OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW TEST GUIDELINE

In Vitro Skin Sensitisation: human Cell Line Activation Test (h-CLAT)

INTRODUCTION

1. A skin sensitiser refers to a substance that will lead to an allergic response following skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). This test guideline (TG) provides an *in vitro* procedure called human Cell Line Activation test, or h-CLAT, to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (1).

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

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

4. More recently mechanistically-based *in chemico* and *in vitro* test methods have been considered scientifically valid for the evaluation of the skin sensitisation hazard potential of chemicals. However, a combination 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 h-CLAT method is proposed to address the third key event (dendritic cell activation) of the skin sensitisation AOP by quantifying changes in the expression of cell surface markers associated with the process of activation of DC (i.e. CD86 and CD54), in the human leukemia cell line THP-1, following exposure to sensitisers (8). The measured expression levels of CD86 and CD54 cell surface markers are

then used for supporting the discrimination between skin sensitisers and non-sensitisers.

6. The h-CLAT method has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-coordinated validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) and was considered scientifically valid (9) to be used as part of an IATA to support the discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling. Examples of the use of h-CLAT data in combination with other information are reported in the literature (10) (11).

7. Definitions are provided in Annex I.

INITIAL CONSIDERATIONS AND LIMITATIONS

8. Skin sensitisers have been reported to induce the expression of cell membrane markers associated with DC activation (2). Consequently test methods such as h-CLAT that are based on DC-like cell lines and which measure markers of DC activation (12) (13) are considered relevant for the assessment of the skin sensitisation potential of chemicals. However, since DC activation represents only one key event of the skin sensitisation AOP, information generated with test methods measuring markers of DC activation may not be sufficient on its own to conclude on the absence of skin sensitisation potential of chemicals. Therefore, data generated with the h-CLAT method should be considered in the context of integrated approaches, such as IATA, and combined 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 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 h-CLAT may be used on its own to classify a chemical into UN GHS category 1.

10. The h-CLAT method proved to be transferable to laboratories experienced in cell culture techniques and flow cytometry analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 80% within and between laboratories (9). Results generated in the validation study (14) and other published studies (15) overall indicate that, compared with LLNA results, the accuracy in distinguishing skin sensitisers (i.e., UN GHS Cat.1) from non-sensitisers is 88% (N=128) with a sensitivity of 95% (87/92) and a specificity of 69% (25/36). The h-CLAT 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) (14) (15). Taken together, this information indicates the usefulness of the h-CLAT method to contribute to the identification of skin sensitisation hazards. However, the accuracy values given here for the h-CLAT 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.

11. The term "test chemical" is used in this Test Guideline to refer to what is being tested[1] and is not related to the applicability of the h-CLAT to the testing of substances and/or mixtures. On the basis of the current data available, the h-CLAT method 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 physicochemical properties (9) (15) (16). Limited information is currently available on the applicability of the h-CLAT method to mixtures (16). The test method is nevertheless technically applicable to the testing of multi-constituent substances and mixtures. However, before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for the testing of the mixture. The h-CLAT method is applicable to test chemicals soluble or that form a stable dispersion (i.e., a colloid or suspension in which the test chemical does not settle or separate from the solvent into different phases) in an appropriate solvent (see paragraph 19). Test chemicals with a Log Kow of up to 3.5 have been successfully assessed by the h-CLAT method (15). However, test chemicals with a Log Kow of greater than 3.5 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 a negative result should be considered as inconclusive. Furthermore, because of the limited metabolic capability of the cell line and because of the experimental conditions, pro-haptens (i.e. chemicals requiring enzymatic activation to exert their sensitisation activity) and pre-haptens (i.e. chemicals activated by auto oxidation) may also provide negative results in the h-CLAT (16). In the light of the above, negative results should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of IATA. In cases where there is evidence demonstrating the non-applicability of the h-CLAT method to other specific categories of chemicals, it should not be used for those specific categories of chemicals.

12. As described, the h-CLAT method supports the discrimination between skin sensitisers from non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency (10) (11) when used in integrated approaches such as IATA. Nevertheless, further work, preferably based on human data, is required to determine how h-CLAT results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

13. The h-CLAT method is an *in vitro* method which quantifies changes of cell surface marker expression (i.e., CD86 and CD54) on a human cell line, THP-1 cells, following 24 hours exposure to test chemical. The changes of surface marker expression are measured by flow cytometry following cell staining with fluorescein isothiocyanate (FITC)-labelled antibodies. Cytotoxicity measurement is also conducted concurrently to assess whether upregulation of surface maker expression occurs at sub-cytotoxic concentrations. The relative fluorescence intensity of surface markers compared to solvent control are calculated and used in the prediction model (see paragraph 31), to support the discrimination 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 10 Proficiency Substances listed in Annex II.

