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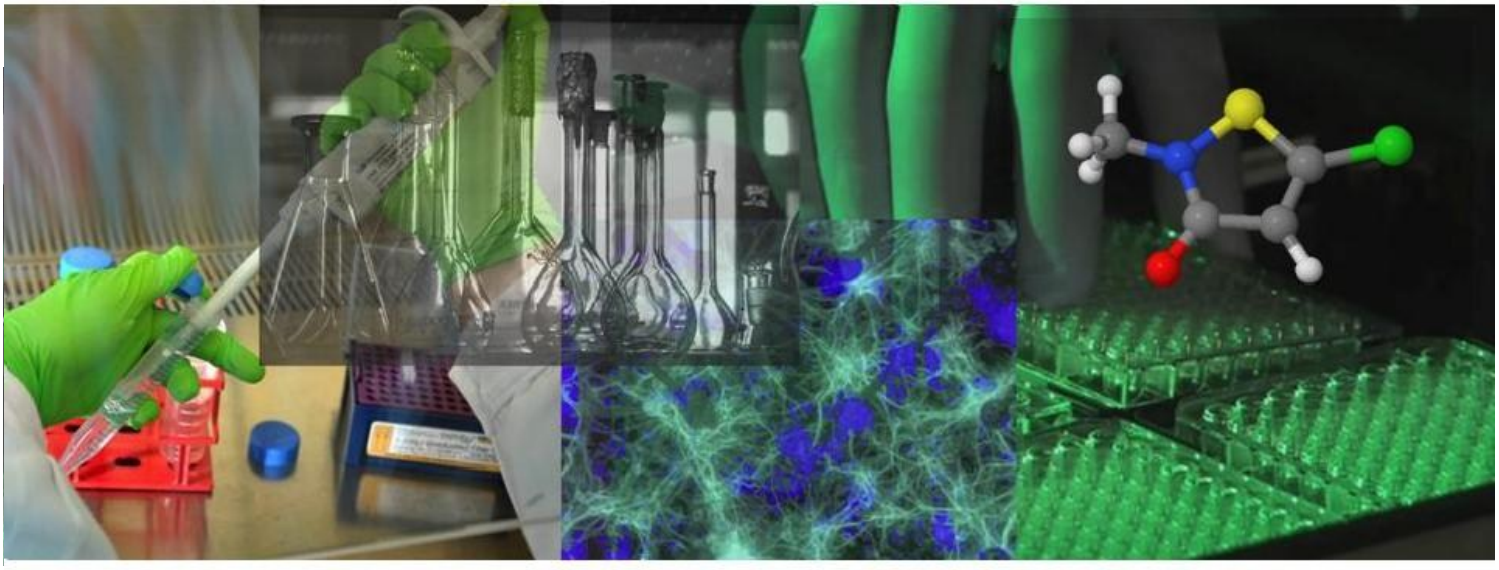
EURL ECVAM Recommendation on the KeratinoSens™ assay for skin sensitisation testing

February 2014



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EURL ECVAM RECOMMENDATION

on the KeratinoSens™ assay for skin sensitisation testing

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This Recommendation was prepared by the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), part of the Institute for Health and Consumer Protection (IHCP), Directorate-General Joint Research Centre (DG JRC) of the European Commission.

The Recommendation was drafted on the basis of the ESAC Opinion and ESAC Working Group Report outlining the detailed scientific peer review of the Givaudan-coordinated study on the KeratinoSens™ assay. The Recommendation further benefitted from comments and suggestions received from members of PARERE (EURL ECVAM's advisory body for Preliminary Assessment of Regulatory Relevance that brings together representatives of Member State regulatory bodies as well as EU agencies including ECHA, EFSA and EMA), and ESTAF (EURL ECVAM's Stakeholder Forum). Input was also provided by partner organisations of EURL ECVAM in the framework of the International Collaboration on Alternative Test Methods (ICATM), and by the general public.

Coordinator of the evaluation of the test submission was Silvia Casati. Coordinator of the ESAC Peer Review and EURL ECVAM Recommendation was Claudius Griesinger.

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BACKGROUND TO EURL ECVAM RECOMMENDATIONS

The aim of a EURL ECVAM Recommendation is to provide EURL ECVAM views on the validity of the test method in question, to advise on possible regulatory applicability, limitations and proper scientific use of the test method, and to suggest possible follow-up activities in view of addressing knowledge gaps.

During the development of its Recommendations, EURL ECVAM consults with its consultation body for Preliminary Assessment of Regulatory Relevance (PARERE) and its EURL ECVAM Stakeholder Forum (ESTAF). Moreover, EURL ECVAM consults with other Commission services and its international validation partner organisations of the International Cooperation on Alternative Test Methods (ICATM). Before finalising its recommendations, EURL ECVAM also invites comments from the general public and, if applicable, from the test method submitter.

EXECUTIVE SUMMARY

The KeratinoSens™ *in vitro* test method for skin sensitisation testing has been developed by Givaudan, a producer of fragrances and flavours. From 2009 to 2010 Givaudan coordinated a validation study on the KeratinoSens™ test method, focusing on its transferability and reproducibility. Following submission to EURL ECVAM of the study data as well as supplementary information, EURL ECVAM charged ESAC to review the KeratinoSens™ validation study which it finalised in December 2012. EURL ECVAM endorses the conclusions of the ESAC opinion (Annex I) on the Givaudan-coordinated study and makes the following recommendations.

(1) The Keap1-Nrf2-ARE pathway is considered a major regulator of cyto-protective responses to electrophile and oxidative stress by controlling the expression of detoxification, antioxidant and stress response enzymes and proteins. Since the majority of chemical skin sensitisers are electrophiles reacting with nucleophilic centres in skin proteins, the pathway is one relevant readout for skin sensitisation (OECD, 2012).

(2) Since activation of the Keap1-Nrf2-ARE pathway addresses only one single biological mechanism, it is likely that information from test methods based on this or similar pathways will not be sufficient to conclude on the skin sensitisation potential of chemicals. Therefore the KeratinoSens™ assay should not be considered a stand-alone full replacement method and data generated with the test method should always be considered in the context of integrated approaches, e.g. Weight-of-Evidence (WoE) or Integrated Testing Strategies (ITS), combining them with complementary information derived from *in vitro* assays addressing other key events of skin sensitisation (e.g. in chemico reactivity assays such as the Direct Peptide Reactivity Assay) as well as non-testing methods including read-across from chemical analogues.

(3) Based on the data generated in the study, the KeratinoSens™ test method proved to be transferable to laboratories experienced in cell culture and reproducible within- and between-laboratories (86% concordance in both cases).

(4) The Givaudan-coordinated validation study generated preliminary information on the test method's predictive capacity and it was found that the accuracy of the KeratinoSens™ to discriminate skin sensitisers from non-sensitisers was 90% (sensitivity 87%, specificity 100%; n=21)[1]. The accuracy calculated for an additional set of chemicals (77 sensitisers and 104 non-sensitisers) tested in-house by Givaudan was 75%. These figures are similar to those recently published by Natsch et al. (2013) based on in-house testing of about 145 chemicals (77% accuracy, 79% sensitivity, 72% specificity). Taken together, this information indicates the usefulness of the KeratinoSens™ assay to contribute to the identification of sensitisers and non-sensitisers.

(5) The KeratinoSens™ assay also provides concentration-response information that may contribute to the assessment of sensitising potency as recently proposed by Jaworska et al. (2013). Further work is required to determine to which extent KeratinoSens™ results relate to potency categories based on, preferentially, human data.

[1] N.B. The values presented here differ from those presented in the ESAC WG report. The Givaudan submission to EURL ECVAM contained data of the Givaudan-coordinated validation study plus data from in-house testing produced under non-validation conditions (e.g. no blind testing). While the ESAC WG calculated the predictive capacity on the basis of all data points irrespective of how they had been generated, the values presented above have been calculated a) on the basis of the validation study and b) on the basis of the additional non-validation data. This ensures a consistent approach with regard to the presentation of the predictive capacity of other skin sensitisation test methods summarised in EURL ECVAM Recommendations (e.g. DPRA), where the preliminary predictive capacity of the assays has been calculated on the basis of a small validation set and compared to the predictive capacity from additional information generated in-house by test submitters under non-validation conditions.

