Standard operating procedure:

Induction of antioxidant-response-element dependent gene activity in the keratinocyte ARE- reporter cell line KeratinoSens

SOP_KeratinoSens_Natsch_March09.doc

Note: The excel-file SOP_KeratinoSens_Natsch_Version1.1.xls forms an integral part of this SOP

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1. INTRODUCTION

The only feature all skin sensitizers have in common is their intrinsic electrophilicity, or their potential to be metabolically transformed to electrophilic chemicals. The signaling pathway with the repressor protein Keap1 and the transcription factor Nrf2, which binds to the antioxidant / electrophile response element (ARE / EpRE), was shown to be a valuable cellular endpoint to detect skin sensitizers in vitro (1,2). This result was confirmed by an independent laboratory (3).

The sensor protein Keap1 (Kelch-like ECH-associated protein 1) contains highly reactive Cys residues. In un-induced conditions, Keap1 is bound to Nrf2 (nuclear factor-erythroid 2-related factor 2), which targets Nrf2 for proteolytic degradation (For review see 4). Covalent modification of crucial Cys residues by small molecules leads to dissociation of Keap1 from the transcriptional regulator Nrf2, which then activates genes (mainly genes coding for phase II detoxifying enzymes) having an antioxidant response element (ARE) in their promoter sequence (4,5). Measurement of the induction of this signaling pathway in a reporter cell line provides a high-throughput cell-based *in vitro* test to screen for the skin sensitization potential of novel chemicals. The *in vivo* relevance of this signalling pathway for contact allergy and in particular for the T_H1 response, has been established by Kim *et al.* (6)

2. PURPOSE

Many phase II genes contain an ARE-element in their promoter. One particular gene is AKR1C2 coding for an aldo-ketoreductase (7). This particular gene was identified as one of the target genes up-regulated by contact sensitizers in dendritic cells (8,9).

The cell line KeratinoSens contains a 56-base-pair insertion shown below containing the ARE sequence from the AKR1C2 gene (7). This genetic element was inserted in front of a SV40 promotor, and the construct was inserted upstream of the luciferase gene in the vector pGL4 from Promega. The resulting vector was transfected into HaCaT keratinocytes, and clones with a stable insertion were selected in the presence of Geneticin / G418.

ARE regulatory sequence from the AKR1C2 gene inserted into the novel reporter vector:

5'-TGGTCGCAAGGTGTGCAAGCT**GC**TGA**GTCA**CCCTGACTGCATCAACCCCAGGAGCT

The selected clone 8 (termed KeratinoSens) was further propagated as a reporter cell line. Induction of luciferase is the single read-out / endpoint currently evaluated to determine sensitization potential in this test. Luciferase induction directly indicates activation of ARE-dependent genes. Cytotoxicity is measured in parallel as background information, but it is not needed for the prediction model.

3. METHOD OUTLINE

The reporter cell line is exposed to the test chemicals for 48 h, and subsequently luciferase induction is evaluated. The assay is run in 96 well plates, and each chemical is tested in parallel at 12 concentrations ranging form 0.98 µM to 2000 µM. Seven compounds are tested in one plate, and each plate contains additionally:

- six wells with solvent control (cells receiving solvent only)
- one blank well with no cells added
- five wells with the standard reference compound cinnamic aldehyde.

Each plate is tested in parallel in triplicate for analysis of luciferase induction and one additional replicate plate is used for cytotoxicity assessment. The full test in triplicate analysis is independently conducted three times. If results are congruent and statistically significant in all repetitions, this is the final result. Compounds with differing results are re-tested.

The principle of the test-setup is shown schematically in the Appendix 1. Details of the plate setup are described in the sheet ‘plate-setup’ in the file SOP_KeratinoSens_Natsch_Version1.1.xls.

4. LIMITATIONS

Currently no interference with specific test compounds are known, as due to the measurement of active visible light emission no interference was found for fluorescent or UV-absorbing compounds. The applicability for dyes absorbing in the visible range has not been assessed yet.

At the maximal dosage of 2000 µM some chemicals are not fully soluble, still even under these conditions gene induction can be measured in many cases. The level of DMSO in the test is 1%, which helps for some co-solubilisation and enhances the sensitivity of the assay. 1% DMSO is not toxic to the test cell line.

The keratinocyte cell line has some basic metabolic capacity detecting a series, but not all, putative pro-haptens.

