European Commission

JRC SCIENCE FOR POLICY REPORT

EURL ECVAM Recommendation on the use of non-animal approaches for skin sensitisation testing

2017

EUR 28553 EN

Contact information
Name: European Union Reference Laboratory for Alternatives to Animal Testing
Address: Joint Research Centre, Via Enrico Fermi 2749, 21027 Ispra (VA), Italy
Email: JRC-ECVAM-CONTACT@ec.europa.eu

JRC Science Hub
https://ec.europa.eu/jrc

JRC106410

EUR 28553 EN

PDF ISBN 978-92-79-67786-1 ISSN 1831-9424 doi:10.2760/588955

Luxembourg: Publications Office of the European Union, 2017

How to cite this report: EURL ECVAM Recommendations on the use of non-animal approaches for skin sensitisation, EUR 28553 EN, Publications Office of the European Union, Luxembourg, 2017, ISBN 978-92-79-67786-1, doi:10.2760/588955, JRC106410.
Available at: http://publications.jrc.ec.europa.eu/repository/handle/JRC106410.

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Abstract
Considerable progress has been made in non-animal methods and approaches for skin sensitisation assessment since the publication in 2013 of the EURL ECVAM strategy for this endpoint. This report illustrates EURL ECVAM views on the regulatory use of individual test methods, including two in vitro methods peer reviewed by ESAC, the LuSens and the U-SENS™ and on defined approaches and provides EURL ECVAM recommendations on future work to be undertaken in the area.

Contents

Executive summary

Considerable progress has been made in non-animal methods and approaches for skin sensitisation assessment since the publication of the EURL ECVAM strategy document for this endpoint in 2013. At the OECD level, three EURL ECVAM validated and/or peer reviewed non-animal methods addressing Key Events (KE) of the skin sensitisation Adverse Outcome Pathway (AOP) have been adopted as OECD Test Guideline (TG) 442C (Direct Peptide Reactivity Assay, DPRA); OECD TG 442D (KeratinoSens™) and, OECD TG 442E (human Cell Line Activation Test, h-CLAT). Other *in vitro* (cell-based) methods, the U937 Cell Line Activation Test (U-SENS™) and the IL-8 Luc assay are at an advanced stage in the regulatory adoption process whereas the LuSens, the SENS-IS and the Genomic Allergic Detection Test (GARD) are under consideration by the OECD for the development of the respective TGs. Among these methods the LuSens and U-SENS™ underwent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC). The outcome of the ESAC peer review was considered by EURL ECVAM when issuing its recommendation on both methods.
Various Defined Approaches (DAs), integrating non-animal data generated with the regulatory adopted methods, have been proposed as valid components of Integrated Approaches to Testing and Assessment (IATA) and documented in OECD Guidance Document (GD) 256 using harmonised templates for their reporting (provided in OECD GD 255). This progress was the driver of the revision of the REACH information requirements for the skin sensitisation endpoint, with *in chemico* and *in vitro* methods becoming the default route, and of the update of the REACH Guidance on Information Requirements and Chemical Safety Assessment for Skin and Respiratory Sensitisation published by ECHA.

This document provides EURL ECVAM views on the regulatory use of non-animal approaches for skin sensitisation and delivers the following key EURL ECVAM recommendations:

- The qualitative and quantitative mechanistic information generated by the *in chemico* and *in vitro* methods adopted by the OECD should be used, together with other relevant information, within DAs and IATA for assessing skin sensitisation hazard and for hazard classification purposes;

- Predictions generated with valid DAs[1] should be used, where applicable and adequate, instead of LLNA data or in conjunction with such data if they already exist, in the context of IATA for assessing skin sensitisation hazard and for hazard classification purposes;

- New DAs used for regulatory purposes should be properly documented using the templates provided in OECD GD 255;

- To promote international applicability and acceptance of alternative non-animal approaches to skin sensitisation, future work should focus on the definition of internationally agreed standards (e.g. OECD TGs) for DAs and individual test methods that provide equivalent or better level of information than the current animal tests for skin sensitisation;

- The LuSens and U-SENS™ methods, that underwent ESAC peer-review in 2016, should be used as valid scientific methods for generating information respectively on KE2 and KE3 of the skin sensitisation AOP to be considered together with other relevant information in the context of DAs and IATA. Inclusion of the LuSens as similar method to the KeratinoSens™ in OECD TG 442D and development of an OECD TG on the U-SENS™ is fully supported.

[1] Examples of DAs which are considered for further evaluation are reported in Annex I to OECD GD 256.

