

## **OECD GUIDELINE FOR THE TESTING OF CHEMICALS**

### **DRAFT PROPOSAL FOR A NEW TEST GUIDELINE**

#### **In Vitro Skin Sensitisation: U937 Cell Line Activation Test (U-SENS™)**

#### **INTRODUCTION**

1. A skin sensitizer refers to a substance that will lead to an allergic response following a secondary skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). This Test Guideline (TG) describes the *in vitro* procedure called U937 cell line activation Test (U-SENS™), to be used for supporting the discrimination between skin sensitizers and non-sensitizers in accordance with the UN GHS (1).

2. There is general agreement regarding the key biological events underlying skin sensitization. The current immunotoxicological view of the chemical and biological mechanisms associated with skin sensitization 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. In this instance, the molecular initiating event (i.e. the first key event) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell activation and proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (3).

3. The assessment of skin sensitization has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman, and the Buehler Test (TG 406) (4), assess both the induction and elicitation phases of skin sensitization. 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 sensitization.

4. More recently mechanistically-based *in chemico* (OECD TG 442C; Direct Peptide Reactivity Assay addressing the first key event of the skin sensitization AOP) (7), *in vitro* (OECD TG 442D; ARE-Nrf2 Luciferase Test Method addressing the second key event of the skin sensitization AOP) (8), and *in vitro* (OECD TG 442E; human Cell Line Activation Test (h-CLAT) addressing the third key event of the skin sensitization AOP) (9), test methods have been adopted for contributing to the evaluation of the skin sensitization 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)(10).

5. The U-SENS™ method is proposed to address the third key event of the skin sensitisation AOP by quantifying the change in the expression of a cell surface marker associated with the process of activation of monocytes and DC (i.e. CD86), in the human histiocytic lymphoma cell line U937, following exposure to sensitisers (11). The measured expression levels of CD86 cell surface marker in the cell line U937 is then used for supporting the discrimination between skin sensitisers and non-sensitisers.

6. The U-SENS™ method has been evaluated in a validation study and subsequently independent peer reviewed by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) Scientific Advisory Committee (ESAC) (12). Considering all available evidence and input from regulators and stakeholders, the U-SENS™ was recommended by EURL ECVAM (13) 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 U-SENS™ data in combination with other information, including historical data and eventually existing valid human data (14), are reported in the literature (15)(16)(17).

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 U-SENS™ which measure markers of monocyte activation and may be related to DC activation (18)(19)(20)(21)(22) 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 U-SENS™ 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 (17).

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 U-SENS™ may be used on its own to classify a chemical into UN GHS category 1.

10. The U-SENS™ 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 90% and 84% within and between laboratories, respectively (23). Results generated in the validation study (23) and other published studies (11) overall indicate that, compared with LLNA results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 86% (N=166) with a sensitivity of 91% (118/129) and a specificity of 65% (24/37). Compared with human results, the accuracy in distinguishing skin sensitisers (i.e. UN GHS Cat.1) from non-sensitisers is 77% (N=101) with a sensitivity of 100% (58/58) and a specificity of 47% (20/43). False negative predictions with the U-SENS™ 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) (11)(23)(24). Taken together, this information indicates the usefulness of the U-SENS™ method to contribute to the identification of skin sensitisation hazards. However, the accuracy

values given here for the U-SENS™ 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 humans.

11. The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the U-SENS™ to the testing of mono-constituent substances, multi-constituent substances and/or mixtures. On the basis of the data currently available, the U-SENS™ method was shown to be applicable to test chemicals (including cosmetics ingredients e.g. preservatives, surfactants, actives, dyes) covering a variety of organic functional groups, of physicochemical properties, skin sensitisation potency (as determined in *in vivo* studies) and the spectrum of reaction mechanisms known to be associated with skin sensitisation (i.e. Michael acceptor, Schiff base formation, acyl transfer agent, substitution nucleophilic bi-molecular (SN2), or nucleophilic aromatic substitution (SNAr) (11)(23)(24)(25). Limited information is currently available on the applicability of the U-SENS™ method to multi-constituent substances/mixtures (11). 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<sup>1</sup>. 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, consideration should be given to possible interference of cytotoxic constituents with the observed responses.

12. The U-SENS™ method is applicable to test chemicals that are 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 20). Chemicals in the dataset reported to be pre-haptens (i.e. substances activated by oxidation) or pro-haptens (i.e. substances requiring enzymatic activation for example via P450 enzymes) were correctly predicted by the U-SENS™ (11)(25). Membrane disrupting substances can lead to false positive results due to a non-specific increase of CD86 expression, as 3 out of 7 false positives relative to the *in vivo* reference classification were surfactants (11). As such positive results with surfactants should be considered with caution, negative results, however, could still be used to support the identification of the test chemical as a non-sensitiser. Fluorescent test chemicals can be assessed with the U-SENS™ (11), 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 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 U-SENS™ method to other specific categories of test chemicals, it should not be used for those specific categories.

