Givaudan

Ring study on ARE-induction in the KeratinoSense cell line - Study-setup

Preparation of this document: Andreas Natsch, Givaudan Schweiz AG, Dübendorf, 17.7.2009

Participating Labs:

- Givaudan (Lead Lab), repetition of historical data for intra-Laboratory repeatability
- Procter & Gamble, Cindy A. Ryan /Leslie Foertsch/ Frank Gerberick
- Beiersdorf, Andreas Schepky / Stefan Onken
- Institute for *in vitro* science IIVS, Rodger Curren / Erin Hill / Hans Raabe
- BASF, Robert Landsiedel, Caroline Bauch. (Joined in December 09)

SOP:

The SOP has been defined and already used for the screening of 67 chemicals of known sensitisation potential within the Givaudan laboratory. The SOP was reviewed by Cindy A. Ryan, and some small clarifications were inserted (new version 1.1).

The main changes in the updated SOP:

- in the viability assay, the medium is replaced before adding the MTT reagent to avoid possible interference from reducing test chemicals with MTT
- The internal positive reference is changed. Before, *tert*-butyl-hydroquinone was used as it is a very well known standard Nrf2-inducer, but not a reference allergen. After a large screening we observe that t-BHQ is reliably always positive, but gives a relatively shallow dose-response curve which gives a higher variation in the EC1.5 values as compared to most other chemicals. Thus the positive control now is cinnamic aldehyde, which is a typical sensitizer, and also the positive control in the peptide reactivity assay and which gives a steeper dose-response curve.

In the current SOP all chemicals are tested in the range between 0.98 µM and 2000 µM. The recent experience from a large screening indicates that the highest concentration is not needed for the sensitivity of the method and the range may be reduced if we go for formal validation, yet the range is left as it is for the time being., as its is better to test a method to its limits.

The SOP also includes an updated Excel file, which allows for rapid evaluation and visualisation of the Luciferase and the cytotoxicity data.

Cytotoxicity measurements were discussed prior to the ring study:. The SOP was adjusted, such that the medium is changed prior to the MTT reaction to avoid interference in the MTT test by reducing chemicals.
In parallel the NRU technique and the resazurin technique were tested. Results indicate that the NRU method gave very similar IC 50 values (see figure 1), yet we observed some edge effects and thus we recommend to continue using the MTT method. However, in the MTT method we sometimes observe enhanced MTT reducing activity in stressed cells at subtoxic concentrations, which is not the case in the NRU technique. Thus the value of the NRU approach will be further tested, but we stick to MTT for the time being. If a Lab wishes to do both in parallel, this would be

useful information. The Beiersdorf Lab can indicate the details of the NRU technique for HaCaT cells, and we had used their protocol.

