

**OECD GUIDELINE FOR THE TESTING OF CHEMICALS*****In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method****INTRODUCTION**

1. A skin sensitiser refers to a substance that will lead to an allergic response following skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). This Test Guideline (TG) provides an *in vitro* procedure (the ARE-Nrf2 luciferase test method) to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (1).

2. There is general agreement regarding the key biological events underlying skin sensitisation. The existing knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP) (2), going from the molecular initiating event through the intermediate events up to the adverse health effect, i.e. allergic contact dermatitis in humans or contact hypersensitivity in rodents (2) (3). The molecular initiating event is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (4).

3. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods based on guinea-pigs, the Magnusson Kligman Guinea Pig Maximisation Test (GMPT) and the Buehler Test TG 406 (5), study both the induction and elicitation phases of skin sensitisation. A murine test, the Local Lymph Node Assay (LLNA) (TG 429) (4) and its two non-radioactive modifications, LLNA: DA (TG 442A) (6) and LLNA: BrdU-ELISA (TG 442B) (7), which all assess the induction response exclusively, have also gained acceptance since they provide advantages over the guinea pig tests in terms of both animal welfare and objective measurement of the induction phase of skin sensitisation.

4. More recently, mechanistically-based *in chemico* and *in vitro* test methods have been considered scientifically valid for the evaluation of the skin sensitisation hazard of chemicals. However, combinations of non-animal methods (*in silico*, *in chemico*, *in vitro*) within Integrated Approaches to Testing and Assessment (IATA) will be needed to be able to fully substitute for the animal tests currently in use given the restricted AOP mechanistic coverage of each of the currently available non-animal test methods (2) (3).

5. The test method described in this Test Guideline (ARE-Nrf2 luciferase test method) is proposed to address the second key event as explained in paragraph 2. Skin sensitisers have been reported to induce genes that are regulated by the antioxidant response element (ARE) (8) (9). Small electrophilic substances  
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such as skin sensitisers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by e.g. covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes (8) (10) (11).

6. Currently, the only *in vitro* ARE-Nrf2 luciferase test method covered by this Test Guideline is the KeratinoSens™ test method for which validation studies have been completed (9) (12) (13) followed by an independent peer review conducted by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) (14). The KeratinoSens™ test method was considered scientifically valid to be used as part of an IATA, to support the discrimination between skin sensitisers and non-sensitisers for the purpose of hazard classification and labelling (14). Laboratories willing to implement the test method can obtain the recombinant cell line used in the KeratinoSens™ test method by establishing a licence agreement with the test method developer (15).

7. Definitions are provided in Annex 1.

### INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

8. Since activation of the Keap1-Nrf2-ARE pathway addresses only the second key event of the skin sensitisation AOP, information from test methods based on the activation of this pathway is unlikely to be sufficient when used on its own to conclude on the skin sensitisation potential of chemicals. Therefore data generated with the present Test Guideline should be considered in the context of integrated approaches, such as IATA, combining them with other complementary information e.g. derived from *in vitro* assays addressing other key events of the skin sensitisation AOP as well as non-testing methods including read-across from chemical analogues. Examples on how to use the ARE-Nrf2 luciferase test method in combination with other information are reported in literature (13) (16) (17) (18) (19).

9. The test method described in this Test Guideline can be used to support the discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers in the context of IATA. This TG cannot be used on its own, neither to sub-categorise skin sensitisers into subcategories 1A and 1B as defined by the UN GHS (1), for authorities implementing these two optional subcategories, nor to predict potency for safety assessment decisions. However, depending on the regulatory framework, a positive result may be used on its own to classify a chemical into UN GHS category 1.

10. Based on the dataset from the validation study and in-house testing used for the independent peer-review of the test method, the KeratinoSens™ test method proved to be transferable to laboratories experienced in cell culture. The level of reproducibility in predictions that can be expected from the test method is in the order of 85% within and between laboratories (14). The accuracy (77% - 155/201), sensitivity (78% - 71/91) and specificity (76% - 84/110) of the KeratinoSens™ for discriminating skin sensitisers (i.e. UN GHS Cat. 1) from non-sensitisers when compared to LLNA results were calculated by considering all of the data submitted to EURL ECVAM for evaluation and peer-review of the test method (14). These figures are similar to those recently published based on in-house testing of about 145 test substances (77% accuracy, 79% sensitivity, 72% specificity) (13). The KeratinoSens™ is more likely to under predict chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (13) (14). Taken together, this information indicates the usefulness of the KeratinoSens™ assay to contribute to the identification of skin sensitisation hazard. However, the accuracy values given here for the KeratinoSens™ as a stand-alone test method are only indicative since the test method should be considered in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraph 9 above. Furthermore when evaluating non-animal methods for skin sensitisation, it should be

kept in mind that the LLNA as well as other animal tests, may not fully reflect the situation in the species of interest i.e. humans.

