Section 4
Health effects

Test Guideline No. 442D
In Vitro Skin Sensitisation

ARE-Nrf2 Luciferase Test Method

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OECD KEY EVENT BASED GUIDELINE FOR THE TESTING OF CHEMICALS

In vitro skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on Keratinocyte activation

GENERAL INTRODUCTION

Keratinocyte activation Key Event based Test Guideline

1. A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). There is general agreement on the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised as an Adverse Outcome Pathway (AOP) (2), starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with thiol (i.e. cysteine) and primary amines (i.e. lysine) such as organic chemicals. In this instance, the molecular initiating event (i.e. the first key 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 changes in 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 (3).

2. This Test Guideline describes in vitro assays that address mechanisms described under the second Key Event of the AOP for skin sensitisation, namely keratinocyte activation (2). The Test Guideline comprises test methods to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (1). The test methods currently described in this Test Guideline are:

- The ARE-Nrf2 luciferase KeratinoSens™ test method (Appendix IA), and
- The ARE-Nrf2 luciferase LuSens test method (Appendix IB).

3. These two in vitro ARE-Nrf2 luciferase test methods have been considered scientifically valid. The KeratinoSens™ test method first underwent a validation study followed by an independent peer-review by EURL ECVAM Scientific Advisory Committee (ESAC) and positive recommendations by EURL ECVAM, and is considered the validated reference method (VRM) (3) (4) (5) (6). The LuSens test method later underwent a Performance Standard-based validation study based on which it was also reviewed and received positive opinion by ESAC (7) (8) (9) (10).

4. The test methods included in this Test Guideline may differ in relation to the procedure used to generate the data and the readouts measured but can be used indiscriminately to address countries' requirements for test results on the keratinocytes activation Key Event of the AOP for skin sensitisation while benefiting from the Mutual Acceptance of Data.

Background and principles of the test methods included in the Key Event based Test Guidelines

5. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman and the Buehler Test (OECD TG 406) (11), assess both the induction and elicitation phases of skin sensitisation. The murine tests, the LLNA (OECD TG 429) (12) and its three non-radioactive modifications, LLNA: DA (OECD TG 442A) (13) as well as LLNA: BrdU-ELISA and BrdU-FCM (OECD TG 442B) (14), all assess the induction response exclusively, and have gained acceptance since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitisation.

6. Mechanistically-based in chemico and in vitro test methods addressing the first three key events of the skin sensitisation AOP have been adopted for contributing to the evaluation of the skin sensitisation hazard potential of chemicals: the OECD TG 442C describes the Direct Peptide Reactivity Assay (15) addressing the first key event; the present Test Guideline assesses keratinocyte activation addressing the second key event and the OECD TG 442E addresses the activation of dendritic cells, the third key event of the skin sensitisation AOP (16). Finally, the fourth key event representing T-cell proliferation is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (12).

7. As keratinocyte activation represents only one key event of the skin sensitisation AOP (2) (17), information generated with test methods developed to address this specific key event may not be sufficient to conclude on the presence or absence of skin sensitisation potential of chemicals. Therefore data generated with the test methods described in this Test Guideline are proposed to support the discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers when used within Integrated Approaches to Testing and Assessment (IATA), together with other relevant 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 (17). Examples on the use of data generated with these methods within Defined Approaches, i.e. approaches standardised both in relation to the set of information sources used and in the procedure applied to derive predictions have been published (17) and can be employed as useful elements within IATA.

8. The test methods described in this Test Guideline cannot be used on their own, neither to sub-categorise skin sensitisers into subcategories 1A and 1B as defined by UN GHS (1), for authorities implementing these two optional subcategories, nor to predict potency for safety assessment decisions. However, depending on the regulatory

framework, positive results generated with these methods may be used on their own to classify a chemical into UN GHS category 1.

9. The term "test chemical" is used in this Test Guideline to refer to what is being tested[1] and is not related to the applicability of the test methods to the testing of mono-constituent substances, multi-constituent substances and/or mixtures. When testing in submerged cultures, it should be determined that the test chemical is dissolved in the exposure medium or at least forms a stable dispersion (e.g. by visual inspection of the test chemical dissolved/prepared at the maximal final test concentration in the exposure medium, showing that no undissolved residues remain and that no precipitate or phase separation forms if the solution is left to settle for several hours).

10. Limited information is currently available on the applicability of the test methods to multi-constituent substances/mixtures (18) (19) (20). Although not evaluated in the validation studies, the test methods may nevertheless be technically applicable to the testing of multi-constituent substances and mixtures. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. Moreover, when testing multi-constituent substances or mixtures, consideration should be given to possible interference of cytotoxic constituents with the observed responses (e.g. the presence of a high content of non-sensitising cytotoxic constituents may mask the response of weakly sensitising components or sensitising components present at low concentration). It might, depending on the particular case, be scientifically justified to test either single main constituents forming the major fraction or several fractions of the mixture to conclude on the sensitisation potential of the complex mixture.

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Annex: 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 (3).

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 Nfr2, it mediates the transcriptional induction of these genes.

CV: Cell viability

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.

CV75: The estimated concentration resulting in 75% cell viability.

EC1.5: Interpolated concentration resulting in a 1.5 fold luciferase induction.

Fold luciferase activity induction: Represents the ratio of luminescence of treated cells (minus blank) over the luminescence of the cells exposed to the concurrent solvent/vehicle control (minus blank).

IC30: Concentration effecting a reduction of cellular viability by 30%.

IC50: 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.

Imax: 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 (4) (5) (6).

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 ≥ 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.

Negative control: A sample containing all components of a test system and treated with a substance known not to induce a positive response in the test system. This sample is processed with test chemical-treated samples and other control samples.

Nrf2: nuclear factor (erythroid-derived 2)-like 2, is a transcription factor involved in the antioxidant response pathway. When Nrf2 is not ubiquitinylated, 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 (4) (5) (6).

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (i) essential test method components; (ii) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of reference chemicals (3).

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.

Proficiency chemicals (substances): A subset of the Reference Chemicals included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardised test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Reference chemicals (substances): A set of chemicals to be used to demonstrate the ability of a new test method to meet the acceptability criteria demonstrated by the validated reference test method(s). These chemicals should be representative of the classes of chemicals for which the test method is expected to be used, and should represent the full range of responses that may be expected from the chemicals for which it may be used, from strong, to weak, to negative.

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 (3).

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 (3).

Reproducibility: The agreement among results obtained from testing the same substance using the same test protocol (see reliability) (3).

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 (3).

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 (3).

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.

Validated Reference Method (VRM): the first method(s) endorsed as scientific valid and used as a reference for performance-based validation studies.

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 (3).

Xeno-free: which does not contain any element that is not from the same species as the cells used, in this case, human.

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Appendix IA: In Vitro Skin Sensitisation: The ARE-Nrf2 Luciferase KeratinoSens™ Test Method

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The test method described in this Appendix to Test Guideline 442D addresses the second key event of the skin sensitisation AOP (1), namely keratinocytes activation, by assessing with the help of luciferase, the Nrf2-mediated activation of antioxidant response element (ARE)-dependent genes. Skin sensitisers have been reported to induce genes that are regulated by the ARE (2) (3). Small electrophilic substances 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 (2) (4) (5).

2. The in vitro ARE-Nrf2 luciferase KeratinoSens™ test method (hereafter called the KeratinoSens™ test method) underwent validation studies (3) (6) (7) followed by an independent peer review conducted by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) (8). 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 identification (8).

3. 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 techniques (8). The level of reproducibility in predictions that can be expected from the KeratinoSens™ test method is in the order of 85% within and between laboratories (8). The accuracy (77% - 155/201), sensitivity (78% - 71/91) and specificity (76% - 84/110) of the KeratinoSens™ test method 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 (8). These figures are similar to those published based on in-house testing of about 145 test substances (77% accuracy, 79% sensitivity, 72% specificity) (7). This information indicates the usefulness of the KeratinoSens™ test method to contribute to the identification of skin sensitisation hazard. However, the accuracy values given here for KeratinoSens™ test method 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 a Defined Approach or an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General Introduction of this Test Guideline. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in humans.

