European Commission

JRC SCIENCE AND POLICY REPORT

EURL ECVAM Recommendation on the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing

2015

Report EUR 27022 EN

Joint Research Centre

European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information
EURL ECVAM
Address: Joint Research Centre, via Enrico Fermi 2749, 21027 Ispra (Va), Italy
E-mail: JRC-ECVAM-CONTACT@ec.europa.eu

https://ec.europa.eu/jrc/en/institutes/ihcp
https://ec.europa.eu/jrc

JRC93307

EUR 27022 EN

ISBN 978-92-79-44706-8 (PDF)

ISSN 1831-9424 (online)

doi:10.2788/29986

Luxembourg: Publications Office of the European Union, 2015

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 summarised in the OECD report on: "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins". Within this AOP the activation of dendritic cells (DC), typically assessed by expression of cell surface markers, chemokines and cytokines, is considered to be a key event. Therefore, test methods able to provide information on the ability of a chemical to up-regulate markers of DC activation may contribute to skin sensitisation hazard assessment. The human Cell Line Activation Test (h-CLAT) measures the upregulation of the CD86 and CD54 markers of DC activation in THP-1 cells, a human monocytic leukemia cell line. The test method has undergone a validation study addressing the test method's transferability and within- and between-laboratory reproducibility. Following independent peer-review by the EURL ECVAM's Scientific Advisory Committee (ESAC) and having considered input from regulators, stakeholders, international partners and the general public, EURL ECVAM concluded that the h-CLAT test method should prove valuable within Integrated Approaches to Testing and Assessment (IATA) for hazard assessment. The h-CLAT may also be able to contribute to the assessment of sensitising potency, however it is recognised that further efforts are required to explore how h-CLAT data may contribute to potency assessment.

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM RECOMMENDATION

on the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing

February 2015

Table of Contents

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 advisory 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.

Enquiries related to this EURL ECVAM Recommendation should be sent to:

EU Reference Laboratory for Alternatives to Animal Testing
European Commission DG Joint Research Centre,
Institute for Health and Consumer Protection,
Via E. Fermi 2749,
I-21027 Ispra (VA), Italy.

Executive Summary

The human Cell Line Activation Test (h-CLAT) for skin sensitisation testing was developed by Kao Corporation and Shiseido (Japan). With a view to facilitating its use as a component of integrated approaches to assessing the skin sensitisation potential of chemicals, EURL ECVAM coordinated a validation study to assess the reliability of the h-CLAT method and to gain some preliminary insight into its predictive capacity. On completion of the study, EURL ECVAM requested ESAC to conduct a scientific peer review of the validation study report and the resulting ESAC opinion was delivered in May 2014. Following consideration of the ESAC opinion EURL ECVAM makes the following observations and recommendations:

1) The h-CLAT addresses one of the key events of the skin sensitisation Adverse Outcome Pathway (AOP) by measuring markers of dendritic cell (DC) activation in THP-1 cells, a human monocytic leukemia cell line. Therefore information generated by the h-CLAT is considered relevant for the assessment of the skin sensitisation potential of chemicals.

2) The validation study demonstrated that the h-CLAT test method is transferable to laboratories experienced in cell culture techniques and flow cytometry analysis. The within-laboratory and between-laboratory reproducibly, as characterised on the basis of concordant classifications of the chemicals employed (n=15 and n=24 respectively), were both in the order of 80%.

3) The accuracy of h-CLAT to discriminate sensitisers from non-sensitisers was calculated to be 76% (sensitivity 81% and specificity 66%) with the chemicals tested (n=24). However this result is only an approximation since the validation study was clearly not designed to fully assess the predictive capacity of the h-CLAT as a stand-alone method. Published information actually reports a higher accuracy (80%) for a larger set of chemicals (n=143; Takenouchi et al., 2013).

4) Based on the outcome of the validation study and reports from the scientific literature, data generated with the h-CLAT method should prove valuable as part of Integrated Approaches to Testing and Assessment (IATA) together with complementary information (e.g. *in chemico* or other *in vitro* data, QSAR or read-across predictions).

5) Besides providing information that contributes to the assessment of the skin sensitisation potential of chemicals, the h-CLAT assay also generates concentration-response information that may contribute to the assessment of potency. Nevertheless, additional work is still required to determine to which extent h-CLAT results can contribute to potency prediction.

6) The h-CLAT method should be further assessed with respect to its response to chemicals that need to be activated (e.g. through biotransformation or auto-oxidation) before eliciting their sensitisation effect, and to its applicability to chemical mixtures and polymers.

7) EURL ECVAM fully supports the development of an OECD Test Guideline for the h-CLAT.

8) Respecting the provisions of Directive 2010/63/EU (EU, 2010) on the protection of animals used for scientific purposes, h-CLAT data should be considered before embarking on animal experiments for assessing skin sensitisation potential. As described in Annex XI of the REACH Regulation (EC, 2006), h-CLAT data may be used to adapt the standard information requirement in the context of Weight-of-Evidence (WoE) judgments (point 1.2) or by the use of *in vitro* methods (point 1.4).

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 Biocidal Products Regulation (EU, 2012) and the Cosmetics Regulation (EC, 2009b). Determining skin sensitisation hazard according to the Globally Harmonised System to Classification and Labelling (GHS) is actually sufficient to satisfy the majority of regulatory needs (EURL ECVAM, 2013a). 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 classification of mixtures, appropriate risk management measures (e.g. setting of appropriate exposure levels) and eventually a full risk assessment.

2) Traditionally, skin sensitisation hazard assessment has involved the use of laboratory animals. In the framework 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). Following the ESAC peer review of the validation studies and the publication of the EURL ECVAM Recommendations on the *in chemico* Direct Peptide Reactivity Assay (DPRA) (EURL ECVAM, 2013b) and the *in vitro* KeratinoSens™ test method (EURL ECVAM, 2014a), OECD Test Guidelines on these two assays have been developed and are in the acceptance process.

3) The key biological events underpinning the skin sensitisation process are well established and have been summarised in the OECD report on "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins" (OECD, 2012a, 2012b). These key events include 1) the covalent binding of the chemical to skin proteins (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. Following the ESAC peer review of the validation studies and the publication of the EURL ECVAM Recommendations on the *in chemico* Direct Peptide Reactivity Assay (DPRA) (EURL ECVAM 2013b), and the *in vitro* KeratinoSens™ test method (EURL ECVAM 2014a), OECD Test Guidelines on these two non-animal methods have been recently adopted (TG 442C, OECD 2015a and TG 442D, OECD 2015b).