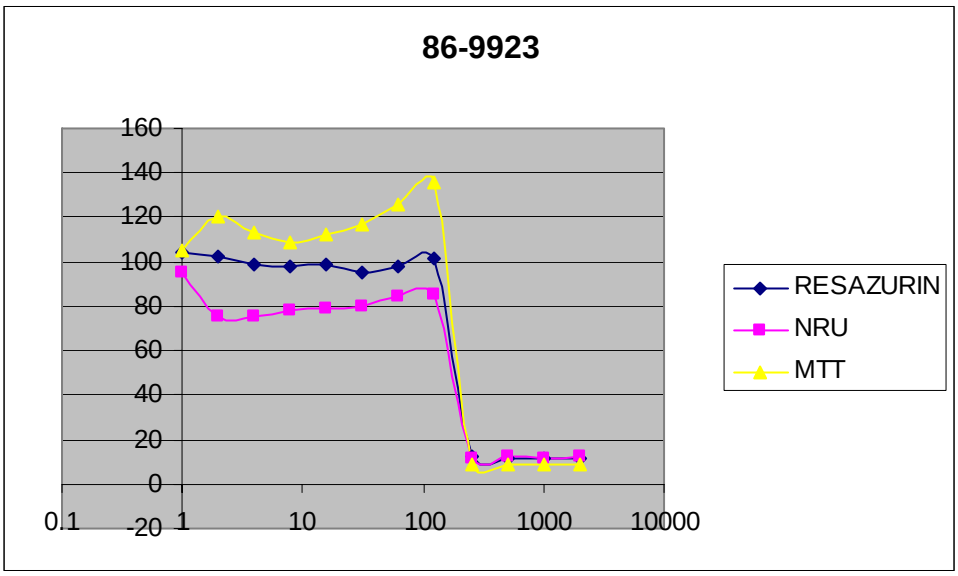


Figure 1: Example of cytotoxicity measurement with three methods on one test chemical. Note that with MTT we se an increase of the MTT-reducing capacity at subtoxic concentrations, whereas NRU gives an edge effect with wells in the middle having only 80% of the control value at the plate edge. Still the effect on IC 50 is very minor.

Alternative Luciferase measurements: As not all laboratories have a luminometer equipped with an injector, we have measured a plate in parallel with the Promega SteadyGlow kit. This allows for a higher throughput, as the substrate is added to all wells simultaneously, and the plate is then measured. Test price however is significantly higher. Results (Figure 2) indicate that the results are quite similar, but overall induction values are a bit lower and EC1.5 a bit higher. However, the absolute luciferase values were also about 10 times lower, and this could be correct for with a longer integration.
If no injector is available and cannot be purchased, we recommend using this method just with a longer integration time.

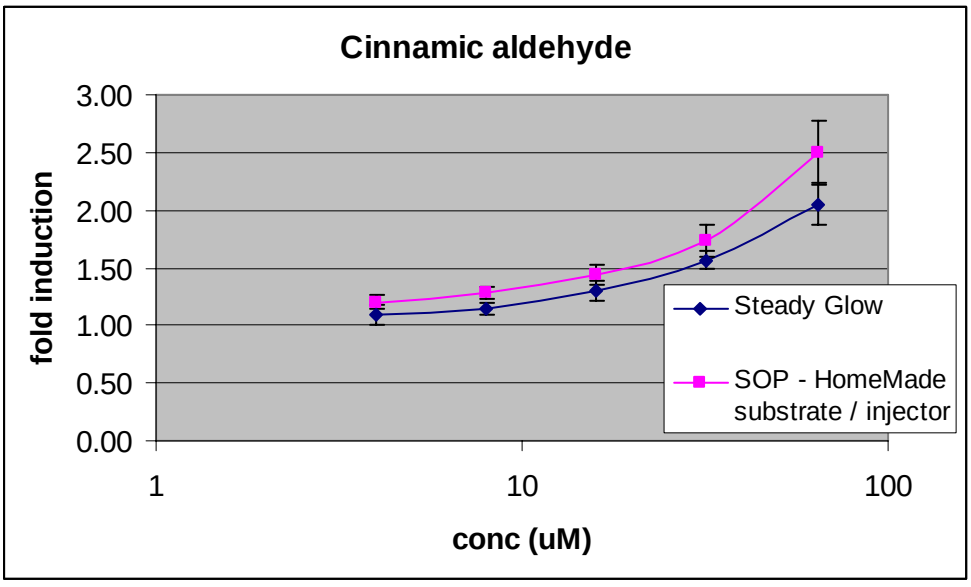


Figure 2. Comparision of the SOP procedure with the Promega SteadyGlow kit. The reference cinnamic aldehyde was done in 14 replicates in two parallel plates with the two methods. Note: This experiment was also done to check for position effects in the plate. We do not note any position effects for the Luciferase measurements.

Attachment7b_ Ring study set-up and communication at start of ring study

Current prediction model:

A chemical is rated positive if there is a statistically significant induction of the Luciferase above the 1.5 fold threshold (50% enhanced activity over background solvent control). Based on the 67 chemicals screened so far, this is a very valid prediction model, yet two things will need to be considered in the future:

- some chemicals from our research process not included in standard datasets appear to give a weak induction just at cytotoxic concentrations. Whether a weak induction just at cytotoxic levels may in some cases yield false-positive results needs to be assessed in the future, and thus an alternative prediction model based only on non-cytotoxic concentrations will be evaluated once more data are available
- If the test is used in a battery of assay, the specificity may need to be maximized, rather then the sensitivity. In this case also a higher threshold (EC_2 or EC_3) may be required. The data can in the future be re-analyzed in this context. However, with the reference chemicals in the current standard sets, specificity is already very good (see below)

Test chemicals:

The overall aim is to test the 28 chemicals. All chemicals are supplied by Givaudan to assure that the same batch and qualities are used. The test Laboratories are blind for the results of the lead Lab which are not published at the time of the study, with the exception of the results for two chemicals shown below.