4. On the basis of the current data available, the KeratinoSens™ 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 (3) (6) (7) (8). The test method is applicable to test chemicals soluble or that form a stable dispersion in the exposure medium (i.e. a colloid or suspension in which the test chemical does not settle or separate from the solvent into different phases). Test chemicals that do not fulfil these conditions at the highest final required concentration of 2 000 µM may still be tested at lower concentrations. In such a case, results fulfilling the criteria for positivity could still be used to support the identification of the test chemical as a skin sensitiser. In cases where a negative result is obtained in a test with a maximal concentrations < 1000 µM and no cytotoxicity is reached, the result should be considered as inconclusive (see prediction model in paragraph 32). If cytotoxicity (< 70% viability) is reached at a maximal soluble test concentration < 1000 µM, criteria for negativity can still be applied. In general mono constituent substances with a LogP above 7 may be insoluble in the exposure medium, however, if solubility or stable dispersion can be obtained and documented, testing may still be conducted.

5. 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 as the key mechanism leading to the activation of the Keap1-Nrf2-ARE pathway appears to be the electrophilic reaction of stressors with nucleophilic thiols (cysteine sulfhydryl groups) of Keap-1. Complementary information from peptide reactivity assays may help addressing this uncertainty, in particular assays able to distinguish between cysteine and lysine reactivity. Furthermore, because of the limited metabolic capability of the cell line used (10) 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. However, it has been shown that the majority of pre-haptens (i.e. chemicals activated by auto-oxidation) and pro-haptens (i.e. chemicals requiring enzymatic activation for example via P450 enzymes) are sufficiently well identified by a combination of test methods covering key events 1, 2 and 3 on the AOP so that negative results can in general be used to support classification (12) (20) (34). On the other hand, test chemicals that do not act as a sensitiser but are nevertheless chemical stressors may lead to false positive results (8). 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 (13). 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 (14) 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 (14). In cases where evidence can be demonstrated on the non-applicability of the KeratinoSens™ test method to other specific categories of test chemicals, the test method should not be used for those specific categories.

6. In addition to supporting discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers, the KeratinoSens™ test method also provides concentration-response information that may potentially contribute to the assessment of sensitising potency when used in integrated approaches such as IATA (11) (15). Examples on how to use the KeratinoSens™ test method results in combination with other information sources are reported in the literature (7) (11) (16) (17) (18) (19) (20). Specifically, the use of KeratinoSens™ test method dose-response data along with quantitative peptide reactivity data to assess potency in the LLNA and in human tests has been described (21) and has been used in Bayesian integrated testing strategies on LLNA potency (11) (22). Furthermore, evaluation has been conducted on how to specifically address potency in humans (23). Finally, the use of KeratinoSens™ test method to assess potency of specific chemical classes has also been described (21) (24).

7. Definitions are provided in the Annex 1 of the General Introduction.

PRINCIPLE OF THE TEST

8. The KeratinoSens™ test method makes use of an immortalised adherent cell line derived from human keratinocytes stably harbouring a luciferase reporter gene under the control of the antioxidant response element of the human AKR1C2 gene (25). This gene is known to be up-regulated by skin sensitisers (26) (27). The cell line contains the luciferase gene under the transcriptional control of a constitutive promoter fused with the ARE element. 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 (28). 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.

9. Test chemicals are considered positive in the KeratinoSens™ test method if they induce a statistically significant induction of the luciferase activity above a given threshold (i.e. $\geq$ 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% (3) (6). 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 obtained from the series of tested concentrations of the test chemical (see paragraph 26 for calculations). Finally, parallel cytotoxicity measurements should be conducted to assess whether luciferase induction occurs at sub-cytotoxic concentrations.

10. Prior to routine use of the KeratinoSens™ test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency, using the ten Proficiency Substances listed in Annex 1 of this Appendix.

11. Performance standards (PS) (29) are available to facilitate the validation of new or modified in vitro ARE-Nrf2 luciferase test methods similar to the KeratinoSens™ VRM 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

12. A DB-ALM protocol for the KeratinoSens™ test method is available and should be employed when implementing and using the test method in the laboratory (9). Laboratories implementing the test method can obtain the recombinant cell line used in the KeratinoSens™ test method by signing a standard agreement with the test method developer[2] which includes the licence for the commercial use of the luciferase gene. The luciferase reporter gene assay is also subject to a Promega limited use licence that requires the use of luminescent assay reagents purchased from Promega. The following paragraphs provide with a description of the main components and procedures of the KeratinoSens™ test method. Furthermore, an adaptation of the KeratinoSens™ test method to xeno-free culture conditions using human reagents is described in Annex 2 of this Appendix (33).

[2] Givaudan Schweiz AG, CH-8310 Kemptthal

However, it is recommended that the relevant regulatory authorities be consulted before deciding on the type of serum to be used in the KeratinoSens™ test method.

Preparation of the keratinocyte cultures

13. The KeratinoSens™ transgenic cell line having a stable insertion of the luciferase reporter gene under the control of the ARE-element should be used. Upon receipt, KeratinoSens™ cells are propagated as defined by the test method protocol (e.g. 2 to 4 passages) and stored frozen as a homogeneous stock. Cells from this original stock can be propagated up to maximum 25 passages and are employed for routine testing using the maintenance/growth medium (Dulbecco's Modified Eagle's medium (DMEM) containing serum and Geneticin to allow maintaining the gene) as described within the test method's DB-ALM protocol (9).

14. 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 at a cell density of 10,000 cells/well. 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 at least one parallel replicate is used for the cell viability assay.

Preparation of the test chemical and control substances

15. The test chemical and control substances are prepared on the day of testing. Test chemicals are dissolved in dimethyl sulfoxide (DMSO, CAS No. 67-68-5, ≥ 99% purity) 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 chemicals not soluble in DMSO are 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 the default concentration of 40 mg/mL or 4% (w/v). In case solvents other than DMSO, water or the culture medium are used, appropriate scientific rationale should be provided.

16. Based on the stock solutions of the test chemical, serial dilutions are made using DMSO or a suitable solvent (i.e. sterile water or culture medium) to obtain 12 master concentrations of the chemical to be tested (from 0.098 to 200 mM). 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 (based on a dilution factor of 2). Alternative concentrations may be used upon justification (e.g. in case of cytotoxicity or poor solubility). For a test chemical which has no defined MW, serial dilutions are made using DMSO or a suitable solvent to obtain the desired final concentrations of the test chemical (e.g. 12 concentrations ranging from 0.196 to 400 µg/ml).

17. A concurrent solvent/vehicle control should be tested within each repetition (i.e. DMSO), for which a sufficient number of wells should be prepared per plate (i.e. six). The solvent/vehicle control undergoes the same dilutions as described for the master concentrations in paragraph 16, so that the final solvent/vehicle 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. This solvent/vehicle control (i.e. DMSO) also represents the negative control for the KeratinoSens™ test method.

18. A concurrent positive control should also be tested in a sufficient number of wells within each repetition as described within the DB-ALM protocol (9) to demonstrate appropriate response of the test system. For example, five concentrations of cinnamic aldehyde (CAS No. 14371-10-9, ≥ 98% purity) are used within each replicate in the KeratinoSens™ test method, 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 16, so that the final concentration of the positive control range from 4 to 64 µM. Other suitable positive controls, preferentially providing $EC_{1.5}$ 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

19. 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.

20. After seeding as described in paragraph 14, 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 as described within the DB-ALM protocol (9)) 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.

21. The treated plates are then incubated for about 48 hours at 37±1°C in the presence of 5% CO_2. 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 during incubation with the test chemicals.

Luciferase activity measurements

22. The following 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,
- the use of a luciferase substrate with sufficient light output to ensure sufficient sensitivity and low variability; and
- an appropriate and stable background level.

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

23. After the 48 hour exposure time with the test chemical and control substances, cells are washed with a phosphate buffered saline, and the relevant lysis buffer for luminescence readings added to each well for a sufficient time (e.g. 20 min at room temperature).

24. Plates with the cell lysate are then placed in the luminometer for reading which 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 of this Appendix is successfully fulfilled.

Cytotoxicity Assessment

25. For the KeratinoSensTM cell viability assay, medium is replaced after the 48 hour exposure time with fresh medium containing 5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue tetrazolium bromide; CAS No. 298-93-1) and cells are incubated for 4 hours at 37±1°C in the presence of 5% CO_2. The MTT medium is then removed and cells are lysed by using an appropriate lysing agent for a sufficient amount of time (e.g. 10% SDS overnight). After shaking, the absorption is then measured at i.e. 600 nm with a photometer as described in the test method protocols (9).

