

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

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

INTRODUCTION

1. A skin sensitizer refers to a substance that will lead to an allergic response following skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). This Test Guideline (TG) provides an *in chemico* procedure (Direct Peptide Reactivity Assay – DPRA) to be used for supporting the discrimination between skin sensitizers and non-sensitizers in accordance with the UN GHS (1).

2. There is general agreement regarding the key biological events underlying skin sensitization. The existing knowledge of the chemical and biological mechanisms associated with skin sensitization has been summarised in the form of an Adverse Outcome Pathway (AOP) (2), from the molecular initiating event through the intermediate events to the adverse effect namely allergic contact dermatitis in humans or contact hypersensitivity in rodents. Within the skin sensitization AOP, the molecular initiating event is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins.

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

4. More recently mechanistically based *in chemico* and *in vitro* test methods have been considered scientifically valid for the evaluation of the skin sensitization hazard of chemicals. However, combinations of non-animal methods (*in silico*, *in chemico*, *in vitro*) within Integrated Approaches to Testing and Assessment (IATA) will be needed to be able to fully substitute for the animal tests currently in use given the restricted AOP mechanistic coverage of each of the currently available non-animal test methods (2) (7).

5. The DPRA is proposed to address the molecular initiating event of the skin sensitization AOP, namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine (8). 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 sensitizers and non-sensitizers (9).

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6. 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) and was considered scientifically valid (10) to be used as part of an IATA to support the discrimination between skin sensitisers and non-sensitisers for the purpose of hazard classification and labelling. Examples on the use of DPRA data in combination with other information are reported in the literature (11) (12) (13) (14).

7. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

8. The correlation of protein reactivity with skin sensitisation potential is well established (15) (16) (17). Nevertheless, since protein binding represents only one key event, albeit the molecular initiating event of the skin sensitisation AOP, protein reactivity information generated with testing and non-testing methods may not be sufficient on its own to conclude on the absence of skin sensitisation potential of chemicals. Therefore, data generated with this Test Guideline should be considered in the context of integrated approaches such as IATA, combining them with other complementary information e.g., derived from *in vitro* assays addressing other key events of the skin sensitisation AOP as well as non-testing methods including read-across from chemical analogues.

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

10. 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 (10). Results generated in the validation study (18) and published studies (19) overall indicate that the accuracy of the DPRA in discriminating sensitisers (i.e. UN GHS Cat. 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) (18) (19). 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 and in accordance with the provisions of paragraph 9 above. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA 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 (8) (9) (10) (19). Taken together, this information indicates the usefulness of the DPRA to contribute to the identification of skin sensitisation hazard.

11. 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. This Test Guideline is

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.

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 paragraph 18). 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 sensitiser 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 (18) (19). The DPRA is nevertheless considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition (see paragraph 18). Before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for testing of the mixture. The current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances) due to the defined molar ratio of test chemical and peptide. For this purpose a new prediction model based on a gravimetric approach will need to be developed. In cases where evidence can be demonstrated on the non-applicability of the Test Guideline to other specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

12. The test method described in this 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 (18). 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 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 29 and 30).

13. As described, the DPRA assay supports the discrimination between skin sensitisers and non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency (11) when used in integrated approaches such as IATA. However further work, preferably based on human data, is required to determine how DPRA results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

14. 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 $25\pm 2.5^\circ\text{C}$. 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 29) which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitisers and non-sensitisers.

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

PROCEDURE

16. This Test Guideline is based on the DPRA DB-ALM protocol n° 154 (20) 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

17. Stock solutions of cysteine (Ac-RFAACAA-COOH) and lysine (Ac-RFAAKAA-COOH) containing synthetic peptides of purity higher than 85% and preferably in the range of 90-95%, 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 Guideline, 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

18. 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 (20). 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 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). As a last option if the test chemical is not soluble in any of these solvents attempts should be made to solubilise it in 300 µL of DMSO and dilute the resulting solution with 2700 µL of acetonitrile and 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. For mixtures and multi-constituent substances of known composition, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent 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 apparent 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. However, when testing mixtures, multi-constituent substances or polymers of known composition, it should be considered to also test the neat chemical. For liquids, the neat chemical should be tested as such without any prior dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. For solids, the test chemical should be dissolved to its maximum soluble concentration in the same solvent used to prepare the apparent 100 mM solution. It should then be tested as such without any further dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. Concordant results (reactive or non-reactive) between the apparent 100 mM solution and the neat chemical should allow for a firm conclusion on the result.