4) There is general agreement that it is unlikely that alternative (non-animal) methods designed to address a single key event of the skin sensitisation pathway will be able to provide sufficient information to fully replace the use of animals for this endpoint (Adler et al., 2011). Instead, what is likely needed is some combination of information from complementary alternative methods (Jowsey et al., 2006; Adler et al., 2011). 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 2008, EURL ECVAM received a joint submission by Kao Corporation and Shiseido (Japan) on the h-CLAT test method that described the extensive work performed to develop and optimise the method. This included the results of multiple laboratories studies (Sakaguchi et al., 2006, 2010; Ashikaga et al., 2006, 2008; Kosaka et al., 2008; Sono et al., 2008; Mizuno et al., 2008) and h-CLAT data for 100 chemicals. As a consequence, in the period between November 2009 and November 2012, EURL ECVAM coordinated a validation study on the h-CLAT (EURL ECVAM 2013c). The study was designed to generate information according to the modular approach to validation (Hartung et al., 2004) with the primary objective of fully assessing the reliability of the h-CLAT (i.e. its transferability and within and between laboratory reproducibility). Only as a secondary study objective, the experimental data generated were used to perform a preliminary evaluation of the ability of the h-CLAT to discriminate between skin sensitising and non-sensitising chemicals, as defined by the United Nations (UN) Globally Harmonised System (GHS) to classification and labelling (UN GHS, 2013). Assessment of the preliminary predictive capacity of the h-CLAT was performed as a step towards determining the potential contribution of the method within integrated approaches to skin sensitisation hazard assessment. In addition, where possible, the experimental data were used to derive preliminary considerations on the ability of the test method to sub-categorise sensitising chemicals, e.g. into sub-categories 1A and 1B as defined by the UN GHS.

6) Following completion of the study and finalisation of the Validation Study Report (EURL ECVAM, 2013c), EURL ECVAM requested the ECVAM Scientific Advisory Committee (ESAC) to provide an ESAC Opinion on the study. The ESAC Working Group (WG) "Skin Sensitisation" prepared a detailed WG report (EURL ECVAM, 2013d) which formed the basis of the ESAC Opinion (EURL ECVAM, 2014b; see Annex 1), endorsed by members at an ESAC meeting in March 2014 and formally delivered to EURL ECVAM in May 2014.

2. Test Method definition

7) The important role played by Dendritic Cells (DC) in the initiation of adaptive immune responses is well established, including the cutaneous immune response to chemical allergens. DC can recognise and internalise antigens such as haptenated proteins, transport them via the lymphatic system to the regional lymph nodes and present them via major histocompatibility complex (MHC) molecules to naïve T lymphocytes to induce differentiation and proliferation of specific memory T-cells. Recognition and processing of the antigen by DC requires the local production of various danger signals including inflammatory mediators resulting from the activation of the innate immunity of the skin (Ainscough et al., 2013; Kaplan et al., 2012; Vocanson et al., 2009). Once activated, DC migrate from the skin to the lymph nodes and undergo a process of maturation characterised by phenotypic and functional changes resulting in the loss of their capability to process antigens and in the acquisition of the functionality of antigen presentation. The maturation process involves the decrease of phagocytic activity, increased expression of MHC molecules on the cell surface, changes in cytokines and chemokines secretion and up-regulation of several co-stimulatory (e.g. CD80, CD86, and CD40) and intercellular adhesion molecules (e.g. CD11a, and ICAM-1/CD54) (Quah and O'Neill, 2005; Vocanson et al., 2009; Kimber et al., 2011).

   Some of these biomarkers have been considered in the development of cell-based assays for assessing the skin sensitisation potential of chemicals (Aiba et al., 1997; Casati et al., 2005; dos Santos et al., 2009; Vandebriel et a.l., 2010). It is recognised that the mechanisms that lead *in vitro* to the augmentation of these membrane markers by sensitising chemicals

may only partially reflect the complexity of the mechanisms inducing DC maturation in integral biological models such as rodents and humans (Kimber et al., 2011; 2013). However, assays based on human myeloid cell lines and measuring markers known to be over-expressed during DC maturation *in vivo*, are considered to provide mechanistic information that can contribute to the *in vitro* assessment of the skin sensitisation potential of chemicals (OECD 2012a; 2012b; Adler et al., 2011; Vanderbriel et al., 2010; van Helden et al., 2008) .

8) In the h-CLAT method the modulation of the CD86 and CD54 membrane markers in THP-1 cells (Tsuchiya et al., 1980), a human monocytic leukemia cell line used as a surrogate model for DC (van Helden et al., 2008), is measured by flow cytometry following 24 hours of exposure to eight serial concentrations of test chemical selected on the basis of a pre-determined CV75 (i.e. the concentration of test chemical that allows 75% of cell survival). The h-CLAT test method is designed to discriminate between sensitising and non-sensitising chemicals whereby chemicals are classified as sensitisers if the relative fluorescence intensity (RFI) of either CD86 and/or CD54 exceeds a defined threshold (i.e. RFI CD86≥150 and RFI CD54≥200; Sakaguchi et al., 2009) compared to the vehicle control wells at any tested concentration, in at least two out of three independent measurements (i.e. repetitions). Cell viability is measured concurrently by Propidium Iodide (PI) staining and RFI values are considered for the prediction only if cell viability is above 50%.

9) Since the THP-1 cells are exposed to 8 serial concentrations of test chemicals, for positive chemicals it is generally possible to calculate from the concentration-response curve an Estimated Concentration (EC)[1] value for the CD86 and the CD54 representing the concentration of test chemical needed to induce an RFI equal to the respective threshold values, i.e. CD86 EC150 and CD54 EC200. Proposals have been made on how to use these values for potency prediction (Nukada et al., 2011, 2013), nevertheless additional work is still required to determine how h-CLAT data may inform potency assessment.

10) As a result of the validation study a revised and more detailed Standard Operating Procedure (SOP) was defined (EURL ECVAM, 2013c) which EURL ECVAM will disseminate, together with a comprehensive description of the h-CLAT method through its database on alternative methods (DB-ALM, see http://ecvam-dbalm.jrc.ec.europa.eu; protocol No. 158). The SOP contains all the necessary technical details (including electronic data reporting templates) needed by an end-user laboratory to implement it in a reliable and self-sufficient manner. In addition, EURL ECVAM intends to make available an online video tutorial with practical demonstration of how to perform the most critical steps of the h-CLAT SOP.

