



Section 4
Health effects

Test Guideline No. 442C

In Chemico Skin Sensitisation

*Assays addressing the Adverse Outcome
Pathway key event on covalent binding to
proteins*

4 July 2023

**OECD Guidelines for the
Testing of Chemicals**



*OECD KEY EVENT BASED GUIDELINE FOR THE
TESTING OF CHEMICALS*

In chemico skin sensitisation assays addressing the Adverse Outcome Pathway Key Event
on Covalent Binding to Proteins

INTRODUCTION

Covalent binding to proteins 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 a molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with amino-acid residues (i.e. cysteine or 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.
2. 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, such as the LLNA (OECD TG 429) (12) and its three non-radioactive modifications — LLNA:DA (OECD TG 442A) (13), 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.
3. 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 present Test Guideline assesses covalent binding to proteins, addressing the first key event; the OECD TG 442D assesses keratinocyte activation (15), the second key event and the OECD TG 442E addresses the activation of dendritic cells (16), the third key event of the skin sensitisation AOP. Finally, the fourth key event representing T-cell proliferation is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (12).

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

4. This Test Guideline (TG) describes *in chemico* assays that address mechanisms described under the first key event of the AOP for skin sensitisation, namely covalent binding to proteins (2). The test methods currently included in this Test Guideline are:
 - The Direct Peptide Reactivity Assay (DPRA) (Appendix I),
 - The Amino Acid Derivative Reactivity Assay (ADRA) (Appendix II), and
 - The kinetic Direct Peptide Reactivity Assay (kDPRA) (Appendix III).
5. The test methods are based on *in chemico* covalent binding to proteins and are considered to be scientifically valid. The DPRA has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-lead validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) (3) (4) (5). The ADRA underwent a validation study coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6) (7) (8) (9) followed by an independent peer-review (10). The kDPRA underwent an industry-coordinated validation study followed by an independent peer-review (17).
6. The test methods included in this Test Guideline might differ with regard to the procedures used to generate the data but can each be used to address countries' requirements for test results on protein reactivity, while benefiting from the Mutual Acceptance of Data.
7. The correlation of protein reactivity with skin sensitisation potential is well established (18) (19) (20). Nevertheless, since protein reactivity represents only one key event of the skin sensitisation AOP (2) (21), information generated with test methods developed to address this specific key event may not be sufficient as stand-alone methods 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 be used within Integrated Approaches to Testing and Assessment (IATA), together with other relevant complementary information from *in vitro* assays addressing other key events of the skin sensitisation AOP as well as non-testing methods, including *in silico* modelling and read-across from chemical analogues (21). Examples on the use of data generated with these methods within Defined Approaches (DAs), 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 (21) and are implemented in an OECD TG on defined approaches for skin sensitisation (22).
8. The DPRA and ADRA described in Appendixes I and II to this Test Guideline, respectively, support the discrimination of skin sensitisers (Category 1) from non-sensitisers. 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. However, these test methods do not allow on their own, the sub-categorisation of skin sensitisers into subcategories 1A and 1B (23), as defined by UN GHS (1) for authorities implementing these two optional subcategories, or potency prediction for safety assessment decisions.

9. In contrast, the kDPRA described in Appendix III of this Test Guideline, allows discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) i.e., subcategory 1B or no category (1) but does not allow to distinguish sensitisers (Category 1) from non-sensitisers. Depending on the regulatory framework, positive results generated with the kDPRA may be used on their own to classify a chemical into UN GHS subcategory 1A.
10. Definitions are provided in the Annex. Performance Standards for the assessment of proposed similar or modified *in vitro* skin sensitisation DPRA and ADRA test methods have been developed (24).

Literature for introduction

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Annex 1.A. 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 (1). The formula used to derive accuracy is shown under “Calculation” of predictive capacity.

ADRA: Amino acid Derivative Reactivity Assay.

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).

Balanced accuracy: The average of sensitivity and specificity. This metric is particularly useful when a different number of *in vivo* positive and *in vivo* negative chemicals were tested. It is an important consideration in assessing the relevance of a test method. The formula used to derive balanced accuracy is shown under “Calculation” of predictive capacity.

Calculation

Calculating predictive capacity

Sensitivity, specificity, accuracy, and balanced accuracy are calculated based on the true positive (TP), true negative (TN), false negative (FN), and false positive (FP) values as follows:

$$\text{Sensitivity} = \frac{\text{Number of true positives (TP)}}{\text{Number of all positive chemicals (TP+FN)}} \times 100$$

$$\text{Specificity} = \frac{\text{Number of true negatives (TN)}}{\text{Number of all negative chemicals (TN+FP)}} \times 100$$

$$\text{Accuracy} = \frac{\text{Number of correct predictions (TP+TN)}}{\text{Number of all chemicals (TP+FN+TN+FP)}} \times 100$$

$$\text{Balanced accuracy} = \frac{\text{Sensitivity} + \text{Specificity}}{2}$$

Calibration curve: The relationship between the experimental response value and the analytical concentration (also called standard curve) of a known substance.

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.

Defined Approach (DA): a DA consists of a fixed data interpretation procedure (e.g. statistical, mathematical models) applied to data (e.g. *in silico* predictions, *in chemico*, *in vitro* data) generated with a defined set of information sources to derive a prediction.

DPRA: Direct Peptide Reactivity Assay.

EDTA: Ethylenediaminetetraacetic acid.

EURL ECVAM: the European Union Reference Laboratory for Alternatives to Animal Testing.

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 hazards, risks, and the need for further targeted and therefore minimal testing.

JaCVAM: Japanese Center for the Validation of Alternative Methods.

kDPRA: kinetic Direct Peptide Reactivity Assay.

k_{max}: is the maximum rate constant (in s⁻¹M⁻¹) determined from the reaction kinetics for a tested substance in the kDPRA (see Appendix III, paragraph 24).

LLNA: murine Local Lymph Node Assay issued as OECD TG 429 in 2010.

Molecular Initiating Event: Chemical-induced perturbation of a biological system at the molecular level identified to be the starting event in the adverse outcome pathway.

Mixture: A solid or liquid comprising two or more substances which do not react chemically (3).

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent comprises at least 80% (w/w) of the whole.

Multi-constituent substance: A substance, defined by its quantitative composition, in which two or more main constituents are present in concentrations ≥ 10% (w/w) and < 80% (w/w). Multi-constituent substances are the result of a manufacturing process. The difference between a mixture and a multi-constituent substance is that a mixture comprises two or more substances which do not react chemically, whereas a multi-constituent substance comprises two or more substances that do react chemically.

NAC: N-(2-(1-naphthyl) acetyl)-L-cysteine (4) (5) (6).

NAL: α-N-(2-(1-naphthyl) acetyl)-L-lysine (4) (5) (6).

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.

Pre-haptens: chemicals which become sensitisers through abiotic transformation.

Pro-haptens: chemicals requiring enzymatic activation to exert skin sensitisation potential.

Reference control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent negative control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

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 (1).

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 (1).

Reproducibility: The concordance of results obtained from testing the same substance using the same test protocol (see reliability). (1)

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 (1). The formula used to derive sensitivity is shown under "Calculation" of predictive capacity.

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 (1). The formula used to derive specificity is shown under "Calculation" of predictive capacity.

Substance: Chemical elements and their compounds in the natural state or resulting from a manufacturing process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process, but excluding solvents that may be separated without affecting the stability of the substance or changing its composition (3).

System suitability: Determination of instrument performance (e.g., sensitivity) by analysis of reference standards prior to running the analytical run (7).

Test chemical: The term test chemical is used to refer to the substance being tested.

TFA: Trifluoroacetic acid.

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 (3).

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

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (1).

Literature for definitions

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APPENDIX I

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The DPRA is proposed to address the molecular initiating event of the skin sensitisation AOP, namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine (1). Cysteine and lysine percent peptide depletion values are then used to categorise a substance in one of four classes of reactivity for supporting the discrimination between skin sensitisers and non-sensitisers (2).
2. The DPRA test method proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 85% within laboratories and 80% between laboratories (3). Results generated in the validation study (4) and published studies (5) overall indicate that the accuracy of the DPRA in discriminating sensitisers (i.e. UN GHS Category 1) from non-sensitisers is 80% (N=157) with a sensitivity of 80% (88/109) and specificity of 77% (37/48) when compared to LLNA results. The DPRA is more likely to under predict chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (4) (5). However, the accuracy values given here for the DPRA as a stand-alone test method are only indicative since the test method should be considered in combination with other sources of information in the context of an IATA or a DA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. 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 the species of interest, i.e. humans. On the basis of the overall data available, the DPRA was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction

mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physico-chemical properties (1) (2) (3) (5). Taken together, this information indicates the usefulness of the DPRA to contribute to the identification of skin sensitisation hazard.

3. The term "test chemical" is used in this Test Guideline to refer to what is being tested¹ and is not related to the applicability of the DPRA to the testing of substances and/or mixtures (see a summary of the known limitations of the DPRA in Annex 1 of this Appendix). This test method is not applicable for the testing of metal compounds since they are known to react with proteins with mechanisms other than covalent binding. A test chemical should be soluble in an appropriate solvent at a final concentration of 100 mM (see paragraphs 10 and 11). However, test chemicals that are not soluble at this concentration may still be tested at lower soluble concentrations. In such a case, a positive result could still be used to support the identification of the test chemical as a skin sensitizer but no firm conclusion on the lack of reactivity should be drawn from a negative result. Limited information is currently available on the applicability of the DPRA to mixtures of known composition (4) (5). The DPRA is nevertheless considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition (see paragraphs 4 and 11). When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Appendix of the Test Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. In cases where evidence can be demonstrated on the non-applicability of the test method to specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

4. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be detected by the test method. Chemicals that become sensitizers after abiotic transformation (i.e. pre-haptens) are reported to be in most cases correctly detected by the test method (4) (9) (10). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA or a DA. Test chemicals that do not covalently bind to the peptide but promote its oxidation (i.e. cysteine dimerisation) could lead to a potential over estimation of peptide depletion, resulting in possible false positive predictions and/or assignment to a higher reactivity class (see paragraphs 23 and 24).

5. As described, the DPRA assay supports the discrimination between skin sensitizers and non-sensitizers. However, it may also potentially contribute to the assessment of sensitizing potency (6) (11) when used in integrated approaches such as IATA or DA (12). However further

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

work, preferably based on human data, is required to determine how DPRA results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

6. The DPRA is an *in chemico* method which quantifies the remaining concentration of cysteine- or lysine-containing peptide following 24 hours incubation with the test chemical at 22.5-30°C. The synthetic peptides contain phenylalanine to aid in the detection. Relative peptide concentration is measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm. Cysteine- and lysine peptide percent depletion values are then calculated and used in a prediction model (see paragraph 23) which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitisers and non-sensitiser.

7. Prior to routine use of the method described in this Appendix, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 2.

PROCEDURE

8. This test method is based on the DPRA DB-ALM protocol n° 154 (7) which represents the protocol used for the EURL ECVAM-coordinated validation study. It is recommended that this protocol is used when implementing and using the method in the laboratory. The following is a description of the main components and procedures for the DPRA. If an alternative HPLC set-up is used, its equivalence to the validated set-up described in the DB-ALM protocol should be demonstrated (e.g. by testing the proficiency substances in Annex 2).

Preparation of the cysteine or lysine-containing peptides

9. Stock solutions of cysteine (Ac-RFAACAA-COOH) and lysine (Ac-RFAAKAA-COOH) containing synthetic peptides of purity higher than 85% and preferably > 90%, should be freshly prepared just before their incubation with the test chemical. The final concentration of the cysteine peptide should be 0.667 mM in pH 7.5 phosphate buffer whereas the final concentration of the lysine peptide should be 0.667 mM in pH 10.2 ammonium acetate buffer. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this test method, up to 26 analysis samples (which include the test chemical, the positive control and the appropriate number of solvent controls based on the number of individual solvents used in the test, each tested in triplicate), can be accommodated in a single HPLC run. All of the replicates analysed in the same run should use the

identical cysteine and lysine peptide stock solutions. It is recommended to prove individual peptide batches for proper solubility prior to their use.

