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LuSens: A keratinocyte based ARE reporter gene assay for use in integrated testing strategies for skin sensitization hazard identification

Tzutzuy Ramirez [a], Annette Mehling [b], Susanne N. Kolle [a], Christoph J. Wruck [c], Wera Teubner [d], Tobias Eltze [e], Alexandra Aumann [a], Daniel Urbisch [a], Ben van Ravenzwaay [a], Robert Landsiedel [a,*]

[a] BASF SE, Experimental Toxicology and Ecology, Germany
[b] BASF Personal Care and Nutrition GmbH, Germany
[c] RWTH Aachen, Germany
[d] BASF Schweiz AG, Switzerland
[e] BASF SE, Paper Chemicals, Germany

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ABSTRACT

Allergic contact dermatitis can develop following repeated exposure to allergenic substances. To date, hazard identification is still based on animal studies as non-animal alternatives have not yet gained global regulatory acceptance. Several non-animal methods addressing key-steps of the adverse outcome pathway (OECD, 2012) will most likely be needed to fully address this effect. Among the initial cellular events is the activation of keratinocytes and currently only one method, the KeratinoSens™, has been formally validated to address this event. In this study, a further method, the LuSens assay, that uses a human keratinocyte cell line harbouring a reporter gene construct composed of the antioxidant response element (ARE) of the rat NADPH:quinone oxidoreductase 1 gene and the luciferase gene. The assay was validated in house using a selection of 74 substances which included the LLNA performance standards. The predictivity of the LuSens assay for skin sensitization hazard identification was comparable to other non-animal methods, in particular to the KeratinoSens™. When used as part of a testing battery based on the OECD adverse outcome pathway for skin sensitization, a combination of the LuSens assay, the DPRA and a dendritic cell line activation test attained predictivities similar to that of the LLNA.

1. Introduction

In a regulatory context, the sensitizing potential of substances is generally evaluated using animal tests, such as the murine local lymph node assay (LLNA; OECD TG 429) or the guinea pig-based tests described in OECD TG 406 (OECD TG 406). As of March 11, 2013, the European Union imposed an animal testing ban on both cosmetic products and their ingredients which is accompanied by a concomitant marketing ban, if animal tests conducted after this date for the purpose of the cosmetics legislation (EU, 2009).

In addition, under the European chemicals legislation REACH, skin sensitization data for any substances registered under the European Chemicals Legislation (REACH, EC 1907/2006) is mandatory, animal testing should only be performed only a last resort (http://echa.europa.eu/documents/10162/13639/alternatives_test_animals_2014_en.pdf).

Currently, there is no non-animal alternative test method for the endpoint of skin sensitization yet available that has gained full regulatory acceptance. During the last decades, extensive work has been conducted to develop *in vitro* assays able to replace current animal test methods for the predictive identification of skin sensitizers (reviewed in Mehling et al., 2012). Given the complexity of the sensitization pathway, a combination of tests will be needed to achieve reliable predictions of the skin sensitization potential of a substance.

In 2012 the OECD published the adverse outcome pathway (AOP) for skin sensitization (OECD 2012a, 2012b, 2012c) in which the key steps in the sensitization process are defined. According to the AOP, one of the early key events of the sensitization process is the induction of cytoprotective gene pathways that occur within keratinocytes (KCs) upon contact with a sensitizer. KCs are the dominant cells in the epidermis and are among the first cells to come into contact with a sensitizer. In this context, various studies have shown that the Nrf2-Keap1 pathway plays an important role in skin sensitization (Ade et al., 2009; Natsch and Emter, 2008;

* Corresponding author.
E-mail address: robert.landsiedel@basf.com (R. Landsiedel).

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Vandebriel et al., 2010). Under physiological conditions, the transcription factor nuclear factor erythroid 2 (Nrf2) is constitutively expressed but is complexed and targeted for ubiquinylation in the cytosol by the cytosensor protein Kelch-like ECH-associated protein 1 (Keap1). In response to covalent modification of the highly reactive cysteine residues of Keap1 via stressors, Nrf2 is released. Free Nrf2 translocates to the nucleus, where it heterodimerizes with other molecules (e.g., small Maf or Jun proteins). This complex then binds to the so called "antioxidant response element (ARE)" in the promoter region of several genes, including *hmox1* and *nqo1*, subsequently initiating transcription of the downstream genes (Ade et al., 2009). The Keap1 protein therefore constitutes an intracellular sensor protein for electrophilic and oxidative stress. Skin sensitizers are thought to directly or indirectly react with cysteine residues of Keap1, thereby enhancing Nrf2 release (Emter et al., 2010; Motohashi and Yamamoto, 2004). The principle of this pathway has been used to develop reporter cells lines including AREc32; ARE-HepG2 (Emter et al., 2010; Simmons et al., 2011) and KeratinoSens™ (Emter et al., 2010; Natsch and Emter 2008).

In light of the urgent need to evaluate non-animal approaches capable of reliably identifying skin sensitization hazards thereby providing the basis for classification and labelling, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) has recently published its strategy on skin sensitization (EURL ECVAM, 2013a) describing its plan for the next five years towards achieving these goals: the two main points of focus will be the development of integrated testing strategies (ITS) to be used within integrated assessment and testing approaches (IATA) for skin sensitization and the facilitation of global acceptance of the new approaches for skin sensitization hazard identification (EURL ECVAM, 2013a). Currently, the assessment of the reliability and reproducibility of several test methods for skin sensitization, namely the Direct Peptide Reactivity Assay (DPRA), human cell line activation test (h-CLAT), KeratinoSens™) under the formal validation process at ECVAM has progressed to an advanced stage. The ECVAM Scientific Advisory Committee (ESAC) EURL ECVAM recently issued their recommendations on the DPRA (EURL ECVAM, 2013b) and KeratinoSens™ (EURL ECVAM, 2013c) and the methods appear adequate to be considered for inclusion in an ITS using test methods that address various key events of the OECD skin sensitization AOP (EURL ECVAM, 2013a). A pragmatic approach to assess the skin sensitization without the use of animals and using these nonanimal alternative test methods has previously been proposed and a very good accuracy using a substantial number of substances was found (Bauch et al. 2012 (n = 54); Natsch et al., 2013 (n = 145)).

In this study, the development and performance of the LuSens assay is described. The LuSens assay utilizes a similar principle as the KeratinoSens™ assay: human keratinocytes harbouring the luciferase reporter gene under the control of an antioxidant response element (ARE) are used to assess the induction of the cytoprotective responses elicited by the genes controlled by the ARE. The luciferase activity is used as a measure for this response. The individual results obtained with 74 substances are reported and the predictivity when the results are used as part of an *in vitro* test battery when assessing 50 and 53 substances in comparison to human and LLNA data, respectively.

2. Materials and methods

2.1. Generation of a transgenic cell line for identification of skin sensitizers

A human keratinocyte cell line (provided by RWTH, Aachen, Germany) was genetically modified at the Institute of Anatomy and Cell Biology of the RWTH, Aachen (laboratory of Wruck). Briefly, the modification was achieved by transfection of the cells with the pGL4.20 [luc2/Puro] vector (Promega, Germany) carrying the regulatory antioxidant response element (ARE) upstream of the luciferase gene (Luc2, Promega, Germany). The ARE itself was derived from the NADPH:quinone oxidoreductase 1 gene from rat (ggtaccagtctagagtcacagtgacttggcaaaatcgctagc) cloned using the KpnI (GGTACC) and NheI (GCTAGC) sites of the multiple cloning site. Transfection was performed with JetPrime™ transfection reagent and according to manufacturer's protocol (Polyplus-transfection SA, USA). Apart from the transfection of the ARE-Luc2 sequence, no further modifications were performed to this cell line. Two days after transfection, cells were cultured in selection media containing different concentrations of puromycin (0.25; 0.5; 1.0 and 1.5 μg/mL). In parallel, non-transfected cells were also incubated in the same selection media in order to determine the concentration of antibiotic needed to select transfected cells. From these experiments it was found that 1 μg/mL was the optimal puromycin concentration to obtain only cells expressing the resistance marker and concomitantly the ARE-Luc2 construct. After clonal selection, transfected cells were maintained in culture with puromycin (0.45 μg/mL).