[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.

PROCEDURE

15. This test guideline is based on the h-CLAT DB-ALM protocol no. 158 (17) 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 h-CLAT method in the laboratory. The following is a description of the main components and procedures for the h-CLAT method, which comprises two steps: *dose finding assay* and *CD86/CD54 expression measurement*.

Preparation of cells

16. The human leukemia cell line, THP-1, should be used for performing the h-CLAT method. It is recommended that cells (TIB-202) are obtained from a well-qualified cell bank, such as American Type Culture Collection.

17. THP-1 cells are cultured, at 37°C under 5% CO_2 and humidified atmosphere, in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 100 units/mL penicillin and 100 µg/mL streptomycin. THP-1 cells are routinely passaged every 2-3 days at the density of 0.1 to 0.2 $\times 10^6$ cells/mL and should be maintained at densities from 0.1×10^6 to 0.8×10^6 cells/mL. The cell density should not exceed 1×10^6 cells/mL. The reactivity check of the cells should be performed using the positive controls, 2,4-dinitrochlorobenzene (DNCB) (CAS n. 97-00-7, ≥ 99% purity) and nickel sulfate (CAS n. 10101-97-0, 99% purity) and the negative control lactic acid (CAS n. 50-21-5, ≥ 99% purity), two weeks after thawing. Both DNCB and NiSO4 should produce a positive response of both CD86 and CD54, and LA should produce negative response of both CD86 and CD54. Only the cells which passed the reactivity check are to be used for the assay. Cells can be propagated up to two months after thawing (passage number should not exceed 30).

18. For testing, THP-1 cells are seeded between 0.1 and 0.2×10^6 cells/mL, and pre-cultured for 48 hours or for 72 hours in culture flasks. In the day of testing, cells harvested from culture flask are resuspended with fresh culture medium at 2×10^6 cells/mL. Then, cells are distributed into a 24 well flat-bottom plate with 500 µL (1×10^6 cells/well) or a 96-well flat-bottom plate with 80 µL (1.6×10^5 cells/well).

Dose finding assay

Preparation of test chemicals and control substances

19. The test chemicals and control substances are prepared on the day of testing. For the h-CLAT method, test chemicals are dissolved in saline, medium or dimethyl sulfoxide (DMSO, ≥ 99% purity) to final concentrations of 100 mg/mL or 500 mg/mL. The test chemicals, which are not soluble in saline, are dissolved in DMSO and diluted. However, other solvents may be used if sufficient scientific rationale is provided.

20. Based on the 100 mg/mL (in saline) or 500 mg/mL (in DMSO) solutions of the test chemicals, 2-fold serial dilutions are made using corresponding solvent to obtain the stock solutions (eight doses) to be tested in the h-CLAT method. These stock solutions are then further diluted 50-fold (for saline) or 250-fold (for DMSO) into the culture medium (working solutions). These working solutions are finally used for treatment with a further 2-fold dilution factor.

21. The negative control used in the h-CLAT method is culture medium (for chemicals solubilised either with medium or saline) or DMSO (for chemicals solubilised in DMSO). It undergoes the same dilution as

described for the working solutions in paragraph 20, so that the final concentration of saline is 1%, and that of DMSO is 0.2%.

Application of test chemicals and control substances

22. The culture medium or working solutions described in paragraph 20 and 21 are mixed 1:1 (v/v) with the cell suspensions prepared in the 24-well or 96-well flat-bottom plate (see paragraph 18). The treated plates are then incubated for 24 hours at 37°C under 5% CO_2. Care should be taken to avoid evaporation of volatile test chemicals and cross-contamination between wells by test chemicals, e.g., by sealing the plate prior to the incubation with the test chemicals.

Propidium iodide (PI) staining

23. After 24 hours of exposure, cells are transferred into sample tubes and collected by centrifugation. The supernatants are discarded and the remaining cells are resuspended with 600 μL of a phosphate buffered saline containing 0.1% bovine serum albumin (FACS buffer). 200 μL of cell suspension is transferred into 96-well round-bottom plate and washed twice with 200 uL of FACS buffer. Finally, cells are resuspended in 200 μL of FACS buffer and 10 μL of PI solution is added (final concentration of PI is 0.625 μg/mL).

