ECHA
EUROPEAN CHEMICALS AGENCY

GUIDANCE FOR THE IMPLEMENTATION OF REACH

Guidance on information requirements and Chemical Safety Assessment

Chapter R.7a: Endpoint specific guidance

Draft Version 5.0

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NOTE

Please note that the present document is a proposed amendment to specific extracts **only** of the *Guidance on IR&CSA, Chapter R.7a*. This document was prepared by the ECHA Secretariat for the purpose of this consultation and includes only the parts open for the current consultation, i.e. section R.7.3 only.

The full document (version before proposed amendments) is available on the ECHA website at http://echa.europa.eu/documents/10162/13632/information_requirements_r7a_en.pdf (version 4.0 published in July 2015).

The numbering and headings of the sub-sections that are displayed in the document for consultation correspond to those used in the currently published guidance document; this will enable the comparison of the draft revised sub-sections with the current text if necessary.

After conclusion of the consultation and before final publication the updated sub-sections will be implemented in the full document.

Document history

Version	Changes	Date
[…]	[…]	[…]
Version 5.0	Full revision addressing the content of Section R.7.3 related to *Skin and Respiratory sensitisation*. The update includes the following: • Modification of Section R.7.3 structure and subdivision by endpoint: Skin sensitisation (Sections R.7.3.2 to R.7.3.6) and Respiratory sensitisation (Sections R.7.3.7 to R.7.3.10). • Update of the information on new/revised EU test methods and OECD test guidelines for skin sensitisation; • Update of the information on respiratory sensitisation; • Update of the information on non-testing methods; • Update of the recommended testing and assessment strategy for skin and respiratory sensitisation in Sections R.7.2.6 and R.7.2.10, respectively; • Replacement of the terms "Integrated Testing Strategy (ITS)" by "testing and assessment strategy" to account for the non-testing part of the evaluation strategy; • Update of the information on Classification and Labelling to reflect changes coming from the 2nd and 4th Adaptations to Technical and Scientific Progress of the CLP Regulation, and to align the text with the revised Section 3.4 *Respiratory or skin sensitisation* of the *Guidance on the Application of the CLP Criteria* (version 4.0, November 2013).	XX 2015

R.7.3 Skin and respiratory sensitisation

R.7.3.1 Introduction

A number of diseases are recognised as being, or presumed to be, allergic in nature. These include asthma, rhinitis, conjunctivitis, allergic contact dermatitis, urticaria and food allergies (the latter is not discussed in this document). In this Section, the endpoints discussed are those traditionally associated with occupational and consumer exposure to chemical substances (proteins are not discussed in this document). Photosensitisation is potentially important but its mechanism of action is poorly understood, and it is not discussed in this document.

R.7.3.1.1 Definition of skin and respiratory sensitisation

A sensitiser is an agent that is able to cause an allergic response in susceptible individuals. The consequence of this is that following subsequent exposure via the skin the characteristic adverse health effects of allergic contact dermatitis or atopic dermatitis may be provoked. After inhalation exposure to respiratory sensitisation, adverse health effects include asthma (and related respiratory symptoms such as rhinitis) or extrinsic allergic alveolitis.

Respiratory hypersensitivity is a term that is used to describe asthma and other related respiratory conditions, irrespective of the mechanism (immunological or non-immunological) by which they are caused. In contrast, dermal allergy is based on an immunological mechanism.

It is perhaps helpful to attempt to define the term chemical respiratory hypersensitivity. One approach taken by the UK Health and Safety Executive was to describe the induction phase as the process of rendering the airways unusually sensitive (hypersensitive) such that following subsequent inhalation exposure an asthmatic reaction might be elicited associated with classical symptoms of airway narrowing, chest-tightening and bronchial restriction (HSE, 1997). Other approaches to definition of relevant terms are available elsewhere. For instance, various definitions are provided for specific sensitising agents in the workplace – all of which imply a mechanism whereby hypersensitivity of the respiratory tract is induced as the result of workplace exposure – and that this may result later in the development of occupational asthma (Bernstein *et al.*, 1993). The lists of substances cited here, by the HSE, and elsewhere, as causes of respiratory sensitisation and occupational asthma are very similar, and in some instances identical (Chan-Yeung *et al.*, 1993). Among the substances populating these lists are: diisocyanates, acid anhydrides, certain platinum salts, some reactive dyes, cyanuric chloride, and plicatic acid (from Western Red Cedar).

When directly considering human data in this document, the clinical diagnostic terms asthma, rhinitis and extrinsic allergic alveolitis have been retained.

These definitions are reflected in the criteria for the classification of skin and respiratory sensitisers, which provide a useful tool against which the hazardous properties of a substance can be judged. These criteria are given in the Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP Regulation).

Classification and labelling under the CLP Regulation

Substances and mixtures causing skin sensitisation and/or respiratory sensitisation can be further characterised by their classification under the CLP Regulation.

Detailed information on the classification and labelling of substances and mixtures can be found in the *Guidance on the Application of the CLP criteria and in the CLP Regulation*.

a) For skin sensitisation

- Skin sensitisers are classified in Category 1 with the Hazard statement H317 *"May cause an allergic skin reaction".* Where data are sufficient, skin sensitisers can be divided into sub-categories. If data are not sufficient for sub-categorisation, Category 1 must be chosen.
    - **Sub-category 1A**: Substances showing a high frequency of occurrence in humans and/or a high potency in animals can be presumed to have the potential to produce significant sensitisation in humans. Severity of reaction may also be considered.
    - **Sub-category 1B**: Substances showing a low to moderate frequency of occurrence in humans and/or a low to moderate potency in animals can be presumed to have the potential to produce sensitisation in humans. Severity of reaction may also be considered.

b) For respiratory sensitisation

- Respiratory sensitisers are classified in Category 1 with the Hazard statement H334 *"May cause allergy or asthma symptoms or breathing difficulties if inhaled"*. Where data are sufficient, respiratory sensitisers can be divided into sub-categories. If data are not sufficient for sub-categorisation, Category 1 must be chosen.
    - **Sub-category 1A**: Substances showing a high frequency of occurrence in humans; or a probability of occurrence of a high sensitisation rate in humans based on animal or other tests. Severity of reaction may also be considered.
    - **Sub-category 1B**: Substances showing a low to moderate frequency of occurrence in humans; or a probability of occurrence of a low to moderate sensitisation rate in humans based on animal or other tests. Severity of reaction may also be considered.

R.7.3.1.2 Objective of the guidance on skin and respiratory sensitisation

The general objectives are to determine:

- whether there are existing *in chemico, in silico, in vitro* or *in vivo* data, or human evidence indicating that the agent has skin or respiratory sensitisation potential or the lack thereof; or

- whether new information needs to be generated to assess the skin sensitisation potential or the lack thereof for the substance according to the testing and assessment strategy as presented in this document[1].

Therefore, in the sections on skin sensitisation and respiratory sensitisation firstly an overview of types of data is given that may provide information on sensitisation, followed by guidance on the process of judging the available data in terms of adequacy, completeness and remaining uncertainty. In Sections R.7.3.5 and R.7.3.9 guidance is given on application of the data to reach a conclusion on suitability for classification and labelling, including potency, if possible. Finally in Sections R.7.3.6 and R.7.3.10 a testing and assessment strategy is presented for skin sensitisation and respiratory sensitisation, respectively.

[1] The testing and assessment strategies are also referred to as Integrated Approaches on Testing and Assessment (IATAs).

SKIN SENSITISATION

The mechanisms leading to skin sensitisation are relatively well understood. In 2012 the OECD published an **Adverse Outcome Pathway** (AOP), which describes the biological mechanisms of skin sensitisation initiated by the covalent binding of substances to skin proteins (OECD, 2012). It should be noted that this AOP does not cover metals or biological allergens, and only substances that form a covalent binding to skin proteins. The key events of the skin sensitisation pathway are: 1) covalent binding of the electrophilic chemical substance into the skin carrier proteins; 2) the release of pro-inflammatory cytokines and the induction of cyto-protective pathways in keratinocytes; 3) the activation and maturation of dendritic cells, and their migration into the local lymph nodes; 4) presentation of the chemical allergen by the dendritic cells to naïve T-cells, which leads to their differentiation into allergen-specific memory T-cells and their subsequent clonal expansion. Even though not considered as being a part of the key events from one to four leading to the adverse outcome, dermal bioavailability (penetration and/or metabolism) is a prerequisite for a substance to cause skin sensitisation i.e. the substance needs to reach the viable dermis.

Traditionally the development of skin sensitisation has been divided in two phases, i.e. induction and elicitation. In the induction phase the naïve individual becomes sensitised to the allergenic agent, e.g. through the molecular events as described above, leading to the formation of allergen specific memory T-cells. Those specific memory T-cells migrate into the dermis for the repeated encounter with the specific allergen. In the elicitation phase the memory T-cells, created in the induction phase, re-encounter the specific allergen which leads to the quick proliferation and activation of those allergen specific T-cells. The activated T-cells start secreting specific cytokines, which in turn induce the release of inflammatory cytokines and mobilization of inflammatory cells and cytotoxic T-cells from the circulating blood. When those cells migrate into the epidermis of the skin a local inflammatory response is triggered.

R.7.3.2 Information requirements for skin sensitisation[2]

The information on skin sensitisation that is required to be submitted for registration and evaluation purposes is specified in Annexes VI to XI to the REACH Regulation. According to Annex VI, the registrant should gather and evaluate all existing available information before considering further testing. This includes physico-chemical properties, (Q)SAR ((Quantitative) Structure-Activity Relationship), grouping, *in vitro/in chemico* data, animal studies, and human data. For classified substances, information on exposure, use and risk management measures should also be collected and evaluated in order to ensure safe use on the substance.

If these data are inadequate for hazard and risk assessment, including classification and labelling, further testing should be carried out in accordance with the **requirements of Annex VII (≥1 tpa) to the REACH Regulation**.

The standard information requirements at this tonnage level for skin sensitisation (see Section 8.3 in Column 1 of Annex VII) can be fulfilled by following two consecutive steps:

[2] Please note that the information requirements in REACH Annex VII in relation to skin sensitisation are currently under revision. This revision is expected to…XXX

1. *an assessment of the available human, animal and alternative data,*

2. *In vivo testing.*

Column 2 of Annex VII lists specific rules according to which the required standard information may be omitted, replaced by other information, or adapted in another way. If the conditions are met under which column 2 of this Annex allows adaptations, the fact and the reasons for each adaptation should be clearly indicated in the registration. For skin sensitisation column 2 reads:

Step 2 does not need to be conducted if:

- *the available information indicates that the substance should be classified for skin sensitisation or corrosivity; or*
- *the substance is a strong acid (pH<2.0) or base (pH>11.5); or*
- *the substance is flammable in air at room temperature* (Please note that this rule should actually read: "the substance is **spontaneously** flammable in air at room temperature").

General provisions for the generation of information on intrinsic properties of substances are contained in REACH Article 13 which states that this information may be generated by means other than tests, provided that the conditions specified in Annex XI are met.

In addition to the specific rules of adaptation (column 2), Annex XI 1.2 to 1.5 to the REACH Regulation lays out general rules of adaptation to the standard information requirements, which may be based on the use of non-animal test methods (e.g. *in vitro/in chemico*) within a *Weight-of-Evidence* approach (section 1.2), or the use of read-across (section 1.5). In the case of Annex XI adaptation as well, the fact and the reasons for each adaptation should be clearly indicated in the registration dossier.

Guidance on application of these rules is given in the testing and assessment strategy described in Section R.7.3.6 of this Guidance.

The Murine Local Lymph Node Assay (LLNA) is the first-choice method for *in vivo* testing. Only in exceptional circumstances should another test be used This means that in certain cases other *in vivo* methods may be conducted. In such cases convincing scientific justification for the use of another test must be provided in the registration dossier.

R.7.3.3 Information sources on skin sensitisation

R.7.3.3.1 Non-human data on skin sensitisation

Non-testing data on skin sensitisation

Non-testing methods for skin sensitisation cover a breadth of different approaches namely read-across/chemical categories, chemistry considerations and (Q)SARs. Read-across/chemical categories are described in Sections R.6.1 and R.6.2 of Chapter R.6 of the *Guidance on IR&CSA*.

The adaptation of standard information requirements can be used for the assessment of skin sensitisation, if it provides relevant and reliable data for the substance of interest. As specified in Annex XI of the REACH regulation, the use of non-testing methods needs to be justified and sufficiently documented. In the case of QSARs and expert systems, registrants need to prepare property predictions by completion of a QSAR Prediction Reporting Format (QPRF). The QPRF is a harmonised template for summarising and reporting substance-specific predictions generated by (Q)SAR models. For filling a data gap under REACH, it is also necessary to provide information on the prediction model employed following a QSAR Model Reporting Format (QMRF) document. The QMRF is a harmonised template for summarising and reporting key information on (Q)SAR model validity, including the results of any validation studies. The information is structured according to the OECD (Q)SAR validation principles (for further information see http://www.oecd.org/env/ehs/risk-assessment/validationofqsarmodels.htm). The JRC QSAR Model Database is an inventory of information on available QMRFs, freely accessible online (https://eurl-ecvam.jrc.ec.europa.eu/databases/jrc-qsar-model-database). More detailed guidance on QSAR models, their use and reporting formats, including the QMRF, is provided in Section R.6.1 of Chapter R.6 of the *Guidance on IR&CSA*.

A non-exhaustive list of available (Q)SARs models that may be useful for predicting several REACH relevant endpoints, including skin sensitisation, was compiled within ANTARES, an EU LIFE project whose results are freely available online (http://www.antares-life.eu/index.php?sec=modellist). The OECD Guidance on grouping of chemicals (OECD, 2014) also provides a summary of tools that might be useful in predicting endpoints of regulatory relevance, including skin sensitisation (see also: http://www.oecd.org/chemicalsafety/risk-assessment/groupingofchemicalschemicalcategoriesandread-across.htm).

Exploring the reaction chemistry of compounds forms the basis of most read-across justifications and many of the available skin sensitisation (Q)SARs. The skin sensitisation potential of a substance is related in the first place to its ability to react with skin proteins to form covalently linked conjugates and recognition of these by the immune system. In the vast majority of cases, this is dependent on electrophilic reactivity of the skin sensitiser or a derivative produced (usually by oxidation) *in vivo* or abiotically (Barratt *et al.*, 1997). There are various types of electrophile-nucleophile reactions in skin sensitisation, perhaps the most frequently encountered are: Michael-type reactions; S_N2 reactions; S_NAr reactions; acylation reactions and Schiff-base formation. These chemical reaction mechanisms can serve as a means of describing the domain of applicability (the scope) of a (Q)SAR or form the basis for grouping substances into chemical categories. Recent work in this area has been described (Aptula *et al.*2005; Aptula and Roberts 2006; Roberts *et al.*, 2007a, 2011; Schultz *et al*., 2009; Natsch *et al*., 2012; Enoch and Roberts, 2013).

The freely downloadable OECD QSAR Toolbox software (http://www.qsartoolbox.org/) encodes several mechanistic and skin sensitisation endpoint specific profilers. They allow the user to group substances which share common structural alerts and to predict their skin sensitisation potential *via* read-across. ECHA has published illustrative examples on how to make skin sensitisation read-across predictions using the OECD QSAR Toolbox (https://echa.europa.eu/documents/10162/21655633/illustrative_example_qsar_part2_en.pdf).

There are also some (Q)SAR models for skin sensitisation reported in the peer reviewed literature. Available models include local and global (Q)SARs as well as expert systems. If not implemented in a software tool, their use might be restricted due to accessibility issues of technical nature.

OECD QSAR Toolbox

The OECD QSAR Toolbox software (current version 3.3) covers the skin sensitisation endpoint with dedicated databases and profilers.

The two dedicated databases for skin sensitisation are "Skin sensitisation", which includes 1 036 substances and 1 573 experimental data points (includes the OASIS skin sensitisation database and the Liverpool John Moores University skin sensitisation database) and "Skin sensitisation ECETOC", with 39 substances and 42 experimental data points. ECHA Chem database, which collects the information found in REACH dossiers, contains also some data on skin sensitisation.

There are four relevant profilers for skin sensitisation. They are all based on protein binding. Three of these profilers can be found under the general mechanistic profiler branch: Protein binding by OASIS v1.3, Protein binding by OECD, Protein binding potency. The fourth profiler is under the endpoint-specific branch: Protein binding alerts for skin sensitisation by OASIS v1.3.

The users can use profilers for the identification of analogues based on mechanistic commonalities and retrieve experimental information from the dedicated databases. Several data gap filling techniques can be used to predict skin sensitisation for the substance of interest: read-across, trend analysis and QSAR models.

The OECD QSAR Toolbox also encodes an Adverse Outcome Pathway (AOP) for skin sensitisation. This is the first attempt in the QSAR Toolbox to allow predictions through AOPs, and at this stage it is premature to advise the use of the AOP functionality within the OECD QSAR Toolbox for predicting skin sensitisation.

