# 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 (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 through intermediate events to the adverse effect, namely allergic contact dermatitis. The molecular initiating event (i.e. the first key event) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as 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 (LLNA) (3).

3. The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs—namely, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman, and the Buehler Test - TG 406 (4)—assess both the induction and elicitation phases of skin sensitisation. The murine tests—the 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 together with an objective measurement of the induction phase of skin sensitisation.

4. More recently mechanistically-based *in chemico* (OECD TG 442C; Direct Peptide Reactivity Assay addressing the first key event of the skin sensitisation AOP) (7) and *in vitro* (OECD TG 442D; ARE-Nrf2 Luciferase Test Method addressing the second key event of the skin sensitisation AOP) (8) test methods have been adopted for contributing to 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)(9).

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 monocytic leukaemia cell line THP-1, following exposure to sensitisers (10). 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). Considering all available evidence and input from regulators and stakeholders, the h-CLAT was recommended by EURL ECVAM (11) 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 (12) (13) (14) (15) (16).

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). Test methods such as the h-CLAT that are based on DC-like cell lines and which measure markers of DC activation (17) (18) are therefore 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 (11). Results generated in the validation study (19) and other published studies (20) overall indicate that, compared with LLNA results, the accuracy in distinguishing skin sensitisers (i.e., UN GHS Cat.1) from non-sensitisers is 85% (N=142) with a sensitivity of 93% (94/101) and a specificity of 66% (27/41) (based on a re-analysis by EURL ECVAM considering all existing data). False negative predictions with the h-CLAT are more likely to concern 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) (16) (17). 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 mono-constituent substances, multi-constituent 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 (11) (20) (21). Limited information is currently available on the applicability of the h-CLAT method to multi-constituent substances/mixtures (21). 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. Moreover, when testing multi-constituent substances or mixtures, detection of their potential sensitisation properties may be affected by the cytotoxicity of the constituents.

12. 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/vehicle into different phases) in an appropriate solvent/vehicle (see paragraph 21). Test chemicals with a Log Kow up to 3.5 have been successfully assessed by the h-CLAT method (20). Test chemicals with a Log Kow greater than 3.5 tend to produce false negative results. Therefore negative results with these test chemicals should be considered as inconclusive whereas positive results could still be used to support the identification of the test chemical as a skin sensitiser. Furthermore, because of the limited metabolic capability of the cell line used (22) and because of the experimental conditions, pro-haptens (i.e., substances requiring enzymatic activation for example via P450 enzymes) and pre-haptens (i.e., substances activated by oxidation) in particular with a slow oxidation rate may also provide negative results in the h-CLAT (21). Fluorescent test chemicals can be assessed with the h-CLAT (23), nevertheless, strong fluorescent test chemicals emitting at the same wavelength as fluorescein isothiocyanate (FITC) or as propidium iodide (PI), will interfere with the flow cytometric detection and thus cannot be correctly evaluated using FITC-conjugated antibodies or PI. In such a case, other fluorochrome-tagged antibodies or other cytotoxicity markers, respectively, can be used as long as it can be shown they provide similar results as the FITC-tagged antibodies (see paragraph 31) or PI (see paragraph 25) e.g. by testing the proficiency substances in Annex II. In the light of the above, negative results should be interpreted in the context of the stated limitations and together 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 test chemicals, it should not be used for those specific categories.

13. 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 (12) (13) 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.

[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 be applied in new and updated Test Guidelines.

## PRINCIPLE OF THE TEST

14. The h-CLAT test method is an *in vitro* assay that quantifies changes of cell surface marker expression (i.e., CD86 and CD54) on a human monocytic leukemia cell line, THP-1 cells, following 24 hours exposure to the test chemical. These surface molecules are typical markers of DC activation that play a critical role in T cell priming by DCs. The changes of surface marker expression are measured by flow cytometry following cell staining with fluorochrome-tagged 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/vehicle control are calculated and used in the prediction model (see paragraph 33), to support the discrimination between sensitisers and non-sensitisers

## DEMONSTRATION OF PROFICIENCY

15. 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. Moreover, test method users should maintain an historical database of data generated with the reactivity checks (see paragraph 18) and with the positive and solvent/vehicle controls (see paragraphs 27-29), and use these data to confirm the reproducibility of the test method in their laboratory.

