

Skin sensitisation

A. TITLE OF THE TEST GUIDELINES (YEAR OF APPROVAL)

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA), **OECD 442C** (adopted 2015)

In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method, **OECD 442D** (adopted 2015)

In Vitro Skin Sensitisation: Human Cell Line Activation Test (h-CLAT) (draft OECD TG under discussion, EURL ECVAM recommendation published 2015)

In vivo tests:

Skin sensitisation: Local Lymph Node Assay (LLNA), **EU B.42, OECD 429** (adopted by OECD 2002, revised by OECD 2010)

Skin Sensitisation: Local Lymph Node Assay: DA, **OECD 442A** (2010)

Skin Sensitisation: Local Lymph Node Assay: BrdU-ELISA, **OECD 442B** (2010)

Skin Sensitisation, **EU B.6, OECD 406** (adopted by OECD 1981, revised by OECD 1992)

Note: In all cases, the most recent version of the test guideline should be used.

B. OECD GUIDELINES FOR TESTING OF CHEMICALS

» <http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>

C. EC TEST METHODS REGULATION

» <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32008R0440:en:NOT>

D. WHICH REACH INFORMATION REQUIREMENTS MAY BE MET WITH THE TEST(S)?

Currently, *in vivo* testing is the standard information requirement for skin sensitisation of Annex VII, section 8.3 to the REACH Regulation (i.e. usually LLNA). It may be possible to adapt the standard information

requirement of Annex VII by alternative methods, if conditions specified under the specific rules of adaptation (column 2) or general rules of adaptation (Annex XI) are met (see the next chapter “How to use these non-animal tests)”

***In chemico/in vitro* methods**

Test method OECD 442C - **Direct Peptide Reactivity Assay (DPRA)** is an *in chemico* test method which addresses peptide reactivity, postulated to be the molecular initiating event (the first key event) of the skin sensitisation adverse outcome pathway (AOP) (OECD 2012). Reactivity is measured by quantifying how much of the substance being tested does not bind to the synthetic heptapeptides containing either cysteine or lysine.

Test method OECD 442D - **ARE-Nrf2 Luciferase Test Method (KeratinoSens™)** is an *in vitro* test method which addresses keratinocyte induction of cyto-protective gene pathways linked to skin sensitisation, i.e. the second key event of the skin sensitisation AOP (OECD 2012). The test method uses luminescence detection to measure gene expression of the antioxidant/electrophile response element (ARE)-dependent pathway.

Test method - **human Cell Line Activation Test (h-CLAT)** is an *in vitro* method which addresses the third key event of the skin sensitisation AOP (OECD 2012), i.e. activation of the dendritic cells. The assay measures quantitatively changes in the expression of cell surface markers, associated with the activation of dendritic cells i.e. CD86 and CD54, in a human monocytic leukaemia cell line by flow cytometry analysis. At present, there is an EURL ECVAM recommendation and a draft OECD test guideline available.

These tests cover specific key events within the skin sensitisation AOP, which is a sequence of events from the molecular initiating event(s) to the adverse outcome(s) in the whole organism (OECD 2012).

However, none of these three *non-animal* methods, DPRA, KeratinoSens™ or h-CLAT should be used alone. They should always be considered in combinations and/or with other information in the context of integrated approaches such as weight of evidence (WoE) or testing and assessment strategies. This is especially the case, when negative predictions are obtained.

As the test methods each have limitations that may lead into false negative predictions, several non-animal testing methods should be used in combination within a weight of evidence approach.

Complementary information may be derived from *chemico/in vitro* assays addressing other biological mechanisms of the AOP than those covered by the three methods described above and/or from non-testing methods (e.g. *in silico* approaches to assess skin metabolism).

The confidence on the use of these methods if used in combination is supported by the scientifically accepted biological mechanisms described in the skin sensitisation AOP, which has been accepted by the OECD member countries.