1. Introduction

Skin sensitisation is the regulatory endpoint aiming at the identification of chemicals able to elicit an allergic response in susceptible individuals. Following repeated exposure to a sensitising agent, the adverse health effect of allergic contact dermatitis (ACD) may be provoked. Thus the development of ACD is characterised by two distinct phases: a) the induction of specialised immunological memory following the initial exposure to an allergen, called sensitisation and b) elicitation of the clinical allergic response following subsequent exposure to the allergen. Skin sensitisation assessment is an important component of the safety evaluation of chemicals.

In 2013 EURL ECVAM undertook an analysis of the standard regulatory requirements for skin sensitisation within pieces of EU chemicals legislation. This analysis, reported in the EURL ECVAM skin sensitisation strategy document (EURL ECVAM, 2013a), clearly indicated that the availability of non-animal approaches capable of identifying skin sensitisation hazard and generating information that would satisfy chemicals' classification needs (i.e. potency sub-categorisation) would have the biggest impact in terms of reduction in total number of animal used in the area.

The EURL ECVAM strategy document also outlined the actions EURL ECVAM planned to undertake in the short (2013-2014), medium (2014-2015) and long term in order to advance progress in the area: a) to finalise the validation and peer review of non-animal test methods for skin sensitisation and lead activities for their regulatory adoption b) to develop non-animal testing strategies suitable for hazard identification and potency sub-categorisation of sensitising chemicals and c) to take a leading role at the OECD in the development of Test Guidelines (TG) and Guidance Documents (GD) that would facilitate a globally harmonised approach to skin sensitisation assessment.

Despite the fact that in the last decade regulations in the cosmetics and chemicals sectors have provided a strong impetus to assess potential toxic effects of chemicals with non-animal methods, at the time of writing of the EURL ECVAM skin sensitisation strategy the assessment of the skin sensitisation potential of chemicals still relied on the use of animal tests (i.e. mainly the Local Lymph Node Assay or LLNA) since no regulatory adopted non-animal methods were available for the purpose. Nevertheless, *in chemico* and *in vitro* (cell-based) methods, addressing mechanisms described under the first three Key Events (KEs) of the skin sensitisation Adverse Outcome Pathway (AOP) initiated by covalent binding to proteins (OECD 2012), were under development by industry and academia.

Three of these methods, namely, the Direct Peptide Reactivity Assay (DPRA), KeratinoSens™ and the human Cell Line Activation Test (h-CLAT) were being formally evaluated by EURL ECVAM through validation and/or independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC). Subsequent to the ESAC peer review and the publication of the EURL ECVAM Recommendation on the three methods (EURL ECVAM 2013b; 2014; 2015), EURL ECVAM took a leading role on behalf of the EU at the OECD in the development of the corresponding TGs. In 2015 the OECD adopted the DPRA and the KeratinoSens™ as TGs 442C and 442D respectively (OECD 2015a; 2015b) and in 2016 the h-CLAT as TG 442E (OECD 2016a).

In addition to the adopted test methods, knowledge of the skin sensitisation pathway has prompted the development of a wide range of other alternative methods (*in silico*, *in chemico*, *in vitro*), addressing specific KEs of the AOP (OECD 2012). Some of these non-animal tests: the SENS-IS (Cottez et al., 2016) and the LuSens (Ramirez et al., 2016), a similar method to the KeratinoSens™, addressing KE2, the U-SENS™ (Piroird et. al. 2015; Alépée et al., 2015), the IL-8 Luc Assay (Kimura et al., 2015) and the GARD (Johansson et al., 2013; 2014), the latter three all addressing mechanisms under KE3 of the skin sensitisation AOP, have been included in the OECD TG programme. The U-SENS™ and the LuSens underwent an industry-led validation study and have recently been peer-reviewed by the ESAC (EURL ECVAM Scientific Advisory Committee 2016a; 2016b). A description of the U-SENS™ and the LuSens including the EURL ECVAM

Recommendations on both methods are provided under the respective sections at the end of this document.

Information generated by these methods can contribute to informing regulatory skin sensitisation assessment and hazard categorisation (e.g. according to the United Nations Globally Harmonised System of Classification and Labelling of Chemicals, GHS, Category 1, 1A and 1B) when used in combination with other relevant evidence, i.e. in the context of Integrated Approaches to Testing and Assessment (IATA) and Defined Approaches (DAs) to testing and assessment (OECD 2016b).

DAs are based on a fixed set of information sources and a fixed data interpretation procedure (DIP) to convert inputs from the different information sources into a prediction (OECD, 2016b) and are therefore standardised both in relation to the set of information sources used and in the DIP applied to the data to derive predictions intended to be used within IATA (OECD, 2016b). In contrast, the assessment process within IATA cannot be standardised since it is always underpinned by a weight of evidence approach (judgment –based approach) (OECD, 2017).

Various DAs for skin sensitisation which integrate data from *in silico*, *in chemico* and *in vitro* methods have been proposed in the past few years. Generally, they are designed to enable the use of mechanistic data from these methods together with other relevant information to predict responses in the LLNA.