2.1.1. Cell maintenance

Cells were maintained in T75 flasks (TPP, Switzerland) with 20 mL of growth media (DMEM with 10% FBS Superior, 1% penicillin/streptomycin, and 0.005% puromycin, all components from Biochrom (Germany)) except puromycin, which was obtained from Sigma (Germany), at 37 °C in a humidified atmosphere containing 5% CO_2 to a confluence of 80–90%. Cells were propagated twice a week as follows; cells were trypsinized, seeded at a density of 0.4×10^6 cells in T75 culture flasks containing 20 mL of culture media. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.1.2. Selection of stable cell clones

Follow antibiotic selection, clonal colony selection was performed in order to obtain a cell population homogeneously expressing luciferase. For this purpose, single cells were seeded in individual wells of 96 well plates. 24 single colonies were isolated, further propagated, aliquots frozen and the clones tested for their proficiency to activate ARE-Luc2. Proficiency was evaluated by measuring the relative increase in luciferase activity following exposure to two concentrations of ethylene glycol dimethacrylate (EGDMA, 75 μM and 150 μM) at non-cytotoxic concentrations. The viability of the cells was evaluated using the MTT assay. Briefly, for luminescence analysis, 200 μL of cell suspension corresponding to 1×10^4 cells per well were seeded in 96 white flat-bottom well plates and incubated for 24 h. All incubation steps were carried out in a humidified atmosphere at 37 °C and 5% CO_2. After incubation, culture media was replaced with 150 μL of fresh media, 50 μL of EGDMA stock solution was added to a final concentration of 75 μM or 150 μM and the cells were incubated for 48 h. After treatment, cell culture media was removed and cells were washed twice with 300 μL PBS (with Ca^{2+}/Mg^{2+}). After washing, 100 μL PBS (Ca^{2+}/Mg^{2+} free) and 100 μL Steady-Glo®-Mix reagent (Promega, Germany) were added to each well. Plates were gently shaken in the dark for 10 min and luminescence measured using a luminometer (Perkin Elmer "Victor 3" 1420 Multilabel counter or GloMax®, Promega). For analysis of cell viability, 200 μL of cell suspension, corresponding to 1×10^4 cells per well, were seeded into clear 96 well plate and incubated for 24 h. After 24 h, cell culture media was replaced by 180 μL of fresh media and 20 μL of MTT solution added to each well. Plates were sealed with breathable tape and incubated for 2 h. After incubation, media and MTT solution were removed,

100 μL of lysis buffer added to each well and plates gently shaken for 5 min. Absorptions at 570 nm and at 690 nm were measured in a spectrophotometer; the latter wavelength was used as reference.

2.2. Test substances

Table 1 summarizes 74 test substances and their following properties; molecular weights, purities, supplier, CAS number, chemical classes, proposed reaction mechanisms, information about known pro- or prehapten properties, human literature data, EC3 (%) value of LLNA data and the respective literature reference.

2.3. LuSens assay

The LuSens assay consists of a cytotoxicity range finding experiment from which the concentrations to be used in the main experiment are calculated. In the case no cytotoxicity is observed the recommended maximum concentration to be tested is 2000 μM.

2.3.1. Cytotoxicity range finder experiment

Cells were 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 were seeded into clear flat bottom 96 well plates (TPP, Switzerland; 1×10^4 in 200 μL per well). Test substances were dissolved in DMSO in a series of 1:2 dilutions starting at 2000 mM (100× stock solution). Substances were further diluted (1:25) in medium to obtain 4× stock solution. Final DMSO concentrations in the assay did not exceed 1%. Treatment was performed by applying 50 μL of the test substance to each well (final volume: 200 μL) for 48 h. Each substance was tested at twelve concentrations in triplicate. Assessment of cell viability was 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) was calculated. The highest tested concentration in the main experiment was then 1.2× CV75 (or 2000 μM if no cytoxicity was observed).

2.3.2. Main experiment for luciferase expression and cell viability

For analysis of luciferase expression, cells were seeded into white flat bottom 96 well plates (TPP, Switzerland; 1×10^4 in 200 μL per well). Test substances were dissolved in DMSO (100× stock solution) at concentrations according to the preliminary cytotoxicity data. Substances were further diluted (1:25) in medium to obtain 4× stock solution. Final DMSO concentration on the cells did not exceed 1%. The highest tested concentration was 1.2× CV75. Treatment was performed by applying 50 μL of the test substance dilution to each well (final volume: 200 μL) for 48 h. Each substance was tested at six concentrations in triplicate. If the classification in both tests differed, a third test was conducted. After treatment, cell culture media was removed and cells were washed twice with 300 μL PBS (with Ca^{2+}/Mg^{2+}). After washing, 100 μL PBS (Ca^{2+}/Mg^{2+} free) were added to each well and also 100 μL Steady-Glo®-Mix reagent (Promega, Germany). Plates were gently shaken in the dark for 10 min and luminescence then measured using a Perkin Elmer "Victor 3" 1420 Multilabel counter. For analysis of cell viability, cells were seeded in clear flat bottom 96 well plates (TPP, Switzerland; 1×10^4 in 200 μL per well). Test substances were dissolved in DMSO (100× stock solution). Substances were further diluted (1:25) in medium to obtain 4× stock solution. Final DMSO concentration on the cells did not exceed 1%. Treatment was performed by applying 50 μL of the test substance dilution to each well (final volume: 200 μL) for 48 h. Each substance was tested in six concentrations (each concentration in triplicate). In addition, the assay was performed in at least 2 independent experiments. Concentrations were chosen according to preliminary MTT cytotoxicity assays. Assessment of viable cells was also performed by via MTT cytotoxicity assays. In parallel to the test substances, a positive control (EGDMA, 120 or 150 μM) was also tested in all cases and in most cases a negative control was also included (lactic acid (LA), 5000 μM).

2.3.3. Acceptance criteria and prediction model

For acceptance of the assay, at least 3 tested concentrations with a viability above or equal to 70% must be available. The positive control, EGDMA, must lead to a 2.5 fold or higher induction of luciferase in comparison to the vehicle control (VC). The luciferase activity induced by lactic acid must be below 1.5 fold of the VC and at viabilities above 70%. Moreover, average standard deviation of the VC should not exceed 20%.

The fold induction (FI) of the luminescent signal was calculated by dividing the relative luminescence units (RLU) of the treated cells (TC) by the RLU of VC cells using following equation: FI = (RLU TC)/(RLU VC). A test compound was considered to have ARE activating potential (sensitizing potential) when the luciferase induction was statistically significant above or equal to 1.5 fold compared to the VC in more than 2 consecutive non-cytotoxic tested concentrations (according to the *t*-test) whereby three concentrations must be non-cytotoxic. A test compound is considered to not to have sensitizing potential if the above criteria for an ARE activating potential were not met.

In order to come to a conclusion on the ARE activating potential of a substance, one complete experiment needs to be conducted. A complete experiment consists of two valid independent repetitions. If both 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 ARE 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 to be a nonsensitizer/sensitizer.

In addition, in accordance to the above prediction models, the predictivity of LuSens was determined according to Cooper statistics (Cooper et al., 1979) using the following to assess sensitivity, specificity, positive and negative predictive value and accuracy: sensitivity: (TP/[TP + FN] × 100); specificity: (TN/[TN + FP] × 100); positive predictivity: (TP/[TP + FP] × 100); negative predictivity: (TN/[TN + FN] × 100); accuracy: (TP + TN/[TN + TP + FP + FN] × 100) with FN being the number of false negative calls, FP the number of the false positive calls, TN the number of the true negative calls and TP the number of the true positive calls.

3. Results

Herein, we report on the development of the LuSens assay, which utilizes a reporter gene cell line based on the ARE pathway activation to assess the skin sensitization potential of a chemical. Cells transfected with the pGL4.20-ARE-Luc2 construct were selected via resistance to the antibiotic puromycin. From these experiments, a population expressing luciferase under the control of ARE was obtained and the clone with the most suitable characteristics selected.

3.1. Generation of cell line with ARE-dependent reporter gene function

Following transfection with the reporter gene construct, a clonal selection was performed in order to generate a stably transfected cell line which expresses homogeneous levels of luciferase and

Table 1
Overview of the test substances used for the validation of the LuSens assay. Information about the sensitization potential of the test substances in mice (LLNA) and humans is indicated by "+" (sensitizing) and "−" (not sensitizing); "NC" indicates not calculated.