***In vivo* methods**

Test method B.42 / OECD 429 - **Local Lymph Node Assay (LLNA)** provides information on the potential of chemicals to induce sensitisation as a function of lymphocyte proliferative responses induced in regional lymph nodes in mice. Due to clear animal welfare benefits and scientific advantages compared with the guinea pig tests (e.g. guinea pig maximisation test (GPMT)), the LLNA is the preferred test method where it is justified that an *in vivo* test is necessary. The standard guinea pig tests should only be used in exceptional circumstances and would require scientific justification.

Test method B.6 / OECD 406 – **Skin Sensitisation** i.e. the GPMT and the Buehler test provide information on skin sensitisation potential by measuring challenge-induced dermal hypersensitivity reactions elicited in test animals compared with controls. The test guideline specifies guinea pigs as the species for testing. The GPMT test is an adjuvant test in which the acquisition of sensitisation is potentiated by the use of Freund's complete adjuvant (FCA) and in which both intradermal and topical exposure are used during the induction phase. The Buehler test is a non-adjuvant test involving topical application only.

E. HOW TO USE THESE NON-ANIMAL TEST METHODS

Note: The REACH Guidance R7a is under revision.

It is recognised that due to the complexity of the skin sensitisation endpoint no single non-animal test method would be able to provide information that would fully substitute the animal tests currently in use.

The *in vitro* and *in chemico* test methods described below have not been developed for use as stand-alone methods due to a lack of or limited metabolic capacity that may lead to potential false negative predictions. Therefore, a combination of these non-animal methods (e.g. *in silico*, *in chemico* and *in vitro*) should be used within a weight of evidence approach.

Information that may complement the weight of evidence may be derived from test methods addressing other biological mechanisms on the basis of skin sensitisation or non-testing methods e.g. read-across.

There are steps to be taken before any testing (*in vitro* or *in vivo*) is conducted. The steps are specified in the introductory paragraph to Annex VII and in column 1 of section 8.3 of the REACH Regulation and include:

- Assessing all available *in vitro* data, *in vivo* data, historical human data, data from valid (Q)SARs and data from structurally related substances (read-across approach).

Testing does not need to be conducted if the conditions specified in column 2 of Annex VII, section 8.3 of the REACH Regulation are present and include:

- Available information indicating that the substance should be classified for skin corrosion or skin sensitisation, or
- Substance is a strong acid ($\text{pH} \leq 2.0$) or base ($\text{pH} \geq 11.5$), or
- Substance is (spontaneously) flammable in air at room temperature.

Due to recent developments in the non-animal testing methods for skin sensitisation, registrants are recommended to explore in line with Article 13(1) and the introductory paragraph to Annex VII of the REACH Regulation whether the information requirement for skin sensitisation can be fulfilled by using results from the newly developed test methods in a weight of evidence approach.

It is important to note, that it is the registrant's responsibility to make sure that the chosen non-animal test methods (e.g. *in vitro*, *in chemico* or *in silico*) are suitable for the substance in order to obtain adequate information. For example, there may be limitations such as low solubility or log Kow of the test substance

that would hinder the use of a particular *in vitro* method.

The main limitations of the *in chemico* or *in vitro* methods are related to the absence of, or limited, metabolic capacity of the test system and hence pre- and pro-haptens (chemicals activated by auto oxidation or chemicals requiring enzymatic activation to exert their sensitisation activity, respectively) may not be correctly identified and therefore, in the case of a negative outcome the prediction may be a false negative.

When the non-animal testing methods are used to fulfil the Annex VII information requirement for skin sensitisation, the registrant should provide a case-specific justification on why and how the *in vitro* data, taken within a weight of evidence approach, can be used to cover the REACH information requirement. In that weight of evidence justification, e.g. coverage of the key events (see above), quality and reliability of data (positive evidence has more weight than the negative), limitations and the scope of each test methods used, and consistency of the results need to be considered. The legal provisions on weight of evidence are given in Annex XI, 1.2 of the REACH Regulation and further guidance can be found in the ECHA Guidance R.4.4.

ECHA notes that it may assess the compliance with the information requirements under dossier evaluation (Title VI of the REACH Regulation), and that any adaptations will be assessed against its criteria on a case-by-case basis.