3. Overall performance of the h-CLAT test method

Reference data

11) A key criterion employed for selecting the validation test chemicals was availability of high quality *in vivo* data from the murine LLNA and GPMT or Buehler test, with concordant classification from these assays. In addition, chemicals with available human data and/or which are known to produce misleading responses in the animal tests (e.g. Nickel chloride and Xylene which produce false negative and false positive responses in the LLNA test,

[1] Estimated Concentrations are not to be confused with Effect Concentrations which are also usually abbreviated "EC".

respectively) were considered in the selection. The set of chemicals used in the study comprised one third of non-sensitisers and two thirds of sensitisers, with a balanced representation of potency classes (weak, moderate, strong and extreme) for the sensitisers. Also included in the reference set were: chemicals from the LLNA performance standards (OECD 2010a), two well characterised pre-haptens (i.e. chemicals requiring abiotic activation to exert their sensitisation potential), 4-Phenylendiamine and R(+)-Limonene, a well-known pro-hapten (i.e. a chemical requiring metabolic activation to act as sensitiser), Dihydroeugenol, to challenge the potential of THP-1 cells to metabolically activate inert substances, and two metal salts, Berillium sulphate and Nickel chloride. Additional details on chemical selection can be found in the Validation Study Report (EURL ECVAM, 2013c).

12) When interpreting the data from alternative non-animal methods such as the h-CLAT that have been largely developed and validated using animal reference data such as LLNA or GPMT, it should be kept in mind that the animal tests are not fully reflective of the human situation. Notably, an evaluation of the LLNA in comparison to human data has shown an accuracy of about 72% (Anderson et al., 2011) indicating an appreciable risk of both false negative and false positive predictions for humans. 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

13) EURL ECVAM concludes that the h-CLAT test method is transferable to laboratories sufficiently experienced in cell culture techniques and flow cytometry analysis and that have received proper training. The h-CLAT procedure is composed of several tasks which need to be performed sequentially, i.e., the qualification of the cell batch, the determination of an accurate CV75 value, the cell staining and measurement of CD86 and CD54 expression by flow cytometry and the data analysis and interpretation. EURL ECVAM recommends therefore that a step-wise approach similar to the one implemented in the transferability phase of the validation study is used when implementing the method before the test is performed for routine testing.

Reproducibility

14) The between laboratory reproducibility, assessed in the validation study by testing a set of 24 coded chemicals and determining concordant predictions of sensitiser versus non-sensitiser, met the expected value of 80% set a priori by the Validation Management Team (VMT). The overall within laboratory reproducibility (calculated from 15 of the 24 chemicals tested) was found to be 80%, which was lower than the expected value of 85% set by the VMT.

15) ESAC raised concerns in relation to within laboratory reproducibility since the VMT target value was not met. EURL ECVAM acknowledges this but notes that the VMT targets were derived from quite limited historical data on between laboratory reproducibility only, generated under non-blinded conditions (Sakaguchi et al., 2010). EURL ECVAM believes that these VMT target values should not be interpreted as 'cut-off' validation criteria since what can be considered as acceptable in terms of reproducibility typically depends on the context of use, such as within an IATA. However, as with data from any other experimental method, the reproducibility of h-CLAT needs to be taken into account when it is applied in any decision-making context. In this respect, it is worth noting that the reproducibility of the h-CLAT for discriminating between skin sensitisers and non-sensitisers appears to be

comparable to that of the LLNA (i.e. 70-80%, as calculated from the data available in the NICEATM database, see: http://ntp.niehs.nih.gov).

16) The ESAC peer review of the h-CLAT study included valuable expert discussion of various statistical approaches to assess within and between laboratory reproducibility. As a follow-up, EURL ECVAM proposes to re-analyse the data from the validation study with a view to exploring the merits of various statistical methods for describing the reproducibility of a test method that produces a classification-based prediction.

17) As indicated by the ESAC, further fine-tuning of the h-CLAT testing protocol and additional characterisation of the test system (THP-1 cells) may lead to an improved performance of the test method including the level of reproducibility that can be achieved. Nevertheless, the validation of the h-CLAT did not highlight any specific feature of the test method that would require additional optimisation in the short term to substantially improve its performance.

Predictive Capacity

18) Full evaluation of the predictive capacity of the h-CLAT was not within the scope of the EURL ECVAM study since the test method is not proposed as a stand-alone full replacement method. Nevertheless, the accuracy of the h-CLAT in predicting the *in vivo* classification (sensitiser/non-sensitiser) determined on the basis of concordant results in the LLNA, guinea pig tests and where available human data (see paragraph 11), was determined as 76% (sensitivity 81% and specificity 66%) (EURL ECVAM, 2013c). A recently published study that reported data on 143 chemicals (Takenouchi et al., 2013) suggested an accuracy of 80% in predicting LLNA classifications indicating that the actual performance of the h-CLAT test in discriminating between sensitisers and non-sensitisers may thus be actually higher. The accuracy of the h-CLAT in predicting human skin sensitising potential is indicated in the scientific literature to be 83% (sensitivity 88%, specificity 67%) for a set of 66 chemicals for which human patch test data and case reports are available (Nukada et al., 2011) and for a smaller set of chemicals (n=23; sensitivity 81%, specificity 86%) (Bauch et al., 2011).

4. Limitations

4.1 Technical limitations

19) **Solubility of test substances**: The test chemicals should be dissolved in a solvent compatible with the cell culture conditions. Therefore, chemicals which are not soluble in either medium, saline or DMSO, these being the solvents prescribed by the SOP, cannot be tested in the h-CLAT assay.

20) **Test substance stability:** As with many *in vitro* and *in chemico* assays, chemicals which are not stable in the prescribed solvents because of hydrolysis or other chemical reactions cannot be reliably tested.

21) **Maximum testable concentration:** In order to prevent osmotic stress of the cells, the maximum concentration of test substance should not exceed 5000 µg/mL.

22) **Interference with flow cytometry analysis:** Since the h-CLAT uses a fluorescein isothiocyanate (FITC)-labelled antibody, strong fluorescent test chemicals emitting at the same wavelength as FITC may interfere with the flow cytometry light-signal acquisition. To circumvent the problem, antibodies labelled with alternative fluorescent dyes may be used

provided that it can be shown that equivalent results to those obtained with the FITC-labelled antibodies are obtained. Also, flow cytometry analysis cannot be conducted correctly in the case of excessive cytotoxicity due to artefacts arising from diffuse labelling of cytoplasmic structures.