In 2013, mindful of the several DAs for skin sensitisation under development, EURL ECVAM took a leading role for the EU at the OECD in the definition of guidance on the reporting of such approaches and proposed to the OECD Task Force on Hazard Assessment (TFHA), now the Working Party on Hazard Assessment (WPHA), to set up an expert group charged with the development of such guidance with the aim to bring to regulatory attention as many approaches as possible.

Discussions within the OECD expert group led to the conclusion that at that point in time, merging or harmonisation of such approaches into a single solution that would satisfy all possible regulatory needs was impossible. Instead, the diverse structures and DIPs underlying the different DAs was acknowledged as providing flexibility in the choice of the most appropriate approach to satisfy a specific need. It was also decided that priority should be given to the development of guidance to ensure a harmonised reporting of DAs since when used as components within IATA this would ultimately facilitate the application and evaluation of the IATA themselves for regulatory purposes.

In June 2016 the OECD TFHA endorsed two GD on the reporting of DAs. The OECD GD 255 (OECD, 2016b) provides a set of principles for the reporting of DAs and provides reporting templates to enable their structured documentation. Beside other elements, emphasis is put in the templates on the proper reporting of the limitations in the application of the DA, on their predictive performance and on the sources of uncertainty that may impact on the final prediction. The OECD GD 256 (OECD, 2016c; OECD 2016d; OECD 2016e) exemplifies how the reporting templates have been used to document a number of DAs developed in the area of skin sensitisation. It is envisaged that these templates will be applied to document DAs developed in other areas of toxicology.

The international regulatory adoption of the first three non-animal test methods for skin sensitisation and proposals on how to use these methods in combination within DAs, paved the way in the EU to a substantial revision of the information requirements for skin sensitisation as laid down in Annex VII of the REACH regulation (EC, 2006; EU, 2016). *In chemico* and *in vitro* test methods have become the default requirement and the *in vivo* methods can now only be used if the non-animal tests are shown to be unsuitable for testing a specific substance or cannot be used for classification and risk assessment (EU, 2016). The revised provisions also foresee consideration of whether a substance can be presumed to have the potential to induce significant sensitisation in humans (i.e. GHS Category 1A). The amended requirements, which entered into force in October 2016, will therefore have a substantial impact in replacing and reducing animal testing in view of the 2018 REACH registration deadline. Advances in the area have also prompted the

revision of the European Chemicals Agency's (ECHA) guidance to industry on Information Requirements and Chemical Safety Assessment (Chapter R 7.a, section R.7.3 Skin sensitisation; ECHA, 2016). The revised ECHA draft guidance describes the scope and limitations of the adopted alternative methods to help registrants using them to fulfil the information requirements under REACH. In addition, it proposes a testing and assessment strategy for skin sensitisation assessment which also illustrates how information on the first three key events of the skin sensitisation AOP (i.e. information generated with the validated alternative methods) can be considered in a weight-of-evidence (WoE) approach. Although the guidance recommends the testing and assessment strategy to be followed, it acknowledges that other approaches may be more appropriate depending on the specific case.

2. Use of non-animal approaches

The *in chemico* and *in vitro* test methods recently adopted by the OECD address the first three KEs of the skin sensitisation AOP (OECD 2012). The DPRA (TG 442C; OECD 2015a) provides information on a chemical's reactivity towards peptides, considered to model the molecular initiating event (MIE) or KE1 within the skin sensitisation AOP. The KeratinoSens™ (TG 442D; OECD 2015b) detects activation of a relevant pathway (the Keap1/antioxidant/electrophile response element (ARE)-dependent pathway) in human-derived keratinocytes providing information on KE2 and h-CLAT (TG 442E; OECD 2016a) addresses KE3 by measuring, in a human monocytic leukemia cell line, the up-regulation of markers of dendritic cell (DC) activation following exposure to sensitising agents.

Based on the data generated during the validation studies and historical evidence, the three OECD-adopted test methods have been shown to be transferable to laboratories that have sufficient experience in the techniques involved and to be reproducible for positive/negative predictions in the order of 80-85%. In addition, they demonstrated considerable accuracy, of about 80%, in predicting LLNA responses despite the fact that they are not meant to be used as stand-alone replacement methods. Moreover, they have been shown in recent analyses to have the ability to correctly detect as positives the majority of chemicals that need to be air oxidised (pre-haptens) or enzymatically transformed (pro-haptens) to act as sensitisers (Casati et al., 2016; Urbisch et al., 2016; Patlewicz et al., 2016).