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

---

<sup>1</sup> This sentence was proposed and agreed at the April 2014 WNT meeting

## PRINCIPLE OF THE TEST

14. The U-SENS™ method is an *in vitro* assay that quantifies changes of CD86 cell surface marker expression on a human histiocytic lymphoma cell line, U937 cells, following 45 hours exposure to the test chemical. The CD86 surface marker is one typical marker of U937 activation known to be a co-stimulatory molecule that may mimic monocytic activation, which plays a critical role in T-cell priming. The changes of CD86 cell surface marker expression are measured by flow cytometry following cell staining typically with fluorescein isothiocyanate (FITC)-labelled antibodies. Cytotoxicity measurement is also conducted (e.g. using PI) concurrently to assess whether upregulation of CD86 cell surface marker expression occurs at sub-cytotoxic concentrations. The stimulation index (S.I.) of CD86 cell surface marker compared to solvent/vehicle control is calculated and used in the prediction model (see paragraph 26), to support the discrimination between sensitisers and non-sensitisers.

## DEMONSTRATION OF PROFICIENCY

15. Prior to routine use of the test method described in this Test Guideline, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Annex II in compliance with the Good *in vitro* Method Practices (26). Moreover, test method users should maintain a historical database of data generated with the reactivity checks (see paragraph 18) and with the positive and solvent/vehicle controls (see paragraphs 22-23), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

## PROCEDURE

16. This Test Guideline is based on the U-SENS™ DataBase service on ALternative Methods to animal experimentation (DB-ALM) protocol 183 (27). The Standard Operating Procedures (SOP) should be employed when implementing and using the U-SENS™ method in the laboratory. An automated system to run the U-SENS™ can be used if it can be shown to provide similar results, for example by testing the proficiency substances in Annex II. The following is a description of the main components and procedures for the U-SENS™ method.

### *Preparation of cells*

17. The human histiocytic lymphoma cell line, U937 (28) should be used for performing the U-SENS™ method. It is recommended that cells (CRL1593.2) are obtained from a well-qualified cell bank such as the American Type Culture Collection and a licence agreement is established with the developer of this cell line, when used (28). Other U937 cellular clones may be used if sufficient scientific rationale is provided and if it can be shown to provide similar results, for example by testing the proficiency substances in Annex II.

18. U937 cells are cultured, at 37°C under 5% CO<sub>2</sub> and humidified atmosphere, in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (complete medium). U937 cells are routinely passaged every 2-3 days at the density of 1.5 or 3 × 10<sup>5</sup> cells/mL, respectively. The cell density should not exceed 2 × 10<sup>6</sup> cells/mL and the cell viability measured by trypan blue exclusion should be ≥ 90% (not to be applied at the first passage after thawing). Prior to using them for testing, every batch of cells, FCS or antibodies should be qualified by

conducting a reactivity check. The reactivity check of the cells should be performed using the positive control, picrylsulfonic acid (TNBS - 2,4,6-Trinitro-benzene-sulfonic acid) (CASRN 2508-19-2,  $\geq 99\%$  purity) and the negative control lactic acid (LA) (CASRN 50-21-5,  $\geq 85\%$  purity), at least one week after thawing. Six concentrations should be tested for each of the 2 controls (TNBS: 1-100 $\mu\text{g}/\text{mL}$  and LA: 1-200 $\mu\text{g}/\text{mL}$ ). TNBS at 50  $\mu\text{g}/\text{mL}$  solubilised in complete medium should produce a positive and concentration-related response of CD86, and LA at 200  $\mu\text{g}/\text{mL}$  solubilised in complete medium should produce negative response of CD86 (see paragraph 28). Only the batch of cells which passed the reactivity check 2 times should be used for the assay. Cells can be propagated up to seven weeks after thawing. Passage number should not exceed 21. The reactivity check should be performed according to the procedures described in paragraphs 25-29.

19. For testing, U937 cells are seeded at a density of either  $3 \times 10^5$  cells/mL or  $6 \times 10^5$  cells/mL, and pre-cultured in culture flasks for 2 days or 1 day, respectively. Other pre-cultured conditions than those described above may be used if sufficient scientific rationale is provided and if it can be shown to provide similar results, for example by testing the proficiency substances in Annex II. In the day of testing, cells harvested from culture flask are resuspended with fresh culture medium at  $5 \times 10^5$  cells/mL. Then, cells are distributed into a 96-well flat-bottom plate with 100  $\mu\text{L}$  (final cell concentration of  $0.5 \times 10^5$  cells/well

#### ***Preparation of test chemicals and control substances***

20. Assessment of solubility is conducted prior to testing. For this purpose, test chemicals are dissolved or stably dispersed at a concentration of 50 mg/mL in complete medium as first solvent option or dimethyl sulfoxide (DMSO,  $\geq 99\%$  purity) as a second solvent/vehicle option if the test chemical is not soluble in the previous solvent/vehicle. For the testing, the test chemical is dissolved to a final concentration of 0.4 mg/mL in complete medium if the chemical is soluble in this solvent/vehicle or if no suitable vehicle (neither complete medium nor DMSO) is found. If the chemical is soluble only in DMSO, the chemical is dissolved at a concentration of 50 mg/mL. Other solvents/vehicles than those described above may be used if sufficient scientific rationale is provided. Stability of the test chemical in the final solvent/vehicle should be taken into account.