Preparation of the test chemical

10. Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay following the solubilisation procedure described in the DPRA DB-ALM protocol (7). An appropriate solvent will dissolve the test chemical completely. Since in the DPRA the test chemical is incubated in large excess with either the cysteine or the lysine peptides, visual inspection of the forming of a clear solution is considered sufficient to ascertain that the test chemical (and all of its components in the case of testing a multi-constituent substance or a mixture) is dissolved. Suitable solvents are, acetonitrile, water, 1:1 mixture water:acetonitrile, isopropanol, acetone or 1:1 mixture acetone:acetonitrile. Other solvents can be used as long as they do not have an impact on the stability of the peptide as monitored with reference controls C (i.e. samples constituted by the peptide alone dissolved in the appropriate solvent; see Annex 3). If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts. It is important to note that DMSO may lead to peptide dimerisation and as a result, it may be more difficult to meet the acceptance criteria. If DMSO is chosen, attempts should be made to first solubilise the test chemical in 300 µL of DMSO and dilute the resulting solution with 2700 µL of acetonitrile. If the test chemical is not soluble in this mixture, attempts should be made to solubilise the same amount of test chemicals in 1500 µL of DMSO and dilute the resulting solution with 1500 µL of acetonitrile. The test chemical should be pre-weighed into glass vials and dissolved immediately before testing in an appropriate solvent to prepare a 100 mM solution.

11. This molecular weight approach should apply if the test chemical is a mono-constituent substance with a known molecular weight or a mixture or multi-constituent substance of known composition. For mixtures and multi-constituent substances of known composition, a single aggregated purity value should be determined by the sum of the proportion of its constituents (excluding water), and a single aggregated molecular weight should be determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and aggregated molecular weight should then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution. For polymers for which a predominant molecular weight cannot be determined, the molecular weight of the monomer (or the apparent molecular weight of the various monomers constituting the polymer) may be considered to prepare a 100 mM solution.

12. For mixtures and multi-constituent substances of unknown composition (i.e. UVCB substances of unknown or variable composition, complex reaction products or biological materials), the test solution can be prepared with a gravimetric approach to a concentration of 20 mg/mL on the basis of the weight of the total components (excluding solvent) in an appropriate solvent. This value is based on a default molecular weight of 200 g/mol. If the mixture to be investigated is known to contain a chemical class with a typical molecular weight which is significantly higher, this default

molecular weight and the test solution concentration should be adjusted accordingly (see e.g. approach for agrochemical formulations (13)). In addition, this gravimetric approach should only be applied as a last resort if no molecular weight is available and no aggregated molecular weight can be determined.

Preparation of the positive control, reference controls and coelution controls

13. Cinnamic aldehyde (CAS 104-55-2; $\geq 95\%$ food-grade purity) should be used as positive control (PC) at a concentration of 100 mM in acetonitrile. Other suitable positive controls providing mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition reference controls (i.e. samples containing only the peptide dissolved in the appropriate solvent) should also be included in the HPLC run sequence and these are used to verify the HPLC system suitability prior to the analysis (reference controls A), the stability of the reference controls over time (reference control B) and to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion (reference control C) (see Annex 3). The appropriate reference control for each substance is used to calculate the percent peptide depletion for that substance (see paragraph 20). In addition, a co-elution control constituted by the test chemical alone for each of the test chemicals analysed should be included in the run sequence to detect possible co-elution of the test chemical with either the lysine or the cysteine peptide.

Incubation of the test chemical with the cysteine and lysine peptide solutions

14. Cysteine and lysine peptide solutions should be incubated in glass autosampler vials with the test chemical at 1:10 and 1:50 ratio respectively. If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, one cannot be sure how much test chemical remained in the solution to react with the peptide. Therefore, in such a case, a positive result could still be used, but a negative result is uncertain and should be interpreted with due care (see also provisions in paragraph 10 for the testing of chemicals not soluble up to a concentration of 100 mM). The reaction solution should be left in the dark at 22.5-30°C for 24 \pm 2 hours before running the HPLC analysis. Each test chemical should be analysed in triplicate for both peptides. Samples have to be visually inspected prior to HPLC analysis. If a precipitate or phase separation is observed, samples may be centrifuged at low speed (100-400xg) to force precipitate to the bottom of the vial as a precaution since large amounts of precipitate may clog the HPLC tubing or columns. If a precipitation or phase separation is observed after the incubation period, peptide depletion may be underestimated and a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result.

Preparation of the HPLC standard calibration curve

15. A standard calibration curve should be generated for both the cysteine and the lysine peptides. Peptide standards should be prepared in a solution of 20% or 25% acetonitrile:buffer

using phosphate buffer (pH 7.5) for the cysteine peptide and ammonium acetate buffer (pH 10.2) for the lysine peptide. Using serial dilution standards of the peptide stock solution (0.667 mM), 6 calibration solutions should be prepared to cover the range from 0.534 to 0.0167 mM. A blank of the dilution buffer should also be included in the standard calibration curve. Suitable calibration curves should have an $r^2 > 0.99$.

HPLC preparation and analysis

16. The suitability of the HPLC system should be verified before conducting the analysis. Peptide depletion is monitored by HPLC coupled with an UV detector (photodiode array detector or fixed wavelength absorbance detector with 220 nm signal). The appropriate column is installed in the HPLC system. The HPLC set-up described in the validated protocol uses a Zorbax SB-C-18 2.1 mm x 100 mm x 3.5 micron as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated at 30°C with 50% phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for at least 2 hours before running. The HPLC analysis should be performed using a flow rate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 minutes, followed by a rapid increase to 90% acetonitrile to remove other materials. Equal volumes of each standard, sample and control should be injected. The column should be re-equilibrated under initial conditions for 7 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the cysteine and lysine peptides, including the injection volume, which may vary according to the system used (typically in the range from 3-10 μ L). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated (e.g. by testing the proficiency substances in Annex 2). Absorbance is monitored at 220 nm. If a photodiode array detector is used, absorbance at 258 nm should also be recorded. It should be noted that some supplies of acetonitrile could have a negative impact on peptide stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 220 peak area and the 258 peak area can be used as an indicator of co-elution. For each sample a ratio in the range of $90\% < \text{mean}^2 \text{ area ratio of control samples} < 100\%$ would give a good indication that co-elution has not occurred.

17. There may be test chemicals which could promote the oxidation of the cysteine peptide. The peak of the dimerised cysteine peptide may be visually monitored. If dimerisation appears to have occurred, this should be noted as percent peptide depletion may be over-estimated leading

² For mean it is meant arithmetic mean throughout the document.

to false positive predictions and/or assignment to a higher reactivity class (see paragraphs 23 and 24).

18. The HPLC analysis should be timed to assure that the injection of the first sample starts 22 to 26 hours after the test chemical was mixed with the peptide solution. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this test method, up to 26 analysis samples can be accommodated in a single HPLC run (see also paragraph 9). An example of HPLC analysis sequence is provided in Annex 3.

DATA AND REPORTING

Data evaluation

19. The concentration of cysteine or lysine peptide is photometrically determined at 220 nm in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of peptide using the linear calibration curve derived from the standards.

20. The percent peptide depletion is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant reference controls C (see Annex 3) according to the formula described below.

$$\text{Percent peptide depletion} = \left[1 - \left(\frac{\text{Peptide peak area in replicate injection}}{\text{Mean peptide peak area in reference controls } C} \right) \right] \times 100$$

Acceptance criteria

21. The following criteria should be met for a run to be considered valid:

a) the standard calibration curve should have an $r^2 > 0.99$,

b) the mean percent peptide depletion value of the three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69.0% for the lysine peptide (for other positive controls a reference range needs to be established) and the maximum standard deviation (SD) for the positive control replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion and

c) the mean peptide concentration of reference controls A should be 0.50 ± 0.05 mM and the coefficient of variation (CV) of peptide peak areas for the nine reference controls B and C in acetonitrile should be $<15.0\%$.

If one or more of these criteria is not met the run should be repeated.

22. The following criteria should be met for a test chemical's results to be considered valid:

a) the maximum standard deviation for the test chemical replicates should be $<14.9\%$ for the percent cysteine depletion and $<11.6\%$ for the percent lysine depletion,

b) the mean peptide concentration of the three reference controls C in the appropriate solvent should be 0.50 ± 0.05 mM.

If these criteria are not met the data should be rejected and the run should be repeated for that specific test chemical.

Prediction model

23. The mean percent cysteine and percent lysine depletion value is calculated for each test chemical. Negative depletion is considered as "0" when calculating the mean. By using the cysteine 1:10/lysine 1:50 prediction model shown in Table 1, the threshold of 6.38% average peptide depletion should be used to support the discrimination between skin sensitisers and non-sensitisers in the framework of an IATA or DA. Application of the prediction model for assigning a test chemical to a reactivity class (i.e. low, moderate and high reactivity) may perhaps prove useful to inform potency assessment within the framework of an IATA or DA.

Table 1: Cysteine 1:10/lysine 1:50 prediction model¹

Mean of cysteine and lysine % depletion	Reactivity Class	DPRAs Prediction ²
$0\% \leq \text{mean \% depletion} \leq 6.38\%$	No or minimal reactivity	Negative
$6.38\% < \text{mean \% depletion} \leq 22.62\%$	Low reactivity	Positive
$22.62\% < \text{mean \% depletion} \leq 42.47\%$	Moderate reactivity	
$42.47\% < \text{mean \% depletion} \leq 100\%$	High reactivity	

¹ The numbers refer to statistically generated threshold values and are not related to the precision of the measurement (2).

² A DPRAs prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

24. There might be cases where the test chemical (the substance or one or several of the components of a multi-constituent substance or a mixture) absorbs significantly at 220 nm and has the same retention time of the peptide (co-elution). Co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and the peptide. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated (e.g. by testing the proficiency substances in Annex 2). When co-elution occurs the peak of the peptide cannot be integrated and the calculation of the percent peptide depletion is not possible. If co-elution of such test chemicals occurs with both the cysteine and the lysine peptides, or with the cysteine peptide only, then the analysis should be reported as “inconclusive”. In cases where co-elution occurs only with the lysine peptide, then the cysteine 1:10 prediction model reported in Table 2 can be used.

Table 2: Cysteine 1:10 prediction model¹

Cysteine (Cys) % depletion	Reactivity class	DPRA prediction ²
$0\% \leq \text{Cys \% depletion} \leq 13.89\%$	No or minimal reactivity	Negative
$13.89\% < \text{Cys \% depletion} \leq 23.09\%$	Low reactivity	Positive
$23.09\% < \text{Cys \% depletion} \leq 98.24\%$	Moderate reactivity	
$98.24\% < \text{Cys \% depletion} \leq 100\%$	High reactivity	

¹ The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.

² A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

25. There might be other cases where the overlap in retention time between the test chemical and either of the peptides is incomplete. In such cases percent peptide depletion values can be estimated and used in the cysteine 1:10/lysine 1:50 prediction model, however assignment of the test chemical to a reactivity class cannot be made with accuracy.

26. A single HPLC analysis for both the cysteine and the lysine peptide should be sufficient for a test chemical when the result is unequivocal. However, in cases of results close to the threshold used to discriminate between positive and negative results (i.e. mean percent depletion falls in the range of 3% to 10% for the cysteine 1:10/lysine 1:50 prediction model or cysteine percent depletion falls in the range of 9% to 17% for the cysteine 1:10 prediction model), additional testing is recommended. In particular, in case of negative results in these ranges (i.e. 3% to 6.38% for the cysteine 1:10/lysine 1:50 prediction model or 9% to 13.89% for the cysteine 1:10 prediction model), a second run should be conducted, as well as a third one in case of discordant results between the first two runs.

Test report

27. The test report should include the following information

Test chemical and Controls (positive control and solvent/vehicle)

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

- Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Additional information for solvent/vehicle control
 - Solvent/vehicle used and ratio of its constituents, if applicable;
 - Justification for choice of solvent for each test chemical;
 - For acetonitrile, results of test of impact on peptide stability.

Peptides

- Supplier, lot, purity

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, detector, autosampler;
- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient.

System suitability

- Peptide peak area at 220 nm of each standard and reference control A replicate;
- Linear calibration curve graphically represented and the r^2 reported;
- Peptide concentration of each reference control A replicate;
- Mean peptide concentration (mM) of the three reference controls A, SD and CV;
- Peptide concentration of reference controls A and C.

Analysis sequence

- For reference controls:
 - Peptide peak area at 220 nm of each B and C replicate;

- Mean peptide peak area at 220 nm of the nine reference controls B and C in acetonitrile, SD and CV (for stability of reference controls over analysis time);
- For each solvent used, the mean peptide peak area at 220 nm of the three appropriate reference controls C (for the calculation of percent peptide depletion);
- For each solvent used, the peptide concentration (mM) of the three appropriate reference controls C;
- For each solvent used, the mean peptide concentration (mM) of the three appropriate reference controls C, SD and CV.
- For positive control:
 - Peptide peak area at 220 nm of each replicate;
 - Percent peptide depletion of each replicate;
 - Mean percent peptide depletion of the three replicates, SD and CV.
- For each test chemical:
 - Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;
 - Presence of co-elution;
 - Description of any other relevant observations, if applicable;
 - Peptide peak area at 220 nm of each replicate;
 - Percent peptide depletion of each replicate;
 - Mean of percent peptide depletion of the three replicates, SD and CV;
 - Mean of percent cysteine and percent lysine depletion values;
 - Prediction model used and DPRA prediction.