Local (Q)SAR models

The majority of local models available have been developed for direct-acting electrophiles using the relative alkylation index (RAI) approach. This is a mathematical model derived by Roberts and Williams (1982). It is based on the concept that the degree of sensitisation produced at induction, and the magnitude of the sensitisation response at challenge, depends on the degree of covalent binding (haptenation; alkylation) to carrier protein occurring at induction and challenge. The RAI is an index of the relative degree of carrier protein haptenation and was derived from differential equations modelling competition between the carrier haptenation reaction in a hydrophobic environment and removal of the sensitiser through partitioning into polar lymphatic fluid. In its most general form the RAI is expressed as:

$$RAI = \log D + a \log k + b \log P \quad (1)$$

Thus the degree of haptenation increases with increasing dose D of sensitiser, with increasing reactivity (as quantified by the rate constant or relative rate constant *k* for the reaction of the sensitiser with a model nucleophile) and with increasing hydrophobicity (as quantified by log P, P being the octanol/water partition coefficient). This RAI model has been used to evaluate a wide range of different datasets of skin sensitising substances. Examples include sulfonate esters (Roberts and Basketter 2000), sulfones (Roberts and Williams 1982), primary alkyl bromides (Basketter *et al.*, 1992), acrylates (Roberts, 1987), aldehydes and diketones (Patlewicz *et al.*, 2001; Patlewicz *et al.*, 2002; Patlewicz *et al.*, 2004; Roberts *et al.*, 1999; Roberts and Patlewicz 2002; Patlewicz *et al.*, 2003).

This approach has shown that local models tend to be transparent, simple and mechanistically derived but are labour-intensive to develop and restricted to local areas of chemistry (Cronin *et al.*, 2011).

The covalent hypothesis has served and continues to be the most promising way of developing mechanistically based robust QSARs. These are local in that their scope is characterised by a mechanistic reactivity domain as outlined in Aptula *et al*., 2005; Aptula and Roberts, 2006; Roberts *et al*., 2007a. An example of this type of mechanistic model has been recently published (Roberts *et al.*, 2006). In the RAI model, log*k*, has been typically modelled by experimental rate constants, substituents' constants or molecular orbital parameters. More effort is needed to encode reactivity into descriptors, this could be achieved through the systematic generation of *in vitro* reactivity data as outlined by Aptula and Roberts (2006), Aptula *et al*. (2006), Schultz *et al*. (2006), Gerberick *et al*. (2004) and in the next section.

Global statistical models

Global Statistical models usually involve the development of empirical QSARs by application of statistical methods to sets of biological data and structural descriptors.

These are perceived to have the advantage of being able to make predictions for a wider range of substances. In some cases, the scope/domain of these models are well described, in most other cases a degree of judgement is required in determining whether the training set of the model is relevant for the substance of interest. Criticism often levied at these types of models is that they lack mechanistic interpretability. The descriptors might appear to lack physical meaning or are difficult to interpret from a chemistry perspective. The sorts of descriptors used may encode chemical reactivity/electrophilicity e.g. LUMO (the energy of the lowest molecular orbital) and partitioning effects e.g. Log P, but more commonplace is that a large number of descriptors are calculated that encode structural, topological and/or geometrical information. A number have been reported in the recent literature, examples include those developed using LLNA data (Devillers, 2000; Estrada *et al*., 2003; Fedorowicz *et al*., 2004; Fedorowicz *et al*., 2005; Li *et al*., 2005; Miller *et al*., 2005; Ren *et al*., 2006; Li *et al*., 2007; Golla *et al*., 2009; Chaudhry *et al*., 2010).

Expert systems

Softwares like VEGA are free to download and use. There are also several commercial (Q)SAR models for skin sensitisation available. Examples include TOPKAT, CASE, Derek Nexus (DN), TIMES (TIssue MEtabolism Simulator), Molcode, and HazardExpert.

- Statistical Models:

TOPKAT (included in Discovery Studio package) marketed by BIOVIA Foundation (formerly Accelrys Enterprise Platform 'AEP') is a suite of two models; one for Non-sensitisers *vs.* Sensitisers and the other for Weak/Moderate vs. Strong sensitisers. The first model calculates the probability of a chemical structure to be a sensitiser. If the probability is greater than or equal to 0.7, the substance is predicted to be a sensitiser, a non-sensitiser would have a probability of less or equal to 0.30. The second model applies to structures predicted as sensitisers by the first model and resolves the potency: weak/moderate *vs.* strong where a probability of 0.7 or more indicates a strong sensitiser and a probability below 0.30 indicates a weak or moderate sensitiser. Probability values between 0.30 and 0.70 are referred to as indeterminate. An optimum prediction space algorithm ensures that predictions are only made for substances within the model applicability domain. Please note that the models are all based on the guinea-pig

maximization test (Enslein *et al*., 1997; http://accelrys.com/solutions/scientific-need/predictive-toxicology.html).

CASE methodology and all its variants were developed by Klopman and Rosenkranz. There are a multitude of models for a variety of endpoints and hardware platforms. The CASE approach uses a probability assessment to determine whether a structural fragment is associated with toxicity (Cronin *et al.*, 2003). The MCASE models that have been developed for skin sensitisation are described further in primary articles (Gealy *et al.*, 1996, Graham *et al.*, 1996, Johnson *et al.*, 1997). There are two sensitisation modules available for purchase from MultiCase Inc (Ohio, USA) (http://www.multicase.com/case-ultra-models). In addition the (Q)SAR estimates for one MCASE skin sensitisation model are included in the Danish Environmental Protection Agency (EPA) (Q)SAR database (http://qsar.food.dtu.dk/).

VEGA platform, freely available for download (http://www.vega-qsar.eu/), incorporates a model (Chaudhry *et al*., 2010) developed using an Adaptive Fuzzy Partition (AFP) algorithm based on eight descriptors. The AFP assigns the substances to two classes, sensitisers and non-sensitisers. An in-depth assessment of the applicability domain of the prediction, mainly based on similarity with substances in the training set of the model, is also provided.

- Knowledge-based systems:

Derek Nexus (DN) is a knowledge-based expert system created with knowledge of structure-toxicity relationships and an emphasis on the need to understand mechanisms of action and metabolism. It is marketed and developed by LHASA Ltd (Leeds, UK) a not-for-profit company and educational charity (http://www.lhasalimited.org/index.php).

Within DN (version 9), there are 361 alerts covering a wide range of toxicological endpoints. An alert consists of a toxicophore, a substructure known or thought to be responsible for the toxicity alongside associated literature references, comments and examples. The skin sensitisation knowledge base in DN was initially developed in collaboration with Unilever in 1993 using its historical database of guinea pig maximisation test (GPMT) data for 294 substances and contained approximately forty alerts (Barratt *et al.*, 1994). Since that time, the knowledge base has undergone extensive improvements as more data have become available (Payne and Walsh 1994). The current version (version 9) contains seventy alerts for skin sensitisation and the closely-related endpoint of photoallergenicity (Barratt *et al*., 2000; Langton *et al*., 2006). The predictivity of Derek Nexus for skin sensitisation was recently assessed by Guesne *et al*. (2014). As a reminder, alert-based systems should not be assessed for their specificity and overall accuracy, contrary to discriminant models.

- Hybrids:

TIssue MEtabolism Simulator (TIMES) software has been developed to integrate a Skin metabolism Simulator (SS) with 3D-QSARs for evaluating reactivity of substances in order to predict their skin sensitisation potency (Dimitrov *et al.,* 2005,). The current version of the simulator (version 2.27.16) contains more than 200 hierarchically ordered spontaneous and enzyme controlled reactions. Covalent interactions of substances/metabolites with skin proteins are described by 47 alerting groups. 3D-QSARs (COREPA) are applied for some of these alerting groups. Characterisation and evaluation of TIMES-SS can be found in Patlewicz *et al*. (2007) and Roberts *et al*. (2007b), respectively. New research with TIMES includes the work of Patlewicz *et al*. (2014a).

Clearly there are a breadth of different (Q)SARs and expert systems available for the estimation of skin sensitisation hazard. The approaches are quite varied and each has been developed on different sets of *in vivo* data (principally GPMT and LLNA). Whilst efforts have been made to characterise a number of the literature based models in terms of the OECD principles for QSAR validation (see Roberts *et al.*, 2007a as an example), further work is still required for some of the commercial systems (ECETOC, 2003). In addition, in many cases these models have been demonstrated to be reasonable for predicting skin sensitisers correctly but are limited in predicting non-sensitisers correctly (Roberts *et al.*, 2007a; ECETOC, 2003). For this reason, careful interpretation of model predictions needs to be considered in light of other information e.g. analogue read-across (other similar substances with respect to their mechanistic domain).

Further work should explore encoding more knowledge/rules for non-reactive substances as well as those substances likely to undergo chemical or metabolic transformation.

Consideration of which model(s) to apply will be dependent on the specific substances of interest, the underlying training set data and the applicability domain. These issues are described more fully in Section R.6.1 of Chapter R.6 of the *Guidance on IR&CSA*. An example is illustrated here; if the substances falls into a chemistry reactivity domain that is well characterised, then a local (Q)SAR model developed for this domain (such as those previously described) will give rise to the most robust prediction of skin sensitisation. Where the mechanism is not understood or not known *a priori* one or more of the expert systems such as TOPKAT, Derek for Windows or the others already described will be best placed to provide an estimate. These systems whilst not wholly transparent do provide a reasonable amount of supporting information to enable the robustness of a prediction to be evaluated. This is discussed in more detail in Section R.7.3.4.1.

Testing data on skin sensitisation

Internationally adopted test methods for skin sensitisation are described in the Annex to the EU Test Methods (TM) Regulation (Council Regulation (EC) No 440/2008) and in OECD Test Guidelines (TGs) (available at http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm).

Please note that the latest version of an adopted test guideline should always be used when generating new data, independently from whether it is published by EU or OECD.

The testing and assessment strategy developed for skin sensitisation (see Section R.7.3.6 of this Guidance) emphasises the need to evaluate all available information (including physico-chemical properties) before attempting any *in vivo* testing.

In chemico/in vitro data

Internationally adopted *in chemico*/*in vitro* test methods to assess whether a substance is a skin sensitiser (i.e. category 1 under CLP) or not are listed in Table R.7.3–1. More information on the specific scope and limitations of these tests is provided in Section R.7.3.4.1 under "Testing data on skin sensitisation".

Table R.7.3–1 Adopted and scientifically valid *in chemico*/*in vitro* methods for skin sensitisation [3]

AOP Key event	Test method	Validation status, regulatory acceptance	EU Test Methods/ OECD test guideline	Classification according to CLP Regulation	EURL ECVAM DB-ALM protocol Nr.
Skin sensitisation					
Key Event 1 Protein binding	DPRA	Validated and regulatory acceptance	N.A/TG 442C	Cat. 1 or NC	154
Key Event 2 Keratinocyte response	KeratinoSens™	Validated and regulatory acceptance	N.A/TG 442D	Cat. 1 or NC	155
	LuSens[4]	Validated	N.A/N.A	Cat. 1 or NC	N.A.
Key Event 3 Dendritic cell response	h-CLAT	Validated and regulatory acceptance	N.A/TG xxx	Cat. 1 or NC	158
	U-SENS™[4]	Validated	N.A/N.A	Cat. 1 or NC	N.A.
	IL-8 Luc Assay[5]	Validated	N.A/N.A	Cat. 1 or NC	N.A.

NOTE: Scientifically valid means that the test method has gone through a validation process and concluded to be scientifically valid for specific purposes.
Abbreviations: N.A. = not available; NC = not classified; DPRA = Direct Peptide Reactivity Assay; h-CLAT = human Cell Line Activation Test; KE: Key Event; TG: Test Guideline.

The test methods indicated in Table R.7.3–1 are either *in chemico* assay(s) (DPRA), or cell-based assays (Keratinosens™, h-CLAT). These test methods were developed to address specific events of the skin sensitisation AOP (OECD, 2012). The AOP for skin sensitisation describes the current understanding of key events linked to skin sensitisation. As the test methods only address a specific key event of skin sensitisation, they should not be used in isolation to identify a skin sensitiser or a non-sensitiser. More

[3] Note: The test methods have been validated to be used together with other information within a *Weight-of-Evidence* approach and not as stand-alone test method independent whether a positive or negative result is obtained.

[4] The LuSens and the U-SENS™ test methods have undergone industry-led validation studies. The information generated in the validation studies has been submitted to EURL ECVAM and is currently under evaluation.

[5] The IL-8 Luc Assay underwent a validation study coordinated by JaCVAM. The test method is currently under peer-review. A standard project submission form (SPSF) for the development of a Test Guideline was submitted to the OECD in 2014. The project will be included in the OECD work programme of 2015 pending revision of the SPSF.

information on how these test methods can be used in REACH context can be found in section R.7.3.6.2 of this guidance.

It is important to note, that currently several non-animal test methods are under development or evaluation. It is advised to monitor the status of current developments e.g. via EURL ECVAM website (https://eurl-ecvam.jrc.ec.europa.eu/).

Animal data

- Guideline-compliant tests

For new *in vivo* testing of skin sensitisation potential, the murine local lymph node assay (LLNA) is the REACH Annex VII-endorsed method. This assay has been validated internationally and has been shown to have clear animal welfare benefits and scientific advantages compared with the guinea pig tests described below. The LLNA is designed to detect the potential of substances to induce sensitisation as a function of lymphocyte proliferative responses induced in regional lymph nodes (induction phase). This method is described in EU B.42/OECD TG 429. In addition, there are different variants of the LLNA adopted by the OECD, i.e. OECD TG 442A (Local Lymph Node Assay: DA) and OECD TG 442B (Local Lymph Node Assay: BrdU-ELISA). The main difference compared to the OECD TG 429 is that these test methods do no use radioactive labelling.

Two further animal test methods for skin sensitisation are described in EU B.6/OECD TG 406: the guinea pig maximisation test (GPMT) and the Buehler test. The GPMT is an adjuvant-type 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 method involving for the induction phase topical application only. Both test methods assess the elicitation phase, i.e. adverse outcome of skin sensitisation.

Both the GPMT and the Buehler test are able to detect substances with moderate to strong sensitisation potential, as well as those with relatively weak sensitisation potential. In such methods activity is measured as a function of challenge-induced dermal hypersensitivity reactions elicited in test animals compared with controls. Since the LLNA is the preferred method for new *in vivo* testing, the use of the standard guinea pig tests to obtain new data on skin sensitisation potential will be acceptable only in exceptional circumstances and will require scientific justification. However, existing data of good quality deriving from such tests will be acceptable and will, if providing clear results, preclude the need for further *in vivo* testing.

ECETOC Monograph 29 (2000) contains a useful discussion of these tests.

- Non-guideline compliant tests and refinements to the standard assays

Existing data may be available from tests that do not have an OECD guideline, for example:

i. other guinea pig skin sensitisation test methods (such as the Draize test, optimisation test, split adjuvant test, open epicutaneous test);

ii. additional tests (such as the mouse ear swelling test).

Information may also be available from other endpoints, for example, repeated dose dermal studies that show effects indicative of an allergic response, such as persistent erythema and/or oedema.

R.7.3.3.2 Human data on skin sensitisation

Human data on cutaneous (allergic contact dermatitis and urticarial) reactions may come from a variety of sources:

- consumer experience and comments, preferably followed up by professionals (e.g. diagnostic patch tests);
- diagnostic clinical studies (e.g. patch tests, repeated open application tests);
- records of workers' experience, accidents, and exposure studies including medical surveillance;
- case reports in the general scientific and medical literature;
- consumer tests (monitoring by questionnaire and/or medical surveillance);
- epidemiological studies;
- human experimental studies such as the human repeat insult patch test (Stotts, 1980) and the human maximisation test (Kligman, 1966), although it should be noted that *new* experimental testing for hazard identification in humans, including HRIPT and HMT, is not acceptable for ethical reasons.

R.7.3.4 Evaluation of available information on skin sensitisation

For both steps of the effects assessment, i.e. hazard identification and dose (concentration)-response (effect) assessment, it is very important to evaluate the data with regard to their adequacy and completeness. The evaluation of adequacy should address the reliability and relevance of the data. The completeness of the data refers to the conclusion on the comparison between the available adequate information and the information that is required under the REACH proposal for the applicable tonnage level of the substance. Such a conclusion relies on *Weight-of-Evidence* approaches, mentioned in REACH Annex XI Section 1.2, which categorise available information based on the methods used: *guideline tests, non-guideline tests*, and other types of information which may justify adaptation of the standard testing regime. Such a *Weight-of-Evidence* approach also includes an evaluation of the available data as a whole, i.e. both over or across endpoints: i.e. for a sensitive evaluation of sensitisation effects, it is necessary to efficiently integrate the information gathered for sensitisation with that obtained from the study of skin and eye irritation (and acute dermal toxicity).

This approach provides a basis to decide whether further information is needed on endpoints for which specific data appear inadequate or not available, or whether the requirements are fulfilled.

For this specific endpoint some additional remarks are made on the adequacy of the various types of data that may be available.

R.7.3.4.1 Non-human data on skin sensitisation

Non-testing data on skin sensitisation

When evaluating the non-testing data on the substance, the evaluation and assessment of a substance using (Q)SARs is dependent on both the substances of interest and the (Q)SAR model(s) used to make a prediction. Here we attempt to provide some specific advice for skin sensitisation. More general advice on (Q)SARs including evaluation of OECD principles is described in Section R.6.1.3 of Chapter R.6 of the *Guidance on IR&CSA*.