(6) To support the development of integrated approaches employing information from cyto-protective signalling pathways such as Keap1-Nrf2-ARE, the applicability of the KeratinoSens™ and its limitations should be further characterised. Based on the available data from the validation study and in-house testing of the submitter, the KeratinoSens™ assay seems applicable to a wide range of chemicals. Nevertheless, negative results should be interpreted with some caution due to (a) the cysteine-dependent mechanism of activation of the signalling pathway; (b) although some pro-haptens are reported to be correctly predicted, those requiring biotransformation by P450 enzymes are not detected; (c) while a variety of pre-haptens are reported to be detected, pre-haptens with a slow oxidation rate may go undetected unless oxidised before the actual experiment (Givaudan, 2011).

(7) The KeratinoSens™ test method can be considered as a valuable component of integrated approaches for skin sensitisation testing although further work is required to fully understand its limitations and to be specific about what complementary data would be desirable depending on the use case. Furthermore, its capacity to contribute to subcategorisation of sensitisers according to UN GHS (UN, 2007) and to potency assessment needs to be defined, the latter preferentially on the basis of human reference data.

(8) Respecting the provisions of Directive 2010/63/EU (EU, 2010) on the protection of animals used for scientific purposes, before embarking on animal experiments to identify substances with skin sensitisation potential, data from the KeratinoSens™ test method should be considered in combination with complementary information in order to reduce and possibly avoid animal testing. As provided for in Annex XI (point 1.2) of the REACH Regulation (EC, 2006), data from non-standard testing methods, such as the KeratinoSens™, may be used to adapt the standard information requirement in the context of Weight-of-Evidence (WoE) judgments.

1. Introduction

1) The assessment of skin sensitisation potential is an important component in the safety evaluation of substances and represents a standard information requirement of legislation on chemicals in the EU. These include: the Classification Labelling and Packaging of substances and mixtures (CLP) Regulation (EC, 2008a), the REACH Regulation, the Plant Protection Products (PPP) Regulation (EC, 2009a), the Biocides Directive (EC, 2012) and the Cosmetics Regulation (EC, 2009b). Determining skin sensitisation hazard in terms of GHS classification is actually sufficient to satisfy the majority of regulatory needs (EURL ECVAM, 2013). However, a more complete characterisation of the potency of a skin sensitiser with regard to both induction as well as elicitation of contact dermatitis is often required for a full risk assessment and the definition of appropriate risk management measures (e.g. setting of appropriate thresholds).

2) Currently only *in vivo* test methods are accepted by regulatory bodies for the generation of data satisfying regulatory requirements on skin sensitisation. For instance, in the frameworks of the Organisation for Economic Cooperation and Development (OECD) and the EU Test Methods Regulation (EC, 2008b), there are four accepted guidelines, describing: the Buehler Test and Guinea-pig Maximisation Test, TG406 (OECD, 1992; EU test method B.6), the Local Lymph Node Assay, TG429 (OECD, 2010a; EU test method B.42) and its non-radio-isotopic variants, the Local Lymph Node Assay: DA (TG 442A; OECD, 2010b) and the Local Lymph Node Assay: BrdU Elisa (TG 442B; OECD, 2010c).

3) The key mechanistic events underpinning the skin sensitisation process that leads to Allergic Contact Dermatitis (ACD) in humans have been identified and recently summarised in the OECD report on "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins"(OECD, 2012). These key events include 1) the covalent binding of the chemical to the skin protein (haptenation), 2) the release of pro-inflammatory cytokines and the induction of cyto-protective pathways in keratinocytes 3) the maturation and mobilisation of dendritic cells (DC), immuno-competent cells in the skin, and 4) the antigen presentation to naïve T-cells and proliferation of memory T-cells. Considerable progress has been made in recent years towards the development of alternative non-animal methods that address these key mechanisms.

4) There is general agreement that it is unlikely that a single alternative method will be able to provide sufficient information to fully replace the use of animals for this endpoint (Adler et al., 2011). Instead it is held that information from different alternative testing and non-testing methods used in combination will need to be integrated to address this health endpoint (Jowsey et al., 2006; Adler et al., 2011). These methods should address different key events involved in skin sensitisation thus covering the mechanistic complexity of this endpoint. Against this background, activities are being pursued by academia, industry and the European Commission to evaluate mechanistically-based test methods that can contribute to skin sensitisation hazard identification and characterisation.

5) In May 2010, EURL ECVAM received a full submission reporting the experimental results generated by five laboratories participating in a Givaudan-coordinated study for the evaluation of the protocol transferability and the within- and between-laboratory reproducibility of the KeratinoSens™ *in vitro* assay. Following the evaluation of the submitted information, EURL ECVAM judged that the within-laboratory reproducibility (WLR) was not sufficiently addressed to progress the study into peer-review and requested Givaudan to generate additional experimental data on the WLR with eight coded chemicals provided by EURL ECVAM. In December 2010 EURL ECVAM received a revised full submission reporting the requested data plus results generated with an additional six

chemicals, not previously tested with the KeratinoSens™ method. The revised full submission was complemented with supplementary information which included an updated analysis of the KeratinoSens™ predictive capacity (PC) based on data from 47 chemicals in addition to the 67 chemicals originally considered for this purpose. Additional information on the PC of the method for 80 non-sensitising chemicals with LLNA reference data was provided by Givaudan in the phase of peer review. Besides the experimental data obtained with 21 coded chemicals in the Givaudan-coordinated study, most of the information on the PC of the KeratinoSens™ has been generated in-house by the test developer.

6) On the basis of the revised submission EURL ECVAM requested the ECVAM Scientific Advisory Committee (ESAC) to provide an ESAC Opinion on the study and supportive information. The ESAC Working Group (WG) "Skin Sensitisation", charged with reviewing validation studies on skin sensitisation test methods, was requested to prepare a detailed WG report (EURL ECVAM, 2012a) on which basis ESAC adopted its Opinion (EURL ECVAM, 2012b; see Annex 1), endorsed on 17. 12. 2012.

2. Test Method definition

The important role of the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2) in promoting the expression of genes coding for cyto-protective proteins (mainly phase 2 enzymes) following electrophilic or oxidative stress is extensively described in the literature (e.g. Baird & Dinkova-Kistova, 2011; Kensler et al., 2007). The activity of Nrf2 is considered to be primarily regulated by the cysteine-rich Keap1 sensor protein (Kelch-like ECH associated protein 1) although other signalling pathways are reported to be involved in its regulation (Baird & Dinkova-Kistova, 2011). Under un-induced conditions the Keap1 protein targets the Nrf2 transcription factor for ubiquitin-dependent proteasomal degradation (Itoh et al., 1999). It is proposed that covalent modification of the cysteine residues in the Keap-1 protein by electrophiles/oxidants leads to the dissociation of the Keap-1 protein from the Nrf2 transcription factor and induces the translocation of the Nrf2 from the cytoplasm to the nucleus where it promotes the activation of cyto-protective genes which have an antioxidant or electrophile response elements (AREs/EpREs) in their promoter sequence (Itoh et al., 1997; Suzuki et al., 2013).

7) Although direct covalent binding to certain Keap1 cysteine residues is considered to be one of the plausible mechanisms through which the Keap1-Nrf2-ARE pathway is activated, other types of modifications of the protein, like oxidation or glutathionylation, are reported to be responsible for its activation. In addition it is proposed that all electrophiles/oxidants may shift the redox balance of the cell through reaction with glutathione (GSH) which may in turn generate an oxidative burst able to modify Keap1 cysteines (Holland & Fishbein, 2010).

8) As reviewed by Natsch (2010) there is increasing evidence that ARE-regulated genes are induced in different cell types after challenge with skin sensitisers. The relevance of the Keap1-Nrf2-ARE regulatory pathway in the *in vivo* reaction to sensitisers was shown in studies with Nrf2 knockout mice (Kim et al., 2008; El Ali et al., 2013; van der Veen et al., 2013).

9) The KeratinoSens™ test method is a reporter gene assay which uses an immortalised adherent cell line derived from an expanded clone of HaCaT human keratinocytes transfected with a selectable plasmid. The plasmid contains the luciferase gene under the transcriptional control of the SV40 promoter fused with the ARE from the AKR1C2 gene which was identified as one of the genes up-regulated by contact sensitisers in dendritic cells (Gildea et al., 2006; Ryan et al., 2004). This allows

to quantitatively measure (by luminescence detection) luciferase gene induction, using well established light producing luciferase substrates, as an indicator of the activity of the Nrf2 transcription factor in cells following exposure to electrophilic chemicals.

