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Skin sensitisation

1. Which of the REACH information requirements may be met with the tests?

Annex VII to the REACH Regulation includes a requirement for *in chemico/in vitro* tests as a first step for addressing skin sensitisation (Section 8.3.1). An *in vivo* skin sensitisation study (preferably Local Lymph Node Assay, EU B.42 / OECD TG 429) can only be performed (Section 8.3.2) if the *in chemico/in vitro* methods are not applicable for the substance or the results are not adequate for classification and risk assessment.

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 often have limitations and cannot be used for all kinds of substances. Therefore, registrants and test houses are advised to check Section 4 on "Specific scope and limitations of the *in chemico/in vitro* tests" below, before deciding on a new test/study.

1.1 Summary of the *in chemico/in vitro* methods

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 events to the adverse outcomes in the whole organism (OECD 2012).

However, none of these eight non-animal methods – DPRA, ADRA, kDPRA, KeratinoSens™, LuSens, h-CLAT, U-SENS™ or IL-8 Luc Assay – should be used alone to fulfil REACH information requirements. They should always be considered in combinations and/or with other information. However, the kDPRA assay can be used as a standalone assay if the result indicates that Sub-category 1A would need to be applied. More information on the scope and known limitations of an individual test method can be found under Section 3.

A guideline for defined approaches (DAs) for skin sensitisation was adopted by the OECD, which combines information from three methods i.e. DPRA, KeratinoSens™ and/or h-CLAT with or without *in silico* tools (DEREK or QSAR Toolbox) and applies a fixed interpretation procedure to those results to generate a prediction.

To use the results of *in chemico*, *in vitro* or *in silico* tools, the substance must fit into the applicability domain of a given method or tool.

Complementary information may be derived from e.g. *in silico* approaches to assess skin metabolism. In addition, information obtained from analogue substance e.g. through thevia 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.

1.1.1. Key event one – molecular interaction with skin proteins

Test method OECD 442C - Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins currently contains three different test methods that address peptide reactivity, postulated to be the molecular initiating event (the first key event) of the skin sensitisation adverse outcome pathway (AOP) (OECD 2012) by measuring covalent binding to peptides.

The methods in the test guideline are: **i)** Direct Peptide Reactivity Assay (DPRA); **ii)** Amino Acid Derivative Reactivity Assay (ADRA); and **iii)** kinetic Direct Peptide Reactivity Assay (kDPRA).

The DPRA and ADRA methods are intended for hazard identification and the kDPRA assay for the identification of strong sensitisers (Sub-category 1A).

1.1.2. Key event two – inflammatory response in keratinocytes

Test method OECD 442D – *in vitro* skin sensitisation assays addressing the AOP key event on keratinocyte activation currently contains two different test methods, which address the second key event of the skin sensitisation AOP i.e. keratinocyte activation. The test methods use luminescence detection to measure gene expression of antioxidant/electrophile response element (ARE)-dependent pathways.

The methods in the test guideline are: **i)** ARE-Nrf2 luciferase KeratinoSens™ test method; and **ii)** ARE-Nrf2 luciferase LuSens test method.

1.1.3. Key event three – activation of dendritic cells

Test method OECD 442E – *in vitro* skin sensitisation assays addressing AOP key event 3: activation of dendritic cells currently contains three different test methods that address the activation of dendritic cells (DC) i.e. the third key event of the skin sensitisation AOP.

The methods in the test guideline are: **i)** human Cell Line Activation Test (h-CLAT) that measures the expression of the 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.

1.1.4. Defined approaches

Guideline – OECD 497 – Defined Approaches on Skin Sensitisation contains fixed data interpretation procedures on how to combine data obtained from different *in chemico*, *in vitro* and *in silico* methods to conclude whether a substance is a skin sensitiser and, if so, what is the skin sensitisation potency.

The *in chemico* and *in vitro* methods that can be used in these defined approaches are: DPRA, KeratinoSens™ and h-CLAT.

The Guideline currently contains three different defined approaches: **i)** 2o3 that uses solely *in chemico/in vitro* data and can be used only for hazard identification; **ii)** ITS v1 that uses information from DPRA, h-CLAT and the *in silico* tool DEREK, and can be used for both hazard identification and potency categorisation; and **iii)** ITS v2 that uses information from DPRA, h-

CLAT and the QSAR Toolbox and can be used for both hazard identification and potency categorisation.