## PROCEDURE

16. This test guideline is based on the h-CLAT DB-ALM protocol no. 158 (24) 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*

17. The human monocytic leukaemia 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 the American Type Culture Collection.

18. 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. The use of penicillin and streptomycin in the culture medium can be avoided if good cell culture practices are followed and if it can be guaranteed that the cells are free of any type of contamination at the time of testing. Users should also consider verifying that the absence of antibiotics in the culture medium has no impact on the results, for example by testing the proficiency substances listed in Annex II. THP-1 cells are routinely passaged every 2-3 days at the density of 0.1 to 0.2 × $10^6$ 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. Prior to using them for testing the cells should be qualified by conducting a reactivity check. 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 (LA) (CAS n. 50-21-5, ≥ 85% purity), two weeks after thawing. Both DNCB and $NiSO_4$ 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.

19. For testing, THP-1 cells are seeded at a density of either $0.1 \times 10^6$ cells/mL or $0.2 \times 10^6$ cells/mL, and pre-cultured in culture flasks for 72 hours or for 48 hours, respectively. It is important that the cell density in the culture flask just after the pre-culture period be as consistent as possible in each experiment (by using one of the two pre-culture conditions described above), because the cell density in the culture flask just after pre-culture could affect the CD86/CD54 expression induced by allergens (26). In the day of testing, cells harvested from culture flask are resuspended with fresh culture medium at $2 \times 10^6$ cells/mL. Then, cells are distributed into a 24 well flat-bottom plate with 500 µL ($1 \times 10^6$ cells/well) or a 96-well flat-bottom plate with 80 µL ($1.6 \times 10^5$ cells/well).

***Dose finding assay***

20. A *dose finding assay* is performed to determine the CV75, being the test chemical concentration that results in 75% cell viability compared to the solvent/vehicle control. The CV75 value is used to determine the concentration of test chemicals for the *CD86/CD54 expression measurement* (see paragraphs 27-31).

*Preparation of test chemicals and control substances*

21. The test chemicals and control substances are prepared on the day of testing. For the h-CLAT method, test chemicals are dissolved or stably dispersed (see also paragraph 12) in saline or medium as first solvent/vehicle options or dimethyl sulfoxide (DMSO, ≥ 99% purity) as a second solvent/vehicle option if the test chemical is not soluble or does not form a stable dispersion in the previous two solvents/vehicles, to final concentrations of 100 mg/mL (in saline or medium) or 500 mg/mL (in DMSO). Other solvents/vehicles than those described above may be used if sufficient scientific rationale is provided.

22. Starting from the 100 mg/mL (in saline or medium) or 500 mg/mL (in DMSO) stock solutions of the test chemicals, the following dilution steps should be taken:

- For saline or medium as solvent/vehicle: Eight stock solutions (eight concentrations) are prepared, by two-fold serial dilutions using the corresponding solvent/vehicle. These stock solutions are then further diluted 50-fold into culture medium (working solutions). If the top final concentration in the plate of 1000 µg/mL is non-toxic, the maximum concentration should be re-determined by performing a new cytotoxicity test. The final concentration in the plate should not exceed 5000 µg/mL for test chemicals dissolved or stably dispersed in saline or medium.
- For DMSO as solvent/vehicle: Eight stock solutions (eight concentrations) are prepared, by two-fold serial dilutions using the corresponding solvent/vehicle. These stock solutions are then further diluted 250-fold into culture medium (working solutions).

The working solutions are finally used for exposure by adding an equal volume of working solution to the volume of THP-1 cell suspension in the plate (see paragraph 19 above) to achieve a further two-fold dilution (usually, the final range of concentrations in the plate is 7.81–1000 µg/mL).