Besides the qualitative information (positive/negative predictions), DPRA, KeratinoSens™ and h-CLAT also provide quantitative readouts that can inform hazard classification (i.e. potency categorisation). Nevertheless, the three methods cannot be used in isolation for potency categorisation or to predict potency for safety assessment.

EURL ECVAM recommends using the qualitative and quantitative mechanistic information generated by the OECD adopted methods, together with other relevant information, in the context of DAs and IATA for assessing skin sensitisation hazard and for hazard classification purposes.

In the past few years advancements have also been made in the integration of data from different non-animal tests in the context of DAs to improve accuracy in predictions with respect to the individual methods. Twelve of these DAs are documented in Annex I to OECD GD 256 (OECD, 2016d). These DAs are based on the use of information sources addressing key mechanisms/events of the skin sensitisation AOP and make use of a variety of specific methodologies, i.e. DIP, for converting the input data into a final prediction.

The documented DAs provide a good overview of the different set of information sources and DIP that can be used for skin sensitisation hazard assessment and/or classification. The DIP can range from very simple rule-based sequential decision steps to mathematical and statistical approaches. Besides those reported in Annex I to OECD GD 256 (OECD, 2016d), other DAs have been documented in the scientific literature (e.g. Luechtefeld et al., 2015; Macmillan et al., 2016; Strickland et al., 2017; Zang et al., 2017) and recently reviewed by Ezendam et al. (2016).

Table 1 provides an overview of the DAs in relation to their proposed use, AOP coverage, type of information sources used within, number of chemicals tested and predictive performances. Note that the information provided is meant to give a flavour of each DA and does not allow a comprehensive understanding of the DA structure and actual performance. The reader should refer to OECD GD 256 (OECD 2016c) and its two annexes (OECD 2016d; 2016e) for a detailed description of the different DAs. In addition, it is beyond the scope of this document to offer any detailed comparison of the different DAs especially in relation to their predictive performance knowing that performance statistics are very much dependent on the dataset used which differs among the reported DAs.

Nevertheless, from the information summarised in Table 1 some general observations can be made:

1) All DAs make use of mechanistic data addressing one or more key events (KE) of the skin sensitisation AOP (OECD 2012). Despite non-animal experimental data on KE4 (T cell priming and proliferation) being included in any of the DAs due to the lack of standardised alternative methods addressing this KE, the different DAs already show a high level of accuracy in predicting binary (i.e. sensitiser/non-sensitiser) LLNA classification.

2) Besides that derived from validated and regulatory adopted methods, other relevant information such as physicochemical properties and *in silico* predictions, contribute to skin sensitisation hazard assessment and classification.

3) Information on KE1 (i.e. the MIE in the skin sensitisation AOP) is used in all DAs (either derived with *in silico* models and/or with *in chemico*, *in vitro* methods) and in some cases reactivity information has proven by the underlying analyses to have the highest power in discriminating between sensitising and non-sensitising chemicals (e.g. Natsch et al., 2015; Asturiol et al., 2016).

4) All of the DAs make use of cell-based assays and/or *in silico* descriptors that account for skin metabolism and autoxidation processes. In fact, as detailed in Annex I to OECD GD 256 (OECD, 2016d), the number of pre- and pro-haptens erroneously classified as being non-sensitisers is generally limited.

5) The majority of the DAs have been developed/tested with a substantial number of chemicals (in certain cases more than 200) for which *in vivo* skin sensitisation data are available, therefore it is likely that their domain of applicability covers the main reaction mechanisms relevant to skin sensitisation.

6) The accuracy of the different DAs specifically designed to predict binary (i.e. sensitiser/non-sensitiser) LLNA classifications is high and in the range of 79-93% (with sensitivity in the range of 79-98% and specificity in the range of 72-94%).

7) Where an evaluation of the predictive capacity against human data was performed, this shows that the DAs tend to predict human responses more accurately than the animal model (LLNA) does (e.g. Urbisch et al., 2015; Asturiol et al., 2016; Strickland at al., 2017; Zang et al., 2017).

8) Six of the DAs are designed for potency assessment. When considering their accuracy in predicting sensitisation potency, an important aspect to consider is the variability of the reference in *vivo* data. Consistent with what was already known about the variability of the LLNA (e.g. ICCVAM 2011), recent analyses have confirmed that this is far from being negligible (Hoffmann, 2015; Dumont et al., 2016) making it difficult to assign a chemical to a specific potency class with sufficient confidence on the basis of a single LLNA study result.

In light of the above, it is evident that some of the DAs developed in the area of skin sensitisation have comparable performance to the LLNA for the identification of skin sensitisation hazard. Moreover, some of them appear to be more accurate than the LLNA in predicting hazard responses in humans. Although it is recognised that further work is needed to achieve a more detailed definition of the relative potency of identified skin sensitising chemicals for risk assessment purposes, the DAs summarised in Table 1 already provide useful information for the purpose of classification and labelling. It is acknowledged that the current GHS criteria for hazard classification are not based on *in vitro* data. However work has been initiated at GHS level on how to make use of non-animal test methods for the purpose of hazard classification.