Table 1: summary of the available *in chemico/in vitro* skin sensitisation test methods and defined approaches

Latest update	AOP key event measured	Test method	Validation status, regulatory acceptance	OECD test guideline	Outcome according to the test method/guideline
2021	Key Event 1 (peptide /protein binding	DPRA	Validated and regulatory acceptance	OECD TG 442C	SS or NS with complementary information
2021		ADRA	Validated and regulatory acceptance	OCD TG 442C	SS or NS with complementary information
2021		kDPRA	Validated and regulatory acceptance	OECD TG 442C	Cat 1A or Cat 1B/NS
2018	Key Event 2 (Keratinocyte response)	Keratinosens™	Validated and regulatory acceptance	OECD TG 442D	SS or NS with complementary information
		LuSens	Validated/under regulatory review	OECD TG 442D	SS or NS with complementary information
2018	Key Event 3 (Monocytic / dendritic cell response)	h-CLAT	Validated and regulatory acceptance	OECD TG 442E	SS or NS with complementary information
2018		U-SENS™	Validated and regulatory acceptance	OECD TG 442E	SS or NS with complementary information
2018		IL-8 Luc	Validated and regulatory acceptance	OECD TG 442E	SS or NS with complementary information
2021	Defined approach	2 out of 3	Validated and regulatory acceptance	OECD TG 497	SS or NS
2021		ITS v1 or v2	Validated and regulatory acceptance	OECD TG 497	SS (Cat 1A or 1B) or NS

Abbreviations: SS = skin sensitiser, NS = non-sensitiser, Cat 1A = extreme/strong sensitiser according to CLP, Cat 1B = moderate sensitiser according to CLP.

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

All of the methods above have been validated by an international validation body before adoption by the OECD or EU.

2. How to use these non-animal test methods

2.1. Information requirement

Testing for skin sensitisation must always start with *in chemico/in vitro* test methods when new testing is required. *In vivo* testing is only needed if *in vitro* methods are not suitable for the substance or if the 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 to the REACH Regulation are met including:

- 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* tests 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.
- 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 (Sub-category 1A).

2.2. Use of *in chemico/in vitro* methods

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 either within a weight of evidence approach or within a defined approach depending which assays have been performed, 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 methods are suitable for the substance 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. If information is provided only from one or two of the required three key events, a justification needs to be submitted why it is not necessary to provide information on all the required three key events. If 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, unless information by using a defined approach is provided.

When **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 versus sensitiser) should be possible. When **inconsistent** data are obtained, a scientific explanation needs to be provided to justify the decision on the classification, 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 whether the substance has the potential to cause significant sensitisation in humans (Sub-category 1A). If Sub-category 1A can be excluded, it can be presumed that the substance merits Sub-category 1B (moderate skin sensitiser) classification.

If significant sensitisation (Sub-category 1A) cannot be excluded, additional information (*in silico*, *in chemico*, *in vitro*) is needed. This means that a self-classification as a Category 1 skin sensitiser does not fulfil the REACH information requirement. 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 for identifying similar substances and predicting the EC3 value used for potency prediction.

2.3. How to use defined approaches

A defined approach (DA) to testing and assessment consists of a fixed data interpretation procedure (DIP) used to interpret data generated with a defined set of information sources, that can either be used alone or together with other information sources, to satisfy a specific regulatory need.

The currently adopted DAs are using three *in chemico/in vitro* methods i.e. DPRA, KeratinoSens™ and h-clat. Other *in chemico/in vitro* cannot currently be used within a DA and need to be used in a weight of evidence approach. In addition, the ITS DAs are using *in silico* tools (DEREK or QSAR Toolbox).

The 2o3 DA can be used to make a prediction whether the substance is a skin sensitiser (Category 1) or not, however, it does not provide information on the skin sensitisation potency (Sub-category 1A vs 1B). Therefore, if the substance is predicted to be a skin sensitiser based on the 2o3 DA, for REACH information requirements, further information needs to be generated to conclude on the skin sensitisation potency.