10) At present, the KeratinoSens™ test method is designed for the identification of sensitisers/non sensitisers. Chemicals are classified as sensitisers if they induce a statistically significant induction of the luciferase gene above a given threshold in two out of three experiments performed on different days. This is established in parallel to cytotoxicity measurements to assess gene induction levels at sub-cytotoxic concentrations. Since cells are exposed to 12 concentrations of the test chemicals, the concentration needed for a statistically significant luciferase gene induction above the threshold (EC1.5 value) can be extrapolated from the dose response curve. In addition, the maximal fold induction of the luciferase gene over solvent control (I_{max}) is determined.

11) As a result of the Givaudan-coordinated study (Natsch et al., 2011) and additional information provided in the submission to EURL ECVAM, the standardised protocol was found to be transferable (to laboratories with cell culture technique experience) and reproducible within and between laboratories.

12) EURL ECVAM will disseminate a comprehensive description of the KeratinoSens™ method through its database on alternative methods (DB-ALM, at http://ecvam-dbalm.jrc.ec.europa.eu; protocol No. 155), together with all the necessary technical details (e.g. electronic data reporting formats) needed by an end-user laboratory to implement it in a reliable and self-sufficient manner.

3. Overall Performance of the KeratinoSens™ test method

Reference data

13) Reference classifications associated with the test chemicals were selected on the basis of a weight of evidence approach considering different data, i.e. the murine Local Lymph Node Assay (LLNA), the Guinea Pig Maximisation Test (GPMT) and, where available, human data. Reference chemicals from the LLNA performance standards (OECD, 2010a) were included in the chemical set. Additional details can be found in the submission (Givaudan, 2011).

    When interpreting the data of alternative methods, such as the KeratinoSens™ that have been largely developed and validated using animal reference data such as LLNA or GPMT, it should be kept in mind that the predictive relevance of these animal tests may not fully reflect the situation in the species of interest, i.e. humans. Notably, an evaluation of the LLNA in comparison to human data has shown an accuracy of about 72% (Anderson et al., 2011), i.e. there is a risk of false negative and false positive results. Moreover there is indication that the LLNA is deficient in detecting low to moderate sensitisers as well as metals and organometal compounds (EC, 2000).

Transferability

14) EURL ECVAM concludes that the KeratinoSens™ test method is transferable to laboratories sufficiently experienced in cell culture techniques. Since stable background levels of the luciferase gene are critical for the generation of reliable results, EURL ECVAM recommends that a number of training experiments, as described in the Standard Operating Procedure (SOP), be performed by

new laboratories to ensure optimal luminescence measurements before the test method is used for routine testing.

Reproducibility

15) For the set of coded chemicals tested during the validation study, the KeratinoSens™ protocol yielded concordant predictions within the Givaudan laboratory (86%; N=14) and between the five laboratories participating in the ring trial (86%, N=21).

Predictive Capacity

16) The accuracy of the test method in predicting the *in vivo* classification (sensitiser/non-sensitiser) determined on the basis of existing evidence from LLNA, GPMT, Buehler Test and human data for the 21 (coded) chemicals evaluated in the validation study was 90% (sensitivity 87%, specificity 100%). However, since the chemicals selected by Givaudan to be used in the validation study have already been used to develop and optimise the KeratinoSens™ prediction model, it is likely that these values reflect a best-case scenario. When calculating the predictive capacity on the basis of a larger set of data generated in-house by Givaudan, sensitivity and specificity are about 75% (n=77 sensitisers and 104 non-sensitisers). A recently published study correlating KeratinoSens™ data with classifications in the LLNA reported an accuracy of 77% (sensitivity 79% and specificity 72%) for a set of 145 chemicals (Natsch et al., 2013). Thus, it is plausible that these figures might reflect the actual performance of the test in discriminating between sensitisers and non-sensitisers.

4. Limitations

4.1 Technical limitations

17) **Solubility of test substances**: Chemicals which are not soluble in either water or DMSO, being these the two solvents prescribed by the SOP, cannot be tested in the KeratinoSens™. Chemicals with a calculated octanol/water partition coefficient (cLogP) up to 5 were reported by the test developer to be successfully tested with the method.

18) **Solvent effects:** As with many *in vitro*/*in chemico* assays, chemicals which are not stable in the prescribed solvents because of hydrolysis or other chemical reactions cannot be reliably tested.

4.2 Limitations with regard to applicability – negative results

19) As the key mechanism leading to the activation of the Keap1-Nrf2-ARE pathway appears to be the electrophilic reaction of stressors with nucleophilic thiols (cysteine sulfhydryl groups) of Keap-1 it is possible that skin sensitising chemicals with selective reactivity towards other nucleophiles may not be reliably identified by the KeratinoSens™ (e.g. amine reactive chemicals preferentially reacting with lysine residues), thereby leading to false negative results. However, there is scientific evidence that the pathway can be activated by other types of modification of Keap-1 cysteine residues, such as oxidation or conjugation with glutathione, and that, moreover, the Nrf2 transcription factor may be controlled by other signaling pathways. It is therefore plausible that sensitising chemicals not covalently modifying Keap-1 cysteine residues (e.g. amine-reactive chemicals) can nevertheless activate the Nrf2 pathway, leading to true positive responses in the KeratinoSens™ assay.

Complementary information from peptide reactivity assays may help addressing this uncertainty, in particular assays able to distinguish between cysteine and lysine reactivity.

20) While a number of pro-haptens requiring enzymatic oxidation or deamination are reported to be correctly classified by the KeratinoSens™, pro-haptens requiring P450 activation are reported not to be identified by the assay. According to the test developer, attempts to incorporate a metabolic system in the KeratinoSens™ assay have recently been published (Natsch & Haupt, 2013).

21) A variety of pre-haptens have been reported as correctly predicted by the assay (e.g. 1,4-phenylenediamine, hydroquinone and isoeugenol). However, some pre-haptens reported to have a slower rate of spontaneous oxidation (e.g. limonene) may require an oxidation step before the actual experiment.

22) Most of the misclassifications generated by the KeratinoSens™ concerns chemicals that are moderate and weak sensitisers *in vivo* (see ESAC WG report, page 31,), while the false negative rate for strong sensitisers is lower. This should be kept in mind when interpreting negative results.

4.3 Limitations with regard to applicability – positive results

23) Considering the pathway monitored (i.e. electrophilic / oxidative stress), chemicals that do not act as sensitisers but are nevertheless chemical stressors may lead to false positive results in the KeratinoSens™ test method. This could for example include reactive chemicals that cause dermal corrosion / irritation without, however, being skin sensitisers. Nevertheless, it was shown that irritating surfactants, which often are predicted positive in the LLNA, are negative in the KeratinoSens (Ball et al., 2011, Emter et al., 2010).

5. Suggested regulatory use

24) Due to the complexity of the mechanisms underlying skin sensitisation, it is likely that information from different methods (*in silico*, *in chemico*, *in vitro*) is needed to reduce or replace the need for animal testing, both for hazard identification and potency characterisation purposes.

25) Based on the validation study results and other available information, the KeratinoSens™ appears to be a reliable test method that provides information on the ability of a chemical to activate the Nrf2 electrophilic and oxidative-stress response signalling pathway which has been shown to be a relevant pathway in the induction of skin sensitisation as demonstrated by studies in Nrf2-knockout mice (Kim et al., 2008; El Ali et al., 2013; van der Veen et al., 2013). Therefore, Nrf2–dependent luciferase induction measurements in the KeratinoSens™ assay when combined with information from other non-animal methods in the context of a Weight-of-Evidence (WoE) approach or Integrated Testing Strategy (ITS) may provide useful information about the sensitisation potential of chemicals. Taking into consideration the dose-response information generated by the assay, it is plausible that KeratinoSens™ data may also contribute to characterisation of skin sensitisation potency within integrated approaches. The extent of information needed to complement a KeratinoSens™ result will depend on the intended application (e.g. hazard identification, classification or potency assessment) and context (availability and quality of other information). An example of the use of KeratinoSens™ data in a WoE approach for hazard assessment is published in the scientific literature (Ball et al., 2011).

26) Notably, due to the nature of the pathway monitored (i.e. general electrophilic and oxidative stress), KeratinoSens™ provides information on reactivity of chemicals that elicit protective stress responses in exposed cells. Such data may be relevant for other health endpoints such as, for example, dermal irritation and cancer (Reuter et al., 2010, Kansanen et al., 2013).

