

i. Incubate the plates for 48h at 37°C in a humidified atmosphere containing 5% CO_2.

Important: for optimal cell culture conditions please bear in mind that the cell culture plates inside the incubator should be placed away from the ventilator.

2.1.5. Viability assay, MTT (Day 25)

a. Prepare MTT working solution by mixing 9 parts of Medium No.3 with 1 part of MTT solution

b. Aspirate cell culture media from all wells.

c. Add 200 µL MTT working solution to each well.

d. Incubate for 2 h at 37°C in a humidified atmosphere containing 5% CO_2.

e. After incubation, remove the solution and add 100 µL of lysis buffer to each well, agitate the plate for 5 min and measure absorption at 570 nm and at 690 nm using a photometer (690 nm is used as reference wavelength to correct for the background of the plate, and this can be dependent on the photometer used).

f. Calculate the concentration at which the viability is reduced to no more than 75% (**CV75**) compared to the vehicle control in order to identify the concentrations to be tested for the main test (For **CV75 calculation** see the section "**Data analysis**" below).

2.2. LuSens Assay (main test)

2.2.1. Pre-culture (Day 26)

a. Cells from p6 (cells up to p15 can be used) are pre-cultured on Friday. 0.68 x10 ^{6} cells are seeded in a T75 culture flask. Optional: for main test starting at Tuesday seed a T75 flask with 0.4 x10 ^{6} cells

2.2.2 . Preparation of plates for treatment (Day 29 or Day 30)

a. At the time of splitting, cells should be 80-90% confluent.

b. Cells are washed twice with 10 mL PBS containing 0.05% EDTA.

c. Cell are trypsinized by adding 1 mL Trypsin/EDTA to the flask and incubating at 37°C for 6- 7 minutes until the cells detach as assessed by microscopic examination.

d. Cells are then resuspended in 9 mL Medium No. 2 per T75 flask.

e. Cells are quantified and the cell suspension is adjusted to 83 000 cells per 1 mL.

f. 120 µL of cell suspension (10 000 cells) are seeded as follows:

- One clear flat bottom 96 well plate and one white flat bottom 96 plate are needed for each test substance. Cells are seeded into a 96-well plates (see plate layout in **Annex 3.2**).

Important: no cells should be seeded into well "H12"

- Before and during seeding, the cells are gently but thoroughly mixed to avoid sedimentation and to ensure equal distribution of cells per well.

g. Cells are incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2

Important: for optimal cell culture conditions please bear in mind that the cell culture plates inside the incubator should be placed away from the ventilator in the incubator.

2.2.3. Preparation of test and control substances for treatment (Day 30 or Day 31)

a. Substances are dissolved to create stock solution concentrations of up to 1.2 x CV75 in DMSO. Positive and negative controls are dissolved to prepare a stock solution of 12 mM EGDMA (positive control) and 500 mM DL-Lactic acid (negative control) in DMSO. The DMSO concentration in the test is adjusted to 1%.

Test substances, **positive and negative control substances** have to be prepared always fresh as described below and in **Annex 2** and **Annex 3**.

b. Preparation of test substances:

1. From the stock solution, a 100x master plate is prepared in DMSO, as described in the **Annex 2** (**2.1** and **2.4**) .
2. The 100x DMSO master plate is then used to prepare a 4x master plate in Medium No.3 as described in the **Annex 2** (**2.1** and **2.5**).
3. Finally the following test concentrations are used for the main test: CV75/2.074, CV75/1.728, CV75/1.44, CV75/1.2, CV75 and CV75x1.2 µM (see **plate layout** in **Annex 3** (**3.2**)) .

c. Preparation of positive and negative controls:

4. Positive control (4x EGDMA): From the 12 mM EGDMA stock solution it is prepared a 1:25 dilution with Medium No.3 (e.g. 8.4 mL Medium No.3 + 350 µL EGDMA stock solution).
5. Negative control (4x DL-Lactic acid): From the 500 mM DL-Lactic acid stock solution it is prepared a 1:25 dilution with Medium No.3 (e.g. 8.4 mL Medium No.3 + 350 µL DL-Lactic acid stock solution).
6. Vehicle Control (4x DMSO): DMSO is 1:25 diluted with Medium No.3 (e.g. 8.4 mL Medium No.3 + 350 µL DMSO).