23. The solvent/vehicle control used in the h-CLAT method is culture medium (for test chemicals solubilised or stably dispersed either with medium or saline) or DMSO (for test chemicals solubilised or stably dispersed in DMSO) tested at a single final concentration in the plate of 0.2%. It undergoes the same dilution as described for the working solutions in paragraph 22.

*Application of test chemicals and control substances*

24. The culture medium or working solutions described in paragraphs 22 and 23 are mixed 1:1 (v/v) with the cell suspensions prepared in the 24-well or 96-well flat-bottom plate (see paragraph 19). The treated plates are then incubated for 24±0.5 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 (25).

*Propidium iodide (PI) staining*

25. After 24±0.5 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 (in case of 96-well) or 1mL (in case of 24-well) of a phosphate buffered saline containing 0.1% bovine serum albumin (staining buffer). 200 µL of cell suspension is transferred into 96-well round-bottom plate (in case of 96-well) or micro tube (in case of 24-well) and washed twice with 200 µL (in case of 96-well) or 1mL (in case of 24-well) of staining buffer. Finally, cells are resuspended in staining buffer (e.g. 400 µL) and PI solution (e.g. 20 µL) is added (for example, final concentration of PI is 0.625 µg/mL). Other cytotoxicity markers, such as 7-Aminoactinomycin D (7-AAD), Trypan blue or others may be used if the alternative stains can be shown to provide similar results as PI, for example by testing the proficiency substances in Annex II.

*Cytotoxicity measurement by flow cytometry and estimation of CV75 value*

26. 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, data can be acquired for one minute after the initiation of the analysis.

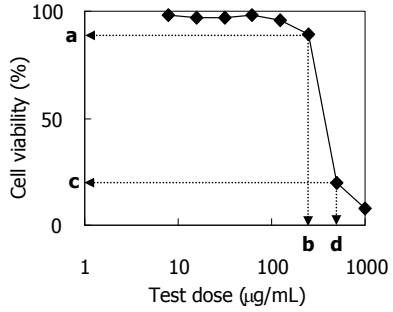
$$\textbf{Cell Viability} = \frac{\textbf{Number of living cells}}{\textbf{Total Number of acquired cells}} \times \textbf{100}$$

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:

$$\textbf{Log CV75} = \frac{\textbf{(75 – c) × Log (d) – (75 – a) × Log (b)}}{\textbf{a – c}}$$

Where:

a is the minimum value of cell viability over 75% in testing groups
c is the maximum value of cell viability below 75% in testing groups
b and d are the concentrations showing the value of cell viability a and c respectively



***CD86/CD54 expression measurement***

*Preparation of the test chemicals and control substances*

27. The appropriate solvent/vehicle (saline, medium or DMSO; see paragraph 21) is used to dissolve or stably disperse the test chemicals. The test chemicals are first diluted to the concentration corresponding to 100-fold (for saline or medium) or 500-fold (for DMSO) of the 1.2 × CV75 determined in the *dose finding assay* (see paragraph 26). If the CV75 cannot be determined (i.e., if sufficient cytotoxicity is not observed in the *dose finding assay*), the highest soluble or stably dispersed concentration of test chemical prepared with each solvent/vehicle should be used as starting concentration. Please note that the final concentration in the plate should not exceed 5000 μg/mL (in case of saline or medium) or 1000 μg/mL (in case of DMSO). Then, 1.2-fold serial dilutions are made using the corresponding solvent/vehicle to obtain the stock solutions (eight concentrations 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 medium) or 250-fold (for DMSO) into the culture medium (working solutions). These working solutions are finally used for exposure with a further final two-fold dilution factor in the plate. If the results do not meet the acceptance criteria described in the paragraphs 35 and 36 regarding cell viability, the *dose finding assay* may be repeated to determine a more precise CV75.