11. The term "test chemical" is used in this Test Guideline to refer to what is being tested<sup>1</sup> and is not related to the applicability of the ARE-Nrf2 luciferase test method to the testing of substances and/or mixtures. On the basis of the current data available the KeratinoSens<sup>TM</sup> test method was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined with *in vivo* studies) and physico-chemical properties (9) (12) (13) (14). Mainly mono-constituent substances were tested, although a limited amount of data also exist on the testing of mixtures (20). The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. However, before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture. Moreover, when testing multi-constituent substances or mixtures, consideration should be given to possible interference of cytotoxic constituents with the observed responses. The test method is applicable to test chemicals soluble or that form a stable dispersion (i.e. a colloid or suspension in which the test chemical does not settle or separate from the solvent into different phases) either in water or DMSO (including all of the test chemical components in the case of testing a multi-constituent substance or a mixture). Test chemicals that do not fulfil these conditions at the highest final required concentration of 2000 µM (cf. paragraph 22) may still be tested at lower concentrations. In such a case, results fulfilling the criteria for positivity described in paragraph 39 could still be used to support the identification of the test chemical as a skin sensitizer, whereas a negative result obtained with concentrations < 1000 µM should be considered as inconclusive (see prediction model in paragraph 39). In general test substances with a LogP of up to 5 have been successfully tested whereas extremely hydrophobic substances with a LogP above 7 are outside the known applicability of the test method (14). For test substances having a LogP falling between 5 and 7, only limited information is available.

12. Negative results should be interpreted with caution as substances with an exclusive reactivity towards lysine-residues can be detected as negative by the test method. Furthermore, because of the limited metabolic capability of the cell line used (21) and because of the experimental conditions, pro-haptens (i.e. chemicals requiring enzymatic activation for example via P450 enzymes) and pre-haptens (i.e. chemicals activated by auto-oxidation) in particular with a slow oxidation rate may also provide negative results. Test chemicals that do not act as a sensitizer but are nevertheless chemical stressors may lead on the other hand to false positive results (14). Furthermore, highly cytotoxic test chemicals cannot always be reliably assessed. Finally, test chemicals that interfere with the luciferase enzyme can confound the activity of luciferase in cell-based assays causing either apparent inhibition or increased luminescence (22). For example, phytoestrogen concentrations higher than 1 µM were reported to interfere with the luminescence signals in other luciferase-based reporter gene assays due to over-activation of the luciferase reporter gene (23). As a consequence, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene needs to be examined carefully (23). In cases where evidence can be demonstrated on the non-applicability of the Test Guideline to other specific categories of test chemicals, the test method should not be used for those specific categories.

13. In addition to supporting discrimination between skin sensitizers and non-sensitizers, the KeratinoSens<sup>TM</sup> assay also provides concentration-response information that may potentially contribute to the assessment of sensitizing potency when used in integrated approaches such as IATA (19). However,

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<sup>1</sup> In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should now be applied in new and updated Test Guidelines.

further work preferably based on reliable human data is required to determine how KeratinoSens™ results can contribute to potency assessment (24) and to sub-categorisation of sensitisers according to UN GHS (1).

## PRINCIPLE OF THE TEST

14. The ARE-Nrf2 luciferase test method makes use of an immortalised adherent cell line derived from HaCaT human keratinocytes stably transfected with a selectable plasmid. The cell line contains the luciferase gene under the transcriptional control of a constitutive promoter fused with an ARE element from a gene that is known to be up-regulated by contact sensitisers (25) (26). The luciferase signal reflects the activation by sensitisers of endogenous Nrf2 dependent genes, and the dependence of the luciferase signal in the recombinant cell line on Nrf2 has been demonstrated (27). This allows quantitative measurement (by luminescence detection) of luciferase gene induction, using well established light producing luciferase substrates, as an indicator of the activity of the Nrf2 transcription factor in cells following exposure to electrophilic test substances.

15. Test chemicals are considered positive in the KeratinoSens™ if they induce a statistically significant induction of the luciferase activity above a given threshold (i.e. > 1.5 fold or 50% increase), below a defined concentration which does not significantly affect cell viability (i.e. below 1000 µM and at a concentration at which the cellular viability is above 70% (9) (12)). For this purpose, the maximal fold induction of the luciferase activity over solvent (negative) control ( $I_{max}$ ) is determined. Furthermore, since cells are exposed to series of concentrations of the test chemicals, the concentration needed for a statistically significant induction of luciferase activity above the threshold (i.e.  $EC_{1.5}$  value) should be interpolated from the dose-response curve (see paragraph 32 for calculations). Finally, parallel cytotoxicity measurements should be conducted to assess whether luciferase activity induction levels occur at sub-cytotoxic concentrations.

16. Prior to routine use of the ARE-Nrf2 luciferase test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency, using the ten Proficiency Substances listed in Annex 2.

17. Performance standards (PS) (28) are available to facilitate the validation of new or modified *in vitro* ARE-Nrf2 luciferase test methods similar to the KeratinoSens™ and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the PS, if these test methods have been reviewed and included in this Test Guideline by the OECD.

## PROCEDURE

18. Currently, the only test method covered by this Test Guideline is the scientifically valid KeratinoSens™ test method (9) (12) (13) (14). The Standard Operating Procedures (SOP) for the KeratinoSens™ is available and should be employed when implementing and using the test method in the laboratory (15). Laboratories willing to implement the test method can obtain the recombinant cell line used in the KeratinoSens™ test method by establishing a licence agreement with the test method developer. The following paragraphs provide with a description of the main components and procedures of the ARE-Nrf2 luciferase test method.

### *Preparation of the keratinocyte cultures*

19. A transgenic cell line having a stable insertion of the luciferase reporter gene under the control of the ARE-element should be used (e.g. the KeratinoSens™ cell line). Upon receipt, cells are propagated

(e.g. 2 to 4 passages) and stored frozen as a homogeneous stock. Cells from this original stock can be propagated up to a maximum passage number (i.e. 25 in the case of KeratinoSens™) and are employed for routine testing using the appropriate maintenance medium (in the case of KeratinoSens™ this represents DMEM containing serum and Geneticin).