2.2.4. Treatment (Day 30 or Day 31)

a. Aspirate cell culture media from the wells.

b. Add 150 µL Medium No. 3 to each well.

c. Add 50 µL (dilution 1:4) of the 4x master plate to A-C, 1-6 (see **Annex 3** (**3.2**)).

d. Add 50 µL (dilution 1:4) of the Medium No.3 to row E, 1-12

e. Add 50 µL (dilution 1:4) of 4x DMSO to row F+G, 1-12

f. Add 50 µL (dilution 1:4) of 4x DL-Lactic acid to row H, 1-6

g. Add 50 µL (dilution 1:4) of 4x EGDMA to row H, 7-11

h. Seal the plates with adhesive foil to avoid evaporation of volatile compounds and to avoid cross contamination between wells.

i. Incubate the plates for 48 h at 37°C in a humidified atmosphere containing 5% CO_2.

Important: for optimal cell culture conditions please place the culture plates away from the ventilator in the incubator.

2.2.5. Viability assay, MTT (Day 32 or Day 33)

a. Prepare MTT working solution by mixing 9 parts of Medium No.3 with 1 part of MTT solution.

b. Aspirate cell culture media from all wells.

c. Add 200 µL MTT working solution to each well.

d. Incubate for 2 h at 37°C in a humidified atmosphere containing 5% CO_2.

e. After incubation, remove the solution and add 100 µL of lysis buffer to each well and shake the plate for 5 min and measure the absorption at 570 nm and at 690 nm with a photometer to correct for the background of the plate.

2.2.6. Luciferase expression (Steady-Glo® Luciferase Assay System, Promega, alternatively One- Glo® Luciferase Assay System can be also used, here it is described the use of Steady-Glo®) (Day 32 or Day 33)

a. Steady-Glo® reagent should be thawed at temperatures below 25°C to ensure reagent performance (see manufacturer's protocol). From experience, best thawing conditions are over night at 4°C.

b. Transfer the content of one bottle of Steady-Glo® buffer to one bottle of Steady-Glo® Substrate.

c. Mix by inversion until the substrate is thoroughly dissolved.

d. Supernatant is aspirated from the white plate and discarded.

e. Wash each well twice with 300 µL PBS (with Ca^{2+}/Mg^{2+}).

f. Prepare Steady-Glo® working solution by mixing 1 part of PBS (without Ca^{2+}/Mg^{2+}) with 1 part of Steady-Glo®-Mix.

g. Add 200 µL Steady-Glo® working solution to each well.

h. Shake the plate slowly for at least 5 min in the dark and measure luminescence for 2 seconds using a luminometer. Other conditions may be required depending on the luminometer used for the analysis by the individual laboratory.

Important:

In order to ensure optimal luminescence measurements, when performing the assay for the first time, it is recommended to perform one or two runs of the LuSens assay using increasing concentrations of EGDMA as test substance (see **plate layout 3.2** in **Annex 3**).

By performing these repetitions, following aspects should be considered:

a) luciferase expression in a concentration response fashion (in wells A-C:1-6), after treatment with increasing concentrations of EDGMA.
b) no concentration response in wells D: 1-6, and A-D: 7 in comparison to luminescence values in wells A-D: 8-12
c) the average percentage SD of the variability in at least 21 vehicle control wells (F-G: 1-12) should be below 20% and they should not show any “gradient-like” pattern.

ACCEPTANCE CRITERIA

For acceptance of a repetition the average induction for the positive control (120 µM EGDMA) should be ≥ 2.5 and it should have a relative viability of at least 70%. The induction triggered by the negative control (5000 µM DL-Lactic acid) as well as the basal expression of the cells should be <1.5 fold as compared to the induction of the solvent control. The average percentage SD of the variability in at least 21 solvent control wells should be below 20%. At least 3 test concentrations must be within viability limits, i.e. have relative viability of at least 70%.

Moreover, in case a result is to be considered negative, at least one concentration must be cytotoxic, i.e. have a cell viability < 70%, or the maximum concentration of 2000 µM (or 2000 µg/mL when no molecular weight is available) must have been tested. The mean basal expression of the blank (only cells) should be < 1.5 (compared to the blank- corrected solvent control).