DATA AND REPORTING

Data evaluation

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

- the maximal average fold induction of luciferase activity (Imax) 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 which 50% and 30% reduction of cellular viability occur respectively.

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.

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

where

L_{sample} is the luminescence reading in the test chemical well
L_{blank} is the luminescence reading in the blank well containing no cells and no treatment
$L_{solvent}$ 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.

$$\text{Equation 2:} \qquad EC1.5 = (C_b - C_a) \times \left(\frac{1.5 - I_a}{I_b - I_a}\right) + C_a$$

where

C_a is the lowest concentration in µM with > 1.5 fold induction
C_b is the highest concentration in µM 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:

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

where

V_{sample} is the MTT-absorbance reading in the test chemical well
V_{blank} is the MTT-absorbance reading in the blank well containing no cells and no treatment
$V_{solvent}$ 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.

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_{50} and IC_{30})
C_a is the lowest concentration in µM with > x% reduction in viability
C_b is the highest concentration in µM with < x% reduction in viability
V_a is the % viability at the lowest concentration with > x% reduction in viability
V_b is the % viability at the highest concentration with < x% reduction in viability

27. For each concentration showing a luciferase activity induction equal or higher (≥) than 1.5 fold, statistical significance is determined (e.g. using a two-tailed Student's t-test) by comparing the luminescence values of the three replicate samples with the luminescence values in the solvent/vehicle control wells to assess whether the luciferase activity induction is statistically significant ($p < 0.05$). Furthermore, it should be checked that no significant cytotoxic effects occur at the lowest concentration leading to ≥1.5 fold luciferase induction and that this concentrations is below the IC_{30} value, indicating that there is less than or equal to 30% reduction in cellular viability. In addition, at least two consecutive concentrations should have > 70% viability, otherwise the concentration range should be adjusted.

28. 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 concentration, i.e. when the threshold of 1.5 is crossed the first time should be reported.

29. In the KeratinoSens™ test method, in the rare cases where a statistically non-significant luciferase induction equal or 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 equal or above the threshold of 1.5 was obtained for a non-cytotoxic concentration.

30. Finally, for test chemicals generating in the KeratinoSens™ test method a 1.5 fold or higher induction already at the lowest tested concentration (i.e. 0.98 µM), the EC1.5 value of <0.98 is set based on visual inspection of the dose-response curve.

Acceptance criteria

31. The following acceptance criteria should be met when using the KeratinoSens™ test method.

- 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 (4 to 64 µM).
- The EC1.5 value of the positive control 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.

- The average coefficient of variation of the luminescence reading for the solvent/vehicle control (i.e. DMSO) should be below 20% in each repetition. If the variability is higher, results should be discarded.

Interpretation of results and prediction model

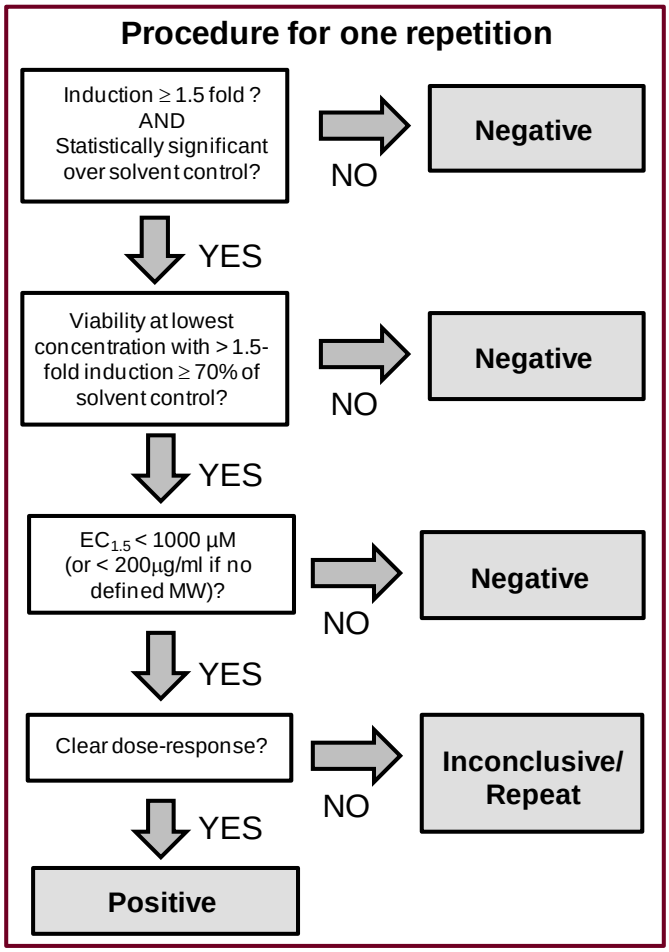
32. A KeratinoSens™ prediction is considered positive if the following 4 conditions are all met in 2 of 2 or in the same 2 of 3 repetitions, otherwise the KeratinoSens™ prediction is considered negative (Figure 1):

- the I_{max} is equal or higher than (≥) 1.5 fold and statistically significantly different as compared to the solvent/vehicle control (as determined by a two-tailed, unpaired Student's T-test);
- the cellular viability is higher than (>) 70% at the lowest concentration with induction of luciferase activity ≥1.5 fold (i.e. at the $EC_{1.5}$ determining concentration);
- the $EC_{1.5}$ value is less than (<) 1000 µM (or < 200 µg/mL for test chemicals with no defined MW);
- there is a dose-dependent increase in luciferase induction (or a biphasic response as mentioned under paragraph 28).

If in a given repetition, all of the three first conditions are met but a clear dose-dependent increase in 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 test chemicals tested at a maximal test concentration < 1000 µM (or 200 µg/mL for test chemicals with no defined MW) and which do not reach cytotoxicity (< 70% viability) at the maximal tested concentration should also be considered as inconclusive (see paragraph 4).

Figure 1. Prediction model used in the KeratinoSens™ test method.

A KeratinoSens™ prediction should be considered in the framework of a Defined Approach or of an IATA and in accordance with the provisions of paragraphs 7 and 8 of the general introduction



Perform at least two independent repetitions

- **If the two repetitions are positive, final outcome is: POSITIVE**
- **If the two repetitions are negative, final outcome is: NEGATIVE**

In case the first two repetitions are not concordant, perform a third repetition and conclude on the basis of the mode of the outcomes (i.e., 2 out of 3).

33. In cases when test chemicals induce the luciferase activity very close to the cytotoxic levels, they can be positive in some repetitions at non-cytotoxic levels (i.e. $EC_{1.5}$ determining concentration below (<) the IC_{30}), and in other repetitions only at cytotoxic levels (i.e. $EC_{1.5}$ determining concentration above (>) the IC_{30}). Such test chemicals shall be retested with more narrow dose-response analysis using a lower dilution factor (e.g. 1.33 or √2 (=1.41) fold dilution between wells), to determine if induction has occurred at cytotoxic levels or not (3).

Test report

34. 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, like batch/ lot number and expiry date;
  - Physical appearance, water solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
  - Statement on (in)solubility or stable dispersion in exposure media;
  - 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;
  - Statement on (in)solubility or stable dispersion in exposure media;
  - 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.

- Solvent/vehicle/negative 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 solvents/vehicles /negative controls than those mentioned in this Appendix are used and to the extent available;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle 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 14);
- 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 of this Appendix;
- 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 Imax, $EC_{1.5}$ 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 (Imax: 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 solvent/vehicle/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™ test method;
- Consideration of the test method results within the context of an IATA, if other relevant information is available.

