How to use new or revised *in vitro* test methods to address skin sensitisation

(Revised in February 2018)

WHICH OF THE REACH INFORMATION REQUIREMENTS MAY BE MET WITH THE TEST(S)

Annex VII of the REACH Regulation includes a requirement for *in chemico*/*in vitro* tests as a first step for addressing skin sensitisation (section 8.3.1). Only in the case that the *in chemico*/*in vitro* methods are not applicable for the substance, or the results are not adequate for classification and risk assessment, can an *in vivo* skin sensitisation study (preferably Local Lymph Node Assay, EU B.42 / OECD TG 429) be performed (section 8.3.2). An overview of the available internationally validated *in chemico*/*in vitro* methods is presented in Table 1.

Those methods can be used to meet the REACH information requirements for a specific key event, as specified in section 8.3.1. The methods have often limitations and cannot be used for all kinds of substances. Therefore, registrants and test houses are advised to check the chapter "Specific scope and limitations of the *in chemico*/*in vitro* tests" below, before deciding on a new test/study.

The *in chemico*/*in vitro* test methods can be summarised as follows:

These tests described below cover specific key events within the skin sensitisation adverse outcome pathway (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 five non-animal methods – DPRA, KeratinoSens™, h-CLAT, U-SENS™ or IL-8 Luc Assay – should be used alone but always be considered in combinations and/or with other information. The tests results should be integrated, normally under a *weight-of-evidence* approach.

Complementary information may be derived from e.g. *in silico* approaches to assess skin metabolism. In addition, information obtained from analogue substance e.g. via OECD QSAR Toolbox may be helpful in determining the skin sensitisation potency of the substance, however a justification of the analogue substances to support the prediction needs to be provided.

Test method EU B.59 / OECD TG 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 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 EU B.60 / OECD 442D – ARE-Nrf2 Luciferase Test Method (KeratinoSens™) is an *in vitro* test method which addresses keratinocyte induction of a 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 antioxidant/electrophile response element (ARE)-dependent pathways. Additional test method LuSens has gone through validation and is being considered in the OECD test guideline programme. The LuSens test method is based on the same principle as the KeratinoSens™ method as it measures the activation of the Nrf2-KEAP1 pathway.

Test method OECD TG 442E – *in vitro* skin sensitisation assays addressing AOP key event 3 – activation of dendritic cells contains currently three different test methods that address the activation of dendritic cells (DC), i.e. the third key event of the skin sensitisation AOP. The test methods included in the test guideline are (i) human cell line activation test (h-CLAT) that measures the expression of specific cell surface markers linked to DC maturation i.e. CD86 and CD54 by using flow cytometry; (ii) U937 cell line activation test (U-SENS™) that measures the expression of specific cell surface marker CD86; and (iii) Interleukin-8 Reporter Gene Assay (IL-8 Luc Assay) that measures changes in a cytokine linked to activation of DCs by measuring induction of IL-8 mRNA.

Table 1: Summary of the available *in chemico*/*in vitro* skin sensitisation test methods.

Latest update	AOP key event measured	Test method	Validation status, regulatory acceptance	EU test method / OECD Test Guideline	Outcome according to the test method/guideline	EURL ECVAM DB-ALM protocol number
Skin sensitisation						
2015	Key event 1 (peptide/protein binding	DPRA	Validated and regulatory acceptance	B.59 / OECD TG 442C	SS or NS with complementary information	154
2015	Key event 2 (keratinocyte response)	KeratinoSens™	Validated and regulatory acceptance	B.60 / OECD TG 442D	SS or NS with complementary information	155
		LuSens	Validated/under regulatory review	N.A. / draft TG available	SS or NS with complementary information	184
2017	Key event 3 (monocytic/dendritic cell response)	h-CLAT	Validated and regulatory acceptance	N.A. / OECD TG 442E	SS or NS with complementary information	158
2017		U-SENS™	Validated and regulatory acceptance	N.A. / OECD TG 442E	SS or NS with complementary information	183

2017		IL-8 Luc	Validated and regulatory acceptance	N.A. / OECD TG 442E	SS or NS with complementary information	N.A.[1]

Abbreviations: SS = skin sensitiser, NS = non-sensitiser

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

STATUS OF THE VALIDATION BY EURL ECVAM OR OTHER BODIES, WHEN NECESSARY

These test methods have been validated before adoption by the OECD and EU.

