KeratinoSens™

In Vitro Skin Sensitisation Assay (ARE-Nrf2 Luciferase Test Method)

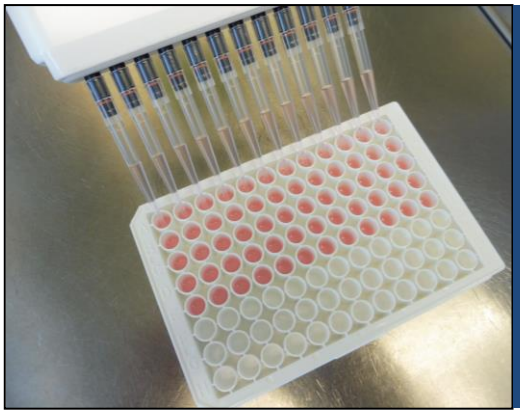
The *in vitro* KeratinoSens™ assay adresses the second molecular key event of the adverse outcome pathway (AOP) of skin sensitisation [1]. The UN GHS (United Nations Globally Harmonized System of Classification and Labelling of Chemicals) defines a skin sensitiser as a substance that will cause an allergic response after skin contact [2].

The KeratinoSens™ assay is validated by the EURL ECVAM (European Union Reference Laboratory for Alternatives to Animal Testing) and is performed in accordance with the OECD guidance OECD 442D at Eurofins BioPharma Product Testing Munich GmbH [1, 3] with chemicals, cosmetics or personal care products and pharmaceuticals.

The KeratinoSens™ is one of three test methods (DPRA and h-CLAT) for the assessment of skin sensitisation potential.

Assessment of Skin Sensitisation Potential with the KeratinoSens™

- The second molecular key event of skin sensitisation addresses the induction of cyto-protective signaling pathways like the Keap1-Nrf2-ARE pathway in keratinocytes in responds to electrophiles and oxidative stress.
- The effect on the antioxidant response element (ARE) dependent pathway is assessed by measuring the induction of the luciferase gene, an ARE-dependent gene product, with luminescence detection.
- The luciferase signal reflects the activation of endogenous Nrf2-dependent genes which are triggered by sensitisers. [1, 3, 4]
- An MTT assay is performed, too, to see if the cells are stressed or if the elevation of the luminescence is due to sensitising properties of the test item.



"Skin sensitisers have been reported to induce genes that are regulated by the antioxidant response element (ARE). Small electrophilic substances such as skin sensitisers can act on the sensor protein Keap1, by *e.g.* covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2. The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes." [1] As a result, a higher luciferase activity is present and the cells are viable >70%.

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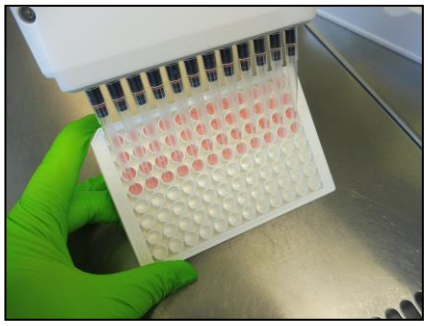

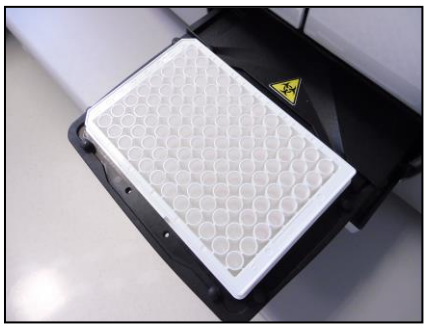

Procedure

Principle of the KeratinoSens™

Protocol	
Cell line	KeratinoSens™ cell line Immortalised adherent human keratinocytes (HaCaT) stably transfected with a selectable plasmid
Analysis	Induction of luciferase reporter gene expression measured by luminescence Cell viability determination
Concentrations	Triplicates of 12 stock solutions Final concentrations range from 0.98 to 2000 µM Alternative concentrations possible (*e.g.* bad solubility)
Exposure time	48 h
Quality controls	Positive control: cinnamic aldehyde (4 to 64 µM) Solvent control: 1% dimethylsulfoxid, medium, 1% tetrahydrofuran
Solvents of test chemical	Dimethylsulfoxid (Sterile) water Tetrahydrofuran
Application	Two independently performed experiments Equivocal results require a third repetition
Data delivery	Maximal fold induction (I_{max}) $EC_{1.5}$ value of luciferase activity IC_{30} and IC_{50} values of cell viability Dose response curves for luciferase activity induction and cell viability
Positive prediction	Significant luciferase activity induction >1.5 fold $EC_{1.5}$ is <1000 µM Cell viability >70% Dose response for luciferase induction

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Data

Eurofins data for demonstration technical proficiency of the KeratinoSens™ Assay

Chemical	$EC_{1.5}$* (OECD)	IC_{50} # (OECD)	Prediction (OECD)	$EC_{1.5}$* (EF)	IC_{50} # (EF)	Prediction (EF)
Non-sensitising Chemicals						
Isopropanol	>1000	>1000	**negative**	>2000	>2000	**negative**
Salicylic acid	>1000	>1000	**negative**	>2000	>2000	**negative**
Lactic acid	>1000	>1000	**negative**	>2000	>2000	**negative**
Glycerol	>1000	>1000	**negative**	>2000	>2000	**negative**
Sensitising Chemicals						
Cinnamyl alcohol	25-175	>1000	**positive**	139.01	>2000	**positive**
Ethylene glycol dimethacrylate	5-125	>500	**positive**	35.53	1811.39	**positive**
2-Mercapthobenzothiazole	25-250	>500	**positive**	91.92	>2000	**positive**
Methyldibromo glutaronitrile	<20	20-100	**positive**	9.74	28.46	**positive**
4-Methylaminophenol sulfate	<12.5	20-200	**positive**	8.07	25.12	**positive**
2,4-Dinitro-chlorbenzene	<12.5	5-20	**positive**	3.19	12.16	**positive**

* = µg/mL; # = µM EF = Eurofins Munich GmbH

Table 1: Eurofins data of the KeratinoSens™ – ten tested proficiency chemicals compared to the data of the OECD guideline [1].