27) As outlined in more detail in section 4.2, negative KeratinoSens™ results should be interpreted with care, taking into due consideration the possibility of false negatives due to (1) possible selective reactivity of the chemical with amino acids other than cysteine, (2) the limited metabolic capacity of the assay leading to possible misclassification of pro-haptens (especially those requiring biotransformation by P450 enzymes), (3) the uncertain capacity to identify pre-haptens, (4) the uncertain capacity to correctly identify moderate and especially weak sensitisers.

28) Chemicals able to activate the Keap1-Nrf2-ARE pathway by other mechanisms than covalent binding to the Keap-1 cysteine residues may give false positive results in the KeratinoSens™ (see section 4.3).

29) Employed within an integrated approach, the KeratinoSens™ may be useful to satisfy information requirements for Cosmetics (Regulation EC/1223/2009), Chemicals (Regulation EC/1907/2006), Biocides (Regulation EC/528/2012) and Plant Protection Products (Regulation EC/1107/2009).

6. Follow-up activities recommended by EURL ECVAM

(1) In view of further prospective testing with the KeratinoSens™ method, EURL ECVAM recommends that the revised protocol available at EURL ECVAM's DB-ALM service (http://ecvam-dbalm.jrc.ec.europa.eu) be used: [DB-ALM protocol on KeratinoSens™ No. 155].

(2) Further testing should investigate possible limitations of the assay that relate to the cellular pathway chosen and the need for abiotic or biotic activation of some sensitisers (i.e. pre- and

pro-haptens). Moreover, since there is at present limited information on the applicability of the KeratinoSens™ to chemical mixtures including plant extracts (Andres et al., 2013), additional data may be helpful.

(3) Predictive capacity, applicability and limitations of the assay should be further evaluated in the context of its use as part of integrated approaches to testing and assessment. When doing so, the limitations of available reference data e.g. from LLNA (EC, 2000) with regard to reproducibility and relevance to the human situation should be however kept in mind. In particular, the capability of the method to detect accurately weak and moderate skin sensitisers should be further investigated.

(4) Further attention should be given to: (a) an evaluation of the possible contribution of KeratinoSens™ data to sub-categorisation of sensitisers according to GHS (i.e. sub category 1A and 1B); (b) an evaluation of whether and how the dose-response information generated by the assay could contribute to potency assessment allowing quantitative risk assessment. For such evaluation, the use of human reference data will be particularly useful.

(5) Considering the limitations of the assay, integrated approaches using Nrf2–dependent luciferase induction measurements should also make use of other information sources, in particular peptide reactivity assays able to distinguish between cysteine and lysine reactivity. In addition, *in silico* methods (expert systems and QSAR models) may prove useful. *In silico* methods that explicitly incorporate metabolic considerations (e.g. TIMES-SS: Patlewicz et al., 2007) may help to identify pre- and pro-haptens. Analogues which have a similarly predicted mechanism of action, based on protein binding, can be found using the OECD QSAR Toolbox (www.qsartoolbox.org). The Toolbox also includes a specific profiler for the KeratinoSens™ assay. A variety of proposals concerning the use of KeratinoSens™ data in combination with other information sources have been published and may support further work (Natsch et al., 2009; Bauch et al., 2012; Jaworska et al., 2013).

(6) EURL ECVAM supports the development of an OECD Test Guideline for the KeratinoSens™. As this test may be best employed in combination with complementary methods, it should be considered in the current initiative being undertaken at OECD to develop a guidance document on Integrated Approaches for Testing and Assessment (IATA) for skin sensitization.

(7) Since the assay is amenable for automation, the development of an automated version of the protocol is recommended.

(8) As the assay addresses a key signaling pathway of cyto-protective responses following electrophilic and oxidative stress, the relevance of the test system for assessing other toxicological endpoints should be considered.

7. PROPRIETARY ASPECTS

The 'KeratinoSens' name is a trade mark of the test method developer (Givaudan SA, Switzerland). EURL ECVAM has received confirmation from Givaudan that the Keratinosens™ test method will be made available to third parties subject to specific conditions including a one-time transfer fee.

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Annex 1 ESAC OPINION

Opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) on a Givaudan-coordinated study on the transferability and reliability of the KeratinoSens assay for skin sensitisation testing.

Ispra, 07.11.2012

Summary of the ESAC Opinion

The ESAC was asked to provide an opinion on a Givaudan-led study assessing the transferability and reproducibility (within- and between-laboratories) of the KeratinoSens (primary objective of the study) in view of its possible future use as part of a non-animal testing strategy for skin sensitization. The ESAC was also asked to provide an opinion on the predictive capacity of the test method.
A wealth of information about the test chemicals, and the assessment of with laboratory reproducibility (WLR), transferability, between laboratory reproducibility (BLR) and predictive capacity of the test were presented. Also the applicability domain of the test was addressed in detail. The evaluation by the ESAC WG was complicated by the lack of detail in the body of the report and the excessive reliance on annexes.
On the basis of the submitted and additionally requested information, the ESAC came to the following conclusions:

Test chemicals:
The 114 selected chemicals represented a sufficient number of materials, reasonable structural diversity and a variety of sensitising potency classes. Pre- and pro-haptens were included. Therefore, the selection of chemicals was considered sufficient to gain information on the applicability domain and limitations of the test method.
The small number of non-sensitizers (N=4) in the list of additional chemicals (N=47) considered eligible for assessing the predictive capacity of the test was supplemented with 80 chemicals with negative LLNA data.

WLR (14 chemicals/1 laboratory):
The ESAC considers the level of concordance acceptable and in agreement with target values (85%) for WLR performance.

Transferability (7 chemicals/4 laboratories):
Concordant predictions between the lead laboratory and the 4 naive laboratories were obtained, demonstrating that the test method can be transferred to naive laboratories that are experienced with cell culture techniques.

BLR (21 chemicals/5 laboratories):
The predictions were concordant for the large majority of chemicals, demonstrating an acceptable level of between laboratory reproducibility.

Predictive capacity:
The conclusions regarding the predictivity are sound. A positive point is that reference information from several *in vivo* tests were used for comparison as opposed to a single assay outcome. Since approximately 1 in 5 sensitizers are likely to be missed, the test method should

be considered in the future as part of an integrated testing strategy and not as a standalone assay.

Applicability domain:
In principle, the applicability domain of this method is limited to cysteine reactive chemicals. However, the evidence indicates that the applicability domain is wider, so it would be advisable to assess this further by testing additional chemicals. Specific amine reactivity and metabolic activation are among the key issues that need to be addressed.

1. Mandate of the ESAC

The opinion of ESAC should support ECVAM with respect to the evaluation of the validity status of the test method and possible necessary further work required to characterize the test method's performance (predictive capacity, applicability and limitations of the test method). Moreover, based on the evaluation of the data submitted, the ESAC should provide advice on the potential usefulness of the KeratinoSens test method within a testing strategy for skin sensitization testing.

2. Detailed opinion of the ESAC

Following a request from ECVAM to ESAC for peer review of and scientific advice on an ECVAM-coordinated prevalidation study concerning the KeratinoSens assay, an ESAC Working Group (ESAC WG) was set up by ESAC. The ESAC WG was charged with conducting a detailed scientific peer review the ECVAM study concerning the transferability and reliability of the KeratinoSens assay.
The ESAC WG had been set up by the ESAC during its meeting on March 2011 (ESAC 34). Basis for the scientific review was the ECVAM request to ESAC concerning a scientific review (ESAC request ER2011-04).
The date for the opinion was set to be 4-5 October 2011 (ESAC 35). However, unclarities and inconsistencies in the report required clarification by the test submitter. Two WG requests were sent: 16.12.2011 and 08.02.2012. These extra steps resulted in substantial additional information that had to be reviewed and caused a 1 year delay.
The ESAC WG conducted the peer review from December 2011 to April 2012. Two face-to-face meetings were organized (December 2011, and February 2012), followed by two telephone conferences (February and April 2012) and finalized by written procedure.
The WG was a presented a wealth of information about the test chemicals, and the assessment of WLR, transferability, BLR and predictive capacity of the test. Also the applicability domain of the test was addressed in detail.
The data and the flow of events would have been more transparent if the report had followed the EURL-ECVAM guidance and reporting template more closely. It would have been very helpful if the test submitters had formulated their own conclusions/opinions when referring to any of the numerous attachments that had followed the report. By plane referral to the attachments, the WG had to figure out itself what was meant and how data had to be interpreted.
The WG identified a number of unclarities and inconsistencies which added hurtles to the evaluation of the report, without explanations being provided.
Issues that needed clarification:

- It was not clear why the applied statistical approach was chosen for the evaluation of the test results.
- The test design was not clear.