	Chemical information									Human data		LLNA data			
No.	Substance	Molecular weight [g/mol]	Purity	Supplier	CAS #	Chemical class	Mechanism	Literature	Pro/prehapten	Human	Literature	EC3 (%)	Potency class	LLNA	Literature
1	Oxazolone	217.2	98%	Sigma	15646-46-5	Oxazole	Acylating agent	Patlewicz et al. (2008)		+	Basketter et al. (1999)	0.003	Extreme	+	Kimber et al. (2003)
2	MCI/MI	Mixture	99%	Sigma–Aldrich	26742-55-4/2682-20-4	Aromatic halide	Michael acceptor	Patlewicz et al. (2008)		+	Basketter et al. (1999)	0.009	Extreme	+	Kimber et al. (2003)
3	p-Benzoquinone	108.1	99%	Sigma–Aldrich	106-51-4	quinone	Michael acceptor	Patlewicz et al. (2008)		+	Basketter et al. (1999)	0.0099	Extreme	+	Gerberick et al. (2005)
4	1-Chloro-2,4-dinitrobenzene	202.6	95%	Aldrich	97-00-7	Nitroaromatic, aromatic halide	SN_{Ar} agent	Patlewicz et al. (2008)		+	Basketter et al. (1999)	0.049	Extreme	+	Kimber et al. (2003)
5	Potassium dichromate	294.2	⩾99.5%	Sigma–Aldrich	7778-50-9	Inorganic salt	SN_1 agent	Roberts et al. (2007)		+	Basketter et al. (2014)	0.08	Extreme	+	Basketter et al. (1994)
6	Metol	344.4	⩾98.0%	Fluka	55-55-0	Aromatic alcohol, aromatic amine	Quinone precursor	Aptula et al. (2009), Roberts et al. (2007)	Pre/pro-MA	+	Basketter et al. (2014)	0.8	Strong	+	Basketter et al. (2014)
7	Glutaraldehyde	100.1	25% in H_2O	Sigma-Aldrich	111-30-8	Ketone	Schiff base	Aptula and Roberts (2006)		+	Basketter et al. (2014)	0.1	Strong	+	Basketter et al. (2014)
8	4-Phenylenediamine	108.1	97%	Sigma	106-50-3	Aromatic amine	Quinone precursor	Patlewicz et al. (2008)	Pre/pro-MA	+	Basketter et al. (1999)	0.16	Strong	+	Gerberick et al. (2005)
9	Propyl gallate	212.2	99%	Fluka	121-79-9	Polysubstituted aromatic alcohol	Quinone precursor	Aptula et al. (2009)	Pre/pro-MA	+	Basketter et al. (1999)	0.32	Strong	+	Natsch and Emter (2008)
10	2,4,6-Trinitro benzenesulfonic acid	293.2	98%	Sigma	2508-19-2	Nitroaromatic	SN_{Ar} agent	Roberts et al. (2007)			No suitable data available	0.36	Strong	+	Robinson et al. (1989)
11	Phthalic anhydride	148.1	5%	Fluka	85-44-9	Aromatic carboxylic acid anhydride	Acylating agent	Aptula and Roberts (2006)		+	Basketter et al. (1999)	0.36	Strong	+	Kimber et al. (2003)
12	Formaldehyde > 36% (1% in DMSO)	30.0	99%	Sigma	50-00-0	Aliphatic aldehyde	Schiff base	Patlewicz et al. (2008)		+	Basketter et al. (1999)	0.61	Strong	+	Gerberick et al. (2005)
13	Methyldibromo glutaronitrile	265.9	98%	Aldrich	35691-65-7	Nitrile/alkyl halide	SN_2 agent	Author's data		+	SCCP (2005)	0.9	Strong	+	Basketter et al. (2008)
14	Glyoxal solution	58.0	~40% in H_2O	Sigma	107-22-2	Aliphatic aldehyde	Schiff base	Roberts et al. (2007)		+	Basketter et al. (2014)	1.4	Moderate	+	Basketter et al. (2014)
15	Isoeugenol	164.2	99%	Sigma-Aldrich	97-54-1	Phenylpropanoid	Quinone precursor	Aptula and Roberts (2006)	Pre/pro-MA	+	Basketter et al. (1999)	1.5	Moderate	+	Kimber et al. (2003)
16	Diethyl maleate	172.2	99%	Sigma	141-05-9	α, ß-unsaturated ester	Michael acceptor	Patlewicz et al. (2008)		+	Ryan et al. (2000)	2.1	Moderate	+	Kimber et al. (2003)
17	Ethylene diamine	60.1	99%	Sigma	107-15-3	Aliphatic amine	Schiff base	Patlewicz et al. (2008)	Pre/pro-SB	+	Basketter et al. (1999)	2.2	Moderate	+	Gerberick et al. (2005)
18	Benzylidene acetone	146.2	99%	Aldrich	122-57-6	α, ß-unsaturated ketone	Michael acceptor	Aptula and Roberts (2006)		+	Schneider and Akkan (2004)	3.7	Moderate	+	Gerberick et al. (2005)

(*continued on next page*)

Table 1 (*continued*)

No.	Chemical information									Human data		LLNA data			
	Substance	Molecular weight [g/mol]	Purity	Supplier	CAS #	Chemical class	Mechanism	Literature	Pro/prehapten	Human	Literature	EC3 (%)	Potency class	LLNA	Literature
19	Cinnamic aldehyde	132.2	>95%	Aldrich	104-55-2	α, ß-unsaturated aldehyde	Michael acceptor	Roberts et al. (2007)		+	Basketter et al. (2014)	3	Moderate	+	Basketter et al. (2014)
20	Cobalt chloride	129.8	98%	Aldrich	7791-13-1	Inorganic salt	Coordination bonds	Author's data		+	Basketter et al. (1999)	0.57	Strong	+	OECD (2010)
21	Thiram	240.4	97%	Aldrich	137-26-8	Dithiocarbamate	SN_2 agent	Roberts et al. (2007)		+	Basketter et al. (2014)	5.2	Moderate	+	Basketter et al. (2014)
22	2-Phenyl propionaldehyde	134.2	99%	Sigma–Aldrich	93-53-8	Aldehyde	Schiff base	Roberts et al. (2007)		+	Schneider and Akkan (2004)	5.9	Moderate	+	Natsch and Emter (2008)
23	Resorcinol	110.1	99%	Sigma–Aldrich	108-46-3	Aromatic alcohol	Quinone precursor	Aptula et al. (2009)	Pre/pro-MA	–	Aptula et al. (2009)	6.3	Moderate	+	Basketter et al. (2014)
24	α-Hexyl-cinnamic aldehyde	216.3	98%	Aldrich	101-86-0	Aldehyde	Michael acceptor	Patlewicz et al. (2008)		+	Basketter et al. (1999)	8	Moderate	+	Kimber et al. (2003)
25	Tartaric acid	150.1	98%	Aldrich	133-37-9	Aliphatic carboxylic acid/glycol	Non-binding	Patlewicz et al. (2008)		-	Basketter et al. (1999)	8.7	Moderate	+	Gerberick et al. (2005)
26	2-Mercapto benzothiazole	167.2	99.50%	Sigma	149-30-4	Thiazole/heterocyclic	SN_2 agent	Roberts et al. (2007)		+	Basketter et al. (1999)	9.7	Moderate	+	Kimber et al. (2003)
27	2.3-Butanedione	86.1	10.15%	Sigma	431-03-8	(1.2-di-) ketone	Schiff base	Patlewicz et al. (2008)			No suitable data available	11	Weak	+	Gerberick et al. (2005)
28	Citral	152.2	98.10%	BASF	5392-40-5	Aldehyde/terpenoids	Schiff base	Patlewicz et al. (2008)		+	Basketter et al. (1999)	13	Weak	+	Kimber et al. (2003)
29	Eugenol	164.2	90%	Fluka	97-53-0	Phenylpropanoid	Quinone precursor	Patlewicz et al. (2008)	Pre/pro-MA	+	Basketter et al. (1999)	13	Weak	+	Gerberick et al. (2005)
30	Farnesal	220.4	>99.5%	Sigma	19317-11-4	Sesquiterpenalkohol	Schiff base	Patlewicz et al. (2008)			No suitable literature	13	Weak	+	Gerberick et al. (2005)
31	Sodium lauryl sulfate	288.4	99%	Sigma–Aldrich	151-21-3	Alkyl sulfate	Non-binding	Patlewicz et al. (2008)		-	Basketter et al. (1999)	14	Weak	+	Gerberick et al. (2005)
32	4-Allylanisole	148.2	95%	Aldrich	140-67-0	Aromatic alkyl ether/phenylpropanoid	Michael acceptor	Patlewicz et al. (2008)	Pro		No suitable data available	18	Weak	+	Gerberick et al. (2005)
33	Hydroxycitronellal	172.3	99%	Sigma	107-75-5	Terpene aldehyde	Schiff base	Patlewicz et al. (2008)		+	Basketter et al. (1999)	20	Weak	+	Kimber et al. (2003)
34	Phenyl benzoate	198.2	99.4%	Fluka	93-99-2	Benzoate (ester)	Acylating agent	Patlewicz et al. (2008)		+	OECD (2010)	20	Weak	+	Gerberick et al. (2005)
35	Cinnamic alcohol	134.2	97%	Alfa Aesar	104-54-1	α, ß-unsaturated alcohol	Michael acceptor	Patlewicz et al. (2008)	Pro	+	OECD (2010)	21	Weak	+	Gerberick et al. (2005)
36	Imidazolidinyl urea	388.3	99%	Sigma	39236-46-9	Imidazoline	Acylating agent	Patlewicz et al. (2008)		+	Basketter et al. (1999)	24	Weak	+	Gerberick et al. (2005)
37	Undecylenic acid	184.3	>95%	Fluka	112-38-9	Carboxylic acid	Non-binding	Author's data		+	Kreiling et al. (2008)	25	Weak	+	Kreiling et al. (2008)
38	Ethylene glycol dimethacrylate	198.2	99%	Aldrich	97-90-5	α, ß-unsaturated ester	Michael acceptor	Patlewicz et al. (2008)		+	Basketter et al. (1999)	35	Weak	+	Kimber et al. (2003)
39	Pyridin	79.1	99%	Sigma	110-86-1	Aromatic heterocyclic	Non-binding	Patlewicz et al. (2008)		–	Basketter et al. (1999)	71.2	Weak	+	Gerberick et al. (2005)