21. The test chemicals and control substances are prepared on the day of testing. Because a dose finding assay is not conducted, for the first run, 6 concentrations should be tested (1, 10, 20, 50, 100 and 200  $\mu\text{g}/\text{mL}$ ) into the corresponding solvent/vehicle either in complete medium or in 0.4% DMSO in medium. Starting from the 0.4 mg/mL in complete medium or 50 mg/mL in DMSO, solutions of the test chemicals, at least 4 working solutions (at least 4 concentrations) are prepared using the corresponding solvent/vehicle. The concentrations (at least 4 concentrations) for any further run are chosen based on the individual results of all previous runs (23). The usable concentrations are 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180 and 200  $\mu\text{g}/\text{mL}$ . The maximum final concentration is 200  $\mu\text{g}/\text{mL}$ . In the case of a CD86 positive value at 1  $\mu\text{g}/\text{mL}$  is observed, then 0.1  $\mu\text{g}/\text{mL}$  is evaluated in order to try and find a negative concentration. For each run, the EC150 (concentration at which a chemical reaches the CD86 positive threshold of 150%, see paragraph 26) and CV70 (concentration at which a chemical reaches the cytotoxicity threshold of 70%, see paragraph 26) are calculated if applicable. To investigate the concentration response effect of CD86 increase, any concentrations from the usable concentrations should be chosen evenly spread between the EC150 (or the highest CD86 negative non cytotoxic concentration) and the CV70 (or the highest concentration allowed i.e. 200  $\mu\text{g}/\text{mL}$ ). A minimum of 4 concentrations should be tested per run with at least 2 concentrations being common with the previous run(s), for comparison purposes. The working solutions are finally used for treatment by adding an equal volume of U937 cell suspension (see paragraph 18 above) to the volume of working solution in plate to achieve a further 2-fold dilution (27).

22. The solvent/vehicle control used in the U-SENS™ method is complete medium (for test chemicals solubilised or stably dispersed) (see paragraph 12) or 0.4% DMSO in complete medium (for test chemicals solubilised or stably dispersed in DMSO).

23. The positive control used in the U-SENS™ method is TNBS (see paragraph 18), prepared in complete medium. TNBS should be used as the positive control for CD86 expression measurement at a final single concentration in plate (50 µg/mL) yielding > 70% of cell viability. To obtain a 50 µg/mL concentration of TNBS in plate, a 1 M (i.e. 293 mg/mL) stock solution of TNBS in complete medium is prepared and further diluted 2930-fold with complete medium to a 100 µg/mL working solution. Lactic acid (LA, CAS 50-21-5) should be used as the negative control at 200 µg/mL solubilised in complete medium (from a 0.4 mg/mL stock solution). In each plate of each run, three replicates of complete medium untreated control, solvent/vehicle control, negative and positive controls are prepared (27). Other suitable positive controls may be used if historical data are available to derive comparable run acceptance criteria. The run acceptance criteria are the same as described for the test chemical (see paragraph 29).

#### ***Application of test chemicals and control substances***

24. The solvent/vehicle control or working solutions described in paragraphs 21 and 22 are mixed 1:1 (v/v) with the cell suspensions prepared in the 96-well flat-bottom plate (see paragraph 19). The treated plates are then incubated for 45±3 hours at 37°C under 5% CO<sub>2</sub>. 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 with e.g. semi permeable membrane, prior to the incubation with the test chemicals (27).

#### ***Cell staining***

25. After 45±3 hours of exposure, cells are transferred into V-shaped microtiter plate and collected by centrifugation. Solubility interference is defined as crystals or drops observed under the microscope at 45 ± 3h post treatment (before the cell staining). The supernatants are discarded and the remaining cells are washed once with 100 µL of an ice-cold phosphate buffered saline containing 5 % foetal calf serum (staining buffer). After centrifugation, cells are re-suspended with 100 µL of staining buffer and stained with 5 µL of FITC-labelled anti-CD86 or mouse IgG1 (isotype) antibodies at 4°C for 30 min protected from light. The antibodies described in the U-SENS™ DB-ALM protocol 183 (27) should be used (for CD86: BD-PharMingen #555657 Clone: Fun-1, or Caltag/Invitrogen # MHCD8601 Clone: BU63; and for IgG1: BD-PharMingen #555748, or Caltag/Invitrogen # GM4992). Based on the experience of the test method developers, the fluorescence intensity of the antibodies is usually consistent between different lots (see paragraph 18). However, users may consider titrating the antibodies in their own laboratory's conditions to define the best concentration for use. Other detection system e.g., fluorochrome-tagged anti-CD86 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 U-SENS™ DB-ALM protocol may affect the results.

