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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW TEST GUIDELINE

In Vitro Skin Sensitization: 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 (UN) Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (GHS)] (1). This Test Guideline (TG) provides an *in chemico* procedure (Direct Peptide Reactivity Assay – DPRA) to be used for supporting the identification of the skin sensitization hazard of chemicals.

2. There is a 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 postulated to be the covalent binding of electrophilic chemicals to nucleophilic centres in skin proteins.

3. So far the assessment of skin sensitization has typically involved the use of laboratory animals. The classical methods based on guinea-pig, 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 and are in use since decades. More recently, a murine test, the Local Lymph Node Assay (LLNA) - TG 429 (4) and the two non-radioactive modifications, LLNA: DA -TG 442 A (5) and LLNA: BrdU-ELISA - TG 442 B (6) all measuring the induction response, have gained acceptance since they provide an advantage over the guinea pig tests in terms of animal welfare and scientific benefits.

4. More recently *in chemico* and *in vitro* test methods have been considered scientifically valid for the evaluation of the skin sensitization hazard potential of chemicals. Given the complexity of the biological mechanisms underlying skin sensitization and the limitations of the currently available non-animal test methods (*in silico*, *in chemico*, *in vitro*), it is likely that combinations of mechanistically-based test methods within Integrated Approaches to Testing and Assessment (IATA) are needed to be able to substitute the regulatory animal tests currently in use (2)(7).

5. The DPRA is proposed to address the molecular initiating event of the skin sensitization AOP by quantifying the reactivity of 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 allowing discriminating between skin sensitising and non-sensitising chemicals (9).

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 (EASC) and was considered scientifically valid (10) to be used as part of IATA to support the identification of the skin sensitization potential of chemicals 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 sensitization potential is well established (15)(16)(17). However, since protein binding represents only one single biological mechanism of the skin sensitization AOP, information from test methods based on peptide reactivity such as the DPRA is unlikely to be sufficient to conclude on the skin sensitization potential of chemicals. Therefore, data generated with the present Test Guideline should be considered in the context of integrated approaches such as IATA, combining them with complementary information derived from *in vitro* assays addressing other key events of skin sensitization (e.g. in *vitro assay* measuring the activation of the Keap1-Nrf2-ARE pathway such as the KeratinoSens^{TM} assay) as well as non-testing methods including read-across from chemical analogues.

9. The test method described in this Test Guideline allows supporting the identification of skin sensitising and non-sensitising chemicals. The test method proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis and reproducible within-and between laboratories (10). Results generated in the validation study (18) and published studies (19) overall indicate that the accuracy of the DPRA in discriminating between sensitisers and non-sensitisers is 80% (N=157) with a sensitivity of 80% (88/109) and specificity of 77% (37/48) when compared to LLNA data. On the basis of the overall data available, the DPRA was shown to be applicable to a broad range of chemicals covering relevant ranges of organic functional groups (Annex 2), reaction mechanisms, skin sensitization potency (as determined in *in vivo* studies) and physico-chemical properties. Taken together, this information indicates the usefulness of the DPRA to contribute to the identification of skin sensitization hazard.

10. This Test Guideline is not applicable for the testing of metal compounds since they are known to react with proteins with different mechanisms than covalent binding by organic chemicals. 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. Limited information is currently available on the applicability of the DPRA to simple mixtures of known composition (18)(19). The current prediction model cannot be used for complex mixtures due to the defined molar ratios of test chemical and peptide.

11. The test method described in this Test Guideline is an *in chemico* method that does not encopass a metabolic system. Chemicals that require to be enzymatically bioactivated to exert their skin sensitization 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 cautiously due to potential under-prediction. Potential over-predictions may be due to chemicals that do not covalently bind to peptide but do promote its oxidation (i.e. Cysteine dimerisation).

12. In addition to supporting the discrimination between skin sensitisers and non-sensitisers the DPRA, when used in integrated approaches such as IATA, may also be able to contribute to the assessment of sensitising potency (11), however further work, based on preferentially human data, is required to determine to which extent DPRA results relate to potency categories.