40	Aniline	93.1	99%	Sigma	62-53-3	Aromatic amine	Non-binding	Roberts et al. (2007)	+	Basketter et al. (1999)	89	Weak	+	Gerberick et al. (2005)
41	Methyl methacrylate	100.1	99%	SAFC	80-62-6	α, ß-unsaturated ester	Michael acceptor	Author's data	+	OECD (2010)	90	Weak	+	OECD (2010)
42	4-Nitrobenzyl bromide	216.0	99%	Aldrich	100-11-8	Benzyl halide	SN_2 agent	Roberts et al. (2007)		No suitable data available	n.d.	n.d.	+	NIH (1999)
43	Butyl paraben	194.2	>99.5%	Aldrich	94-26-8	Aromatic ester	Non-binding	Roberts et al. (2007)	+	Cosmetic review expert panel (2008)	n.d.	n.d.	+	Cosmetic review expert panel (2008)
44	Methyl paraben	152.2	⩾98.0%	Sigma	99-76-3	Aromatic ester	Non-binding	Roberts et al. (2007)	+	Cosmetic review expert panel (2008)	n.d.	n.d.	+	Cosmetic review expert panel (2008)
45	Sorbic acid	112.1	99.7%	Fluka	110-44-1	α, ß-unsaturated acid	Non-binding	Author's data	+	Menné et al. (2009)	n.d.	Weak	+	Patrizi et al. (1999)
46	Benzoylperoxide	242.2	75%	Aldrich	94-36-0	Peroxy ester	Acylating agent	Author's data	+	Haustein et al. (1985), Schneider and Akkan (2004)	41	Weak	+	Schneider and Akkan (2004)
47	Butyl glycidyl ether	130.2	95%	Aldrich	2426-08-6	Epoxide	SN_2 agent	Roberts et al. (2007)	+	Schneider and Akkan (2004)	28	Weak	+	Basketter et al. (1996)
48	Ethylparaben	166.2	na	Fluka	120-47-8	Aromatic ester	Non-binding	Roberts et al. (2007)	+	Cosmetic review expert panel (2008)	n.d.	n.d.	+	Marzulli and Maibach (1974)
49	2-Bromo-2-nitro-1.3-propanediol	200.0	98%	Aldrich	52-51-7	Halogenated nitro alcohol	$SN_{1/2}$ agent	Author's data	+	Hahn et al. (2005), Marzulli and Maibach (1974), Maibach (1977)				No suitable data available
50	Xylene	106.2	95%	Aldrich	1330-20-7	Aromatic hydrocarbon	Non-binding	Author's data	–	Basketter et al. (1999)	95.8	Weak	–	OECD (2010)
51	Glycerol	92.1	90%	Fluka	56-81-5	Aliphatic alcohol	Non-binding	Patlewicz et al. (2008)	–	Basketter et al. (1999)	100.02	Non	–	Natsch and Emter (2008)
52	1.2-Propanediol	76.1	99.5%	Supelco	57-55-6	Aliphatic alcohol	Non-binding	Patlewicz et al. (2008)	–	Basketter et al. (1999)	100.11	Non	–	Natsch and Emter (2008)
53	4-Hydroxybenzoic acid	138.1	99%	Aldrich	99-96-7	Phenolic acid	Non-binding	Patlewicz et al. (2008)	–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
54	4-Methoxy acetophenone	150.2	99%	Sigma–Aldrich	100-06-1	Ketone ether	Non-binding	Patlewicz et al. (2008)	–	Gerberick et al. (2001)	NC	Non	–	Gerberick et al. (2005)
55	6-Methylcoumarin	149.6	99%	Sigma	92-48-8	α, ß-unsaturated ester/lactone	Michael acceptor	Patlewicz et al. (2008)	–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
56	Chlorobenzene	112.6	98.5%	Fluka	108-90-7	Aromatic halide	Non-binding	Patlewicz et al. (2008)	–	OECD (2010)	NC	Non	–	Gerberick et al. (2005)
57	DL-lactic acid	90.1	99.80%	Sigma	50-21-5	Organic acid, aliphatic alcohol	Non-binding	Patlewicz et al. (2008)	–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
58	Fumaric acid	116.1	99.70%	Sigma	110-17-8	Dicarbonic acid	Non-binding	Author's data	–	Hansson and Thoerneby-Andersson (2003)	NC	Non	–	Kreiling et al. (2008)

(*continued on next page*)

Table 1 (*continued*)

No.	Chemical information									Human data		LLNA data			
	Substance	Molecular weight [g/mol]	Purity	Supplier	CAS #	Chemical class	Mechanism	Literature	Pro/prehapten	Human	Literature	EC3 (%)	Potency class	LLNA	Literature
59	Glucose	180.2	98%	Aldrich	55-99-7	Carbohydrate	Non-binding	Author's data		–	Bauch et al. (2012)			–	Bauch et al. (2012)
60	Isopropanol	60.1	99%	Fluka	67-63-0	Aliphatic alcohol	Non-binding	Patlewicz et al. (2008)		–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
61	Methyl salicylate	152.1	99%	Aldrich	119-36-8	Aromatic ester	Non-Binding	Patlewicz et al. (2008)		–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
62	n-Butanol	74.1	99%	Sigma–Aldrich	71-36-3	Aliphatic alcohol	Non-binding	Patlewicz et al. (2008)		–	Ryan et al. (2000)	NC	Non	–	Gerberick et al. (2005)
63	n-Hexane	86.2	99%	Sigma	110-54-3	Hydro carbon	Non-binding	Patlewicz et al. (2008)		–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
64	Nickel chloride	129.6	98.5%	Fluka	7718-54-9	Inorganic salt	Coordination bonds	Author's data		+	Basketter et al. (1999)	NC	Non	–	OECD (2010)
65	p-Aminobenzoic acid	137.1	98%	Fluka	150-13-0	Aromatic amine organic acid	Non-binding	Aptula and Roberts (2006)		–	Basketter et al. (1999)	NC	Non	–	Basketter et al. (2008)
66	Propyl paraben	180.2	99.80%	Sigma	94-13-3	Aromatic ester	Non-binding	Roberts et al. (2007)		–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
67	Salicylic acid	138.1	98%	Sigma-Aldrich	69-72-7	Aromatic alcohol, organic acid	Non-binding	Patlewicz et al. (2008)		–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
68	Sulfanilamide	172.2	99%	Sigma	63-74-1	Aromatic sulfon, aromatic amine	Non-binding	Patlewicz et al. (2008)		–	Basketter et al. (1999)	NC	Non	–	Gerberick et al. (2005)
69	Vanillin	152.2	98.5%	T.J. Baker	121-33-5	Phenolic aldehyde	Non-binding	Patlewicz et al. 2008		–	Uter et al. (2010)	NC	Non	–	Gerberick et al. (2005)
70	Hexadecyltrimethyl ammonium bromide	364.45	99%	Sigma	57-09-0	Aliphatic cation (quaternary ammonium)	Non-binding	Author's data		–	Andersen and Frankild (1997)				No suitable data available
71	*Tween 80*	1310.0	⩾58.0%	Sigma–Aldrich	9005-65-6	Polyfunctional ester	Non-binding	Author's data		–	Basketter et al. (2014)	NC	Non	–	Emter et al. (2010)
72	Diethyl phthalate	222.2	99.5%	Aldrich	84-66-2	Aromatic ester	Non-binding	Roberts et al. (2007)		–	Schneider and Akkan (2004)	NC	Non	–	Ryan et al. (2000)
73	Sodium benzoate	144.1	99.5%	Aldrich	532-32-1	Organic carboxylate	Non-binding	Author's data		–	SCCP (2005)	NC	Non	–	SCCP (2005)
74	Benzyl alcohol	108.1	99.80%	Sigma–Aldrich	100-51-6	Benzyl alcohol	Non-binding	Author's data		+	Schnuch et al. (1998)	NC	Non	–	RIFM database

the optimal signal to noise ratio of luciferase induction. Single cell clones ($n = 24$) were selected and propagated. The relative basal expression level of these clones was compared (Fig. 1): 17 out of 24 colonies expressed basal luciferase levels which changed only marginally to 0.5–1 fold when treated with solvent only (VC). 5 out of 24 colonies expressed luciferase levels between >1 and 1.5 fold compared to VC, one clone expressed basal luciferase levels below 0.5 fold compared to VC (Fig. 1), and only one clone (clone 18) did not show any luciferase expression. To determine the range of induction of luciferase following exposure to sensitizing substances, the clones were exposed to a weak sensitizer, EGDMA, at two concentrations (75 μM and 150 μM). As illustrated in Fig. 2, the clones exhibited a concentration dependent induction of luciferase. The lower concentration of EGDMA, 75 μM, induced luciferase expression from 1.5 to 4.6 fold in 22 of 24 clones; an induction of only 1.1 fold was observed for one clone (24). In addition to luciferase induction, viability was also measured: in 21 of 24 clones EGDMA did not induce cytotoxicity above 20% and in only two clones (1 and 13) the viability was below 80% when treated with 75 μM EGDMA (Fig. 2). Luciferase induction was higher in cells treated with 150 μM EGDMA, ranging from 2 to 9 fold in 22 of 24 clones; one clone (clone 24) exhibited an induction below 2 fold. Viability was reduced below 70% in three clones (1, 5 and 9), while the rest of the clones retained good viability. Based on these experiments clone 16 was selected as the best clone because of its low basal activity and its good capacity to be activated when exposed to EGDMA and was selected to further develop the LuSens assay.