Inconsistencies:

- Data analysis apparently moved from a test result oriented (e.g. Imax, EC1.5) to a prediction (S/NS) oriented approach.
- Test acceptance criteria changed over time without explanation as to why this was introduced.
- Acceptance criteria were not consistently applied.
- Chemicals that were used for test development and refinement were inappropriately included in the assessment of the BLR and the predicitive capacity.
- The WG addressed these issues by requesting additional information and re-analysis of the data from the test submitter (See Annexes).

The provided information did not provide any clarity about the statistical approach applied in the study. The WG decide not to go into further discussion, and to focus on the outcome of the prediction model (S/NS).
The test design was sufficiently clarified, and the data were re-analysed on the basis of the various identified test acceptance criteria. This allowed the WG to properly assess reproducibility, transferability and predictive capacity.
The WG attempted to recalculate the predictive capacity of the KeratinoSens based upon the chemicals that had not been included in test development and refinement. Since the number of well-characterized non-sensitizers (i.e. chemicals with negative LLNA outcome) among the eligible chemicals was considered too low, the WG requested data on more negative compounds.

On the basis of the submitted and upon request acquired information the WG came to the following conclusion:

Test chemicals:
The 114 selected chemicals were representing a sufficient number of materials, reasonable structural diversity and a variety of sensitising potency classes. Pre- and pro-haptens were included. Therefore, the selection of chemicals was considered sufficient to gain information on the applicability domain and limitation of the test method.
The number of test items was considered sufficient to draw conclusions about the transferability (N=7) and reproducibility (N=21) of the test.
The small number of non-sensitizers (N=4) in the extended list of chemicals (N=47) for assessing the predictive capacity of the test was considered too low. The 67 chemicals used for development, refinement and evaluation of the test were not taken into consideration for assessing the predictive capacity by the WG.

WLR (14 chemicals, 1 laboratory):
Including all available data concordant results were obtained for 12/14 chemicals (85.7%). The WG endorsed the conclusion of the VMG that the test is reproducible with laboratories. WG considered this concordance in agreement with target values (85%) for WLR performance standards as published in international accepted guidelines (e.g. Performance standards of TG439 in vitro skin irritation).
The ESAC WG agreed that the re-analysis that was resubmitted upon request (see section 6.1) was satisfying with regard to answering the question to which extent non-qualified test results might have influenced the WLR analysis**.** The impact on WLR was felt to be negligible as even under the most stringent criteria (set 2 in Annex 4, p60) only 3 individual laboratory predictions had not qualified.

Transferability (7 chemicals, 4 laboratories):
The conclusion on transferability was justified on the basis of concordant predictions (S/NS) between the lead laboratory and the naive laboratories. The WG endorses the conclusion that the test method can be transferred to naive laboratories that are experienced with cell culture techniques.
Concerns were raised about the reliability of luciferase measurements for transferability. Differences in brand of luminometer or substrate were demonstrated by the test submitters not to affect the liability of luminescence measurement. Based on this fact, it seems obvious to the WG that the observed variation in luminescence measurements between laboratories is due to lack of experience, stressing the necessity of operating a number of training experiments in the naïve laboratory before the test method can be used to identify skin sensitizers.
Regarding dose-response curve or EC1.5 (Attachment 8a & 8b), certain variability among the laboratories was observed to cinnamic aldehyde and ethylene glycol dimethacrylate. But, no further explanation was given whether these variabilities originated from the chemicals' own physico-chemical characteristics or luminescence measurement issues.

BLR (21 chemicals, 5 laboratories):
The S/NS prediction gave congruent results for the majority of chemicals (85.7 – 90.5%), taking into consideration the explanations give for the outliers, also between laboratories. (See section 6.1).
The test acceptance criteria provided to the participating laboratories during the ring trial had not been applied consequently when analysing the data. The reason for this inconsistency was that the criteria were found too stringent. In contrast to WLR and transferability assessment, these nonqualified data had an effect on the concordance of predictions (Annex 4, p62 (C. 2)). There were no provisions made for re-testing in case of nonqualified predictions.

Predictive capacity:
The conclusions regarding the predictivity are sound given the overall value of 76.6%, the key here is that weight of evidence data were used for comparison as opposed to a single assay outcome.
The WG was impressed by the wealth of information that was provided by the test submitter on the 114 chemicals assessed in this study. Based upon the 114 chemicals included in the study, the predicitive capacity of the KeratinoSens was 78%. However, the 114 chemicals included the 67 chemicals of the Silver list. Including chemicals that were used for development, refinement and evaluation of a test system might induce a bias in the assessment of the predictive capacity and was therefore considered by the WG as inappropriate.
Considering only the new chemicals (43 sensitizers and 4 non-sensitizers), the calculations showed that the predictive capacity (69%) was considerably lower than the 78% presented by the submitter. It was noted that the number of new qualified non-sensitizers used in this study was considered insufficient (N = 4).
The submitters were requested to submit additional data on chemicals with negative LLNA reference data. Such data were provided for an additional 80 chemicals. Compiling all the data provided by the submitters, the KeratinoSens revealed a sensitivity, specificity and accuracy of 79.3%, 79.8% and 79.5%. Omission of the seven reactive, peptide alkylating chemicals, for which the LLNA data were not trusted despite absence of human data, the remaining chemicals resulted in a sensitivity, specificity and accuracy of 79.3%, 84.5% and 81.7% (Annex 4, p64 (C8)).
The WG observed a poor performance of the test on weak sensitizers. Based on the predictions using the 114 chemicals, 41% of the weak and 86% of the very weak sensitizers were missed (Table 4). Furthermore, the frequency false negative results were found to increase with decreasing potency of the test chemical. This limitation is not clearly indicated in the submission.

Applicability domain:
The applicability domain was well described in the section 1.6 of KeratinoSens report. The authors stated a variety of chemical classes which were expected to be successfully tested in the KeratinoSens assay. These limitations were mainly limited to the issues of solubility or stability in vehicle (e.g. interactions with vehicle, such as hydrolysis).
The WG discussed this issue (See section 2.2) and came to the conclusion that there is indirect evidence that the applicability domain of test may extent to chemicals that not (only) react with the cysteine residues of Keap1. Alternative mechanisms may lead to Nrf2 activation.
Study design allowed testing of some of the limitations of the applicability domain.

Readiness for standardized use:
The WG considered the test method sufficiently mature for classification and labelling of chemicals (relevant to Regulation EC N° 1271/2008).

Negative results have to be considered with care as weak sensitizers (and possibly also moderate sensitizers at the lower end of the scala) will be probably missed (see section 9). Unless this issue gets solved, the KeratinoSens has to be seen as a brick in an integrated testing strategy of weight-of-evidence approach. The consideration of the chemistry /reactivity must be included either by combination with a peptide reactivity test or predictive chemistry assessment. This reactivity assessment should include consideration concerning activating mechanism(s). The KeratinoSens was considered useful for screening purposes, to identify molecular initiators and to gain mechanistic information on the role of e.g. oxidative stress in sensitization.

Identified gaps:
Weak and low-moderate sensitizers, as well as pro-haptens were performing poorly.
When considering cytotoxicity, more emphasis could have been paid to GSH status of the cells and their GSH regenerating capacity. This system may have an impact the inherent chemical reactivity whether directly conjugating to GSH or oxidising it (ref.).
The data do not support the expectation that this test can be used as a stand-alone (preliminary, waiting for PC and reproducibility assessment).
It appears that the correlation between *in vivo* and *in vitro* data needs further improvement as there was a relative high variation among the *in vitro* scores of chemicals belonging to the same potency class (Natsch et al., 2009).

Recommendations:
The test method can be used for S/NS identification of chemicals. Therefore, the test was considered ready for the next steps in the ECVAM process. A Validation study should however include more well-defined non-sensitizing compounds. Furthermore, a consistent use of acceptance criteria nr 3 should be assured.
Since the test revealed issues around weak and low moderate sensitizers, negative results cannot rule out a sensitization potential. This problem should be clearly flagged and/or addressed to be solved.
At SOP level, the test submitters were recommended to modify the 96-well plate design, which currently is prone to bias.
Integration of this assay with other predictive tests as they emerge needs to be based on the better defined applicability domain.
Eventual combination of the KeratinoSens assay with a reactivity based approach needs to include unambiguous identification of reactivity and any specificity associated with it.
Training should be considered.