Cytotoxicity measurement by flow cytometry and estimation of CV75 value.

24. The PI uptake is analysed using flow cytometry with the acquisition channel FL-3. A total of 10,000 living (PI negative) cells are acquired. The cell viability can be calculated using the following equation by the cytometer analysis program. When the cell viability is low, up to 30,000 cells including dead cells should be acquired. Alternatively, the data acquisition can be finished one minute after the initiation.

$$\textbf{Cell Viability} = \frac{\textbf{Number of living cells}}{\textbf{Total Number of acquired cells}} \times 100$$

The CV75 value, i.e. a concentration showing 75% of THP-1 cell survival (25% cytotoxicity), is calculated by log-linear interpolation using the following equation:

$$\textbf{Log CV75} = \frac{(75 - B) \times \text{Log}(C) - (75 - A) \times \text{Log}(D)}{A - B}$$

Where:

A is the minimum value of cell viability over 75% in testing groups
B is the maximum value of cell viability below 75% in testing groups
C or D is the concentration showing the value of cell viability A or B

The CV75 value is used to determine the concentration of test chemicals in *CD86/CD54 expression measurement*.

CD86/CD54 expression measurement

Preparation of the test chemicals and Control Substances.

25. The appropriate solvent (saline or DMSO; see paragraph 19) is used to dissolve the test chemicals. The test chemicals are first diluted to the concentration corresponding to 100-fold (for saline) or 500-fold (for DMSO) of the 1.2 × CV75 determined in the *dose finding assay* (see paragraph 24). If the CV75 is not determined (i.e. if sufficient cytotoxicity is not observed in the *dose finding assay*), the highest soluble concentration of test chemical prepared with each solvent should be used as starting dose. Then, 1.2-fold serial dilutions are made using the corresponding solvent to obtain the stock solutions (eight doses ranging from 0.335 × CV75 to 1.2 × CV75) to be tested in the h-CLAT method. The stock solutions are then further diluted 50-fold (for saline) or 250-fold (for DMSO) into the culture medium (working solutions)). These working solutions are finally used for treatment with a further 2-fold dilution factor. Alternative concentrations may be used upon justification (e.g. in case of poor solubility or cytotoxicity).

26. The negative (solvent) control is prepared as described in paragraph 21. The positive control used in the h-CLAT method is DNCB (see paragraph 17), for which stock solutions are prepared in DMSO and diluted as described for the stock solutions in paragraph 25. The final concentration of the positive control, 4.0 μg/mL, should yield approximately 70-90% of cell viability. Alternatively, the CV75 of DNCB, which is determined in each test facility, could be also used as the positive control dose. Other suitable positive controls may be used if historical data are available to derive comparable run acceptance criteria.

Application of test chemicals and control substances

27. For each test chemicals and control substances, one experiment is needed to derive a prediction. Each experiment consists of at least two independent runs (n=2) (see paragraphs 31 and 33). Test chemicals and control substances prepared as working solutions are mixed with suspended cells at 1:1 ratio, and cells are incubated for 24 hours as described in paragraphs 25 and 26.

Cell staining and analysis

28. After 24 hours of exposure, cells are transferred into sample tubes and collected by centrifugation. The supernatants are discarded and the remaining cells are resuspended with 600 μL of FACS buffer. Cells are split in three aliquots of 180 μL into a 96-well round-bottom plate. After centrifugation, cells are resuspended in 200 μL of blocking solution (FACS buffer containing 0.01% (w/v) globulin) and incubated at 4°C for 15 min.

29. After centrifugation, cells are stained with 50 μL of FITC-labelled anti-CD86, anti-CD54 or mouse IgG1 (isotype) antibodies at 4°C for 30 min. The antibodies described in the h-CLAT DB-ALM protocol no. 155 (17) should be used by diluting 3:25 (v/v, for CD86) or 3:50 (v/v, for CD54 and IgG1) with FACS buffer. After washing with 200 μL of FACS buffer three times, cells are resuspended in 200 μL of FACS buffer and 10 μL of PI solution is added (final concentration of PI is 0.625 μg/mL). The expression levels of CD86 and CD54, and cell viability are analysed using flow cytometry.