If any of these criteria is not met, the repetition is considered not valid and needs to be repeated.

Conditions for re-testing

For those repetitions that do not meet the validity criteria, re-testing is possible if:

- the reason for non-valid repetition is known and can be justified (i.e., the use of unqualified cell passages, or technical mistakes such as dilution or pipetting mistakes).
  In this case, the repetition (including the reason for disregarding it) will be documented and reported as **non-qualified**. The number of non-qualified runs is not restricted, but is expected to be low.
- the reason for non-valid repetition is **unknown**, the repetition will be documented and reported as **not-valid**. For a given test substance a maximum of three **invalid** repetitions is acceptable. In the case of three **invalid** repetitions without coming to a conclusion (see the section **Prediction model** below) **re-testing is stopped**.

Data Analysis

CV75 calculation

The CV75-value (relative survival rate) is calculated by linear extrapolation. This value is the substance concentration at which cell viability is 75% compared to the control.

The CV75 should be calculated as follows:

a. Two concentrations are selected, one above 75% viability and one below 75% viability as reference points.

Concentration	Replicate 1	Replicate 2	Replicate 3	Mean	Rel.viability [%]	SD
Vehicle control	71.92	69.3	70.2	70.47	**100.00**	1.33
0.976 µM	70.14	65.8	69.4	68.45	97.12	2.32
1.953 µM	68.05	67.19	67.9	67.71	96.08	0.46
3.906 µM	67.85	66.69	67.5	67.35	95.56	0.60
7.812 µM	67.12	65.13	66.4	66.22	93.96	1.01
15.625 µM	56.01	50.70	54.18	53.63	76.10	2.70
31.25 µM	47.76	42.66	45.71	45.38	64.39	2.56
62.5 µM	39.50	34.63	37.23	37.12	52.37	2.44
125 µM	30.12	28.04	29.45	29.20	41.44	1.06
250 µM	25.25	15.66	18.9	19.94	28.29	4.88
500 µM	13.61	11.97	12.5	12.69	18.01	0.84
1000 µM	4.6	3.79	4.3	4.23	6.00	0.41
2000 µM	2.33	3.22	2.87	2.92	4.14	0.28

b. These data are used to calculate the slope as follows:

$$Slope = \frac{value\ (\text{rel. viability} < 75\%) - value\ (rel.viability\ > 75\%)}{value\ (\mu\text{M of the rel. viability} < 75\%) - value\ (\mu M\ of\ the\ rel.viability\ > 75\%)}$$

c. Thereafter, the value of the Y intercept is calculated as follows:

$$Y\ intercept = value\ (rel.viability < 75\%) - (Slope \cdot value\ (\mu M\ of\ the\ rel.viability < 75\%)$$

d. Once obtained, the slope and Y intercept, the CV75 is calculated as follows:

$$CV75 = \frac{75 - Y\ intercept}{slope}$$

In the present example, the calculated slope is -0.74944 and the Y intercept is 87.81, leading to a CV75 of 17.1 µM.

Luciferase fold induction

The fold induction is calculated as follows:

$$Fold\ induction\ = \frac{Results\ from\ the\ measurement\ (luminescene\ 2\ \text{sec}) - Blank\ (value\ from\ well\ H12)}{Mean, blank\ solvent\ (DMSO) - Blank\ without\ cells\ (well\ H12)}$$

The mean is calculated from three independent values.

Statistical analyses of luciferase fold-induction

For the statistical evaluation of luciferase fold-induction the EXCEL-function T.TEST is used:

Parameter	Statistical test	Markers	References
Luciferase fold-induction	A pair-wise comparison of the concentration groups, positive control and negative control group with the vehicle control was performed using the Welch t-test (one-sided) for the hypothesis of equal means	* for $p \leq 0.05$ ** for $p \leq 0.01$	Welch B.L. (1947): The generalization of Student's problem when several different population variances are involved. Biometrika, 34, 28-35

Adjustment of concentration range

In some cases, it is possible that no cytotoxicity is observed. In these cases the maximum concentration used for testing is 2000 µM (or 2000 µg/mL when no molecular weight is available).