One of the first steps to consider is what information already exists on substances *similar* to the one of interest. Chemical similarity is a widely used concept in toxicology, and is based on the hypothesis that similar compounds have similar biological activities. This forms the underlying basis for developing (Q)SARs. In the case of skin sensitisation, the most robust means of comparing two or more substances is through an evaluation of their likely chemical reactivity. Recent work in this area has been investigating means of encoding reactivity for the different mechanistic domains in form of rules (Aptula and Roberts, 2006; Aptula *et al*., 2006; Schultz *et al.*, 2009) [6]. If the chemical reactivity is not known, or cannot be determined through experimentation, then a pragmatic means of identifying similar substances can be through a substructural/analogue search.

There are a number of available computational tools and databases that facilitate the search and retrieval of similar analogues. Some like Leadscope (http://www.leadscope.com) are commercial, others like Chemfinder (www.chemfinder.com), ChemID (http://chem.sis.nlm.nih.gov/chemidplus/), NICEATM LNA Database (http://ntp.niehs.nih.gov/pubhealth/evalatm/test-method-evaluations/immunotoxicity/nonanimal/index.html) or DssTox (http://www.epa.gov/nheerl/dsstox/) are freely available to use on the internet.

Some of the available search engines are linked to databases (through hyperlinks and indexes) whereas other facilities such as DssTox provide a repository of available QSAR datasets which can be downloaded for subsequent use in appropriate QSAR /database software tools.

Many of currently available tools containing public data have focussed on endpoints such as carcinogenicity, mutagenicity or acute toxicity. This means that an additional search is needed to identify skin sensitisation data. Much of the available skin sensitisation experimental data resides in peer reviewed publications. Cronin and Basketter (1994) published the results of over 270 *in vivo* skin sensitisation tests (mainly from the guinea pig maximisation test). All data were obtained in the same laboratory and represent one of the few occasions when large amounts of information from corporate databases were released into the open literature. A larger database of animal and human studies for 1034 compounds is described by Graham *et al.* (1996), the MCASE database. A comparatively large number of data have been published for the local lymph node assay; examples include publications by Ashby *et al.* (1995), Gerberick et al (2005) and Kern *et al*. (2010).

These publications are invaluable to identify analogues with associated skin sensitisation test data.

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[6] This approach might involve the systematic generation of *in vitro* reactivity data for these different mechanistic domains.

The second step involves an assessment of the similarity of the analogues identified. Considerations will include whether:

- the same endpoint is considered;
- there are any additional functional groups or additional substituents that might influence the reactivity and sensitising behaviour (applicability domain considerations);
- the physico-chemical parameters are similar (e.g. LogP, applicability domain considerations);
- there are impurities that influence the sensitisation profile;
- the likely chemical mechanism is the same.

These considerations may help identify an available local (Q)SAR for that chemical class/mechanistic group.

If an appropriate local model cannot be identified then a third step of evaluating a substance using one of the available global models/expert systems is merited.

Here a prediction needs to be evaluated in the context of the likely chemistry and the presence of similar substances within the training set. i.e. is the compound of interest within the scope of the model and are similar substances in the training set of the model well predicted. This type of information provides additional weight to whether the estimate derived is meaningful and relevant. For global models available in the literature, the training sets and the algorithm(s) are usually available to allow such comparisons to be made.

For expert systems such as Derek Nexus, TOPKAT etc, the training sets and to an extent the algorithms or descriptors used are often kept latent within the software. Some supporting information is provided on the robustness and relevance for a given prediction. For example, within DN it is possible to see representative example substances and explanations of the mechanistic basis for the SAR developed.

TOPKAT supports the users in assessing the reliability of the prediction by: 1) evaluating if the substance falls into the applicability domain of the model (based on structural fragments and descriptors), 2) checking if the substance is present in its database, and 3) identifying analogues of the target substance based on chemical similarity. Similar functionalities and features are present in many of the other commercial expert systems available.

Although the main factors driving skin sensitisation (and therefore the (Q)SARs) is the underlying premise of the electrophilicity of a substance, other factors such as hydrophobicity encoded in the octanol/water partition coefficient (log P) may also be considered as playing a role in the modifying the sensitisation response observed. Within DN, an assessment of the likely skin penetration ability is made using the algorithm by Potts and Guy. This relates the Kp value to log P and MW (Potts and Guy 1992). It is then possible to rationalise the output in terms of bands of penetration potential. Some have been described in Howes *et al.* (1996).

Specific model and prediction information can be described in more detail in reporting formats ((Q)SAR Reporting Format). This summarises the pertinent information to consider for a given model when evaluating an estimate as well as the estimate itself. More details are provided in Section R.6.1 of Chapter R.6 of the *Guidance on IR&CSA*.

Other information such as results in other assays, e.g. the Ames test (a common feature of genotoxic substances is that they can bind covalently to DNA and cause direct DNA damage) or aquatic toxicity tests, may provide supporting information about the electrophilicity of the substance of interest and hence its likely sensitisation ability. Some of this work explores correlations between aquatic toxicants and skin sensitisers (Aptula *et al*., 2006) and between experimentally identified mutagens and sensitisers (Wolfreys and Basketter 2004; Patlewicz *et al*., 2014b). More recently, the use of mutagenicity data was proposed as part of an integrated approach to testing and assessment (IATA) for skin sensitisation (Patlewicz *et al*., 2014b).

The increasing necessity for sustainable testing and animal welfare considerations, as well as the information requirements of the REACH Regulation, highly stimulated research on integrated strategies for skin sensitisation in the past few years. Some of these works are limited in scope, combining only *in silico* (Teubner *et al*., 2013) or *in vitro* information (Maxwell *et al*., 2014; Reisinger *et al*., 2015), while others make use of all possible alternatives to animal testing in different combinations (Basketter *et al*., 2013; Rorije *et al*., 2013; Jaworska *et al*., 2013).

Testing data on skin sensitisation

In chemico/in vitro data

There are OECD-adopted test guidelines available for the assessment of skin sensitisation potential *in vitro* (see Section R.7.3.3.1). These test methods have not been developed as stand-alone test methods, but test methods to be used together with other pieces of information in a *Weight-of-Evidence* approach, e.g. by using several *in chemico/in vitro* methods together.

Annex VII to the REACH Regulation specifies that the standard information requirement for skin sensitisation is an *in vivo* study. However the REACH Regulation gives several options for adapting this standard information requirement, e.g. via Column 2 specific rules for adaptations or via Annex XI general rules for adaptations. As a consequence, data from the tests described below may be accepted for Annex VII requirement when used **in combination** with other pieces of information (*in chemico*, *in vitro*, *in silico*, (Q)SARs, etc.) e.g. within a *Weight-of-Evidence* approach according to Annex XI, sections 1.2 – 1.5 to the REACH Regulation (see Section R.7.3.6).

It should be noted that the test methods described below are not suitable **on their own** for sub-categorisation of skin sensitisers into CLP sub-categories 1A and 1B. Potency indicators such as the level of protein depletion and dose-dependent responses can be obtained from the existing *in chemico* and *in vitro* tests, respectively. However, there is currently no prediction model able to integrate these indicators into an adequate potency classification. A few approaches have been recently proposed for potency prediction (Jaworska *et al.*, 2013; Natsch *et al*., 2014; Reisinger *et al*., 2015; OECD, 2015a). It is nevertheless, strongly recommended that when non-animal testing methods are used to fulfil the REACH information requirements to consider the skin sensitisation potency of the substance by all means available, even though at this point of time no proper advise can be provided. The reader is advised to follow on the recent developments on the matter.

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

  - The specific limitations of the test method are: It is applicable to test substances that are soluble in an appropriate solvent at a final concentration of

100 mM. Substances that are not soluble at this final concentration can still be tested at lower soluble concentrations. In such a case, positive results could still be used to identify a test substance as a sensitiser whereas negative results should be considered inconclusive.

- It is not applicable to the testing of metal compounds (known to react with proteins with mechanisms other than covalent binding), complex mixtures of unknown composition, substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances) due to the defined molar ratio of the test substance and peptide;
- The test system has no metabolic capacity and therefore pro-haptens (i.e. substances requiring enzymatic activation to exert their sensitising activity) and pre-haptens (i.e. substances activated by auto-oxidation) may provide (false) negative results;
- Test substances with exclusive reactivity towards amino-acids other than cysteine or lysine (e.g. nucleophilic sites of histidine) may lead to false negative results;
- Potential over-predictions may be due to substances that do not covalently bind to the peptide but do promote its oxidation (e.g. cysteine dimerisation).

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

The specific scope and limitations of the test method are:

- It is applicable to test substances that are soluble or that form a stable dispersion either in water or DMSO, or another appropriate solvent if its choice is scientifically justified. Test substances that do not fulfil these conditions at the highest final required concentration of 2000 µM may still be tested at lower concentrations. In such a case, positive results could be used to identify a test substance as sensitiser whereas negative results obtained with concentrations < 1000 µM should be considered inconclusive;
- The test system has a limited metabolic capacity and therefore pro- and pre-haptens may produce (false) negative results;
- Test substances with exclusive reactivity towards nucleophiles other than cysteine's sulfhydryl group (e.g. lysine residues) can produce negative results in the assay;
- Test substances that do not act as sensitisers but are nevertheless chemical stressors may produce false positive results;
- Highly cytotoxic substances cannot always be reliably assessed;
- Test substances that interfere with the luciferase enzyme can affect its activity by either increasing or inhibiting the luminescence.

- Human Cell Line Activation Test (h-CLAT) - draft OECD TG available (see http://www.oecd.org/env/ehs/testing/section4healtheffects.htm)

The specific scope and limitations of the test method are:

- It is applicable to test substances that are soluble or form a stable dispersion in an appropriate solvent;
- Test substances with Log Kow ≤ 3.5 can be tested whereas substances with Log Kow > 3.5 tend to produce negative results. For such substances positive results could be used to support the identification of a test substance as a sensitiser. Negative results should be considered inconclusive.
- The test system has a limited metabolic capacity and therefore pro- and pre-haptens may produce (false) negative results;
- Higly cytotoxic substances cannot always be reliably assessed;
- Since it uses a fluorescein isothiocyanate (FITC)-labelled antibody, strong fluorescent test substances emitting at the same wavelength as FITC may interfere with the flow cytometry light-signal acquisition.

Concerning the *in chemico/in vitro* test methods, any modification made to the adopted test methods needs to be properly documented and justified. The reporting template in Annex II of the OECD Guidance Document on the Reporting of IATAs (OECD, 2015b) can be used for that purpose. Proper documentation and justification are also needed when the information submitted has been generated with test methods that are used in-house only, without adopted test guidelines.

Animal data

Well reported studies using internationally acceptable protocols, particularly if conducted in accordance with the principles of GLP, can be used for hazard identification. Other studies (see Section R.7.3.3.1 and below), not fully equivalent to OECD test protocols, can, in some circumstances, provide useful information. Particular attention should be paid to the quality of these tests and the use of appropriate positive and negative controls. The specificity and sensitivity of all animal tests should be monitored through the inclusion of appropriate positive and negative controls. In this context, positive controls are the 6-monthly sensitivity checks with an appropriate positive control substance, and negative controls are the vehicle-treated control animals included as part of each test.

- Guideline-compliant tests

Murine Local Lymph Node Assay

For the conduct and interpretation of the LLNA the following points should be considered:

i. the vehicle in which the test material and controls have been applied;
ii. the concentrations of test material that have been used;
iii. any evidence for local or systemic toxicity, or skin inflammation resulting from application of the test material;
iv. whether the data are consistent with a biological dose-response;
v. the submitting laboratory should be able to demonstrate its competency to conduct the LLNA.

EU B.42/OECD TG 429 provides guidance on the recommended vehicles, number of animals per group, concentrations of test substance to be applied and substances to be used as positive control. A preliminary study or evaluation of existing acute toxicity/dermal irritation data is normally conducted to determine the highest concentration of test substance that is soluble in the vehicle but does not cause unacceptable local or systemic toxicity. The submission of historical control data will demonstrate the ability of the test laboratory to produce consistent responses. Based on the use of radioactive labelling (tritiated (3H)-methyl thymidine), substances that result in a stimulation index (SI) ≥3 at one or more test concentrations are considered to be positive for skin sensitisation. Both positive and negative responses in the LLNA conducted as described in EU B.42/OECD TG 429 meet the data requirements for classification of a substance as a skin sensitiser including potency estimations: no further testing is required.

Alternative vehicles to those listed in EU B.42/OECD TG 429 may be used in the LLNA if sufficient scientific justification is provided.

The LLNA: DA test method measures ATP content by luminescence in the proliferating cells and hence does not require the use of radioactive labelling of cells. Substances that result in SI ≥1.8 at one or more testing concentration(s) are considered to be positive for skin sensitisation. In case of borderline positive results (1.8 ≤ SI ≤ 2.5) additional information may be considered such as the dose-response relationship, evidence of systemic toxicity or excessive irritation, and, where appropriate, statistical significance together with SI values to confirm that such results are indeed positives.

The LLNA: BrdU-ELISA test method uses the non-radiolabelled marker 5-bromo-2-deoxyuridine (BrdU) to measure lymphocyte proliferation. Substances that result in SI ≥1.6 at one or more testing concentration(s) are considered to be positive for skin sensitisation. In case of borderline positive results (1.6 ≤ SI ≤ 1.9) additional information may be considered such as the dose-response relationship, evidence of systemic toxicity or excessive irritation, and, where appropriate, statistical significance together with SI values to confirm that such results are indeed positives.

The OECD TG 442A (LLNA: DA) and OECD TG 442B (LLNA: BrdU-ELISA) recommend the use of the same vehicles as in the standard LLNA EU B.42/OECD TG 429.

Limitations of the all above LLNAs include the following:

- False negative predictions can be obtained with certain metals (e.g. nickel) and false positive predictions may be obtained with certain surfactant type substances (Kreiling *et al.*, 2008).
- The solubility of the substance may interfere with the accuracy of the predictions.
- The choice of vehicle may affect the prediction for certain substances. For instance DMSO as a polar solvent may enhance dermal bioavailability of some test substances and propylene glycol may suppress the proliferative effects of some test substances (e.g. DNCB) (Anderson *et al.*, 2011). Therefore, it is important to properly select the vehicle used in the study.

The updated OECD TG 429 of 2010 contains the inclusion of the reduced LLNA (rLLNA), in which only one concentration is tested and less animals are used. It is recommended to use this refinement method only in case a confirmation on a negative result obtained with another testing method is required. Since only one dose is used in the study design, the rLLNA cannot currently be used for estimating the skin sensitisation potency of a substance (Ezendam *et al.*, 2013), even though a proposal has recently been published

for predicting potency from a single dose (Roberts, 2015). The TGs for the LLNA variants, i.e. DA and BrdU-ELISA test methods, do not include the use of the rLLNA study design.

Guinea pig studies

The guinea pig test method described in EU B.6/OECD TG 406, the GPMT (Magnusson *et al.*, 1969; Schlede *et al.*, 1995) and the Buehler test, can also be used for hazard identification. Recommendations on conducting and analysing these methods are provided by Steiling *et al.* (2001). Particular attention should be paid to the quality of these tests with consideration given to the following points:

i. numbers of test and control guinea pigs;

ii. number or percentage of test and control animals displaying skin reactions;

iii. whether skin irritation was observed at the induction phase;

iv. whether the maximal non-irritating concentration was used at the challenge phase;

v. the choice of an appropriate vehicle (ideally, one that solubilises or gives a stable suspension or emulsion of the test material, is free of allergenic potential, is non-irritating, enhances delivery across the stratum corneum, and is relevant to the usage conditions of the test material, although it is recognised that it will not always be possible to meet all these conditions);

vi. whether there are signs of systemic toxicity (a sighting study should be performed to determine an appropriate induction dose that causes irritation but not systemic toxicity);

vii. staining of the skin by the test material that may obscure any skin reactions (other procedures, such as chemical depilation of the reaction site, histopathological examination or the measurement of skin fold thickness may be carried out in such cases);

viii. results of rechallenge treatments if performed;

ix. checking of strain sensitivity at regular intervals by using an appropriate control substance (as specified in OECD guidelines and EU Test Methods). Currently (in 2015), the recommended interval is 6 months.

The investigation of doubtful reactions in guinea pig tests, particularly those associated with evidence of skin irritation following first challenge, may benefit from rechallenge of the test animals. In cases where reactions may have been masked by staining of the skin, other reliable procedures may be used to assist with interpretation; where such methods are used, the submitting laboratory should provide evidence of their value.

A justification for performing a new guinea pig test instead of LLNA could be e.g. that the test substance contains nickel, as it is known that nickel is not correctly predicted in the LLNA.

- Non-guideline compliant tests and refinements to the standard assays

The submitted dossier should include scientific justification for conducting any new test that is a modification or deviation from guideline methods. In such cases, it would be advisable to seek appropriate expert advice on the suitability of the assay before testing is begun.