Proficiency testing

- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals.

Discussion of the results

- Description of any unintended modifications to the test procedure.
- Discussion of the results obtained with the DPRA test method and if it is within the ranges described in paragraph 26.

Conclusion

LITERATURE FOR APPENDIX I

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APPENDIX I, ANNEX 1

KNOWN LIMITATIONS OF THE DIRECT PEPTIDE REACTIVITY ASSAY

The table below provides a summary of the known limitations of the DPRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Pro-haptens	Test Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential; cannot be detected by the test method unless activation is caused by auto-oxidation to a similar degree as in vivo /in humans. It will however normally not be known whether this will be the case	May lead to false negatives. Negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA or a DA	Diethylenetriamine; 111-40-0 (1A chez l'homme, LLNA n/a)
Pre-haptens	Chemicals that become sensitisers after abiotic transformation are reported to be in most cases correctly detected by the test method		Linalool: 78-70-6
Test chemicals absorbing significantly at 220 nm and having the same retention time of the peptides (co-elution)	When co-elution occurs the peak of the peptide cannot be integrated and the calculation of the percent peptide depletion is not possible	If co-elution of such test chemicals occurs with both the cysteine and the lysine peptides, or with the cysteine peptide only, then the analysis should be reported as "inconclusive" and alternative HPLC set up should be considered (see paragraph 22). In cases where co-elution occurs only with the lysine peptide, then the cysteine 1:10 prediction model reported in Table 2 can be used.	Salicylic acid: 69-72-7
Complex mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials	The molecular weight approach cannot apply - See paragraph 12 for conditions of application of the gravimetric approach	See paragraph 12	UVCBs, chemical emissions, products or formulations with variable or not fully known composition
Test chemicals which cannot be dissolved in an appropriate solvent at a final	Not sure if sufficient exposure can be achieved	Test chemicals that are not soluble at this concentration may still be tested at lower soluble concentrations. In such a case, a positive result could be used to support the identification of the test	n/a

concentration of 100 mM

chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.

Chemicals which precipitate in reaction solution	Not sure if sufficient exposure can be achieved	A conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result	Isopropyl myristate CAS: 110-27-0
Test chemicals that do not covalently bind to the cysteine-peptide but promote its oxidation (i.e. cysteine dimerisation)	Could lead to a potential over-estimation of cysteine-peptide depletion, resulting in possible false positive predictions.		DMSO Oxidant
Test chemicals that are only soluble in DMSO	DMSO causes excessive peptide depletion due to cysteine dimerization resulting in high background cysteine depletion.	May lead to false negative results	n/a

APPENDIX I, ANNEX 2

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay

Prior to routine use of the test method described in this test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected DPRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining cysteine and lysine depletion values that fall within the respective reference range for 8 out of the 10 proficiency substances for each peptide. These proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the DPRA are available, and that they were used in the EURL ECVAM-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Table 1: Recommended proficiency substances for demonstrating technical proficiency with the Direct Peptide Reactivity Assay

Proficiency substances	CASRN	Physical state	<i>In vivo</i> prediction ¹	DPRA prediction ²	Range ³ of % cysteine peptide depletion	Range ³ of % lysine peptide depletion
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Positive	90-100	15-45
Oxazolone	15646-46-5	Solid	Sensitiser (extreme)	Positive	60-80	10-55
Formaldehyde	50-00-0	Liquid	Sensitiser (strong)	Positive	30-60	≤ 24
Benzylideneacetone	122-57-6	Solid	Sensitiser (moderate)	Positive	80-100	≤ 7
Farnesal	19317-11-4	Liquid	Sensitiser (weak)	Positive	15-55	≤ 25
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	Positive	60-100	10-45
1-Butanol	71-36-3	Liquid	Non-sensitiser	Negative	≤ 7	≤ 5.5
6-Methylcoumarin	92-48-8	Solid	Non-sensitiser	Negative	≤ 7	≤ 5.5
Lactic Acid	50-21-5	Liquid	Non-sensitiser	Negative	≤ 7	≤ 5.5

4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	Negative	≤ 7	≤ 5.5
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¹The *in vivo* hazard and (potency) predictions are based on LLNA data (5). The *in vivo* potency is derived using the criteria proposed by ECETOC (8).

² A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

³ Ranges determined on the basis of at least 10 depletion values generated by 6 independent laboratories.

APPENDIX I, ANNEX 3

EXAMPLES OF ANALYSIS SEQUENCE

Calibration standards and reference controls	STD1 STD2 STD3 STD4 STD5 STD6 Dilution buffer Reference control A, rep 1 Reference control A, rep 2 Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1 Reference control B, rep 2 Reference control B, rep 3
First set of replicates	Reference control C, rep 1 Cinnamic aldehyde, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Cinnamic aldehyde, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Cinnamic aldehyde, rep 3 Sample 1, rep 3 Sample 2, rep 3
Reference controls	Reference control B, rep 4 Reference control B, rep 5 Reference control B, rep 6

Three sets of reference controls (i.e. samples constituted only by the peptide dissolved in the appropriate solvent) should be included in the analysis sequence:

Reference control A: used to verify the suitability of the HPLC system.

Reference control B: included at the beginning and at the end of the analysis sequence to verify stability of reference controls over the analysis time.

Reference control C: included in the analysis sequence to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion.

APPENDIX II

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The ADRA is proposed to address the molecular initiating event of the skin sensitisation AOP - namely, protein reactivity - by quantifying the reactivity of test chemicals towards model synthetic amino acid derivatives containing either lysine or cysteine (1) (2) (3). Depletion values of the cysteine derivative N-(2-(1-naphthyl)acetyl)-L-cysteine (CAS. 32668-00-1), which is known as NAC, and the lysine derivative α -N-(2-(1-naphthyl)acetyl)-L-lysine (CAS. 397841-92-8), known as NAL are then used to support the discrimination between skin sensitisers and non-sensitisers (1) (2) (3).
2. The reproducibility and transferability of the ADRA protocol were confirmed using validation studies coordinated by the Japanese Center for validation of alternative methods (JaCVAM) (4) (5) (6) (7) (8) (9) (10). There are two detection types of ADRA: ultraviolet (UV) detection and fluorescence (FL) detection (11) (12). Within-laboratory reproducibility (WLR) and between-laboratory reproducibility (BLR) of ADRA were 100% each determined using both the UV detection and fluorescence detection (9) (10). Prediction of skin sensitisation potential based on local lymph node assay (LLNA) data indicated that ADRA with UV-detection identified sensitisers and non-sensitisers with an accuracy of 76 % (104/136), a sensitivity of 76% (74/98), a specificity of 79% (30/38) and a balanced accuracy of 77% (8). In addition, the prediction of the skin sensitisation potential based on human data indicated that ADRA with UV detection has an accuracy of 84% (67/80), a sensitivity of 83% (48/58), a specificity of 86% (19/22) and a balanced accuracy of 84% (8). However, the accuracy values given here for ADRA as a stand-alone test method are for reference only, since it is recommended that the test method be used in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General Introduction. Furthermore, when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA as well as other animal tests may not fully reflect the situation in humans. On the basis of the overall data available, ADRA's

applicability domain was shown to include a variety of organic functional groups, reaction mechanisms, skin sensitisation potencies (as determined in in vivo studies), and physicochemical properties (1) (2) (3) (4). Following an independent peer review, the ADRA validation studies were considered to demonstrate that this method should be acceptable as part of an integrated testing strategy for the predictive identification of skin sensitisation hazard (6) (13) (14).

3. Co-elution occurs when the test chemical (the substance or one or several of the constituents of a multi-constituent substance or a mixture) was detected significantly at an OD of 281 nm (UV detector) or Ex/Em 284/333 nm (FL detector) and has the same retention time as NAC or NAL (15). Co-elution of UV absorbing-compounds using with the nucleophiles NAC and NAL can lead to inconclusive results when using conventional ultraviolet (UV) detection (11) (12). This problem can be prevented by an alternative or parallel measurement using a fluorescence (FL) detector; thus, the depletion values obtained by simultaneous measurement using both detectors were also collected in the validation studies (9) (10) and equivalent results to those obtained with UV-detection were obtained, indicating that both detection methods are valid, but FL-detection may lead to fewer inconclusive results. Known limitations of the ADRA are tabulated in Appendix II, Annex 1.

4. The term "test chemical" is used in this Test Guideline to refer to what is being tested³. This test method is not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding. The test method described in this Appendix of the Test Guideline is an in chemico method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e., pro-haptens) cannot be detected by the test method. Chemicals that become sensitisers after abiotic transformation (i.e., pre-haptens) are reported to be in some cases correctly detected by the test method (1) (2) (3) (4) (7) (8). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA. Test chemicals that promote the oxidation of the N-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) reagent (i.e. cysteine dimerisation) could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions (see paragraph 22 and Appendix II, Annex 1); it may be possible to detect and quantify any NAC dimer formed by high-performance liquid chromatography (HPLC) using a UV detector, thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and covalent bonding to the test item substance(s).

³ In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should now be applied in new and updated Test Guidelines.

5. The ADRA test method allows testing of poorly soluble chemicals (16). To be tested, a test chemical should be soluble in an appropriate solvent at a final concentration of 4 mM (see paragraph 14). Test chemicals that are not soluble at this concentration may still be tested at lower concentrations. In such cases, a positive result could still be used to support identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.

6. The nucleophilic reagents used in ADRA are quantified at 281 nm (1) (2). In the case of co-elution of the nucleophilic reagent and the UV-absorbing test chemical, this might result in inconclusive predictions. However, substances that absorb UV in this range of the spectrum are generally limited to those having conjugated double bonds, which significantly lowers the potential for inconclusive results due to co-elution of UV-absorbing components (15). Furthermore, NAC and NAL are fluorescent and thus, they can be detected using a FL detector (11) (12). Since test chemicals rarely have fluorescence at the specific excitation/emission wavelengths, it is possible to further reduce frequency of inconclusive results by using a FL detector. This is particularly useful in the case of multi-constituent substances with UV absorbance.

7. When assessing the sensitisation potential of a test chemical by using ADRA, there are two options for the preparation of the stock solution (see Figure 1 and paragraphs 15-16): a) If the test chemical is a mono-constituent substance with a known molecular weight or a mixture or multi-constituent substance of known composition, ADRA should be performed using a stock solution prepared at a concentration of 4 mM (8); b) If the test chemical is a mono-constituent substance of unknown molecular weight or a mixture and there is no defined molecular weight (mixtures of unknown or variable composition, complex reaction products, or biological materials (UVCB)), ADRA should be performed using a gravimetric approach based on a stock solution prepared at 0.5 mg/mL. In addition, the gravimetric approach with ADRA (0.5 mg/mL) can also be used for polymers. Assessment of the predictive capacity of ADRA conducted with this gravimetric approach indicated that ADRA (0.5 mg/mL) identified sensitisers and non-sensitisers with an accuracy of 76 % (103/136), a sensitivity of 74% (73/98), a specificity of 79% (30/38) and a balanced accuracy of 77% when compared to LLNA data (8). In addition, the predictive capacity for human data indicated that the gravimetric ADRA (0.5 mg/mL) has an accuracy of 83% (66/80), a sensitivity of 81% (47/58), and a specificity of 86% (19/22) (8). The molecular weight range of the test chemicals used in the validation study of ADRA (0.5 mg/mL) was 60.10 - 388.29, and the ratio of nucleophilic reagent to test chemical in the reaction solution at that time was 1:416 - 1:64 (9).

8. ADRA can be used to support the discrimination between skin sensitisers and non-sensitisers. Further work, preferably based on human data, is necessary to determine whether ADRA results can contribute to potency assessment when considered in combination with other information sources (13) (14).

PRINCIPLE OF THE TEST

9. ADRA is an in chemico test method that quantifies residual concentrations of the NAC and NAL, following a 24 ± 1 hour incubation at $25 \pm 1^\circ\text{C}$ in the presence of a test chemical. Both these derivatives include a naphthalene ring that is introduced to their N-terminal in order to facilitate UV detection and FL detection. The relative concentrations of NAC and NAL are measured by HPLC using UV detection (optical density, 281 nm), optionally in combination with FL detection (excitation/emission [Ex/Em], 284/333 nm) and with gradient elution (see paragraph 19). To ultimately support the discrimination between skin sensitisers and non-sensitisers, percent depletion values are then calculated for both NAC and NAL and compared to a prediction model (see paragraph 27).