28. The solvent/vehicle control is prepared as described in paragraph 23. The positive control used in the h-CLAT method is DNCB (see paragraph 18), for which stock solutions are prepared in DMSO and diluted as described for the stock solutions in paragraph 27. DNCB should be used as the positive control for *CD86/CD54 expression measurement* at a final single concentration in the plate (typically 4.0 μg/mL) yielding approximately 70-90% of cell viability. To obtain a 4.0 μg/mL concentration of DNCB in the plate, a 2 mg/mL stock solution of DNCB in DMSO is prepared and further diluted 250-fold with culture medium to a 8 μg/mL working solution. Alternatively, the CV75 of DNCB, which is determined in each test facility, could be also used as the positive control concentration. Other suitable positive controls may be used if historical data are available to derive comparable run acceptance criteria. For positive controls, the final single concentration in the plate should not exceed 5000 μg/mL (in case of saline or medium) or 1000 μg/mL (in case of DMSO). The run acceptance criteria are the same as those described for the test chemical (see paragraph 35, except for the last point).

*Application of test chemicals and control substances*

29. For each test chemical and control substance, one experiment is needed to obtain a prediction. Each experiment consists of at least two independent runs for *CD86/CD54 expression measurement* (see paragraphs 33 and 34). 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 chemical and antibody solutions are prepared and b) independently harvested cells are used (i.e. cells are collected from different culture flasks); however, cells may come from the same passage. Test chemicals and control substances prepared as working solutions are mixed with suspended cells at 1:1 ratio, and cells are incubated for 24±0.5 hours as described in paragraphs 27 and 28. In each run, a single replicate for each concentration of the test chemical and control substance is sufficient because a prediction is obtained from at least two independent runs.

*Cell staining and analysis*

30. After 24 hours of exposure, cells are transferred into sample tubes, collected by centrifugation and then

washed twice with 1mL of staining buffer. After washing, cells are blocked with 600 µL of blocking solution (staining buffer containing 0.01% (w/v) globulin) and incubated at 4°C for 15 min. After blocking, cells are split in three aliquots of 180 µL into a 96-well round-bottom plate or micro tube.

31. 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. 158 (24) should be used by diluting 3:25 (v/v, for CD86) or 3:50 (v/v, for CD54 and IgG1) with staining buffer. These antibody dilution factors were defined by the test method developers as those providing the best signal-to-noise ratio. Based on the experience of the test method developers, the fluorescence intensity of the antibodies is usually consistent between different lots. However, users may consider titrating the antibodies in their own laboratory's conditions to define the best concentrations for use. Other fluorochrome-tagged anti-CD86 and/or anti-CD54 antibodies may be used if they can be shown to provide similar results as FITC-conjugated antibodies, for example by testing the proficiency substances in Annex II. It should be noted that changing the clone or supplier of the antibodies as described in the h-CLAT DB-ALM protocol no. 158 (24) may affect the results. After washing with 200 µL of staining buffer three times, cells are resuspended in staining buffer (e.g. 400 µL), and the PI solution (e.g. 20 µL to obtain a final concentration of 0.625 µg/mL) or another cytotoxicity marker's solution (see paragraph 25) is added. The expression levels of CD86 and CD54, and cell viability are analysed using flow cytometry.

## DATA AND REPORTING

### *Data evaluation*

32. 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 (ctrl) cells and chemical-treated cells are calculated according to the following equation:

$$\mathbf{RFI} = \frac{\mathbf{MFI\ of\ chemical\text{-}treated\ cells} - \mathbf{MFI\ of\ chemical\text{-}treated\ isotype\ control\ cells}}{\mathbf{MFI\ of\ solvent/vehicle\text{-}treated\ ctrl\ cells} - \mathbf{MFI\ of\ solvent/vehicle\text{-}treated\ isotype\ ctrl\ cells}} \times \mathbf{100}$$

The cell viability from the isotype control (ctrl) cells (which are stained with mouse IgG1 (isotype) antibodies) is also calculated according to the equation described in paragraph 26.