The ITS DAs (v1 and v2) provide a prediction on both hazard identification (sensitiser vs non-

sensitiser) and hazard characterisation (Sub-category 1A vs 1B). Therefore, the positive predictions can be used to fulfil the REACH information requirements, as they consider the potency as well.

The predictions obtained from the DAs give either **conclusive** (high confidence) or **inconclusive** (low confidence) predictions. The conclusive prediction can be used on their own to make a similar conclusion as one would when using a standard *in vivo* assay such as the Local Lymph Node Assay (LLNA). However, as is the case with the LLNA, if conflicting information is available for the substance from other sources, a weight of evidence assessment needs to be made based on all available information to conclude on the correct hazard classification.

If an inconclusive prediction is obtained, no standalone prediction can be made based on the DA. However, the information generated from the individual information sources can still be used in a weight of evidence approach to conclude on the skin sensitisation potential if adequate information is available. The weight of evidence assessment may, however, indicate the need to generate additional information e.g. through further experimental studies, from different *in silico* tools or by using a read-across approach.

2.4. *In silico* tools within the ITS DAs

The *in silico* tools included in the ITS DAs provide positive or negative skin sensitisation predictions to be used in combination with other information sources of the DA.

Information about the applicability domain is automatically provided by the tools together with the prediction. A brief description of the tools is given here, while a more comprehensive description can be found in the OECD Guideline document.

For REACH purposes, the *in silico* predictions have to cover the whole composition of the registered substance. This means that more than one structure might need to be predicted, as follows:

a. For mono-constituent substances, in addition to the main constituent, individual predictions have to be run for eventual impurities and/or additives present at significant concentrations in the composition of the substance;

b. For multi-constituent substances, all constituents have to be predicted individually;

c. For UVCB substances, one or more representative substances have to be selected and individually predicted. The selection has to be justified.

The result is considered negative if all individual predictions are negative.

2.4.1. Derek Nexus (ITS v1)

Derek Nexus is a module of the commercial software Lhasa Knowledge Suite. It allows the generation of predictions for a multitude of endpoints using the chemical structure as input. Skin sensitisation predictions from Derek Nexus v6.1.0 are used in ITS v1. All positive predictions (likelihood = certain, probable, plausible or equivocal) are considered to be inside the applicability domain. Negative predictions (likelihood = doubted, improbable, impossible or non-sensitiser) are also considered to be in the applicability domain unless they contain misclassified and/or unclassified features, which are automatically flagged by the software.

2.4.2. OECD QSAR Toolbox (ITS v2)

The OECD QSAR Toolbox is a freely available software co-owned and co-developed by ECHA and the OECD. It provides tools to perform or support chemical hazard assessment for the majority of endpoints of regulatory interest. Using the chemical structure or other identifiers as input, the "automated workflow" functionality identifies analogues of a target and makes predictions based on automated read across or structural alerts. Skin sensitisation predictions from the QSAR Toolbox automated workflow "Skin sensitisation for defined approaches" are used in ITS v2. The calculation of the applicability domain of the predictions is automatically provided by the Toolbox and consists of three layers: structural, parametric and mechanistic. The applicability domain layers considered for each individual prediction depend on the type and outcome of the prediction.

2.5. How to do risk assessment

The REACH information requirement specifies in Section 8.3, column 1 that risk assessment needs to be performed if a skin sensitisation hazard has been identified.

2.5.1. Qualitative approach

Normally it is not possible to establish a threshold for skin sensitisation, therefore, a respective derived no-effect level (DNEL) cannot be determined. The registrant is required to undertake a qualitative human health assessment and document the assessment in the chemical safety report. ECHA has developed Practical Guide 15 which explains how to provide solid and consistent justification to support the conclusion that the operational conditions (OCs) and risk management measures (RMMs) described in the exposure scenarios are sufficient to avoid the likelihood of adverse health effects.

Since sensitisation is essentially systemic in nature, it is important for the purposes of risk management to acknowledge that skin sensitisation may be acquired by other routes of exposure than dermal. There is, therefore, a need for cautious use of known contact allergens in products to which consumers or workers may be exposed by inhalation.

It should be verified whether or not the RMMs/OCs proposed are sufficient to also cover for other relevant effects for which DNELs can be derived (e.g. reproduction toxicity or repeated dose toxicity). Exposures should be controlled at least to these levels, not only for the dermal route of exposure, but also for the inhalation and oral routes of exposure (when relevant).