Should the cytotoxicity in the main test be substantially lower than in the range finder test and no luciferase induction is observed, then the next repetition should be performed using a higher 1.2-fold of the CV75 determined in the range finder (e.g. 1.2^2x CV75, 1.2^3 x CV75 etc.) µM. If in the second repetition toxicity and luciferase induction are still not observed, an additional repetition should be run with the maximum concentration of 2000 µM (or 2000 µg/mL when no molecular weight is available). This repetition should then be confirmed (4th repetition).

Adjustment of positive control concentration

In case the above specified positive control concentration of 120 µM is too toxic or not able to induce luciferase ≥ 2.5 (see the section **Acceptance criteria** above, p.15), the performing laboratory should run a range finder experiment with EGDMA in order to set the concentration at which luciferase induction is ≥ 2.5 folds compared to VC and viability is above 70%. This concentration should be confirmed in at least two more runs and should be used for the following experiments. In order to avoid variations in the positive control due to the use of new batches/charge of EGDMA, every time a new batch/charge is used, its toxicity and capacity to induce luciferase should be tested.

Prediction Model

Each valid repetition (i.e. meeting all acceptance criteria, according to the procedure described above) is interpreted as follows:

A test compound is considered to have keratinocyte activating potential when the luciferase induction is above or equal to 1.5 fold and statistically significant compared to the vehicle control in 2 (or more than) consecutive non-cytotoxic tested concentrations whereby at least three tested concentrations must be non-cytotoxic. A test compound is considered to not to have keratinocyte activating potential if the above effects are not observed. In order to come to a conclusion on the keratinocyte activating potential of a substance, one complete experiment needs to be conducted. A complete experiment consists of two valid independent repetitions according to acceptance criteria. If the first two repetitions come to the same result (i.e. either being negative or being positive) no further testing is required. In case that the first two repetitions give discordant results (i.e. one is negative and the other is positive), a third independent repetition needs to be conducted to complete the experiment.

The keratinocyte activating potential of a test substance is determined by the result of the majority of the repetitions of an experiment. If two of two or two of three repetitions are negative/positive, the substance is considered as negative/positive.

In **Annex 4** the prediction model including a borderline range based on the validation study data is presented.

LuSens predictive capacity.

The predictivity of the LuSens assay has been evaluated using Cooper statistics (Cooper et al., 1979) for determination of accuracy, specificity, sensitivity as follows:

Parameter	Mathematical Equation
Sensitivity	$\frac{CP}{CP + FN} \cdot 100$
Specificity	$\frac{CN}{CN + FP} \cdot 100$
Positive Predictive Value	$\frac{CP}{CP + FP} \cdot 100$
Negative Predictive Value	$\frac{CN}{CN + FN} \cdot 100$
Accuracy	$\frac{CN + CP}{CN + CP + FN + FP} \cdot 100$

Note: CP: correct positive, **CN**: correct negative, **FP**: false positive and **FN**: false negative

For this purpose, the data obtained from the LuSens assay were compared to the literature data from human or local lymph node assay (LLNA). The predictivity is summarized in **Table 1**. The overview of the effect per test substance compared to human or LLNA are summarized in (Ramirez et al., 2014 and corrected in Kolle 2019).

Table 1. Summary of predictivity of LuSens based on Cooper statistics (Ramirez et al., 2014 and corrected in Kolle 2019).

	Compared to human data	Compared to LLNA data
No. compounds analyzed	69	72
Sensitivity	83%	75%
Specificity	78%	71%
Positive Predictive Value	85%	84%
Negative Predictive Value	75%	59%
Accuracy	81%	74%

Method Performance

The LuSens assay based on luciferase reporter cell lines and ARE pathway activation can also be used to indirectly assess the intracellular cysteine reactivity of a substance and the resulting activation of the keap-1/Nrf2 signalling pathway.