The cytogenetic analysis of clone 16 was also performed and the results were consistent with a cell-line of human origin, presenting a hypertriploid karyotype with an average number of 77 chromosomes (modal range from 74 to 80) and 6 marker chromosomes (M1–M6), of which 3 showed 2 copies each. In addition, genomic DNA of this clone was isolated for verification of the presence of the ARE sequence. Sequencing demonstrated that the sequence of the ARE of the reporter gene construct was present without any modification (data not shown). When a Blast search of the ARE sequence using whole genomic DNA sequences from the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome) was conducted, the sequence from LuSens clone 16, provided a 100% identity with the NAD(P)H dehydrogenase [quinone] 1 gene (*Nqo1*) sequence of *Rattus norvegicus* strain BN; Sprague-Dawley

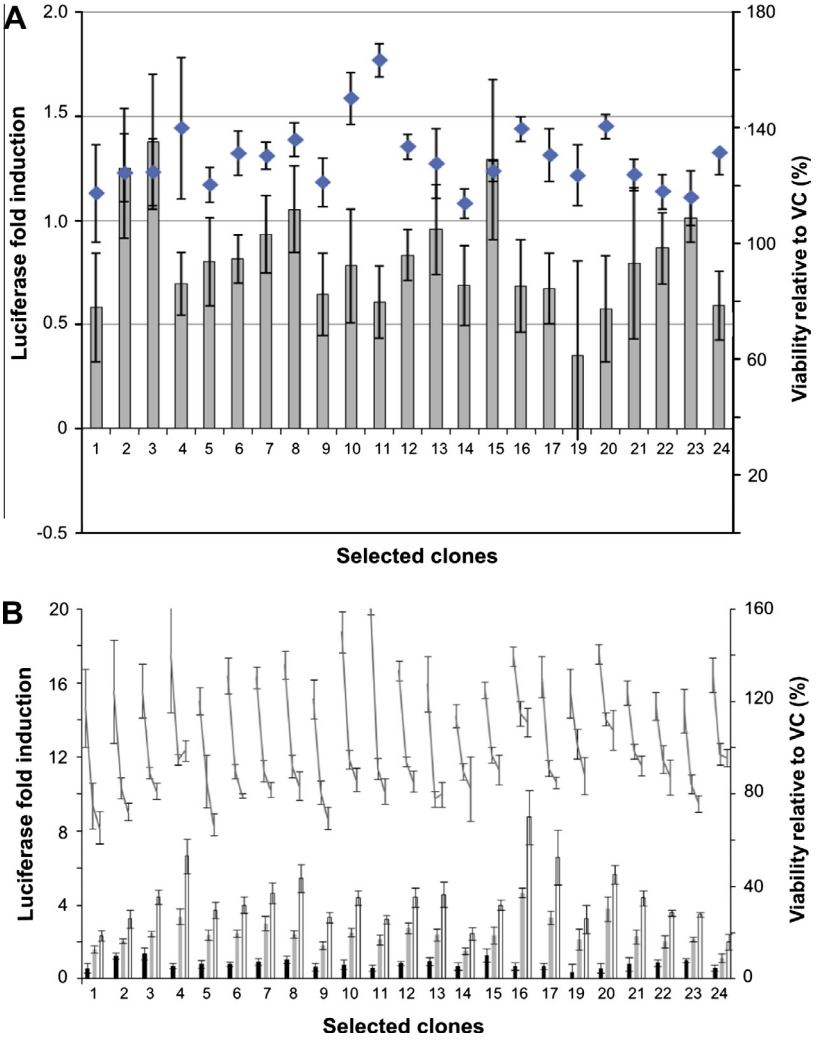

Fig. 1. (A) Basal luciferase activity of 23 clones of LuSens cells (grey bars), the expression of single clones were compared to the expression of each clone treated with the vehicle control (VC, DMSO 1%). Data for clone 18 are not displayed since this clone did not show detectable basal luciferase activity. In addition, the viability of the clones without DMSO (blue diamonds) was compared to the viability of each clone treated with VC. (B) Effect of increasing concentrations of the weak sensitizer, ethylene glycol dimethacrylate (EGDMA, 75 μM white bars; 150 μM grey bars, the black bars correspond to VC) towards the luciferase expression of 23 selected clones of LuSens cells. Viability relative to VC is also illustrated per clone (black lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

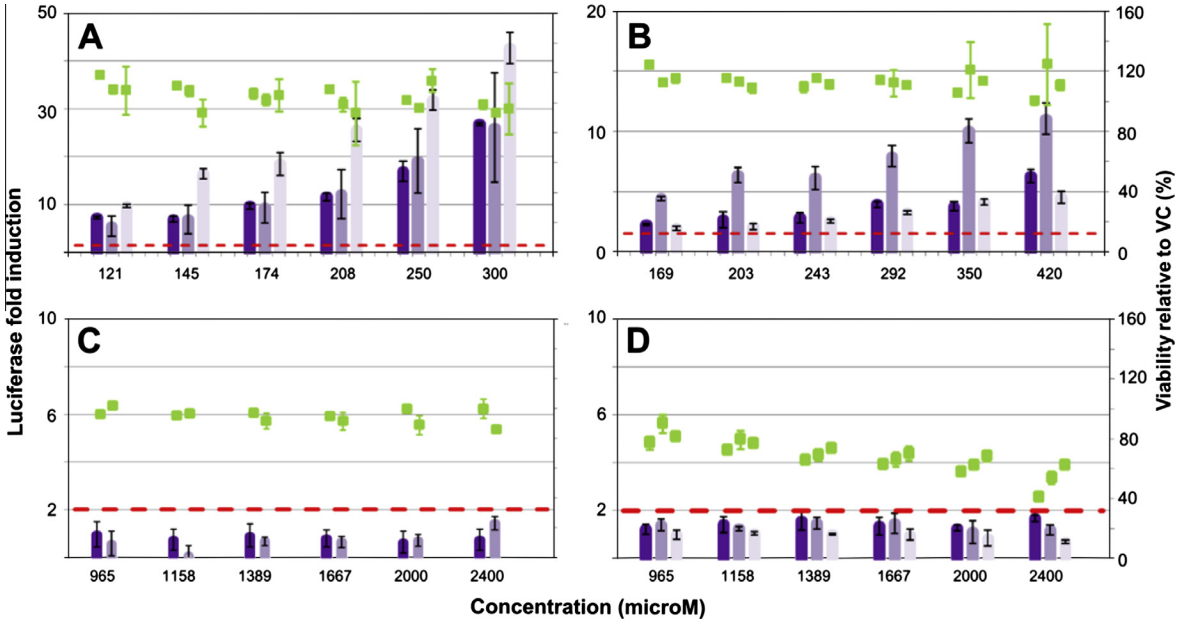

Fig. 2. Exemplary performance of LuSens cells clone 16 towards skin sensitizers and non-sensitizers. Effect of increasing concentrations of EGDMA (A), cinnamyl alcohol (B), sulfanilamide (C) and vanillin (D) on the luciferase expression (bars) and viability (green squares) of LuSens cells clone 16, illustrated are results of three independent experiments. After exposure with skin sensitizers (A and B) an increase in the luciferase expression above the 1.5 fold threshold is observed in a dose dependent manner in non-cytotoxic concentrations. Contrastingly, no luciferase induction is observed above the 1.5 fold threshold, even at concentrations close to cell toxicity, indicating no activation of LuSens cells.

(Sequence ID: ref AC_000087.1). It is important to highlight that ARE consensus sequence, responsible for ARE functionality among species (i.e., human, mouse, rat), TMAnnRTGAYnnnGCRwww (where M = A or C, R = A or G, Y = C or T, W = A or T, S = G or C) (Wasserman and Fahl, 1997) is present in the rat ARE sequence found in LuSens cells.

3.2. Assay development and predictivity according to Cooper statistics

In order to determine the predictive capacity of the LuSens assay, 74 test compounds with animal and/or human sensitization data available (Table 1) were tested in at least two independent experiments in order to compare to human and LLNA data from the literature. The LuSens assay correctly predicted 57 of 69 or 53 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. Five substances were incorrectly rated as positive when compared to human data: 4 methoxyacetophenone, 6-methylcoumarin, methyl salicylate, propyl-4-hydroxybenzoate, 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 sulfate (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. Eight substances would be incorrectly rated to be positive when compared to LLNA data: 4-methoxyacetophenone, 6-methlycoumarin, benzyl alcohol, methyl salicylate, propyl 4-hydroxybenzoate, and tween 80 (Table 2). 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 3). From this analysis the following predictivity values were calculated: Sensitivity of 83% or 73%, specificity of 81% or 74% and an overall accuracy of 83% or 74% when compared to human or LLNA data, respectively.

3.3. Intralaboratory reproducibility

The intralaboratory reproducibility of the LuSens assay was assessed based on the concordance of the classification of independent valid runs testing the same substance. All valid runs, namely those that adhered to the acceptance criteria, were considered even if an overall conclusion was already reached after two runs. From this analysis it was found that the assessment of 69 of 74 compounds was consistently reproducible (93%) and the assessment of 5 compounds, 4-allylanisole, benzyl alcohol, glyoxal solution, Luperox A75 and n-butanol, were not reproducible along the independent runs (Table 2).

