KeratinoSens® - Skin Sensitization Testing using Prequalified Assay Ready Cells

summary

The activation of the ARE/Nrf2 pathway is one of the key events in the complex cascade of skin sensitization. To evaluate the skin sensitizing potential of cosmetic ingredients, Givaudan has developed a luciferase reporter gene cell line, based on human keratinocytes (KeratinoSens®), which monitors the activation of the Nrf2 antioxidant response pathway by sensitizing chemicals. The assay protocol has been validated by the ECVAM and was adopted by OECD (TG 442D) as one alternative method for *in vitro* skin sensitization testing. According to Annex 3 of the DB-ALM protocol n° 155, viability and luciferase expression can be measured simultaneously on the same cells (multiplexing). Here we demonstrate, that the KeratinoSens® assay can be successfully performed with prequalified Assay Ready Cells, which are used like a reagent instantly after thawing instead of continuously cultured cells. Furthermore, the use of Assay Ready KeratinoSens® cells in a multiplexed approach is shown. To proof technical proficiency, skin sensitizing chemicals recommended by the OECD guideline were used.

KeratinoSens® Assay Ready Cells

The KeratinoSens® cells have been developed by stable transfection of a human keratinocyte cell line (HaCaT) with a luciferase reporter gene (pGL2, Promega) controlled by a Nrf2 responsive enhancer element from the AKR1C2 gene. Under non-stressed conditions, the Nrf2 transcription factor is kept in the cytoplasm by binding to Keap1. This binding leads to ubiquitination and degradation of Nrf2 and therefore to inhibition of the antioxidant response. Sensitizing chemicals bind to Keap1 and release Nrf2 into the nucleus, where it binds to the promoter region of the antioxidant response element (ARE). It binds to the promoter region of the antioxidant response element (ARE) and transcriptionally activates the expression of detoxifying enzymes and transporters, and in the reporter cell line luciferase (Fig. 1).

For the preparation of Assay Ready KeratinoSens®, the cells were expanded close to passage 20 according to the DB-ALM protocol. The KeratinoSens® cells were enzymatically detached by Accutase®, resuspended in cryopreservation medium containing 5% DMSO and quickly dispensed into cryovials at 10 million cells/vial using an automated decapping and filling device (FluidX XSD, Brooks). Cryopreservation was performed in a controlled rate freezer (Cryomed 7452, ThermoFisher) using a specifically optimized freezing protocol. The ready-to-use aliquots of KeratinoSens® cells were stored in liquid nitrogen until use.

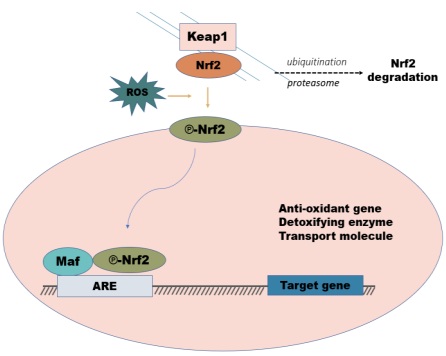

Fig. 1: Nrf2-Keap1-ARE Pathway

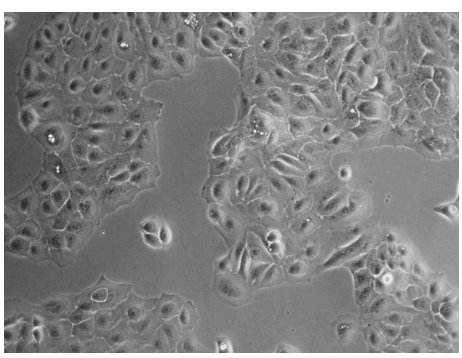

Fig. 2: Assay Ready KeratinoSens® Cells

prequalification of Assay Ready Cells

To control the quality of the Assay Ready Cells aliquots of the cells were quickly thawed in a water bath at 37°C. The cells recovered well and had an average viability of 97% after thawing and entered proliferation without a significant lag phase, whereby the growth was at 95% compared to a logarithmically growing culture. The cells attached quickly overnight and display a typical morphology of epithelial cells (Fig. 2).

To proof technical proficiency 8 of 10 proficiency substances (Tab. 1) must be correctly determined. Therefore, Assay Ready KeratinoSens® Cells were seeded directly after thawing into 96-well plates at 10.000 cells/well. For comparison, cells from a continuously passaged culture were prepared the same way. The cells were incubated at 37°C for 24h to attach before the supernatant was replaced by serial dilutions of the proficiency substances. After 48 hours of incubation with the sensitizers cell viability and the activation of the Nrf2 pathway was analyzed either in parallel plates (classic) or multiplexed in the same plate and well. Viability was determined by Resazurin, a dye which is metabolized in

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living cells to fluorescent Resorufin and which was quantified in a multimode reader (Safire 2, Tecan) at 540nm/590nm. For the multiplex approach the dye was removed afterwards and luciferase substrate in lysis buffer was added to the cells (One-Glo™ luciferase system, Promega). Luminescence was detected after 20min with an integration time of 1s (Safire 2, Tecan).