Conclusion

Literature

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APPENDIX IA - ANNEX 1: PROFICIENCY SUBSTANCES

In Vitro Skin Sensitisation: The ARE-Nrf2 Luciferase KeratinoSensTM Test Method

Prior to routine use of a test method that adheres to this Appendix of Test Guideline 442D, 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 $EC_{1.5}$ and IC_{50} 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	LLNA Prediction (1)	Human category (2)	KeratinoSens™ Prediction (3)	$EC_{1.5}$ (µM) Reference Range (4)	IC_{50} (µM) Reference Range (5)
Salicylic acid	69-72-7	Solid	Non-sensitiser	Cat. 6	Negative	> 1000	> 1000
Lactic acid	50-21-5	Liquid	Non-sensitiser	Cat. 6	Negative	> 1000	> 1000
Glycerol	56-81-5	Liquid	Non-sensitiser	Cat. 6	Negative	> 1000	> 1000
Isopropanol	67-63-0	Liquid	Non-sensitiser	Cat. 5	Negative	> 1000	> 1000
Ethylene glycol dimethacrylate	97-90-5	Liquid	Sensitiser (weak)	Cat. 4	Positive	5 - 125	> 500
Cinnamyl alcohol	104-54-1	Solid	Sensitiser (weak)	Cat. 3	Positive	25 - 175	> 1000
2-Mercaptobenzothiazole	149-30-4	Solid	Sensitiser (moderate)	Cat. 3	Positive	25 - 250	> 500
4-Methylaminophenol sulfate	55-55-0	Solid	Sensitiser (strong)	Cat. 3	Positive	< 12.5	20 - 200
Methyldibromo	35691-65-7	Solid	Sensitiser	Cat. 2	Positive	< 20	20 - 100

glutaronitrile			(strong)				
2,4-Dinitro-chlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Cat. 1	Positive	< 12.5	5 - 20

Notes: (1) The in vivo hazard (and potency) predictions are based on LLNA data (7). The in vivo potency is derived using the criteria proposed by ECETOC (15); (2) According to Basketter and co-workers (32). Cat. 1 represents clear evidence of contact allergy, Cat. 2 a frequent cause of contact allergy, Cat. 3 a common cause of contact allergy, Cat. 4 an infrequent cause of contact allergy, Cat. 5 a rare cause of contact allergy, and Cat. 6 essentially absent evidence of contact allergy (32). (3) A KeratinoSensTM prediction should be considered in the framework of a Defined Approach or of an IATA and in accordance with the provisions of paragraphs 7 and 8 of the general introduction. (4) Based on the historical observed values (6).

APPENDIX IA - ANNEX 2: ADAPTATION OF THE KERATINOSENS™ TEST METHOD USING HUMAN REAGENTS TO ACHIEVE XENO-FREE CELL CULTURE

The following adaptation to the KeratinoSens™ test method may be performed using human reagents (human serum and recombinant human trypsin) to achieve xeno-free cell culture, subject to demonstration of technical proficiency (as described in Annex 1) using the adapted method (33).

Table 2. Summary of adaptations

Aspect of the Method	Validated Reference Method (KeratinoSens™) (Appendix 1A)	Xeno-Free Adaptation (this Annex)
Serum[1]	States "serum" (DB-ALM protocol 155 states Foetal Calf Serum) (paragraph 13)	Specifies 10% human serum
Cytotoxicity measurement[2]	MTT: 4hrs incubation; solubilise in 10% SDS overnight; read at 600nm (paragraph 25)	MTT (1mg/ml): 3hrs incubation; solubilise in isopropanol; read at 570nm
Positive control[2]	Cinnamic aldehyde 4-64µM (paragraph 18)	Cinnamic aldehyde 8-128µM.
Trypsin[1]	Not specified (DB-ALM protocol 155 states Trypsin EDTA)	Non-animal recombinant trypsin (TrypZean, Sigma-Aldrich T3499)

Note: [1]adaptations to achieve xeno-free conditions; [2]other adaptations to the method (33).

Prior to use for testing purposes, the KeratinoSens™ cell line should be adapted to routine culture using 10% human serum. Human serum (from pooled donors) should be obtained from a reliable commercial source, with appropriate donor consent and QC testing for cell culture applications. As with any type of serum, when a new batch is used, an internal validation of the batch including cell morphology, growth rates and Imax / $EC_{1.5}$ values with at least the positive control, and preferably representative reference chemicals (at least one sensitiser and one non sensitiser) should be conducted, with subsequent reservation of successfully performing batches for long term use. If the cells have previously been cultured in foetal calf serum, they should be weaned into human serum over at least 3 passages. Provided that the cells are showing healthy morphology and comparable growth rates with those in foetal calf serum, a cell bank should then be created for future use. It should be noted that the KeratinoSens™ cell line, when cultured in human serum, should be cultured up to a maximum passage number of 22 for optimal performance, including the number taken to adapt them to human serum. To achieve fully xeno-free cell culture, a non-animal source of recombinant trypsin (for example, Trypzean™) should be used to harvest the cells during sub-culture (33). In all other respects, the cells should be cultured in the same way as described in this Appendix to Test Guideline 442D and the DB-ALM protocol (9) for the reference KeratinoSens™ cell line

With reference to paragraph 18, the xeno-free adaptation of the KeratinoSens™ test method using human reagents has been optimised using cinnamic aldehyde (CAS No. 14371-10-9, >98% purity) as a positive control, at a final concentration range from 8 to 128µM. Other positive controls, preferentially providing EC1.5 values in the mid-range, may be used if historical data are available to derive comparable run acceptance criteria (33).

With reference to paragraph 25, the xeno-free adaptation of the KeratinoSens™ test method using human reagents has been optimised using the following method for cytotoxicity assessment. Medium is replaced after the 48 hour exposure time with fresh medium containing MTT (3-(4,5-dimethyl2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; CAS No. 298-93-1) at a concentration of 1mg/ml, and cells incubated for 3 hours at 37 ± 1°C in the presence of 5% CO_2. The MTT medium is then removed and cells are solubilised by the addition of isopropanol. After shaking for 30 minutes, the absorption is measured at 570 nm with a spectrophotometer.

All other aspects the xeno-free adaptation of the KeratinoSens™ test method using human reagents should be conducted in the same way as described for the standard method described in this Appendix to Test Guideline 442D and the DB-ALM protocol (9) for the reference KeratinoSens™ cell line.

APPENDIX IA - ANNEX 3: QUALITY CONTROL OF LUMINESCENCE MEASUREMENTS

Basic experiment for ensuring optimal luminescence measurements in the KeratinoSensTM test method

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).

Table 1. 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**

Notes: EGDMA = Ethylene glycol dimethacrylate (CAS No.: 97-90-5) a strongly inducing compound. C = Cinnamic aldehyde, positive reference (CAS No.: 104-55-2). Concentrations are given in µM

The quality control analysis should demonstrate:

- a dose-dependent increase in luciferase induction in row D, with the I_{max} > 20 fold above background (in most cases Imax values between 100 and 300 are reached);
- a dose-dependent increase in luciferase induction in wells H7 to H11, with a fold induction of 2 to 8 in well H11;
- no dose-dependent increase in luciferase induction in row C and E (no induction value equal or 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).

Appendix IB: In Vitro Skin Sensitisation: The ARE-Nrf2 Luciferase LuSens Test Method

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The test method described in this Appendix to Test Guideline 442D addresses the second key event of the skin sensitisation AOP (1), namely keratinocytes activation, by assessing with the help of luciferase, the Nrf2-mediated activation of antioxidant response element (ARE)-dependent genes. Skin sensitisers have been reported to induce genes that are regulated by the ARE (2) (3). Small electrophilic substances 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 (2) (4) (5).

2. The in vitro ARE-Nrf2 luciferase LuSens test method (hereafter called the LuSens test method) underwent a Performance Standard-based validation study based on the KeratinoSens™ Validated Reference Method (VRM) (6) (7) (8) (9), followed by an independent peer review conducted by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) (10). The LuSens 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 identification (10).

3. The LuSens test method proved to be transferable to laboratories experienced in cell culture techniques and met the reproducibility performance standards required both within and between laboratories (10). Additional information from earlier in-house study on 72 test chemicals showed similar predictive capacity as the VRM (74% accuracy, 74% sensitivity, and 74% specificity) for discriminating skin sensitisers (i.e. UN GHS Cat. 1) from non-sensitisers when compared to LLNA results (7) (10), indicating the usefulness of the LuSens test method to contribute to the identification of skin sensitisation hazard. However, the accuracy values given here for LuSens test method 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 a Defined Approach or an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General Introduction of this Test Guideline. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in humans.