10. Prior to routine use of the method described in this test method, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Appendix II, Annex 2.

PROCEDURE

11. This test method is based on the protocol (17) used for the JaCVAM-coordinated ADRA validation study and is recommended for use when implementing ADRA at a laboratory. The main components and procedures for the ADRA are described below. Before using an alternative HPLC set-up, its equivalence to the validated set-up described in the protocol should be demonstrated, preferably by testing the proficiency substances in Appendix II, Annex 2.

Quality of NAC and NAL

12. The Nucleophilic Reagents can be obtained as an ADRA Kit for Skin Sensitisation Test, from FUJIFILM Wako Pure Chemical Corporation, Catalogue No. 296-80901. The use of NAC/NAL as reagent for detecting sensitisation is patented in Japan only, by Fujifilm Corporation. Therefore, in other countries, NAC/NAL can be used without permission. In case other manufacturer's NAC/NAL are used, these should satisfy three quality criteria described below. Quality checks can be obviated and ADRA testing can be performed without delay by purchasing NAC and NAL that have been manufactured specifically to satisfy these quality criteria.

Quality required for NAC and NAL:

1) Purity: Both NAC and NAL are to be at least 98% pure.

2) Stability: Using NAC and NAL stock solution, prepare a reference control free of any test chemical and quantify the residual levels of NAC and NAL both immediately after preparation (0 hours) and after a 24 hour incubation. The residual level of NAC and NAL is calculated as follows:

$$\text{Residual levels of NAC} = \frac{\text{Peak area of NAC}}{\text{Total peak area of NAC and NAC dimer}} \times 100$$

$$\text{Residual levels of NAL} = \frac{\text{Peak area of NAL at 24 hour}}{\text{Peak area of NAL at 0 hour}} \times 100$$

The main cause of NAC stability degradation is dimerisation, which may affect reactivity with the test chemical and test reproducibility (3). Therefore, the residual level of NAC should be calculated with respect to the total amount of NAC and dimer. Since the dimers may be formed over time or may have already been formed during the preparation of the stock solution, residual level of NAC is calculated at the time of stock solution preparation and after 24 hours. Residual levels of NAC (both of 0 hour and 24 hour) and NAL (24 hour) should be a minimum of 90% in either case (17).

3) Reactivity: NAC and NAL are to be evaluated for reactivity with the ten proficiency substances given in Appendix II, Annex 2 and should satisfy the requirement given therein.

Preparation of the NAC and NAL stock solution

13. The solubility of individual NAC and NAL batches should be verified prior to use. NAC stock solution should be prepared to a concentration of 2 mM in 100 mM of pH 8.0 phosphate buffer, including 0.333 μM of EDTA, as well as NAL stock solution to a concentration of 2 mM in 100 mM of pH 10.2 phosphate buffer. These two stock solutions are then diluted in buffer to prepare 6.667 μM stock solutions. Both NAC and NAL stock solutions should be used as soon as possible after preparation (3). In the event that they are to be stored, these stock solutions may be frozen and stored for up to twelve months time at less than -75°C prior to use. The final concentration of the NAC in the incubation mixture is 5 μM in pH 8.0 phosphate buffer, and the final concentration of the NAL in the incubation mixture is 5 μM in pH 10.2 phosphate buffer.

Preparation of the test chemical solution

14. Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay in accordance with the solubilisation procedure described in the ADRA JaCVAM protocol (17). An appropriate solvent should dissolve the test chemical completely. Since the ADRA protocol stipulates that either NAC or NAL are incubated in an excess volume of the test chemical, visual inspection of the clear test chemical solution is considered sufficient to confirm that the test chemical (and all its constituents, if testing a multi-constituent substance or a mixture) is dissolved (17). Suitable solvents are distilled water, acetonitrile and acetone. If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts (19). It is important to note that DMSO may lead to dimerisation of the nucleophilic reagent NAC (18) (19) and as a result, it may be more difficult to meet the acceptance criteria. If a

DMSO-acetonitrile solvent is chosen (5% DMSO in acetonitrile), the test chemical should be dissolved at 80 mM in DMSO, and then this solution should be diluted 20-fold with acetonitrile to prepare a 4 mM test chemical solution. In case the use of DMSO leads to increased dimerisation of the NAC reagent, this can be checked analytically as the NAC dimer can be detected by HPLC. If a solvent other than those already considered appropriate for the ADRA is used for the test chemical, it is necessary to confirm that the solvent itself does not lead to NAC or NAL depletion (e.g., dimerisation, oxidation) and does not degrade or disrupt the integrity of the test substances or mixture components. The test chemical should be pre-weighed into a disposable polypropylene tube and dissolved immediately before testing in an appropriate solvent to prepare a 4 mM stock solution (See paragraph 5).

15. This molecular weight approach should apply if the test chemical is a mono-constituent substance with a known molecular weight or a mixture or multi-constituent substance of known composition (See Figure 1). For mixtures and multi-constituent substances of known composition, a single aggregated purity value should be determined by the sum of the proportion of its constituents (excluding water), and a single aggregated molecular weight should be determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and aggregated molecular weight should then be used to calculate the weight of test chemical necessary to prepare a 4 mM solution.

16. Mono-constituent substances of unknown molecular weight should be tested based on a test chemical stock solution at a concentration of 0.5 mg/mL rather than 4 mM (7) (See Figure 1 and paragraph 7). Polymers can also be tested at a concentration of 0.5 mg/mL. For mixtures and multi-constituent substances of unknown composition (i.e. UVCB substances of unknown or variable composition, complex reaction products or biological materials), the test solution can be prepared with a gravimetric approach. The substance should then be dissolved in the stock solution at 0.5 mg/mL on the basis of the weight of the total components (excluding solvent) in an appropriate solvent (See paragraph 14 and Figure 1). This 0.5 mg/mL of test chemical concentration corresponds to a molecular weight of 125 g/mol when ADRA (4 mM) is performed. The ADRA gravimetric approach with ADRA (0.5 mg/mL) has been shown to be almost as accurate in prediction as ADRA (4 mM) for 136 chemicals in a wide molecular weight range (30.03 - 512.60) (8) (see paragraph 7). This assessment of the predictive capacity of the gravimetric approach is based on testing chemicals with defined molecular weight and not based on the testing of mixtures, as no reference data for mixtures are available. Therefore, if the mixture to be investigated is known to contain a chemical class with a typical molecular weight which is significantly higher, this default molecular weight and the test solution concentration should be adjusted accordingly [see e.g. approach for agrochemical formulations in (24)]. The gravimetric approach should only be applied as a last resort if no aggregated molecular weight can be calculated. As for any testing with mixtures, as much as possible, information should be gathered on the sensitization potential and reactivity of individual constituents.

Preparation of the positive control, reference controls and co-elution controls

17. Either phenylacetaldehyde (CAS 122-78-1, purity $\geq 90\%$) or squaric acid diethyl ester (CAS 5231-87-8, purity $> 95\%$) should be used as the positive control (PC) at a concentration of 4 mM in acetonitrile (10). Phenylacetaldehyde is prone to oxidation and polymerisation and integrity of the sample has to be assured by proper storage or by using fresh samples. Squaric acid diethyl ester should be stored protected from high temperature or humidity, since it is prone to hydrolysis. Other suitable positive controls that provide mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition, reference controls comprising only NAC or only NAL dissolved in the appropriate solvent should also be included in the HPLC run sequence, and these are used to verify the HPLC system suitability prior to analysis (Reference Control A), the stability of the reference controls over time (Reference Control B), and any effects of the solvent used on depletion of NAC or NAL (Reference Control C) (See Appendix II, Annex 3). The percent NAC and NAL depletion for a test chemical is calculated using an appropriate reference control for that test chemical (see paragraph 23). Also, a co-elution control comprising only the test chemical should be included in the run sequence to detect possible co-elution of the test chemical with either the NAC or NAL.

Incubation of the test chemical with the NAC and NAL solutions

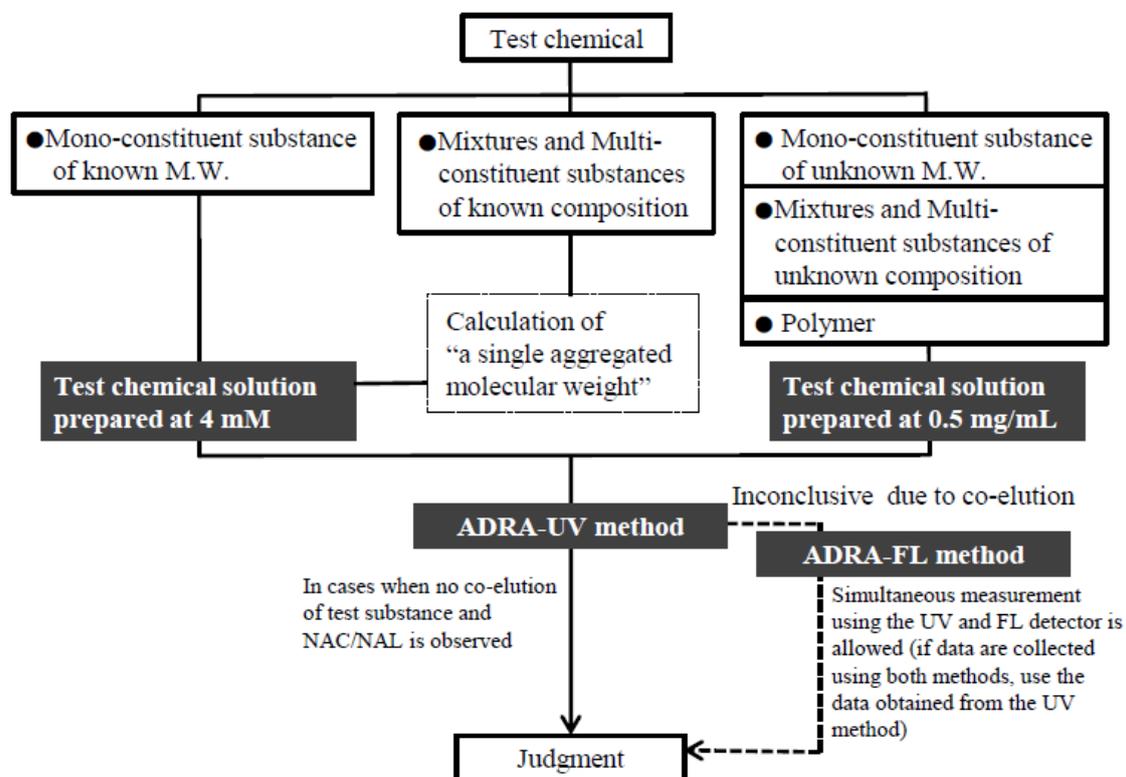
18. Both the NAC and the NAL stock solutions are incubated with the test chemical stock solution in a 3:1 ratio in a 96-well microplate. For the 4 mM test chemical stock solution this gives a final concentration of 1 mM test chemical and 5 μM NAC/NAL (17). For the 0.5 mg/ml test chemical stock solution, the final level of the test chemical is 0.125 mg/ml. The observation of precipitate immediately upon addition of the test chemical solution to the NAC and the NAL solutions is an indication of poor solubility, which means that there is no way to know exactly how much test chemical is contained in the solution. Thus, although positive results can be used with confidence, negative results are uncertain and no firm conclusion on the lack of reactivity should be drawn from a negative result (see also paragraph 5 regarding the testing of chemicals not soluble at concentrations as high as 4 mM). The reaction solution should be incubated in the dark at $25\pm 1^\circ\text{C}$ for 24 ± 1 hours before performing HPLC analysis. After incubation, trifluoroacetic acid (TFA) ($\geq 98\%$) should be added to reaction solution as a fixing solution to stop the reaction (3). 2.5% (v/v) TFA aqueous solution is added to the reaction solution in a 1:4 ratio. Thus, final concentration of NAC/NAL and TFA are 4 μM and 0.5%, respectively.

