Validation and Application of the KeratinoSens Assay, a Novel *In Vitro* Skin Sensitization Assay

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ABSTRACT

Background

Determination of the potential for individual chemicals and product ingredients to induce allergic contact dermatitis (skin sensitization) is a key toxicological endpoint for the screening of novel ingredients used in consumer and industrial products. Although *in vivo* methods exist to evaluate the skin sensitization potential of chemicals, *in vitro* non-animal test methods have been developed using human cell lines to predict human skin sensitizers. *In vitro* human cell-based systems have been developed in response to international regulatory requirements prohibiting the use of animals in research, and to meet the needs of corporations proactively choosing to eliminate the use of animals in safety testing. The KeratinoSens assay was developed by Givaudan, and recently evaluated in an international multi-laboratory validation exercise. The KeratinoSens assay is a human immortalized keratinocyte cell-based reporter gene assay which is designed to identify chemicals likely to induce skin sensitization in humans. A feature of all chemical allergens is their intrinsic electrophilicity (or their potential to be transformed to electrophilic chemicals) and their reactivity with skin proteins to form haptens.

Materials and Methods

Mechanistically, the intercellular Nrf-2-electrophile sensing pathway comprised of the repressor protein Keap1, the transcription factor Nrf2, and the antioxidant response element (ARE), is capable of detecting skin sensitizers. In the KeratinoSens assay, the induction of a luciferase gene, under the control of the antioxidant response element (derived from the human gene AKR1C2 gene) is determined by measuring the relative light output of treated cells. In parallel, viability of the treated cells is measured using the MTT assay.

Results and Discussion

In the "ring trial" validation, 28 chemicals (19 sensitizers of varying potencies, and 9 non-sensitizers) were evaluated in 5 laboratories, and at least 3 experiments per chemical. The predictive capacity of the assay was found to be similar between labs and ranged from 85.7% to 96.4%. Subsequent application of the assay is targeted at further defining the applicability and predictivity of the assay by testing more neat chemicals, chemical mixtures, industrial solvents, and complex product matrices. Thus far, over 150 chemicals have been evaluated using the KeratinoSens assay and the results indicate a good predictive value (~79.5%). The results indicate that the KeratinoSens assay may be a relevant and reliable method for evaluating a broad range of materials. The presentation will highlight the assay performance and lessons learned from the validation program.