In Table 1 the obtained data from the KeratinoSens™ assay of four non-sensitising and six sensitising chemicals are shown. The prediction of all tested chemicals was correct in comparison to the classification of the OECD guideline.

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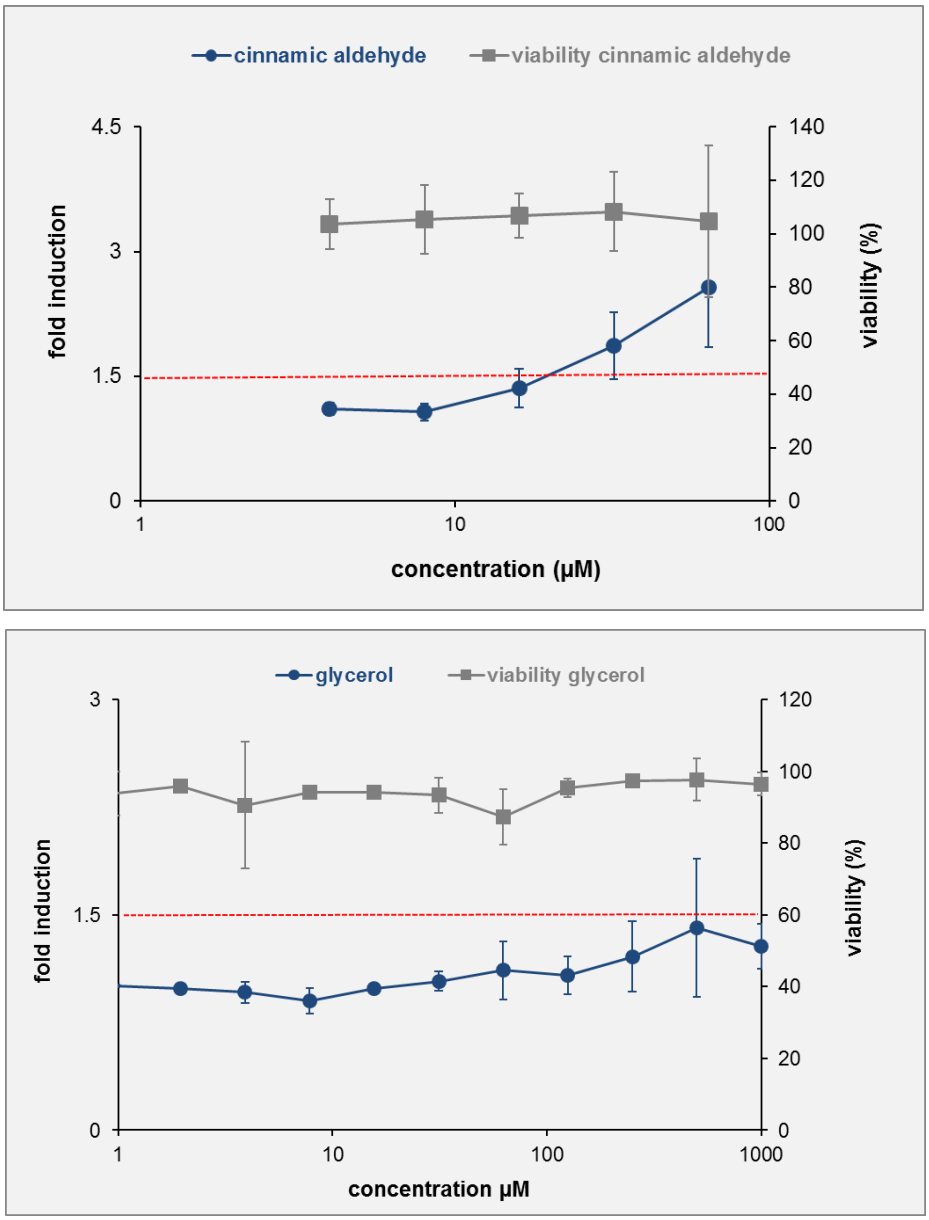

Figures 1 and 2: Eurofins KeratinoSens™ data of the fold induction and the viability of the sensitiser cinnamic aldehyde (Figure 1) and the non-sensitiser glycerol (Figure 2).

The graphs show the induction of the luciferase gene expression (blue line) and MTT data (grey line) used to determine cytotoxicity of the chemicals cinnamic aldehyde and glycerol. The red line represents the threshold of luciferase induction, at which chemicals are classified as sensitisers.

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References

1) OECD Guidelines for Testing of Chemicals, number 442d “In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method” (adopted: June 25, 2018).

2) UN (2015), United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Sixth revised edition, UN New York and Geneva.

3) EURL-ECVAM (2014). Recommendation on the KeratinoSens™ assay for skin sensitization testing, 42 pp.

4) Emter R., Ellis G., Natsch A.(2010). Performance of a novel keratinocyte-based reporter cell lineto screen skin sensitizers in vitro. Toxicology and Applied Pharmacology 245, 281-290.