HPLC preparation and analysis

19. NAC/NAL depletion is monitored by HPLC coupled with an UV-detector. In case of co-elution of NAC/NAL with an UV-absorbing component in the test chemical solution, a fluorescence detector is used (11) (12). There are two options for NAC/NAL detection: Successive measurement should be started with UV-detection and fluorescent detection is used only if inconclusive results due to co-elution are obtained. Alternatively, simultaneous measurement is performed by

connecting both the UV and FL detector to the HPLC system for parallel detection. If no co-elution of UV-absorbing components is observed, only the UV data are used. If inconclusive results due to co-elution are observed, FL data will be used (see Figure 1). In the unlikely event that a co-elution also appears in ADRA-FL, the operation should be performed according to paragraph 28. Each test chemical should be analysed in triplicate to determine percent depletion for both NAC and NAL. Although adding the fixing solution does stop the reaction, measurement of the reaction solution is to be performed as soon as possible and in any case within three days after adding the fixing solution. For example, when HPLC analysis of NAC and NAL are performed separately using two 96-well microplates, up to 34 samples may be analysed at one time, including the test chemical, the positive control, and the appropriate number of solvent controls based on the number of individual solvents used in the test, each in triplicate. All of the replicates analysed in a single run should use identical batches of NAC and NAL stock solution. Test chemical and control solutions are to be visually inspected prior to HPLC analysis and may be centrifuged at low speed (100–400 × g) to force any precipitate to the bottom of the vial as a precaution against large amounts of precipitate clogging the HPLC tubing or columns. Observation of precipitation or phase separation after the incubation period is an indication that NAC and NAL depletion could be misleading, and negative results in that case are uncertain and should be interpreted with due care, as well as for any precipitate observed at the beginning of the incubation period (see above).

Figure 1: Procedure to assess NAC/NAL depletion in ADRA including a gravimetric approach for mixtures and alternative fluorescent detection in case of co-elution with UV-absorbing components.



MW, molecular weight; ADRA, amino acid derivative reactivity assay; UV, ultraviolet; FL, fluorescence

20. A standard calibration curve should be generated for both NAC and NAL. Standard solutions of both NAC and NAL should be prepared in 20% acetonitrile in buffer and containing 0.5% trifluoroacetic acid. For NAC, a phosphate buffer at pH 8.0, and for NAL, a phosphate buffer at pH 10.2 should be used. Using the NAC and NAL stock solutions (6.667 μM), six calibration solutions should be prepared in concentrations from 5.0 to 0.156 μM . A blank of the dilution buffer should also be included in the standard calibration curve. Suitable calibration curves should have an $R^2 > 0.990$.

21. The suitability of the HPLC system should be verified before conducting the analysis. Both NAC and NAL depletion is monitored by HPLC coupled with an UV-detector (photodiode array detector or fixed wavelength absorbance detector with 281 nm signal) and a FL detector (Ex, 284 nm and Em, 333 nm) (see paragraph 19). The appropriate column is installed in the HPLC system. The recommended HPLC set-up described in the validated protocol uses a column with the

following specifications. Base particle: core-shell type silica gel, Particle size: 2.5~2.7 μm , column size: 3.0 \times 150 mm as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated for at least 30 minutes at 40°C with 50% phase A (0.1% (v/v) trifluoroacetic acid in water), 50% phase B (0.1% (v/v) trifluoroacetic acid in acetonitrile) before use. Then, the column is conditioned by running the gradient at least twice before actual use. The HPLC analysis should be performed using a flow rate of 0.30 mL/min and a linear gradient from 30% to 55% acetonitrile for NAC and from 25% to 45% acetonitrile for NAL within 10 minutes, followed by a rapid increase to 100% acetonitrile to remove other materials. Equal volumes of the standard solutions, test chemical solutions, and control solutions should be injected. The column should be re-equilibrated under initial conditions for 6.5 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the NAC and NAL, including the injection volume, which may vary according to the system used (typically in the range from 10–20 μL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated, preferably by testing the proficiency substances in Appendix II, Annex 2. Using the UV detection method, absorbance is monitored at 281 nm. If a photodiode array detector is used, absorbance at 291 nm should also be recorded. It should be noted that some batches of acetonitrile could have a negative impact on NAC and NAL stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 281 nm peak area and the 291 nm peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90% < mean area ratio of control samples < 100% would give a good indication that co-elution has not occurred. An example of HPLC analysis sequence is provided in Appendix II, Annex 3.

22. There are some test chemicals that could potentially promote oxidation of NAC. The peak of the dimerised NAC may be monitored visually in the case of ADRA-UV. However, since the NAC dimer does not exhibit fluorescence, it cannot be detected in the fluorescent detection mode. Any apparent dimerisation should be noted, since overestimation of NAC depletion could result in false-positive predictions (See paragraphs 4, 14 and Appendix II, Annex 1).

DATA AND REPORTING

Data evaluation

23. The concentration of both NAC and NAL is photometrically determined at 281 nm (UV detector) and if needed by fluorescence detection with Ex/Em, 284/333 nm (FL detector) (see paragraph 21) in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of both NAC and NAL using the linear calibration curve derived from the standards.

24. The percent depletion for both NAC and NAL is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant Reference Controls C (See Appendix II, Annex 3) according to the formula described below.

$$\text{Percent NAC or NAL depletion} = \left[1 - \left[\frac{\text{NAC or NAL peak area in replicate injection}}{\text{Mean NAC or NAL peak area in reference controls C}} \right] \right] \times 100$$

Acceptance criteria

25. The following criteria should be met for a run to be considered valid:

a) the standard calibration curve should have an $R^2 > 0.990$,

b) the mean percent NAC and NAL depletion value and the maximum standard deviation (SD) of the three replicates for the positive control (phenylacetaldehyde or squaric acid diethyl ester) should meet the following criteria:

- NAC depletion:

Phenylacetaldehyde: 30 - 80%; Squaric acid diethyl ester: 30 - 80 %

- NAL depletion:

Phenylacetaldehyde: 70 - 100%; Squaric acid diethyl ester: 70 - 100 %

- Maximum standard deviation (SD) for NAC and NAL depletion for both phenylacetaldehyde and squaric acid diethyl ester: < 10%,

c) the mean NAC and NAL concentration of both Reference Controls A and C should be 3.2–4.4 μM and the coefficient of variation (CV) of NAC and NAL peak areas for the nine Reference Controls B and C in acetonitrile should be < 10%.

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated for that specific test chemical.

26. The following criteria should be satisfied for a test chemical's results to be accepted as valid:

a) the maximum standard deviation for the test chemical replicates should be < 10% for the percent depletion of both NAC and NAL,

b) the mean NAC and NAL concentration of the three Reference Controls C in the appropriate solvent should be 3.2–4.4 μM . The permissible range of the mean NAC concentration of Reference Control C when 5% DMSO in acetonitrile is used as a solvent is 2.8 to 4.0 μM (19).

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated for that specific test chemical.

Prediction model

27. The mean percent depletion of NAC and NAL is calculated for each test chemical. Negative depletion is considered to be “0” when calculating the mean. By using the NAC/NAL prediction model shown in Table 1, the threshold of 4.9% mean depletion should be used to support the discrimination between skin sensitizers and non-sensitizer in the framework of an IATA or a DA. The 4.9% of cut-off value for the mean percent depletion of NAC and NAL was set by using 2 class classification model so that the sensitizer and non-sensitizer could be predicted most appropriately.

Table 1: NAC/NAL prediction model¹

Mean NAC and NAL percent depletion	ADRA prediction ²
Less than 4.9%	Negative
4.9% or higher	Positive

¹ The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.

² An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 13 and 14.

28. If co-elution is observed using either the UV or the FL detector, the depletion value measured using the detector in which co-elution is not observed should be used (See Figure 1). If co-elution is observed with both detectors, co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and NAC or NAL. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated, preferably by testing the proficiency substances in Appendix II, Annex 2. When co-elution occurs, it is not possible to integrate the peak of the NAC or NAL, thereby preventing calculation of the percent depletion of NAC or NAL. If co-elution of test chemicals occurs with both the NAC and NAL and separation of elution time is not feasible, then the analysis should be reported to be inconclusive. In cases where co-elution occurs only with NAL and separation of elution time is not feasible, the NAC-only prediction model (See Table 2) can be used to make a prediction. In this case, the NAC data of ADRA-UV should still be preferentially adopted than that of ADRA-FL. The 5.6% cut-off value for the percent depletion of NAC was set by using 2 class classification model so that the sensitizer and non-sensitizer could be predicted most appropriately.

Table 2: NAC-only prediction model¹

Mean NAC percent depletion	ADRA prediction ²
Less than 5.6%	Negative
5.6% or higher	Positive

¹ The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.

² An ADRA prediction should be considered in the framework of an IATA (13) (14).

29. When a result is unequivocal, a single HPLC analysis for both NAC and NAL should be sufficient for a test chemical. However, in case of results close to the threshold used to discriminate between positive and negative results (i.e. in the range of 3% to 10% for NAC/NAL prediction model or NAC percent depletion falls in the range of 4% to 11% for NAC-only prediction model), additional testing is recommended. In particular, in case of negative results in these ranges (i.e. 3% to 4.9% for NAC/NAL prediction model or 4% to 5.6% for NAC-only prediction model), a second run should be conducted, as well as a third one in case of discordant results between the first two runs. In the above cases, the majority of the three test results is adopted.

Test report

30. The test report should include the following information:

Test chemical and Controls (positive control and solvent/vehicle)

- For all mono-constituent substance (test and control chemicals)
 - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers
 - Physicochemical properties such as physical state, appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available
 - Purity, chemical identity of impurities as appropriate and practically feasible, etc.
 - Treatment prior to testing, if applicable (warming, grinding)
 - Concentration(s) tested
 - Storage conditions and stability to the extent available
- Multi-constituent substance, UVCB, and mixtures
 - Characterisation by chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available
 - Physical appearance, water solubility, and additional relevant physicochemical properties, to the extent available
 - Molecular weight (or apparent molecular weight) for mixtures or polymers of known composition, or other information relevant to the study

- Treatment prior to testing, if applicable (warming, grinding)
- Concentration(s) tested
- Storage conditions and stability, to the extent available.
- Additional information for positive control
 - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Additional information for solvent/vehicle control
 - Solvent used and ratio of its constituents, if applicable
 - Justification for choice of solvent for each test chemical
 - Impact on NAC and NAL stability when using acetonitrile

Preparation of NAC and NAL, positive control and test chemical solution

- Characterisation of NAC and NAL solutions (supplier, lot, exact weight of NAC and NAL, volume added for the stock solution)
- Characterisation of positive control solutions (exact weight of positive control reagent, volume added for the control solution)
- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test chemical solution)

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, UV or FL detector, autosampler
- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient

System suitability

- NAC and NAL peak area at OD 281 nm (UV detector) or Ex/Em 284/333 nm (FL detector) of each standard and reference control A replicate
- Linear calibration curve graphically represented and the R2 reported
- NAC and NAL concentration of each Reference Control A replicate
- Mean NAC and NAL concentration (μM) of the three reference controls A, SD and CV
- NAC and NAL concentration of Reference Controls A and C.

Analysis sequence

- For Reference Controls
 - NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of each replicate of Reference Controls B and C
 - Mean NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of the nine Reference Controls B and C in acetonitrile, SD and CV (for stability of reference controls over analysis time)
 - For each solvent used, the mean NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of the three appropriate Reference Controls C (for the calculation of percent NAC and NAL depletion)
 - For each solvent used, the NAC and NAL concentration (μM) of the three appropriate Reference Controls C
 - For each solvent used, the mean NAC and NAL concentration (μM) of the three appropriate Reference Controls C, SD and CV.
- For positive controls
 - NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of each replicate
 - Percent NAC and NAL depletion of each replicate
 - Mean percent NAC and NAL depletion of the three replicates, SD and CV.
- For each test chemical

- Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;
- Presence of co-elution
- Description of any other relevant observations, if applicable
- NAC and NAL peak area at an OD of 281 nm (UV detector) or an Ex/Em of 284/333 nm (FL detector) of each replicate
- Percent NAC and NAL depletion of each replicate
- Mean of percent NAC and NAL depletion of the three replicate, SD and CV
- Mean of percent NAC and percent NAL depletion values
- Prediction model used and ADRA prediction

Proficiency testing

- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals

Discussion of the results

- Description of any unintended modifications to the test procedure.
- Discussion of the results obtained with the ADRA test method and if it is within the ranges described in paragraph 29.