20. For testing, cells should be 80-90% confluent, and care should be taken to ensure that cells are never grown to full confluence. One day prior to testing cells are harvested, and distributed into 96-well plates (10,000 cells/well in the case of KeratinoSens™). Attention should be paid to avoid sedimentation of the cells during seeding to ensure homogeneous cell number distribution across wells. If this is not the case, this step may give rise to high well-to-well variability. For each repetition, three replicates are used for the luciferase activity measurements, and one parallel replicate used for the cell viability assay.

#### *Preparation of the test chemical and control substances*

21. The test chemical and control substances are prepared on the day of testing. For the KeratinoSens™ test method, test chemical are dissolved in dimethyl sulfoxide (DMSO) to the final desired concentration (e.g. 200 mM). The DMSO solutions can be considered self-sterilising, so that no sterile filtration is needed. Test chemical not soluble in DMSO is dissolved in sterile water or culture medium, and the solutions sterilised by e.g. filtration. For a test chemical which has no defined molecular weight (MW), a stock solution is prepared to a default concentration (40 mg/mL or 4% (w/v)) in the KeratinoSens™ assay. In case solvents other than DMSO, water or the culture medium are used, sufficient scientific rationale should be provided.

22. Based on the stock DMSO solutions of the test chemical, serial dilutions are made using DMSO to obtain 12 master concentrations of the chemical to be tested (from 0.098 to 200 mM in the KeratinoSens™ test method). For a test chemical not soluble in DMSO, the dilutions to obtain the master concentrations are made using sterile water or sterile culture medium. Independent of the solvent used, the master concentrations, are then further diluted 25 fold into culture medium containing serum, and finally used for treatment with a further 4 fold dilution factor so that the final concentrations of the tested chemical range from 0.98 to 2000 µM in the KeratinoSens™ test method. Alternative concentrations may be used upon justification (e.g. in case of cytotoxicity or poor solubility).

23. The negative (solvent) control used in the KeratinoSens™ test method is DMSO (CAS No. 67-68-5, ≥ 99% purity), for which six wells per plate are prepared. It undergoes the same dilution as described for the master concentrations in paragraph 22, so that the final negative (solvent) control concentration is 1%, known not to affect cell viability and corresponding to the same concentration of DMSO found in the tested chemical and in the positive control. For a test chemical not soluble in DMSO, for which the dilutions were made in water, the DMSO level in all wells of the final test solution must be adjusted to 1% as for the other test chemicals and control substances.

24. The positive control used in the case of KeratinoSens™ is cinnamic aldehyde (CAS No. 14371-10-9, ≥ 98% purity), for which a series of 5 master concentrations ranging from 0.4 to 6.4 mM are prepared in DMSO (from a 6.4 mM stock solution) and diluted as described for the master concentrations in paragraph 22, so that the final concentration of the positive control range from 4 to 64 µM. Other suitable positive controls, preferentially providing EC<sub>1.5</sub> values in the mid-range, may be used if historical data are available to derive comparable run acceptance criteria.

#### *Application of the test chemical and control substances*

25. For each test chemical and positive control substance, one experiment is needed to derive a prediction (positive or negative), consisting of at least two independent repetitions containing each three

replicates (i.e. n=6). In case of discordant results between the two independent repetitions, a third repetition containing three replicates should be performed (i.e. n=9). Each independent repetition is performed on a different day with fresh stock solution of test chemicals and independently harvested cells. Cells may come from the same passage however.

26. After seeding as described in paragraph 20, cells are grown for 24 hours in the 96-wells microtiter plates. The medium is then removed and replaced with fresh culture medium (150 µl culture medium containing serum but without Geneticin in the case of KeratinoSens™) to which 50 µl of the 25 fold diluted test chemical and control substances are added. At least one well per plate should be left empty (no cells and no treatment) to assess background values.

27. The treated plates are then incubated for about 48 hours at  $37\pm 1^{\circ}\text{C}$  in the presence of 5% CO<sub>2</sub> in the KeratinoSens™ test method. Care should be taken to avoid evaporation of volatile test chemicals and cross-contamination between wells by test chemicals by e.g. covering the plates with a foil prior to the incubation with the test chemicals.

#### *Luciferase activity measurements*

28. Three factors are critical to ensure appropriate luminescence readings:

- the choice of a sensitive luminometer,
- the use of a plate format with sufficient height to avoid light-cross-contamination; and
- the use of a luciferase substrate with sufficient light output to ensure sufficient sensitivity and low variability.

Prior to testing, a control experiment setup as described in Annex 3 should be carried out to ensure that these three points are met.

29. After the 48 hour exposure time with the test chemical and control substances in the KeratinoSens™ test method, cells are washed with a phosphate buffered saline, and the relevant lysis buffer for luminescence readings added to each well for 20 min at room temperature.

30. Plates with the cell lysate are then placed in the luminometer for reading which in the KeratinoSens™ test method is programmed to: (i) add the luciferase substrate to each well (i.e. 50 µl), (ii) wait for 1 second, and (iii) integrate the luciferase activity for 2 seconds. In case alternative settings are used, e.g. depending on the model of luminometer used, these should be justified. Furthermore, a glow substrate may also be used provided that the quality control experiment of Annex 3 is successfully fulfilled.”

#### *Cytotoxicity Assessment*

31. For the KeratinoSens™ cell viability assay, medium is replaced after the 48 hour exposure time with fresh medium containing MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue tetrazolium bromide; CAS No. 298-93-1) and cells incubated for 4 hours at 37°C in the presence of 5% CO<sub>2</sub>. The MTT medium is then removed and cells are lysed (e.g. by adding 10% SDS solution to each well) overnight. After shaking, the absorption is measured at i.e. 600 nm with a photometer.