Assay Ready KeratinoSens® Cells treated with the substances displayed a response very much comparable to cells from a continuous culture, in the classic as well as in the multiplexed approach. Viability was not differently affected, and the sensitizers activated the Nrf2 pathway to the same extent in Assay Ready Cells as in continuously cultured cells. Non-sensitizers did not activate the pathway in any way (Fig. 3). Assay Ready Cells also did not display elevated levels of basal Nrf2 activation. For all tested substances, the correct KeratinoSens® prediction, $EC_{1.5}$ and IC_{50} values according to the OECD guideline could be obtained, except MBT had a lower IC_{50} value than expected (Tab. 1).

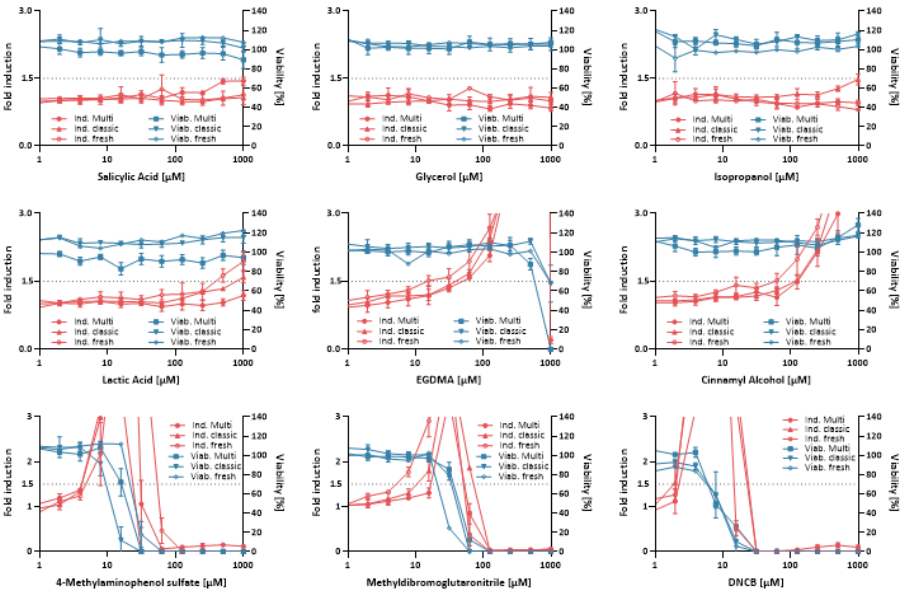

Fig. 3: Comparison of Assay Ready Cells in the OECD adopted approach (classic) and in the multiplex approach (Multi) with cells from a continuous cell culture (fresh)
Luciferase induction is displayed on the left axis (red), viability is on the right axis (blue)

discussion

Assay Ready KeratinoSens® Cells, which are used instantly after thawing like a reagent and were not cultured before use, respond identically to proficiency sensitizers as KeratinoSens® cells from a continuously passaged culture. Technical proficiency could be demonstrated no matter the classic approach adopted by the OECD or the multiplex approach of the amended DB-ALM protocol was used since 9 of 10 proficiency substances were determined correctly.

Since Assay Ready KeratinoSens® cells can be produced in large homogenous batches of thousands of vials and stored in liquid nitrogen for a long time, the same prequalified cells can be used for repeated skin sensitization testing or even in different testing labs around the world. By this, the reproducibility from assay-to-assay can be easily increased, because of all uncertainties in cell culture like variations in culture medium, supplements, and in particular serum or slight difference in handling of different operators, the cells have the most significant impact on the assay performance.

Proficiency substances	KeratinoSens® Prediction	$EC_{1.5}$ (µM) OECD	$EC_{1.5}$ (µM) classic	$EC_{1.5}$ (µM) Multi	$IC_{1.5}$ (µM) OECD	$IC_{1.5}$ (µM) classic	$IC_{1.5}$ (µM) Multi
Salicylic Acid	negative	>1000	>1000	>1000	>1000	>1000	>1000
Lactic Acid	negative	>1000	>1000	>1000	>1000	>1000	>1000
Glycerol	negative	>1000	>1000	>1000	>1000	>1000	>1000
Isopropanol	negative	>1000	>1000	>1000	>1000	>1000	>1000
EGDMA	positive	5 - 125	49	53	>500	>500	>500
Cinnamyl alcohol	positive	25 - 175	118	121	>1000	>1000	>1000
MBT	positive	25 - 250	125	107	>500	303	383
Metol	positive	<12.5	8.8	4.1	20-200	23.7	20.4
MDBGN	positive	<20	11.2	17.3	20-100	37	46
DNCB	positive	< 12.5	1.9	2.3	5 - 20	9.3	7.8

Tab. 1: Proficiency substances tested on Assay Ready KeratinoSens® Cells

related products

KeratinoSens® Cell Line
master vial of KeratinoSens® cell line for continuous cultivation. Passage <10.
5 million cells/vial. (Cat.N° RE242)

KeratinoSens® Assay Ready Cells
prequalified cells for instant use, no propagation required. Passage <25.
Including recovery buffer & assay medium.
5 million cells/vial. (Cat.N° RE232)

instaCELL® KeratinoSens® assay kit
validated kit with prequalified Assay Ready KeratinoSens® Cells, media & buffer, control substances, 96-well assay plates, Resazurin viability dye, and Promega's One-Glo™ luciferase system (Cat.N° SF220-01)