Historically, guinea pig studies that are not fully equivalent to OECD test protocols have been conducted and can provide useful hazard information. These studies include, but are not limited to, the following: Draize test, optimisation test, split adjuvant test, open epicutaneous test and the cumulative contact enhancement test. In the case of positive results the substance may be considered as a potential skin sensitiser. If, taking into account the above quality criteria, especially the positive and negative control data, there is a clear negative result, i.e. no animals displaying any signs of sensitisation reactions, then no further animal testing is required. Where there is a low level of response, the quality of the study is questionable, or where unacceptably low concentrations of the test material have been used for induction and/or challenge, further testing may be required.

R.7.3.4.2 Human data on skin sensitisation

When reliable and relevant human data are available, they can be useful for hazard identification and even preferable over animal data. However, lack of positive findings in humans does not necessarily overrule positive and good quality animal data.

Well conducted human studies can provide very valuable information on skin sensitisation. However, in some instances (due to lack of information on exposure, a small number of subjects, concomitant exposure to other substances, local or regional differences in patient referral, etc.) there may be a significant level of uncertainty associated with human data. Moreover, diagnostic tests are carried out to see if an individual is sensitised to a specific agent, and not to determine whether the agent can cause sensitisation.

For evaluation purposes, existing human experience data for skin sensitisation should contain sufficient information about:

- the test protocol used (study design, controls);
- the substance or preparation studied (should be the main, and ideally, the only substance or preparation present which may possess the hazard under investigation);
- the extent of exposure (magnitude, frequency and duration);
- the frequency of effects (versus number of persons exposed);
- the persistence or absence of health effects (objective description and evaluation);
- the presence of confounding factors (e.g. pre-existing dermal health effects, medication; presence of other skin sensitisers);
- the relevance with respect to the group size, statistics, documentation;
- the *healthy worker* effect.

Evidence of skin sensitising activity derived from diagnostic testing may reflect the induction of skin sensitisation to that substance or cross-reaction with a chemically very similar substance. In both situations, the normal conclusion would be that this provides

positive evidence of the skin sensitising activity of the substance used in the diagnostic test.

Human experimental studies on skin sensitisation are not normally conducted and are generally discouraged. Where human data are available, then quality criteria and ethical considerations are presented in ECETOC monograph no 32 (ECETOC, 2002).

Ultimately, where a very large number of individuals (e.g. 10^5) have frequent (daily) skin exposure for at least two years and there is an active system in place to pick up complaints and adverse reaction reports (including via dermatology clinics), and where no or only a very few isolated cases of allergic contact dermatitis are observed then the substance is unlikely to be a significant skin sensitiser. However, information from other sources should also be considered in making a judgement on the substance's ability to induce skin sensitisation.

It is emphasised that testing with human volunteers is strongly discouraged, but when there are good quality data already available they should be used as appropriate in well justified cases.

R.7.3.5 Conclusions on skin sensitisation

R.7.3.5.1 Remaining uncertainty on skin sensitisation

Reliable data on skin sensitisation can be generated from well designed and well conducted studies in animals. However, it should be noted that no toxicological test is perfect and each test method has to balance between the sensitivity (false negatives) and specificity (false positives) of the prediction. The use of adjuvant in the GPMT may lower the threshold for irritation and so lead to false positive reactions, which can therefore complicate interpretation (running a pre-test with FCA treated animals can provide helpful information). In international trials, the LLNA has been shown to be reliable, but like the guinea pig tests it is dependent on the vehicle used. It has been claimed that LLNA may create false positives for (irritating) surfactants (non-specific lymphocyte proliferation, Garcia *et al*., 2010). However, Basketter & Kimber, 2011 states that if the study is performed according to the dose selection criterion as specified in the OECD TG 429, no false positives results should be obtained based only on the irritating properties of the substance. A vehicle selected in the assay may cause variability in the response (lymphocyte proliferation) as vehicle may enhance or supress the response (Anderson *et al.*, 2011).Careful consideration should be given to circumstances where exposure may be sub-optimal due to difficulties in achieving a good solution and/or a solution of sufficient concentration. In some circumstances inconsistent results from guinea pig studies, or between guinea pig and LLNA studies, might increase the uncertainty of making a correct interpretation. Finally, for existing human data consideration must be given to whether inter-individual variability is such that it is not scientifically sound to generalize from a limited population.

The non-animal test methods (*in chemico/in vitro*) currently available have no or limited metabolic capacity, therefore substance requiring enzymatic activation before becoming sensitisers may not be correctly identified by such test methods. Also, some chemicals requiring auto-oxidation before becoming active may not be detected. More information on these limitations can be found in section R.7.3.6 of this Guidance.

R.7.3.5.2 Concluding on suitability for Classification and Labelling

REACH demands that all available information for a substance is gathered and any lack of information is reported.

Standard information required for skin sensitisation is described in Annex VII of REACH, i.e. for any substance manufactured or imported in quantity of 1 tonne or more per year.

Classification as skin sensitiser must be considered following the flow chart for the testing and assessment strategy reported in Section R.7.3.6 of this Guidance.

According to the CLP Regulation, labelling for skin sensitisation uses the signal word *"Warning"* and the hazard statement H317 *("May cause allergic skin reaction"*).

The CLP Regulation specifies that skin sensitising substances must be allocated into sub-categories (i.e. 1A or 1B) and appropriate specific concentration limits must be set whenever possible. In case the data are not sufficient for sub-categorisation, the substance must be classified in the general Category 1 (for further information, see Section 3.4 of the *Guidance on the Application of the CLP criteria*).

Measurement of potency

Appropriate dose-response data can provide important information on the potency of the material being tested. This can facilitate the development of more accurate risk assessments. This section refers to potency in the induction phase of sensitisation.

Neither the standard LLNA nor the GPMT/Buehler test is specifically designed to evaluate the skin sensitising potency of test compounds, instead they are used to identify sensitisation potential for classification purposes. However, they can all be used to estimate of potency to a varying degree. The relative potency of substances may be indicated by the percentage of positive animals in the guinea pig studies in relation to the concentrations tested. Likewise, in the LLNA, the EC3 value (the dose estimated to cause a 3-fold increase in local lymph node proliferative activity) is used as a measure of potency (CLP Regulation, table 3.4.3 and 3.4.4. and CLP Guidance Table 3.4.2.f). Often, linear interpolation of a critical effect dose from the EC3 is proposed (ECETOC, 2000), but more advanced statistical approaches basing conclusions on the characteristic of the dose-response curve and variability of the results is also used (Basketter *et al.*, 1999; van Och *et al.*, 2000). The dose-response data generated by the LLNA makes this test more informative than guinea pig assays for the assessment of skin sensitising potency. EC3 data correlate well with human skin sensitisation induction thresholds derived from historical predictive testing (Schneider and Akkan, 2004; Griem, 2003; Basketter *et al.*, 2005b). In the CLP regulation there are criteria for determining potency based on both LLNA and GPMT/Buehler tests.

In the case of the GPMT and Buehler test, due to the dose selection criteria specified in the OECD TG 406, it is usually not possible to firmly conclude that a substance is a Category 1B sensitiser since classification in Category 1A cannot be excluded. Therefore, in case classification in Category 1A cannot be excluded the general Category 1 classification must be chosen.

Concerning classification based on non-animal test data, currently (in 2015) it is not possible to classify skin sensitising substances into a sub-category or to set specific concentration limits and hence only the general Category 1 can be used. However, there is currently no prediction model able to integrate these into an adequate sensitisation

potency classification. Few approaches have been proposed for potency prediction (Jaworska *et al*., 2013; Natsch *et al*., 2014; Reisinger *et al*., 2015; OECD, 2015a). However, work is ongoing in order to address the lack of potency characterisation based on non-animal approaches, therefore the reader is advised to follow-up the recent and future developments in the field.

The lack of potency information and sub-categorisation possibility may result in a lower level of protection of humans, especially if the substance is used in a mixture and correct concentration limits are not used, leading to incorrect labelling of the mixture. In case it is not possible to assess the skin sensitising potency of the substance based on the information available, it is strongly recommended to classify the substance in Cat 1A until a reliable prediction model becomes available or new data is generated to allow sub-categorisation.

Derivation of a DNEL

Potency information, such as the LLNA EC3 value, can be utilised for the derivation of no-effect levels, that is – in this instance – the threshold required for the induction of skin sensitisation. It should be noted that thresholds for skin sensitisation should be expressed in terms of dose per unit area. As mentioned above, the EC3 value correlates well with thresholds observed in previously published human predictive test data and with clinical experience (reviewed in Basketter *et al.*, 2007). The EC3 value can then be extrapolated by the application of assessment factors (reflecting e.g. intra and inter-individual variability and vehicle matrix effects) to derive no-effect levels (expressed in $\mu g/cm^2$ of skin) for use of specific skin sensitisers in defined exposure situations (Gerberick *et al.*, 2001; Felter *et al.*, 2002 and 2003; Basketter *et al.*, 2006). The approach is commonly referred to as quantitative risk assessment (QRA) and has been deployed, with considerable effect, to identify safe exposure levels for a range of skin sensitising chemicals (Zachariae *et al.*, 2003; Basketter *et al.*, 2003). This has been reported extensively for fragrance and preservative sensitisers (Api *et al.*, 2008; Basketter *et al.*, 2008).

Even though EC3 values can be used for DNEL derivation, the first step should always be the qualitative approach to assess and control the risks that may arise. The DNEL obtained from the LLNA could then be used to assess the remaining likelihood of risks. It should be noted that currently quantitative assessment cannot be performed by using guinea pig data or non-animal testing approaches. Guidance on how to use the potency information for qualitative assessment (see also Section E.3.4.2 of *Part E* of the *Guidance on IR&CSA*) and how to derive a DNEL as a second step in the safety assessment of sensitisers is given in Section R.8.6 and Appendix R.8-10 of *Chapter R.8* of the *Guidance on IR&CSA*.

R.7.3.5.3 Additional considerations

Chemical allergy is commonly designated as being associated with skin sensitisation (allergic contact dermatitis), or with sensitisation of the respiratory tract (asthma and rhinitis). In view of this it is sometimes assumed that allergic sensitisation of the respiratory tract will result only from inhalation exposure to the causative substance, and that skin sensitisation necessarily results only from dermal exposure. This is misleading, and it is important for the purposes of risk management to acknowledge that sensitisation may be acquired by other routes of exposure. Since adaptive immune responses are essentially systemic in nature, sensitisation of skin surfaces may theoretically develop from encounter with contact allergens via routes of exposure other than dermal contact (although in practice this appears to be uncommon). Similarly, there is evidence from both experimental and human studies which indicate that effective sensitisation of the respiratory tract can result from dermal contact with a chemical

respiratory allergen. Thus, in this case, it appears that the quality of immune response necessary for acquisition of sensitisation of the respiratory tract can be skin contact with chemical respiratory allergens (Kimber *et al.*, 2002). Such considerations have important implications for risk management. Thus, for instance, there is a growing view that effective prevention of respiratory sensitisation requires protection of both skin and respiratory tracts. This includes the cautious use of known contact allergens in products to which consumers are (or may be) exposed via inhalation, such as sprays. The generic advice is that appropriate strategies to minimise the risk of sensitisation to chemical allergens will require consideration of providing protection of all relevant routes of exposure.

R.7.3.5.4 Information not adequate

A *Weight-of-Evidence* approach, comparing available adequate information with the tonnage-triggered information requirements by REACH, may result in the conclusion that the requirements are not fulfilled. In order to proceed in further information gathering the testing and assessment strategy given in the next Section R.7.3.6 can be adopted.

R.7.3.6 Testing and assessment strategy for skin sensitisation

R.7.3.6.1 Objective / General principles

The following testing and assessment strategy is recommended for developing adequate and scientifically sound data for the assessment and classification of the skin sensitisation properties of a substance. For existing substances with insufficient data, this strategy can also be used to decide which additional data, besides those already available, are needed. The strategy is aimed at assessing skin sensitisation by using different elements where appropriate and depending on the information available. The key principle of the strategy is that the available information and results of one study/test battery or from one information source are evaluated before another study is initiated. The strategy seeks to ensure that the data requirements are met in the most efficient and humane manner so that animal usage and costs are minimised.

The different elements provided in Figure R.7.3–1 describe information sources that can be used to conclude on a substance's hazard potential towards skin sensitisation. The elements described in Figure R.7.3–2 can be rearranged as appropriate, especially those in Part 1 (elements 1 to 5). This may be particularly helpful in cases where a conclusion can be drawn from certain elements without having to consider all of them.

The specific rules for adaptation of standard information requirements are described in column 2 of Annex VII to the REACH Regulation, whereas the general rules for adaptation from standard information requirements are given in Annex XI.

The new elements in the strategy are the recently OECD adopted and/or internationally validated *in chemico/in vitro* test methods for skin sensitisation. These methods are based on the mechanistic understanding of the biological key events of skin sensitisation, initiated by the covalent binding of the substance onto skin proteins. These key events have been codified in the Adverse Outcome Pathway (AOP) for skin sensitisation approved by OECD (OECD, 2012). Three of these key events, i.e. protein reactivity, keratinocyte response and dendritic cell response, correspond to elements 5b (existing data), and to elements 7b, 7c and 7d (generation of new data) of Figure R.7.3–2 below.

The **strategy** aims to help the Registrant to find out how these *in chemico*/*in vitro* test methods for skin sensitisation can be used in a *Weight-of-Evidence* approach according to the Annex XI, 1.2 – 1.5 to the REACH Regulation to enable hazard identification and

appropriate classification decision on a substance. Also other types of data, such as QSAR, read-across and human data can be used in combination with the *in chemico/in vitro* test results. The key strengths and limitations of the *in chemico/in vitro* tests and other types of data are addressed below.

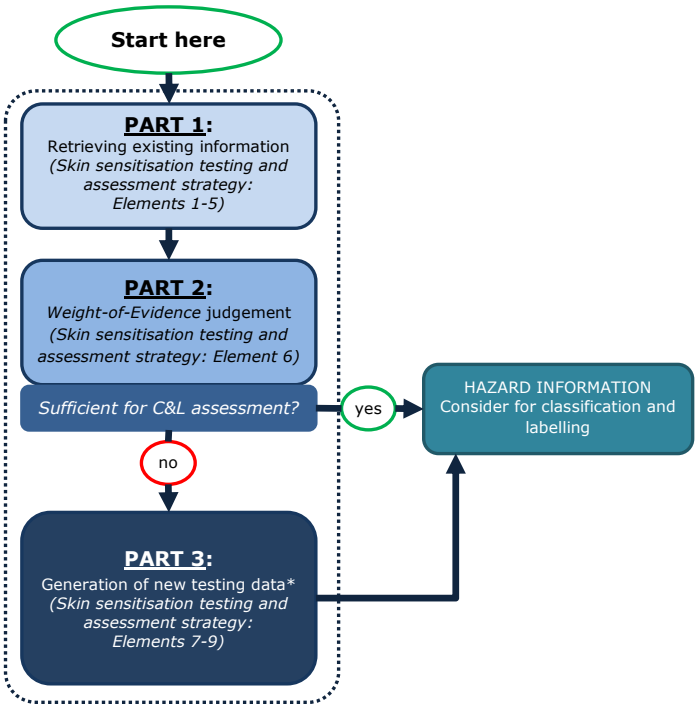


Figure R.7.3–1 Overview of the testing and assessment strategy for skin sensitisation

R.7.3.6.2 Application of the Testing and Assessment Strategy

The testing and assessment strategy presented here comprises three parts (see Figure R.7.3–2): Part 1 (elements 1 to 5) is about retrieving existing information, Part 2 (element 6) represent *Weigh-of-Evidence* analysis and expert judgement, and Part 3 (elements 7 to 9) is about generation of new information by testing.

According to Step 1 of Annex VI to the REACH Regulation, all existing available test data should be gathered before any new testing is initiated. In Part 1 of this strategy, existing and available information from the literature and databases is gathered and considered in the strategy approach. The order of the different elements, i.e. 1 to 5, is only indicative and they may be arranged as appropriate. This may especially be helpful in cases where a realiable conclusion can drawn from certain elements without having to consider all of them. For instance, if there are adequate human data (element 2) available that indicate that the substance should be classified as skin sensitiser accoring to the CLP Regulation, further testing is not required. At the end of the Part 1, and if no final conclusion can be derived directly from one or several of the available pieces of information, all the

information collected should be analysed using a *Weight-of-Evidence* approach (element 6).

In the information generation part (elements 7 to 9), new information on the skin sensitisation potential of the substance is produced either by means of non-animal test methods or, as a last resort (see Articles 13(1), 25(1) and Annex XI to the REACH Regulation), *in vivo* testing (element 9).

While it is recommended that this approach be followed, other approaches may be more appropriate and efficient on a case-by-case basis.

Due to the complexity of the skin sensitisation endpoint, a combination of alternative test methods would need to be provided in order to provide more confidence in the results for assessing skin sensitisation. The *in vitro* and *in chemico* test methods described in Sections R.7.3.3.1 and R.7.3.4.1 and in Figure R.7.3–2 below (as elements 5 and 7) have not been developed as stand-alone methods. Some *in silico* methods aim at predicting the final endpoint (e.g. LLNA outcome) and thus could, in theory, be used as stand alone methods. However, additional evidence (such as read-across from analogues) is crucial to confirm the reliability of the (Q)SAR prediction, which would be otherwise difficult to assess and accept. Therefore, a combination of these non-animal test methods (e.g. *in silico*, *in chemico* and *in vitro*) in a *Weight-of-Evidence* approach is considered the best approach. Supporting information may be derived from test methods addressing other biological mechanisms at the basis of skin sensitisation or from non-testing methods e.g. read-across.