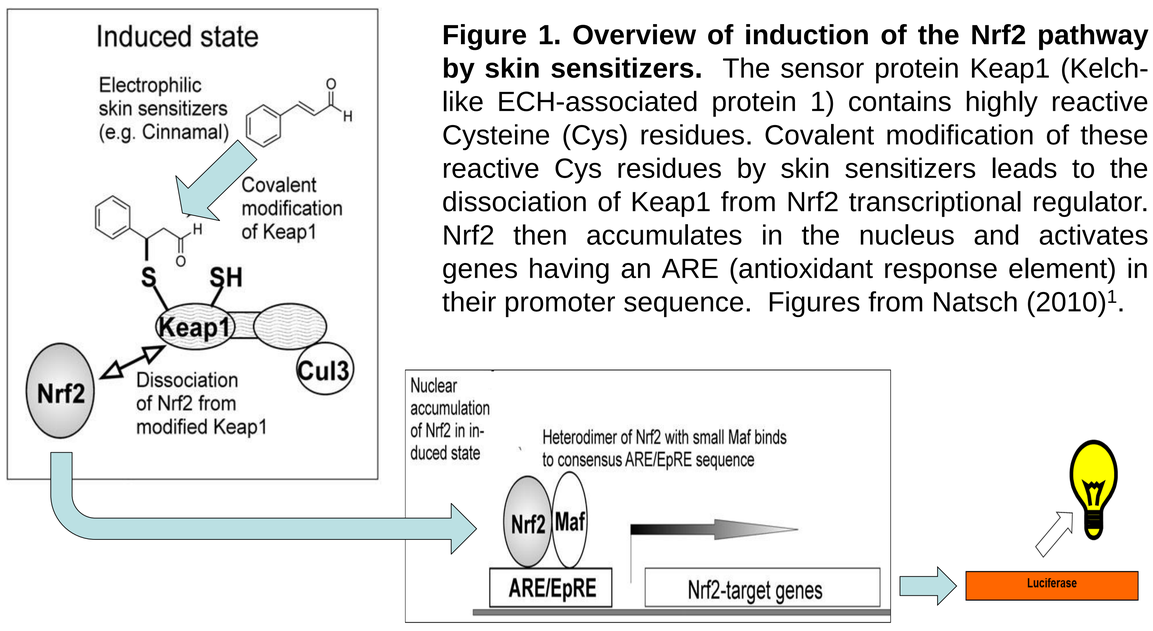

Figure 1. Overview of induction of the Nrf2 pathway by skin sensitizers. The sensor protein Keap1 (Kelch-like ECH-associated protein 1) contains highly reactive Cysteine (Cys) residues. Covalent modification of these reactive Cys residues by skin sensitizers leads to the dissociation of Keap1 from Nrf2 transcriptional regulator. Nrf2 then accumulates in the nucleus and activates genes having an ARE (antioxidant response element) in their promoter sequence. Figures from Natsch (2010)[1].

MATERIALS AND METHODS

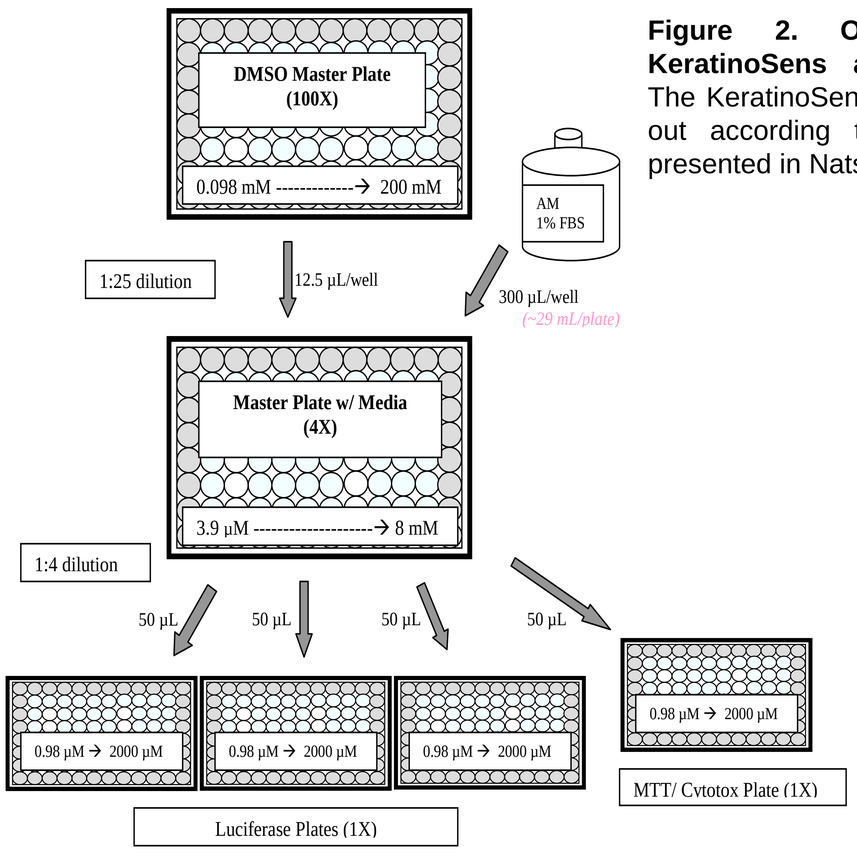

Figure 2. Overview of the KeratinoSens assay procedures. The KeratinoSens Assay was carried out according to Givaudan SOPs presented in Natsch *et al*.(2011)[2].

Cells: Transfected HaCaT cells were obtained from Givaudan.

Compounds: 28 chemicals selected from Casati, *et al*., 2009, including many chemicals from the Sens-it-iv consortium and all chemicals from the LLNA Performance Standards (ICCVAM).

Testing Labs: Lead Laboratory (Givaudan Schweiz), and 4 naïve laboratories.