DATA AND REPORTING

Data evaluation

30. The expression of CD86 and CD54 is analysed with flow cytometry with the acquisition channel FL-1. Based on the geometric mean fluorescence intensity (MFI), the relative fluorescence intensity (RFI) of

CD86 and CD54 for positive control cells and chemical-treated cells are calculated according to the following equation:

$$\mathbf{R}\text{FI} = \frac{\textbf{MFI of chemical-treated cells} - \textbf{MFI of chemical-treated isotype control cells}}{\textbf{MFI of solvent-treated control cells} - \textbf{MFI of solvent-treated isotype control cells}}$$

The cell viability from the isotype control cells is also calculated according to the equation described in paragraph 24.

Prediction model

31. Each chemical is tested in at least two independent runs to derive a single prediction (positive or negative). Each independent run is performed on a different day or on the same day provided that for each run: a) independent fresh stock solutions and working solutions of the test chemicals and antibody solutions are prepared and b) independently harvested cells are used (i.e. cells are collected from different culture flasks), cells may come from the same passage however. If the RFI of CD86 is equal to or greater than 150% at any tested dose (≥ 50% of cell viability) in at least two independent runs and/or if the RFI of CD54 is equal to or greater than 200% at any tested dose (≥ 50% of cell viability) in at least two independent runs, the prediction is considered as positive. Otherwise, it is considered as negative. In case the first two independent runs are not concordant a third run needs to be performed and the final prediction will be based on the mode of the conclusions from the three individual runs (i.e. 2 out of 3).

32. Test chemicals with a Log Kow of up to 3.5 have been successfully tested by the test method (15). Test chemicals with a Log Kow of greater than 3.5 may still be tested at lower soluble concentrations. In such a case, a negative result should be considered inconclusive, whereas a positive result could still be used to support the identification of the test chemical as a skin sensitiser.

33. For the test chemicals considered to be sensitisers, two Effective Concentrations (EC) values, the EC150 for CD86 and EC200 for CD54, i.e. the concentration at which the test chemicals induced a RFI of 150 or 200, can be calculated by the following equations:

$$\textbf{EC150 (for CD86)} = B_{dose} + [(150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$

$$\textbf{EC200 (for CD54)} = B_{dose} + [(200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$

where

A_{dose} is the lowest concentration in μg/mL with RFI > 150 (CD86) or 200 (CD54)
B_{dose} is the highest concentration in μg/mL with RFI < 150 (CD86) or 200 (CD54)
A_{RFI} is the RFI at the lowest concentration with RFI > 150 (CD86) or 200 (CD54)
B_{RFI} is the RFI at the highest concentration with RFI < 150 (CD86) or 200 (CD54)

For the purpose of more precisely deriving the EC150 and EC200 values, three independent runs should be performed. The EC150 and EC200 values are the median value calculated from three independent runs. When only two of three independent runs meet the positive criteria (See paragraph 31), the higher EC150 or EC200 value is adopted. Whereas it is not always possible to derive the EC150 and/or EC200 value for positive chemicals, the value could potentially contribute to the assessment of sensitising potency when

used in integrated approaches such as IATA (10)(11).

Acceptance criteria

34. The following acceptance criteria should be met when using the h-CLAT method.

- The cell viabilities of medium and solvent control are more than 90%.

- In the positive control (DNCB), RFI values of both CD86 and CD54 are over the positive criteria (CD86 RFI ≥ 150 and CD54 RFI ≥ 200) and cell viability is more than 50%.

- In the solvent control (DMSO), RFI values of both CD86 and CD54 should not exceed the positive criteria (CD86 RFI ≥ 150 and CD54 RFI ≥ 200).

- For both medium and DMSO controls, the MFI ratio of both CD86 and CD54 to isotype control should be > 105%.

- The cell viability of tested chemicals at more than four tested doses in each run should be ≥ 50%.

35. Negative results are acceptable only for test chemicals exhibiting cell viability at 1.2 × CV75 of less than 90%. Negative results with cell viability of 90% or higher are discarded. The dose finding study should be retested to determine the CV75 determination. Positive results for test chemicals of any cell viability at 1.2 × CV75 are acceptable. It should be noted that when 5000 μg/mL in saline, 1000 μg/mL in DMSO, or the highest soluble concentration is used as the maximal test concentration of a test chemical, the results are acceptable.

Test report

36. The test report should include the following information.

Test Chemical

- Mono-constituent substance

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI
  - code, structural formula, and/or other identifiers;
  - Physical appearance, water solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;
  - Treatment prior to testing, if applicable (e.g., warming, grinding);
  - Concentration(s) tested;
  - Storage conditions and stability to the extent available.