Due to the recent developments in the field of non-animal test methods for skin sensitisation, and in line with Article 13(1) and the introductory paragraph of Annex VII to the REACH Regulation, Registrants are advised to investigate whether the information requirement for skin sensitisation can be fulfilled by using results from the new test methods in a *Weight-of-Evidence* approach.

In case no information on skin sensitisation is available for a substance it is recommended to start the assessment by using the OECD QSAR Toolbox (see Section R.7.3.3.1). The Toolbox can be used for many purposes. First, it facilitates the identification of existing *in chemico*, *in vitro* and *in vivo* data already available for the substance of interest. Second, it identifies skin sensitisation specific alerts and protein-binding alerts using profilers. Third, it can be used to predict and characterise metabolic and auto-oxidation products of the substance. Fourth, it facilitates the identification of analogues with experimental data for read-across. In addition, the existing *in vivo* data for the substance and/or analogue substance may provide useful information on the skin sensitisation potency, e.g. via EC3 values obtained from the exsiting LLNA studies. Note that the predictions can address the *in vivo* endpoints as well as *in vitro* ones (although for the moment there are not many *in vitro* data included in the Toolbox and the identification of analogues with data can be difficult). In addition to the Toolbox, expert systems and (Q)SAR softwares may provide further valuable information.

In case the use of the OECD QSAR Toolbox does not enable to conclude on the skin sensitisation hazard including the sensitising potency, of a substance, it is strongly recommended to investigate **at least** three key events (elements 7b, c and d in Figure R.7.3–2) as described in the AOP for skin sensitisation by providing information from non-animal test methods or by other sources of information. This is due to the fact that the test methods that are currently available (adopted by the OECD and/or considered to be scientifically valid) are not stand-alone methods and should be used together with other supporting information.

It is important to note that it is the responsibility of the registrant to ensure that the chosen test method (e.g. *in vitro, in chemico* or *in silico)* is suitable for testing the substance and obtain adequate information. So before performing a specific non-animal test the registrant should consider whether there are substance-specific limitations that may hinder the performance of the test (e.g. low solubility or log Kow, UVCB nature of the substance while for instance the DPRA is not applicable to UVCBs). There may also be some limitations of the test system like the absence of or limited metabolic capacity and hence pre- and pro-haptens may not be correctly detected and may give false negative results.

The OECD Guidance Document on the reporting of integrated approaches to testing and assessment (IATA) (OECD, 2015b) aims to provide a harmonised approach for the reporting for an AOP-based IATA (see Annexes I and II of the OECD Guidance Document). Within such an AOP-based IATA, the different pieces of information would target key events along the defined toxicity pathway and the results used to inform a regulatory decision, as pointed out in Figure R.7.3–2.

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

It is also to be noted that in case the subtance does not fall into the applicability domain of the non-animal test methods, an *in vivo* test (i.e. an LLNA) would need to be performed.

Figure R.7.3–2 Testing and assessment strategy for evaluating the skin sensitisation potential of substances (footnotes a to c are detailed below the figure)

Element	Information	Conclusion
Existing data on physico-chemical properties		
1	Is the substance a strong acid (pH< 2.0) or base (pH> 11.5), corrosive to the skin or (spontaneously) flammable in air at room temperature?	**YES**: No *in vivo* testing required (Column 2 adaptation of Annexes VII, section 8.3) Note: extreme pH values/corrosive properties do not prevent from performing *in chemico/in vitro* test(s) and it is recommended to assess skin sensitisation hazard in sub-corrosive concentrations.
Existing human data		
2	Are there adequate existing human data[a], which provide evidence that the substance is a skin sensitiser?	**YES**: Consider classifying according CLP criteria (Cat 1, 1A or 1B). If not conclusive on its own, use this information for *Weight-of-Evidence* analysis under point 6.
Existing animal data from sensitisation studies		
3	Are there data from existing studies *on skin sensitisation* in laboratory animals (LLNA, GPMT, or Buehler test, OECD TGs 429 and 406), which provide sound conclusive evidence that the substance is a sensitiser, or non-sensitiser?	**YES**: Consider classifying according CLP criteria (Cat 1, 1A or 1B) or consider no classification. If not conclusive on its own, use this information for *Weight-of-Evidence* analysis under point 6.
Existing (Q)SAR data and read-across		
4	Do "read-across" from structurally and mechanistically related substances or do suitable (Q)SAR predictions indicate some skin sensitisation potential of the substance? [b]	**YES**: Consider classifying as Skin Sensitiser Cat. 1, 1A or 1B. If not conclusive on its own, use this information for *Weight-of-Evidence* analysis under point 6
Existing in chemico and in vitro data		
5a	Has the substance demonstrated **dermal bioavailability** properties in an EU/OECD	**YES/NO:**

	adopted *in vivo/in vitro* test (OECD TG 427 or 428)?	Use this information for *Weight-of-Evidence* analysis
5b	Has the substance demonstrated **protein binding** properties in an OECD adopted *in vitro* test (OECD TG 442c)? (*Key event 1 of the AOP*), and/or Has the substance demonstrated **activation of biochemical pathways in Keratinocytes** in an OECD adopted *in vitro* test (OECD TG 442d)? *(Key event 2 of the AOP*), and/or Has the substance demonstrated chemokine and **cytokine expressions in dendritic cells** in a validated *in vitro* test, h-CLAT? (*Key event 3 of the AOP*). Data from *in vitro* test methods that have been validated and are considered scientifically valid but are not yet adopted by the EU and/or OECD may also be used if the provisions defined in Annex XI to the REACH Regulation are met.	**YES/NO:** Use this information for *Weight-of-Evidence* analysis.
5c	Are there data from (a) non-validated *in vitro* test(s), which provide evidence that the substance may be a skin sensitiser?	**YES/NO:** Use this information for *Weight-of-Evidence* analysis.
Weight-of-Evidence analysis		
6	The "elements" described above may be arranged as appropriate. Taking all existing and relevant data (elements 1-5) into account, is there sufficient information to meet the information requirement of Section 8.3 of Annex VII and to make a decision on whether classification and labelling are warranted? For specific guidance on *Weight of Evidence see below*.	**YES**: Classify according to CLP criteria (Skin Sensitiser Cat. 1, 1A or 1B) or consider no classification. If discrimination between Skin Sensitiser Cat 1A, 1B is not possible, it is strongly recommended that Cat 1A be chosen or new data generation be considered. **NO:** Consider the next elements of the strategy.
Generation of new non-animal data [c]		
7a	Consider generating data according to EU/OECD adopted in vitro test (OECD TG 428) for dermal bioavailability to support the overall *Weight-of-Evidence*. Does the substance demonstrate **dermal bioavailability?**	**YES/NO:** Use this information for *Weight-of-Evidence* analysis

7b	Does the substance demonstrate **protein binding** properties in an OECD adopted *in vitro* test (OECD TG 442c)? (*Key event 1 of the AOP*) Data from *in vitro* test methods that have been validated and are considered scientifically valid but are not yet adopted by the EU and/or OECD may also be used if the provisions defined in Annex XI to the REACH Regulation are met.	**YES/NO:** Use this information for *Weight-of-Evidence* analysis.
7c	Does the substance demonstrate **activation of biochemical pathways in Keratinocytes** in an OECD adopted *in vitro* test (OECD TG 442d)? *(Key event 2 of the AOP*) Data from *in vitro* test methods that have been validated and are considered scientifically valid but are not yet adopted by the EU and/or OECD may also be used if the provisions defined in Annex XI to the REACH Regulation are met.	**YES/NO:** Use this information for *Weight-of-Evidence* analysis.
7d	Does the substance demonstrate chemokine and **cytokine expressions in dendritic cells** in an validated *in vitro* test (h CLAT)? (*Key event 3 of the AOP*) Data from *in vitro* test methods that have been validated and are considered scientifically valid but are not yet adopted by the EU and/or OECD may also be used if the provisions defined in Annex XI to the REACH Regulation are met.	**YES/NO:** Use this information for *Weight-of-Evidence* analysis.
7e	Is any additional testing/generation of data considered necessary in order to conclude on classification, to explain the inconsistent data obtained in previous elements or to address the *Key event 4 of the AOP* (T cell proliferation) with an *in vitro* test?	**YES**: Use this information for *Weight-of-Evidence* analysis.
Weight-of-Evidence analysis		
8	The "elements" described above may be arranged as appropriate. Taking all existing and relevant data (elements 1-7) into account, is there sufficient information to meet the respective information requirement of Section 8.3 of Annex VII and to make a decision on whether classification and labelling are warranted? For specific guidance on *Weight of Evidence*	**YES**: Classify accordingly (Skin Sensitiser Cat. 1A or 1B) or consider no classification. If discrimination between Skin Sensitiser Cat 1A, 1B is not possible, it is strongly recommended that Cat 1A be chosen or new data generation be considered.

	see below.	**NO:** Consider the next element of the strategy.
Generation of new in vivo data for sensitisation as a last resort (Annex VII to the REACH Regulation)		
9	Does the substance demonstrate sensitising properties in an EU/OECD adopted *in vivo* test, the LLNA (OECD TG 429)? →	**YES**: Classify according to CLP criteria (Skin Sensitiser Cat. 1A or 1B). **NO:** No classification needed.

Notes to the information scheme on skin sensitisation:

a) Data from case reports, occupational experience, poison information centres, HPTs or from clinical studies.

b) It is worthwhile to apply the OECD QSAR Toolbox (see Section R.7.3.3.1) to check whether there are existing data available for the substance or for potential analogue substances that may have existing and good quality data available for skin sensitisation. It should be noted that in case read-across or a category approach is to be used, adequate justification must be provided (for further information see http://echa.europa.eu/support/grouping-of-substances-and-read-across). The use of available and suitable (Q)SAR models for skin sensitisation is also recommended.

c) In case (a) non-animal testing approach(es) is (are) used, information needs to be generated at least for elements 7b to 7d unless not already available.

Predictive capacity of the existing *in vivo* and non-animal tests when compared to human data

Urbisch *et al*. (2015) compared the predictive capacity of the LLNA and that of non-animal *(in chemico/in vitro)* testing strategies towards skin sensitisers in humans. The authors showed that for LLNA *vs.* human data, the accuracy of prediction was 82%, with a sensitivity (i.e. true positive rate) of 91% and a specificity (i.e. true negative rate) of 64 %. For non-animal test methods used in combination the accuracy was 90% with a sensitivity and a specificity of 90 % (n~100 chemicals). So, there is some indication that, when *in chemico* and *in vitro* methods are used in combination, non-animal tests methods exhibit good predictivities and are even slightly more accurate than the LLNA in the identification of human sensitisers and non-sensitisers (i.e. Cat 1 vs. non-classified). However, the individual tests on their own were not as sensitive as the LLNA.

How to deal with the lack of or limited metabolic capacity of the non-animal test methods?

The *in chemico* Direct Peptide Reactivity Assay does not have any metabolic capacity and the *in vitro* Keratinosens™ assay and h-CLAT assay have only limited metabolic capacity in the test systems. Due to the lack of or limited metabolic capacity, these test methods may not correctly identify sensitisers that would require enzymatic activation or auto oxidation to exert their sensitisation activity and therefore may provide false negative results.

Thus, it is strongly recommended to run computational tools such as the OECD QSAR Toolbox or TIMES-SS that can partially cover for the lack of metabolic or auto-oxidation information. These softwares have modules for simulating (skin) metabolism and auto-oxidation of substances. In case the substance is predicted to be a non-sensitiser but the simulated metabolites or products have positive experimental data or trigger skin sensitisation alerts, then the latter might be responsible for sensitisation and need a specific assessment and might require the generation of new experimental data.

Are there experimental data available from endpoints (e.g. from in vitro mutagenicity) that could provide additional information to support the conclusions on skin sensitisation obtained from the non-animal test methods? In case of negative *in chemico/in vitro* test results, positive results from an Ames test or *in vitro* chromosomal aberration studies may provide useful information on the electrophilic reactivity of a given substance and information on the likely reactivity may be a useful indicator of sensitisation potential. It is good to note that not all skin sensitisers give positive results in *in vitro* mutagenicity studies. Therefore, a negative prediction in the Ames test and/or *in vitro* chromosomal aberration study should be assessed with care, e.g. by assessing the modes and mechanisms of action of the substance (Patlewicz *et al*., 2010). For example, in case negative predictions are obtained from non-animal tests for skin sensitisation and (a) positive(s) result is (are) obtained from an Ames test and/or an *in vitro* chromosomal aberration study with metabolic activation, it is advised to examine more in detail if a similar metabolism could occur in the viable epidermis (i.e. the substance could be a pro-hapten); this would allow to confirm or rule out a potentially false-negative prediction for skin sensitisation based on the non-animal test predictions. In case, negative results are obtained from the *in vitro/in chemico* test methods as well from Ames and/or an *in vitro* chromosomal aberration study with metabolic activation, this could be useful in the *Weight-of-Evidence* assessment when used in combination with the computational tools.

Use of non-animal data (e.g. *in vitro* methods) to support a category approach

In case a category approach is used to fulfil the REACH information requirements and data are available for some category members only, the generation of data by using e.g. *in chemico/in vitro* test methods could be used to support the category approach for this endpoint. This is especially the case when similar results on the skin sensitisation potential (or the lack thereof) are obtained from one (or more) non-animal testing method(s). In practice, it may be possible to perform only one or two *in chemico*/*in vitro* tests for the target substance of the read-across. In case of conflicting results, it is important to consider why they occurred: the reason might be that the specific substance does not belong to the category because of sensitising properties different from that of category members with good quality animal and/or human data, or that the substance does not fit into the applicability domain of the specific non-animal test. In those cases, *in vivo* testing may be required to assess the skin sensitisation potential of the substance.

Whenever a category approach is applied, it is essential to always justify why data can be read across from the category member substances to the target substance, which does not have good quality animal and/or human data). This justification also needs to be endpoint specific. Advice on how to build and report a category can be found on ECHA website http://echa.europa.eu/support/grouping-of-substances-and-read-across.

Sub-categorisation

Currently (in 2015), the results of the adopted/scientifically valid non-animal test methods cannot be used alone for the classification into skin sensitisation sub-categories i.e. Cat 1A or 1B as required by the CLP regulation. Potency indicators can be obtained from the existing *in chemico* (level of protein depletion) and *in vitro* tests (dose-dependent responses); however, there is currently no prediction model able to integrate these data into an adequate sensitisation potency classification. Few approaches have been proposed for potency prediction (Jaworska *et al.*, 2013; Natsch *et al.*, 2014; Reisinger *et al.*, 2015; OECD, 2015a). As the current first choice method, i.e. the LLNA (EU B.42/OECD TG 429), allows potency estimation and the setting of specific concentration limits, the registrant is strongly advised when using non-animal test methods (*in chemico/in vitro*) to fulfil the REACH information requirement, to assess potency information by all means possible.

The current lack of sub-categorisation potential when using non-animal test methods within a *Weight-of-Evidence* approach may result in the lower level of protection of humans in respect to mixture classification. This is due to the fact that, depending on the skin sensitisation potency, different concentration limits are to be applied, i.e. for Cat 1 and Cat 1B the generic concentration limit (GCL) is 1%, for Cat 1A (strong or extreme) the GCL is 0.1% and for extreme sensitisers a specific concentration limit (SCL) of 0.001% is recommended according to the CLP Regulation (for further information, see Section 3.4 of the *Guidance on the Application of the CLP criteria*). In short, this may lead to that potent sensitisers are not correctly classified in the mixture, if the general Cat 1 is used and the GCL of 1% is applied instead of the 0.1 % (or the SCL of 0.001%). This would mean a lowering of the safety level as compared to current provisions, which may lead to an increased incidence of human sensitisation to potent sensitisers. In case it is not possible to assess the skin sensitising potency of the substance based on the information available, it is strongly recommended to classify the substance as Cat 1A until a reliable prediction model becomes available or new data is generated to allow sub-categorisation.

However, there is currently (in 2015) work on-going to try and raddress the potency characterisation by using non-animal approaches and therefore the reader is advised to follow the recent and future developments in the field. The reader is also advised to follow any updates to the ECHA webpage concerning Testing methods and alternatives (see: http://echa.europa.eu/support/oecd-eu-test-guidelines).

Comment [LR1]: **Placeholder**, if developments occur during the guidance update, the relevant sections needs to be reformulated.

How to perform and report a *Weight-of-Evidence* analysis

When *in chemico*/*in vitro* studies are used to fulfil the Annex VII information requirement for skin sensitisation by means of the general rules of adaptation as specified in sections 1.2 – 1.5 of Annex XI to the REACH Regulation, the Registrant should provide a case-specific justification on why and how the *in chemico/in vitro* data, used within a *Weight-of-Evidence* approach, can cover for the information requirement. In that *Weight-of-Evidence* justification, e.g. coverage of the **key events** (see "Testing and assessment strategy for skin sensitisation" above), the quality and reliability of the data, scope and

limitations of each test method used, and consistency of the results need to be considered. Further provisions on *Weight of Evidence* can be found in Section R.4.4 of Chapter R.4 of the *Guidance on IR&CSA* and in Art. 9(3) of the CLP Regulation.