5. DEFINITIONS/ABBREVIATIONS

ARE = antioxidant response element

KeapI = Kelch-like ECH-associated protein

Nrf2 = nuclear factor-erythroid 2-related factor

AKR1C2 = Aldoketoreductase

DMSO = Dimethylsulfoxide

FCS = Foetal calf serum

PBS = Phosphate buffered saline

MTT = Thiazolyl Blue Tetrazolium bromide

6. MATERIALS

6.1. Cells

The transgenic cell line KeratinoSens with a stable insertion of the Luciferase-construct is supplied by Givaudan on dry ice. Upon receipt, it should be propagated to passage 2 – 4 and multiple vials of the resulting cell population stored in liquid nitrogen as a homogeneous stock. Cells from this stock are then used for routine testing. The cells propagated from this original stock can then be kept in culture for a maximum passage number of 25.

6.2. Technical Equipment

- Sterile hood for cell culture work
- CO_2 incubator
- 8 channel pipettes for volumes between 10 µl and 200 µl
- 96 well plate Luminometer with an injector (single injector sufficient, no need for double luciferase measurement), different models useable, possible model is the GloMax™ 96 Microplate Luminometer (Promega)
- 96 well plate absorbance reader (equipped for reading at 600 nm)

6.3. Reagents, Media, Sera, Culture Plates

Below are listed the reagents used for the routine testing. For most cell-culture products, alternative products from other manufacturers will work equally. Lysis buffer is the only complex reagent which is specific to the indicated supplier and where no alternative products were tested yet.

	Product	**Company**	**Catalog Number**
Medium	D-MEM (Dulbecco's Modified Eagle Medium), liquid with GlutaMAX™ I, 1000 mg/L D-Glucose, Sodium Pyruvate	Gibco	21885-025
Serum	Foetal calf serum Origin: South America An alternative source of the serum can be used with the standard supplier for each Laboratory	AMIMED	2-01F10-I
Trypsin	0.05% Trypsin-EDTA	Gibco	25300
Phosphate buffered saline	DPBS	Gibco	14190
G-418	Geneticin (G418)	Gibco	10131-027
EDTA	Ethylenediamin-tetra-acetic acid trinatrium salt	FLUKA	03710
Solvent	DMSO	Sigma	41650
Lysis buffer	Passive Lysis Buffer, 5x	Promega	E1941

Luciferase substrate	Luciferase Assay System 10-Pack	Promega	E1501
Luciferin	D-Luciferin potassium salt	Synchem	bc219
Coenzyme A	Coenzyme A sodium salt hydrate, from Yeast	Sigma	C3144
ATP	Adenosine-5'-triphosphate disodium salt hydrate	Fluka	02055
MTT	Thiazolyl Blue Tetrazolium bromide	Fluka	88415
White 96 well culture plates	Lia-Plate, white, Tissue culture (TC), 96 well, flat bottom, with lid, sterile	Greiner Bio-One	655 083
Transparent 96 well culture plates	Tissue culture (TC) test plate, 96 well, flat bottom	Orange Scientific	5530100
Addhesive foils to cover plates during 2 day incubation period	Sealing tape SI	Nunc	0236366
Culture plates	Culture Dishes 100 x 20 mm	Milian	TP-93100
CryoTubes	CryoTube 1,8 ml SI	Nunc	368632

6.4. Preparation of media and solutions

Maintenance medium

The maintenance medium for the KeratinoSens cell line is prepared by supplementing 500 ml D-MEM with 50 ml FCS (final FCS concentration: 9.1 %) and 5.5 ml Geneticin Gibco (final concentration 500 µg/ml). The medium is stored at 4°C and used within 28 days.

Home-made Luciferase substrate

Compound	Stock conc.		final conc.		for 100 ml	
Tricine, pH 7.8	100	mM	20	mM	20.000	ml
$MgSO_4$	1000	mM	2.67	mM	0.267	ml
EDTA, pH 8	250	mM	0.1	mM	0.040	ml
DTT	154	g/mol	33.3	mM	0.513	g
Coenzyme A	811.51	g/mol	0.27	mM	0.022	g
D-Luciferin	318.41	g/mol	0.47	mM	0.015	g
ATP	100	mM	0.53	mM	0.530	ml
Water					78.613	ml
Total					100.000	ml

The luciferase substrate can be stored at -80° C but should not be prepared more than one month prior to use.

EDTA solution 10%, pH 8

10 g EDTA is dissolved in 100 ml H_2O and NaOH is added to bring the solution to pH8.