After washing with 100 µL of staining buffer two times and once with 100 µL of an ice-cold phosphate buffered saline, cells are resuspended in ice-cold PBS (e.g. 125 µL for samples being analysed manually tube by tube, or 50 µL using an auto-sampler plate) and PI solution is added (final concentration of 3 µg/mL). Other cytotoxicity markers, such as 7-Aminoactinomycin D (7-AAD) or Trypan blue 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.

**Flow cytometry analysis**

26. Expression level of CD86 and cell viability are analysed using flow cytometry. Cells are displayed within a size (FSC) and granularity (SSC) dot plot set to log scale in order to clearly identify the population in a first gate R1 and eliminate the debris. A targeting total of 10,000 cells in gate R1 are acquired for each well. Cells from the same R1 gate are displayed within a FL3 or FL4 / SSC dot plot. Viable cells are delineated by placing a second gate R2 selecting the population of propidium iodide-negative cells (FL3 or FL4 channel). The cell viability can be calculated using the following equation by the cytometer analysis program. When the cell viability is low, up to 20,000 cells including dead cells could be acquired. Alternatively, data can be acquired for one minute after the initiation of the analysis.

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

Percentage of FL1-positive cells is then measured among these viable cells gated on R2 (within R1). Cell surface expression of CD86 is analyzed in a FL1 / SSC dot plot gated on viable cells (R2). For the complete medium / IgG1 wells, the analysis marker is set close to the main population so that the complete medium controls have IgG1 within the target zone of 0.6 to 0.9%. Colour interference is defined as a shift of the FITC-labelled IgG1 dot-plot (IgG1 FL1 Geo Mean S.I.  $\geq$  150%).

The stimulation index (S.I.) of CD86 for controls cells (untreated or in 0.4% DMSO) and chemical-treated cells are calculated according to the following equation:

$$\text{S.I.} = \frac{\% \text{ of CD86}^+ \text{ treated cells} - \% \text{ of IgG1}^+ \text{ treated cells}}{\% \text{ of CD86}^+ \text{ control cells} - \% \text{ of IgG1}^+ \text{ control cells}} \times 100$$

% of IgG1+ untreated control cells: referred to as percentage of FL1-positive IgG1 cells defined with the analysis marker (accepted range of  $\geq$  0.6% and  $<$  1.5%, see paragraph 29) among the viable untreated cells.  
 % of IgG1+/CD86+ control/treated cells: referred to as percentage of FL1-positive IgG1/CD86 cells measured without moving the analysis marker among the viable control/treated cells.

**DATA AND REPORTING****Data evaluation**

27. The following parameters are calculated in the U-SENS<sup>TM</sup> test method: CV70 value, i.e. a concentration showing 70% of U937 cell survival (30% cytotoxicity) and the EC150 value, i.e. the concentration at which the test chemicals induced a CD86 stimulation index (S.I.) of 150%.

CV70 is calculated by log-linear interpolation using the following equation:

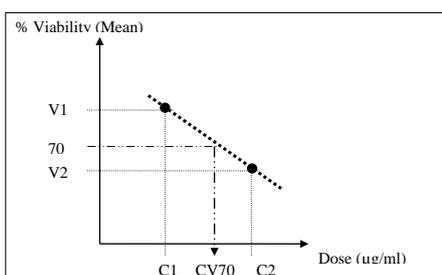
$$\text{CV70} = C1 + [(V1 - 70) / (V1 - V2) * (C2 - C1)]$$

Where:

V1 is the minimum value of cell viability over 70%

V2 is the maximum value of cell viability below 70%

C1 and C2 are the concentrations showing the value of cell viability V1 and V2 respectively.



Other approaches to derive the CV70 can be used as long as it is demonstrated that this has no impact on the results (e.g. by testing the proficiency substances).

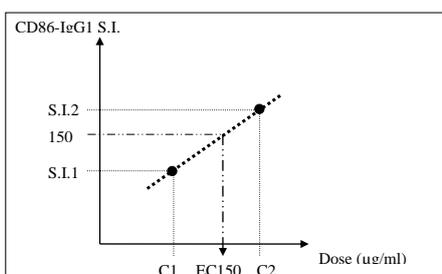
EC150 is calculated by log-linear interpolation using the following equation:

$$EC150 = C1 + [(150 - S.I.1) / (S.I.2 - S.I.1) * (C2 - C1)]$$

Where:

C1 is the highest concentration in  $\mu\text{g/mL}$  with a CD86 S.I. < 150% (S.I. 1)

C2 is the lowest concentration with a CD86 S.I.  $\geq$  150% (S.I. 2).