This list of chemicals contains the chemicals from the ICCVAM list for validation of alternative endpoints in the LLNA and the chemicals from the submitted publication of Casati *et al.* . (COLIPA/ECVAM list). Many chemicals of these latter list are extensively tested also in the Sens-it-IV project. From the Sens-it-IV chemical list diethylphtalate was added as further negative control.

Cooper statistics for the study chemicals in the Givaudan Lab

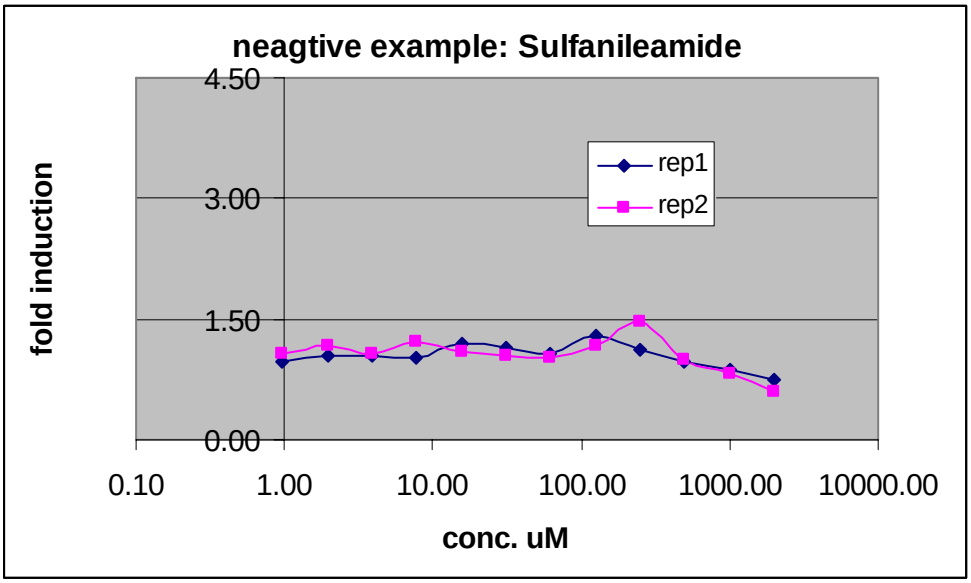
	ICCVAM list	ECCVAM list	All 28 chemicals
sensitivity	84.6	91.7	89.5
specificity	100.0	100.0	100.0
accuracy	90.0	93.8	92.9

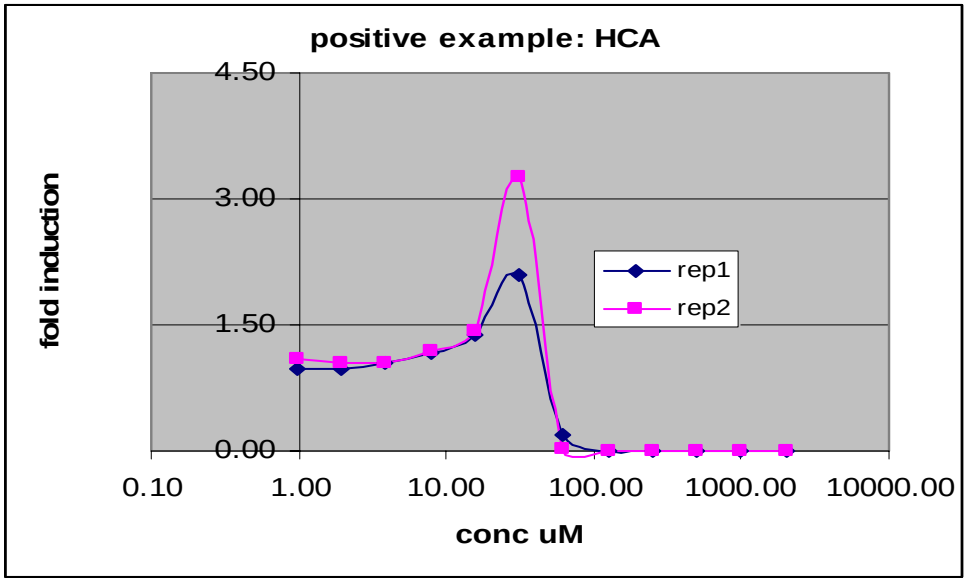
Study phase 1:

The chemicals in below Table are tested first (test transfer phase). They can be tested in one master plate, and **three independent repetitions each with three replicates** are made.

Chemical Name	**MW**	**Solvent**
2,4-Dinitrochlorobenzene	202.6	DMSO
Ethylene glycol dimethacrylate	198.2	DMSO
Hexyl cinnamic aldehyde	216.3	DMSO
Citral	152.2	DMSO
Methyl salicylate	152.1	DMSO
Chlorobenzene	112.6	DMSO
Sulfanilamide	172.2	DMSO

As example results, below the results for α-Hexyl-cinnamic aldehyde (HCA) and Sulfanilamide are shown.





- **Once the results from this phase are completed, they are shared and compared with both the new and the historical data of the lead Lab.**
- **An *interim* report is prepared to compare the data.**
- **A telephone conference will be held at this stage to exchange the experiences.**

Study phase 2:

If results are satisfying and the test protocol appears as defined properly, phase 2 is started with the remaining 21 chemicals (lab-to-lab reproducibility phase). After discussion with Silvia Casati from ECVAM, it is more valuable to already perform this phase with chemicals blindly coded. Thus the test laboratories are blind for the identity of the chemicals tested in this phase, and also blind for the results obtained in the lead Lab.

List of test chemicals for Phase II

CODE	Preparation of solution	
BC_04	Dissolve 22.1 mg in 1 ml sterile H2O, gives 100 mM solution (thus maximal test conc. is 1000 micromolar), this solution is tested instead of a 200 mM solution please adjust results accordingly!	
BC_09	Dilute 200 microliter with 120 microliter water, gives 50 mM solution (thus maximal test conc. is 500 micromolar), this solution is tested instead of a 200 mM solution, please adjust results accordingly!	
BC_07	Dissolve 43.4 mg in 1 ml DMSO	Final solution is 200 mM
BC_01	Dissolve 21.6 mg in 1 ml DMSO	Final solution is 200 mM
BC_02	Dissolve 26.4 mg in 1 ml DMSO	Final solution is 200 mM
BC_05	Dissolve 32.8 mg in 1 ml DMSO	Final solution is 200 mM
BC_20	Dissolve 59.3 mg in 1 ml DMSO	Final solution is 200 mM
BC_08	Dissolve 33.5 mg in 1 ml DMSO	Final solution is 200 mM
BC_06	Dissolve 32.8 mg in 1 ml DMSO	Final solution is 200 mM
BC_18	Dissolve 26.8 mg in 1 ml DMSO	Final solution is 200 mM
BC_14	Dissolve 11.6 mg in 1 ml DMSO	Final solution is 200 mM
BC_03	Dissolve 43.2 mg in 1 ml DMSO	Final solution is 200 mM
BC_13	Dissolve 53.2 mg in 1 ml DMSO	Final solution is 200 mM
BC_11	Dissolve 27.6 mg in 1 ml DMSO	Final solution is 200 mM
BC_10	Dissolve 57.7 mg in 1 ml DMSO	Final solution is 200 mM
BC_19	Dissolve 18 mg in 1 ml DMSO	Final solution is 200 mM
BC_15	Dissolve 18.4 mg in 1 ml DMSO	Final solution is 200 mM
BC_21	Dissolve 39.6 mg in 1 ml DMSO	Final solution is 200 mM
BC_12	Dissolve 77.7 mg in 1 ml DMSO	Final solution is 200 mM
BC_17	Dissolve 12 mg in 1 ml DMSO	Final solution is 200 mM
BC_16	Dissolve 44.4 mg in 1 ml DMSO	Final solution is 200 mM