4.2 Limitations with regard to applicability

23) A recently published analysis of h-CLAT data suggests that chemicals with an octanol-water partition coefficient (log Kow) value lower than 3.5 can be tested in the assay and provide accurate predictions, whereas chemicals with a log Kow greater than 3.5 tend to produce false negative results (Takenouchi et al., 2013). For this reason, it was suggested that positive h-CLAT predictions obtained with chemicals with a log Kow greater than 3.5 are likely to be trustworthy whereas a negative prediction should be considered inconclusive.

24) As for many other assays based on an individual cellular model, the metabolic capacity (biotransformation) of the h-CLAT only partially represents the skin metabolism in vivo (Hennen et al, 2011; Chipinda et al., 2011; Fabian et al., 2013). Therefore, pro-haptens such as Isoeugenol may not be correctly identified by the assay. Nevertheless, putative pro-haptens such as 2-Aminophenol, Eugenol, 1-Naphtol and 2-Methoxy-4-methylphenol (Gerberick et al., 2009) have been reported in the h-CLAT submission to EURL ECVAM as being correctly predicted by the assay. In addition, Dihydroeugenol, a well characterised pro-hapten, was correctly classified as a sensitiser by all of the laboratories participating in the validation study (EURL ECVAM, 2013c).

25) Some pre-haptens are reported to be false negative in the h-CLAT (e.g. Abietic Acid) whereas others are reported as being correctly predicted by the assay (e.g. Geraniol and Linalool) (Ashikaga et al., 2010). The two pre-haptens evaluated in the EURL ECVAM validation study, 1,4-Phenylendiamine and R(+)Limonene, were correctly detected as potential sensitisers by all of the laboratories.

26) Most of the misclassifications generated by the h-CLAT in the EURL ECVAM study (EURL ECVAM 2013c), and in other published studies (Ashikaga et al., 2010) concerns chemicals that are weak sensitisers *in vivo* while the false negative rate for strong sensitisers is much lower. This should be kept in mind when interpreting negative results.

5. Suggested regulatory use

27) 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.

28) Based on the validation study results and other available information, the h-CLAT test method appears to be effective in providing information on the ability of a chemical to enhance the expression of the CD54 and/or CD86 cell membrane markers in THP-1 cells. Such markers are considered useful readouts for the identification of skin sensitising chemicals (OECD 2012a; 2012b). In addition, evidence in the literature clearly indicates the predictive value of h-CLAT data when combined with complementary information (Bauch et al., 2012; Nukada et al., 2013; Hirota et al., 2013; Tsujita-Inoue et al., 2014; van der Veen et al., 2014). Therefore results from the h-CLAT assay can be used within an IATA to determine the sensitisation potential of chemicals.

29) Taking into consideration the concentration-response information generated by the assay, it is plausible that h-CLAT may potentially contribute within an IATA to the characterisation of skin sensitisation potency. The extent of additional evidence needed to complement a h-CLAT result will depend on the intended application (e.g. hazard identification or potency assessment) and context (availability and quality of other information). Examples of the use of h-CLAT data in integrated non-animal approaches for hazard and potency assessment have been published in scientific literature (Bauch et al., 2012; Nukada et al., 2013; Hirota et al., 2013; Tsujita-Inoue et al., 2014; van der Veen et al., 2014).

30) Negative h-CLAT results should be interpreted with care, taking into due consideration (1) the limited capacity of the assay to metabolise (biotransform) pro-haptens, (2) the fact that some pre-haptens may not be sufficiently oxidised under the h-CLAT experimental conditions, and (3) the high rate of false negative predictions obtained with chemicals with a log Kow greater than 3.5.

31) Employed within an appropriate IATA, the h-CLAT assay 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

32) When applying the h-CLAT method, EURL ECVAM recommends that the revised protocol available at EURL ECVAM's DB-ALM service (http://ecvam-dbalm.jrc.ec.europa.eu, protocol No. 158) be used.

33) EURL ECVAM will undertake additional statistical analysis of the validation study results to better describe and understand aspects of reproducibility of this method.

34) Further testing to assess the performance of the h-CLAT method should include emphasis on assessing pre-and pro-haptens. In addition, its applicability to chemical mixtures and polymers (Jung YS et al., 2011) should be further investigated.

35) Predictive capacity of the assay for the discrimination between sensitisers and non-sensitisers should be further evaluated in the context of its inclusion within IATA. 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.

36) Integrated approaches using the h-CLAT method should also make use of other information sources, in particular from testing and non-testing methods (e.g. chemoinformatics, read-across and QSAR models). In silico methods that incorporate metabolic considerations (e.g. TIMES-SS: Patlewicz et al., 2007) may also help to identify pre- and pro-haptens. Analogues which have a similarly predicted mechanism of action, e.g. based on protein binding, can be found using the OECD QSAR Toolbox (www.qsartoolbox.org). The Toolbox also includes a specific profiler based on the h-CLAT assay. A variety of proposals concerning the use of h-CLAT data in combination with other information sources to discriminate between sensitising and non-sensitising chemicals have been published (Bauch et al., 2012; Nukada et al., 2013; van der Veen et al., 2014) and may support further work.

37) The possible contribution of h-CLAT CD86 EC150 and CD54 EC200 values derived from the concentration-response curve to support sub-categorisation of sensitisers according to GHS (i.e. sub category 1A and 1B) and to contribute to potency assessment should be evaluated in the context of integrated approaches. Examples are published in the scientific literature

on how these values can contribute to both purposes (Ashikaga et al., 2010; Hirota et al., 2013; Nukada et al., 2013; Tsujita-Inoue et al., 2014). For such evaluation, the use of human reference data (Basketter et al., 2014) will be particularly useful.

38) To reduce the cost and time needed for deriving a h-CLAT prediction for the purpose of skin sensitisation hazard identification, consideration should be given to adapting the h-CLAT SOP to eliminate the need for a third run in case of consistent and unequivocal predictions in the first two runs.

39) EURL ECVAM supports the development of an OECD Test Guideline for the h-CLAT. 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 IATA for skin sensitisation.

References

Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Andersen KE, Angers-Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S, Coecke S, Corvi R, Cronin M, Daston G, Dekant W, Felter S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Leite SB, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhler S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, Tähti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM (2011). Alternative (non-animal) methods for cosmetics testing: current status and future prospects. Arch. Toxicol., 85:367-485.