### *Prediction model*

33. For *CD86/CD54 expression measurement*, each test chemical is tested in at least two independent runs to derive a single prediction (POSITIVE or NEGATIVE). An h-CLAT prediction is considered POSITIVE if at least one of the following conditions is met in 2 of 2 or in at least 2 of 3 independent runs, otherwise the h-CLAT prediction is considered NEGATIVE (Figure 1):

- The RFI of CD86 is equal to or greater than 150% at any tested concentration (with cell viability ≥ 50%);
- The RFI of CD54 is equal to or greater than 200% at any tested concentration (with cell viability ≥ 50%).

Based on the above, if the first two runs are both positive for CD86 and/or are both positive for CD54, the h-CLAT prediction is considered POSITIVE and a third run does not need to be conducted. Similarly, if the first two runs are negative for both markers, the h-CLAT prediction is considered NEGATIVE (with due consideration of the provisions of paragraph 36) without the need for a third run. If however, the first two runs are not concordant for at least one of the markers (CD54 or CD86), a third run is needed and the final prediction will be based on the majority result of the three individual runs (i.e., 2 out of 3). In this respect, it should be noted that if two independent runs are conducted and one is only positive for CD86 (hereinafter referred to as $P_1$) and the other is only positive for CD54 (hereinafter referred to as $P_2$), a third run is required. If this third run is negative for both markers (hereinafter referred to as N), the h-CLAT prediction is considered NEGATIVE. On the other hand, if the third run is positive for either marker ($P_1$ or $P_2$) or for both markers (hereinafter referred to as $P_{12}$), the h-CLAT prediction is considered POSITIVE.