3. Informative background to the Mandate and Opinion

Skin sensitization is the toxicological endpoint associated with substances that have the intrinsic ability to cause Allergic Contact Dermatitis, ACD in humans. ACD represents the most common manifestation of immunotoxicity in humans, i.e. adverse effects of xenobiotics involving the immune system. The identification of the skin sensitization potential represents an important component of
the safety assessment of any new substance and especially those intended for topical use (e.g. cosmetics). Current regulatory predictive tests for skin sensitization rely on the use of animals, these
include:
a) the traditional guinea pig tests: *Buehler Test* and *Guinea-pig Maximization Test* (OECD TG 406, Ref.1),

b) the *Local Lymph Node Assay* (LLNA, OECD TG 429, Ref.2) and its recently OECD adopted non-radioactive variants (OECD TG 422A, Ref.3 and OECD TG 422B, Ref.4).
Despite the progress that has been made in the development of alternative methods for skin sensitization hazard identification, there are currently no validated methods available. In addition none of the tests under development/evaluation is able to fully characterize the relative potency of sensitizing substances and therefore, none of these assays is considered a stand-alone method, capable of fully replacing current animal procedures.
The current view therefore is to combine different test methods in order to address different key mechanisms of skin sensitization, these includes: skin bioavailability, haptenation (the protein binding of chemicals which triggers immunological responses), epidermal inflammation, dendritic cell activation and migration, T cell proliferation. Before these test methods can be routinely used, their capacity to produce reproducible results needs to be demonstrated as a first step.

There is evidence in the literature showing that the Nrf2-Keap1-ARE regulatory pathway is induced by electrophilic chemicals. Since a considerable proportion of chemicals that lead to skin sensitization have these properties, the Nrf2-Keap1-ARE regulatory pathway is considered one of the most relevant pathways for the identification of potential skin sensitizers (recently reviewed by Natsch A, Ref.5). This knowledge was exploited by Givaudan to develop the KeratinoSens assay which uses an adherent cell line derived from an expanded clone of HaCaT keratinocytes transfected with a selectable plasmid. The plasmid contains the luciferase gene under the transcription control of the SV40 promoter fused with the ARE (antioxidant response element) from the AKR1C2 gene.

Using well-established light-producing luciferase substrates, the activity of ARE-binding transcription factors in the cells in response to exposure with soluble chemicals can be easily measured. Luciferase induction is the read-out of the KeratinoSens test method and the concentration at which the induction is 50% above the background level (EC1.5) is established in parallel to the IC50 value to classify chemicals as having skin sensitization potential.

The test method submitter proposes this method to be used in future as part of an integrated approach for the full replacement of the animal tests or as a stand-alone method for skin sensitisation hazard identification. In relation to the ability of the test method to differentiate between sensitizing and non-sensitizing chemicals, the test method submitter reported an accuracy of 85.1% (sensitivity 86.4%, specificity 82.6%) with respect to *in vivo* data for a set of 67 chemicals tested in-house.

The KeratinoSens test method has been evaluated in a ring study involving 5 laboratories including Givaudan who acted as the study coordinator. The transfer of the protocol was evaluated with a set of 7 chemicals. 21 additional chemicals (15 sensitizers and 6 non sensitizers) have been tested coded to generate information on the test method reliability and predictive capacity. The laboratories consistently classified 18 of the 21 coded chemicals. The accuracy of the in vitro classification with respect to the *in vivo* classification is reported to vary between 85.4% and 96.7% for the different laboratories. Following the formal submission of the KeratinoSens assay to ECVAM, Givaudan was asked to generate additional information on the within-laboratory reproducibility. In order to achieve this, ECVAM supplied Givaudan with 8 coded chemicals which have not been tested before with the KeratinoSens test method. Data for these additional chemicals were generated at the Givaudan laboratories and were submitted to ECVAM middle of March 2011.

With respect to the modular approach of validation (Hartung et al., 2004, Ref.6) the study provides information on module 1) test definition, module 2) within laboratory reproducibility, module 3) transferability and module 4) between laboratory reproducibility. Information for module 5), predictive capacity, is only partially fulfilled.

4. References

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5. Natsch A. The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers-functional relevance and a hypothesis on innate reactions to skin sensitizers. Toxicological Sciences 2010 113(2):284-92.
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ANNEX 2 EURL ECVAM request for ESAC advice

EURL ECVAM request to ESAC for scientific advice on the Givaudan-coordinated study on the transferability and reliability of the KeratinoSens assay for skin sensitisation testing

Title page information	
Abbreviated title of ESAC request	ESAC peer review of and ESAC opinion on the Givaudan-led study on the KeratinoSens test method.
ESAC REQUEST Nr.	2011-04
Template used for preparing request	EP 2.01
Date of finalising request	2011-03-07
Date of submitting request to ESAC	2011-03-09
Request discussed through	ESAC 34. 22-23 March 2011 and ESAC 35. 4-5 October 2011 (mandate adopted: objective/questions of review and ESAC WG)
Opinion expected at (date)	ESAC 36. 20-21 March 2012
File name of this request	**ER2011-04_KeratinoSens_ESACadopted.doc**

1. TYPE OF REQUEST

Request Type		**Identify request ("YES")**
R1 ESAC Peer Review **of a Prevalidation Study or Validation Study**		**YES**
If R1)applies please specify further:		
►Prevalidation Study	**YES** The KeratinoSens assay for skin sensitisation testing has been evaluated in a ring study involving five laboratories and led by Givaudan, a producer of fragrances and flavours. The study has been designed to generate information on the test method's transferability and reproducibility to allow recommendations to be made on these two aspects in view of the future use of this test method in an integrated approach for the full replacement of the currently used regulatory animal tests. In addition the data generated in this study will inform possible future evaluations of the test method's predictive capacity.	
►Prospective Validation Study	**No**	
►Retrospective Validation Study	**No**	
►Validation Study based on Performance Standards	**No**	
R2 Scientific Advice on a test method submitted to ECVAM for validation **(e.g. the test method's biological relevance etc.)**		**No**
R3 Other Scientific Advice **(e.g. on test methods, their use; on technical issues such as cell culturing, stem cells etc.)**		**No**

2. TITLE OF STUDY OR PROJECT FOR WHICH SCIENTIFIC ADVICE OF THE ESAC IS REQUESTED

Givaudan study on the KeratinoSens assay for skin sensitisation testing.

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

1) Background to skin sensitization and current predictive tests

Skin sensitisation is the toxicological endpoint associated with substances that have the intrinsic ability to cause Allergic Contact Dermatitis, ACD in humans. ACD represents the most common manifestation of immunotoxicity in humans, i.e. adverse effects of xenobiotics involving the immune system. The identification of the skin sensitization potential represents an important component of the safety assessment of any new substance and especially those intended for topical use (e.g. cosmetics). Current regulatory predictive tests for skin sensitization rely on the use of animals, these include:

a) the traditional guinea pig tests: *Buehler Test* and *Guinea-pig Maximisation Test* (OECD TG 406, Ref.1),

b) the *Local Lymph Node Assay* (LLNA, OECD TG 429, Ref.2) and its recently OECD adopted non-radioactive variants (OECD TG 422A, Ref.3 and OECD TG 422B, Ref.4).

Despite the progress that has been made in the development of alternative methods for skin sensitisation hazard identification, there are currently no validated methods available. In addition none of the tests under development/evaluation is able to fully characterise the relative potency of sensitising substances and therefore, none of these assays is considered a stand-alone method, capable of fully replacing current animal procedures.

The current view therefore is to combine different test methods in order to address different key mechanisms of skin sensitisation, these includes: skin bioavailability, haptenation (the protein binding of chemicals which triggers immunological responses), epidermal inflammation, dendritic cell activation and migration, T cell proliferation. Before these test methods can be routinely used, their capacity to produce reproducible results needs to be demonstrated as a first step.

2) Background to the KeratinoSens

There is evidence in the literature showing that the Nrf2-Keap1-ARE regulatory pathway is induced by electrophilic chemicals. Since a considerable proportion of chemicals that lead to skin sensitisation have these properties, the Nrf2-Keap1-ARE regulatory pathway is considered one of the most relevant pathways for the identification of potential skin sensitisers (recently reviewed by Natsch A, Ref.5). This knowledge was exploited by Givaudan to develop the KeratinoSens assay which uses an adherent cell line derived from an expanded clone of HaCaT keratinocytes transfected with a selectable plasmid. The plasmid contains the luciferase gene under the transcription control of the SV40 promoter fused with the ARE (antioxidant response element) from the AKR1C2 gene. Using well established light-producing luciferase substrates, the activity of ARE-binding transcription factors in the cells in response to exposure with soluble chemicals can be easily measured.