PRINCIPLE OF THE TEST

13. 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 room temperature. The peptide is a custom material containing 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 27) which allows assigning the substance to one of four reactivity classes used to discriminate between sensitisers and non-sensitisers.

14. Prior to routine use of the method described in this Test Guideline, laboratories should demonstrate technical proficiency, using the Proficiency Chemicals listed in Annex 2. A laboratory can use these chemicals to demonstrate their technical competence in performing the DPRA prior to submitting results for regulatory purposes.

PROCEDURE

15. This Test Guideline is based on the DPRA DB-ALM protocol n. 154 (20) which represents the protocol used for EURL ECVAM-coordinated validation study. It is recommended that this protocol il consulted when implementing and using the method in the laboratory. The following is a description of the main components and procedures for the DPRA.

Preparation of the Cysteine or Lysine-containing peptides

16. Stock solutions of Cysteine (Ac-RFAA**C**AA-COOH) and Lysine (Ac-RFAA**K**AA-COOH) containing synthetic peptides should be prepared fresh immediately before use. 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 can accommodate from 1 to 26 test chemicals in addition to the positive control and the reference controls for each solvent used to solubilise the test chemicals, each tested in triplicate. All of the replicates analysed in the same run should use the identical Cysteine and Lysine peptide stock solutions.

Preparation of the Test Chemicals

17. Solubility of the test chemicals in a suitable solvent should be assessed before performing the assay. An appropriate solvent will dissolve the test chemical completely. Suitable solvents are, in order of preference, acetonitrile, water, 1:1 mixture water:acetonitrile, isopropanol, acetone or 1:1 mixture acetone:acetonitrile, 1: 11 mixture DMSO:acetonitrile and 1:1 mixture DMSO:acetonitrile. Test chemicals should be pre-weighted into clean, dry 4 mL glass vials and dissolved immediately before testing in 3 mL of the appropriate solvent to prepare a 100 mM solution.

Preparation of the Positive Control, Reference Controls and Coelution Controls

18. Cinnamic aldehyde (CAS 104-55-2) should be used as positive control (PC) at a concentration of 100 mM in acetonitrile. In addition Reference Controls (i.e. samples constituted only by 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, the stability of the reference controls over time and to verify that the solvent used to dissolve the test chemical does not impact the Percent Peptide Depletion. The appropriate Reference Control for each substance is used to calculate the Percent Peptide Depletion for that substance (see paragraph 24). In addition a Coelution Control constituted by the test chemical alone for each test chemical analysed should be included in the run sequence to detect possible co-elution of the test chemicals with either the Lysine or the Cysteine peptide.

Incubation of the Test Chemicals with the Cysteine and Lysine peptide solutions

19. Cysteine and Lysine peptide solutions should be incubated in glass autosampler vials with the test chemical at 1:10 and 1:50 ratio respectively. The mixture should be left in the dark at room temperature 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 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.

Preparation of the HPLC Standard Calibration Curve

20. A standard calibration curve should be generated for both the Cysteine and the Lysine peptides. Cysteine peptide standards should be prepared in a solution of 20% 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

21. 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 (Preferred Column: Zorbax SB-C-18 2.1 mm x 100 mm x 3.5 micron). The entire system is 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 is performed using a flow 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. The injection volume may vary according to the system used (typically in the range from 3-10 µL). 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.

22. 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 made fresh for both assays on each day. The analysis should be timed to assure that the injection of the first sample starts 24±2 hours after the test chemical was mixed with the peptide solution. Samples are analysed in triplicate in batches of up to 26 test chemicals (including controls) to keep the HPLC analysis time less than 30 hours. An example of HPLC analysis sequence is provided in Annex 4.

DATA AND REPORTING

Data Evaluation

23. The concentration of Cysteine or Lysine peptide is determined in each sample from absorbance at 220 nm, by measuring the peak area of the appropriate peaks by peak integration and by calculating the concentration of peptide using the linear calibration curve derived from the standards.