The *Weight-of-Evidence* based adaptation of the standard information requirement, i.e. LLNA, is based on the OECD AOP for skin sensitisation and its key events (OECD, 2012). It is recognised that in the LLNA key events 1 to 4 are addressed since the biological response, i.e. induction of skin sensitisation, is caused by the cascade of this key events. Therefore in the *Weight-of-Evidence* approach these key events should be covered to the extent possible. At present, three *in chemico/in vitro* tests that each closely correspond to a specific key event have been adopted by the OECD and/or validated by EURL ECVAM. It is strongly recommended, these three key events should be covered either by an *in chemico/in vitro* test or by other types of information (e.g. (Q)SAR, read-across). There is currently no scientifically valid or internationally adopted *in vitro* method to cover the fourth key event, i.e. lymhocyte proliferation. However, the available studies on the predictivity of different combinations of *in chemico/in vitro* methods/other information type show that a good predictivity for hazard identification (Cat. 1 *vs.* non-sensitiser) can be achieved by covering the first three key events (Urbisch *et al.*, 2015): the use of the non-animal test methods in combination showed good accuracies when predicting skin sensitisers (Cat. 1) when compared to human or LLNA data and their accuracy even slightly exceeded that of the LLNA when compared to human data.

It should be noted that the data used to cover the first three key events, be they *in chemico/in vitro* results or other data, can be inconsistent. For example it may happen that two tests/data points are negative and one is positive for skin sensitisation. In case of inconsistent or conflicting data, a scientific explanation should be provided. The explanation may be, for example, that the substance needs metabolic activation to become a skin sensitiser and the test system misses the required metabolic competence. It may also be that the test substance does not fall into the applicability domain(s) of one or more of the *in chemico/in vitro* methods used. If the conflicting information/results cannot be explained, the registrant will need to generate/collect further information in order to support the prediction of the skin sensitisation potential of the substance. If in the end the registrant is not able to conclude on this endpoint due to inconsistent or inconclusive data, there may be a need to perform an LLNA study.

As pointed out in elements 6 and 8 (*Weight-of-Evidence* analysis) of the testing and assessment strategy above, in case the skin sensitisation potential of a substance cannot be properly characterised based on the available data, generation of new data is necessary. This data can be e.g. (Q)SAR, data that is specific to a key event, read-across or, as a last resort, the *in vivo* study, i.e. LLNA. The LLNA may have to/must be performed in any case e.g.:

- The test substance does not fall into the applicability domain of the *in chemico/in vitro* tests for skin sensitisation,
- The results of the *in chemico/in vitro* tests are inconsistent and this inconsistency cannot be explained scientifically, or
- The registrant may have some existing or structural or (Q)SAR information indicating that the substance may be a strong or extreme skin sensitiser and cannot conclude on it based on existing information or by new data generation using non-animal test methods, and therefore the registrant aims to ensure an appropriate classification and the consequent high level of risk management measures.

Comment [LR2]: Placeholder: the last bullet may change if potency assessment becomes available.

At the end of the *Weight-of-Evidence* analysis, the data obtained, justification of the choice of the test methods, analysis of data consistency, conclusion made on hazard and on classification according to CLP criteria should be reported clearly and transparently. For the reporting of the *Weight of Evidence* and testing and assessment strategy it is recommended to use the template provided in Appendix R.7.3–3 of this Guidance and which is based on Annex I of the OECD Guidance Document on the Reporting of IATA (OECD, 2015b).

RESPIRATORY SENSITISATION

For substances that sensitise via the respiratory tract, the relevant mechanisms are believed to be essentially similar to those leading to skin sensitisation, although due to the lack of a stratum corneum gaining access to the respiratory epithelium may be somehow easier than to the skin. Moreover, because the lining of the respiratory tract, the professional antigen presenting cells, and regulatory mechanisms in the respiratory tract differ from those in the skin, they may all have an impact on the type of immune response triggered. Although the site of induction of an adaptive immune response to a chemical allergen may be influenced by local conditions and local immuno-regulatory mechanisms, the fact remains that the inherent properties of the substance itself play a major role in determining whether an immune responses is induced and the qualitative characteristics of that response.

In the respiratory tract, chemical respiratory allergens appear to preferentially elicit Th_2-immune responses (Maestrelli *et al.*, 1997), observations that are consistent with studies in mice (Dearman *et al*., 2002; Herrick *et al*., 2003; Farraj *et al*., 2004), and possibly also rats (Arts *et al*., 1998). Recently it has been hypothesised that Th17 cells would also play a crucial role in respiratory sensitisation via secretion of IL-17 (Lambrecht and Hammad, 2013). Th2-type immune responses are characterised by the production of cytokines such as IL4 and IL5 and by the production of IgE antibodies. However, the mechanisms through which substances are able to induce sensitisation of the respiratory tract are not fully understood and there remains controversy about the roles played by IgE antibody-mediated mechanisms, and whether IgE represents a mandatory universal requirement for the induction by substances of allergic sensitisation of the respiratory tract. The area is complicated because although for all chemical respiratory allergens there are patients who display serum IgE antibodies of the appropriate specificity, in other instances (and particularly with respect to the diisocyanates) there are symptomatic subjects in whom it is not possible to detect IgE antibody. There are two, non-mutually exclusive, possibilities. The first is that IgE does play a central role but that for one or more of various reasons it is not being detected accurately in the serum of patients with occupational asthma. The second is that allergic sensitisation of the respiratory tract by substances can be effected through IgE antibody-independent immunological mechanisms (Kimber *et al.*, 2002 and 2005). These may also include Th1-type immune responses. In this context it has been reported, for instance, that inhalation challenge of sensitised rodents with contact allergens may elicit respiratory allergic reactions (Garssen *et al.*, 1991; Garcia *et al.*, 1992; Buckley *et al.*, 1994; Zwart *et al*., 1994; Satoh *et al.*, 1995; Arts *et al.*, 1998). This comes as no surprise because it is clear that contact sensitisation is systemic in nature and that there is no reason to suppose that encounter of sensitised animals with the relevant contact allergen at respiratory epithelial surfaces will not cause an adverse immunologic reaction. However, it is important to note that in reality only a very few precedents for the elicitation of pulmonary reactions by skin sensitising chemicals in humans have been observed, and in practice it may not represent a significant health issue.

In addition, there is a growing body of evidence that effective sensitisation of the respiratory tract by chemicals defined as respiratory allergens (such as for instance the acid anhydrides, diisocyanates and others) can and does occur in response to dermal contact (reviewed by *Kimber et al.*, 2002). There are also experimental animal data and human evidence for sensitisation by inhalation and skin effects following dermal challenge (Kimber *et al.*, 2002, Baur *et al.*, 1984, Ebino *et al.*, 2001, Stadler *et al.*, 1984). Therefore, it is not necessarily the case that chemicals that cause allergic dermal reactions require sensitisation via the skin, or that chemicals that cause allergic airway reactions require sensitisation via the respiratory tract.

R.7.3.7 Information and its sources on respiratory sensitisation

R.7.3.7.1 Non-human data on respiratory sensitisation

Non-testing data on respiratory sensitisation

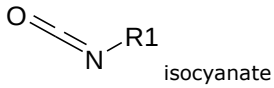
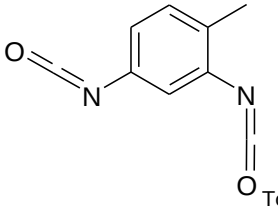
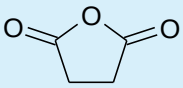
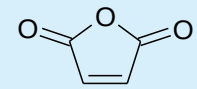
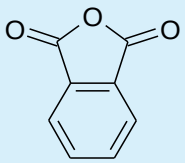
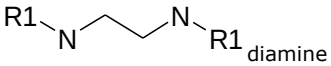
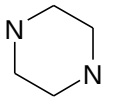
Attempts to model respiratory sensitisation have been hampered by a lack of a predictive test protocol for assessing chemical respiratory sensitisation. (Q)SAR models are available but these have largely been based on data for substances reported to cause respiratory hypersensitivity in humans. Examples of some structural alerts are shown in Table R.7.3–2.

Agius *et al.* (1991) made qualitative observations concerning the chemical structure of substances causing occupational asthma. This work drew attention to the large proportion of chemical asthmagens with at least two reactive groups, e.g., ethylene diamine and toluene diisocyanate. The earlier work was followed up by a simple statistical analysis of the occurrence of structural fragments associated with activity, with similar conclusions (Agius *et al.*, 1994 and 2000).

The MCASE group has developed three models for respiratory hypersensitivity (Karol *et al.*, 1996; Graham *et al.*, 1997, Cunningham *et al.*, 2005). The Danish (Q)SAR Database has an in-house model for respiratory hypersensitivity for which estimates can be extracted from the on-line database (available at http://qsar.food.dtu.dk/). Derek Nexus contains several alerts derived from a set of respiratory sensitisers/asthmogens (Payne *et al.*, 1995).

Whilst the available structural alerts (SAR) are transparent and easy to apply (Aigus *et al.*, 1991, 1994 and 2000; Payne *et al.*, 1995), it should be stressed that these are derived from chemical asthmagens not specifically chemical respiratory allergens. A need therefore remains to develop new (Q)SARs as and when a robust predictive test method becomes available.

Table R.7.3–2 Examples of structural alerts for respiratory sensitisation

Structural Alert Description	Examples of structures
 isocyanate	 Toluene-2,4-diisocyanate
 cyclic anhydride	 maleic anhydride  trimellitic anhydride
 diamine	 piperazine

Recent work on the mechanism of respiratory sensitisation in humans and on the identification of structural alerts specific to respiratory sensitisation has been described in Enoch *et al*. (2009, 2010, 2012 and 2014). In these papers, the authors investigated a common molecular initiating event and mechanism for low molecular weight respiratory sensitisers (found to be the formation of a covalent bond in the lung) and applied their findings to predict respiratory sensitisation by read-across. The authors have proposed a set of 52 structural alerts which define the chemistry associated with covalent protein binding in the lung. Each structural alert is also characterised by a mechanistic domain ("mechanistic alert") and some data indicating presence of effect. Most of these alerts (a total of 41) have been encoded in the OECD QSAR Toolbox (ver. 3.3) profiler "Respiratory sensitisation". The full list of the encoded structural alerts for respiratory sensitisation is available under the new OECD QSAR Toolbox feature "documentation", together with the description, applicability domain, mechanism, set of substances used for the profile training set, profile/alert analysis. Some examples of structural alerts are di-isocyanates, anhydrides and lactams.

Testing data on respiratory sensitisation

In vitro data

No validated or widely recognised *in vitro* test methods specific to respiratory sensitisation are available yet, owing to the complexity of the mechanisms of the sensitisation process. This is most likely due to the fact that there are still some

uncertainties concerning the underlying immunological mechanisms, in particular with respect to the role of IgE antibody.

Efforts are still needed to identify the most relevant endpoints in the optimisation of existing tests. However, a combination of several *in vitro* tests, covering the relevant mechanistic steps of respiratory sensitisation, into a test battery could eventually lead to replacement of the *in vivo* tests.

Animal data

At present, although a number of test protocols has been published to detect respiratory allergenicity of low molecular weight compounds, none of these are validated nor are these widely accepted. One approach that might be of some value in characterising the likely respiratory sensitising activity of substances is application of the LLNA, or of other tests for measuring skin sensitisation potential. Although the LLNA was developed and validated for the identification of contact allergens, there is evidence that chemical respiratory allergens will also elicit positive responses in this assay (Kimber, 1995). That is, substances known to cause respiratory allergy and occupational asthma have been shown to test positive in the LLNA. Among such substances are acid anhydrides (such as trimellitic anhydride and phthalic anhydride), diisocyanates (including diphenylmethane diisocyanate and hexamethylene diisocyanate) and certain reactive dyes. In fact, the view currently is that most, if not all, chemical respiratory allergens are able to elicit positive responses in the LLNA, or in other tests for skin sensitisation, such as the M&K (guinea pig maximisation) test. This is true even of those chemical respiratory allergens, such as phthalic anhydride, for instance, that are implicated virtually exclusively with the induction of chemical respiratory allergy and have rarely, if ever, been shown to cause allergic contact dermatitis. Against this background and in combination with other data it might be possible to conclude in a *Weight-of-Evidence* assessment that substances that (at an appropriate test concentration and test conditions, i.e. skin penetration should have occurred) are negative in the LLNA, as well as being considered as not being skin sensitisers, can also be regarded as lacking the potential to cause allergic sensitisation of the respiratory tract.

One approach that has been proposed for the identification of substances that have the potential to cause allergic sensitisation of the respiratory tract is one in which activity is measured as a function of the profiles of cytokines produced by draining lymph node cells in mice exposed more chronically (over a 2 week period) topically to the test substance (Dearman *et al.*, 2002) or a shorter period (3 days) via inhalation exposure (Arts *et al.*, 2008). This method is predicated on an understanding that allergic sensitisation of the respiratory tract is favoured by selective Th2-type immune responses and that in many instances chemical respiratory allergy and occupational asthma are associated with IgE antibody. Using this approach chemical respiratory allergens are identified as a function of their ability to stimulate in mice the selective development of preferential Th2-type immune responses associated with a predominance of type 2 cytokine secretion by draining lymph node cells (Dearman *et al.*, 2002 and 2003). Specifically, chemical contact allergens promote Th1 responses characterised by an enhanced production of IFN-gamma, whereas chemical respiratory allergens promote Th2 responses characterised by enhanced production of IL-4, IL-5 and IL-13. Many variables other than the substance itself, such as the concentration used to induce sensitisation, duration of the sensitisation period, and presence or absence of mitogens to reveal differences in cytokine expression, have all been noted to have an impact on the outcome (Van Och *et al.*, 2002). There are general guidelines now available for the conduct of the method (Dearman *et al.*, 2003), however, this method has not yet been formally validated nor is it widely accepted.

Another, relatively simple, approach may serve the purpose to specifically predict sensitisation of the respiratory tract: i.e. increases in total serum IgE antibodies after induction. This method is based on statistically significant increases in total serum IgE (see review by Arts and Kuper, 2007).

Methods that use both an induction and an inhalation elicitation or challenge phase and which include different parameters such as total and/or specific IgE antibody determinations, lung function testing, tests for a specific hyperreactivity (e.g. methacholine challenges), bronchoalveolar lavage measurements, and histopathological examination of the entire respiratory tract, may provide (additional) information on the potential of substances to cause respiratory sensitisation. These methods usually use high IgE-responding animal strains; to test for Th1-mediated responses low IgE-responding strains should typically be used. Several of these models have been reviewed by Arts and Kuper (2007).

There are currently no predictive methods to identify substances that induce asthma through non-immunological mechanisms, however, when performing challenge tests including non-sensitised but challenged controls, information can be obtained on non-immunological effects of these substances.

R.7.3.7.2 Human data on respiratory sensitisation

Human data on respiratory reactions (asthma, rhinitis, alveolitis) may come from a variety of sources:

- consumer experience and comments, preferably followed up by professionals (e.g. bronchial provocation tests, skin prick tests and measurements of specific IgE serum levels)
- records of workers' experience, accidents, and exposure studies including medical surveillance
- case reports in the general scientific and medical literature
- consumer tests (monitoring by questionnaire and/or medical surveillance)
- epidemiological studies

R.7.3.8 Evaluation of available information on respiratory sensitisation

R.7.3.8.1 Non-human data on respiratory sensitisation

Non-testing data on respiratory sensitisation

The freely downloadable OECD QSAR Toolbox software (http://www.qsartoolbox.org/) encodes a profiler (set of rules and structural domains) specific for respiratory sensitisation. The profiler offers support to the user in grouping substances which share common structural alerts and possibly predict the respiratory sensitisation potential *via* read-across. The current version of the profiler encodes 41 structural alerts for respiratory sensitisation.

This profiler is intended to be used for the assessment of the respiratory sensitisation potential of low molecular weight substances. The profiler has been developed based on the mechanistic knowledge of the elicitation phase of respiratory sensitisation, and thus identifies substances able to covalently bind to proteins in the lung. Presence of activity could be predicted from positive predictions. Absence of effect however cannot be

predicted from the lack of alert because the lack of alert might be due to the lack of effect or lack of knowledge.

This profiler should also be used with caution due to the limited data available for the development of structural alerts. This is due to the lack of a standardised assay (*in vivo* or *in vitro*) suitable for identifying potential respiratory sensitisers. The available data are drawn from clinical reports of occupational asthma, which in a number of cases results in structural alerts defined based on a low number of substances. However, all structural alerts have a clear mechanistic rationale associated with them (in terms of covalent protein binding).

Experimental data on respiratory sensitisation can be found in two of the OECD QSAR Toolbox databases: Skin sensitisation ECETOC and ECHA Chem.