HOW TO USE THESE NON-ANIMAL TEST METHODS

Testing for skin sensitisation must always start with *in chemico*/*in vitro* test methods, in case new testing is required. *In vivo* testing is only needed if *in vitro* methods are not suitable for the substance or if results of the *in vitro* tests are not adequate for classification and risk assessment.

Certain steps need to take place before any testing (*in vitro* or *in vivo*) is conducted as described in the introductory paragraph to Annex VII, i.e. assessment of all available information, which could be e.g. existing *in vitro*, *in vivo*, 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 met and include:

- the substance is classified as skin corrosion (Cat. 1); or
- the substance is a strong acid (pH $\leq$2) or base (pH $\geq$11,5); or
- the substance is spontaneously flammable in air, or in contact with water or moisture at room temperature.

If a conclusion on classification cannot be made based on existing information or the column 2 adaptation criteria cannot be applied, the following information *in chemico*/*in vitro* test(s) addressing each of the following key events needs to be performed:

1) molecular interaction with skin proteins;

2) inflammatory responses in keratinocytes;

3) activation of dendritic cells.

After these steps, no new *in vivo* test is necessary unless:

- the *in chemico*/*in vitro* tests available are not applicable for the test substance;

[1] Protocol available at: http://www.jacvam.jp/files/doc/03_08/03_08_E1.pdf.

- the results obtained from such methods are not adequate for classification and risk assessment, e.g. for skin sensitising substances it cannot be concluded whether the substance can be presumed to produce significant sensitisation in humans (CLP Cat. 1A).

As the available *in chemico*/*in vitro* methods, as specified above, provide information only on one mechanistic event, i.e. key event from the AOP, combinations of the methods are needed and should be used within a *weight-of-evidence* approach to conclude on the skin sensitisation hazard potential. 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 or *in silico* approaches.

The registrant should ensure that the chosen non-animal test method(s) 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, section 8.3.1 information requirement for skin sensitisation, information on three key events needs to be provided, unless a conclusion on classification and risk assessment can be made by using information obtained from one or two key events. In case information on one or more key events is obtained by using e.g. (Q)SARs or read-across, then an Annex XI adaptation needs to be submitted.

In the case that **consistent** data have been obtained from *in vitro* tests and potentially from other relevant sources, e.g. OECD QSAR Toolbox, then a conclusion on skin sensitisation hazard (non-sensitiser vs. sensitiser) should be possible. In the case that **inconsistent** data are obtained, a scientific explanation needs to be provided, which could be e.g. that the substance needs metabolic activation to become a sensitiser. If the conflicting information cannot be explained, the registrant needs to generate/collect additional information to ensure a correct prediction of skin sensitisation potential.

For skin-sensitising substances, an assessment needs to be made on whether the substance has the potential to cause significant sensitisation in humans (CLP Cat. 1A). If Cat. 1A can be excluded, it can be presumed that the substance merits Cat. 1B (moderate skin sensitiser) classification.

In case significant sensitisation (Cat. 1A) cannot be excluded, additional information (*in silico*, *in chemico*, *in vitro*) is needed. Information obtained from *similar substances* having e.g. LLNA data may help in assessing the skin sensitisation potential. The OECD QSAR Toolbox can be helpful in the identification of similar substances and to predict the EC3 value used for potency prediction.

Currently, there is no generally approved and/or validated way how to combine results obtained from *in chemico* and *in vitro* methods to assess the skin sensitisation **potency**. Some approaches on how to combine the *in vitro* data have been described in the ECHA

Guidance (Appendix R.7.3-4) and in the OECD Guidance Document 256, Annex 1. Due to this, an OECD project has been launched in 2017 to assess how *in vitro* data obtained from these three key events and other data can be combined to conclude on the skin sensitisation potency classification within a **Defined Approach**[2]. Depending on the outcome of the OECD work, potency assessment solely based on key event-specific *in chemico*/*in vitro* data may become possible. In addition, other test methods that may be useful for potency prediction are currently being validated[3]. ECHA will update its guidance once (draft) results of this project are available.