4. On the basis of the current data available, the LuSens 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 (7) (8). The test method is applicable to test chemicals soluble or that form a stable dispersion in the exposure medium (i.e. a colloid or suspension in which the

test chemical does not settle or separate from the solvent into different phases). Test chemicals that do not fulfil these conditions at the highest final required testing concentration (i.e. 2000 µM or 2000 µg/mL if no molecular weight is available) may still be tested at lower concentrations. In such a case, results fulfilling the criteria for positivity could still be used to support the identification of the test chemical as a skin sensitiser. In cases where a negative result is obtained in a test with a maximal concentrations < 2000 µM (or < 2000 µg/mL if no molecular weight is available) and no cytotoxicity is observed, the result should be considered as inconclusive (see prediction model in paragraph 32). If cytotoxicity (<70% viability) is reached at a test concentration < 2000 µM (or < 2000 µg/mL if no molecular weight is available), criteria for negativity can still be applied. In general mono constituent substances with a LogP above 7 may be insoluble in the exposure medium, however, if solubility or stable dispersion can be obtained and documented, testing may still be conducted.

5. 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 as the key mechanism leading to the activation of the Keap1-Nrf2-ARE pathway appears to be the electrophilic reaction of stressors with nucleophilic thiols (cysteine sulfhydryl groups) of Keap-1. Complementary information from peptide reactivity assays may help addressing this uncertainty, in particular assays able to distinguish between cysteine and lysine reactivity. Furthermore, because of the limited metabolic capability of the cell line used (12) 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. However, it has been shown that the majority of pre-haptens (i.e. chemicals activated by auto-oxidation) and pro-haptens (i.e. chemicals requiring enzymatic activation for example via P450 enzymes) are sufficiently well identified by a combination of test methods covering key events 1, 2 and 3 on the AOP so that negative results can in general be used to support classification (13) (14) (15). On the other hand, test chemicals that do not act as a sensitiser but are nevertheless chemical stressors may lead to false positive results as shown with the VRM (11). 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 (16). 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 (17). 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 (17). In cases where evidence can be demonstrated on the non-applicability of the LuSens test method to other specific categories of test chemicals, the test method should not be used for those specific categories.

6. In addition to supporting discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers, the LuSens test method also provides information (e.g. dose- response) that may potentially contribute to the assessment of sensitising potency when used in integrated approaches such as IATA such as described for the VRM (13). However, further work, preferably based on human data, is required to determine how the LuSens test method results can contribute to potency assessment, especially in the context of an IATA (18). Examples on how to use the ARE-Nrf2 luciferase test methods in combination with other information are reported in literature (15) (18).

7. Definitions are provided in Annex 1 of the General Introduction.

PRINCIPLE OF THE TEST

8. The LuSens test method makes use of an immortalised adherent cell line derived from human keratinocytes stably harbouring a luciferase reporter gene under the control of

the antioxidant response element of the rat NQO1 gene (20). Genes dependent on the ARE such as NQO1 are known to be up-regulated by contact sensitisers (21) (22). The cell line contains the luciferase gene under the transcriptional control of a promoter fused with the ARE element (7). 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 directly demonstrated for the VMR (23), and indirectly demonstrated for the LuSens (7). 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.

9. Test chemicals are considered positive in the LuSens test method if they induce a statistically significant induction of the luciferase activity above a given threshold (i.e. $\geq$ 1.5 fold, or 50% increase) in at least two consecutive concentrations which do not significantly affect cell viability (i.e. at which the cellular viability is above 70%) (7) (8). For this purpose, induction of the luciferase activity over solvent/vehicle control is determined. Furthermore, parallel cytotoxicity measurements should be conducted to assess whether luciferase activity induction levels occur at sub-cytotoxic concentrations.

10. Prior to routine use of the LuSens test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency, using the ten Proficiency Substances listed in Annex 1 of this Appendix.

PROCEDURE

11. A DB-ALM protocol for the LuSens test method is available and should be employed when implementing and using the test method in the laboratory (24). A summary of the main protocol steps of the LuSens test method as compared to the VRM is given in Annex 2 of this Appendix. Laboratories implementing this Test Guideline can obtain the recombinant cell line used in the test method by requests to the test developers[3]. The luciferase reporter gene assay is subject to a Promega limited use licence requiring i) the use of luminescent assay reagents purchased from Promega; or ii) to contact Promega to obtain a free license for commercial use. The following paragraphs provide with a description of the main components and procedures of the LuSens test method.

Preparation of the keratinocyte cultures

12. The LuSens transgenic cell line having a stable insertion of the luciferase reporter gene under the control of the ARE-element should be used. Upon receipt, cells are propagated as defined by the test method protocol (e.g. 1 to 3 passages) and stored frozen as a homogeneous stock. Cells from this original stock can be propagated up to a maximum of 20 passage number and are employed for routine testing using the appropriate maintenance/growth medium (e.g. Dulbecco's Modified Eagle's medium (DMEM) containing serum and antibiotics such as puromycin in the maintenance medium (for selection) and penicillin/streptomycin (to prevent contamination)) as described within the test method's protocol (24). No antibiotics are added however to the medium during testing.

13. 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 at the appropriate cell density (i.e. 10 000 cells/well). 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 result in high well-to-well variability. For each repetition of the main luciferase test for each

[3] BASF SE, 67056 Ludwigshafen, Germany.

test chemical concentration, three replicates are used for the luciferase activity measurements, and three replicates used for the cell viability assay.

Preparation of the test chemical and control substances

14. The test chemical and control substances are prepared (or thawed in case of stable frozen solutions) on the day of testing. Test chemicals are dissolved in a suitable solvent, e.g. dimethyl sulfoxide (DMSO, CAS No. 67-68-5, ≥ 99% purity) to the final concentration that allows reaching the maximum concentration tested (e.g. 200 mM). The DMSO solutions can be considered self-sterilising, so that no sterile filtration is needed. Test chemicals not soluble in DMSO are dissolved in sterile water or culture medium, in which the appropriate measures should be taken to ensure that the final solutions are sterile. For a test chemical which has no defined molecular weight (MW), a stock solution is prepared to a default concentration of 200 mg/mL or 20% (w/v). In case solvents other than DMSO, water or the culture medium are used, appropriate scientific rationale should be provided.

15. Based on the stock solutions of the test chemical, serial dilutions are made using DMSO or for test chemicals not soluble in DMSO, using sterile water or culture medium, to obtain master concentrations of the chemical to be tested (e.g. 12 concentrations ranging from 0.098 to 200 mM). 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 are reached (e.g. ranging from 0.98 to 2000 µM based on a dilution factor of 2). For a test chemical which has no defined MW, serial dilutions are made using DMSO or a suitable solvent to obtain the desired final concentrations of the test chemical (e.g. from 0.98 to 2000 µg/ml).

16. A cytotoxicity pre-range dose finding test is first performed, based e.g. on the above concentrations, to determine the concentration at which cell viability is reduced to 75% (CV_{75}). The CV_{75} is then used as a basis for determining the concentrations to be tested in the main luciferase test and the parallel cytotoxicity test (e.g., one concentration above CV_{75}, the CV_{75} and four concentrations below CV_{75} using a serial dilution factor of 1.2 resulting in the concentrations CV_{75}/2.07, CV_{75}/1.73, CV_{75}/1.44, CV_{75}/1.2, CV_{75} and CV_{75}x1.2 µM). Alternative concentrations may be used upon justification (e.g. in case of too low or too high cytotoxicity or poor solubility) (24).

17. A concurrent solvent/vehicle control should be tested within each repetition (e.g. DMSO), for which a sufficient number of wells should be prepared per plate (e.g. 12 for the cytotoxicity pre-range dose finding test and 24 for the main luciferase test as described in the protocol (24)). The solvent/vehicle control undergoes the same dilutions as described for the master concentrations in paragraph 15, so that the final solvent/vehicle control concentration should correspond to the same concentration as in the tested chemicals and in the positive control (i.e. 1%), and should not significantly affect cell viability. For a test chemical not soluble in the used solvent (e.g. DMSO), for which the dilutions were made in water, the solvent level in all wells of the final test solution of this test chemical must be adjusted to be equal to the solvent concentration used for the other test chemicals and control substances (i.e. 1%).

18. A concurrent negative control should also be tested within each repetition, for which a sufficient number of wells should be prepared per plate (e.g. 3 for the cytotoxicity pre-range dose finding test and 6 for the main luciferase test as described in the protocol (24)). In the LuSens test method, the concurrent negative control tested is 5 000 µM (or 450 µg/mL) DL-Lactic acid (CAS No. 50-21-5, ≥ 99% purity), known to be a non-sensitiser and to result in a negative prediction with the LuSens test method. Other suitable negative controls may be used if historical data are available to derive comparable run acceptance criteria. Furthermore, in the LuSens test method a sufficient number of wells (e.g. 6 for the cytotoxicity pre-range dose finding test and 12 for the main luciferase test as described in

the protocol (24)) containing blank medium controls are prepared consisting of untreated cells and culture medium only.