The EC150 and CV70 values are calculated

- for each run : the individual EC150 and CV70 values (if applicable) are used as tools to investigate the concentration response effect of CD86 increase (see paragraph 21),
- based on the average viabilities and S.I. of CD86 values: if applicable, the overall EC150 is determined for the test chemical predicted as POSITIVE with the U-SENS<sup>TM</sup> (see paragraph 28) and the overall CV70 is determined

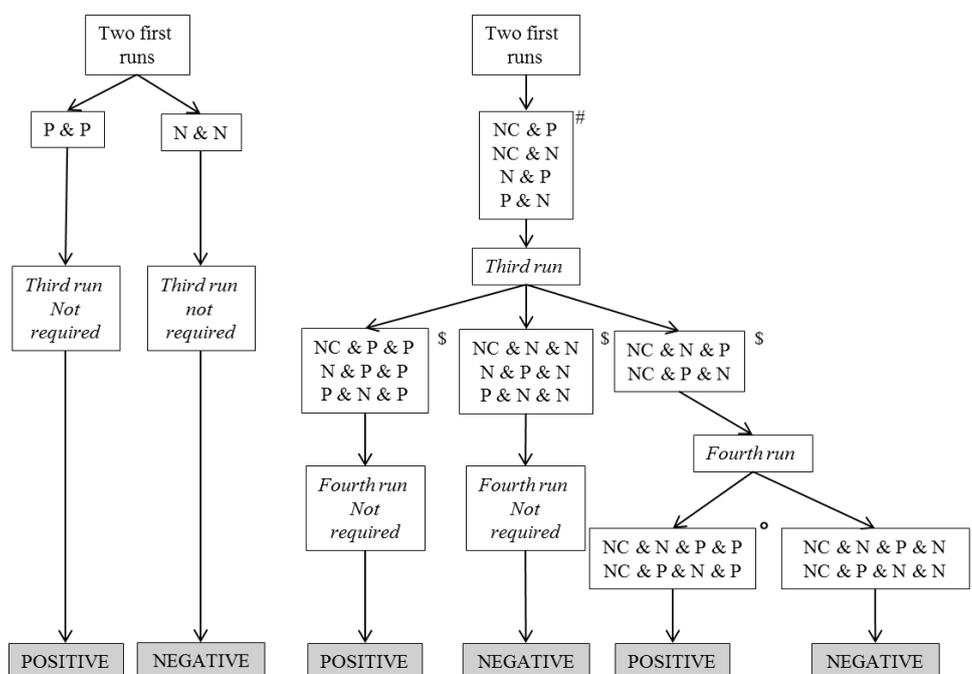
For the test chemical predicted as POSITIVE with the U-SENS<sup>TM</sup>, optionally, the EC150 value potentially could contribute to the assessment of sensitising potency (3) when used in integrated approaches such as IATA (15).

### ***Prediction model***

28. For CD86 expression measurement, each test chemical is tested in at least two independent runs (performed on a different day) to derive a single prediction (NEGATIVE or POSITIVE).

- The individual conclusion of an U-SENS<sup>TM</sup> run is considered Negative (hereinafter referred to as N) if the S.I. of CD86 is less than 150% at all non-cytotoxic concentrations (cell viability  $\geq$  70%) and if no interference (cytotoxicity, solubility: see paragraph 25 or colour: see paragraph 26) is observed. In all other cases: S.I. of CD86 higher or equal to 150% and/or interferences observed, the individual conclusion of an U-SENS<sup>TM</sup> run is considered Positive (hereinafter referred to as P).

- An U-SENS™ prediction is considered NEGATIVE if at least two independent runs are negative (N) (Figure 1). If the first two runs are both negative (N), the U-SENS™ prediction is considered NEGATIVE and a third run does not need to be conducted.
- An U-SENS™ prediction is considered POSITIVE if at least two independent runs are positive (P) (Figure 1). If the first two runs are both positive (P), the U-SENS™ prediction is considered POSITIVE and a third run does not need to be conducted.
- There is an exception if, in the first run, the S.I. of CD86 is higher or equal to 150% at the highest non-cytotoxic concentration only. The run is then concluded NO CONCLUSION (NC), and additional concentrations (between the highest non cytotoxicity concentration and the lowest cytotoxicity concentration - see paragraph 27) should be tested in additional runs. A run NC conducts automatically to the need of at least 2 more runs, and to a fourth run in case runs 2 and 3 are not concordant (N and/or P independently) (Figure 1). Follow up runs will be considered positive even if only one non cytotoxic concentration gives a CD86 equal or above 150%, since the dose setting has been adjusted for the specific test chemical. The final prediction will be based on the majority result of the three or four individual runs (i.e. 2 out of 3 or 2 out of 4) (Figure 1).