3.4. Comparison of the LuSens and KeratinoSens™ assays

In general KeratinoSens™ and LuSens assays possess similar capacity to predict sensitizers. In KeratinoSens™ the luciferase gene is regulated by the ARE-element of the human aldoketoreductase (AKR1C2), whereas in LuSens it is under the control of ARE element from the rat NADPH:quinone oxidoreductase (*nqo1*). Another major difference between both assays is that LuSens uses a cytotoxicity range finder experiment to select 6 testing concentration to analyze the luciferase activity, KeratinoSens™ in contrast uses a range of 12 fixed concentrations from 1 to 200 μM. In addition, while LuSens plate set up uses all replicates for all concentrations in one plate in order to avoid potential plate differences effect on the replicates, KeratinoSens™ distribute the three replicates of each testing concentrations in 3 independent plates. The last major difference will be validity controls of both assays, for LuSens a negative and positive control is used, DL-Lactic acid and EGDMA, respectively, while KeratinoSens™ uses as positive control cinnamic aldehyde. The analysis of same data set in comparison to the data reported for KeratinoSens™ (Natsch et al., 2013; Natsch et al., 2011; Bauch et al., 2012) indicates a good concordance of

Table 2
Summary results of the LuSens assay. Depicted are the I_{max} (maximal induction), the concentration at which the 1.5 fold and 2 fold induction was observed (μM), the number of experiments showing a statistical significant 1.5 fold and 2 fold induction, the concentration (μM) at which 50% cytotoxicity was induced. Generally no cytotoxicity was observed at the 1.5 fold induction. In addition, the reproducibility is indicated by "R" for reproducible and "NR" for not reproducible. The results obtained from the LuSens assay were compared to those reported on the literature for KeratinoSens™ assay. Where results from both assays were concordant, a match is indicated as "M", in contrast, when data from both assays were discordant, a mismatch is indicated by "MM". When literature data from KeratinoSens™ was inconclusive (different results reported in the literature), no "M" or "MM" could be assessed, then this was indicated as "?". Where no KeratinoSens™ data were available, no comparison was made; indicated by "ND".

Test substance	I_{max} (fold)	Concentration (μM) for 1.5 fold induction	Number of EC1.5 positives	Concentration (μM) for 2 fold induction	Number of EC2.0 positives	Concentration (μM) causing 50% cytotoxicity	LuSens prediction	Reproducibility	Comparison with KeratinoSens™	References
Oxazolone	7.49	<743.47	2 of 2	<743.47	2 of 2	>1850	+	R	M	Natsch et al. (2013)
MCI/MI	6.78	<4.6	3 of 3	<4.6	3 of 3	>900	+	R	M	Natsch et al. (2013)
p-Benzoquinone	2.58	9.69	2 of 2	<11.11	2 of 2	>16	+	R	M	Natsch et al. (2013)
1-Chloro-2,4-dinitro benzene	3.34	3.23	2 of 4	3.65	2 of 4	>5	+	NR	M	Natsch et al. (2013)
Potassium dichromate	2.95	0.82	2 of 2	1.19	2 of 2	>1.56	+	R	M	Natsch et al. (2013)
Metol	7.82	<10.99	4 of 4	<10.99	4 of 4	>27.35	+	R	M	Natsch et al. (2013)
Glutaraldehyde	5.99	<45.05	2 of 2	45.05	2 of 2	104.24	+	R	M	Natsch et al. (2013)
4-Phenylenediamine	11.44	<53.05	4 of 4	<53.05	4 of 4	>132	+	R	M	Natsch et al. (2013)
Propyl gallate	0.89	>140	0 of 2	>140	0 of 2	>140	–	R	MM	Natsch et al. (2013)
Pricrysulfonic acid	5.94	<964.51	2 of 2	<964.51	2 of 2	>2400	+	R	ND	
Phthalic anhydride	0.33	>2400	0 of 2	>2400	0 of 2	>2400	–	R	M	Natsch et al. (2013)
Formaldehyde	3.99	184.38	3 of 3	214.28	3 of 3	>288	+	NR	M	Natsch et al. (2013)
Methyldibromo glutaronitrile	2.71	<16.08	3 of 3	18.98	2 of 3	>34.72	+	R	M	Natsch et al. (2013)
Glyoxal solution	4.64	<180.84	2 of 3	278.47	1 of 3	>450	+	NR	M	Natsch et al. (2013)
Isoeugenol	25.41	<120.56	3 of 3	<120.56	3 of 3	>300	+	R	M	Natsch et al. (2013)
Diethyl maleate	13.83	<24.11	2 of 2	<24.11	2 of 2	>60	+	R	M	Natsch et al. (2013)
Ethylene diamine	1.12	>750	0 of 2	>750	0 of 2	>750	–	R	MM	Natsch et al. (2013)
Benzylidene acetone	21.81	<10.05	2 of 2	<10.05	2 of 2	>25	+	R	M	Natsch et al. (2013)
Cinnamic aldehyde	23.25	<44.21	3 of 3	<44.21	3 of 3	>110	+	R	M	Natsch et al. (2013)
Cobalt chloride	7.48	<241.13	3 of 3	<241.13	3 of 3	>600	+	R	M	Natsch et al. (2013)
Thiram	5.29	<20.82	4 of 4	<20.82	4 of 4	49.50	+	R	ND	
2-Phenyl propionaldehyde	3.45	48.23	4 of 4	70.35	4 of 4	112.03	+	R	M	Natsch et al. (2013)
Resorcinol	0.58	>920.74	0 of 4	>920.74	0 of 4	>1104.89	–	R	M	Natsch et al. (2013)
α-Hexyl-cinnamic aldehyde	3.30	14.53	2 of 2	18.99	2 of 2	29.56	+	R	?	Natsch et al. (2013), Bauch et al. (2012)
Tartaric acid	1.28	>2400	0 of 4	>2400	0 of 4	>2400	–	R	?	Natsch et al. (2013), Bauch et al. (2012)
2-Mercapto benzothiazole	23.48	<361.69	3 of 3	<361.69	3 of 3	>900	+	R	M	Natsch et al. (2013)
2.3-Butanedione	8.30	<160.75	2 of 2	189.50	2 of 2	>400	+	R	M	Nat sch et al. (2013)
Citral	32.32	<48.23	3 of 3	<48.23	3 of 3	>120	+	R	M	Natsch et al. (2013)
Eugenol	5.49	<241.13	4 of 4	<241.13	4 of 4	>600	+	R	?	Natsch et al. (2013), Bauch et al. (2012)
Farnesal	0.85	>11.11	0 of 2	>11.11	0 of 2	13.51	–	R	MM	Natsch et al. (2013)
Sodium lauryl sulfate	0.77	>30	0 of 3	>30	0 of 3	>36	–	R	M	Natsch et al. (2013)
4-Allylanisole	2.00	>540	1 of 4	540.00	1 of 4	>540	-	NR	MM	Natsch et al. (2013)
Hydroxycitronellal	2.03	189.04	2 of 3	424.68	1 of 3	>434	+	NR	ND	

(*continued on next page*)

Table 2 (*continued*)

Test substance	I_{max} (fold)	Concentration (μM) for 1.5 fold induction	Number of EC1.5 positives	Concentration (μM) for 2 fold induction	Number of EC2.0 positives	Concentration (μM) causing 50% cytotoxicity	LuSens prediction	Reproducibility	Comparison with KeratinoSens™	References
Phenyl benzoate	1.14	>250	0 of 3	>250	0 of 3	>360	–	R	M	Natsch et al. (2013)
Cinnamyl alcohol	7.28	<168.79	3 of 3	<168.79	3 of 3	>420	+	R	M	Natsch et al. (2013)
Imidazolidinyl urea	4.72	31.10	3 of 4	37.20	3 of 4	55.56	+	NR	M	Natsch et al. (2013)
Undecylenic acid	8.05	<172.36	2 of 2	<172.36	2 of 2	>428.88	+	R	M	Natsch et al. (2013)
Ethylene glycol dimethacrylate	31.84	<120.56	3 of 3	<120.56	3 of 3	>300	+	R	M	Natsch et al. (2013)
Pyridine	1.46	>2400	0 of 4	>2400	0 of 4	>2400	–	R	M	Natsch et al. (2013)
Aniline	1.04	>2400	0 of 2	>2400	0 of 2	>2400	–	R	?	Natsch et al. (2013), Bauch et al. (2012)
Methyl methacrylate	7.33	<1205.63	2 of 2	<1205.63	2 of 2	>3000	+	R	?	KeratinoSens Invittox (2010), Bauch et al. (2012)
4-Nitrobenzyl bromide	3.18	<0.88	3 of 3	<0.88	3 of 3	>2.18	+	R	M	Natsch et al. (2013)
Butyl 4-hydroxy benzoate	4.69	<22.10	3 of 3	<22.10	3 of 3	>55	+	R	ND	
Methyl 4-hydroxy benzoate	6.70	<381.78	3 of 3	<381.78	3 of 3	>950	+	R	M	Natsch et al. (2013)
Sorbic acid	4.20	<964.51	3 of 3	1893.00	3 of 3	>2400	+	R	ND	
Luperox A75	2.28	41.50	1 of 4	46.82	0 of 4	>49.8	–	NR	ND	
Butyl glycidyl ether	4.80	<72.34	3 of 3	<72.34	3 of 3	>180	+	R	M	Natsch et al. (2013)
Ethylparaben	3.67	<108.51	3 of 3	<108.51	3 of 3	>270	+	R	ND	
2-Bromo-2-nitro-1,3-propanediol	5.47	<29.18	3 of 3	38.84	3 of 3	72.10	+	R	ND	
Xylene	1.43	>578.70	0 of 4	>578.70	0 of 4	814.45	–	R	M	Natsch et al. (2013)
Glycerol	1.16	>2400	0 of 3	>2400	0 of 3	>2400	–	R	M	Natsch et al. (2013)
1,2-Propanediol	1.21	>2400	0 of 2	>2400	0 of 2	>2400	–	R	M	Natsch et al. (2013)
4-Hydroxybenzoic acid	1.06	>2400	0 of 2	>2400	0 of 2	>2400	–	R	M	Natsch et al. (2013)
4-Methoxy acetophenone	8.97	<964.51	2 of 2	<964.51	2 of 2	>2400	+	R	M	Natsch et al. (2013)
6-Methylcoumarin	3.54	<120.56	3 of 3	141.48	3 of 3	>300	+	R	M	Natsch et al. (2013)
Chlorobenzene	1.48	>2000	0 of 4	>2000	0 of 4	1989.00	–	R	M	Natsch et al. (2013)
DL-lactic acid	0.98	>7000	0 of 2	>7000	0 of 2	>7000	–	R	M	Natsch et al. (2013)
Fumaric acid	1.27	>2400	0 of 3	>2400	0 of 3	>2400	–	R	M	Natsch et al. (2013)
D(+)glucose	1.07	>3000	0 of 3	>3000	0 of 3	>3000	–	R	M	Natsch et al. (2013)
Isopropanol	1.23	>3000	0 of 3	>3000	0 of 3	>3000	–	R	M	Natsch et al. (2013)
Methyl salicylate	2.22	<803.76	3 of 4	1851.96	1 of 4	>2000	+	NR	MM	Natsch et al. (2013)
n-Butanol	1.62	2400.00	1 of 4	>2400	0 of 4	>2400	–	NR	M	
n-Hexane	1.23	>2400	0 of 3	>2400	0 of 3	>2400	–	R	?	Natsch et al. (2013), Bauch et al. (2012)
Nickel chloride	0.79	>261.20	0 of 3	>261.20	0 of 3	>261.20	–	R	?	Natsch et al. (2013), Bauch et al. (2012)
4-Aminobenzoic acid	1.11	>2400	0 of 2	>2400	0 of 2	>2400	–	R	M	Natsch et al. (2013)
Propyl-4-hydroxybenzoate	3.94	<65.104	2 of 2	<65.104	2 of 2	>162	+	R	ND	
Salicylic acid	0.80	>3000	0 of 3	>3000	0 of 3	>3000	–	R	M	Natsch et al. (2013)
Sulfanilamide	1.08	>2400	0 of 2	>2400	0 of 2	>2400	–	R	M	Natsch et al. (2013)
Vanillin	1.48	>1650	0 of 3	>1650	0 of 3	>1650	–	R	M	Natsch et al. (2013)
Hexadecyltrimethylamonium bromide	0.95	>0.69	0 of 4	>0.69	0 of 4	1.03	–	R	ND	