EURL ECVAM recommends that the predictions generated using valid DAs[2] be used, where applicable and adequate, instead of LLNA data or in conjunction with such data if they already exist, in the context of IATA for assessing skin sensitisation hazard and for hazard classification purposes.

EURL ECVAM recommends that new DAs used for regulatory purposes be properly documented using the templates provided in OECD GD 255.

[2] Examples of DAs which are considered for further evaluation are reported in Annex I to OECD GD 256

3. Future developments

Table 1 indicates that some of the DAs have comparable or even better performance than the LLNA for skin sensitisation hazard assessment and classification. It is nevertheless recognised that additional work is needed to refine sensitisation potency prediction for the purpose of risk assessment. At the international level, progress was made to guarantee harmonised reporting of DAs in view of facilitating their regulatory application. However, harmonised reporting is not sufficient to guarantee their effective implementation and acceptance of the DAs' predictions by different jurisdictions and regions.

EURL ECVAM recommends that future work should focus on the definition of internationally agreed standards (e.g. OECD TGs) for DAs and individual test methods that provide equivalent or better level of information than the current animal tests for skin sensitisation.

To this end, the European Commission, United States and Canada have submitted a project proposal to the OECD Test Guidelines Programme for the development of a performance-based test guideline (PBTG) for DAs and test methods for skin sensitisation. According to the proposal work should be undertaken to develop assessment criteria to objectively and systematically evaluate the DAs reported in Annex I to OECD GD 256 as well as other candidate DAs and upcoming individual test methods. Such assessment criteria should be informed by a comprehensive understanding of the performance of the LLNA in terms of reproducibility and relevance in predicting human responses.

As part of the previous OECD activities on the documentation of DAs (OECD 2016b, 2016c), emphasis has been given to systematically report the possible sources of uncertainty associated with the application of a specific DA. For example, uncertainties can be associated with the structure of the DA itself, the information sources used within (e.g. variability of the input data) and the *in vivo* (animal and/or human) benchmark data used to assess the performance of the DA. In fact the predictive performance of the DAs listed in Table 1, including those proposed for potency categorisation, has been evaluated using as benchmark data individual LLNA predictions and potency estimates (i.e. EC3 values). Thus the calibration of the DIP associated with each DA did not take into account either the variability of the animal test or the variability associated with the model input parameters (e.g. non-animal data). The impact of the combined effect of these sources of uncertainties on the final DA prediction should be further characterised as part of future activities on the evaluation of DAs.

4. The LuSens test method

The LuSens is an *in vitro* test method proposed to contribute to the assessment of the skin sensitisation potential of chemicals when used in conjunction with other information (i.e. in the context of DAs and IATA).

The method quantifies luciferase gene induction as a measure of the activation of the Keap1/antioxidant/electrophile response element (ARE)-dependent pathway in a keratinocyte cell line stably transfected with a selectable plasmid. The LuSens is addressing the mechanism of induction of cyto-protective pathways in keratinocytes, covered by KE2 in the skin sensitisation AOP (OECD, 2012). The LuSens method is considered similar to the KeratinoSensTM for which an OECD TG is available (OECD TG 442D) (OECD, 2015b) supplemented by "Performance standards (PS) for assessment of proposed similar or modified *in vitro* skin sensitisation ARENrf2 luciferase test methods" (OECD, 2015c).

The LuSens underwent an industry-led PS-based validation study involving four laboratories and conducted to fulfil the requirements detailed in the OECD PS for demonstrating comparable performance to that of the validated KeratinoSens™ and adherence to the essential test methods components that would assure similarity with the validated reference method.

The validation study demonstrated that the method is easily transferable to laboratories experienced in cell culture techniques. The within-laboratory reproducibility (WLR) and the between-laboratory reproducibility (BLR), as calculated on the basis of concordant classifications for the chemicals tested (n=12 for WLR and n=20 for BLR) was 100%. The LuSens also complied with the performance standards requirements for accuracy ≥ 80% (LuSens 85%) and sensitivity ≥ 80% (LuSens 92%) but not with the one for specificity ≥ 80% (LuSens 75%). Potential differences between the LuSens and the KeratinoSens™ assays were only observed for methyl salicylate and eugenol, which resulted in apparently higher sensitivity and lower specificity.

The ESAC Opinion on the method, delivered in June 2016 (EURL ECVAM Scientific Advisory Committee, 2016a), highlighted the fact that the two substances giving different results in the two methods are borderline substances (i.e. give both positive and negative predictions in repeated runs in several methods, including LuSens, KeratinoSens™, DPRA, h-CLAT and also in the LLNA and humans).