2.5.2. Semi-quantitative approach

Extreme and strong skin sensitisers (classified in Sub-category 1A in CLP) are allocated to the high hazard band on the basis that exposure to such potent skin sensitising substances should be strictly contained and dermal contact avoided. Moderate skin sensitisers (classified in Sub-category 1B in CLP) are allocated to the moderate hazard category band on the basis that exposure to these moderate skin sensitising substances should be well-controlled.

Where, even after extended testing, the available data does not allow potency categorisation of a sensitising substance, the substance should be classified as Category 1, thus, the risk management measures (RMMs) and operational conditions (OCs) applicable to the high hazard band should be considered. Further information can be found in ECHA Guidance Part E – Risk Characterisation.

Even when a DNEL can be determined for skin sensitisation, the risk assessment should start with a qualitative approach. This is because deriving the safe use levels for skin sensitisation can be problematic and may be associated with considerable uncertainty. Uncertainty

assessment approaches have been published, e.g. by ECHA (see Chapter R.19 of the *Guidance on IR&CSA*).

When developing a quantitative exposure assessment, it should be noted that dermal exposure data are rarely available, and often difficult to interpret because of missing contextual information and/or information on the measurement method. In most cases, the default approach in quantitatively assessing dermal exposure is using dermal exposure models e.g. ECETOC TRA or RiskofDerm. Biomonitoring can be used to estimate total exposure if suitable monitoring methods are available.

Further information on occupational exposure assessment is available in ECHA's R.14 Guidance and on consumer exposure assessment in ECHA's R.15 Guidance.

ECHA has developed Chesar (Chemical Safety Assessment and Reporting Tool) which enables registrants to import substance information from the IUCLID dossier as a basis for exposure assessment and risk characterisation.

3. Specific scope of the test method, including scope and know limitations

3.1. Key event one - molecular interaction with skin proteins

3.1.1. Direct Peptide Reactivity Assay (DPRA) (OECD 442C, Appendix I)

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach or DA and not as a standalone test method for fulfilling REACH information requirements.
- Can be used to support the discrimination between sensitisers and non-sensitisers; currently, the test method is not suitable on its own for sub-categorisation of skin sensitisers into classification Sub-categories 1A and 1B.
- Can be used for potency categorisation of a skin sensitising substance within ITS DA.
- 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. If there is a 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).

- If borderline results are obtained (i.e. mean percent depletion falls in the range of 3 % to 10 % for the cysteine 1:10/lysine 1:50 prediction model or cysteine percent depletion falls in the range of 9 % to 17 % for the cysteine 1:10 prediction model), additional testing is recommended. In particular, if negative results are obtained in these ranges (i.e. 3 % to 6.38 % for the cysteine 1:10/lysine 1:50 prediction model or 9 % to 13.89 % for the cysteine 1:10 prediction model), a second run should be conducted, as well as a third one if there are discordant results between the first two runs.

3.1.2. Amino Acid Derivative Reactivity Assay (ADRA) (OECD 442C, Appendix II)

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach and not as a standalone test method for fulfilling REACH information requirements.

- Can be used to support the discrimination between sensitisers and non-sensitisers; currently, the test method is not suitable on its own for sub-categorisation of skin sensitisers into classification Sub-categories 1A and 1B.

- The test method allows testing with poorly soluble substances and a test chemical solubility of 1 mM is required in an appropriate solvent. If solubility of 1 mM cannot be reached, positive results of lower test concentrations can still be accepted.

- 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.

3.1.3. Kinetic Direct Peptide Reactivity Assay (kDPRA) (OECD 442C, Appendix III)

- Information obtained from this test method should be used either as a follow-up test method for sub-categorisation of skin sensitising chemicals (Category 1), or as a standalone assay to identify a chemical as a Sub-category 1A skin sensitiser.

- Method only measures reactivity with the cysteine peptide, so strong sensitisers with an exclusive lysine-reactivity, such as some acyl-halides, phenol esters or aldehydes, are outside of the applicability domain of kDPRA.