- Multi-constituent substance, UVCB and mixture:

  - Characterisation as far as possible by e.g., chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;

  - Physical appearance, water solubility, DMSO solubility and additional relevant physicochemical properties, to the extent available;

  - Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;

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

  - Concentration(s) tested;

  - Storage conditions and stability to the extent available.

Controls

- Positive control

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

  - Physical appearance, water solubility, DMSO solubility, molecular weight, and additional relevant physicochemical properties, to the extent available and where applicable;

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

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

  - Concentration(s) tested;

  - Storage conditions and stability to the extent available;

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

- Negative (vehicle) control

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;

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

  - Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other negative controls / vehicles than those mentioned in the Test Guideline are used and to

the extent available;

- Storage conditions and stability to the extent available;
- Justification for choice of solvent for each test chemical.

Test method Conditions

- Name and address of the sponsor, test facility and study director
- Description of test method used
- Cell line used, its storage conditions and source (e.g., the facility from which they were obtained)
- Passage number and confluence of cells used for testing
- Flow cytometry used (e.g., model), including instrument settings, globulin, antibodies and propidium iodide used
- 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.

Results

- Tabulation of the data, including CV75 (if applicable), individual geometric MFI, RFI, cell viability values, EC150/EC200 values (if applicable) obtained for the test chemical and for the positive control in each run, and an indication of the rating of the test chemical according to the prediction model
- Description of any other relevant observations, if applicable.

Discussion of the Results

- Discussion of the results obtained with the h-CLAT method
- Consideration of the test method results within the context of an IATA, if other relevant information is available

Conclusions

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 (18).

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).

CV75: The estimated concentration showing 75% cell viability.

EC150: the concentrations showing the RFI values of 150 in CD86 expression

EC200: the concentrations showing the RFI values of 200 in CD54 expression

FACS buffer: A phosphate buffered saline containing 0.1% bovine serum albumin.

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.

Medium control: An untreated replicate containing all components of a test system. This sample is processed with test chemical-treated samples and other control samples to determine whether the solvent interacts with the test system.

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

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

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

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

Relative fluorescence intensity Relative values of geometric mean fluorescence intensity (MFI) in chemical-treated cells compared to MFI in solvent-treated cells.

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 (18).

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 (18).

Run: A run consists of one or more test chemicals tested concurrently with a negative control and with a positive control.

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. 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 (18).

Solvent control: An untreated sample containing all components of a test system, including the solvent 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. When tested with a concurrent medium control, this sample also demonstrates whether the solvent interacts with the test system.

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. 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 (18).

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, inducing 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 it composition.

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

United Nations Globally Harmonized System of Classification and Labeling 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).

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 (18).

ANNEX II

PROFICIENCY SUBSTANCES

Prior to routine use of a test method that adheres to this Test Guideline, laboratories should demonstrate technical proficiency by correctly obtaining the expected h-CLAT prediction for the 10 chemicals recommended in Table 1.. Proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that the substances are commercially available, and that high-quality *in vivo* reference data as well as high quality *in vitro* data generated with the h-CLAT method are available. Also, published reference data are available for the h-CLAT method (9)(15).

Table 1: Recommended substances for demonstrating technical proficiency with the h-CLAT method

Proficiency substances	**CASRN**	**Physical state**	***In vivo* prediction[1]**	**h-CLAT prediction[2]**
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Positive
Chloramin T	127-65-1	Solid	Sensitiser (strong)	Positive
Nickel sulfate	10101-97-0	Solid	Sensitiser (moderate)	Positive
Phenylacetaldehyde	122-78-1	Liquid	Sensitiser (moderate)	Positive
hydroxycitronellal	107-75-5	Liquid	Sensitiser (weak)	Positive
Imidazoidinyl urea	39236-46-9	Solid	Sensitiser (weak)	Positive
1-Butanol	71-36-3	Liquid	Non-sensitiser	Negative
Glycerol	56-81-5	Liquid	Non-sensitiser	Negative
Lactic acid	50-21-5	Liquid	Non-sensitiser	Negative
Vanillin	121-33-5	Solid	Non-sensitiser	Negative

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

[1] The *in vivo* hazard and (potency) prediction is based on LLNA data (9) (15). The *in vivo* potency is derived using the criteria proposed by ECETOC (19).

[2] Predictions made with the h-CLAT method should be considered in the framework of IATA and in accordance with the provision of paragraph 9.