Aiba S, Terunuma A, Manome H, Tagami H (1997). Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. Eur J Immunol., 27:3031-8.

Ainscough JS, Frank Gerberick G, Dearman RJ, Kimber I (2013). Danger, intracellular signaling, and the orchestration of dendritic cell function in skin sensitization. J Immunotoxicol, 10:223-34.

Anderson SE, Siegel PD, Meade BJ (2011). The LLNA: A Brief Review of Recent Advances and Limitations. J. Allergy (Cairo), doi: 10.1155/2011/424203.

Ashikaga T, Yoshida Y, Hirota M, Yoneyama K, Itagaki H, Sakaguchi H, Miyazawa M, Ito Y, Suzuki H, Toyoda H (2006). Development of an in vitro skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol. Toxicol In Vitro, 20:767-73.

Ashikaga, T., Sakaguchi, H., Okamoto, K., Mizuno, M., Sato, J., Yamada, T., Yoshida, M., Ota, N., Hasegawa, S., Kodama, T., Okamoto, Y., Kuwahara, H., Kosaka, N., Sono, S., Ohno, Y. (2008). Assessment of the human Cell Line Activation Test (h-CLAT) for skin sensitization; results of the first Japanese inter-laboratory study. AATEX, 13: 27–35.

Ashikaga T, Sakaguchi H, Sono S, Kosaka N, Ishikawa M, Nukada Y, Miyazawa M, Ito Y, Nishiyama N, Itagaki H (2010). A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). Altern Lab Anim., 38:275-84.

Basketter DA, Alépée N, Ashikaga T, Barroso J, Gilmour N, Goebel C, Hibatallah J, Hoffmann S, Kern P, Martinozzi-Teissier S, Maxwell G, Reisinger K, Sakaguchi H, Schepky A, Tailhardat M, Templier M (2014). Categorization of chemicals according to their relative human skin sensitizing potency. Dermatitis., 25:11-21.

Bauch C, Kolle SN, Fabian E, Pachel C, Ramirez T, Wiench B, Wruck CJ, van Ravenzwaay B, Landsiedel R (2011). Intralaboratory validation of four in vitro assays for the prediction of the skin sensitizing potential of chemicals. Toxicol In Vitro, 25:1162-1168.

Bauch C, Kolle SN, Ramirez T, Eltze T, Fabian E, Mehling A, Teubner W, van Ravenzwaay B, Landsiedel R (2012). Putting the parts together: combining in vitro methods to test for skin sensitizing potentials. Regul Toxicol Pharmacol., 63:489-504.

Casati S, Aeby P, Basketter DA, Cavani A, Gennari A, Gerberick GF, Griem P, Hartung T, Kimber I, Lepoittevin JP, Meade BJ, Pallardy M, Rougier N, Rousset F, Rubinstenn G, Sallusto F, Verheyen GR, Zuang V (2005). Report and Recommendations of ECVAM Workshop 51. Dendritic cells as a tool for the predictive identification of skin sensitisation hazard. Altern Lab Anim., 33:47-62.

Chipinda I, Ruwona TB, Templeton SP, Siegel PD. (2011). Use of the human monocytic leukemia THP-1 cell line and co-incubation with microsomes to identify and differentiate hapten and prohapten sensitizers. Toxicology. 27:135-43.

EC, 2000. Opinion on the Murine Local Lymph Node Assay (LLNA) adopted by the SCCNFP during the 12th plenary meeting of 3 May 2000. http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/sccnfp_opinions_97_04/sccp_out114_en.htm

EC, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal No L 396, p. 1-849.

EC, 2008a. Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Official Journal No L 353, p. 0001-1355.

EC, 2008b. Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal L 142 , 31/05/2008 P. 0001 – 0739.

EC, 2009a. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC Official Journal No L 309, p. 01-50.

EC, 2009b. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Official Journal No L 342, p. 01-59.

EU, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal L 276, 20/10/2010 P. 0033 – 0079.

EU, 2012. Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. Official Journal No L167, p.01-116.

EURL ECVAM, 2013a. EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification. JRC Scientific and Policy Reports. Publication Office of the European Union. Accessible at: http://publications.jrc.ec.europa.eu/repository/handle/111111111/27708

EURL ECVAM, 2013b. Recommendation on the Direct Peptide Reactivity Assay (DPRA) for skin sensitisation testing. Accessible at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations

EURL ECVAM, 2013c. Human Cell Line Activation Test (h-CLAT) Validation Study Report. Accessible at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations

EURL ECVAM, 2013d ESAC Working Group Peer Review Consensus Report on an ECVAM-coordinated study concerning the transferability and reliability of the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing. Accessible at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations

EURL ECVAM, 2014a. EURL ECVAM Recommendation on the KeratinoSens™ assay for Skin Sensitisation Testing. Accessible at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations

EURL ECVAM, 2014b. ESAC Opinion on the ECVAM-led study of the human Cell Line Activation Test (h-CLAT). Accessible at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations

Fabian E, Vogel D, Blatz V, Ramirez T, Kolle S, Eltze T, van Ravenzwaay B, Oesch F, Landsiedel R (2013). Xenobiotic metabolizing enzyme activities in cells used for testing skin sensitization in vitro. Arch Toxicol., 87:1683-1696.

Gerberick GF, Troutman JA, Foertsch LM, Vassallo JD, Quijano M, Dobson RL, Goebel C, Lepoittevin JP (2009). Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. Toxicol Sci., 112:164-74.

Hartung T, Bremer S, Casati S, Coecke S, Corvi R, Fortaner S, Gribaldo L, Halder M, Hoffmann S, Roi AJ, Prieto P, Sabbioni E, Scott L, Worth A, Zuang V (2004). A modular approach to the ECVAM principles on test validity. Altern Lab Anim., 32:467-72.

Hennen, J., Aeby, P., Goebel, C., Schettgen, T., Oberli, A., Kalmes, M., Blömeke, B. (2011). Cross talk between keratinocytes and dendritic cells: impact on the prediction of sensitization. Toxicol Sci,. 123:501-10.

Hirota M, Kouzuki H, Ashikaga T, Sono S, Tsujita K, Sasa H, Aiba S (2013). Artificial neural network analysis of data from multiple in vitro assays for prediction of skin sensitization potency of chemicals. Toxicol In Vitro, 27:1233-46.