LINK TO THE OECD SITE

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

LINK TO THE EC TEST METHODS REGULATION
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32008R0440:en:NOT

REFERENCE TO THE RELEVANT GUIDANCES

1) Information toolkit
http://echa.europa.eu/en/support/information-toolkit

This web page provides practical information and tools in relation to help using 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, section R.7.3 (ECHA Guidance R7a)
http://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf

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

5) OECD Guidance Document on Reporting of Defined Approaches and individual information sources to be used within Integrated Approaches to Testing and Assessment (IATA) for skin sensitisation (OECD GD 256, 2016)

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[2] A defined approach to testing and assessment consists of a fixed data interpretation procedure (DIP) applied to data generated with a defined set of information sources to derive a result that can either be used on its own, or together with other information sources within an IATA, to satisfy a specific regulatory need. Thus, a defined approach to testing and assessment can be used to support the hazard identification, hazard characterisation and/or safety assessment of chemicals.

[3] Two *in vitro* methods being validated are SENS-IS (Cottrez *et al*., 2016, SENS-IS, a 3D reconstituted epidermis based model for quantifying chemical sensitization potency: Reproducibility and predictivity results from an inter-laboratory study, *Toxicology in vitro*) and GARD (Zeller *et al.*, 2017, The GARD Platform for Potency Assessment of Skin Sensitizing Chemicals, *Altex*). Please note that the scientific validity of these methods have not been established yet.

http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)29&doclanguage=en

6) Annex I: Case studies to the 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 (OECD GD 256, 2016)
http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)29/ann1&doclanguage=en

7) Practical Guide "How to use alternatives to animal testing to fulfil your information requirements for REACH registration"
https://echa.europa.eu/documents/10162/13655/practical_guide_how_to_use_alternatives_en.pdf/148b30c7-c186-463c-a898-522a888a4404

This practical guide provides practical information and tools in relation to help using existing information and non-test methods as a first step to meeting the REACH information requirements.

8) Webinar "Use of alternative methods to animal testing in your REACH registration", held on 22 September 2016

https://echa.europa.eu/-/use-of-alternative-methods-to-animal-testing-in-your-reach-registration

9) 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 an alternative test, whether the test method is a replacement and in which context the method should be used.

10) EURL ECVAM – a 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.

11) EURL ECVAM recommendations
https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations

THE SPECIFIC SCOPE AND LIMITATIONS OF THE TEST GUIDELINES

For example, limitations on chemical categories covered, if any, and limitation on classification and labelling are addressed below.

All methods listed below address a specific key event as identified in the skin sensitisation AOP.

Key event 1 – protein/peptide binding

Direct Peptide Reactivity Assay (DPRA), OECD TG 442C

- Information obtained from this test method should be used **in combination** with other information within a *weight-of-evidence* approach and not as stand-alone test method.

- Can be used to support the discrimination between sensitisers and non-sensitisers. Currently the test method is not suitable on its own for subcategorisation of skin sensitisers in to classification subcategories 1A and 1B; however, work is ongoing at OECD level to see whether sub-categorisation would be feasible within a defined approach.

- 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 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 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 a peptide but do promote its oxidation (i.e. cysteine dimerisation).

Key event 2 – responses in keratinocytes

ARE-Nrf2 Luciferase Test Method (KeratinoSens™), OECD TG 442D

- Information obtained from this test method should be used **in combination** of other information within a *weight-of-evidence* approach and not as stand-alone test method.

- Can be used to support the discrimination between sensitisers and non-sensitisers; currently the test method is not suitable on its own to sub-categorise

skin sensitisers in to 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 dimethyl sulfoxide (DMSO). The highest concentration required in the test method is 2000 µM. However, in case the highest concentration of 2000 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 <1000 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. Also pre-haptens (i.e. chemicals activated by auto-oxidation), especially those with slow oxidation rate, may result in (false) negative results.

- Substances with exclusive reactivity towards nucleophiles other than cysteine's sulfhydryl group (e.g. lysine residues) can be detected as false 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 $\geq$70 %.

- Substances that interfere with the luciferase enzyme can affect the luciferase activity either by increasing (e.g. phytoestrogens) or inhibiting the luminescence.

LuSens ARE-Nrf2 Luciferase Test Method (LuSens), draft OECD TG 442E available

- Information obtained from this test method should be used **in combination** of other information within a *weight-of-evidence* approach and not as stand-alone test method.