Conclusion

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APPENDIX II, ANNEX 1

Known limitations of the Amino acid Derivative Reactivity Assay (ADRA)

The table below provides a summary of the known limitations of the ADRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Pro-haptens	Test Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential cannot be detected by the test method unless activation is caused by auto-oxidation to a similar degree as in vivo /in humans. It will however normally not be known whether this will be the case	May lead to false negatives. Negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA	Diethylenetriamine; 111-40-0 (human 1A, LLNA n/a)
Pre-haptens	Chemicals that become sensitisers after abiotic transformation are reported to be in some cases correctly detected by the test method		Linalool: 78-70-6
Test chemicals that have a UV absorption (OD, 281 nm) or FL (Ex/Em, 284/333 nm) and have the same retention time than NAC or NAL (co-elution)	When co-elution occurs the peak of the NAC or NAL cannot be integrated and the calculation of the percent NAC or NAL depletion is not possible.	The substances that absorb UV in this range of the spectrum are generally limited to those having conjugated double bonds, which significantly lowers the potential for co-elution. The substances that have a FL in this range are generally limited to polyaromatic or polyheterocyclic compounds, including naphthalene derivatives. If co-elution of such test chemicals occurs with both the NAC and the NAL or with the NAC only, then the analysis should be reported as "inconclusive" and alternative HPLC set up should be considered (see paragraph 28). In cases where co-elution occurs only with the NAL, then the NAC-only prediction model reported in Table 2 can be used."	Safranal; 116-26-7
Complex mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials	ADRA using a 4 mM chemical solution needs for defined molar ratio of test chemical and nucleophilic reagent, but ADRA using a 0.5 mg/mL solution does not need the defined molar ratio of a test chemical and can predict sensitisation for	Since plant extract contains various polyphenols, which react with NAC, it may be judged as a sensitiser when a solution containing a high concentration of the plant extract is evaluated using ADRA. Therefore, these results should be considered with reference to results obtained using alternative methods for other	n/a

	test chemicals, which are prepared at a weight concentration of 0.5 mg/mL. When the mixture is a liquid, the evaluation of sensitisation using ADRA cannot be performed if the total weight of the mixture components dissolved in solvent (water, dissolving solution, extraction solvent, etc) is not known, since it is then impossible to prepare a 0.5 mg/mL test chemical solution.	key events and <i>in vivo</i> results of similar substances.	
Test chemicals which cannot be dissolved in an appropriate solvent at a final concentration of 4 mM	Not sure if sufficient exposure can be achieved If the mixture is liquid and the total weight of the mixed components dissolved in a solvent (e.g., water, dissolving solution, extraction solvent) is not known, it is not possible to prepare a 0.5 mg/mL test substance solution, and thus the sensitisation potential cannot be evaluated by ADRA.	The ADRA test method allows testing of poorly soluble chemicals. Test chemicals that are not soluble at this concentration though may still be tested at lower soluble concentrations. In such a case, a positive result could be used to support the identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.	n/a
Chemicals which precipitate in reaction solution	Not sure if sufficient exposure can be achieved	Test chemicals that precipitate in the reaction solution even if dissolved in the solvent may still be tested at lower soluble concentrations. In such a case, a positive result could still be used to support the identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.	Isopropyl myristate CAS: 110-27-0
Test chemicals that do not covalently bind to the NAC but promote its -oxidation (i.e. NAC dimerisation)	Could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions.	It may be possible to detect and quantify any NAC dimer formed by HPLC (UV detector), thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and covalent bonding to the test item substance(s) Therefore, ADRA may prevent erroneous judgement due to the oxidizing action of the test chemical. However, since the NAC dimer does not have fluorescence, it can only be detected by ADRA-UV.	DMSO Oxidant
Test chemicals that are only soluble in DMSO	DMSO causes excessive NAC depletion due to NAC dimerization resulting in high background NAC depletion.	DMSO is allowed to be contained in the test chemical solution up to 5%. If DMSO is chosen, attempts should be made to solubilise the test chemical in a 1:20 mixture of DMSO and acetonitrile (5% DMSO in acetonitrile).	n/a

APPENDIX II, ANNEX 2

Proficiency Substances

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

Prior to routine use of the test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected ADRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining NAC and NAL depletion values that fall within the respective reference ranges for 8 out of the 10 proficiency substances. The test to demonstrate technical proficiency in ADRA is basically ADRA with 4 mM (10). If ADRA with 4 mM has been proven to be mastered by performing proficiency substances, ADRA with 0.5 mg/mL can be exempt from demonstrating the technical proficiency (9). These proficiency substances were selected to represent the full range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality ADRA data are available, and that they were used during the JaCVAM-coordinated validation study to demonstrate successful implementation.

Table 1. Recommended chemicals for demonstrating technical proficiency with ADRA_4 mM

No.	Test chemicals	CAS No.	Physical state	Molecular weight	<i>In vivo</i> Prediction ¹	ADRA 4 mM prediction ²	Range of % depletion	
							NAC ³	NAL ³
1	<i>p</i> -Benzoquinone	106-51-4	Solid	108.09	Sensitiser (extreme)	Positive	90-100	70-100
2	Diphenylcyclopropenone	886-38-4	Solid	206.24	Sensitiser (extreme)	Positive	50-90	≤ 10
3	2-Methyl-2H-isothiazol-3-one	2682-20-4	Solid	115.15	Sensitiser (strong)	Positive	80-100	≤ 10
4	Palmitoyl Chloride	112-67-4	Liquid	274.87	Sensitiser (moderate)	Positive	≤ 40	70-100
5	Imidazolidinyl urea	39236-46-9	Solid	388.29	Sensitiser (weak)	Positive	40-70	≤ 20
6	Farnesal	19317-11-4	Liquid	220.35	Sensitiser (weak)	Positive	60-100	5-40
7	Glycerol	56-81-5	Liquid	92.09	Non-sensitiser	Negative	≤ 7	≤ 7
8	Isopropanol	67-63-0	Liquid	60.10	Non-sensitiser	Negative	≤ 7	≤ 7
9	Dimethyl isophthalate	1459-93-4	Solid	194.19	Non-sensitiser	Negative	≤ 7	≤ 7
10	Propyl paraben	94-13-3	Solid	180.20	Non-sensitiser	Negative	≤ 7	≤ 7

¹The *in vivo* hazard (and potency) predictions are based on LLNA data (20) (21) (22). The *in vivo* potency is derived using the criteria proposed by ECETOC (23).

² An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 3 and 5.

³ Ranges determined on the basis of at least 10 depletion values generated by 5 independent laboratories.

APPENDIX II, ANNEX 3

EXAMPLES OF ANALYSIS SEQUENCE

Each sample of HPLC analysis should be analysed in number order below. Refer to the table showing Examples of HPLC Sample Analysis Sequences for more practical sequences about HPLC analysis.

1. Start to analyse calibration standards and Reference Control A (N = 3).
2. The co-elution Control does not need to be analysed by turns if it is analysed after analysis of standard solution and Reference Control A.
3. Reference Control B should be analysed three times (total six times) before and after the analysis of sample, Reference Control C and Positive Control.
4. The Reference Control C, Positive Control and Test chemical solutions are analysed. (After the first set of replicates of each sample is analysed, the second set of replicates of each should be analysed).

Calibration standards and reference controls	STD1 STD2 STD3 STD4 STD5 STD6 Dilution buffer Reference control A, rep 1 Reference control A, rep 2 Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1 Reference control B, rep 2 Reference control B, rep 3
First set of replicates	Reference control C, rep 1 Positive control, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Positive control, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Positive control, rep 3 Sample 1, rep 3 Sample 2, rep 3

Reference controls	Reference control B, rep 4 Reference control B, rep 5 Reference control B, rep 6
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Three sets of reference controls (NAC or NAL dissolved in the appropriate solvent) should be included in the analysis sequence:

Reference control A: Control for verifying validity of the HPLC system. Reference Control A is used to verify concentration of NAC and NAL from each calibration curve after addition of acetonitrile rather than test chemical.

Reference control B: Control for verifying stability of reaction solution under analysis. Reference Control B is used to verify variability (CV) of each three NAC/NAL peak areas in the solution after addition of acetonitrile rather than test chemical at the start of analysis and at the end of analysis.

Reference control C: Control for calculating NAC/NAL depletion of each test chemical solution. To calculate depletion of NAC/NAL, measure three Reference Controls C after addition of solvent instead of test chemical. Prepare reference Control C for all solvents used to dissolve the test chemicals.

APPENDIX III

In Chemico Skin Sensitisation: kinetic Direct Peptide Reactivity Assay (kDPRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The kDPRA is proposed to address the molecular initiating event of the skin sensitisation AOP - namely, protein reactivity - by quantifying the reactivity of test chemicals towards a synthetic model peptide containing cysteine in a time- and concentration dependent manner (1) (2). Kinetic rate constants are calculated and the logarithm of the maximum rate constant ($\log k_{\max}$ value in $s^{-1}M^{-1}$) for a tested substance is then used to support the discrimination of UN GHS subcategory 1A skin sensitisers (subcategory 1A) from those not categorised as subcategory 1A (non-subcategory 1A) i.e., subcategory 1B or no category according to UN GHS (3). Based on theoretical consideration, the rate constant of the reaction between a test chemical and skin proteins will determine the amount of epitope formed from a given amount of chemical or, vice-versa, determine the dose needed to form the amount of epitope needed for induction of sensitization to occur and it is thus a rate limiting and potency determining step. Based on empirical evidence when evaluating 180 chemicals, the rate constant was shown to be the strongest determinant of potency among all evaluated parameters measured in OECD 442C, 442D and 442E (3).
2. The kDPRA proved to be transferable to laboratories without hands-on training (4). For the 24 test chemicals tested during the validation study, the overall within-laboratory reproducibility of kDPRA for assigning UN GHS subcategory 1A was 96% and the average between-laboratory reproducibility was 88% (4). Results from the validation study (4) as well as from other published studies (3) encompassing 180 test chemicals that fall within kDPRA's applicability domain indicate that kDPRA allows to discriminate UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) according to UN GHS with a balanced accuracy of 85%, a sensitivity of 84% (38/45), and a specificity of 86% (116/135) relative to LLNA results (3). Similar performances were obtained when comparing kDPRA outcomes with the OECD LLNA database compiled within the context of the Test Guideline on Defined

Approaches for Skin Sensitization (15)⁴. In addition, the prediction for 123 test chemicals (out of the 180) having human skin sensitisation data (5) (6) has a balanced accuracy of 76%, a sensitivity of 64% (21/33), and a specificity of 89% (80/90) (3), although the human reference data are subject to a significant uncertainty⁵. 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 the species of interest, which is humans. For comparison, based on a data set of 123 chemicals used to evaluate the kDPRA vs. human sensitising potential, the LLNA showed a 73% balanced accuracy, a 55% (18/33) sensitivity and a 91% (82/90) specificity for the identification of UN GHS subcategory 1A. On the basis of the overall data available, kDPRA's applicability domain was shown to include a variety of organic functional groups, reaction mechanisms, skin sensitisation potencies (as determined in *in vivo* studies), and physicochemical properties (3). Following an independent peer review (16), the kDPRA was considered to be scientifically valid to discriminate UN GHS subcategory 1A skin sensitisers from those not categorised as 1A (non-subcategory 1A) according to UN GHS (7). The kDPRA can therefore be used (i) as a follow-up test method for sub-categorisation of chemicals identified as UN GHS Category 1 skin sensitisers, or (ii) on its own by using positive results for direct classification of a chemical into UN GHS subcategory 1A, depending on the regulatory framework.

3. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the kDPRA to the testing of substances and/or mixtures. This test method is not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding. Furthermore, kDPRA only measures reactivity with the cysteine peptide, so that strong sensitisers having an exclusive lysine-reactivity, such as some acyl-halides, phenol-esters or aldehydes are outside of the applicability domain of kDPRA. However, only few UN GHS subcategory 1A skin sensitisers are known currently to react exclusively with lysine residues. In addition, considering exclusive strong Lysine-reactivity from the DPRA or ADRA in a tiered strategy may reduce this uncertainty. Test chemicals that do not covalently bind to the peptide but promote its oxidation (i.e. cysteine dimerisation) could lead to a potential over estimation of peptide depletion, resulting in possible false positive predictions and/or assignment to a higher reactivity class. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Reactivity of chemicals that require enzymatic bioactivation to

⁴ A balanced accuracy of 85%, a sensitivity of 82% (31/38), and a specificity of 88% (102/116) were found relative to LLNA dataset compiled within the context of the Test Guideline on Defined Approaches for Skin Sensitization (15).