Jowsey IR, Basketter DA, Westmoreland C, Kimber I (2006). A future approach to measuring relative skin sensitising potency: a proposal. J. Appl. Toxicol., 26:341-350.

Jung YS, Kato R, Tsuchiya T (2011). Biodegradable Polymers Induce CD54 on THP-1 Cells in Skin Sensitization Test. Int J Biomater., 424571.

Kaplan DH, Igyártó BZ, Gaspari AA (2012). Early immune events in the induction of allergic contact dermatitis. Nat Rev Immunol., 13:114-24.

Kimber I, Basketter DA, Gerberick GF, Ryan CA, Dearman RJ (2011). Chemical allergy: translating biology into hazard characterization. Toxicol Sci., 1:238-68.

Kimber I, Dearman RJ, Basketter DA (2013). Dendritic cells and the assessment in vitro of skin sensitizing potential. Cutan Ocul Toxicol., 32:54-9.

Kosaka, N., Okamoto, Y., Mizuno, M., Yamada, T., Yoshida, M., Kodama, T., Sakiko, S., Ashikaga, T., Sato, J., Ota, N., Hasegawa, S., Okamoto, Y., Kuwahara, H., Sakaguchi, H., Ohno, Y. (2008). A study of the criteria for selection of THP-1 cells in the human Cell Line Activation Test (h-CLAT). Results of 2nd Japanese inter-laboratory study. AATEX, 13: 55-62.

Mizuno, M., Yoshida, M., Kodama, T., Kosaka, N., Okamoto, K., Sono, S., Yamada, T., Hasegawa, S., Ashikaga, T., Kuwahara, H., Sakaguchi, H., Sato, J., Ota, N., Okamoto,Y., Ohno, Y. (2008). Effects of pre-culture conditions on the human Cell Line Activation Test (h-CLAT) results: Results of the 4th Japanese inter-laboratory study. AATEX, 13: 70-82.

Nukada Y, Ashikaga T, Sakaguchi H, Sono S, Mugita N, Hirota M, Miyazawa M, Ito Y, Sasa H, Nishiyama N (2011). Predictive performance for human skin sensitizing potential of the human cell line activation test (h-CLAT). Contact Dermatitis, 65:343-53.

Nukada Y, Ashikaga T, Miyazawa M, Hirota M, Sakaguchi H, Sasa H, Nishiyama N (2012). Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency. Toxicol In Vitro, 26:1150-60.

Nukada Y, Miyazawa M, Kazutoshi S, Sakaguchi H, Nishiyama N (2013). Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Toxicol In Vitro, 27:609-18.

OECD (1992). OECD Guideline for the Testing of Chemicals No. 406: Skin Sensitisation. Paris, France: Organisation for Economic Cooperation and Development. Accessible at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

OECD (2010a). OECD Guideline for the Testing of Chemicals No. 429: Skin Sensitisation: Local Lymph Node Assay. Paris, France: Organisation for Economic Cooperation and Development. Accessible at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

OECD (2010b). OECD Guideline for the Testing of Chemicals No. 442A: Skin Sensitisation: Local Lymph Node Assay DA. Paris, France: Organisation for Economic Cooperation and Development. Accessible at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

OECD (2010c). OECD Guideline for the Testing of Chemicals No. 442B: Skin Sensitisation: Local Lymph Node Assay BrdU-ELISA. Paris, France: Organisation for Economic Cooperation and Development. Accessible at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

OECD (2012a). The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168. Accessible at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)10/PART1&docLanguage=En

OECD (2012b). The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 2: Use of the AOP to Develop Chemical Categories and Integrated Assessment and Testing Approaches. Series on Testing and Assessment No. 168. Accessible at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2012)10/PART2&docLanguage=En

OECD (2015a). OECD Guideline for the Testing of Chemicals No. 442C: *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA). Paris, France: Organisation for Economic Cooperation and Development. Accessible at http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

OECD (2015b). OECD Guideline for the Testing of Chemicals No. 442D: *In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method. Paris, France: Organisation for Economic Cooperation and Development. Accessible at http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

Patlewicz G, Dimitrov SD, Low LK, Kern PS, Dimitrova GD, Comber MIH, Aptula AO, Phillips RD, Niemela J, Madsen C, Wedebye EB, Roberts DW, Bailey PT, Mekenyan OG (2007). TIMES-SS – A promising tool for the assessment of skin sensitization hazard. A characterisation with respect to the OECD Validation Principles for (Q)SARs. Reg. Toxicol. Pharmacol., 48:225–239.

Quah BJ, O'Neill HC (2005). Maturation of function in dendritic cells for tolerance and immunity. J Cell Mol Med., 9:643-54.

Sakaguchi H, Ashikaga T, Miyazawa M, Yoshida Y, Ito Y, Yoneyama K, Hirota M, Itagaki H, Toyoda H, Suzuki H. (2006). Development of an in vitro skin sensitization test using human cell lines;

human Cell Line Activation Test (h-CLAT) II. An inter laboratory study of the h-CLAT. Toxicol In Vitro, 20:774-84.

Sakaguchi H, Ashikaga T, Miyazawa M, Kosaka N, Ito Y, Yoneyama K, Sono S, Itagaki H, Toyoda H, Suzuki H (2009). The relationship between CD86/CD54 expression and THP-1 cell viability in an in vitro skin sensitization test--human cell line activation test (h-CLAT). Cell Biol Toxicol., 25:109-126.

Sakaguchi H, Ryan C, Ovigne JM, Schroeder KR, Ashikaga T. (2010). Predicting skin sensitization potential and inter-laboratory reproducibility of a human Cell Line Activation Test (h-CLAT) in the European Cosmetics Association (COLIPA) ring trials. Toxicol In Vitro, 24:1810-20.

dos Santos GG, Reinders J, Ouwehand K, Rustemeyer T, Scheper RJ, Gibbs S (2009). Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound. Toxicol Appl Pharmacol., 236:372-82.

Sono S., Yamada T., Kosaka N., Ikamoto K., Mizuno M., Sato J., Yoshida M., Ota N., Kidama T., Okamoto Y., Kuwahara H., Sakaguchi H., Hasegawa S., Ashikaga T., Ohno Y. (2008). A study on serum difference on test results in the human Cell Line Activation Test (h-CLAT): Results of 3rd Japanese inter-laboratory study. AATEX, 13:63-69.