- Can be used to support the discrimination between sensitisers and non-sensitisers. Currently the test method is not suitable on its own to subcategorise skin sensitisers in to subcategories 1A and 1B; however, work is ongoing at OECD level to see whether sub-categorisation would be feasible within a defined approach.

- 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 2000 µM. However, in case the highest concentration of 2000 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 < 2000 µM should be considered as inconclusive.

- Substances with log Kow above 7 are outside the applicability domain, information on substances with log Kow between 5 and 7, limited information is available.
- Substances with exclusive reactivity towards nucleophiles other than cysteine's sulfhydryl group (e.g. lysine residues) can be detected as false negative in the assay.
- 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. Also pre-haptens (i.e. chemicals activated by auto oxidation), especially those with slow oxidation rate, may result in (false) negative results.
- 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 $\geq$70 %.
- Substances that interfere with the luciferase enzyme can affect the luciferase activity either by increasing (e.g. phytoestrogens) or inhibiting the luminescence.

Key event 3 – activation of dendritic cells

Human Cell Line Activation Test (h-CLAT), OECD TG 442E, Annex I

- Information obtained from this test method should be used **in combination** of other information within a *weight-of-evidence* approach and not as stand-alone test method.
- Can be used to support the discrimination between sensitisers and non-sensitisers. Currently the test method is not suitable on its own to sub-categorise skin sensitisers in to sub- categories 1A and 1B; however, work is ongoing at OECD level to see whether sub-categorisation would be feasible within a defined approach.
- Applicable to test chemicals that are soluble or that form a stable dispersion in an appropriate solvent.
- 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. Also pre-haptens (i.e. chemicals activated by auto-oxidation) may provide (false) negative results.
- 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 sensitiser. Negative results should not be considered.

- Highly cytotoxic chemicals cannot always be reliably assessed as the viability of the cells needs to be $\geq$70 %.

- Strong fluorescence substances emitting the same wavelength as FITC or propidium iodide (PI) will interfere with flow cytometric detection and thus cannot be correctly evaluated by using FITC-labelled antibodies. Other fluorochromes can be used, in case it can be proven that similar results are obtained as with FITC and PI.

U937 Cell Line Activation Test (U-SENS^{TM}), OECD TG 442E, Annex II

- Information obtained from this test method should be used in combination of other information within a *weight-of-evidence* approach and not as stand-alone test method.

- Can be used to support the discrimination between sensitisers and non-sensitisers; currently the test method is not suitable on its own to sub-categorise skin sensitisers in to sub- categories 1A and 1B; however, work is ongoing at OECD level to see whether sub-categorisation would be feasible within a defined approach.

- Applicable to test chemicals that are soluble or that form a stable dispersion in an appropriate solvent.

- 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. Also pre-haptens (i.e. chemicals activated by auto oxidation) may provide (false) negative results.

- Membrane-disrupting substances, e.g. surfactants, may lead to false positive predictions due to non-specific increase of CD86.

- Highly cytotoxic chemicals cannot always be reliably assessed as the viability of the cells needs to be $\geq$50 %.

- Strong fluorescence substances emitting the same wavelength as FITC or PI will interfere with flow cytometric detection and thus cannot be correctly evaluated by using FITC-labelled antibodies. Other fluorochromes can be used, in case it can be proven that similar results are obtained as with FITC and PI.

IL8-Luc Assay, OECD TG 442E, Annex III

- Information obtained from this test method should be used in combination of other information within a weight-of-evidence approach and not as stand-alone test method.

- Can be used to support the discrimination between sensitisers and non-sensitisers. Currently the test method is not suitable on its own to sub-categorise skin sensitisers in to sub- categories 1A and 1B; however, work is ongoing at

OECD level to see whether sub-categorisation would be feasible within a defined approach.

- Applicable to test chemicals that are soluble or that form a stable dispersion in an appropriate solvent.
- Negative results obtained with substances not dissolved at 20 mg/ml in an appropriate solvent should not be considered.
- 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. Also pre-haptens (i.e. chemicals activated by auto-oxidation) may provide (false) negative results.
- Following substance types are outside the applicability domain of this assay: surfactants (false positive predictions), anhydrides (false negative predictions) and substances interfering with luciferase activity e.g. phytoestrogen (inhibition or increased luminescence).