**Figure 1: Prediction model used in the U-SENS™ test method.** An U-SENS™ prediction should be considered in the framework of an IATA and in accordance with the provision of paragraphs 9, 11 and 12.

N: Run with no CD86 positive or interference observed;

P: Run with CD86 positive and/or interference(s) observed;

NC: First run with No Conclusion when CD86 is positive at the highest non-cytotoxic concentration only;

# : A No Conclusion (NC) individual conclusion attributed only to the first run conducts automatically to the need of a third run to reach a majority of Positive (P) or Negative (N) conclusions in at least 2 of 3 independent runs.

\$: 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.

°: The boxes show the relevant combinations of results from the four runs on the basis of the results obtained in the three first runs shown in the box above.

### ***Acceptance criteria***

29. The following acceptance criteria should be met when using the U-SENS™ method (27).

- At the end of the 45±3 h incubation treatment period, the mean viability of the triplicate untreated U937 cells had to be > 90% and no drift in CD86 expression is observed. The CD86 basal expression of untreated U937 cells had to be comprised within the range of ≥ 2% and ≤ 25%.
- When DMSO is used as a solvent, the validity of the DMSO vehicle control is assessed by calculating a DMSO S.I. compared to untreated cells, and the mean viability of the triplicate cells had to be > 90%. The DMSO vehicle control is valid if the mean value of its triplicate CD86 S.I. was smaller than 250% of the mean of the triplicate CD86 S.I. of untreated U937 cells.
- The runs are considered valid if at least two out of three IgG1 values of untreated U937 cells fell within the range of ≥ 0.6% and < 1.5%.
- The concurrent tested negative control (lactic acid) is considered valid if at least two out of the three replicates were negative (CD86 S.I. < 150%) and non-cytotoxic (cell viability ≥ 70%).
- The positive control (TNBS) was considered as valid if at least two out of the three replicates were positive (CD86 S.I. ≥ 150%) and non-cytotoxic (cell viability ≥ 70%).

### ***Test report***

30. 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, complete medium 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, complete medium 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, 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, 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 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 and CD86 S.I values obtained with the solvent/vehicle control in comparison to the acceptance ranges;
- Cell viability and S.I. 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;
- Description of evaluation and decision criteria used;
- Description of any modifications of the test procedure.

#### *Results*

- Tabulation of the data, including CV70 (if applicable), S.I., cell viability values, EC150 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 U-SENS™ method;
- Consideration of the test method results within the context of an IATA, if other relevant information is available.

#### *Conclusions*

## LITERATURE

1. United Nations UN (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Fifth revised edition. New York & Geneva: United Nations Publications. ISBN: 978-92-1-117006-1. Available at: [[http://www.unece.org/trans/danger/publi/ghs/ghs\\_rev05/05files\\_e.html](http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html)].
2. OECD. (2012). Series on Testing and Assessment No. 168: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
3. OECD. (2010). Guideline for the Testing of Chemicals No. 429: The Local Lymph Node Assay. Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
4. OECD. (1992). Guideline for the Testing of Chemicals No. 406: Skin Sensitisation. Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
5. OECD. (2010). Guideline for the Testing of Chemicals No. 442A: Skin Sensitisation: Local Lymph Node Assay: DA. Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
6. OECD. (2010). Guideline for the Testing of Chemicals No. 442B: Skin sensitisation: Local Lymph Node Assay: BrdU-ELISA. Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
7. OECD. (2015). Guideline for the Testing of Chemicals No. 442C: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA). Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
8. OECD. (2015). Guideline for the Testing of Chemicals No. 442D: *In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method. Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
9. OECD. (2016). Guideline for the Testing of Chemicals No. 442E: *In Vitro* Skin Sensitisation: human Cell Line Activation Test (h-CLAT). Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
10. Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., Andersen, K.E., Angers-Loustau, A., Aptula, A., Bal-Price, A., Benfenati, E., Bernauer, U., Bessems, J., Bois, F.Y., Boobis, A., Brandon, E., Bremer, S., Broschard, T., Casati, S., Coecke, S., Corvi, R., Cronin, M., Daston, G., Dekant, W., Felner, S., Grignard, E., Gundert-Remy, U., Heinonen, T., Kimber, I., Kleinjans, J., Komulainen, H., Kreiling, R., Kreysa, J., Leite, S.B., Loizou, G., Maxwell, G., Mazzatorta, P., Munn, S., Pfuhler, S., Phrakonkham, P., Piersma, A., Poth, A., Prieto, P., Repetto, G., Rogiers, V., Schoeters, G., Schwarz, M., Serafimova, R., Tähti, H., Testai, E., van Delft, J., van Loveren, H., Vinken, M., Worth, A., Zaldivar, J.M. (2011). Alternative (non-animal) methods for cosmetics testing: current status and future prospects. *Archives of Toxicology* 85, 367-485.
11. Piroird, C., Ovigne, J.M., Rousset, F., Martinozzi-Teissier, S., Gomes, C., Cotovio, J., Alépée, N. (2015). The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization. *Toxicol. In Vitro* 29, 901-916.
12. EC EURL ECVAM. (2016). ESAC Opinion No. 2016-03 on the L'Oréal-coordinated study on the transferability and reliability of the U-SENS™ test method for skin sensitisation testing. EUR 28178

EN; doi 10.2787/815737. Available at:  
[<http://publications.jrc.ec.europa.eu/repository/handle/JRC103705>].