Takenouchi O, Miyazawa M, Saito K, Ashikaga T, Sakaguchi H (2013). Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients. J Toxicol Sci., 38:599-609.

Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer., 26:171-176.

Tsujita-Inoue K, Hirota M, Ashikaga T, Atobe T, Kouzuki H, Aiba S (2014). Skin sensitization risk assessment model using artificial neural network analysis of data from multiple in vitro assays. Toxicol In Vitro, 28:626-39.

UN, United Nations N (2011). Globally Harmonised System of Classification and Labelling of Chemicals (GHS). Fifth revised edition, UN NY and Geneva, 2013. Accessible at: http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html

Vocanson M, Hennino A, Rozières A, Poyet G, Nicolas JF (2009). Effector and regulatory mechanisms in allergic contact dermatitis. Allergy, 64:1699-714.

Vandebriel RJ, van Loveren H (2010). Non-animal sensitization testing: state-of-the-art. Crit Rev Toxicol,. 40:389-404.

Van Helden SF, van Leeuwen FN, Figdor CG (2008). Human and murine model cell lines for dendritic cell biology evaluated. Immunol Lett. 15;117: 191-197.

Van der Veen JW, Rorije E, Emter R, Natsch A, van Loveren H, Ezendam J (2014). Evaluating the performance of integrated approaches for hazard identification of skin sensitizing chemicals. Regul Toxicol Pharmacol., 69:371-379.

ANNEX 1 ESAC OPINION

on the ECVAM-led study of the human Cell Line Activation Test (h-CLAT)

TABLE OF CONTENTS

Ispra, 11 March 2014

Summary of the ESAC Opinion

The ESAC was requested to provide a scientific opinion on an EURL-ECVAM led validation study assessing mainly the transferability and reproducibility (within- and between-laboratories) of the h-CLAT test method (primary objective of the study) in view of its possible future use as part of a non-animal testing strategy for skin sensitization. The study had also been designed to provide *preliminary* information on a) the predictive capacity of the test method and b) its potential use for contributing to sub categorisation of sensitizing chemicals.

Overall, the conclusions made by the ESAC based on the ESAC WG report correspond well with the conclusions drawn by the Validation Management Group overseeing the study and as described in the Validation Study Report, indicating that, generally, the conclusions are supported by the results shown in the report (see Section 15.1).

The ESAC disagreed, however, with the VMG conclusion concerning the Within Laboratory Reproducibility (WLR).

- Acceptance criteria were determined at the start of the study by the VMG. WLR was assessed using 15 chemicals in three independent experiments. The average reproducibility of 80% (KAO (86.7%), Shiseido (80.0%), EURL-ECVAM (80.0%) and Bioassay (73.3%)) did not meet the 85% reproducibility target set by the VMG. Actually, only one out of four participants met this target. Despite missing the expected performance level, the VMG nevertheless concluded that the h-CLAT is a reproducible method within laboratories. This conclusion was

partly based on the premise that a subset of chemicals consistently drove the discrepancies in reproducibility, and that some of these problem chemicals might fall outside the applicability domain. While the chemical limitations of the test are appreciated, the ESAC is concerned that there may be other inherent characteristics or critical aspects of the h-CLAT test method, which could be important sources of variability (e.g. the time course for expression of the cell surface markers; the state of cell differentiation be a source of variability). The other reason given by the VMG to support their conclusion is that the h-CLAT assay is intended to be used as part of an ITS. Generally, the ESAC does not support this reasoning: a low reproducibility, i.e. high variability will cause problems when using a test method in practice and this is independent of whether it is used as a stand-alone test method or within integrated approaches. Other information sources within an integrated approach will not be able to remedy the intrinsic variability of one information source. With regard to the h-CLAT assay, the ESAC Working Group is concerned that the poor reproducibility of the assay may actually create difficulties with respect to the interpretation of data generated as part of an ITS, as results are likely to be conflicting.

- The data were considered strong enough to support transferability of the test to properly equipped, trained and staffed laboratories with the appropriate analytical capabilities.
- Five of the 24 chemicals produced a discordant classification by the laboratories resulting in an average BLR reproducibility of 81.3%, meeting the target (80%).
- For S/NS classification, values (accuracy: 76%; sensitivity: 81.3%; specificity: 65.6%) are, overall, lower than the values (84%, 87%, 75%, respectively) resulting from the historical data on 100 chemicals (Ashikaga et al., 2010), which were provided to EURL-ECVAM as part of the test submission. Due to this discrepancy the ESAC concludes that the number of substances and the information available for these substances in the peer reviewed publication was insufficient for allowing more than a purely preliminary indication on the predictive capacity in terms of S/NS.
- For sub-categorization, the data generated and statistically assigned cut-offs propose a maximum accuracy of 58% accuracy, which is in contrast to previously published data (N=100) that reported an accuracy of 72% (Ashikaga et al. 2010). The ESAC does not understand why the VMT considers the values obtained in the validation study as promising. Our conclusion is that the number of substances and the information available for these substances was insufficient for allowing more than a purely preliminary indication on the predictive capacity in terms of potency classification.
- The number of chemicals did not allow us to draw conclusions about the applicability domain of the test (which, notably, was not one of the study objectives). Empirically the applicability domain seems to exclude pro-haptens, auto-fluorescent compounds, chemicals with limited water solubility/stability, metal salts and volatile compounds. However, pre-/pro-haptens were reported as correctly identified.

The predictive capacity, applicability domain and limitations of the test are not, in our view, yet fully defined. The submitted study does not provide strong evidence supporting the usefulness of the h-CLAT for GHS sub-categorisation of sensitizers. However, recent studies substantiate the preliminary data of the VSR (Nukada et al., 2012; Nukada et al., 2013).

- The ESAC recommends that the sources of the unsatisfactory WLR (below the 85% target) be identified and addressed;

- better defining (1) the predictive capacity and (2) the applicability domain of the h-CLAT (to eliminate the uncertainty currently associated with a negative result) either through further testing (i.e. prospective validation) or through retrospective analysis of existing information (retrospective validation: data grouping / meta-analysis);
- to adapt the SOP to reduce resource costs by eliminating the need for a third evaluation run in case where the first two runs are consistent;
- to reassess the amended SOP version 7 using existing/historical results with the purpose to re-evaluate the predictive capacity of this test method.
- that further studies be conducted to determine the potential of the test method to properly sub-categories chemicals with skin sensitisation potential.