19. A concurrent positive control should also be tested in a sufficient number of wells within each repetition to demonstrate appropriate response of the test system (e.g. 2 for the cytotoxicity pre-range dose finding test and 5 for the main luciferase test as described in the protocol (24)). For the LuSens test method, 120 µM Ethylene Glycol Dimethacrylate (EGMDA, CAS No. 97-90-5, ≥ 99% purity) is used. The positive control is prepared using the same dilution steps as described for the master concentrations in paragraph 14 and as described in the test method's protocols (24). If the positive control concentration of 120 µM is too toxic or not able to induce luciferase ≥ 2.5 (see paragraph 31) due to e.g. a new laboratory facility or a new batch of EGMDA, the performing laboratory may run a range finder experiment with EGDMA (confirmed in at least two more runs) in order to set the concentration at which luciferase induction is ≥ 2.5 folds compared to solvent/vehicle control, and for which cell viability is ≥ 70%. Finally, other suitable positive controls, preferentially providing $EC_{1.5}$ 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

20. For each test chemical, 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.

21. After seeding as described in paragraph 13, cells are grown for 24 hours in the 96-wells microtiter plates. The medium is then removed and replaced with fresh culture medium (i.e. 150 µl DMEM containing serum but without antibiotics as described within the method's protocol (24)) 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.

22. The treated plates are then incubated for about 48 hours at 37±1°C in the presence of 5% CO_2. 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 during incubation with the test chemicals.

Luciferase activity measurements

23. The following 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,
- the use of a luciferase substrate with sufficient light output to ensure sufficient sensitivity and low variability; and
- an appropriate and stable background level.

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

24. After the 48 hour exposure time with the test chemical and control substances, cells are washed with a phosphate buffered saline, and the appropriate lysis buffer for luminescence readings added to each well for a sufficient time (e.g. 5-10 min in dark).

25. Plates with the cell lysate are then placed in the luminometer for reading using the specific program prescribed within the test method's protocol (24). 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 of this Appendix is successfully conducted.

Cytotoxicity Assessment

26. For the LuSens cell viability assay, medium is replaced after the 48 hour exposure time with fresh medium containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl) -2,5 diphenyltetrazolium bromide, Thiazolyl blue tetrazolium bromide; CAS No. 298-93-1) and cells are incubated for 2 hours at 37±1°C in the presence of 5% CO_2. The MTT medium is then removed and cells are lysed by using an appropriate lysing agent for a sufficient amount of time (e.g. 10 % (w/v) SDS and 0.4% (v/v) acetic acid solution in DMSO for 5 min). After shaking, the absorption is then measured using the parameters described in the test method protocol (24).

DATA AND REPORTING

Data evaluation

27. The following parameters are calculated in the LuSens test method (see Annex 4 of this Appendix for the detailed equations):

- Fold luciferase activity induction at all concentrations of the tested chemical, positive control and negative control.
- Cellular viability (CV) at all concentrations of the tested chemical and for all controls to determine (by interpolation) the concentration value at which 75% of cell viability occurs (CV_{75}).

28. For each concentration showing a luciferase activity induction equal or higher (≥) than 1.5, statistical significance is determined (e.g. using a two-tailed Student's t-test) by comparing the luminescence values of the three replicate samples with the luminescence values in the solvent/vehicle control wells to assess whether the luciferase activity induction is statistically significant ($p < 0.05$). Furthermore, it should be checked that no significant cytotoxic effects occur at these concentrations (i.e. that the cell viability is ≥ 70% at the concentrations leading to ≥ 1.5 fold luciferase induction).

29. 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 concentration, i.e., when the threshold of 1.5 is crossed the first time should be reported. However, a concentration delivering an $EC_{1.5}$ is not a requirement.

30. Finally, when in the LuSens test method a ≥ 1.5 fold luciferase activity induction is observed only at the lowest tested concentration (e.g. CV_{75}/2.07), re-testing should be conducted using at least one additional lower concentration.

Acceptance criteria

31. The following acceptance criteria should be met when using the LuSens test method. If any of the criteria listed below is not met, the data should be discarded and a new repetition should be performed.

- The average luciferase activity induction obtained with the positive control, 120 µM EGDMA (or comparable concentration – see paragraph 19) should be ≥ 2.5, and the positive control should have a relative cell viability ≥ 70% as compared to the solvent/vehicle control.
- The average luciferase activity induction obtained with the negative control, i.e., 5000 µM DL-Lactic acid, as well as the basal expression of untreated cells should be < 1.5 fold as compared to the average solvent/vehicle control.
- The average coefficient of variation of the luminescence reading for the solvent/vehicle controls (e.g. DMSO) should be below 20% in each repetition.
- At least three test concentrations should have cell viability of at least 70% relative to the solvent/vehicle controls. Moreover, in case a result is to be considered negative, at least one concentration should be cytotoxic, i.e. have a cell viability < 70%, or the maximum concentration of 2000 µM (or 2000 µg/ mL for substances with no defined MW) should have been tested.

32. In some cases, test chemicals may induce no cytotoxicity, in which cases the maximum concentration tested should be 2000 µM (or 2000 µg/ mL for test chemicals having undefined MW). If in the main luciferase test no concentration is cytotoxic, i.e. has a cell viability < 70%, and no luciferase induction is observed, then a second repetition should be performed using e.g. a 1.44 serial dilution factor based on the CV75 (i.e. starting with 1.44 x CV_{75}) instead of the 1.2 serial dilution factor used in the main luciferase test. If in the second repetition cytotoxicity and luciferase induction are still not observed, a third repetition should be run with the maximum concentration of 2000 µM (or 2000 µg/ mL for substances with undefined MW). This repetition should then be confirmed by performing a fourth repetition.

Interpretation of results and prediction model

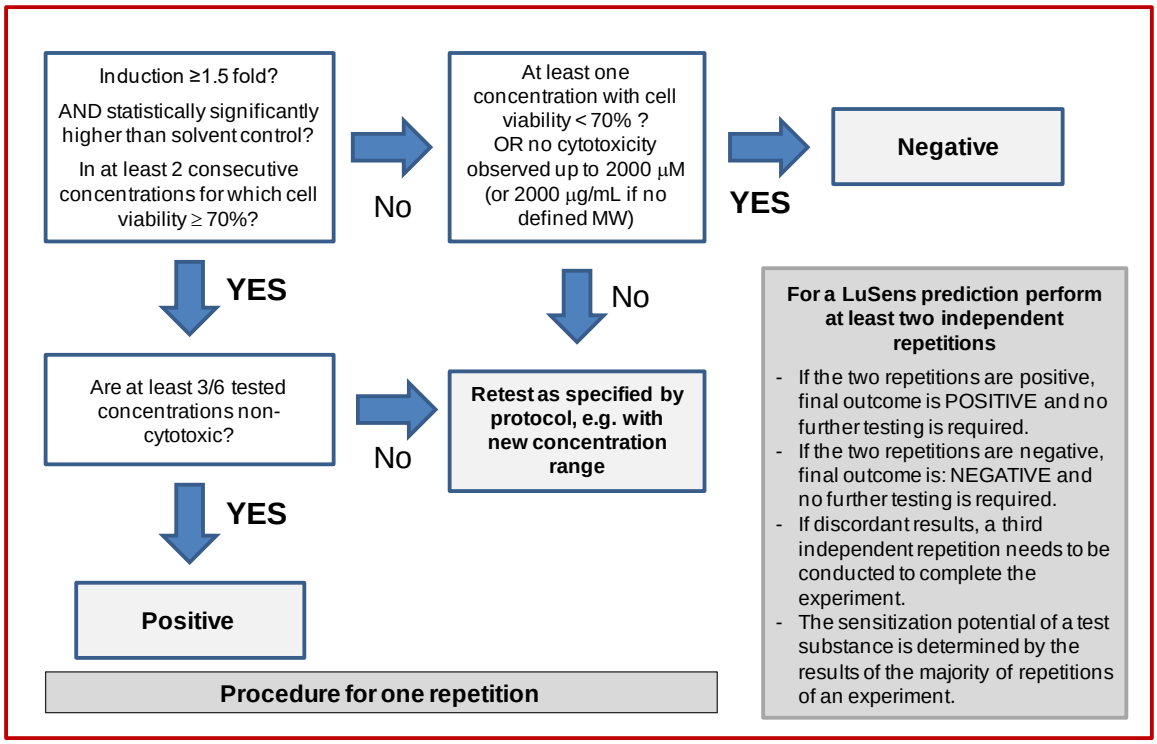
33. A LuSens prediction is considered positive if the following conditions are met in 2 of 2 or in the same 2 of 3 repetitions, otherwise the LuSens prediction is considered negative (Figure 1):

- the luciferase induction is above or equal to (≥) 1.5 fold and is statistically significant compared to the solvent control in at least 2 consecutive non-cytotoxic tested concentrations (i.e. cellular viability is equal or higher than (≥) 70%), whereby at least three tested concentrations should be non-cytotoxic (cellular viability equal or higher than (≥) 70%).