13. EC EURL ECVAM. (2016). Recommendation on the U937 Skin Sensitisation Test (U-SENS™) for skin sensitisation testing. Accessible at: in preparation.
14. Steiling, W. (2016). Safety Evaluation of Cosmetic Ingredients Regarding their Skin Sensitization Potential. doi:10.3390/cosmetics3020014. *Cosmetics* 3, 14.
15. OECD (2016). Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation- ENV/JM/HA(2016)11. Organisation for Economic Cooperation and Development, Paris. Available at: [<https://community.oecd.org/community/iatass>].
16. Urbisch, D., Mehling, A., Guth, K., Ramirez, T., Honarvar, N., Kolle, S., Landsiedel, R., Jaworska, J., Kern, P.S., Gerberick, F., Natsch, A., Emter, R., Ashikaga, T., Miyazawa, M., Sakaguchi, H. (2015). Assessing skin sensitization hazard in mice and men using non-animal test methods. *Regul. Toxicol. Pharmacol.* 71, 337-351.
17. OECD. (2016). Series on Testing & Assessment No. 256: Guidance Document On The Reporting Of Defined Approaches And Individual Information Sources To Be Used Within Integrated Approaches To Testing And Assessment (IATA) For Skin Sensitisation, Annex 1 and Annex 2. ENV/JM/HA(2016)29. Organisation for Economic Cooperation and Development, Paris. Available at: [<https://community.oecd.org/community/iatass>].
18. Aiba, S., Terunuma, A., Manome, H., Tagami, H. (1997). Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol.* 27, 3031–3038.
19. Coutant, K.D., de Fraissinette, A.B., Cordier, A., Ulrich, P. (1999). Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. *Toxicol. Sci.* 52, 189–198.
20. Rousset, F., Verda, D., Arrighi, J. F., Peguet-Navarro, J., Cottin, M. (2000). A strategy for the prediction of skin sensitization: An alternative to animal testing. Proceedings of the 3rd World Congress on Alternatives and Animal Use in the Life Sciences. *Progress in the Reduction, Refinement and Replacement of Animal Experimentation* 31, 635–642.
21. Python, F., Goebel, C., Aeby, P. (2007). Assessment of the U937 cell line for the detection of contact allergens. *Toxicol. Appl. Pharmacol.* 220, 113-124.
22. Ade, N., Martinozzi-Teissier, S., Pallardy, M., Rousset, F. (2006). Activation of U937 cells by contact sensitizers: CD86 expression is independent of apoptosis. *J. Immunotoxicol.* 3,189-197.
23. Alépée, N., Piroird, C., Aujoulat, M., Dreyfuss, S., Hoffmann, S., Hohenstein, A., Meloni, M., Nardelli, L., Gerbeix, C., Cotovio, J. (2015). Prospective multicentre study of the U-SENS test method for skin sensitization testing. *Toxicol In Vitro* 30, 373-382.
24. Reisinger, K., Hoffmann, S., Alépée, N., Ashikaga, T., Barroso, J., Elcombe, C., Gellatly, N., Galbiati, V., Gibbs, S., Groux, H., Hibatallah, J., Keller, D., Kern, P., Klaric, M., Kolle, S., Kuehnl, J., Lambrechts, N., Lindstedt, M., Millet, M., Martinozzi-Teissier, S., Natsch, A., Petersohn, D., Pike, I., Sakaguchi, H., Schepky, A., Tailhardat, M., Templier, M., van Vliet, E., Maxwell, G. (2014). Systematic evaluation of non-animal test methods for skin sensitisation safety assessment. *Toxicol. In Vitro* 29, 259-270.
25. Fabian, E., Vogel, D., Blatz, V., Ramirez, T., Kolle, S., Eltze, T., van Ravenzwaay, B., Oesch, F., Landsiedel, R. (2013). Xenobiotic metabolizing enzyme activities in cells used for testing skin sensitization *in vitro*. *Arch. Toxicol.* 87, 1683-1696.