Preparation of the positive control, reference controls and coelution controls

19. Cinnamic aldehyde (CAS 104-55-2; ≥95% food-grade purity) should be used as positive control (PC) at a concentration of 100 mM in acetonitrile. Other suitable positive controls preferentially 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 26). 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

20. 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, in this case 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 11 for the testing of chemicals not soluble up to a concentration of 100 mM). The reaction solution should be left in the dark at 25±2.5°C for 24±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

21. 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

22. 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 µL). 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% < mean² area ratio of control samples < 100% would give a good indication that co-elution has not occurred.

23. 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 to false positive predictions and/or assignment to a higher reactivity class (see paragraphs 29 and 30).

24. HPLC analysis for the cysteine and lysine peptides can be performed concurrently (if two HPLC systems are available) or on separate days. If analysis is conducted on separate days then all test chemical solutions should be freshly prepared for both assays on each day. The 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 Guideline, up to 26 analysis samples can be accommodated in a single HPLC run (see also paragraph 17). An example of HPLC analysis sequence is provided in Annex 3.

DATA AND REPORTING

Data evaluation

25. 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.

26. 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.

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

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

Acceptance criteria

27. 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 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.

28. 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

29. 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 [Table1](#), the threshold of 6.38% average peptide depletion should be used to support the discrimination between skin sensitizers and non-sensitizers in the framework of an IATA. 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.

Table1: Cysteine 1:10/lysine 1:50 prediction model¹

Mean of cysteine and lysine % depletion	Reactivity Class	DPPRA 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 A DPPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 12.

30. 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 then the analysis should be reported as

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“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.

Table2: Cysteine 1:10 prediction model¹

Cysteine (Cys) % depletion	Reactivity class	DPRAs 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 DPRAs prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 12.

31. 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.

32. 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. borderline results), additional testing may be necessary. If situations where the mean percent depletion falls in the range of 3% to 10% for the cysteine 1:10/lysine 1:50 prediction model or the cysteine percent depletion falls in the range of 9% to 17% for the cysteine 1:10 prediction model, a second run should be considered, as well as a third one in case of discordant results between the first two runs.

Test report

33. The test report should include the following information

Test chemical

- Mono-constituent substance
 - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
 - Physical appearance, water solubility, 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.

Controls

- Positive control
 - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
 - Physical appearance, water solubility, 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;
 - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.
- Solvent/vehicle
 - Solvent/vehicle used and ratio of its constituents, if applicable;
 - Chemical identification(s), such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;
 - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
 - Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other solvents / vehicles than those mentioned in the Test Guideline are used and to the extent available;
 - Storage conditions and stability to the extent available;
 - Justification for choice of solvent for each test chemical;
 - For acetonitrile, results of test of impact on peptide stability.

Preparation of peptides, positive control and test chemical

- Characterisation of peptide solutions (supplier, lot, exact weight of peptide, volume added for the stock solution);
- Characterisation of positive control solution (exact weight of positive control substance, volume added for the test solution);

- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test solution).

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 replicate, SD and CV;
- Mean of percent cysteine and percent lysine depletion values;
- Prediction model used and DPRA prediction.

Proficiency testing

- If applicable, the procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or to demonstrate reproducible performance of the test method over time.

Discussion of the results

- Discussion of the results obtained with the DPRA test method;
- Discussion of the test method results in the context of an IATA if other relevant information is available.

Conclusion

LITERATURE

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ANNEX 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of “relevance.” The term is often used interchangeably with “concordance”, to mean the proportion of correct outcomes of a test method (21).

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).

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.

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

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

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

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

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

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.

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 (21).

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 (21).

Reproducibility: The agreement among results obtained from testing the same substance using the same test protocol (see reliability) (21).

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 (21).

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 (21).

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (1).

System suitability: Determination of instrument performance (e.g., sensitivity) by analysis of a reference standard prior to running the analytical batch (22).

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

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

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

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (21).

PROFICIENCY SUBSTANCES

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay

Prior to routine use of the test method described in this Test Guideline, 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	0-24
Benzylideneacetone	122-57-6	Solid	Sensitiser (moderate)	Positive	80-100	0-7
Farnesal	19317-11-4	Liquid	Sensitiser (weak)	Positive	15-55	0-25
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	Positive	60-100	10-45
1-Butanol	71-36-3	Liquid	Non-sensitiser	Negative	0-7	0-5.5
6-Methylcoumarin	92-48-8	Solid	Non-sensitiser	Negative	0-7	0-5.5
Lactic Acid	50-21-5	Liquid	Non-sensitiser	Negative	0-7	0-5.5
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	Negative	0-7	0-5.5

¹The *in vivo* hazard and (potency) predictions are based on LLNA data (19). The *in vivo* potency is derived using the criteria proposed by ECETOC (23).

² A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 11.

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

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.