34. In addition, a negative result obtained with test chemicals that do not form a stable dispersion and were not tested up to 2000 µM (or 2000 µg/mL for test chemicals with no defined MW) and for which no cytotoxicity is observed in any of the tested concentration (see paragraph 31) should also be considered as inconclusive (see paragraph 4).

Figure 1. Overview of the criteria leading to a prediction in the LuSens test method.

A LuSens prediction should be considered in the framework of a Defined Approach or of an IATA and in accordance with the provision paragraph 4 and paragraphs 7 and 8 of the general introduction.



Test report

35. 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 like batch/ lot number and expiry date;
  - Physical appearance, water solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
  - Statement on (in)solubility or stable dispersion in exposure media
  - 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;
  - Statement of (in)solubility or stable dispersion in exposure media

- 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.
- Solvent/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 solvents/vehicles than those mentioned in this Appendix are used and to the extent available;
  - Storage conditions and stability to the extent available;
  - Justification for choice of solvent/vehicle for each test chemical.
- Negative 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 than those mentioned in this Appendix are used and to the extent available;
  - Storage conditions and stability to the extent available;
  - Justification for choice of the negative control in the case other negative controls than those mentioned in the Test Guideline are used.

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 13);
- 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 of this Appendix;
- 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 fold luciferase induction activity and viability values (i.e.CV75 for the LuSens test method) obtained for the test chemical and for the positive control for each repetition;
- The mean values (i.e. arithmetic means of cell viability and luciferase activity induction) and SD calculated using data from all individual repetitions;
- An indication of the rating of the test chemical according to the prediction model;
- Coefficient of variation obtained with the luminescence readings for the solvent/vehicle 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 LuSens test method;
- Consideration of the test method results within the context of an IATA, if other relevant information is available.

Conclusion

Literature

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APPENDIX IB - ANNEX 1: PROFICIENCY SUBSTANCES

In Vitro Skin Sensitisation: The ARE-Nrf2 Luciferase LuSens Test Method

Prior to routine use of a test method that adheres to this Appendix to Test Guideline 442D, laboratories should demonstrate technical proficiency by correctly obtaining the expected prediction for the 10 Proficiency Substances recommended in Table 1 and by obtaining the raw values that fall within the respective reference range for at least eight out of the ten 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 LuSens test method.

Table 1: Recommended substances for demonstrating technical proficiency with the LuSens test method.

Proficiency Substances	CASRN	Physical Form	LLNA Prediction (1)	Human category (2)	LuSens		
					In Vitro Prediction (3)	$EC_{1.5}$ (µM) Reference Range (4)	CV_{75} (µM) Reference Range (4)
Salicylic acid	69-72-7	Solid	Non-sensitiser	Cat. 6	Negative	> 1000	> 2000
Glycerol	56-81-5	Liquid	Non-sensitiser	Cat. 6	Negative	> 1000	> 2000
Isopropanol	67-63-0	Liquid	Non-sensitiser	Cat. 5	Negative	> 1000	> 2000
Sulfanilamide	63-74-1	Solid	Non-sensitiser	Negative (Basketter et al. 1994)	Negative	> 1000	> 2000
Eugenol	97-53-0	Liquid	Sensitiser (weak)	Cat. 3	Positive	< 500	< 1000
Cinnamyl alcohol	104-54-1	Solid	Sensitiser (weak)	Cat. 3	Positive	< 170	> 420
2-Mercaptobenzothiazole	149-30-4	Solid	Sensitiser (moderate)	Cat. 3	Positive	< 800	< 2000
4-Methylaminophenol sulfate	55-55-0	Solid	Sensitiser (strong)	Cat. 3	Positive	< 30	< 50
Methyldibromo glutaronitrile	35691-65-7	Solid	Sensitiser (strong)	Cat. 2	Positive	< 25	< 50
2,4-Dinitro-chlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Cat. 1	Positive	< 5	< 10

Notes: (1) The in vivo hazard (and potency) predictions are based on LLNA data (25). The in vivo potency is derived using the criteria proposed by ECETOC (18).
(2) According to Basketter and co-workers (26). Cat. 1 represents clear evidence of contact allergy, Cat. 2 a frequent cause of contact allergy, Cat. 3 a common cause of contact allergy, Cat. 4 an infrequent cause of contact allergy, Cat. 5 a rare cause of contact allergy, and Cat. 6 essentially absent evidence of contact allergy.
(3) An ARE-Nrf2 luciferase test method prediction should be considered in the framework of a Defined Approach or of an IATA and in accordance with the provisions of paragraphs 7 and 8 of the general introduction.
(4) Based on the historical observed values (7) (8). Although the EC 1.5 is not part of the LuSens prediction model, it can be calculated from the obtained data, and used to determine the ranges of LuSens response for the Proficiency Substances. The EC 1.5 values were calculated according to Appendix IA (paragraph 26).

APPENDIX IB - ANNEX 2: Comparison of the main protocol steps of the LuSens and the VRM KeratinoSensTM test methods

	VRM (KeratinoSens™)	LuSens
		Preparation of the keratinocyte cultures
Propagation	2 to 4 passages	1 to 3 passages
Cryopreserved storage	2 to 4 passages	3 passages
Cell passages before main experiment	At least 2	At least 5
Maximal passage number propagation from frozen stocks	25 passages	20 passages for cytotoxicity range finding test 15 passages for main luciferase test
Propagation medium	DMEM containing serum and Geneticin	DMEM containing serum, penicillin/streptomycin and puromycin
Cell confluence for testing		80-90%
Harvest of cells prior to testing		1 day
Plate format used for testing		96 well-plates
Cell number seeded for testing	10 000 cells/well, except in the well that is used for measurement of background	
Number of replicates for each test chemical concentration (in each repetition)	3 wells (on independent plates) for luciferase measurement 1 well for cytotoxicity assessment	3 wells (in the same plate) for all tests i.e. the cytotoxicity range finder test and the main luciferase test (including 3 wells for luciferase measurement, and 3 wells for parallel cytotoxicity assessment)
		Preparation of the test chemical and control substances
Preparation		Same day of testing
Solvent	DMSO, sterile water or media for those test items not soluble in DMSO	DMSO or media for those test items not soluble in DMSO
Stock concentration		200 mM
Test items with no defined molecular weight	Stock solution prepared to a default concentrations (40 mg/mL or 4% (w/v))	Stock solution prepared to a default concentrations (200 mg /mL or 20% (w/v))
Final tested concentration range in 96 well-plate	12 concentrations (2 fold dilution) ranging from 0.98 to 2000 µM	Cytotoxicity range finder test: 12 concentrations (2 fold dilution) ranging from 0.98 to 2000 µM Main luciferase test: 6 concentrations (1.2 fold dilution) ranging from CV_{75}/2.074 to CV_{75}x1.2 µM
Solvent control	1% DMSO (18 replicates per repetition)	1% DMSO (12 replicates per repetition for cytotoxicity range finder test, and 24 replicates per repetition for main luciferase test)
Negative control	See solvent control	5000 M DL-Lactic acid (3 replicates per repetition for cytotoxicity range finder test, and 6 replicates per repetition for main luciferase test)
Positive control	Cinnamic aldehyde Four concentrations (2 fold dilution) ranging from 4 to 64 µM (3 replicates per repetition)	120 M EGDMA or alternative concentration that induced luciferase =2.5 folds, and for which cell viability is ≥ 70% (2 replicates per repetition for cytotoxicity range finder test, and 5 replicates per repetition for main luciferase test)
Medium control	Not applicable	6 replicates per repetition for cytotoxicity range finder test, and 12 replicates per repetition for main luciferase test
Blank control (no cells)	3 replicates per repetition	1 replicates per repetition