24. 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 (i.e. peptide alone dissolved in the appropriate solvent) 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

25. The following criteria must 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% 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 c) the mean peptide concentration of Reference Controls A should be 0.50±0.05 mM and the Coefficient of Variability (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

26. The following criteria must be met for a test chemicals'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 run should be repeated for that specific test chemical.

Prediction Model

27. 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. A reactivity category is assigned to each test chemical by using the Cysteine 1:10/Lysine 1:50 prediction model shown in Table1.

Table1: Cysteine 1:10/Lysine 1:50 Prediction Model

Mean of Cysteine and Lysine % depletion	Reactivity Class	Prediction
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitiser
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitiser
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitiser
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitiser

28. There might be cases where the test chemical and the peptide have the same retention time (co-elution). In such cases the peak of the peptide cannot be integrated and the calculation of the Percent Peptide Depletion is not possible. If co-elution of the test chemical occurs with both the Cysteine and the Lysine peptides then the analysis must be reported as "inconclusive" for that test chemical. In cases where co-elution occurs only with the Lysine peptide, than the Cysteine 1:10 prediction model reported in Table 2 can be used for the prediction.

Table2: Cysteine 1:10 Prediction Model

Cysteine (Cys) % depletion	Reactivity Class	Prediction
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitiser
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitiser
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitiser
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitiser

29. 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.

Test report

30. The test report should include the following information

Test and control chemicals

- Chemical(s) name, CAS number, molecular weight, purity
- Positive control name, CAS number, molecular weight, purity
- Physiochemical properties relevant for the conduct of the study
- Storage conditions

Solvent/vehicle

- Characterisation (nature, supplier and lot). For acetonitrile, results of test of impact on peptide stability.
- Justification for choice of solvent for each test chemical

Preparation of peptides, positive control and test chemicals

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

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 an 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 precipitate was re-solubilised or centrifuged.
    - Presence of co-elution
    - 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 ad Percent Lysine Depletion values
    - Reactivity class

Discussion of the results

Conclusion

LITERATURE

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ANNEX I

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*).

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 Approaches to Testing and Assessment): Approaches that integrate existing knowledge bases on classes of chemicals with the results of biochemical and cellular assays, computational predictive methods, exposure studies, and other sources of information to identify requirements for targeted testing or develop assessment conclusions. In some cases, the application of IATA could lead to the refinement, reduction, and/or replacement of selected conventional tests (e.g., animal toxicity tests). IATA also have the potential to further enhance the understanding of mode/mechanism of action including the consideration of relevant adverse outcome pathways (AOPs) that provide biological linkages between molecular initiating events to adverse outcomes in individual organisms and populations that are the bases for risk assessments (22).

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).

Positive control: A replicate containing all components of a test system and treated with a chemical known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the severe 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 (20).

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 (23).

Test chemical: Chemical (substance or mixture) assessed in the test method.

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 standardized 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).

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).

Validated test method: A test method for which validation studies have been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose (21).

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ANNEX 2

ORGANIC FUNCTIONAL GROUPS

In Vitro Skin Sensitization: Direct Peptide Reactivity Assay

Organic functional groups (nested) determined using the OECD QSAR Toolbox (version 3.1.0.21) of n=157 chemicals tested in the DPRA (18)(19). In addition to the 157 chemicals listed, 2 metals have been tested (18) despite these are known to fall outside the applicability domain of the DPRA and for which no organic functional groups can be determined.