Table 2 (*continued*)

Test substance	I_{max} (fold)	Concentration (μM) for 1.5 fold induction	Number of EC1.5 positives	Concentration (μM) for 2 fold induction	Number of EC2.0 positives	Concentration (μM) causing 50% cytotoxicity	LuSens prediction	Reproducibility	Comparison with KeratinoSens™	References
Tween 80	3.76	<65.91	4 of 4	<65.91	3 of 4	>164	+	R	M	Natsch et al. (2013)
Diethyl phthalate	3.37	<516.41	0 of 3	516.41	0 of 3	>1285	–	R	M	Natsch et al. (2013)
Sodium benzoate	1.01	>2400	0 of 3	>2400	0 of 3	>2400	–	R	ND	
Benzyl alcohol	1.93	<964.51	2 of 3	>2400	1 of 3	>2400	+	NR	ND	

Table 3
Summary of predictivity of LuSens assay based on 69 test substances.

In comparison to		
	Human	LLNA
n	69	72
Sensitivity	83%	74%
Specificity	81%	74%
PPV	88%	86%
NPV	76%	56%
Accuracy	83%	74%

92% for 63 compounds in total (for which data for both assays were available). A concordance of 94% was reached for the 52 test compounds for which human data from the literature were available and 93% for the 61 test compounds from which LLNA data were available. From these 52 compounds, for which human data were available, non-concordant data (mismatch) were observed for three substances. In the LuSens methyl salicylate (non to very weak sensitizer in humans) tested positive, ethylenediamine (prohapten) and propyl gallate (both sensitizers in humans) tested negative. The discordant results with methyl salicylate are probably the consequence of borderline readouts in both assays. It is worth noticing that non-concordant data are also reported for substances tested multiple times in the same assay since some variations within the same system are expected under the frame of technical or biological difference (intra-laboratory variation).

In the case of the substances predicted as false negative, ethylenediamine and propyl gallate both substances were rated as positive in the KeratinoSens™ assay but negative in the LuSens assay. KeratinoSens™ only requires one single concentration of the test substance yielding an induction higher than 1.5 fold whereas LuSens requires at least two consecutive concentrations.

Seven substances did not yield consistent results in the data previously published for KeratinoSens™ assay, i.e., tartaric acid, hexane, nickel chloride, methylmethacrylate, aniline, eugenol and α-hexyl cinnamic aldehyde (Natsch et al., 2013; Bauch et al., 2012; KeratinoSens Invittox, 2010). The results of these substances were therefore not used for comparisons to LuSens data (Table 2).

For 57 of the 61 compounds with available LLNA data consistent results in both the LuSens and KeratinoSens™ resulted in 93% concordance among both assays. Only four test substances did not provided concordant data among both assays, i.e., methyl salicylate, farnesal, ethylenediamine and propyl gallate. For these substances the LuSens assay results were consistent with the LLNA results. For additional seven substances no comparison was possible since data in the literature were not concordant for following substances in the KeratinoSens™ assay: tartaric acid, n-hexane, nickel chloride, methylmethacrylate, aniline, eugenol and α-hexyl cinnamic aldehyde (Natsch et al., 2013; Bauch et al., 2012; KeratinoSens Invittox, 2010; Natsch et al., 2011, Table 2).

According to the performance standard guidance for KeratinoSens™, the value for similar or modified test methods is required to be equal to or greater than 80% based on the data of three independent experiments for 12 reference substances (OECD 2014). In the LuSens assay all but one substance of these 12 reference substances were reproducible (92%) and hence the criteria for the intralaboratory reproducibility fully met and almost identical to the overall within laboratory reproducibility described above for the 74 compounds assessed.

3.5. LuSens assay as part of a testing strategy

The pragmatic 'weight of evidence' approach predicts skin sensitization potential based on two of three tests addressing protein reactivity (e.g., DPRA), keratinocyte ARE activation (e.g., LuSens) and dendritic cell activation (e.g., h-CLAT); i.e., concordant results of two tests then determines whether a substance is considered to be a sensitizer or not (if at least two tests are negative the substance is assessed as a being a non-sensitizer and vice versa; Bauch et al., 2012). When the LuSens results are used in combination with previously reported data for DPRA and mMUSST and compared to LLNA data, the sensitivity is 75%, specificity is 94% and the accuracy is 81% (43 of 53 correct predictions) (Table 4). 9 Substances would be rated as false negative: phthalic anhydride, α-hexyl cinnamic aldehyde, tartaric acid, farnesal, SDS, pyridine, aniline, xylene and 6-methylcoumarin, whereby only nickel chloride as false positive. Similar calculations were performed for the combination of DPRA, LuSens and h-CLAT (data are summarized in Table 4). When comparing the results of this set of chemicals to human data, the LLNA had 96% sensitivity, 81% specificity and an accuracy of 90% indicating that the LLNA also did not correctly predict the sensitization potential for humans for all chemicals tested. When compare to human data the combination of LuSens, DPRA and mMUSST yields, the predictivity of the combination yields sensitivity, 82%, specificity of 100% and accuracy of 92% (45 of 50 correct predictions) (Table 4, compare to human data), Five substances would be rated as false negative: phtalic anhydride, tartaric acid, α-hexyl cinnamic aldehyde, undecylenic acid, and aniline.

4. Discussion

Although animal tests encompass all steps leading to an adverse reaction, they remain a "blackbox" as the final reactions are monitored but no knowledge is generated elucidating the pathways leading to these effects. When developing new toxicological tests, the current approach is therefore to understand the key steps leading to the reactions assessed by the toxicological endpoint and to utilize this knowledge to optimize tests and test strategies. According to the OECD, "an adverse outcome pathway (AOP) is the sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an *in vivo* outcome of interest". Skin sensitization is a multistep process and the key steps involved are fairly well understood (EURL ECVAM, 2013a). The OECD recently published a document describing adverse outcome pathway for skin sensitization

Table 4
Summary of predictivities of single assays and combinations thereof: LuSens assay and other assays (OECD QSAR toolbox, Direct Peptide Reactivity Assay (DPRA), modified myeloid U937 skin sensitization test (mMUSST), human cell line activation test (h-CLAT). Data were obtained from Bauch et al., 2012). These figures are based on 50 test substances (out of a total of 69) for which all data are available.