The OECD Guidance Document on Skin Sensitisation Integrated Approaches to Testing and Assessment (IATA) (under preparation) aims to give a harmonised approach for the reporting of an AOP-based IATA.

Within such an AOP-based IATA, the different types of information would target key events along the defined toxicity pathway and the results used to inform a regulatory decision. The future update of ECHA Guidance R.7a aims to give more detailed advice on how these AOP based non-animal testing methods can be used for REACH purposes. The updated R.7a Guidance is planned to be published before summer 2016 i.e. well in advance of the 2018 registration deadline.

The use of positive predictions obtained from *in chemico/in vitro* test methods tends to be more straightforward than when negative or conflicting predictions are obtained. Due to the specific limitations of each of the *in chemico/in vitro* test methods, if a negative prediction has been obtained, it is important to justify in the dossier how a potential false prediction could be ruled out. Supporting information might be a consideration of whether the substance is or is not a pre- or pro-hapten and whether metabolism is expected to occur.

F. STATUS OF THE VALIDATION BY EURL ECVAM OR OTHER BODIES, WHERE NECESSARY

All of the tests were evaluated in validation studies for transferability and reproducibility and recommended by EURL ECVAM to be used as part of a testing and assessment strategy.

Reference to the relevant guidance

1. Information toolkit

» <http://echa.europa.eu/en/support/information-toolkit>

This website provides practical information and tools to help use existing information and non-test methods as a first step to meeting the REACH information requirements.

2. Guidance on information requirements and chemical safety assessment (ECHA Guidance R7a)

» http://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf

Note: The REACH Guidance R7a is under revision.

3. Guidance on information requirements and chemical safety assessment (ECHA Guidance R.4)

» http://echa.europa.eu/documents/10162/13643/information_requirements_r4_en.pdf

4. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 16 (OECD 2012)

» [http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO\(2012\)10/PART1&docLanguage=En](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)10/PART1&docLanguage=En)

5. Practical guides e.g. how to report in vitro data (PG1) and how to avoid unnecessary testing on animals (PG10)

» <http://echa.europa.eu/practical-guides>

6. Webinar on “How to use in vitro data to fulfil REACH information requirements” held on 29 February 2012

» http://echa.europa.eu/view-article/-/journal_content/7def3c04-4b2b-4cfd-86d0-5ce36797faa8

7. Tracking system for alternative test methods review, validation and approval in the context of EU regulations on chemicals (TSAR)

» <http://tsar.jrc.ec.europa.eu/>

This website provides information on the validation and adoption status of alternative tests, whether the test method is a replacement and in which context the method should be used.

8. EURL ECVAM - validation and regulatory acceptance

» http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/validation-regulatory-acceptance

This website provides information on the validation and regulatory acceptance status of alternative methods including information on the validation studies.

9. EURL ECVAM recommendations

» <https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations>

G. THE SPECIFIC SCOPE OF THE TEST METHOD, I.E. LIMITATION ON CHEMICAL CATEGORIES COVERED, IF ANY, AND LIMITATIONS FOR CLASSIFICATION AND LABELLING

All methods listed below address a specific key event as identified in the skin sensitisation AOP. The Direct Peptide Reactivity Assay (OECD 442C) addresses key event 1; the ARE-Nrf2 Luciferase Test Method (OECD 442D) addresses key event 2; and the h-CLAT test method addresses key event 3. More information can be obtained from the OECD TGs (OECD 442C and D) and from the EURL ECVAM recommendations (DPRA, Keratinosens and h-CLAT).