## DATA AND REPORTING

*Data evaluation*

32. The following parameters are calculated in the KeratinoSens™ test method:

- - the maximal average fold induction of luciferase activity ( $I_{\max}$ ) value observed at any concentration of the tested chemical and positive control;
- - the  $EC_{1.5}$  value representing the concentration for which induction of luciferase activity is above the 1.5 fold threshold (i.e. 50% enhanced luciferase activity) was obtained; and
- - the  $IC_{50}$  and  $IC_{30}$  concentration values for 50% and 30% reduction of cellular viability.

Fold luciferase activity induction is calculated by Equation 1, and the overall maximal fold induction ( $I_{\max}$ ) is calculated as the average of the individual repetitions.

$$\text{Equation 1: } \textit{Fold induction} = \frac{(L_{\text{sample}} - L_{\text{blank}})}{(L_{\text{solvent}} - L_{\text{blank}})}$$

where

$L_{\text{sample}}$  is the luminescence reading in the test chemical well  
 $L_{\text{blank}}$  is the luminescence reading in the blank well containing no cells and no treatment  
 $L_{\text{solvent}}$  is the average luminescence reading in the wells containing cells and solvent (negative) control

$EC_{1.5}$  is calculated by linear interpolation according to Equation 2, and the overall  $EC_{1.5}$  is calculated as the geometric mean of the individual repetitions.

$$\text{Equation 2: } \textit{EC}_{1.5} = (C_b - C_a) \times \left( \frac{1.5 - I_a}{I_b - I_a} \right) + C_a$$

where

$C_a$  is the lowest concentration in  $\mu\text{M}$  with  $> 1.5$  fold induction  
 $C_b$  is the highest concentration in  $\mu\text{M}$  with  $< 1.5$  fold induction  
 $I_a$  is the fold induction measured at the lowest concentration with  $> 1.5$  fold induction (mean of three replicate wells)  
 $I_b$  is the fold induction at the highest concentration with  $< 1.5$  fold induction (mean of three replicate wells)

Viability is calculated by Equation 3:

$$\text{Equation 3: } \textit{Viability} = \frac{(V_{\text{sample}} - V_{\text{blank}})}{(V_{\text{solvent}} - V_{\text{blank}})} \times 100$$

where

$V_{\text{sample}}$  is the MTT-absorbance reading in the test chemical well  
 $V_{\text{blank}}$  is the MTT-absorbance reading in the blank well containing no cells and no treatment  
 $V_{\text{solvent}}$  is the average MTT-absorbance reading in the wells containing cells and solvent (negative) control

$IC_{50}$  and  $IC_{30}$  are calculated by linear interpolation according to Equation 4, and the overall  $IC_{50}$  and  $IC_{30}$  are calculated as the geometric mean of the individual repetitions.

$$\text{Equation 4: } IC_x = (C_b - C_a) \times \left( \frac{(100-x) - V_a}{V_b - V_a} \right) + C_a$$

where

- X is the % reduction at the concentration to be calculated (50 and 30 for IC<sub>50</sub> and IC<sub>30</sub>)  
 C<sub>a</sub> is the lowest concentration in µM with > x% reduction in viability  
 C<sub>b</sub> is the highest concentration in µM with < x% reduction in viability  
 V<sub>a</sub> is the % viability at the lowest concentration with > x% reduction in viability  
 V<sub>b</sub> is the % viability at the highest concentration with < x% reduction in viability

For each concentration showing > 1.5 fold luciferase activity induction, statistical significance is calculated (e.g. by a two-tailed Student's t-test), comparing the luminescence values for the three replicate samples with the luminescence values in the solvent (negative) control wells to determine whether the luciferase activity induction is statistically significant ( $p < 0.05$ ). The lowest concentration with > 1.5 fold luciferase activity induction is the value determining the EC<sub>1.5</sub> value. It is checked in each case whether this value is below the IC<sub>30</sub> value, indicating that there is less than 30% reduction in cellular viability at the EC<sub>1.5</sub> determining concentration.

33. It is recommended that data are visually checked with the help of graphs. If no clear dose-response curve is observed, or if the dose-response curve obtained is biphasic (i.e. crossing the threshold of 1.5 twice), the experiment should be repeated to verify whether this is specific to the test chemical or due to an experimental artefact. In case the biphasic response is reproducible in an independent experiment, the lower EC<sub>1.5</sub> value (the concentration when the threshold of 1.5 is crossed the first time) should be reported.

34. In the rare cases where a statistically non-significant induction above 1.5 fold is observed followed by a higher concentration with a statistically significant induction, results from this repetition are only considered as valid and positive if the statistically significant induction above the threshold of 1.5 was obtained for a non-cytotoxic concentration.

35. Finally, for test chemicals generating a 1.5 fold or higher induction already at the lowest test concentration of 0.98 µM, the EC<sub>1.5</sub> value of <0.98 is set based on visual inspection of the dose-response curve.

### ***Acceptance criteria***

36. The following acceptance criteria should be met when using the KeratinoSens™ test method. First, the luciferase activity induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 (e.g. using a t-test) in at least one of the tested concentrations (from 4 to 64 µM).

37. Second, the EC<sub>1.5</sub> value should be within two standard deviations of the historical mean of the testing facility (e.g. between 7 µM and 30 µM based on the validation dataset) which should be regularly updated. In addition, the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8. If the latter criterion is not fulfilled, the dose-response of cinnamic aldehyde should be carefully checked, and tests may be accepted only if there is a clear dose-response with increasing luciferase activity induction at increasing concentrations for the positive control.