- Test chemical should be soluble in an appropriate solvent at a final concentration of 20 mM. Test chemicals that are not soluble at this concentration may still be tested at lower concentrations as long as a kmax value (i.e. the maximum rate constant (in s-

1M-1) determined from the reaction kinetics for a tested substance in the kDPRA), can be derived. In such a case, a positive result leading to a Category 1A skin sensitisation prediction (i.e. log kmax ≥-2.0) could still be used, but no firm conclusion should be drawn from a negative result (i.e. non-reactive or log kmax <-2.0 outcome).

- Test chemicals that do not covalently bind to the peptide but promote its oxidation (i.e. cysteine dimerisation) could lead to a potential overestimation of peptide depletion, resulting in possible false positive predictions and/or assignment to a higher reactivity class.

- 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.

- Aromatic amines, catechols or hydroquinones may require further data to confirm their weak reactivity even under oxidising conditions, and acyl-halides, phenol-esters or aldehydes specifically reacting with lysine-residue according to e.g. the DPRA or ADRA, may require further data to confirm their weak reactivity.

- 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. The majority of chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) were reported to be correctly detected by *in chemico* test methods. However, spontaneously rapidly oxidising pre-haptens may be under-predicted by kDPRA (as in any *in vitro* skin sensitisation assay) due to a lag-phase for oxidation which reduces the overall reaction rate.

3.2. inflammatory response in keratinocytes

3.2.1. ARE-Nrf2 Luciferase Test Method (KeratinoSensTM) (OECD 442D, Appendix 1A)

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach or DA and not as a standalone test method for fulfilling REACH information requirements.

- 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 into classification Sub-categories 1A and 1B.

- Can be used for potency categorisation of a skin sensitising substance within ITS DA.

- 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. Also, pre-haptens (i.e. chemicals activated by auto oxidation) especially with slow oxidation rate may 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 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 ≥70 %.
- Substances that interfere with the luciferase enzyme can affect the luciferase activity either by increasing (e.g. phytoestrogens) or inhibiting the luminescence.

3.2.2. LuSens ARE-Nrf2 luciferase test method (LuSens) (OECD TG 442E, Appendix IB)

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach and not as a standalone test method for fulfilling REACH information requirements.
- 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 soluble chemicals 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 <2 000 µM should be considered as inconclusive.
- Substances with exclusive reactivity towards other nucleophiles than cysteine sulfhydryl group (e.g. lysine-residues) can be detected as false negatives 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 with a 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 ≥70 %.
- Substances that interfere with the luciferase enzyme can affect the luciferase activity either by increasing (e.g. phytoestrogens) or inhibiting the luminescence.

3.3. Key event three – activation of dendritic cells

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

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach or DA and not as a standalone test method for fulfilling REACH information requirements.

- 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 into Sub-categories 1A and 1B.
- Can be used for potency categorisation of a skin sensitising substance within ITS DA.
- Applicable to test chemicals 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 false negative results. For such substances, positive results could be used to support the identification of a test chemical as a 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 if it can be proven that similar results are obtained as with FITC and PI.

3.3.2. U937 Cell Line Activation Test (U-SENSTM) (OECD TG 442E, Annex II)

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach and not as a standalone test method for fulfilling REACH information requirements.
- 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 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 if it can be proven that similar results are obtained as with FITC and PI.

3.3.3. IL8-Luc Assay (OECD TG 442E, Annex III)

- Information obtained from this test method should be used **in combination** with other information within a weight of evidence approach and not as a standalone test method for fulfilling REACH information requirements.
- 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 into 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 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.
- Surfactants (false positive predictions), anhydrides (false negative predictions and substances interfering with luciferase (inhibition or increased luminescence) e.g. phytoestrogen are outside the applicability domain of this assay.

3.4. Guideline for Defined Approaches for Skin Sensitisation (OECD TG 497)

- All of the information sources used to obtain a conclusive prediction need to meet the individual test guideline acceptance criteria, unless otherwise stated in the specific DA.
- DAs use information sources from DPRA (OECD TG 442C), KeratinoSens™ (OECD TG 442D), h-CLAT (OECD TG 442E), DEREK and OECD QSAR Toolbox. Currently, information from other methods cannot be used in the existing DAs in OECD TG 497.