EURL ECVAM recommends the use of the LuSens as a valid scientific method for generating information on KE2 of the skin sensitisation AOP to be used together with other relevant information in the context of DAs and IATA and fully supports the inclusion of the method into OECD TG 442D.

5. The U-Sens™ test method

The U-SENSTM is an *in vitro* test method proposed to contribute to the assessment of the skin sensitisation potential of chemicals when used in conjunction with other information (i.e. in the context of DAs and IATA).

The test method is based on the quantitative cytofluorimetric analysis of the induction of the CD86 protein marker in U937 cells (a cell line established from a diffuse histiocytic lymphoma) after 45h exposure to the test chemical. The test method is proposed to address KE3 (DC activation) of the AOP (OECD, 2012). The U937 cells are human myeloid cells used as a surrogate model for DC. Activated upon contact with skin sensitizers, they increase their CD86 expression. The induction of the CD86 membrane protein following exposure to skin sensitisers is one of the biomarkers indicating activation of DC that is most frequently used in in *vitro* assays. The U-SENS™ is similar to the h-CLAT (OECD, 2016a) although the latter is based on the measure of expression levels of both CD86 and CD54 cell surface markers in THP-1 cells.

The U-SENSTM underwent an industry-led validation study designed primarily to address the reproducibility of the method (WLR and BLR). The WLR assessed in four laboratories on the basis of concordant classifications for 15 chemicals was 73%, 93%, 100% and 100% respectively (average 91.7%) with the lowest reproducibility was observed in the laboratory less familiar with the use of the U-SENSTM method indicating that the method may require expertise and time for proper implementation. The between-laboratory reproducibility was approximately 84% (n=38).

The accuracy of the method in discriminating between sensitisers and non sensitisers on the basis of LLNA classifications was calculated to be 93% (sensitivity 97%, specificity 89%) with the chemical tested in the validation study (n=38). In addition, performance values for a larger set of 166 substances tested in house were provided indicating an accuracy of 85% (sensitivity 95% and specificity 65%) when evaluated against LLNA data. When evaluated against human data (n=101) the U-SENSTM showed an accuracy of 83% (sensitivity 95% and specificity 59%).

The limitations of the U-SENSTM are likely to be very similar to other submerged cell culture assays (e.g. h-CLAT, KeratinoSens). Potential issues may be encountered with substances of low solubility or low stability in an aqueous environment, fluorescent substances interfering with flow cytometry analysis, pro-haptens and volatile substances and substances disrupting cell membranes. Pre-haptens included in the validation chemicals set were correctly detected by the USENSTM.

The ESAC Opinion on the method, delivered in June 2016 (EURL ECVAM Scientific Advisory Committee, 2016b), indicated that the application of six rules to the prediction model to resolve inconclusive results increases the complexity of the method without adding to its predictive performance since in most cases the six rules appears to convert inconclusive results into positive results. This suggestion has been taken into account by the test developer through a revision of the protocol and a supporting analysis of the validation study data and historical data showing that the elimination of the six rules does not impact on the test method's performance.

EURL ECVAM recommends the use of the U-SENS™ as a valid scientific method for generating information on KE3 of the skin sensitisation AOP to be used together with other relevant information in the context of DAs and IATA and fully supports the development of an OECD TG on the method.

Table 1: Overview of the Defined Approaches documented in Annex I to OECD GD 256

	Defined Approach	Proposed use	AOP KEs addressed[3]	Information sources used[4]	DIP	Number of chemicals tested	Predictive capacity parameters (%) evaluated against LLNA and/or human responses[5]	Comments
1	AOP –based "2 out of 3" weight of evidence / integrated testing strategy ("2 out of 3 – Sens ITS") approach to skin hazard identification (BASF)	Hazard identification	1,2,3	OECD TG 442C (KE1) OECD TG 442D or LuSens (KE2) mMUSST or OECD TG 442E (KE3)	Integrated Testing Strategy (ITS) in which concordant results for two KEs drive the prediction	213 (151 S[6]) (62 NS[6])	Against LLNA (n=126-180): Accuracy 79-84 Sensitivity 79-84 Specificity 72-84 Against human (n=75-101): Accuracy 88-91 Sensitivity 84-90 Specificity 89-100	

[3] The Key Events (KE) reported in the column does not necessary imply that all of them are addressed each time a substance is tested with the DA
[4] The information sources are not necessarily listed in the table in the order they are used within the DAs
[5] Predictive capacity parameters are those documented in Annex I to OECD GD 256 (OECD, 2016d) and, where possible, are presented as ranges when more than one value is reported (e.g. in case these have been calculated using different subsets of data)
[6] S=sensitiser, NS= non-sensitiser on the basis of the reference *in vivo* data
*Values calculated by EURL ECVAM

