**Attachment 17e to the KeratinoSens TST**

# Conclusions regarding the Reliability and preliminary predictive capacity of the KeratinoSens assay to detect skin sensitizers

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# 1. Reliability

The studies summarized in the TST were designed to answer the following questions:

- Is the method reproducible in the lead laboratory developing the method
- Does it generate stable results over time
- Is it transferable to naïve laboratories
- Are the results reproducible in other laboratories testing blind-coded items
- Is it reproducible within repetitions performed by other laboratories (preliminary analysis)

## 1.1 Intralaboratory reproducibility in the lead laboratory

The reproducibility of the quantitative dose-response curves for both cytotoxicity and luciferase induction in within-laboratory repetitions appears very high. This is illustrated in attachments 4a, 4b and 4c. Based on this reproducible dose-response data, the yes/no predictions as made with the prediction model demonstrate overall a good intralaboratory reproducibility in the lead laboratory. Exceptions were only encountered for chemicals which give results very close to the cut-off thresholds of the prediction model, i.e for substances with an $I_{max}$ close to 1.5 or substances with a cytotoxic level close to the level at which gene induction starts.

*We can thus conclude that this assay gives a high level of intralaboratory reproducibility in an experienced laboratory.*

## 1.2 Stability of results in the lead laboratory over time

The assay is based on a stable genetically engineered cell line. It is therefore not due to donor-to-donor variation or variation of the sourcing of biological material from primary tissue. It was run with separate batches of the cell line in multiple passages over a time-course of two years. As illustrated in Attachment 17a, no change in the performance for the positive control as a function of time was noted.

*Hence the assay does generate stable data over the time course investigated and there are no obvious reasons to expect that this performance should change in the future.*

## 1.3 Transferability to naïve laboratories

The assay was so far transferred to four laboratories, two in Europe and two in the Unites States. All laboratories were able to perform the test (see attachments 8a and 8b). Limitations encountered were of a technical nature at the level of luminescence detection, which was not a routine technique in these laboratories, and which could be resolved easily.
The biological assay *per se* was found to be readily transferable, and the recombinant cell line gives overall similar results in different laboratories. No direct hands-on / face-to-face training was required to transfer the method.

*We therefore conclude that the test method is transferable to other laboratories experienced in general cell culture techniques.*

## 1.4 Reproducibility in other laboratories

21 blind coded chemicals were tested in the four external laboratories in the second phase of the study. With few exceptions, results in the different laboratories gave congruent yes/no predictions according to the prediction model (Attachment 12b). Statistical analysis of the variance of the

quantitative data (IC50 and EC1.5) within and between laboratories indicated that the between-laboratory variance was only slightly above the intra-laboratory variance (see Attachments 10d and 10e). A statistical analysis comparing the data of the external labs to the lead lab indicated that there was no statistical significant effect on data quality in three of the four external laboratories (see Attachment 10f). One lab had slightly lower performance of the quantitative data, but this laboratory still made an identical yes/no judgment for all the 21 blind-coded chemicals as the lead lab.

*We therefore conclude that the method delivers reproducible results in different test laboratories.*

### 1.5 Within laboratory reproducibility in other laboratories

So far external laboratories have performed three independent repetitions, each repetition with triplicate analysis at 12 concentrations. These laboratories have not repeated this full procedure three times as was done in the extended intra-laboratory study in Attachment 4c. Nevertheless, based on the three independent repetitions a within laboratory reproducibility analysis was possible as presented in Attachments 10d and 10e.

*We thus conclude that preliminary analysis indicates good intra-laboratory reproducibility in the external laboratories.*

### 1.6 Overall conclusion on the reliability

The test system is based on a number of intrinsic features which facilitate reliability: (i) stably engineered cell line, (ii) no primary human tissues needed, (iii) endpoints which involve few experimental steps for quantification and most importantly (iv) relative and not absolute metrics (i.e. fold-induction and % viability as compared to 'absolute light units' or pg/ml of released Interleukin etc.). This last feature appears to greatly facilitate inter-laboratory reliability.