⁵ A balanced accuracy of 67%, a sensitivity of 53% (9/17), and a specificity of 81% (25/31) were found relative to human skin sensitisation dataset compiled within the context of the Test Guideline on Defined Approaches for Skin Sensitization (15).

exert their skin sensitisation potential (i.e. pro-haptens) cannot be reliably detected by the test method. However, the limitation for detecting pro-haptens was found to be less pronounced when identifying strong sensitisers as compared to the identification of weak sensitisers (3). The majority of chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) were reported to be correctly detected by *in chemico* test methods (8) (9). However, spontaneously rapidly oxidizing pre-haptens may be under-predicted by kDPRA (as in any *in vitro* skin sensitisation assay) due to a lag-phase for oxidation which reduces the overall reaction rate. In the light of the above, results obtained with the test method that do not lead to subcategory 1A categorisation should be interpreted in the context of the currently known limitations (see also Annex 1 of this Appendix), i.e.:

- aromatic amines, catechols or hydroquinones may require further data to confirm their weak reactivity even under oxidizing conditions, and
 - acyl-halides, phenol-esters or aldehydes specifically reacting with Lysine-residue according to e.g. the DPRA or ADRA, may require further data to confirm their weak reactivity.
4. To be tested, a test chemical should be soluble in an appropriate solvent at a final concentration of 20 mM (see paragraphs 12-13). Test chemicals that are not soluble at this concentration may still be tested at lower concentrations as long as a k_{max} value (i.e., the maximum rate constant (in $s^{-1}M^{-1}$) determined from the reaction kinetics for a tested substance in the kDPRA (see paragraph 24)), can be derived. In such a case, a positive result leading to a UN GHS subcategory 1A skin sensitization prediction (i.e. $\log k_{max} \geq -2.0$) could still be used, but no firm conclusion should be drawn from a negative result (i.e., non-reactive or $\log k_{max} < -2.0$ outcome).
 5. The kDPRA uses a fluorescence readout which requires attention for potential test chemical autofluorescence, fluorescence quenching or interaction with the reagent (monobromobimane). In particular, it is important to include the respective test chemical controls as described in paragraph 16 and to assess the incubation time dependence of the determined peptide depletion. Furthermore, test chemicals with primary SH-group (thiols) cannot be tested with the kDPRA as the thiol group can interact with the monobromobimane (see paragraph 8) leading to enhanced fluorescence. Finally, chemicals decomposing under the conditions of the assay (neutral, aqueous conditions) and releasing a free SH-group will be prone to the same limitations.
 6. The kDPRA is considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition, although such substances were not tested during the validation studies. In this case, a single purity may be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight may be determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 20 mM solution. Results obtained with mixtures and multi-constituent substances of known composition

can lead to a non-linear behaviour, so that the provisions described in paragraph 27(ii) should be used. Regarding mixtures and substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances), the current model cannot be used due to the need for defined molar ratios. In any case, 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. Finally, in cases where evidence can be demonstrated on the non-applicability of the test method to specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

7. The kDPRA can be used for the discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory1A) according to UN GHS (3). As for any key-event based test method, the performance of kDPRA will have to be further assessed when used in combination with other assays such as DPRA or ADRA, and within integrated approaches such as IATA or DA for a more comprehensive analysis of skin sensitisation (3) (10).

PRINCIPLE OF THE TEST

8. The kDPRA is a modification of the *in chemico* test method DPRA (described in Appendix I of this Test Guideline). The kDPRA uses the cysteine peptide (Ac-RFAACAA-COOH) also used in the DPRA, while it does not use a lysine containing peptide. The final concentration of the test peptide (0.5 mM) and the reaction medium (25% acetonitrile in phosphate buffer) is identical in the kDPRA and in the DPRA. While the DPRA measures only at one concentration of the test chemical (5 mM for the cysteine peptide) and at one time point (≥ 24 h), the kDPRA performs parallel reactions at five concentrations (5, 2.5, 1.25, 0.625 and 0.3125 mM) and at six time-points (10, 30, 90, 150, 210 and 1440 min) at $25 \pm 2.5^\circ\text{C}$. Residual concentration of the cysteine peptide after the respective reaction time is measured after stopping the reaction by the addition of monobromobimane (mBrB; CAS 74235-78-2). The highly reactive and non-fluorescent mBrB rapidly reacts with unbound cysteine moieties of the model peptide to form a fluorescent complex which is measured in order to quantify the non-depleted peptide concentration. If the depletion of the highest concentration surpasses the threshold of 13.89% (cut-off used in the DPRA for positivity in the cysteine only prediction model) and this depletion is statistically significant vs. controls with peptide only, further calculations are performed (otherwise the test chemical is considered to be non-reactive according to the prediction model shown in paragraph 28). The natural logarithm of the non-depleted peptide concentrations is plotted vs. the concentration of the test chemical at each time point. If a linear relationship is observed (correlation coefficient > 0.90), the slope of this curve is determined and divided by the incubation time to calculate the rate constant in $[\text{min}^{-1}\text{mM}^{-1}]$. This value is transformed to the rate constant in $[\text{s}^{-1}\text{M}^{-1}]$ and the logarithm is calculated. The maximum value observed at any time point is taken as the $\log k_{\text{max}}$, and this maximum rate constant is the primary read-out of the test. It gives a quantification of the maximum kinetic rate of the reaction of the test chemical with the test peptide. Kinetic reaction rates of the cysteine peptide depletion are then used to discriminate UN GHS subcategory 1A skin sensitizers from those not categorised as 1A (non-subcategory 1A) according to UN GHS. Chemicals with a $\log k_{\text{max}} \geq -2.0$ are predicted as UN GHS subcategory 1A. The kinetic rate constant may be further used in integrated approaches such as IATA or DA to assess the skin sensitisation potency of a test chemical in a continuous scale as needed for risk assessment (3) (10).
9. Prior to routine use of this test method, laboratories should demonstrate technical proficiency, using the nine proficiency substances listed in Annex 2 of this Appendix.

PROCEDURE

10. This test method is based on the kDPRA DB-ALM protocol no 217 (11) which represents the protocol used for the industry-coordinated validation study. It is recommended that this protocol

is used when implementing and using the method in a laboratory. The main components and procedures for the KDPR are described below.

Preparation of the cysteine-peptide

11. The stock solution of the cysteine containing synthetic peptide (Ac-RFAACAA-COOH) of purity equal to or higher than 95% should be freshly prepared just before the incubation with the test chemical. The final concentration of the cysteine peptide should be 0.667 mM in pH 7.5 phosphate buffer for test chemical soluble in acetonitrile and 1.0 mM for chemicals soluble in pH 7.5 phosphate buffer.

Preparation of the test chemical

12. Solubility of the test chemical in an appropriate vehicle should be assessed before performing the assay. A non-reactive, water-miscible vehicle able to completely dissolve the test chemical should be used. Solubility is checked by visual inspection where the forming of a clear solution is considered sufficient to ascertain that the test chemical is dissolved. The preferred vehicle is acetonitrile. When a substance is not soluble in acetonitrile, solubilisation in pH 7.5 phosphate buffer should be assessed. Further vehicles have not been tested yet but may be used if it is demonstrated that the vehicle does not interfere with the assay, e.g. all controls should be prepared using the same vehicle, and the reaction rates obtained for the positive control and for the proficiency chemicals should fall within the ranges described in paragraph 26 and Annex 2 of this Appendix, respectively. It is important to note that use of DMSO as a vehicle should be avoided as it may lead to peptide dimerisation.
13. The test chemical should be pre-weighed into glass vials and dissolved immediately before testing to prepare a 20 mM solution using the appropriate vehicle as described in paragraph 12. Test chemical dilutions are prepared by serial dilution to obtain concentrations of 20, 10, 5, 2.5 and 1.25 mM.

Preparation of controls

14. Cinnamic aldehyde (CAS 104-55-2; ≥95% food-grade purity) should be used as positive control (PC). It is dissolved at a concentration of 20 mM in acetonitrile immediately before testing. Serial dilutions are then prepared to obtain PC concentrations of 20, 10, 5, 2.5 and 1.25 mM. Use of other positive controls is not recommended since in this assay an exact reaction rate is measured and consistent use of the positive control allows quantitative comparison between laboratories, with validation study data and as intra-laboratory historical control.
15. A vehicle control (VC), considered as the negative control, includes the peptide dissolved in buffer and vehicle respectively but no test chemical nor PC. The peptide-depletion of test chemical or PC incubated samples is calculated relative to the respective VC.

16. The assay also includes test chemical controls at the respective test chemical concentration in the vehicle and buffer but without peptide. This set of controls is used for the identification of interference of the test chemical with the fluorescence measurement (autofluorescence and quenching) to assess e.g., interference with monobromobimane and as a background measurement.
17. A blank control (BC) is used as a background measurement and is prepared with vehicle and buffer but without test chemical, PC, or peptide.

Incubation of the test chemical with the cysteine peptide solution

18. Serial dilutions of the test chemical and PC are prepared in a 96-well microtiter plate referred to as the application plate. Further, a 96-well black assay plate for each exposure time is prepared, referred to as the assay plates, by adding the relevant reagents (i.e., peptide stock solution, vehicle and buffer solution) according to a predefined plate layout such as recommended within the kDPRA protocol (11). Each test chemical concentration should be analysed in triplicate. The reaction is started by adding the test chemical and PC dilutions from the application plates to the assay plates. If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, one cannot be sure how much test chemical remained in the solution to react with the peptide. In such a case, a positive result (i.e. $\log k_{\max} \geq -2.0$) could still be used, but a negative result (i.e., non-reactive or $\log k_{\max} < -2.0$ outcome) should be interpreted with due care (see also provisions in paragraph 4 for the testing of chemicals not soluble up to a concentration of 20 mM in the kDPRA). After adding the test chemical and PC, plates are sealed with gas-tight adhesive foil and shaken at least 200 rpm for 5 min. Assay plates solution should be incubated in the dark at $25 \pm 2.5^\circ \text{C}$ for several incubation (exposure) times, i.e. 10, 30, 90, 150, 210, and 1440 min before addition of mBrB solution. Incubation times may be adapted to investigate the most relevant time points for a specific chemical (e.g., shorter incubation times might be more suitable for fast reacting chemicals). However, 1440 min should always be tested, as it corresponds to the incubation time of the DPRA. The incubation (exposure) time is the time interval from the application of the test chemical and PC dilutions to the assay plate until the addition of mBrB.

Fluorescence measurement

19. When the desired incubation (exposure) time is reached, freshly prepared mBrB solution (3 mM in acetonitrile) is added rapidly to the wells of the assay plates (one per exposure time) in the dark. Plates are sealed with gas-tight adhesive foil and shaken at least 200 rpm for 5 min. Fluorescence intensity is then determined using an excitation filter of 390 nm and an emission filter of 480 nm.

DATA AND REPORTING

Data evaluation

20. An automated Excel-evaluation spreadsheet is available with the DB-ALM protocol and should be used for data evaluation. Detailed instructions are provided in the DB-ALM protocol no. 217 (11).
21. For each incubation (exposure) time 't' the following parameters are calculated:
 - The arithmetic mean and standard deviation of the fluorescence intensity of the 12 blank controls (BC);
 - The arithmetic mean and standard deviation of the fluorescence intensity of the 12 vehicle controls (VC);
 - The mean BC value is subtracted from the VCs to obtain corrected VC values.
 - For each test chemical and PC concentration, the respective test chemical control value is subtracted from their obtained values to calculate corrected test chemical or PC values.
22. To determine the relative peptide depletion in % for each test chemical concentration per exposure time, the following calculation is performed:

$$\text{relative peptide depletion [\%]} = \left[1 - \left(\frac{\text{corrected test chemical or PC value}}{\text{mean of corrected VC}} \right) \right] \times 100\%$$

23. For each test chemical concentration, the arithmetic mean and standard deviation of the three replicates is calculated (per exposure time). A student's t-test is performed to test whether the peptide concentrations measured in the three replicates is statistically significantly lower as compared to the concentration in the 12 VC wells.
24. In the kDPRA, reaction kinetic rate constants are determined as explained below if (i) a peptide depletion of $\geq 13.89\%$ is observed at the highest test chemical concentration (final test chemical concentration 5 mM) at a given time and if (ii) the difference is statistically different from the VC. This 'positivity criterion' is based on the 'positive' criterion for peptide reactivity in the cysteine only prediction model of the DPRA described in Appendix I of this test guideline. If the positive criterion is not met, the test chemical is considered to be non-reactive according to the prediction model shown in paragraph 28.

The natural logarithm of the non-depleted peptide concentrations (100-relative peptide depletion (%)) is plotted vs. the concentration of the test chemical at each time point. If a linear relationship is observed (correlation coefficient > 0.90), the slope of this curve is determined. The absolute value of this negative slope corresponds to the observed reaction kinetic constant (pseudo first order rate constants k_{observed} in mM^{-1}). From the k_{observed} value for each exposure time, the

reaction kinetic constant (k_t) per concentration and incubation (exposure) time 't' is calculated as follows:

$$k_t [M^{-1}s^{-1}] = k_{observed} \cdot \frac{1000}{60 \cdot t}$$

with 't' being the exposure time in minutes. If no linear relationship is observed (i.e., correlation coefficient < 0.90), the recommendations within paragraph 27.ii should be followed.