38. Finally, the average coefficient of variation of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each repetition which consists of 6 wells tested in triplicate. If the variability is higher, results should be discarded.



*Interpretation of results and prediction model*

39. A KeratinoSens™ prediction is considered positive if the following 4 conditions are all met in 2 of 2 or in the same 2 of 3 repetitions, otherwise the KeratinoSens™ prediction is considered negative (Figure 1):

1. the  $I_{\max}$  is higher than ( $>$ ) 1.5 fold and statistically significantly different as compared to the solvent (negative) control (as determined by a two-tailed, unpaired Student's T-test);
2. the cellular viability is higher than ( $>$ ) 70% at the lowest concentration with induction of luciferase activity above 1.5 fold (i.e. at the  $EC_{1.5}$  determining concentration);
3. the  $EC_{1.5}$  value is less than ( $<$ ) 1000  $\mu\text{M}$  (or  $<$  200  $\mu\text{g/mL}$  for test chemicals with no defined MW);
4. there is an apparent overall dose-response for luciferase induction (or a biphasic response as mentioned under paragraph 33).

If in a given repetition, all of the three first conditions are met but a clear dose-response for the luciferase induction cannot be observed, then the result of that repetition should be considered inconclusive and further testing may be required (Figure 1). In addition, a negative result obtained with concentrations  $<$  1000  $\mu\text{M}$  (or  $<$  200  $\mu\text{g/mL}$  for test chemicals with no defined MW) should also be considered as inconclusive (see paragraph 11).

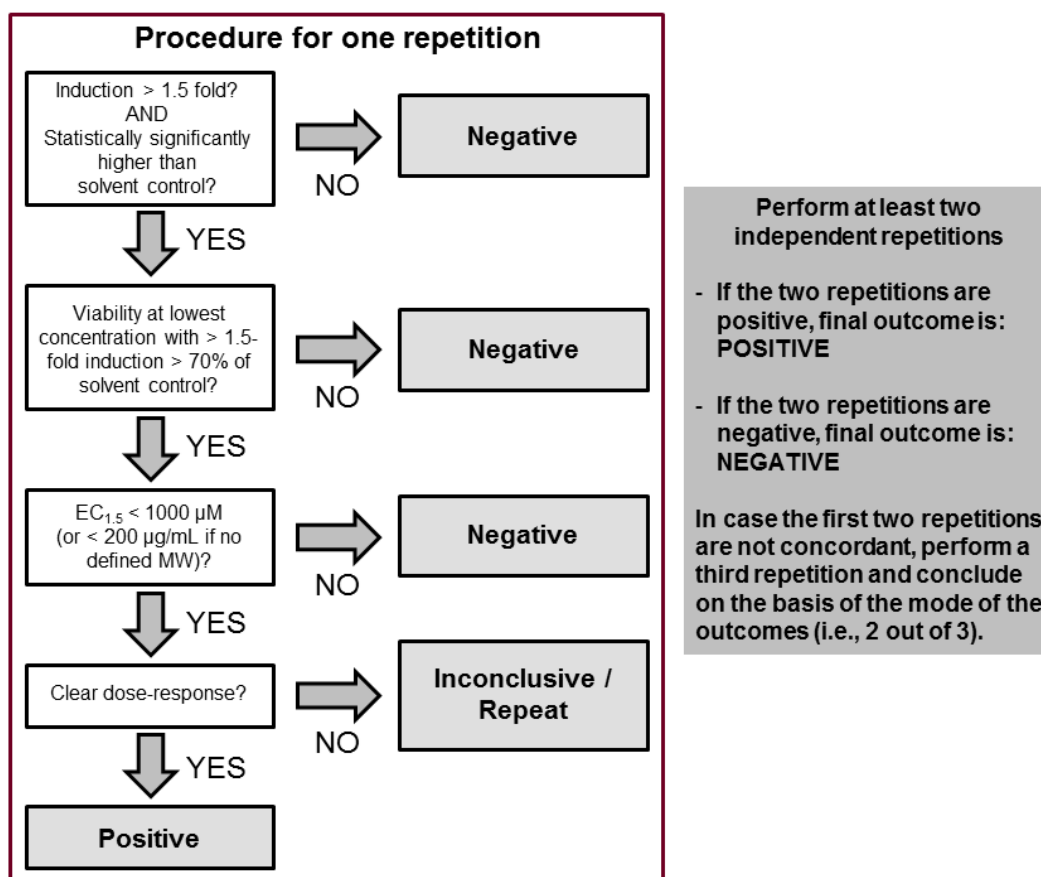


Figure 1: Prediction model used in the KeratinoSens™ test method. A KeratinoSens™ prediction should be considered in the framework of an IATA and in accordance with the provision of paragraphs 9 and 11.

40. In rare cases, test chemicals which induce the luciferase activity very close to the cytotoxic levels can be positive in some repetitions at non-cytotoxic levels (i.e. EC<sub>1.5</sub> determining concentration below (<) the IC<sub>30</sub>), and in other repetitions only at cytotoxic levels (i.e. EC<sub>1.5</sub> determining concentration above (>) the IC<sub>30</sub>). Such test chemicals shall be retested with more narrow dose-response analysis using a lower dilution factor (e.g. 1.33 or  $\sqrt{2}$  (=1.41) fold dilution between wells), to determine if induction has occurred at cytotoxic levels or not (9).

#### *Test report*

41. The test report should include the following information:

##### *Test chemical*

- Mono-constituent substance
  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Physical appearance, water solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available.
- Multi-constituent substance, UVCB and mixture:
  - Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;
  - Physical appearance, water solubility, DMSO solubility and additional relevant physicochemical properties, to the extent available;
  - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available.