Testing data on respiratory sensitisation

In vitro data

Presently (in 2015) there are neither scientifically valid nor regulatory accepted *in vitro* tests available to assess respiratory sensitisation. Several *in vitro* test methods have been described in the literature; however more work is needed for wider acceptance of a given test method.

Animal data

Although the LLNA does not represent a method for the specific identification of chemical respiratory allergens, there is evidence that chemical respiratory allergens will also elicit positive responses in this assay (Kimber, 1995). The interpretation is therefore that a substance which fails to induce a positive response in the LLNA (at an appropriate test concentration) most probably lacks the potential for respiratory allergy. Conversely, it cannot be wholly excluded that a substance that induces a positive response in the LLNA might sensitise the respiratory tract upon inhalation or via dermal exposure. Any potential hazard for respiratory sensitisation could only be positively identified by further testing, although such testing is neither validated nor widely accepted.

One further approach to the identification of substances that have the potential to induce allergic sensitisation of the respiratory tract is *cytokine fingerprinting* (Dearman *et al.*, 2002; Arts *et al.*, 2008; see Section R.7.3.8.1). These methods are predicated on an understanding that allergic sensitisation of the respiratory tract is favoured by selective Th2-type immune responses and that in many instances chemical respiratory allergy and occupational asthma are associated with IgE antibody.

In addition, there are other approaches that have been proposed and these have been reviewed by Arts and Kuper (2007) - although again it is important to emphasise that there are currently no fully evaluated or validated animal models available for the predictive identification of chemical respiratory allergens.

As indicated previously, some substances may have the potential to induce pulmonary reactions via Th1-type immune responses. Studies with typical skin allergens such as DNCB, DNFB and picryl chloride (trinitrochlorobenzene) in BALB/c mice, guinea pigs or Wistar rats have shown the potential of these substances to induce allergic reactions in the lungs that are independent of IgE (Garssen *et al.*, 1991; Garcia *et al.*, 1992; Buckley *et al.*, 1994; Zwart *et al.*, 1994; Satoh *et al.*, 1995; and see for a review Arts and Kuper, 2007). Sensitisation and challenge with DNCB resulted in laryngitis in low IgE-responding Wistar rats (Arts *et al.*, 1998). In addition, cellular immune responses to these sensitisers were shown to be associated with hyperreactivity of the airways to non-specific stimuli

(Garssen *et al.*, 1991). For these reasons, it might be the case that people who are sensitised via the skin might suffer adverse pulmonary reactions if they were to inhale sufficient amounts of the contact allergen to which they were sensitised. As indicated previously, very few precedents for the elicitation of pulmonary reactions by skin sensitising substances in humans have been observed. In practice it appears not to represent a health issue.

R.7.3.8.2 Human data on respiratory sensitisation

Although human studies may provide some information on respiratory hypersensitivity, the data are frequently limited and subject to the same constraints as human skin sensitisation data.

For evaluation purposes, existing human experience data for respiratory sensitisation should contain sufficient information about:

- the test protocol used (study design, controls);
- the substance or preparation studied (should be the main, and ideally, the only substance or preparation present which may possess the hazard under investigation);
- the extent of exposure (magnitude, frequency and duration);
- the frequency of effects (versus number of persons exposed);
- the persistence or absence of health effects (objective description and evaluation);
- the presence of confounding factors (e.g. pre-existing respiratory health effects, medication; presence of other respiratory sensitisers);
- the relevance with respect to the group size, statistics, documentation;
- the healthy worker effect.

Evidence of respiratory sensitising activity derived from diagnostic testing may reflect the induction of respiratory sensitisation to that substance or cross-reaction with a chemically very similar substance. In both situations, the normal conclusion would be that this provides positive evidence for the respiratory sensitising activity of the substance used in the diagnostic test.

For respiratory sensitisation, no clinical test protocols for experimental studies exist but tests may have been conducted for diagnostic purposes, e.g. bronchial provocation test. The test should meet the above general criteria, e.g. be conducted according to a relevant design including appropriate controls, address confounding factors such as medication, smoking or exposure to other substances, etc. Furthermore, the differentiation between the symptoms of respiratory irritancy and allergy can be very difficult. Thus, expert judgement is required to determine the usefulness of such data for the evaluation on a case-by-case basis.

Although predictive models are under validation, there is as yet no internationally recognised animal method for identification of respiratory sensitisation. Thus human data are usually evidence for hazard identification.

Where there is evidence that significant occupational inhalation exposure to a substance has not resulted in the development of respiratory allergy, or related symptoms, then it may be possible to draw the conclusion that the substance lacks the potential for sensitisation of the respiratory tract. Thus, for instance, where there is evidence that a large cohort of subjects have had opportunity for regular inhalation exposure to a substance for a sustained period of time in the absence of respiratory symptoms, or

related health complaints, then this will provide reassurance regarding the absence of a respiratory sensitisation hazard.

More information on how to apply human data for C&L purposes can be found in Section 3.4.2.1.3.1 of the *Guidance on the Application of the CLP criteria*.

R.7.3.9 Conclusions on respiratory sensitisation

R.7.3.9.1 Remaining uncertainty on respiratory sensitisation

When considering whether or not a substance is a respiratory sensitiser, observations of idiosyncratic reactions in only a few individuals with hyper-reactive airways are not sufficient to indicate the need for classification.

Major uncertainties remain in our understanding of the factors that determine whether or not a substance is an allergen, and if so, what makes it a respiratory sensitiser.

R.7.3.9.2 Concluding on suitability for Classification and Labelling

REACH demands that all available information for a substance is gathered and any lack of information is reported.

In REACH, respiratory sensitisers are indicated for harmonised classification and labelling and regulated in Annex VI to Regulation (EC) No 1272/2008. Annex XV to the REACH Regulation lays down general principles for preparing dossiers to propose and justify harmonised classification and labelling of CMRs (carcinogenic, mutagenic, toxic for reproduction) and respiratory sensitisers.

Potential hazard for respiratory sensitisation cannot be easily addressed, as validated test methods are currently not available. A probable hazard for respiratory sensitisation should be mentioned in the Safety Data Sheet.

Although no testing strategy is available, a substance could be classified as *respiratory sensitiser* by following the flow chart for an integrated evaluation reported in Section R.7.3.10 which is based on existing evidence.

According to Regulation (EC) No 1272/2008, the labelling for *respiratory sensitisers* is with the signal word "*Danger*" and the Hazard statement H334: "*May cause allergy or asthma symptoms or breathing difficulties if inhaled*".

R.7.3.9.3 Concluding on suitability for chemical safety assessment: dose-response assessment and potency

The CLP Regulation specifies that respiratory sensitising should be allocated into sub-categories (i.e. 1A or 1B) whenever possible. In case the data are not sufficient for sub-categorisation, the substance must be classified in the general Category 1 (for further information, see Section 3.4 of the *Guidance on the Application of the CLP criteria*).

There is evidence that for both skin sensitisation and respiratory hypersensitivity dose-response relationships exist although these are frequently less well defined in the case of respiratory hypersensitivity. The dose of agent required to induce sensitisation in a previously naïve subject or animal is usually greater than that required to elicit a reaction in a previously sensitised subject; therefore the dose-response relationship for the two

phases will differ. Little or nothing is known about dose-response relationships in the development of respiratory hypersensitivity by non-immunological mechanisms.

It is frequently difficult to obtain dose-response information from either existing human or animal data where only a single concentration of the test material has been examined. With human data, exposure measurements may not have been taken at the same time as the disease was evaluated, adding to the difficulty of determining a dose response.

Measurement of potency

The measurement of potency for respiratory sensitisation is currently (in 2015) solely based on human data (See Section 3.4.2.1 of the *Guidance on the Application of the CLP criteria)*.

Derivation of a DNEL

Currently available methods do not allow the determination of a threshold and establishment of a DNEL. Guidance on how to perform a qualitative safety assessment for respiratory sensitisers can be found in Section E.3.4.2 of Part E and Appendix R.8-10 of *Chapter R.8* of the *Guidance on IR&CSA*.

R.7.3.9.4 Additional considerations

Chemical allergy is commonly designated as being associated with sensitisation of the respiratory tract (asthma and rhinitis). In view of this it is sometimes assumed that allergic sensitisation of the respiratory tract will result only from inhalation exposure to the causative substance, and that skin sensitisation necessarily results only from dermal exposure. This is misleading, and it is important for the purposes of risk management to acknowledge that sensitisation may be acquired by other routes of exposure. Since adaptive immune responses are essentially systemic in nature, sensitisation of skin surfaces may theoretically develop from encounter with contact allergens via routes of exposure other than dermal contact (although in practice this appears to be uncommon). Similarly, there is evidence from both experimental and human studies which indicate that effective sensitisation of the respiratory tract can result from dermal contact with a chemical respiratory allergen. Thus, in this case, it appears that the quality of immune response necessary for acquisition of sensitisation of the respiratory tract can be skin contact with chemical respiratory allergens (Kimber *et al.*, 2002). Such considerations have important implications for risk management. Thus, for instance, there is a growing view that effective prevention of respiratory sensitisation requires protection of both skin and respiratory tracts. This includes the cautious use of known contact allergens in products to which consumers are (or may be) exposed via inhalation, such as sprays. The generic advice is that appropriate strategies to minimise the risk of sensitisation to chemical allergens will require consideration of providing protection of all relevant routes of exposure.

R.7.3.9.5 Information not adequate

A *Weight-of-Evidence* approach, comparing available adequate information with the tonnage-triggered information requirements of REACH, may result in the conclusion that the requirements are not fulfilled. In order to proceed in further information gathering the assessment strategy given in Section R.7.3.10 can be adopted.

R.7.3.10 Assessment strategy for respiratory sensitisation

R.7.3.10.1 Objective / General principles

The objective of this assessment strategy is to give guidance on a stepwise approach to hazard identification with regard to the respiratory sensitisation endpoint. A key principle of the strategy is that the results of one study are evaluated before another is initiated. The strategy should seek to ensure that the data requirements are met in the most efficient and humane manner so that animal usage and costs are minimised.

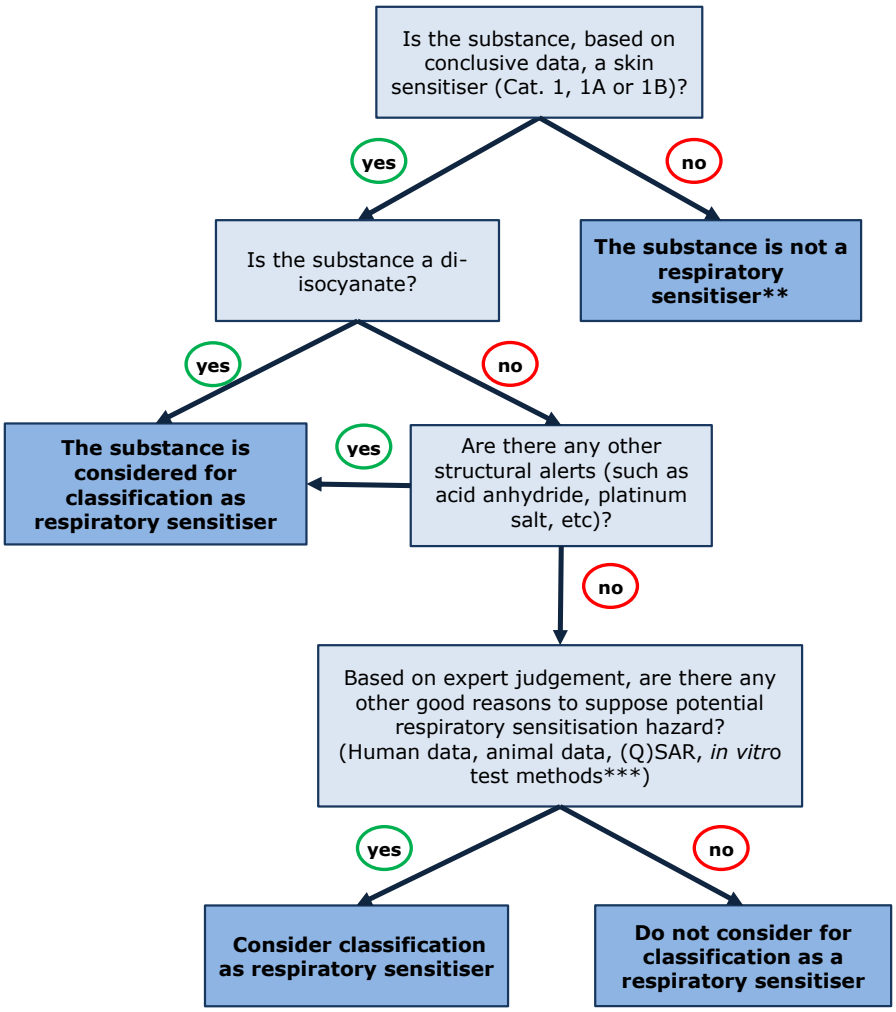
R.7.3.10.2 Preliminary considerations

Careful consideration of existing toxicological data, exposure characteristics and current risk management procedures is recommended to ascertain whether the fundamental objectives of the assessment strategy (see above) have already been met. Give guidance on other factors that might mitigate data requirements for the endpoint of interest e.g. possession of other toxic properties, characteristics that make testing technically not possible.

R.7.3.10.3 Recommended approach

The below strategy for respiratory sensitisation assessment (Figure R.7.3–3) can be followed:

Figure R.7.3–3 Assessment strategy for respiratory sensitisation data*



* In contrast to tests for skin sensitisation, the performance of tests for respiratory sensitisation is currently not required under REACH. Therefore the present strategy scheme depicts a strategy for evaluating existing data.
** This does not discount the possibility that the chemical may induce respiratory hypersensitivity through non-immunological mechanisms. Chemicals that act through such mechanisms are usually identified on the basis of evidence from human exposure.
*** not yet available

R.7.3.11 References

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Appendices R.7.3-1 to 3 to Section R.7.3

Appendix R.7.3–1 Principles of the OECD IATA and the technicalities of the AOP key events

Based on the Adverse Outcome Pathway (AOP), the OECD has adopted a Guidance Document on the reporting of structured approaches to data integration and individual information sources used within an Integrated Approach to Testing and Assessment (IATA) for skin sensitisation (OECD, 2015a). A separate OECD Guidance Document on the reporting of IATAs has also been published (OECD, 2015b). These documents provide a framework/structured approach that can be used for hazard identification, hazard characterisation and/or safety assessment of a substance or group of substances, which strategically integrates and weighs all relevant data in order to make a decision concerning potential hazard and/or risk and/or the need for further targeted testing.

The above-mentioned guidance documents contain the following elements:

- A general framework for IATAs that allows sufficient flexibility in the use of individual information sources to cover multiple regulatory needs;
- General guidance on the evaluation and application of IATA;
- Consistent description of the information sources that can be used within an IATA;
- A template for describing IATA.

The IATA can be divided into separate elements based on the key events specified in the AOP for skin sensitisation and one element can contain multiple potential information sources as described below.

Note: the information sources included in the elements below may differ from the information sources described in the OECD GD on skin sensitisation IATA (OECD, 2015a).

Element 1: Dermal Bioavailability (penetration and metabolism)

Even though dermal bioavailability is not a key event described in the AOP *per se*, dermal bioavailability is an important parameter in the assessment of skin sensitisation potential. A substance cannot exert skin sensitisation-related reactivity in the deeper layers of the epidermis unless it is absorbed and penetrates the upper layer first (Basketter *et al.*, 2007). Potential information sources to assess dermal bioavailabily can be e.g.:

- Physico-chemical properties: e.g. molecular weight , pKa, Log Kow, evaporation rate/vapour pressure, melting point, No or Ho bond donors/acceptors and others. More guidance on dermal bioavailability estimations based on physico-chemical properties can be found in Section R.7.12.2. of Chapter R.7c of the *Guidance on IR&CSA*;
- Non-testing methods for skin penetration: e.g. *in silico* models providing direct estimates of dermal permeability (DERMWIN, Derek Nexus), physiologically based-pharmacokinetic (PBPK) models;
- Testing methods for skin penetration: e.g. EU B.45/OECD TG 428 (skin absorption: *in vitro* method), EU B.44/OECD 427 (skin absorption: *in vivo* method);
- Non-testing methods for skin metabolism: e.g. in silico models e.g. strucutre-metabolisms encoded in the expert system TIMES-SS, Meteor; simulators for skin mentabolism and auto-oxidation within OECD QSAR Toolbox;

- Testing methods for skin metabolism: e.g.Peroxidase Peptide Reactivity Assay (PPRA), S9, metabolic competent system.

Element 2: Protein binding reactions, Reactivity and Metabolism (AOP Key event 1)

Protein binding reactions, i.e. the covalent binding of electrophilic chemical species to selected nucleophilic molecular sites of action in skin proteins, is considered to be the molecular initiating event of skin sensitisation (Gerberick *et al*., 2008; Karlberg *et al*., 2008). Therefore, protein binding reactions can be used to identify different chemical structures associated with skin sensitisation.