25. For each exposure time 't' with a correlation > 0.90, the decimal logarithm ($\log k_t$) is calculated and the highest value is determined as $\log k_{max}$.

Acceptance criteria

26. The following criteria should be met for a run to be considered valid. If one or more of these criteria is not met the run should be repeated.
- PC: the $\log k$ of the PC at 90 min ($\log k_{90 \text{ min}}$) should be within the following range: -1.75 to -1.40 $M^{-1}s^{-1}$. If no $\log k_{90 \text{ min}}$ is obtained in case of e.g., reactivity is not yet statistically significant, the value at 150 min ($\log k_{150 \text{ min}}$) can be taken into account and should lie in the following range: -1.90 to -1.45 $M^{-1}s^{-1}$.
 - VC: The coefficient of variance of the 12 VC values of a plate should be < 12.5% for at least 5 of the 6 exposure times.
27. The data obtained for the test chemical are further assessed to check for possible conditions which may affect results:
- Interrupted time-course: If significant peptide depletion is observed at early time-points but not at following time points, there is either an intrinsic non-linear reaction for the test chemical or an experimental variation. In such cases the run is repeated. If the same pattern is reproducible, a non-linear kinetic is proven and the rate-constant observed at early time points is accepted.
 - Non-linear concentration-response: There are few cases where the concentration-response is not linear, but clear depletion is noted. In such cases no rate constant is calculated by the slope method, as regression coefficient is $R^2 < 0.90$. Alternatively, rate constants can also be calculated based on individual depletion values according to the formula:

$$k = [\ln (100/(100 - dp))]/(E \times t)$$

Where 'dp' is depletion in %, 'E' is the concentration of test chemical and 't' is the incubation (exposure) time. Rate constants according to this formula are calculated at each time point 't' and at each concentration 'E' with depletion values above the threshold of 13.89%. For

each time point 't' the average of the values for the different concentrations is taken, and then again the log k_{\max} for the highest rate at any given time point is reported.

In such a case a repetition should be performed to check whether this non-linear behaviour is intrinsic to the test chemical, or whether an experimental variation is the cause. If the non-linearity is reproducible, this alternative rate calculation based on the individual depletion values is used for the final rating.

- (iii) Fluorescence interference, namely autofluorescence or fluorescence quenching: Based on the control wells with test chemical only in absence of the test peptide, incidences of autofluorescence and fluorescence quenching by the test chemical can be detected. As the values are corrected for the autofluorescence recorded in the test chemical control wells, this shall not be a problem for low autofluorescence, but with a high autofluorescence, the fluorescence of the peptide-adduct and the autofluorescence may not be fully additive, and subtraction of autofluorescence may lead to apparent depletion, which is not due to loss of peptide signal but to this non-additivity. Thus, one should check whether the observed depletion is time dependent. If this is not the case and autofluorescence is observed, then depletion from autofluorescence is assumed to occur. Fluorescence quenching can also lead to 'pseudo-depletion', but this would happen immediately and resulting depletion would not increase with time. If both conditions are met, it is assumed that depletion from quenching occurs. These cases are rare. If this is not clear from the results a run may be repeated, but if the effect is clear-cut no repetition is needed. In such a case, the test chemical cannot be assessed in the kDPRA (technical limitation) unless the reaction can be measured with an alternative fluorescent probe not leading to autofluorescence or quenching (see Section II of the Annex 1 to DB-ALM protocol (11)).
- (iv) All above cases are detailed in the DB-ALM protocol and automatic alerts appear in the Excel template provided with the DB-ALM protocol when evaluating the data.

Prediction model

28. The kDPRA uses kinetic rates of cysteine peptide depletion for discrimination of UN GHS subcategory 1A skin sensitisers from those not categorised as subcategory 1A (non-subcategory 1A) according to UN GHS (3). Results obtained with the test method that do not lead to subcategory 1A categorisation should be interpreted in the context of the limitations stated in paragraph 3 and Annex 1 of this appendix.

Table 1: kDPRA prediction model

Reaction rate	kDPRA Prediction
$\log k_{\max} \geq -2.0$	UN GHS subcategory 1A
Non-reactive or $\log k_{\max} < -2.0$	Not categorised as UN GHS subcategory 1A* (non-subcategory 1A)

* Further information is needed to discriminate UN GHS subcategory 1B from UN GHS No Category. Depending on the context (e.g. IATA, DA) this information can be generated prior to or after performing the kDPRA.

29. In cases of a $\log k_{\max}$ result close to the -2.0 threshold falling in the borderline range calculated for kDPRA (i.e., between -1.93 and -2.06 (12)), no conclusive prediction can be made. In this case, re-testing and/or additional data/information is needed before a conclusive prediction can be made.
30. The kinetic rate constant may be further used in integrated approaches such as IATA or DA to assess the skin sensitisation potency of a test chemical in a continuous scale as needed for risk assessment (3) (10).

Test report

31. The test report should include the following information

Test chemical and Controls (positive control and solvent/vehicle)

For all mono-constituent substance (test and control chemicals)

Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;

Physicochemical properties such as physical state, appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;

Purity, chemical identity of impurities as appropriate and practically feasible, etc;

Treatment prior to testing, if applicable (e.g. warming, grinding);

Concentration(s) tested;

Storage conditions and stability to the extent available.

Additional information for positive control

Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

Additional information for solvent/vehicle control

Solvent/vehicle used and ratio of its constituents, if applicable;

Justification for choice of other solvent than acetonitrile and experimental assessment of the solvent effect on peptide stability.

Peptide

Supplier, lot, purity

Fluorescence analysis

Fluorimeter used (e.g., model and type), including wavelengths settings

Proficiency testing

Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals.

Discussion of the results

Description of any unintended modifications to the test procedure.

Discussion of the results obtained with the kDPRA test method and if it is within the ranges described in paragraph 29.

Description of any relevant observations made, such as appearance of precipitate in the reaction mixture at the end of the incubation time, if precipitate was resolubilised or centrifuged.

Conclusion

LITERATURE FOR APPENDIX III

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APPENDIX III, ANNEX 1

KNOWN LIMITATIONS OF THE KINETIC DIRECT PEPTIDE REACTIVITY ASSAY

The table below provides a summary of the known limitations of the kDPRA.

Substance class / interference	Reason for potential underprediction or interference	Data interpretation	Example substance
Metals and inorganic compounds	Known to react with proteins via mechanisms other than covalent binding	Should not be tested	Nickel sulphate; 7786-81-4
Hydroquinones, catechols and aromatic amines	Lag time of oxidation may reduce apparent reaction rate	Results with $\log k_{\max} < -2.0$ can only be accepted if low reactivity can be confirmed after oxidation	Para-phenylenediamine; 106-50-3; Human and LLNA 1A
Thiols or thiol-releasers	Test chemicals with primary SH-groups and those decomposing under the conditions of the assay can react with the detection probe	Test chemical cannot be tested in the kDPRA with derivatisation by thiol reactive probes: other kinetic data with the test peptide e.g. by HPLC may need to be generated (not part of this guideline)	Thioglycerol; 96-27-5; LLNA UN GHS category 1B; Human n/a
Test chemicals having an exclusive lysine-reactivity as observed in DPRA or ADRA	kDPRA only measures reactivity with the cysteine peptide	Results with $\log k_{\max} < -2.0$ for chemicals which specifically deplete NH_2 -groups, but not SH-groups in DPRA or ADRA are not conclusive	Some acyl-halides, phenol-esters or aldehydes, Dihydrocoumarin, 119-84-6; LLNA UN GHS category 1B; Human n/a, Glutaric aldehyde; 111-30-8; Human and LLNA UN GHS category 1A
Pro-haptens	Test chemicals for which there is evidence that they strictly require enzymatic bioactivation to exert their skin sensitizing potential	Strict pro-haptens may be underestimated. However chemicals which are i) strict pro-haptens (i.e. test chemicals not also acting as direct haptens or pre-haptens, too) and ii) strong allergens were found to be rare	Diethylenetriamine; 111-40-0 (human 1A, LLNA UN GHS category 1)
Fluorescent chemicals with excitation in the range of the fluorescent probe	If fluorescence of test chemicals and of the mBrB-peptide adduct is not additive, pseudo-depletion is observed	Follow the considerations in the DB-ALM Protocol n° 217 to evaluate assay interference	Tetrachlorosalicylanilide; 1154-59-; Human and LLNA UN GHS category 1A
Test chemicals absorbing in	If test chemical quenches	Follow the considerations in the DB-ALM Protocol n° 217 to	Vanillin, 121-33-5;

the emission range of the probe	fluorescence emission of the mBrB-peptide adduct, pseudo-depletion is observed	evaluate assay interference	LLNA NC; Human n/a
Mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials	no information on applicability of kDPRA is available in the published literature		n/a UVCBs, chemical emissions, products or formulations with variable or not fully known composition
Test chemicals which cannot be dissolved in water or acetonitrile or a compatible water-miscible solvent	Not sure if sufficient exposure can be achieved	In such cases, a log $k_{max} > -2.0$ could still be used to support the identification of the test chemical as a UN GHS subcategory 1A skin sensitiser but no firm conclusion should be drawn in case log k_{max} is < -2.0 . Alternative vehicle may be used according to the prescriptions given in paragraph 12.	n/a
Test chemicals which precipitate in reaction solution	Not sure if sufficient exposure can be achieved: If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, one cannot be sure how much test chemical remained in the solution to react with the peptide.	In such a case, a positive result (i.e. log $k_{max} \geq -2.0$) could still be used, but a negative result (i.e., non-reactive or log $k_{max} < -2.0$ outcome) should be interpreted with due care (see also provisions in paragraph 4 for the testing of chemicals not soluble up to a concentration of 20 mM in the kDPRA).	Methyl-2-nonynoate ⁶ ; 111-80-8; LLNA NC
Test chemicals promoting cysteine-peptide oxidation		May lead to a potential over estimation of peptide reactivity.	DMSO

⁶ Roberts, D.W. and A. Natsch, *High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitization potency of michael acceptor electrophiles*. Chem. Res. Toxicol., 2009. **22**(3): p. 592-603

APPENDIX III, ANNEX 2

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: kinetic Direct Peptide Reactivity Assay (kDPRA)

Prior to routine use of the test method described in this appendix, laboratories should demonstrate technical proficiency by correctly obtaining the expected kDPRA prediction for at least 8 of the 9 proficiency substances recommended in Table 1 and by obtaining cysteine rate constants $\log k_{\max}$ that fall within the respective reference range for 7 out of the 9 proficiency substances. These proficiency substances were selected to represent the range of responses for skin sensitisation hazard and potency. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the kDPRA are available, and that they were used in the industry-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Proficiency substances	CASRN	Physical state	<i>In vivo</i> prediction ¹	UN GHS Category LLNA	UN GHS Category human	kDPRA prediction ²	Range of $\log k_{\max}$ ²
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	1A	1A	1A	(-0.8) – (-0.4)
Methylisothiazolinone	2682-20-4	Solid	Sensitiser (extreme)	1A	1A	1A	(-0.5) – (-0.1)
Oxazolone	15646-46-5	Solid	Sensitiser (extreme)	1A	No data	1A	(-0.3) – (0.0)
Methyl-2-octynoate	111-12-6	Liquid	Sensitiser (strong)	1A	1A	1A	(-1.6) – (-1.2)
Isoeugenol	97-54-1	Liquid	Sensitiser (moderate)	1A	1A	1A	(-1.4) – (-1.1)
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	1B	No data	non-1A (1B or NC)	(-3.2) – (-2.1)
Ethylene glycol dimethacrylate (EGDMA)	97-90-5	Liquid	Sensitiser (weak)	1B	1B	non-1A (1B or NC)	(-2.8) – (-2.1)
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	No Cat. ³	No Cat. ³	non-1A (1B or NC)	Not reactive
Chlorobenzene	108-90-7	Liquid	Non-sensitiser	No Cat. ³	No Cat. ³	non-1A (1B or NC)	Not reactive

Table 1: Recommended proficiency substances for demonstrating technical proficiency with the kinetic Direct Peptide Reactivity Assay

¹The *in vivo* hazard and (potency) predictions are based on LLNA data (13). The *in vivo* potency is derived using the criteria proposed by ECETOC (14).

² Rounded ranges determined on the basis of at least 14 log k_{max} determinations generated by 7 independent laboratories.

³ Non sensitisers according to the UN GHS.