3.4.1. 2o3 DA (Part I, OECD TG 497)

- Conclusive predictions can be used to make a distinction between sensitisers (Category 1) and non-sensitisers.
- If a conclusive negative prediction is obtained, a **standalone conclusion** can be made for REACH purposes.
- Does not provide information on potency. Therefore, for REACH purposes, if a positive prediction is obtained, **further information needs to be generated** to conclude on the skin sensitisation potency (Sub-category 1A vs 1B of CLP).
- Two concordant predictions from the DA that are not borderline need to be obtained.
- DA specific borderline values (BR) for each information source are specified:
    - DPRA BR: mean peptide depletion: 4.95 % – 8.32 %, Cys-only depletion (for co-elution with lysine peptide): 10.56 % – 18.47 %;
    - KeratinoSens™ BR: Imax: 1.35-fold – 1.67-fold;

- h-CLAT BR: RFI CD54: 157 % – 255 %; RFI CD86: 122 % – 184 %.

- For inconclusive predictions, **no standalone conclusion** on skin sensitisation potential, or the lack thereof, can be made. However, the information generated from the individual information sources can still be used in a weight of evidence approach to conclude on the skin sensitisation potential if adequate information is available. The weight of evidence assessment may, however, indicate the need to generate additional information e.g. through further experimental studies, from different *in silico* tools or by using a read-across approach.

3.4.2. ITS DAs (Part II, OECD TG 497)

- Conclusive predictions can be used to make a distinction on whether a substance is a skin sensitiser, including potency prediction (Sub-category 1A vs 1B) or a non-sensitiser.

- For conclusive predictions, a **standalone conclusion** can be made for REACH purposes.

- Depending on the scoring obtained from individual information sources, information from all three information sources may not be needed for a conclusive prediction.

- For inconclusive predictions, **no standalone conclusion** on skin sensitisation potential, or a lack thereof, can be made. However, the information generated from the individual information sources can still be used in a weight of evidence approach to conclude on the skin sensitisation potential if adequate information is available. The weight of evidence assessment may, however, indicate the need to generate additional information e.g. through further experimental studies, from different *in silico* tools or by using a read-across approach.

- Currently, no conclusive negative prediction can be made for test chemicals having LogP >3.5, due to the reasons that negative results from h-CLAT should not be considered. Therefore, a false negative prediction for the DA cannot be excluded.

4. References

1) Information toolkit http://echa.europa.eu/en/support/information-toolkit
This website provides practical information and tools in relation to 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) Guidance on information requirements and chemical safety assessment (ECHA Guidance R.14)
https://echa.europa.eu/documents/10162/13632/information_requirements_r14_en.pdf/bb14b581-f7ef-4587-a171-17bf4b332378

5) Guidance on information requirements and chemical safety assessment (ECHA Guidance R.14)

https://echa.europa.eu/documents/10162/13632/information_requirements_r14_en.pdf/bb14b581-f7ef-4587-a171-17bf4b332378

6) Guidance on information requirements and chemical safety assessment (ECHA Guidance R.15)
https://echa.europa.eu/documents/10162/13632/information_requirements_r15_en.pdf/35e6f804-c84d-4962-acc5-6546dc5d9a55

7) Guidance on information requirements and chemical safety assessment (Part E – Risk Characterisation)
https://echa.europa.eu/documents/10162/13632/information_requirements_part_e_en.pdf/1da6cadd-895a-46f0-884b-00307c0438fd

8) Guidance on information requirements and chemical safety assessment (ECHA Guidance R.19)
https://echa.europa.eu/documents/10162/13632/information_requirements_r19_en.pdf/d5bd6c3f-3383-49df-894e-dea410ba4335

9) 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

10) 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):
http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)29&doclanguage=en

11) 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

12) 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 website provides practical information and tools in relation to using existing information and non-test methods as a first step to meeting the REACH information requirements.

13) Practical guide "How to undertake a qualitative human health assessment and document it in a chemical safety report"
https://echa.europa.eu/documents/10162/13655/pg_15_qualitative-human_health_assessment_documenting_en.pdf/26a645d4-a81e-4223-8ca9-20162ae74e72

14) Webinar on "How to use *in vitro* data to fulfil REACH information requirements held on 22 September 2016
https://echa.europa.eu/-/use-of-alternative-methods-to-animal-testing-in-your-reach-registration

15) 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.

16) Chesar http://chesar.echa.europa.eu