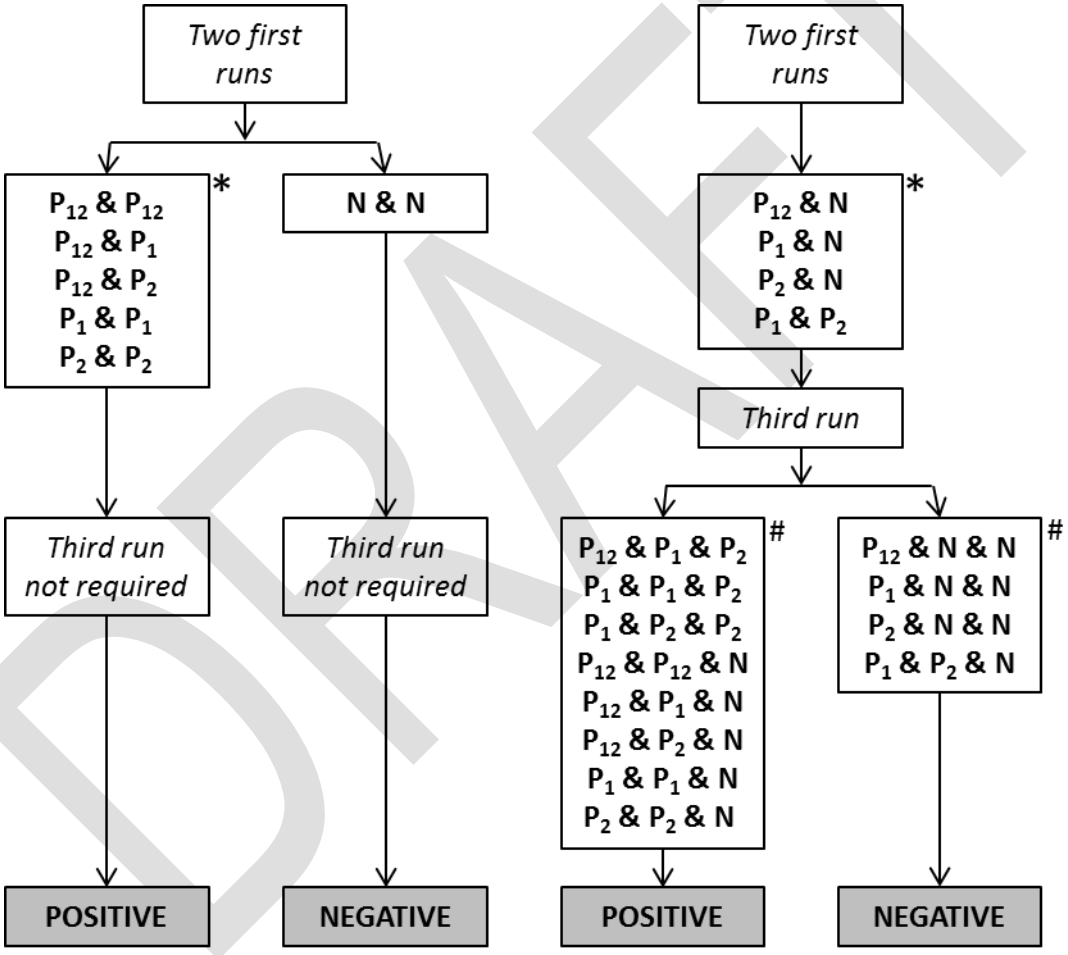



Figure 1: Prediction model used in the h-CLAT test method. An h-CLAT prediction should be considered in the framework of an IATA and in accordance with the provision of paragraphs 9, 11 and 12. $P_1$: run with only CD86 positive; $P_2$; run with only CD54 positive; $P_{12}$: run with both CD86 and CD54 positive; N: run with neither CD86 nor CD54 positive. *The boxes show the relevant combinations of results from the two first runs, independently of the order in which they may be obtained. #The boxes show the relevant combinations of results from the three runs on the basis of the results obtained in the two first runs shown in the box above, but do not reflect the order in which they may be obtained.

34. For the test chemicals predicted as POSITIVE with the h-CLAT, optionally, 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, may be determined. These EC values potentially could contribute to the assessment of sensitising potency (3) when used in integrated approaches such as IATA (12) (13) (14) (15) (16). They can be calculated by the following equations:

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

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

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 for *CD86/CD54 expression measurement* may be required. The final EC150 and EC200 values are then determined as the median value of the ECs calculated from the three independent runs. When only two of three independent runs meet the criteria for positivity (see paragraph 33), the higher EC150 or EC200 of the two calculated values is adopted.

***Acceptance criteria***

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

- The cell viabilities of medium and solvent/vehicle controls should be higher than 90%.

- In the solvent/vehicle control, RFI values of both CD86 and CD54 should not exceed the positive criteria (CD86 RFI ≥ 150% and CD54 RFI ≥ 200%). RFI values of the solvent/vehicle control are calculated by using the formula described in paragraph 32 ("MFI of chemical" should be replaced with "MFI of solvent/vehicle", and "MFI of solvent/vehicle" should be replaced with "MFI of (medium) control").

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

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

- For the test chemical, the cell viability should be more than 50% in at least four tested concentrations in each run.

36. Negative results are acceptable only for test chemicals exhibiting a cell viability of less than 90% at 1.2 × CV75 (or highest concentration). If the cell viability at 1.2 × CV75 is equal or above 90% the negative result should be discarded. In such a case it is recommended to try to refine the dose selection by repeating the CV75 determination. It should be noted that when 5000 µg/mL in saline (or medium or other solvents/vehicles), 1000 µg/mL in DMSO or the highest soluble concentration is used as the maximal test concentration of a test chemical, a negative result is acceptable even if the cell viability is above 90%.

***Test report***

37. 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, Log Kow, 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.
    - Justification for choice of solvent/vehicle for each test chemical.

- 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;
    - Justification for choice of solvent/vehicle for each test chemical.

*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, Log Kow, 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 and solvent/vehicle control

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, 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 control solvent/vehicle 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/vehicle for each test chemical.

*Test method Conditions*

- Name and address of the sponsor, test facility and study director;
- Description of test method used;
- Cell line used, its storage conditions and source (e.g. the facility from which they were obtained);
- Flow cytometry used (e.g. model), including instrument settings, globulin, antibodies and cytotoxicity marker used;
- The procedure used to demonstrate proficiency of the laboratory in performing the test method by testing of proficiency substances and if applicable, the procedure used to demonstrate reproducible performance of the test method over time, e.g. historical control data and/or historical reactivity checks' data.