*Based on the experimental results and the conclusions on the five key questions addressed above, we conclude that the method is reliable, both over time and across different laboratories.*

## 2. Preliminary predictive capacity

The studies summarized in Attachments 12a -12e were designed to address the following questions:

- Determine the predictive capacity on published reference lists
- Evaluate the predictive capacity for chemicals for which congruent LLNA and Guinea pig/human data are available
- Determine the applicability domain
- Determine potential gaps which may need to be filled by testing with complementary methods

### 2.1 Predictive capacity on published reference lists

Three carefully composed lists of reference chemicals for an initial evaluation of non-alternative methods for skin sensitization or alternative endpoints in the LLNA have been compiled and published (ECVAM/COLIPA list [1], Sens-it-iv list [2] and ICCVAM list [3]). The predictive capacity of the test method for these three reference lists was found to be high [4].

*Since these reference lists contain sensitizers acting by a wide range of reaction mechanisms and structural and physicochemical features, we can conclude that the KeratinoSens assay has a high predictive capacity for typical sensitizers and non-sensitizers in published lists.*

### 2.2 The predictive capacity for chemicals for which congruent LLNA and Guinea pig/human data are available

An extended list of reference chemicals was now compiled based on the rationales outlined in Attachment 12c. This extended list is based on published data [5, 6], but it has not been published or reviewed by external experts.
For this list the overall predictivity is lower as compared to the more specific published reference lists, especially the sensitivity of the KeratinoSens assay, if used as stand alone method, is lower (76.6%).

*The lower sensitivity for the chemicals of this extended list indicates that the applicability domain needs to be carefully assessed, and options to improve sensitivity by combining two or more tests need to be considered.*

### 2.3 Applicability domain and gaps which may need to be filled by testing with complementary methods

As outlined in Attachment 12c, two specific groups of false-negative chemicals can be discerned in the extended list:

- Specifically amine-reactive chemicals
- Specific prohaptens requiring putative P450 activation

Besides these two specific groups, the false-negatives include also chemicals which are thought to require an air oxidation or photo-activation step and, some miscellaneous chemicals. We had shown that air oxidation and photoactivation prior to the KeratinoSens is possible to activate these chemicals (see Attachment 12c) but it does not form part of the SOP and is also not done routinely in the LLNA.

Specifically amine-reactive chemicals are probably rather rare in the chemical universe, but significantly represented in the extended list in Attachment 12c. As discussed in detail in previous publications and in Attachment 12c, the directly amine-reactive chemicals are routinely identified with a peptide-binding assay including a Lysine-containing peptide [7, 8].

*We therefore conclude that for optimal predictivity the KeratinoSens assay should be run in parallel with a peptide binding assay, which does use a peptide with a lysine residue.*

While a number of prohaptens requiring enzymatic oxidation or oxidative deamination are correctly classified by the KeratinoSens assay (see Attachment 12c), the limitations for the pro-haptens requiring putative P450 activation in Table 4b of Attachment 12c is not so easily overcome, as no functional, physiologically based, metabolic system has yet been described. As

surrogate systems horse-radish peroxidase [9] and human liver microsomes [10, 11] have been proposed. However these systems may be more active as compared to skin and may thus lead to overpredictions.

*We conclude that for a further improvement, an additional test method may be needed which includes a relevant metabolic system to detect the phenolic prohaptens which require enzymatic activation; yet these chemicals appear to be a minor group of the known skin sensitizers.*

## 3. Recommended further steps

To progress from pre-validation to validation, we consider that the following steps should be pursued:

**1) Testing of additional blind-coded chemicals in different laboratories to further evaluate the predictive capacity in different laboratories.**

We had already tested a substantial number of the chemicals for which high quality animal data exist. Thus such inter-laboratory studies may, in our views, cover also chemicals already tested, and it should mainly be designed to evaluate the predictivity in different laboratories. Since the transferability to four laboratories has been verified, such a study can, but must not exclusively, include naïve laboratories.

**2) Evaluate how the test method should be combined with other tests**

The predictive capacity when combining KeratinoSens with other tests should be further evaluated. In a shorter term, assessments combining KeratinoSens with (a) a direct peptide binding assay including a Lysine residue and (b) a peptide-binding assay in presence of metabolism may be a fast and economically feasible approach. As other cell-based assays proceed through prevalidation, the complimentary predictive capacity with these other tests need to be assessed in detail to further define the optimal test battery.

## References


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