Luciferase induction is the read-out of the KeratinoSens test method and the concentration at which the induction is 50% above the background level (EC1.5) is established in parallel to the IC50 value to classify chemicals as having skin sensitisation potential.

The test method submitter proposes this method to be used in future as part of an integrated approach for the full replacement of the animal tests or as a stand alone method for skin sensitisation hazard identification.

In relation to the ability of the test method to differentiate between sensitising and non-sensitising chemicals, the test method submitter reported an accuracy of 85.1% (sensitivity 86.4%, specificity 82.6%) with respect to in vivo data for a set of 67 chemicals tested in house.

3) Background to the KeratinoSens ring study

The KeratinoSens test method has been evaluated in a ring study involving 5 laboratories including Givaudan who acted as the study coordinator. The transfer of the protocol was evaluated with a set of 7 chemicals. 21 additional chemicals (15 sensitisers and 6 non sensitisers) have been tested coded to generate information on the test method reliability and predictive capacity. The laboratories consistently classified 18 of the 21 coded chemicals. The accuracy of the in vitro classification with respect to the in vivo classification is reported to vary between 85.4% and 96.7% for the different laboratories.

Following the formal submission of the KeratinoSens assay to ECVAM, Givaudan was asked to generate additional information on the within-laboratory reproducibility. In order to achieve this, ECVAM supplied Givaudan with 8 coded chemicals which have not been tested before with the KeratinoSens test method. Data for these additional chemicals are being generated at the Givaudan laboratories and are expected to be submitted to ECVAM middle of March 2011.

With respect to the modular approach of validation (Hartung et al., 2004, Ref.6) the study provides information on module 1) test definition, module 2) within laboratory reproducibility, module 3) transferability and module 4) between laboratory reproducibility. Information for module 5), predictive capacity, is only partially fulfilled.

References

OECD, Organisation for Economic Cooperation and Development (1992) Skin Sensitisation Guidelines for Testing of Chemicals No. 406, Paris.

OECD, Organisation for Economic Cooperation and Development (2002) The Local Lymph Node Assay. Guidelines for Testing of Chemicals No. 429, Paris.

OECD, Organisation for Economic Cooperation and Development (2010a) Skin Sensitization: Local Lymph Node Assay: DA, Guidelines for Testing of Chemicals No. 442A, Paris.

OECD, Organisation for Economic Cooperation and Development (2010b) Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA, Guidelines for Testing of Chemicals No. 442B, Paris.

Natsch A. The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers--functional relevance and a hypothesis on innate reactions to skin sensitizers. Toxicological Sciences 2010 113(2):284-92.

Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi A.J., Prieto, P., Sabbioni, E., Scott, L., Worth, A. and Zuang. V. (2004) A Modular Approach to the ECVAM Principles on Test Validity. ATLA 32, 467-72.

4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does ECVAM require advice on the current issue?*	The opinion of ESAC on the present Prevalidation study of the KeratinoSens test method should support ECVAM with respect to the evaluation of the validity status of the test method at present and with regard to possible necessary further work required to fully characterise the test method's performance (reproducibility, predictive capacity, applicability, limitations of the test method). Moreover, based on the evaluation of the data submitted, the ESAC should provide advice on the potential usefulness of the KeratinoSens test method within a testing strategy for skin sensitisation testing and the proper scientific use of the test method within such a testing strategy (e.g. with respect to its specific applicability and limitations). It is explicitly noted that the ESAC is not requested to suggest the precise placing of the submitted method in a hypothetical ITS, but rather to provide advice on the characteristics of the method relevant for its subsequent integration into an ITS at a later point in time (i.e. when other buildings blocks of such an ITS are known).

4.2 QUESTION(S) TO BE ADDRESSED

Questions *What are the questions and issues that should be addressed in view of achieving the objective of the advice?*	1) **DESIGN & CONDUCT OF STUDY:** The ESAC is requested to review whether the submitted prevalidation study was conducted appropriately in view of the objective of the study (see attachment 17e of the submission). The study objective was to assess (1) the reproducibility of the KeratinoSens method in one (the lead) laboratory (n=14 substances plus further 28 substances which were however not tested in a sufficient number of runs), (2) its transferability to other laboratories (n=7), (3) its reproducibility in other laboratories (BLR) when test items were tested repeatedly, but in deviation from the complete procedure as conducted by the lead laboratory in the intra-laboratory study (n=21). (4) Furthermore, the study aimed at assessing, in a preliminary manner, the predictive capacity of the test method based on the testing of published reference chemicals in the lead laboratory (n=114; this included 67 chemicals used for test development/optimisation and development of the prediction model) and during the ring trial to assess transferability (n=7) and BLR (n=21). When reviewing the design and conduct of the study, the following issues should be addressed in particular: • Clarity of the test definition (module 1) • Clarity of the definition of the study objective (see attachment 17e of the Prevalidation study report). • Appropriateness of the study design in view of study objective, *inter alia:* - Is the **number of chemicals** sufficient for the purposes of the study? - Are the **reference data** used for assessing in particular the predictive capacity appropriate and of good quality? - Was the identification of **chemicals** conducted in an appropriate manner (i.e. presence or absence of selection criteria, justification etc.)? - Is the **adverse effect range of the selected chemicals** appropriate for the purpose of the study - In case of **gaps** (chemical class etc.) – are these justified? - Is the **number of laboratories** sufficient? Appropriateness of the **study execution** (e.g. were there pre-defined acceptance criteria, were these respected? How were exceptions / deviations handled? Were provisions specified for retesting? Were the number of repetitions sufficient? etc.) Appropriateness of the **statistical analysis** used for analysing WLR, transferabilitiy, BLR and (preliminary) predictive capacity. 2) **CONCLUSIONS OF STUDY:** The ESAC is requested to assess whether the conclusions, as presented in the Test Submission Template (TST), Annex 17e, are substantiated by the information generated during prevalidation and are plausible with respect to existing information and current views (e.g. literature).

	In particular: • Do the data on the basis of these chemicals provide sufficient information on **applicability and possible limitations** of the test method, in particular in view of its potential use within an ITS for sensitisation? • Are the conclusions on **reproducibility** (WLR and BLR) as well as transferability justified and plausible? • Are the conclusions on **predictive capacity** justified and plausible with respect to existing information • Are there **possible gaps between study design and study conclusions** which remain to be addressed in view of the suggested conclusions / use (see also point 3)? 3) **SUGGESTED USE OF THE TEST METHOD:** The ESAC is requested (a) to evaluate, on the basis of the data submitted in the Prevalidation study, the possible use of the validated method (also within a strategy) to identify skin sensitisers, (b) to make additional recommendations (as required) on the proper scientific use of the test method within such a strategy taking specific aspects of this method into account (e.g. applicability, limitations etc.) and (c) to identify possible further information required (i.e. are there gaps) to be able to conclude on the plausibility of the suggested use (including within an ITS).

4.3 TIMELINES

Timelines concerning this request *When does ECVAM require the advice?*	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	ESAC 36, 20-21 March 2012
	Request to be presented to ESAC by written procedure (e.g. due to urgency) prior to the next ESAC	NO
	Request to be presented to ESAC at ESAC plenary meeting	ESAC 34, 22-23 March 2011 and ESAC 35, 4-5 October 2011 (mandate)

5. ECVAM PROPOSALS ON HOW TO ADDRESS THE REQUEST WITHIN ESAC

5.1 ECVAM PROPOSAL REGARDING REQUEST-RELATED STRUCTURES REQUIRED

Specific structures required within ESAC to address the request *Does the advice require an ESAC working group, an ESAC rapporteur etc.?*	**Structure(s) required**	**Required according to ECVAM? (YES/NO)**
	S1 ESAC Rapporteur	NO
	S2 ESAC Working Group	**YES**
	S3 Invited Experts	NO
	Ad S3: If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	
	If other than above (S1-S3):	NO

5.2 DELIVERABLES AS PROPOSED BY ECVAM

Deliverables *What deliverables (other than the ESAC opinion) are required for addressing the request?*	**Title of deliverable other than ESAC opinion**	**Required? (YES/NO)**
	D1 ESAC Rapporteur Report and draft opinion	NO
	D2 ESAC Peer Review Report and draft opinion	**YES**
	If other than above (D1-D2):	

6. LIST OF DOCUMENTS TO BE MADE AVAILABLE TO THE ESAC

Count	Description of document	Available (YES/NO)	File name
1	Validation study report (external validation) of Givaudan-coordinated KeratinoSens study based on the ECVAM Test Submission Template following	YES	ER2011-03_Ring_study_KeratinoSens.pdf

	ECVAM's Modular approach.		
2	Review paper on biological relevance of Nrf2-Keap1-ARE toxicity pathway for sensitisation testing	YES	ER2011-03_Toxicol. Sci.-2010-Natsch.pdf

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 34th meeting on March 22-23 the ESAC plenary unanimously decided to establish an ESAC Working Group Sensitisation charged with the detailed scientific review of four test methods for skin sensitisation.