Organic functional groups	n.	Organic functional groups	n.
Acetoxy	2	Fused carbocyclic aromatic	3
Acid anhydride	1	Fused saturated heterocycles	2
Acrylate	6	Glycerol and derivatives	1
Alcohol	12	Heterocyclic spiro rings	1
Aldehyde	14	Hydantoin	1
Aliphatic Amine, primary	3	Isobenzofuran	2
Aliphatic Amine, tertiary	2	Isobenzofurandione	2
Alkane, branched with tertiary carbon	3	Isopropyl	4
Alkene	5	Isothiocyanate	1
Alkenyl halide	2	Ketimine	1
Alkoxy	5	Ketone	4
Alkyl halide	7	Lactone	5
Alkyne	1	Maleate/ Fumarate	1
Allyl	21	Methacrylate	3
Alpha,beta unsaturated aldehyde	11	Naphtalene	1
Aminoaniline, para	3	Nitrile	3
Aniline	11	Nitrobenzene	5
Aromatic amine	3	Nitroso	1
Aromatic heterocyclic halide	7	No functional group found	3
Aromatic perhalogencarbons	1	Overlapping groups	104
Aryl	25	Oxocarboxylic acid	1
Aryl halide	7	Oxolane	1
Azomethine	1	Phenol	15
Benzamide	1	Phenothiazine	1
Benzothiazole/ Benzoisothiazole	2	Precursors quinoid compounds	13
Benzothiazolinone/ Benzoisothiazolinone	1	Pyridine	1
Benzyl	11	Quinoid compounds	1
Carboxamide	1	Rosins	1
Carboxylic acid	11	Saturated heterocyclic amine	1
Carboxylic acid ester	14	Saturated heterocyclic fragment	4
Chromene	2	Sulfate	1
Conjugated system	1	Sulfonamide	4
Cycloalkane	1	Sulfonate ester	1
Cycloalkene	4	Sulfonic acid	2
Diacyl peroxides	1	tert-Butyl	1
Dialdehydes	2	Thiaazabicycloheptane, oxo	1
Dihydrobenzopyranone	1	Thiazolidinone/ Isothiazolone	2

Organic functional groups	n.	Organic functional groups	n.
Dihydroxyl group	5	Thioalcohol	2
Diketone	8	Thiocarbamate	1
Disulfide	1	Unsatureted Heterocyclic fragment	1
Epoxide	1	Urea derivatives	1
Ether	16	Xanthene	1
Furanone/ Furanondione	1		

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ANNEX 3

PROFICIENCY CHEMICALS FOR THE DPRA TEST METHOD

In Vitro Skin Sensitization: Direct Peptide Reactivity Assay

Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency by correctly identifying the sensitization hazard classification of the 15 chemicals recommended in Table 1 and by assigning the 11 sensitizers to the same DPRA reference reactivity class or to one class above or below the reference reactivity class. These chemicals were selected to represent the range of responses for skin sensitization hazards. Other selection criteria were that chemicals are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the DPRA are available, and that 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 chemicals for demonstrating technical proficiency with the Direct Peptide Reactivity Assay

Substance	CASRN	Physical State	In vivo Classification[1]	DPRA Classification	DPRA Reference Reactivity Class
p-Benzoquinone	106-51-4	Solid	Sensitizer (extreme)	Sensitizer	High
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitizer (extreme)	Sensitizer	High
Oxazolone	15646-46-5	Solid	Sensitizer (extreme)	Sensitizer	High
Formaldehyde	50-00-0	Liquid	Sensitizer (strong)	Sensitizer	Moderate
2-Phenylpropionaldehyde	93-53-8	Liquid	Sensitizer (moderate)	Sensitizer	High
Diethyl maleate	141-05-9	Liquid	Sensitizer (moderate)	Sensitizer	High
Benzylideneacetone	122-57-6	Solid	Sensitizer (moderate)	Sensitizer	High
Farnesal	19317-11-4	Liquid	Sensitizer (weak)	Sensitizer	Low
2,3-Butanedione	431-03-8	Liquid	Sensitizer (weak)	Sensitizer	High
4-Allylanisol	140-67-0	Liquid	Sensitizer (weak)	Sensitizer	Low
Hydroxycitronellal	107-75-5	Liquid	Sensitizer (weak)	Sensitizer	Low
1-Butanol	71-36-3	Liquid	Non-sensitizer	Non-sensitizer	Minimal
6-Methylcoumarin	92-48-8	Solid	Non-sensitizer	Non-sensitizer	Minimal
Lactic acid	50-21-5	Liquid	Non-sensitizer	Non-sensitizer	Minimal
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitizer	Non-sensitizer	Minimal

[1]The in vivo hazard classification (and potency classification) is based on LLNA data (19).

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ANNEX 4

Example 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 for chemical 1 Co-elution Control 2 for 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.