26. OECD. (2016). Draft Guidance document: Good *In Vitro* Method Practices (GIVIMP) for the Development and Implementation of *In Vitro* Methods for Regulatory Use in Human Safety Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [[www.oecd.org/env/ehs/testing/OECD\\_Draft\\_GIVIMP\\_in\\_Human\\_Safety\\_Assessment.pdf](http://www.oecd.org/env/ehs/testing/OECD_Draft_GIVIMP_in_Human_Safety_Assessment.pdf)].
27. DB-ALM. (2016). Protocol 183: Myeloid U937 Skin Sensitization Test (U-SENS™), 33pp. Accessible at: [<http://ecvam-dbalm.jrc.ec.europa.eu/>].
28. Sundström, C., Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* 17, 565-577.
29. OECD. (2005). Series on Testing and Assessment No. 34: Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [<http://www.oecd.org/env/testguidelines>].
30. ECETOC. (2003). Technical Report No. 87: Contact sensitization: Classification according to potency. European Centre for Ecotoxicology & Toxicology of Chemicals, Brussels. Available at: [[https://ftp.cdc.gov/pub/Documents/OEL/06.%20Dotson/References/ECETOC\\_2003-TR87.pdf](https://ftp.cdc.gov/pub/Documents/OEL/06.%20Dotson/References/ECETOC_2003-TR87.pdf)].

## 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 (29).

**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).

**CD86 Concentration response:** There is concentration-dependency (or concentration response) when a positive concentration (CD86 S.I.  $\geq 150$ ) is followed by a concentration with an increasing CD86 S.I.

**CV70:** The estimated concentration showing 70% cell viability.

**Drift:** A drift is defined by i) the corrected %CD86<sup>+</sup> value of the untreated control replicate 3 is less than 50% of the mean of the corrected %CD86<sup>+</sup> value of untreated control replicates 1 and 2; and ii) the corrected %CD86<sup>+</sup> value of the negative control replicate 3 is less than 50% of mean of the corrected %CD86<sup>+</sup> value of negative control replicates 1 and 2.

**EC150:** the estimated concentrations showing the 150% S.I. of CD86 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.

**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.

**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  $\geq 10\%$  (w/w) and  $< 80\%$  (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained by blending of two or more substances without chemical reaction. A

multi-constituent substance is the result of a chemical reaction.

**Positive control:** A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

**Pre-haptens:** chemicals which become sensitisers through abiotic transformation, e.g. through oxidation.

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

**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 (29).

**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 (29).

**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 (29).

**S.I.:** Stimulation Index. Relative values of geometric mean fluorescence intensity in chemical-treated cells compared to solvent-treated cells.

**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 (29).

**Staining buffer:** A phosphate buffered saline containing 5% foetal calf serum.

**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.

**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 (29).

**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 U-SENS™ prediction for the 10 substances recommended in Table 1 and by obtaining CV70 and EC150 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 U-SENS™ method are available. Also, published reference data are available for the U-SENS™ method (11) (23).

Table 1: Recommended substances for demonstrating technical proficiency with the U-SENS™ method

Proficiency substances	CASRN	Physical state	<i>In vivo</i> prediction <sup>1</sup>	U-SENS™ Solvent/ Vehicle	U-SENS™ CV70 Reference Range in $\mu\text{g/mL}^2$	U-SENS™ EC150 Reference Range in $\mu\text{g/mL}^2$
4-Phenylenediamine	106-50-3	Solid	Sensitiser (strong)	Complete medium <sup>3</sup>	<30	Positive ( $\leq 10$ )
Picryl sulfonic acid	2508-19-2	Liquid	Sensitizer (strong)	Complete medium	>50	Positive ( $\leq 50$ )
Diethyl maleate	141-05-9	Liquid	Sensitiser (moderate)	DMSO	10-100	Positive ( $\leq 20$ )
Resorcinol	108-46-3	Solid	Sensitiser (moderate)	Complete medium	>100	Positive ( $\leq 50$ )
Cinnamic alcohol	104-54-1	Liquid	Sensitiser (weak)	DMSO	>100	Positive (10-100)
4-Allylanisole	140-67-0	Liquid	Sensitiser (weak)	DMSO	>100	Positive (<200)
Saccharin	81-07-2	Solid	Non-sensitiser	DMSO	>200	Negative (>200)
Glycerol	56-81-5	Liquid	Non-sensitiser	Complete medium	>200	Negative (>200)
Lactic acid	50-21-5	Liquid	Non-sensitiser	Complete medium	>200	Negative (>200)
Salicylic acid	69-72-7	Solid	Non-sensitiser	DMSO	>200	Negative (>200)

Abbreviations: CAS RN = Chemical Abstracts Service Registry Number

<sup>1</sup> The *in vivo* hazard and (potency) prediction is based on LLNA data (11)(23). The *in vivo* potency is derived using the criteria proposed by ECETOC (30).

<sup>2</sup> Based on historical observed values (11)(23).

<sup>3</sup> Complete medium: RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin and 100  $\mu\text{g/mL}$  streptomycin (23).