2	Sequential Testing Strategy (STS) for hazard identification of skin sensitisers (RIVM)	Hazard identification	1,2,3,4	OECD TG 442C (KE1) OECD TG 442D and HaCaT gene signature (KE2) OECD TG 442E (KE3) Bayesian QSAR approach (MultiCASE, CAESAR, DEREK and OECD QSAR Toolbox) (KE4)	Sequential Testing Strategy (TST) in which decision criteria are applied after each tier	41 (27 S) (14 NS)	Against human: Accuracy 95 Sensitivity 96 Specificity 93 LLNA against human Accuracy 78 Sensitivity 93 Specificity 64	Performance of the LLNA in predicting human responses for the same set of chemicals, as reported in van der Veen et al. (2014).
3	A non-testing Pipeline approach for skin sensitisation (G. Patlewicz)	Primarily hazard identification. In certain cases allows sub-categorisation of sensitisers into GHS subcategories 1A and 1B	1,2,3,4,AO	Various physicochemical properties Various *in silico* simulators for abiotic or enzymatic activation Various *in silico* methods (KE1, KE2 and KE4) TG 442C and glutathione depletion assay (KE1) OECD TG 442D (KE2) OECD TG 442E and U-SENS™ (KE3) OECD TG 429 (KE4) OECD TG 406 (AO) Others	Weight-of-evidence	100 (55 S) (45 NS)	Against LLNA, GPMT, Buehler test: Accuracy 88 Sensitivity 89 Specificity 86	This approach represents an IATA workflow rather than a DA. It was used to show that the template provided in OECD GD 255 is flexible enough to be used to document IATA besides DAs.

4	Stacking meta-model for skin sensitisation hazard identification (L'Oréal)	Hazard identification	1,2,3	Various physicochemical properties Various *in silico* methods and OECD TG 442C (KE1) OECD TG 442D (KE2) U-SENS™ (KE3)	Meta-model stacking five different statistical methods (Boosting, Naïve Bayes, Support Vector Machine (SVM), Sparse PLS-DA and Expert Scoring). The model provides a probabilistic output	165 113 training set (66 S) (47 NS) 52 test set (31 S) (21 NS)	Against LLNA for training set: Accuracy 93 Sensitivity 95 Specificity 90 Against LLNA for test set: Accuracy 92 Sensitivity 93 Specificity 90	Overall accuracy values not reported.
5	Integrated decision strategy for skin sensitisation hazard (ICCVAM)	Hazard identification	3,4,AO	Various physicochemical properties OECD TG 442E (KE3) OECD Toolbox (KE4, AO)	Support vector machine	120 (87 S) (33 NS)	Against LLNA: Accuracy 88 Sensitivity 85 Specificity 94	Accuracy values for training and test set reported in the Annex I to OECD GD 256.
6	Classification consensus model of decision trees based on in silico descriptors to predict skin sensitisation hazard (EC- JRC)	Hazard identification	1	Various in silico descriptors generated with TIMES-SS (KE1) and DRAGON software packages	Consensus model of two decision trees	269 (170 S) (99 NS)	Against LLNA (n=269): Accuracy 93 Sensitivity 98 Specificity 85 Against human (n=99): Accuracy 81 Sensitivity 90 Specificity 64	Accuracy values for training and test set reported Annex I to OECD GD 256.

7	Sensitizer potency prediction based on Key event 1 + 2: Combination of kinetic peptide reactivity data and KeratinoSens™ data (Givaudan)	Hazard identification Prediction of a continuous likely LLNA or human potency value Potency sub-categorisation in the two GHS potency classes (1A and 1B)	1,2	Various physicochemical properties TIMES-SS and Cor1C420-assay (KE1) OECD TG 442D (KE2)	Reactivity domain-based regression equations Global regression equation for chemicals that cannot be assigned to a specific domain	244 (133 S) (111 NS) Human GHS class 1B (44 S) Human GHS class 1A (27 S)	Against LLNA for binary classification: Accuracy 83* Sensitivity 89* Specificity 75* Against LLNA GHS potency classes (NS, 1B, 1A) Accuracy: Overall for 3 classes 75 NS 76* 1B 77* 1A 70* a) Predicted by in vitro data: 1B 55 1A 70 b) Predicted by LLNA data: 1B 72 1A 52	The accuracy values reported are calculated combining results from the global and domain models. Classifications derived by estimating human DSA_{05} values (induction dose per skin area, in µg/cm2, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population using a) in vitro data and b) LLNA data.