##### *Controls*

- Positive control

- Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
  - Physical appearance, water solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available and where applicable;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Treatment prior to testing, if applicable (e.g. warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available;
  - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Negative (vehicle) control
- Chemical identification, such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other negative controls / vehicles than those mentioned in the Test Guideline are used and to the extent available;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent for each test chemical.

#### *Test method conditions*

- Name and address of the sponsor, test facility and study director;
- Description of test method used;
- Cell line used, its storage conditions and source (e.g. the facility from which they were obtained);
- Passage number and level of confluence of cells used for testing;
- Cell counting method used for seeding prior to testing and measures taken to ensure homogeneous cell number distribution (cf. paragraph 20);
- Luminometer used (e.g. model), including instrument settings, luciferase substrate used, and demonstration of appropriate luminescence measurements based on the control test described in Annex 3;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or to demonstrate reproducible performance of the test method over time.

#### *Test procedure*

- Number of repetitions and replicates used;
- Test chemical concentrations, application procedure and exposure time used (if different than the one recommended)

- Description of evaluation and decision criteria used;
- Description of study acceptance criteria used;
- Description of any modifications of the test procedure.

*Results*

- Tabulation of  $I_{\max}$ ,  $EC_{1.5}$  and viability values (i.e.  $IC_{50}$ ,  $IC_{30}$ ) obtained for the test chemical and for the positive control for each repetition as well as the mean values ( $I_{\max}$ : average;  $EC_{1.5}$  and viability values: geometric mean) and SD calculated using data from all individual repetitions and an indication of the rating of the test chemical according to the prediction model;
- Coefficient of variation obtained with the luminescence readings for the negative control for each experiment;
- A graph depicting dose-response curves for induction of luciferase activity and viability;
- Description of any other relevant observations, if applicable.

*Discussion of the results*

- Discussion of the results obtained with the KeratinoSens<sup>TM</sup> test method;
- Consideration of the test method results within the context of an IATA, if other relevant information is available.

*Conclusion*

## LITERATURE

1. United nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva, 2013. Available at: [[http://www.unece.org/trans/danger/publi/ghs/ghs\\_rev05/05files\\_e.html](http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html).]
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. OECD Environment, Health and Safety publications, Series on Testing and Assessment No. 168. OECD, Paris.
3. Adler S., Basketter D., Creton S., Pelkonen O., van Benthem J., Zuang V., Andersen K.E., Angers-Loustau A., Aptula A., Bal-Price A., Benfenati E., Bernauer U., Bessems J., Bois F.Y., Boobis A., Brandon E., Bremer S., Broschard T., Casati S., Coecke S., Corvi R., Cronin M., Daston G., Dekant W., Felter S., Grignard E., Gundert-Remy U., Heinonen T., Kimber I., Kleinjans J., Komulainen H., Kreiling R., Kreysa J., Leite S.B., Loizou G., Maxwell G., Mazzatorta P., Munn S., Pfuhrer S., Phrakonkham P., Piersma A., Poth A., Prieto P., Repetto G., Rogiers V., Schoeters G., Schwarz M., Serafimova R., Tähti H., Testai E., van Delft J., van Loveren H., Vinken M., Worth A., Zaldivar J.M. (2011). Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. *Archives of Toxicology* 85, 367-485.
4. OECD (2010). Skin sensitization: Local Lymph Node assay. OECD Guidelines for Chemical Testing No. 429. OECD, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
5. OECD (1992). Skin Sensitisation. OECD Guidelines for the Testing of Chemicals No. 406. OECD, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
6. OECD (2010). Skin sensitization: Local Lymph Node assay: DA. OECD Guidelines for Chemical Testing No. 442A. OECD, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
7. OECD (2010). Skin sensitization: Local Lymph Node assay: BrdU-ELISA. OECD Guidelines for Chemical Testing No. 442B. OECD, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
8. Natsch A. (2010). The Nrf2-Keap1-ARE Toxicity Pathway as a Cellular Sensor for Skin Sensitizers-Functional Relevance and Hypothesis on Innate Reactions to Skin Sensitizers. *Toxicological Sciences* 113, 284-292.
9. Emter R., Ellis G., Natsch A. (2010). Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. *Toxicology and Applied Pharmacology* 245, 281-290.
10. Dinkova-Kostova A.T., Holtzclaw W.D., Kensler T.W. (2005). The role of Keap1 in cellular protective responses. *Chem Res Toxicol.* 18, 1779-1791.
11. Kansanen E., Kuosmanen S.M., Leinonen H., Levonen A.L. (2013). The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biol.* 18,1, 45-49.
12. Natsch A., Bauch C., Foertsch L., Gerberick F., Normann K., Hilberer A., Inglis H., Landsiedel R., Onken S., Reuter H., Schepky A., Emter R. (2011). The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSens assay to predict skin sensitizers *in vitro*: results of a ring-study in five laboratories. *Toxicol. In Vitro* 25, 733-744.