As decribed in Ramirez et al., 2016 the LuSens assay correctly predicted 56 of 69 or 52 of 74 substances when compared to human or LLNA data, respectively. When compared to human data, seven substances were incorrectly rated to be negative: aniline, ethylene diamine, Luperox A75 (benzoyl peroxide), nickel chloride, phenyl benzoate, phthalic anhydride and propyl gallate. Six substances were incorrectly rated as positive when compared to human data: 4 methoxyacetophenone, 6-methylcoumarin, methyl salicylate, propyl-4-hydroxybenzoate, diethyl phthalate, and tween 80. When compared to LLNA data, twelve substances would be incorrectly rated to be negative: 4- allylanisole, aniline, ethylene diamine, farnesal, hexadecyltrimethylammonium bromide, phthalic anhydride, phenyl benzoate, propyl gallate, pyridin, sodium lauryl sulphate (SDS), resorcinol, tartaric acid and xylene. It should be noted that hexadecyltrimethylammonium bromide, pyridine, SDS, tartaric acid, resorcinol and xylene yield false positive results in the LLNA when compared to human data. Nine substances would be incorrectly rated to be positive when compared to LLNA data: 4- methoxyacetophenone, 6-methlycoumarin, benzyl alcohol, methyl salicylate, propyl 4- hydroxybenzoate, diethyl phthalate, and tween 80. A more detailed discussion including the reference data is provided in Kolle et al., 2019.

For the assessment of the predictive capacity of the LuSens assay, the data obtained from the in vitro assay were compared to human or LLNA data from the literature using Cooper statistics **(Table 1).**

From this analysis the following predictivity values were calculated: sensitivity of 83% or 75%, specificity of 78% or 71% and an overall accuracy of 81% or 74% when compared to human or LLNA data, respectively.

Modifications of the method

The here in reported method is the one used for the Phase II of the inter-laboratory validation study (Ramirez et al., 2016) and the intra-laboratory validation study (Ramirez et al., 2014, Kolle 2019).

In comparison to the original protocol (Bauch et al., 2012), the major modifications of the method are listed as follows:

- A **clonal selection** was performed in order to obtain the fittest and purest clone to react against sensitizers.
- **Preliminary cytotoxicity assay (range finder experiment)** dosed from 2000 µM to 12 concentrations at decreasing 2-fold dilution series (2000; 1000; 500; 250; 125; 62.50; 31.25; 15.62; 7.81; 3.90; 1.95;1.97 µM) to identify the CV75.
- **Test compounds** concentrations were adjusted, the top concentration was always 1.2x CV 75 and from it 1.2-fold dilution are prepared as follows: CV 75x1.2; CV75, CV75/1.2x; CV75/1.44x; CV75/1.728; CV75/2.074
- **Positive control** selected was ethylene glycol dimethacrylate (EGDMA) at 120 µM or similar concentration meeting the positive control acceptance criteria.
- **Inclusion of control** for basal expression of luciferase. Inclusion of a negative control, DL-Lactic acid at 5000 µM.
- **Acceptance criteria of the positive control**: Luciferase fold induction ≥ 2.5x with a relative viability ≥70%.
- **Acceptance criteria** of the negative control: Luciferase fold induction < 1.5x
- **Non sensitizers** are to induce a luciferase fold induction < 1.5x, while **sensitizers** will induce a luciferase fold induction equal or above 1.5x.

After inclusion of those modifications, the predictivity of the new assay was evaluated with a set of 74 test substances (Ramirez et al., 2014 and Kolle 2019), leading to similar predictive capacity to the one previously reported by Bauch and co-workers (Bauch et al., 2012).

Annexes

Supporting information to the LuSens Assay protocol are available as **Annexes.**
Select **Downloads** to get access to the following Annexes.

- **Annex 1. LuSens Assay- Test compounds.**
  List of test items used to develop and optimize the test method.
- **Annex 2. LuSens Assay- Experimental setup.**
  Instructions for preparation of experimental plates with indication of test substances dilutions.
  2.1 Preparation of the 100x DMSO master plate and dilutions
  2.2 Preliminary cytotoxicity test: Preparation of Plate 100 x
  2.3 Preliminary Cytotoxicity test: Preparation of Plate 4x
  2.4 Main test: Preparation of Plate 100x
  2.5 Main test: Preparation of Plate 4x
- **Annex 3. LuSens Assay- Plate layouts.**
  Plate layouts for the tests of LuSens assay.
  3.1 Cytotoxicity range finder test, plate layout
  3.2 Main test plate layout
- **Annex 4. LuSens Assay- Prediction model including the borderline range.**
- **Annex 5. The LuSens Assay as a suitable and valid replacement of the KeratinoSens™ assay in the "2 out of 3" Approach.**

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