7. METHODS

7.1. Cell maintenance and culture conditions

Thawing: Upon receipt, The frozen cells should be transferred to a liquid nitrogen tank for longer storage. To thaw the cells, they should be warmed in a 37ºC water bath. The cells are then suspended in 10 ml maintenance medium and pelleted by centrifugation at 125 g for 5 min to get rid of the DMSO used for freezing. The cell pellet is then re-suspended in 10 ml of maintenance medium with 9.1% FCS without G418 and they are finally plated in a 10 cm tissue culture dish. G418-containing medium is only added in the next passage.

Maintenance: Cells are maintained in Dulbecco's modified Eagle's medium containing Glutamax (Gibco/Invitrogen) supplemented with 9.1 % fetal calf serum and 500 µg/ml G418 at 37ºC in the presence of 5% CO_2. 80-90% confluent cells are washed twice with DPBS containing 0.05% EDTA, then Trypsin-EDTA (1 ml / plate) is added and plates are put back into the 37°C incubator. After cells have detached (usually after 5 – 10 min), they are resuspended in 10 medium and split at a ratio of 1: 4 – 1: 16 in fresh medium and grown to 80-90% confluency. With a split ratio of 1:4, cells need 2 days to reach confluency again, in a ratio of 1:8, cells need 3 days (normally done for the weekend) and in a ratio of 1:16 4 -5 days.

Antibiotics against microbial contaminations are not used in the standard cultivation of these cells.

All historical experiments were done in 100 mm culture dishes. However, cells may also be grown in T75 flasks, as HaCaT cells usually grow equally well in flasks and in dishes.

Freezing: For the preparation of frozen stocks, the cells are harvested as described above, pelleted by centrifugation (125 g for 5 min), and re-suspended in growth medium containing 20% FCS and 10% DMSO at a density of 3-4 × 10^6 cells per ml. The cells are aliquoted into CryoTubes and frozen in a -80 ºC freezer using a Freezing Container. After 24 h they are then transferred to liquid nitrogen.

Cell seeding for testing: Cells are split on Friday afternoon in a split ratio of 1:8 and 1:12 and grown for 3 – 4 days in 10 cm culture dishes. On Monday morning the media is replaced with fresh medium. The cells from the 1:8 split are then used to prepare assay plates on Monday afternoon, whereas the cells from the 1:12 split are used on Tuesday afternoon to prepare additional assay plates. At the stage of preparing assay plates cells should be 80- 90 % confluent. They are washed twice with PBS, harvested as described above, re-suspended in DMEM with 9.1% FCS without G- 418 and adjusted to a density of 80'000 cells / ml. The cells are then distributed to the 96-well plates, 125µl (containing 10'000 cells) per well. It is very important to avoid sedimentation of the cells during this step and to assure that the same cell number is distributed to all wells. If this is not assured, this step could give the highest well-to-well variability in the assay.

Four parallel plates are prepared for each batch of seven test chemicals: Three white 96 well plates and one transparent 96 well plate.

7.2. Quality check of cells

No specific phenotypic characterisation of the cells prior to the assay is needed. Each test plate incorporates a relevant control for quality check, and results from plates which do not conform to this quality test are discarded after the experiment (for acceptance criteria see 7.8).

7.3. Handling of chemicals

All chemicals are dissolved to up to a final concentration of 200 mM in DMSO. These DMSO solutions can be stored at -20°C up to 2 weeks. (Note: Chemicals not soluble in DMSO are dissolved and diluted in sterile water. The DMSO level in the test must in these cases also be adjusted to 1% as for the other compounds).

Based on these stock concentrations, a 100 × DMSO master plate is made as described schematically in the Appendix 1 and in detail in the sheet 'plate-setup' in the file SOP_KeratinoSens_Natsch_Version1.1.xls. This master plate can be sealed with a foil and stored at --20°C up to 1 week and used for several repetitions.

7.4. Cell Exposure

After seeding, the cells are grown for 24 h in the 96-wells microtiter plates in presence of 9.1 % FCS without G-418 prior to compound addition. The medium is then removed by aspiration and replaced with 150 µl DMEM-medium without G-418 containing 1% FCS.

The DMSO master plate is replicated into a fresh plate (10 µl solution per well) and the DMSO solution is diluted 25-fold by adding 240 µl of DMEM-medium containing 1% FCS. This resulting 4 × master plate with medium is then distributed to the replicate assay plates: 50 µl each to three white assay plates and 50 µl to one cytotoxicity plate (see Appendix I). All the plates are then covered with a foil (Sealing tape SI, Nunc) to avoid evaporation of volatile compounds and to avoid cross-contamination between wells. The plates are then incubated for a further 48 hours in the CO_2 incubator.