Compared to human						
		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
LLNA		96	81	87	94	90
Single assays	OECD QSAR toolbox	64	100	100	69	80
	DPRA	89	82	86	86	86
	LuSens	79	82	85	75	80
	KeratinoSens™	86	73	80	80	80
	mMUSST	68	100	100	71	82
	h-CLAT	75	77	81	71	76
Combinations	DPRA and LuSens	96	64	77	93	82
	DPRA and KeratinoSens™	100	59	76	100	82
	DPRA and mMUSST	93	82	87	90	88
	DPRA and h-CLAT	96	59	75	93	80
	LuSens and mMUSST	93	82	87	90	88
	LuSens and h-CLAT	93	73	81	89	84
	KeratinoSens™ and MUSST	90	56	77	77	77
	KeratinoSens™ and h-CLAT	93	64	76	88	80
Prediction model (high overall accuracy)	DPRA. LuSens and mMUSST	82	100	96	84	90
	DPRA. KeratinoSensTM and mMUSST	86	95	96	84	90
	DPRA. LuSens and h-CLAT	86	86	89	83	86
	DPRA. KeratinoSensTM and h-CLAT	89	82	86	86	86

Compared to LLNA						
		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Single assays	OECD QSAR toolbox	56	100	100	52	70
	DPRA	81	76	88	65	79
	LuSens	69	82	89	56	74
	KeratinoSens™	83	76	88	68	81
	mMUSST	64	94	96	55	74
	h-CLAT	72	76	87	57	74
Combinations	DPRA and LuSens	89	59	82	71	79
	DPRA and KeratinoSens™	94	59	83	83	83
	DPRA and mMUSST	86	76	89	72	83
	DPRA and h-CLAT	89	53	80	69	77
	LuSens and mMUSST	83	76	88	68	81
	LuSens and h-CLAT	86	71	86	71	81
	KeratinoSens™ and MUSST	89	71	86	75	83
	KeratinoSens™ and h-CLAT	89	65	84	73	81
Prediction model	DPRA, LuSens and mMUSST	75	94	96	64	81
	DPRA, KeratinoSens™ and mMUSST	81	88	94	68	83
	DPRA, LuSens and h-CLAT	81	88	94	68	83
	DPRA, KeratinoSens™ and h-CLAT	83	82	91	70	83

describing key steps in the sensitization process thereby facilitating the development of new toxicological test methods and ITS to assess skin sensitization (ENVJMV/MONO, 2012). Among the steps defined, protein binding, keratinocyte and dendritic cell activation are key initiating steps in the sensitization process.

In this study, a new stable ARE reporter gene assay based on a human keratinocyte cell line, LuSens, was developed and evaluated for its use in the identification of skin sensitizers. The LuSens assay provides information on both protein reactivity and keratinocyte activation. This is accomplished by monitoring the activity of luciferase, which is regulated by the ARE of the rat *Nqo1* gene located upstream the luciferase reporter gene, following contact to a test substance. Protein reactivity of a substance can be indirectly assessed, as reactivity with the cysteine residues of Keap1 leads to the dissociation of Nrf2 and its subsequent binding to ARE and the expression of the downstream genes, in this case luciferase. As described extensively in the literature, ARE plays a crucial role in the activation of cytoprotective genes in the elicitation of the toxicity pathway induced by skin sensitizers (Natsch and Emter, 2008; Johnson et al., 2008) and therefore cell activation can be assessed by the expression of luciferase. Since keratinocytes are the first cells exposed to a substance when skin contact occurs, the LuSens assay was designed using the primary target cells of the skin, namely the keratinocytes, and thereby gives a measure of their activation as cellular event leading to skin sensitization.

The validity criteria of the LuSens assay described here were refined from the initially used criteria (Bauch et al., 2012). The assay avoids the use of concentrations inducing toxicity greater than 30%. For this purpose, the assay includes a range finding experiment to select the concentration range that will be used in the main experiment and thereby avoids testing of unneeded toxic concentrations. In addition, a rigorous prediction model was applied, meaning that sensitizer compounds were only those that had the capacity to induced $\geqslant 1.5\times$ luciferase induction in two consecutive test concentrations and at least half of the tested concentrations should have yielded a viability $\geqslant 70\%$. Substances that induced luciferase expression only in one concentration or in non-consecutive concentrations were considered to be non-sensitizers.

Although the validation of the assay has been designed to be used with the SteadyGlo™ luciferase assay system to measure luciferase activity, recent data from our laboratory with a small set of test compounds (i.e., 8 compounds in at least 2 independent experiments, data not shown) demonstrated the same proficiency when using OneGlo™ luciferase detection system. This detection

system is reported by the producer to be more robust (Promega, 2007), as some detection difficulties can be encountered depending on the equipment used when using the SteadyGlo™ luciferase assay (personal communication). However, in our hands, both luciferase detection system delivered comparable data, but the OneGlo™ system provided a stronger signal.

The LuSens assay shows good accuracy, sensitivity and specificity values that are comparable to those of the KeratinoSens™ assay. The luciferase reporter gene in the LuSens assay is under the control of the ARE-element of *Nqo1* gene from the rat, which is activated by the Nrf2 pathway and is comparable to the ARE from human *AKR1C2* gene that has been cloned into KeratinoSens™ (Nioi and Hayes, 2004; Natsch and Emter, 2008). In addition, LuSens cell-line used in this study was derived from the selected clone which gave the best signal to noise ratio of the light output of luciferase induction, and which showed good luciferase induction when treated with weak sensitizers. The latter is also reflected in the use of the weak sensitizer EGDMA as a positive control of the assay. Recent experiments in which the cells have been cultured in the absence of puromycin for more than 15 cell passages exhibited the same proficiency towards a small set of skin sensitizers (Fig. 3), an indication that the inserted sequence was stably inserted into the cells.

Based on the established validity criteria, an analysis of the predictivity of the method was conducted with a set of 74 substances comprising 42 skin sensitizers with a wide range of potencies and 27 non-sensitizing substances comprised of various industrially used substances and cosmetic ingredients. Human data were available for 69 substances and LLNA data was available for 72 substances. An overall accuracy of 83% was achieved when compared to human data. Seven false negative predictions were found indicating limited applicability for acyl transferases (3 of 7 FN predictions), prohaptens (3 of 9 FN predictions, whereby the pro Michael acceptors, except propyl gallate, were correctly predicted), and the difficulty to predict nickel chloride (false negative in the LLNA). In both the KeratinoSens™ and LuSens assays, phthalic anhydride is not correctly predicted. In the DPRA, phthalic anhydride exhibits virtually no cysteine binding but a strong reactivity to lysine (Gerberick et al., 2008). As the LuSens assay assesses the activation of the ARE dependent gene expression in keratinocytes by modification of a cysteine in the Nrf2 protein, some molecular or cellular events contributing to skin sensitization may be missed. This may, however, be balanced by using a testing battery of three assays addressing several steps of the adverse outcome pathway and in particular the DPRA which assesses both cysteine and lysine reactivity.

Other limitations of the LuSens assay, as is the case with most cell-based methods, solubility and cytotoxicity of the substance can limit the applicability as the cells are cultured in aqueous medium. The metabolic capacity of the cells which is required to activate certain pro-haptens is not identical to the metabolic capacity of native skin (Götz et al., 2012; Jäckh et al., 2012; Fabian et al., 2013).

There are limitations to any toxicological test method, and these must be considered to reliably ensure predictions of potential hazards to human health. The LLNA is the method of choice to assess the skin sensitization potential of a substance under REACH. The predictive accuracy of the LLNA, compared to human data was 72% (n = 74) based on the formal validation conducted by ICCVAM (http://ntp.niehs.nih.gov/iccvam/docs/immunotox_docs/llna/llna-rep.pdf). To date, no non-animal test method has been accepted as a full replacement of the animal based tests for skin sensitization. Even those currently advanced in validation process (DPRA, KeratinoSens™, h-CLAT) will not achieve regulatory acceptance as a stand-alone method but they will find use in ITSs. The combination of these assays should be acceptable to fully replace animal testing for the identification of a skin sensitization potential in the near future in particular in the context of an AOP-based IATA. In a recent workshop with regulators (ECHA and member state representatives) and industry, it was agreed that an ITS should preferably be composed of methods which had a sound mechanistic rationale, e.g., by reflecting key steps in the AOP (Basketter et al., 2013). Two studies with large databases have demonstrated, that a combination of these or similar assays covering three key steps of the AOP offers a high predicitivity of the skin sensitization potential (hazard) even when using a simple prediction model such as "2 out of 3" weight of evidence approach (also sometimes termed the "majority vote" approach; Bauch et al., 2012; Natsch et al., 2013).

The LuSens assay (as does the KeratinoSens™ assay) contributes to the 3R principles as it addresses one step of the adverse outcome pathway, namely keratinocyte activation. ITSs using combinations of the LuSens assay with other non-animal tests can result in predictivities comparable to or even higher than the LLNA. In Phase 3 of REACH an evaluation of the sensitization hazard for thousands of substances produced at 1–100 tons will need to be submitted by spring 2018. Bearing this in mind, the application of the LuSens assay in a testing battery can quite conceivably significantly contribute to the reduction of the number of animals used for REACH.

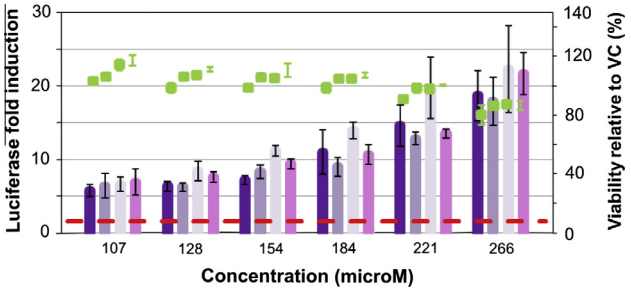

Fig. 3. Effect of increasing concentrations of the weak sensitizer, EGDMA on the luciferase expression (bars) and viability (green squares) of LuSens cells clone 16 that have been culture without the puromycin antibiotic as selection pressure. Illustrated are results of four independent experiments.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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