13. Natsch A., Ryan C.A., Foertsch L., Emter R., Jaworska J., Gerberick G.F., Kern P. (2013). A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. *Journal of Applied Toxicology*, 33, 1337-1352.
14. EURL-ECVAM (2014). Recommendation on the KeratinoSens™ assay for skin sensitisation testing, 42 pp. Available at: [http://ihcp.jrc.ec.europa.eu/our\\_labs/eurl-ecvam/eurl-ecvam-recommendations/recommendation-keratinosens-skin-sensitisation](http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations/recommendation-keratinosens-skin-sensitisation).
15. DB-ALM (INVITTOX) (2013) Protocol 155: KeratinoSens™, 17pp. Available: [<http://ecvam-dbalm.jrc.ec.europa.eu/>].
16. Natsch A., Emter R., Ellis G. (2009). Filling the concept with data: integrating data from different in vitro and in silico assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. *Toxicol Sci.* 107, 106-121.
17. Ball N., Cagen S., Carrillo J.C., Certa H., Eigler D., Emter R., Faulhammer F., Garcia C., Graham C., Haux C., Kolle S.N., Kreiling R., Natsch A., Mehling A. (2011). Evaluating the sensitization potential of surfactants: integrating data from the local lymph node assay, guinea pig maximization test, and in vitro methods in a weight-of-evidence approach. *Regul Toxicol Pharmacol.* 60, 389-400.
18. Bauch C., Kolle S.N., Ramirez T., Eltze T., Fabian E., Mehling A., Teubner W., van Ravenzwaay B., Landsiedel R. (2012). Putting the parts together: combining in vitro methods to test for skin sensitizing potentials. *Regul Toxicol Pharmacol.* 63, 489-504.
19. Jaworska J., Dancik Y., Kern P., Gerberick F., Natsch A., 2013. Bayesian integrated testing strategy to assess skin sensitization potency: from theory to practice. *J Appl Toxicol.* 33, 1353-1364.
20. Andres E., Sa-Rocha V.M., Barrichello C., Haupt T., Ellis G., Natsch A. (2013). The sensitivity of the KeratinoSens™ assay to evaluate plant extracts: A pilot study. *Toxicology In Vitro* 27, 1220-1225.
21. Fabian E., Vogel D., Blatz V., Ramirez T., Kolle S., Eltze T., van Ravenzwaay B., Oesch F., Landsiedel R. (2013). Xenobiotic metabolizing enzyme activities in cells used for testing skin sensitization in vitro. *Arch Toxicol* 87, 1683-1969.
22. Thorne N., Inglese J., Auld D.S. (2010). Illuminating Insights into Firefly Luciferase and Other Bioluminescent Reporters Used in Chemical Biology. *Chemistry and Biology* 17, 646-657.
23. OECD (2012). BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists. OECD Guidelines for Chemical Testing No. 457. OECD, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
24. ECETOC (2003). Contact sensitization: Classification according to potency. European Centre for Ecotoxicology & Toxicology of Chemicals (Technical Report No. 87).
25. Gildea L.A., Ryan C.A., Foertsch L.M., Kennedy J.M., Dearman R.J., Kimber I., Gerberick G.F. (2006). Identification of gene expression changes induced by chemical allergens in dendritic cells: opportunities for skin sensitization testing. *J Invest Dermatol*, 126, 1813-1822.

26. Ryan C.A., Gildea L.A., Hulette B.C., Dearman R.J., Kimber I. And Gerberick G.F. (2004). Gene expressing changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. *Toxicol. Lett.* 150, 301-316.
27. Emter R., van der Veen J.W., Adamson G., Ezendam J., van Loveren H., Natsch A. (2013). Gene expression changes induced by skin sensitizers in the KeratinoSens™ cell line: Discriminating Nrf2-dependent and Nrf2-independent events. *Toxicol In Vitro* 27, 2225-2232.
28. OECD (2014, in preparation). Performance Standards for the assessment of proposed similar or modified in vitro skin sensitisation ARE-Nrf2 luciferase test methods in TG xxx. OECD Environment, Health and Safety publications, Series on Testing and Assessment N.XXX, OECD, Paris.
29. OECD (2005). Guidance Document the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. OECD Environment, Health and Safety publications, OECD Series on Testing and Assessment No.34. OECD, Paris, France.
30. NAFTA (North American Free Trade Agreement) (2012). Technical Working Group on Pesticides – (Quantitative) Structure Activity Relationship ((Q)SAR) Guidance Document. 186 pp. Disponible à l'adresse suivante :  
<http://www.epa.gov/oppfead1/international/naftatwg/guidance/qsar-guidance.pdf>

## ANNEX 1

## DEFINITIONS

**Accuracy:** The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of “relevance.” The term is often used interchangeably with “concordance”, to mean the proportion of correct outcomes of a test method (29).

**AOP (Adverse Outcome Pathway):** sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an *in vivo* outcome of interest (2).

**ARE:** Antioxidant response element (also called EpRE, electrophile response element), is a response element found in the upstream promoter region of many cytoprotective and phase II genes. When activated by Nrf2, it mediates the transcriptional induction of these genes.

**Coefficient of variation:** a measure of variability that is calculated for a group of replicate data by dividing the standard deviation by the mean. It can be multiplied by 100 for expression as a percentage.

**EC<sub>1.5</sub>:** Interpolated concentration for a 1.5 fold luciferase induction.

**IC<sub>30</sub>:** Concentration effecting a reduction of cellular viability by 30%.

**IC<sub>50</sub>:** Concentration effecting a reduction of cellular viability by 50%.

**Hazard:** Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

**IATA (Integrated Approach to Testing and Assessment):** A structured approach used for hazard identification (potential), hazard characterisation (potency) and/or safety assessment (potential/potency and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazard and/or risk and/or the need for further targeted and therefore minimal testing.

**I<sub>max</sub>:** Maximal induction factor of luciferase activity compared to the solvent (negative) control measured at any test chemical concentration.