Validation Study Design: A Ring Trial divided into 2 phases: Phase I (7 compounds) Method Transfer (MT) to evaluate transferability of the method to laboratories, Phase II (21 compounds) Blind Coded chemical study (BC). Three (3) independent trials per chemical.

Endpoints: Gene induction was compared to DMSO controls. Doses with statistically significant induction over the threshold of 1.5 (i.e. 50% enhanced gene activity) were determined (EC1.5).

The maximum fold-induction (Imax); EC1.5 induction; and IC_{50} cytotoxicity values were calculated.

Prediction Model: chemicals were predicted to have sensitization potential if (*i*) an EC1.5 value below 1000 µM in at least 2 of 3 independent trials is determined, (*ii*) viability is >70% at the lowest concentration tested above the EC1.5, and (*iii*) there is an apparent overall dose-response for luciferase induction, which is similar among the independent trials.

RESULTS

Table 1. The predictive capacity in different labs. If ≥2 of 3 trials are positive and overall dose response is given in all trials, chemical is predicted positive (red and orange). If ≤1 trial is positive and dose response is not evident, chemical is predicted negative (light and dark green). The induction at cytotoxic concentrations for SDS was not considered positive.

Phase I: Method Transfer chemicals (MT) Phase II: Blind Coded study chemicals (BC)

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

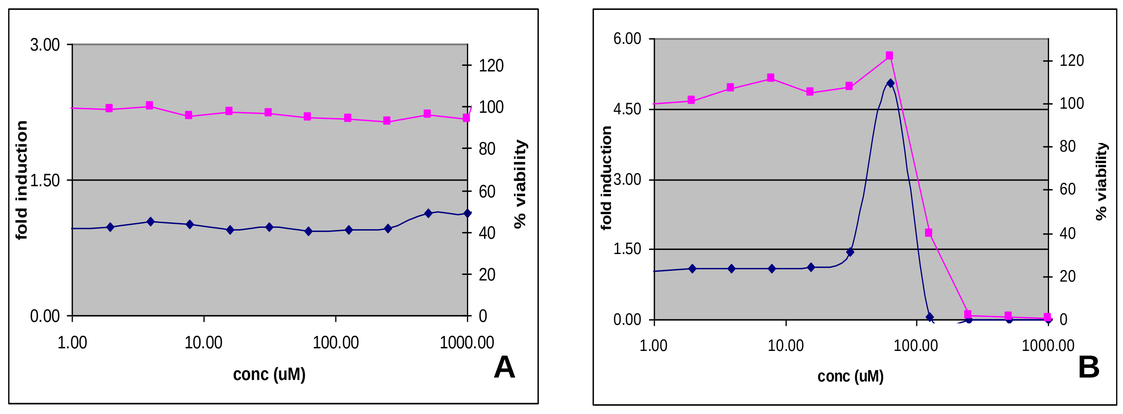

Figure 3. Representative graphs of the induction of luciferase activity (blue) and cellular viability (pink) in dose-response analysis. Panel A is the dose-response of a chemical which is negative (non-sensitizing) and has no affect on cellular viability. Panel B is the dose-response of a chemical which is positive (EC 1.5) and is cytotoxic at higher doses.

CONCLUSIONS

Transferability: Phase I showed that the methods were highly transferable between labs. No "face-to-face" training of naïve labs was required.

Optimization: Lessons learned from Phase I resulted in method improvements, application of "flash" and "glow" luminescence endpoints, and selection of low cross-talk black-wall plates.

Predictive Capacity: Similar between labs, and more importantly, the quantitative dose-response data were reproduced in the participating laboratories.

Reliability: Between-laboratory variability for EC1.5 values was only slightly above the within-laboratory variability, indicating that transfer of the assay did not affect the results significantly.

Post Ring-trial Performance: 150+ chemicals evaluated with continued good predictive value (~79.5%) (includes chemicals outside of fragrance chemical domain).

Regulatory Application: EURL-ECVAM (2013): proposes assay may be used in an integrated testing strategy to identify sensitizers. OECD: Draft Test Guideline in process.

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