	Application of the test chemical and control substances & endpoints assessed	
Number of repetitions for each test chemical concentration	At least two independent repetitions containing each three replicates (n='6),' and in case of discordant results, a third repetition should be performed (n='9).' Each repetition is conducted on a different day with freshly prepared test chemicals and independently harvested cells (but eventually having the same passage number)	
Cell treatment medium	150 µl culture DMEM containing serum but without antibiotics (i.e. Geneticin, penicillin/streptomycin and puromycin) to which 50 µl of the 25 fold test chemical and control substances are added	
Exposure time	48 hours at 37±1°C in the presence of 5% CO_2 Plates are covered with a foil to avoid evaporation of volatile test chemicals and cross-contamination between wells	
Luminescence activity measurement	After exposure, cells are washed with phosphate buffered saline, and the relevant lysis buffer for luminescence readings added to each well for 20 min at room temperature. Plates with the cell lysate are placed in the luminometer for reading which is programmed to: i) add the luciferase substrate to each well, ii) wait for 1 second, and iii) integrate the luciferase activity for 2 seconds.	After exposure, the relevant lysis buffer for luminescence readings added to each well for 5-10 min, under agitation in the dark. Luminescence is measured for 2 seconds using a luminometer. *Other conditions may apply depending on the luminometer used.*
Cytotoxicity assessment	After exposure, 5mg/ml MTT solution is added and cells are incubated 4h at 37±1°C in the presence of 5% CO_2 Cells are then lysed overnight (with 10% SDS solution), agitated and absorption measured at 600 nm	After exposure, 200 µL of MTT working solution (0.5mg/ml) are added and cells are incubated 2h at 37±1°C in the presence of 5% CO_2 Cells are lysed for 5 min (with 10% (w/v) SDS and 0.4% (v/v) acetic acid in a DMSO solution), and absorption measured at 570 and 690 nm
Endpoints evaluated	I_{max}: maximal average fold induction observed at any concentration tested $EC_{1.5}$: interpolated concentration for which there is a 1.5 fold induction of luciferase activity IC_{50} / IC_{30}: interpolated concentration at which 50% and 30% reduction of cell viability occurs respectively	Fold luciferase activity induction as an average of each tested concentration Cellular viability as an average of each tested concentration CV_{75}: interpolated concentration at which 75% cell viability occurs
		Acceptance criteria
Positive control luciferase activity	>1.5 fold statistically significant induction in at least one of the tested concentrations of the positive control (4 to 64 µM cinnamic aldehyde). $EC_{1.5}$ value of positive control should be between 2SDs of historical mean (e.g. 2 to30 µM in validation dataset) Average induction of 64 µM cinnamic aldehyde should be between 2 and 8.	= 2.5 fold induction with the positive control (e.g. 120 M EGDMA) relative to solvent control at a non-cytotoxic concentration, i.e., cell viability = 70% relative to solvent control
Negative control luciferase activity	Not applicable	< 1.5 fold induction with the negative control (5000 µM DL-Lactic acid) relative to solvent control
Solvent control variability	Coefficient of variation = 20% (18 replicates)	Coefficient of variation = 20% (of at least 21 replicates)
Others	Not applicable	Mean basal expression of medium control (cells with medium only) should have < 1.5 fold luciferase activity induction relative to solvent control At least three test concentrations (of 6 in the main luciferase test) should be non-cytotoxic (cell viability = 70%). In addition, in case of a negative result, at least one tested concentration (of 6 in the main luciferase test) should be cytotoxic (cell viability < 70%)
		Prediction model

A prediction is considered positive whenthe following conditions are met in 2 of 2 or in 2 of 3 repetitions, otherwise the prediction is considered negative	1. Imax equal or higher than (≥) 1.5 fold and statistically significantly different to the solvent control (two-tailed, unpaired Student's T-test) 2.The cellular viability is higher than (>) 70% at the lowest concentration with induction of luciferase activity equal or above 1.5 fold (i.e. at the $EC_{1.5}$ determining concentration) 3.The $EC_{1.5}$ value is less than (<) 1000 µM (or < 200 µg/mL for test chemicals with no defined MW) 4. There is an apparent overall dose-dependent increase in luciferase induction	1. A luciferase induction above or equal to (≥) 1.5 fold as compared to the solvent control is observed in at least 2 consecutive non-cytotoxic tested concentrations (i.e. cellular viability is equal or higher than (≥) 70%) 2. At least three tested concentrations should be non-cytotoxic (cellular viability equal or higher than (≥) 70%).
Chemicals that do not form a stable dispersion	Negative result obtained with test chemicals that do not form a stable dispersion < 1000 µM (or < 200 µg/mL for test chemicals with no defined MW), should be considered inconclusive	Negative result obtained with test chemicals that do not form a stable dispersion and were not tested up to 2000 µM (or 2000 µg/mL for test chemicals with no defined MW) should be considered inconclusive

APPENDIX IB - ANNEX 3: QUALITY CONTROL OF LUMINESCENCE MEASUREMENTS

Basic experiment for ensuring optimal luminescence measurements in the LuSens test method

In order to ensure optimal luminescence measurements, when performing the assay for the first time, it is recommended to perform one or two runs of the LuSens test method using increasing concentrations of EGDMA as a test substance and using the plate layout as described below. By performing these repetitions, the following aspects should be considered:

- luciferase induction should be increased in a dose-dependent fashion (in wells A-C:1-6) after treatment with increasing concentrations of EDGMA;
- no dose-dependent increase in luciferase induction should be observed in wells D:1-6, and A-D: 7 (empty wells) in comparison to luminescence values in wells A-D: 8-12;
- the average percentage Standard Deviation of the variability in at least 21 solvent/vehicle control wells (F-G: 1-12) should be below 20% and should not show any “gradient-like” pattern.

Table 1: Plate setup of first training experiment

	1	2	3	4	5	6	7	8	9	10	11	12
A	EGDMA CV75/2.07	EGDMA CV75/1.73	EGDMA CV75/1.44	EGDMA CV75/1.2	EGDMA CV75	EGDMA CV75x1.2						
B	EGDMA CV75/2.07	EGDMA CV75/1.73	EGDMA CV75/1.44	EGDMA CV75/1.2	EGDMA CV75	EGDMA CV75x1.2						
C	EGDMA CV75/2.07	EGDMA CV75/1.73	EGDMA CV75/1.44	EGDMA CV75/1.2	EGDMA CV75	EGDMA CV75x1.2						
D												
E	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium	Medium
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					DL-Lactic acid 5000 M					**EGDMA 120 M**		**Blank**

APPENDIX IB - ANNEX 4: CALCULATIONS USED IN THE LUSENS TEST METHOD

1. **The fold induction of luciferase activity** (I_{max}) is calculated in the LuSens test method by Equation 1, and the overall maximal fold induction (Imax) is calculated as the average of the individual repetitions.

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

where

L_{sample} is the luminescence reading in the test chemical well

L_{blank} is the luminescence reading in the blank well containing no cells and no treatment

$L_{solvent}$ is the average luminescence reading in the wells containing cells and solvent control

2. **Viability** in the LuSens test method is calculated by Equation 2:

$$\text{Equation 2:} \qquad Viability = \frac{(V_{sample} - V_{blank})}{(V_{solvent} - V_{blank})} \times 100$$

where

V_{sample} is the MTT-absorbance reading in the test chemical well

V_{blank} is the MTT-absorbance reading in the blank well containing no cells and no treatment

$V_{solvent}$ is the average MTT-absorbance reading in the wells containing cells and solvent control

3. **The concentration at which cell viability is reduced to 75% (CV_{75})** is then calculated in the LuSens test method by linear interpolation according to Equation 3, and the overall the CV_{75} is calculated as the geometric mean of the individual replicates.

$$\text{Equation 3:} \qquad CV_{75} = (C_b - C_a) \times \left(\frac{75 - V_b}{V_b - V_a}\right) + C_b$$

where

C_a is the tested concentration in µM with cell viability just above 75%

C_b is the tested concentration in µM with cell viability just below 75%

V_a is the % viability obtained with C_a

V_b is the % viability obtained with C_b