7.6. Measure of endpoints

After the incubation time, the supernatant is aspirated from the three white assay plates and discarded. The cells are washed once with DPBS. To each well, 20 µl of passive lysis buffer is added (at this stage, the formation of foam should be avoided by careful pipetting) and the cells are incubated for 20 min at RT**.** The plates with the cell lysate are then placed in the luminometer for reading: The injector is programmed to add 50 µl of the luciferase substrate to each well and to integrate the luciferase activity for 2 seconds**.** Alternative setting may be needed depending on the model of luminometer used.

For the cell viability assay, medium is replaced with 200 µl fresh medium containing 1% FCS , and 27 µl of a MTT solution (5mg/ml in DPBS) is directly added to each well of the transparent 96-well plate. The plates are covered with a sealing tape and returned to the incubator. After 4 hour incubation, the medium is removed and 200 µl of a 10% SDS solution is added to each well. The plate is covered with a sealing tape and placed in the incubator. After overnight incubation to dissolve the cells, the absorption at 600nm is determined for each well.

7.7. Data Analysis

For each set of seven chemicals, a copy of the standard file 'SOP_KeratinoSens_Natsch_version1.1.xls' is made. The fields which need to be filled in are marked yellow. On the 'Summary sheet' the compound identifiers and the plate identifier are inserted. On the sheet 'rep1' the plate readout of the triplicate analysis can directly be inserted in the yellow areas. The second experiment is added to sheet 'rep2' and also the 3th repetition can be evaluated in the same file. The cytotoxicity results from all repetitions are pasted into the same sheet 'Cytotoxicity'.

This file then automatically calculates the gene induction and the wells with statistically significant induction over a given threshold (default value set to 1.5 = 50% enhanced gene activity). Furthermore the maximal induction (I_{max}), the concentration for maximal gene induction (CI_{max}) and the EC value (concentration for induction above threshold), both with linear and log-linear extrapolation, are calculated similar to the LLNA. The results from the different repetitions are then summarized in the 'Summary' file.

The data are also automatically plotted in the graphs on the different repetition sheets. The automatically calculated I_{max} and the EC values should visually be checked with the help of this graph, as uneven dose-response curves or outliers may lead to wrong extrapolations which may need to be corrected manually. Note: Especially in the very rare cases with a non-significant induction above the EC1.5 which is followed by a higher concentration with a significant induction, the automatically calculated value maybe wrong.

Note: The current prediction model rates any chemical with significant gene induction above 1.5 positive and thus likely to be a sensitizer. This model may need adjustment as more data accumulate on larger sets of test chemicals, and an adjusted EC value can automatically be calculated by modifying the threshold in the 'summary sheet'.

7.8. Acceptance criteria

The I_{max} and the EC 1.5 for cinnamic aldehyde is also calculated. For acceptance of the test for a given master plate in a given repetition, the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 4, and the EC 1.5 value should be between 10 µM and 25 µM. The average variability in the 3 × 6 solvent control wells for each master plate/repetition should be below 20% for acceptance of the test. If the variability is higher, one has check whether it is due to a single outlier in the 18 wells, otherwise results are discarded. These acceptance criteria are automatically calculated in the Summary sheet of the Excel file, and results should appear as in below example:

	Criteria		Quality control: Variability blank	
EC 1.5	EC 1.5	Ind. 64 uM	% standard deviation blanks	
12.93	TRUE	TRUE	15.16659	ACCEPTED

7.9. Final Report

- Tested chemicals with CAS number, name of supplier and catalogue number, purity
- Summary of findings
  - I_{max}
  - CI_{max}
  - EC1.5 with linear extrapolation (Log-linear result is only used where linear extrapolation appears unreliable by inspection of the raw data)
  - EC 50 for cytotoxicity

If there were experiments which did not fulfil the acceptance criteria, this should also be reported.

• The standard excel file containing raw data and evaluations is also included and forms part of the final report

8. HEALTH SAFETY AND ENVIRONMENT

Test chemicals must be considered potential skin sensitizers and skin contact with all test chemicals should be avoided. All precautions for untested chemicals should be taken, when testing novel chemicals from the research process. No specific precautions are required for this specific cell line, standard procedures as applied in general cell culture work should be applied.

9. REFERENCES

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10. APPENDIX

Appendix 1: Experimental setup, preparation of the master plate and dilutions.