**Keap1:** Kelch-like ECH-associated protein 1, is a sensor protein that can regulate the Nrf2 activity. Under un-induced conditions the Keap1 sensor protein targets the Nrf2 transcription factor for ubiquitinylation and proteolytic degradation in the proteasome. Covalent modification of the reactive cysteine residues of Keap 1 by small molecules can lead to dissociation of Nrf2 from Keap1 (8) (10) (11).

**Mixture:** A mixture or a solution composed of two or more substances in which they do not react (1).



**Mono-constituent substance:** A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

**Multi-constituent substance:** A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration  $\geq 10\%$  (w/w) and  $< 80\%$  (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

**Nrf2:** nuclear factor (erythroid-derived 2)-like 2, is a transcription factor involved in the antioxidant response pathway. When Nrf2 is not ubiquitinated, it builds up in the cytoplasm and translocates into the nucleus, where it combines to the ARE in the upstream promoter region of many cytoprotective genes, initiating their transcription (8) (10) (11).

**Positive control:** A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

**Relevance:** Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (29).

**Reliability:** Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (29).

**Reproducibility:** The agreement among results obtained from testing the same substance using the same test protocol (see reliability) (29).

**Sensitivity:** The proportion of all positive / active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (29).

**Solvent/vehicle control:** A replicate containing all components of a test system except of the test chemical, but including the solvent that is used. It is used to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent.

**Specificity:** The proportion of all negative / inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (29).

**Substance:** Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities

deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (1).

**Test chemical:** The term "test chemical" is used to refer to what is being tested.

**United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS):** A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

**UVCB:** substances of unknown or variable composition, complex reaction products or biological materials.

**Valid test method:** A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (29).

## ANNEX 2

## PROFICIENCY SUBSTANCES

*In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected KeratinoSens™ prediction for the 10 Proficiency Substances recommended in Table 1 and by obtaining the EC<sub>1.5</sub> and IC<sub>50</sub> values that fall within the respective reference range for at least 8 out of the 10 proficiency substances. These Proficiency Substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were commercial availability, availability of high quality *in vivo* reference, and availability of high quality *in vitro* data from the KeratinoSens™ test method.

Table 1: Recommended substances for demonstrating technical proficiency with the KeratinoSens™ test method

Proficiency Substances	CASRN	Physical Form	<i>In Vivo</i> Prediction (1)	KeratinoSens™ Prediction (2)	EC <sub>1.5</sub> (µM) Reference Range (3)	IC <sub>50</sub> (µM) Reference Range (3)
Isopropanol	67-63-0	Liquid	Non-sensitiser	Negative	> 1000	> 1000
Salicylic acid	69-72-7	Solid	Non-sensitiser	Negative	> 1000	> 1000
Lactic acid	50-21-5	Liquid	Non-sensitiser	Negative	> 1000	> 1000
Glycerol	56-81-5	Liquid	Non-sensitiser	Negative	> 1000	> 1000
Cinnamyl alcohol	104-54-1	Solid	Sensitiser (weak)	Positive	25 - 175	> 1000
Ethylene glycol dimethacrylate	97-90-5	Liquid	Sensitiser (weak)	Positive	5 - 125	> 500
2-Mercaptobenzothiazole	149-30-4	Solid	Sensitiser (moderate)	Positive	25 - 250	> 500
Methyldibromo glutaronitrile	35691-65-7	Solid	Sensitiser (strong)	Positive	< 20	20 - 100
4-Methylaminophenol sulfate	55-55-0	Solid	Sensitiser (strong)	Positive	< 12.5	20 - 200
2,4-Dinitro-chlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Positive	< 12.5	5 - 20

(1) The *in vivo* hazard (and potency) predictions are based on LLNA data (13). The *in vivo* potency is derived using the criteria proposed by ECETOC (24).

(2) A KeratinoSens™ prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 11 of the Test Guideline.

(3) Based on the historical observed values (12).

## ANNEX 3

## QUALITY CONTROL OF LUMINESCENCE MEASUREMENTS

## Basic experiment for ensuring optimal luminescence measurements in the KeratinoSens™ assay

The following three parameters are critical to ensure obtaining reliable results with the luminometer:

- having a sufficient sensitivity giving a stable background in control wells;
- having no gradient over the plate due to long reading times; and
- having no light contamination in adjacent wells from strongly active wells.

Prior to testing it is recommended to ensure having appropriate luminescence measurements, by testing a control plate set-up as described below (triplicate analysis).

## Plate setup of first training experiment

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
B	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
C	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
D	EGDMA 0.98	EGDMA 1.95	EGDMA 3.9	EGDMA 7.8	EGDMA 15.6	EGDMA 31.25	EGDMA 62.5	EGDMA 125	EGDMA 250	EGDMA 500	EGDMA 1000	EGDMA 2000
E	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
F	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
G	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
H	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	CA 4	CA 8	CA 16	CA 32	CA 64	Blank

EGDMA = Ethylene glycol dimethacrylate (CAS No.: 97-90-5) a strongly inducing compound

CA = Cinnamic aldehyde, positive reference (CAS No.: 104-55-2)

## The quality control analysis should demonstrate:

- a clear dose-response in row D, with the  $I_{\max} > 20$  fold above background (in most cases  $I_{\max}$  values between 100 and 300 are reached);
- no dose-response in row C and E (no induction value above 1.5 (ideally not above 1.3) due to possible light contamination especially next to strongly active wells in the EGDMA row;
- no statistically significant difference between the rows A, B, C, E, F and G. (i.e. no gradient over plate); and
- variability in any of the rows A, B, C, E, F and G and in the DMSO wells in row H should be below 20% (i.e. stable background).