*Test Acceptance Criteria*

- Cell viability, MFI and RFI values obtained with the solvent/vehicle control in comparison to the

acceptance ranges;

- Cell viability and RFI values obtained with the positive control in comparison to the acceptance ranges;
- Cell viability of all tested concentrations of the tested chemical;

*Test procedure*

- Number of runs used;
- Test chemical concentrations, application and exposure time used (if different than the one recommended)
- Duration of exposure (if different than the one recommended);
- Description of evaluation and decision criteria used;
- Description of any modifications of the test procedure.

*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*

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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 (27).

**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

**Flow cytometry:** a cytometric technique in which cells suspended in a fluid flow one at a time through a focus of exciting light, which is scattered in patterns characteristic to the cells and their components; cells are frequently labeled with fluorescent markers so that light is first absorbed and then emitted at altered frequencies.

**Staining 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/vehicle 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 (RFI):** Relative values of geometric mean fluorescence intensity (MFI) in chemical-treated cells compared to MFI in solvent/vehicle-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 (27).

**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 (27).

**Run:** A run consists of one or more test chemicals tested concurrently with a solvent/vehicle 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 (27).

**Solvent/vehicle control:** An untreated sample containing all components of a test system except of the test chemical, but including the solvent/vehicle that is used. It is used to establish the baseline response for the samples treated with the test chemical dissolved or stably dispersed in the same solvent/vehicle. When tested with a concurrent medium control, this sample also demonstrates whether the solvent/vehicle 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 (27).

**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 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).

**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 (27).

## 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 substances recommended in Table 1 and by obtaining CV75, EC150 and EC200 values that fall within the respective reference range for at least 8 out of the 10 proficiency substances. 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 (11) (20).

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

| **Proficiency substances** | **CASRN** | **Physical state** | ***In vivo* prediction[1]** | **CV75 Reference Range in µg/mL[2]** | **h-CLAT results for CD86 (EC150 Reference Range in µg/mL)[2]** | **h-CLAT results for CD54 (EC200 Reference Range in µg/mL)[2]** |
|---|---|---|---|---|---|---|
| 2,4-Dinitrochlorobenzene | 97-00-7 | Solid | Sensitiser (extreme) | 2-12 | Positive (0.5-10) | Positive (0.5-15) |
| 4-Phenylenediamine | 106-50-3 | Solid | Sensitiser (strong) | 5-95 | Positive (<40) | Negative (>1.5)[3] |
| Nickel sulfate | 10101-97-0 | Solid | Sensitiser (moderate) | 30-500 | Positive (<100) | Positive (10-100) |
| 2-Mercaptbenzothiazole | 149-30-4 | Solid | Sensitiser (moderate) | 30-400 | Negative (>10)[3] | Positive (10-140) |
| R(+)-Limonene | 5989-27-5 | Liquid | Sensitiser (weak) | >20 | Negative (>5)[3] | Positive (<250) |
| Imidazolidinyl urea | 39236-46-9 | Solid | Sensitiser (weak) | 25-100 | Positive (20-90) | Positive (20-75) |
| Isopropanol | 67-63-0 | Liquid | Non-sensitiser | >5000 | Negative (>5000) | Negative (>5000) |
| Glycerol | 56-81-5 | Liquid | Non-sensitiser | >5000 | Negative (>5000) | Negative (>5000) |
| Lactic acid | 50-21-5 | Liquid | Non-sensitiser | 1500-5000 | Negative (>5000) | Negative (>5000) |
| 4-Aminobenzoic acid | 150-13-0 | Solid | Non-sensitiser | >5000 | Negative (>5000) | Negative (>5000) |

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

[1] The *in vivo* hazard and (potency) prediction is based on LLNA data (11) (20). The *in vivo* potency is derived using the criteria proposed by ECETOC (28).

[2] Based on historical observed values (19) (29).

[3] Historically, a majority of negative results have been obtained for this marker and therefore a negative result is mostly expected. The range provided was defined on the basis of the few historical positive results observed. In case a positive result is obtained, the EC value should be within the reported reference range.