8	The artificial neural network model for predicting LLNA EC3 (Shiseido)	Hazard identification potency sub-categorisation in 3 potency classes: non-sensitisers (N), combined weak and moderate (M), combined strong (S) and extreme (E) sensitisers	1,2,3	Log P cell-surface thiol test (SH test) (KE1) Antioxidant Response Element (ARE) assay (KE2) OECD TG 442E (KE3)	Artificial neural network	62 (48 S) (14 NS)	Against 3 LLNA potency classes (NS, W/M, S/E) Accuracy: Overall for 3 classes 79 NS 64* W/M 93* S/E 67*	
9	Sensitizer potency prediction based on Key event 1+2+3: Bayesian Network ITS/DS for hazard and potency identification of skin sensitizers (P&G)	LLNA potency probabilistic distribution (pEC3), for 4 potency classes: non-sensitisers (N), weak (W), moderate (M), and combined strong (S) and extreme (E) sensitisers	1,2,3	Various parameters for bioavailability In silico simulators for abiotic or enzymatic activation (TIMES) TIMES-SS and OECD TG 442C (KE1) OECD TG 442D (KE2) OECD TG 442E (KE3)	Bayesian Network, the model provides a prediction with either all or partial data inputs	207 (154 S) (53 NS)	Against LLNA for binary classification: Accuracy 96 Against 4 LLNA potency classes (NS, W, M, S/E): Accuracy: Overall 74-89 NS 87-100 W 83-90 M 45-75 S/E 60-87	The accuracy reported is calculated for the test set and considering data inputs and omission of each one of the KE assays. Potency probabilistic distributions are documented in Annex I to OECD GD 256.

10	Sequential testing strategy (STS) for sensitising potency classification based on in chemico and in vitro data (Kao Corporation)	Hazard identification LLNA potency sub-categorisation in 3 potency classes: non-sensitisers (N), weak (W) (combined W and M sensitisers in the LLNA), strong (S) (combined S and E sentisers in the LLNA)	1,3	OECD TG 442C (KE1) OECD TG 442E (KE3)	Sequential Testing Strategy (TST) in which decision criteria are applied after each tier	139 (1O2 S) (37 NS)	Against LLNA for binary classification: Accuracy 81 Sensitivity 90 Specificity 54 Against LLNA potency classification: Strong (EC3<1% in the LLNA) and Weak (EC3≥1% in the LLNA): Accuracy Overall 69 NS 54* Weak 78* Strong 66*	

11	Integrated testing strategy (ITS) for sensitising potency classification based on in silico, in chemico, and in vitro data (Kao Corporation)	Hazard identification LLNA potency sub-categorisation in 3 potency classes: non-sensitisers (N), weak (W) (combined W and M sensitisers in the LLNA), strong (S) (combined S and E sentisers in the LLNA)	1,3	DEREK Nexus and OECD TG 442C (KE1) OECD TG 442E (KE3)	Integrated Testing Strategy (ITS) based on the integration of input parameters converted into scores	139 (1O2 S) (37 NS)	Against LLNA for binary classification: Accuracy 84 Sensitivity 89 Specificity 70 Against LLNA potency classification Strong (EC3<1% in the LLNA) and Weak (EC3≥1% in the LLNA): Accuracy: Overall 71 NS 70* Weak 78* Strong 52*	
12	DIP for skin allergy risk assessment (SARA) (Unilever)	Potency prediction expressed as probability that a specific CD8+ T cell response will be induced following a given skin exposure to a direct-acting sensitising chemical	1,3,4	Modified OECD TG 428 to derive information on skin bioavailability kinetics and protein haptenation kinetics (KE1) Prediction of Class I MHC processing & presentation of haptenated skin protein by dendritic cells (DC) (KE3) Prediction of the extent of human naïve CD8+ T cell activation (KE4)	Ordinary differential equation	1	Not applicable	

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List of abbreviations and definitions

ACD	Allergic Contact Dermatitis
AOP	Adverse Outcome Pathway
DA	Defined Approach
DIP	Data Interpretation Procedure
DPRA	Direct Peptide Reactivity Assay
ECHA	European Chemicals Agency
ESAC	EURL ECVAM Scientific Advisory Committee
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
GARD	Genomic Allergic Detection Test
GD	Guidance Document
GHS	Globally Harmonised System to Classification and Labelling of Chemicals
h-CLAT	human Cell Line Activation Test
IATA	Integrated Approach to Testing and Assessment
KE	Key Event
LLNA	Local Lymph Node Assay
MIE	Molecular Initiating Event
OECD	Organisation for Economic Co-operation and Development
PBTG	Performance Based Test Guideline
PS	Performance Standards
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
TFHT	Task Force on Hazard Assessment (now renamed Working Party on Hazard Assessment, WPHA)
TG	Test Guideline
U-SENS™	U937 Cell Line Activation Test

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doi:10.2760/588955

ISBN 978-92-79-67786-1