Potential information sources for measuring protein reactivity are e.g.:

- Non-testing methods: e.g. protein binding alerts (e.g. OECD QSAR Toolbox, Derek Nexus, Toxtree). These methods have encoded a number of structural alerts that indicate that the molecule has the potential to react with skin proteins. The basis for these alerts varies from chemical considerations (e.g. some alerts in the OECD QSAR Toolbox only indicate that a reaction could theoretically happen) to experimental test results (like most of the alerts in DEREK). Some *in silico* models (TIMES-SS, OECD QSAR Toolbox, but **not Meteor Nexus which is only for liver metabolism**) can also provide predictions of potential skin metabolites which might have a different skin permeability because of different physico-chemical properties or a different ionisation potential. In addition, the OECD QSAR Toolbox contains some alerts and databases which indicate the reactivity of a molecule based on structural alerts derived from datasets of *in chemico* reactivity tests (such as GSH or DPRA). The OECD QSAR Toolbox also provides a prediction of auto-oxidation products and checks the presence of reactive tautomers;
- Testing methods: e.g. *in chemico* Direct Peptide Reactivity Assay (DPRA, OECD TG 442c), and other methods measuring peptide depletion, methods measuring adduct formation, methods measuring relative reactivity rate.

Element 3: Events in Keratinocytes (AOP Key event 2)

Haptens can also react with cell surface proteins and activate pathways in keratinocytes (Welzien *et al.*, 2009). The hapten uptake by keratinocytes activates multiple events, including the release of pro-inflammatory cytokines and the induction of cyto-protective cellular pathways. Keratinocyte exposure to sensitisers also results in the induction of antioxidant/electrophile response element (ARE/EpRE)- dependent pathways (Natsch and Emter, 2008). Therefore, test methods measuring these events in keratinocytes can be used for detecting sensitising substances.

Potential information sources for measuring events in keratinocytes include e.g.:

- Non-testing methods: e.g. OECD QSAR Toolbox profiler for structural alerts for keratinocyte gene expression; the available data themselves can be used for read-across and/or developing e.g. local QSARs for particular chemical classes but the additional uncertainty of using estimated data should be considered;
- Test methods measuring the activation of biochemical pathways: *in vitro* Keratinosens™ assay measuring Keap-1 NrF2-ARE pathway (OECD TG 442d), LuSens assay measuring Keap-1 NrF2-ARE pathway (Ramirez *et al.*, 2014) AREc32 assay measuring Keap-1 NrF2-ARE pathway (Natsch and Emter, 2008);

- Test methods measuring pathways-associated gene expressions: Sens-is assay (Cottrez *et al.*, 2015), SenCeeTox assay (McKim et al., 2012), HaCaT gene signature assay (van der Veen et al., 2013), Epidermal Sensitization Assay (EpiSens, Saito *et al.*, 2013), proteomic signature in keratinocytes (Thierse *et al.*, 2011);
- Test methods measuring release of pro-inflammatory mediators: RhE-IL-18 assay (Gibbs *et al.*, 2013).

Element 4: Events in dendritic cells (AOP Key event 3)

Epidermal dendritic cells, i.e. Langerhans cells, and dermal dendritic cells serve as antigen presenting cells (APCs) (Kimber *et al.*, 2009): they recognise and internalise the hapten-protein complex formed during the covalent binding step. By internalising the hapten-protein complex the APC has the potential to present the allergen-MHC (Major Histocompatibility complex class II) complex to naïve T-cells. The MHC is also called human leukocyte antigen (HLA) in humans. Upon exposure to the sensitisers dendritic cells are activated which leads also to changes in their chemokine and cytokine expressions, changes in the expression of chemokine receptors and up-regulation of co-stimulatory and intercellular adhesion molecules (e.g. CD40, CD 86, and DC11 and CD54). Therefore, testing methods measuring these changes in dendritic cells and/or chemokine and cytokine expressions can be used for detecting sensitising substances.

Potential information sources for measuring events in dendritic cells include e.g.:

- Test methods measuring the expression of co-stimulatory and adhesion molecules: e.g. h-CLAT assay (scientific validity established, draft OECD TG available), U-Sens ™ assay (Piroird *et al*., 2015), modified MUSST assay (Bauch *et al.*, 2012), PBMCD assay (Reuter *et al*., 2011);
- Test methods measuring pathway-associated gene expression: e.g. IL-8 Luc assay (Takahashi *et al.,* 2011), GARD assay (Johansson *et al*., 2013), VitoSens assay (Hooyberghs *et al*., 2008);
- Test methods measuring pathway-associated protein expression: e.g. MUTZ SensDerm assay (Thierse *et al*., 2011).

Element 5: Events in Lymphocytes (AOP Key event 4)

In the lymph nodes, the APCs display the MHC to naïve T-cells, which induces the differentiation and proliferation of allergen-specific memory T-cells. These events e.g. proliferation of allergen specific T-cells can be measured by using specific test methods.

Potential information sources for measuring events in lymphocytes include e.g.:

- Non-testing methods: There is a good understanding of the electrophilic mechanisms that can lead to protein binding and some methods have been adapted to reflect the strength of the reaction. For example, in the OECD QSAR Toolbox there is a protein-binding profiler specific to skin sensitisation: the scope of this profiler is to investigate the presence of alerts within the target molecules responsible for the interaction with skin proteins based on LLNA and GPMT data. Some Quantitative Mechanistic Models able to quantify skin sensitisation potency have been described in literature (e.g. there is a model for the prediction of EC3 values for Michael acceptors based on quantum descriptors by Enoch *et al.*,

2013). There are also some semi-quantitative models that allow to differentiate between weak and strong sensitisers (e.g. TIMES model for skin sensitisation or the descriptor-based models for skin sensitisation in Discovery Studio's TOPKAT). The OECD QSAR toolbox allows both approaches (quantitative and semi-quantitative) by trend analysis or read-across of similar substances, but the predictions are dependent on finding good analogues with reliable data. ECHA has published illustrative examples of EC3 predictions with the OECD QSAR Toolbox (see: https://echa.europa.eu/documents/10162/21655633/illustrative_example_qsar_part2_en.pdf);

- *In vitro* test methods: Human T cell priming/proliferation assay (hTCPA, Moulon *et al.*, 1993; Krasteva *et al*., 1996; Dietz *et al*., 2010; Martin *et al*., 2010, Richter *et al*., 2013; Popple *et al*., 2015);
- *In vivo* test methods: Local Lymph Node Assay (OECD TG 429, 442a and 442b).

Element 6: *In vivo* and human study (adverse outcome)

In vivo studies and studies in humans can be considered to gather information about the occurrence of the adverse outcome of interest, described as allergic contact dermatitis, after exposure to a substance. *In vivo* studies still remain the basis for assessing the skin sensitisation potential of substances.

Potential information sources for measuring the adverse outcome include e.g.:

- (Existing) human data: e.g. Human Repeat Insult Patch Test (HRIPT), clinical data, data from occupational exposure, epidemiological data;
- (Existing) animal data: e.g. Guinea Pig Maximization Test (GPMT) EU method B.6/OECD TG 406.

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Appendix R.7.3–2 Template for the reporting of the individual information sources for a non-animal test method

The following reporting format (Table R.7.3–3) should be considered when information is generated by non-animal test methods to fulfil the REACH information requirement for skin sensitisation. The use of this reporting template is very important in case (a) test method(s) is (are) used which has (have) not been considered scientifically valid in a international validation study and/or there is no internationally adopted test guideline available.

In case a test method has an internationally adopted test guideline available, some of the points described below can already be included in the test guideline itself, hence detailed reporting of such (an) information source(s) is usually not needed. The reporting of each individual information source needs to be included in a separate endpoint study record (ESR) of the IUCLID dossier, i.e. one ESR per individual information source should be filled in.

Note: this reporting template has been modified based on the OECD template for the reporting of individual information sources (OECD, 2015) to be relevant for the skin sensitisation endpoint and REACH information requirements.

Table R.7.3–3 Template for the reporting of the individual information sources describing a non-animal test method used to fulfil the REACH information requirement for skin sensitisation

Name of the information source	Provide the name of the information source and the acronym (if applicable)
Mechanistic basis including AOP coverage	Describe which key event of skin sensitisation is addressed by the information source. A desription of the extent to which the mechanistic basis of the information source relates to the chemical/biological mechanism covered by the (key) event should be provided.
Description	Provide a short description of the information source including the experimental system used and any relevant aspect of the procedure (e.g. time of exposure of the experimental system with the test substance, number of doses/concentrations tested, number of replicates, concurrent testing of control(s) and vehicle(s), laboratory instruments/techniques used to quantify the response).
Response(s) measured	Specify the response(s) measured by the information source and its measure (e.g. *in chemico* binding to synthetic peptides, expressed as % of peptide depletion).
Prediction model	Indicate whether there is a prediction model associated to the information source and its purpose. Briefly describe the prediction model and provide a reference to a paper or document where the prediction model is described (if available).
Metabolic competence (if applicable)	Specify whether the information source encompasses any metabolically competent system/step and, to the extent possible, how this relates to the situation *in vivo*.
Status of development, standardisation, validation	Indicate whether the information source is: a) an officially adopted (standard) test method (e.g. a test method covered by an OECD Test Guideline); b) a validated but non-standard test method; c) a test method undergoing formal evaluation (e.g. prevalidation, validation, others); d) a non-validated test method widely in use; e) a non-validated test method implemented by a small number of users.
Technical limitations and limitations with regard to applicability	Indicate the substance(s) and/or chemical categories (e.g. based on physico-chemical properties or functional groups) for which the information source has been shown **not** to be applicable because of technical limitations, e.g. highly volatile chemicals, poorly water soluble chemicals, solid materials, interference of the chemical with the detection system (e.g. coloured or autofluorescent chemicals interfering with spectrophotometric analysis). Indicate whether the information source is technically applicable to the testing of multi constituent-substances, UVCBs and mixtures. In addition indicate the substance(s) and/or chemical categories for which the information source has been experimentally shown to yield incorrect and/or unreliable predictions with respect to the reference classifications (e.g false negative predictions with substances requiring enzymatic activation, high false positive rate for alcohols).
Strengths and Weaknesses	Provide an indication of the strengths and weaknesses of the information source, compared to existing similar non-testing or testing methods, considering among others the following aspects: a) extent of mechanistic information provided and relevance (i.e. measurement of various responses in the same experimental model, limited or good coverage of the mechanisms at the basis of the effect

	being investigated, predictive of responses in humans); b) level of information provided (single-point estimate or dose-response information); c) level of performance (e.g. higher or lower reproducibility, predictive capacity); d) extent of domain of applicability; e) number of substances with published information.
Reliability (within and between laboratories) (if applicable)	Describe the level of reliability of the information source (i.e. the agreement among results obtained from testing the same substances over time using the same protocol in one or multiple laboratories) and to what extent this has been characterised including the number of substances used for the assessment.
Predictive capacity (if applicable)	Describe the extent to which the information source predicts the key event of interest (as reported in scientific publications and as determined in validation studies). Express the predictive capacity in terms of sensitivity, specificity and accuracy if applicable or by other goodness-of-fit statistics (e.g. linear correlation analysis). Include the number of substances used in this assessment and their predictions using the reference method.
Proposed regulatory use	Indicate the proposed regulatory use of the information source (e.g. stand-alone full replacement method, partial replacement method, screening method, others).
Potential role within a Testing and Assessment Strategy	Indicate the potential weight the information source is expected to carry within a structured approach to data integration (if applicable) and/or within a Testing and Assessment Strategy, and for which specific purpose the information source can potentially be used on its own.

Reference

OECD (2015) Guidance Document On The Reporting Of Integrated Approaches To Testing And Assessment (IATA) (ENV/JM/HA(2015)7). Available at: XXX

Appendix R.7.3–3 Reporting format for structured approaches to data integration

This template aims to provide advice for a structured approach for the reporting of the integration of the individual information sources used to build a *Weight-of-Evidence* approach to fulfil the REACH information requirement for skin sensitisation. The reporting of the structured approaches for the data integration and the conclusions obtained from them should be included in the dossier, e.g. as an attachment to the endpoint summary record of the IUCLID dossier.

Note: the reporting template is based on the OECD reporting format for data integration as described in Annex I of the OECD Guidance Document on the Reporting of Integrated Approaches to Testing and Assessment (IATA) (OECD, 2015), however the template has been adapted to REACH specific purposes.

1 Summary

Summarise the information in the reporting format in order to provide a concise overview of the proposed approach.

2 General information

2.1 Identifier: *Provide a short and informative title for the structured approach.*

2.2 Reference to main scientific papers: *List the main bibliographic references (if any).*

3 Endpoint addressed

Specify the endpoint (here skin sensitisation). Also specify related properties that have been measured or predicted by the proposed approach and indicate whether these address (or partially address) an endpoint, or key event being predicted by an existing test guideline.

4 Definition of the purpose of the *Weight-of-Evidence* approach

Default: meeting the REACH information requirement for skin sensitisation (Annex VII, 8.3) and the relevant classification and/or risk assessment obligations.

5 Rationale underlying the construction of the approach

Describe the rationale used to construct the approach. This should include an assessment of the linkage of the individual information sources used within the approach to the known chemical and the key events being predicted. The reason for the choice of (a) specific information source(s)/test(s) addressing (a) specific key event(s) possibly in the light of other existing similar information sources should be provided.

6 Description of the individual information sources used within the approach (see Appendix R.7.3–1 and Appendix R.7.3–2 **of this Guidance)**

List the information sources employed within the proposed approach (e.g. physico-chemical properties, non-testing (in silico) methods and testing (in chemico, in vitro, in vivo) methods, including the response(s) measured and the respective measure(s) (e.g. in chemico binding to synthetic peptides, expressed as % peptide depletion). A detailed

description for each in chemico, in vitro, and in vivo method should be provided using the endpoint study records (ESRs) in IUCLID (i.e. one ESR per individual information source).

In addition, when QSAR models are used the QSAR Model Reporting Format (QMRF) should be provided and individual predictions, if applicable, should be reported using the QSAR Prediction Reporting Format (QPRF) and included in the ESR of the IUCLID. Both reporting formats are accessible at: https://eurl-ecvam.jrc.ec.europa.eu/laboratories-research/predictive_toxicology/qsar_tools/QRF.

7 Process applied to derive the prediction/assessment

Describe the process used to arrive at the prediction/assessment. This should consist of a pre-defined data interpretation procedure containing a Weight-of-Evidence assessment.

8 Substances used to develop and test the approach (if applicable)

8.1 Availability of training and test sets: *Indicate whether a training set (i.e. chemical data used in the development of the structured approach) and test set (i.e. chemical data used to evaluate the approach) are available (e.g. published in a paper, stored in a database) or appended to this Reporting format. If they are not available, explain why. Example: "It is available and attached"; "It is available and referenced"; "It is not available because the data set is proprietary"; "The data set could not be retrieved".*

8.2 Selection of the training set and test set used to assess the approach: *If the training set and test set are available please describe the rationale for their selection (e.g. availability of high quality in vivo data for the endpoint being predicted, coverage of the range of effects observed in vivo, coverage of diverse physico-chemical properties, coverage of structural diversity, others).*

8.3 Other information on the training and test sets: *If the training and/or the test sets are not available for inclusion as supporting information, indicate any other relevant information about the training and/or test sets (e.g. number and type of substances). This will be useful to gain an appreciation of e.g. the chemical coverage.*

9 Limitations in the application of the approach

*Indicate the type(s) of substances, in terms of their physico-chemical properties, structures and functional groups, for which the approach is considered **not** to be applicable because of technical constraints in the testing of those substances or because such substances have been found to give incorrect and/or unreliable predictions with respect to the reference data or classifications.*

10 Predictive capacity of the approach

Provide an indication of the extent to which the approach overall predicts the skin sensitisation potential by considering all existing evidence and by excluding chemical types identified in the limitations above. Express the predictive capacity in terms of sensitivity, specificity and concordance, if applicable, or by other goodness-of-fit statistics (e.g. linear correlation analysis). Describe and rationalise to the extent possible potential misclassifications or unreliable predictions for substances that are considered to be covered by the applicability domain of the approach (i.e. substances under-predicted or over-predicted with respect to the reference classification).

11 Known uncertainties associated with the application of the approach

11. 1 Sources of uncertainty

Describe the uncertainty(ies) which is (are) known to be associated with the application of the approach by capturing the source(s) of uncertainty that result(s) from:

1. Approach structure

- *What are the uncertainties related to the chosen approach structure?*
- *How does the approach's coverage or weighing of the AOP events affect your confidence in the overall prediction?*
- *How does your confidence in the approach prediction vary across different substances?*

2. Approach information sources

- *How does the variability in approach information source data for a given substance (i.e. reproducibility) affect your confidence in the approach prediction?*

3. Approach benchmark data

- *How does the variability in approach target data (e.g. LLNA, human) affect your confidence in the approach prediction?*

4. Others sources

11.2 Impact of uncertainty on approach prediction

Consider how these sources of uncertainty translate into prediction uncertainty in the context of your defined application.

- *Does the approach prediction for a new substance include an assessment of uncertainty?*

12 References

List relevant references, weblinks etc., including those describing the structured approach itself (also provided under Section 2 on General Information).

Reference

OECD (2015) Guidance Document On The Reporting Of Integrated Approaches To Testing And Assessment (IATA) (ENV/JM/HA(2015)7). Available at: XXX