7.2 TITLE OF THE ESAC WORKING GROUP

Full title:
ESAC Working Group on Skin Sensitisation Test Methods
Abbreviated title:
ESAC WG Sensitisation

7.3 MANDATE OF THE ESAC WG

The EWG is requested to conduct a scientific review of the relevant studies concerning four skin sensitisation test methods (DPRA, MUSST, h-CLAT, KeratinoSens). The review needs to address the questions put forward to ESAC by ECVAM.

The review should focus on the appropriateness of design and conduct of the study in view of the study objective and should provide an appraisal to which extent the conclusions of the Validation Management Team (VMT) / test method submitter are substantiated by the information generated during the study and how the information generated relates to the scientific background available.

7.4 DELIVERABLE OF THE ESAC WG

The ESAC WG is requested to deliver to the chair of the ESAC and the ESAC Secretariat a detailed **ESAC Working Group Report** outlining its analyses and conclusions**.** A reporting template has been appended (Appendix 1) intended to facilitate the drafting of the report.
The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the report should clearly outline the differences in the appraisals and provide appropriate scientific justifications.

7.5 PROPOSED TIMELINES OF THE ESAC WG

The ESAC Coordinator has proposed timelines* which should be agreed upon during the first Teleconference (Item 1 in the table):

Item	Proposed date/time	Action	Deliverable
1	**7. September 2011 (Wednesday)** 13:30 CET	**Kick-off teleconference** Discussion of a) the submission b) the mandate put forward by the Secretariat c) the working procedure (ESAC WG template)	Feedback on the mandate.
2	**14. October 2011 (Friday)**	**Deadline** for submitting first comments within ESAC WG template	Draft observations of each ESAC WG member in the ESAC WG

			template (to be compiled by ECVAM)
3	**24-26 October 2011 (Monday to Wednesday)**	ESAC WG meeting in Ispra. Discussion of contentious items. Drafting of the report.	Draft report
4	Further teleconferences and work progress to be agreed during meeting (Nr. 3).	Progressing of draft report	Draft report
5	**15. February 2012 (Wednesday)**	Final report to be delivered to ESAC Coordinator/Secretariat.	Final report

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WG

The ESAC WG is requested to address the **three questions posed to the ESAC** which have been broken down further in more **specific questions** by Secretariat (see section 4.2) and were discussed with the ESAC WG and approved by the ESAC.

When preparing the final ESAC WG report to address these questions, the ESAC WG is requested to use a pre-defined reporting template. This template (see appendix 1) follows ECVAM's modular approach and addresses to which extent the standard information requirements have been addressed by the study. In addition, the template allows for addressing the specific questions outlined in section 4.2. The Secretariat will provide guidance if necessary.

APPENDIX 1 REPORTING STRUCTURE FOR THE *ESAC WG REPORT*

The following suggested structure follows the ECVAM information requirements ("modules") for scientific review following validation and allows at the same time for the description of the analysis and conclusions concerning more specific questions. A template has been created on the basis of the structure below and this template will be made available to the ESAC.

The template can be used for various types of validation studies (e.g. prospective full studies, retrospective studies, performance-based studies and prevalidation studies). Depending on the study type and the objective of the study, not all sections may be applicable. However, for reasons of consistency and to clearly identify which information requirements have not been sufficiently addressed by a specific study, this template is uniformly used for the evaluation of validation studies.

1. Data collection

1.1 Information / data sources used

1.2 Search strategy

1.3 Selection criteria applied to the available information

2. Study objective and design

2.1 Clarity of the definition of the study objective

2.2 Analysis of the scientific rationale provided

2.3 Analysis of the regulatory rationale provided

2.4 Appropriateness of the study design

2.5 Appropriateness of the statistical evaluation

3. Test definition (Module 1)

3.1 Quality and completeness of the overall test definition

3.2 Quality of the background provided concerning the purpose of the test method

3.3 Quality of the documentation and completeness of (a) standardised protocols (SOPs) and (b) prediction models

4. Data quality

4.1 Overall quality of the evaluated data

4.2 Sufficiency of the evaluated data in view of the study objective

4.3 Quality of the reference data for evaluating reliability and relevance[2]

5. Test materials

5.1 Sufficiency of the number of evaluated test items in view of the study objective

5.2 Representativeness of the test items with respect to applicability

6. Within-laboratory reproducibility (Module 2)

6.1 Assessment of repeatability and reproducibility in the same laboratory

6.2 Conclusion on within-laboratory reproducibility as assessed by the study

7. Transferability (Module 3)

7.1 Quality of design and analysis of the transfer phase

7.2 Conclusion on transferability to a second laboratory/other laboratories as assessed by the study

8. Between-laboratory reproducibility (Module 4)

8.1 Assessment of reproducibility in different laboratories

8.2 Conclusion on reproducibility as assessed by the study

9. Predictive capacity (Module 5)

9.1 Adequacy of the assessment of the predictive capacity in view of the purpose

9.2 Overall relevance (biological relevance and accuracy) of the test method in view of the purpose

[2] OECD guidance document Nr. 34 on validation defines relevance as follows: "Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It sis the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy (concordance) of a test method."

10. Applicability domain (Module 6)
10.1 Appropriateness of study design to conclude on applicability domain, limitations and exclusions
10.2 Quality of the description of applicability domain, limitations, exclusions
11. Performance standards (Module 7)
11.1 Adequacy of the proposed Essential Test Method Components
11.2 Adequacy of the Reference Chemicals
11.3. Adequacy of the defined Accuracy Values
12. Readiness for standardised use
12.1 Assessment of the readiness for regulatory purposes
12.2. Assessment of the readiness for other uses (in house screening etc.)
12.3 Critical aspects impacting on standardized use
12.4 Gap analysis
13. Other considerations
14. Conclusions on the study
14.1 Summary of the results and conclusions of the study
14.2 Extent to which conclusions are justified by the study results alone
14.3 Extent to which conclusions are plausible in the context of existing information
15. Recommendations
15.1 General recommendations concerning the study
15.2 Recommendations concerning the test method (test system, protocol, prediction model)
16. References
17. Annexes

END OF EURL ECVAM RECOMMENDATION

European Commission
EUR 26427 – Joint Research Centre – Institute for Health and Consumer Protection

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Abstract

Identification of the skin sensitisation hazard of chemicals has traditionally relied on the use of animals. Progress in the development of alternative methods has been prompted by the increasing knowledge of the key biological mechanisms underlying this human health effect, as documented by the OECD's recent report summarising the key biological events leading to skin sensitisation ("Adverse Outcome Pathway" (AOP) for skin sensitisation). Within this AOP the activation of cellular signalling pathways, such as the Keap1-Nrf2- antioxidant/electrophile response element (ARE)-dependent pathway, known to play a relevant role in keratinocytes' responses to skin sensitisers, is postulated to be a key event. Therefore, test methods able to provide information on the ability of a chemical to activate this or other relevant pathways in keratinocytes, may contribute to skin sensitisation hazard and safety assessment. The KeratinoSensTM test method measures ARE-Nrf2 activation through a luciferase reporter gene. The test method has undergone a validation study addressing mainly the test method's transferability and within- and between-laboratory reproducibility. Following independent scientific peer review by EURL ECVAM's Scientific Advisory Committee (ESAC) and having considered the input from regulators, stakeholders, international partners and the general public, EURL ECVAM concluded that the KeratinoSensTM may prove a useful component of integrated approaches such as Weight of Evidence (WoE) or Integrated Testing Strategies (ITS) for skin sensitisation hazard assessment. In addition to this, the KeratinoSensTM may also be able to contribute to the assessment of sensitising potency, e.g. by supporting sub-categorisation of sensitisers according to UN GHS. However it is recognised that further efforts are required to explore how KeratinoSensTM data may contribute to potency assessment.

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