H. DIRECT PEPTIDE REACTIVITY ASSAY (DPRA), OECD 442C

- Information obtained from this test method should be used in combination with other information within a weight of evidence approach and not as a stand-alone test method.
- Can be used to support the discrimination between sensitisers and non-sensitisers; currently the test method is not suitable for sub-categorisation of skin sensitisers into classification sub-categories 1A and 1B. However, work is ongoing to see whether sub-categorisation would be feasible within an IATA.
- A test chemical should be soluble in an appropriate solvent at a final concentration of 100 mM. However, test chemicals that are not soluble at this concentration may still be tested at lower soluble concentrations and in such a case positive results could be used to identify a test chemical as a sensitiser. In the case of negative prediction (lack of reactivity), no firm conclusion should be drawn.
- The method is not applicable for the testing of metal compounds (known to react with proteins with mechanisms other than covalent binding) or for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances) due to the unknown and/or variable composition of the test substance as the defined molar ratio of the test chemical and peptide is needed for the assessment of the test results.
- The test system has no metabolic capacity and therefore pro-haptens (i.e. chemicals requiring enzymatic activation to exert their sensitisation activity) cannot be detected in this assay. Pre-haptens (i.e. chemicals activated by auto oxidation) may provide (false) negative results.
- Test chemicals with preferential reactivity towards amino acids other than cysteine or lysine (e.g. nucleophilic sites in histidine), may lead to false negative results. However, when considering this limitation, it should be also kept in mind that the relative percentages of substances reacting preferably with amino acids other than cysteine and lysine is at present unclear and that the cysteine and lysine peptides represent different types of nucleophiles which would cover different reaction mechanisms.
- Potential false positive predictions may be obtained due to chemicals that do not covalently bind to peptide but do promote its oxidation (i.e., cysteine dimerisation).

I. ARE-NRF2 LUCIFERASE TEST METHOD (KERATINSENS™), OECD 442D

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach and not as a stand-alone test method.
- Can be used to support the discrimination between sensitisers and non-sensitisers; currently the test method is not suitable to sub-categorise skin sensitisers into classification sub-categories 1A and 1B. However, work is ongoing to see whether sub-categorisation would be feasible within an IATA.
- The test method is applicable to test chemicals that are soluble or that form a stable dispersion either in water or DMSO. The highest concentration required in the test method is 2 000 µM. However, if the highest concentration of 2 000 µM cannot be obtained e.g. due to limited solubility or cytotoxic properties of the test chemical, lower concentrations can be used. Negative results obtained with concentrations < 1 000 µM should be considered as inconclusive.
- The test system has a limited metabolic capacity and therefore pro-haptens (i.e. chemicals requiring

enzymatic activation to exert their sensitisation activity) may provide (false) negative results. Pre-haptens (i.e. chemicals activated by auto oxidation), especially those with a low oxidation rate, may also result in (false) negative results.

- Substances with exclusive reactivity towards other nucleophiles than the cysteine sulfhydryl group (e.g. lysine-residues) can be detected as negative in the assay.
- Test chemicals that do not act as a sensitiser but are nevertheless chemical stressors may lead to false positive results.
- Highly cytotoxic chemicals within the test systems cannot always be reliably assessed as the viability of the cells needs to be ≥ 70 %.
- Substances that interfere with the luciferase enzyme can affect the luciferase activity either by increasing (e.g. phytoestrogens) or inhibiting the luminescence.

J. HUMAN CELL LINE ACTIVATION TEST (H-CLAT) (DRAFT OECD TG UNDER DISCUSSION)

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach and not as a stand-alone test method.
- Can be used to support the discrimination between sensitisers and non-sensitisers; currently the test method is not suitable to sub-categorise skin sensitisers into sub-categories 1A and 1B. However, work is ongoing to see whether sub-categorisation would be feasible within an IATA.
- Applicable to test chemicals soluble or that form a stable dispersion in an appropriate solvent.
- Substances with Log Kow up to 3.5 can be tested whereas substances with Log Kow higher than 3.5 tend to produce negative results. For such substances, positive results could be used to support the identification of a test chemical as a sensitiser. Negative results should be considered as inconclusive.
- The test system has a limited metabolic capacity and therefore pro-haptens (i.e. chemicals requiring enzymatic activation to exert their sensitisation activity) may provide (false) negative results. Pre-haptens (i.e. chemicals activated by auto oxidation) may also provide (false) negative results.
- Highly cytotoxic chemicals cannot always be reliably assessed as the viability of the cells needs to be ≥ 50 %.
- Since the h-CLAT uses a fluorescein isothiocyanate (FITC)-labelled antibody, strong fluorescent test chemicals emitting at the same wavelength as FITC may interfere with the flow cytometry light-signal acquisition.

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