Recently, Nukada et al. (2013) reported a data integration strategy including the h-CLAT, the Direct Peptide Reactivity Assay (DPRA) and the knowledge-based expert system 'DEREK' for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Using a tiered strategy of h-CLAT and DPRA an accuracy of 86% and 73% for the potential and potency prediction, respectively, was obtained. Further studies are needed to identify the best integrated testing strategy or strategies able to address the different regulatory goals and risk assessments (hazard identification, classification, potency assessment, etc.) in reliable and relevant a manner.

1. Mandate of the ESAC

The opinion of ESAC should support ECVAM with respect to the development of recommendations regarding the reliability (transferability, within and between laboratory reproducibility) of the h-CLAT and the potential regulatory use of the test method.

1. Study design – transferability, reliability and relevance

- The ESAC was requested to review whether the validation study was conducted appropriately in view of the objective of the study:
  - Reproducibility of the h-CLAT method within laboratories (WLR);
  - Transferability;
  - Reproducibility between laboratories (BLR);
  - Predictive capacity of the test method.

- With respect to the design and conduct of the study, the following issues were to be addressed:
  - Clarity of the test definition (module 1)
  - Clarity of the definition of the study objective
  - Appropriateness of the study design in view of study objective
  - Appropriateness of the study execution:
  - Appropriateness of the statistical analysis used for analysing WLR, transferability, BLR and (preliminary) predictive capacity.

2. Conclusions of the study

- The ESAC was requested to assess the justification and plausibility of
  - Reproducibility (WLR and BLR) and transferability;
  - Preliminary predictive capacity;
  - Possible gaps between study design and study conclusions which remain to be addressed in view of the suggested conclusions/use;
  - Applicability and possible limitations of the test method, in particular in view of its potential use within an ITS for sensitisation testing and assessment.

3. The ESAC is requested (a) to evaluate, on the basis of the data submitted in the validation study, the possible use of the test method (also within a strategy) to identify skin sensitizers, (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).

2. Detailed opinion of the ESAC

The ESAC was asked to provide an opinion on a EURL-ECVAM-coordinated study assessing the transferability and reproducibility (within- and between-laboratories) of the h-CLAT (primary objective of the study) in view of its possible future use as part of a non-animal testing strategy for skin sensitization. The study had also been used to provide *preliminary* information on a) the predictive capacity of the test method and b) its potential use for contributing to sub-categorisation of sensitizing chemicals.

1) Study design – transferability, reliability and relevance.

- The Test Definition of the h-CLAT assay would benefit from a more detailed rationale behind the selection of the THP-1 cell line, and CD86 and CD54 membrane markers; in particular as to why both of the markers are required. Furthermore, their biological and mechanistic relevance to the human situation is not sufficiently explained. There is ample evidence showing that CD86 and CD54 are generally up-regulated in response to challenges that cause cell damage, inflammation and cytotoxicity. There is a need to explain what special features of the test or the prediction model are making the test specific for sensitization.
- The WLR was assessed at the level of concordance with a binary prediction (S/NS). An average reproducibility of 80% did not meet the 85% reproducibility target set by the VMG. Actually, only one out of the four participating laboratories met this target. The definition of the reproducibility target (85%) set by the VMG was based on the performance of methods previously evaluated at EURL-ECVAM. The expected performance of the test (WLR) is derived from the BLR calculated from the test submissions. The ESAC does not consider 85% to be an unreasonably high target. Furthermore, the explanation offered according to which it was a small number of compounds with special properties that caused problems with reproducibility was not

further substantiated. Indeed, it was noted that the problem concerned 9 out of 15 chemicals and none of the problem chemicals gave issues in all 4 laboratories.

- The ESAC is concerned, in the absence of evidence, that low WLR was caused by the characteristics of the chemicals tested and that there may be inherent characteristics of the h-CLAT, which could be important sources of WLR variability The low reproducibility of the test, raised also the concern that the h-CLAT as potential ITS building block with poor reproducibility might actually create more difficulties in interpreting data as part of an ITS due to conflicting results.
- The training and transfer phases of the validation study were well planned and executed. All the stages appear well documented. Some key issues have been identified during the process of transfer to the naïve laboratories and effort has been put into identifying and solving these issues. These changes were taken up in SOP version 5 (used for transfer) and resulted in SOP versions 6 and 7. It is clear from the transfer data that adopting this method in a laboratory requires sufficient experience in flow cytometry and cell culture.
- The BLR was assessed in terms of concordance in predictions. Two BLR values were generated by testing 24 chemicals, one comparing the consistency of the two naïve labs with the first lead lab and the second comparing them with the second lead lab. ESAC agreed with the VMG's conclusions with respect to the acceptability of the BLR because of the marginal difference between the lowest BLR (79.2%) and expected performance of 80%. The chemicals that drove discrepancies in the BLR study were the same as those driving discrepancies in the WLR study. The ESAC notes, in the absence of a defined applicability domain, that some of these test chemicals may have physicochemical properties making them incompatible with this test method.
- The ESAC recognizes the fact that this study was not designed to address the predictive capacity of the h-CLAT due to the low number of chemicals. This also applies to the sub-categorization. Three chemicals (methyl methacrylate, DCNB and benzyl alcohol) were consistently and reproducibly wrongly classified.
- The project was described and designed in clearly recognizable and well described phases including Test Definition (Module 1), Transferability (Module 3), Within Laboratory Reproducibility (WLR) (Module 2), Between Laboratory Reproducibility (BLR) (Module 4). The data were also used for a preliminary evaluation of Predictive Capacity (Module 5).
- Overall, the chosen statistical approach was considered appropriate. The 'expected proportion' of concordant classifications (between laboratories) was calculated to be 90% on the basis of available data on between-laboratory reproducibility as submitted to ECVAM (see Appendix 2 of VSR, page 5). However, it was not clear why a power of 75% rather than the more conventional 80% or 90% power had been applied. This power allows for detecting 25% changes in each direction and, as a consequence, leads to a lower limit of the confidence interval of 65 % (90%-25%).

2) Conclusions of the study

- Overall, the study design, including the chemicals and their associated reference data, were considered appropriate for the purpose of addressing the first objective of the study: Assessing the WLR (N=15) and BLR (N=24) of the h-CLAT.
- Overall, the conclusions made by the ESAC correspond well with the conclusions drawn by the VMG as described in the VSR, tending for confirm that these conclusions are supported by the results shown in the report.

- The ESAC disagreed, however, with the VMG conclusion concerning the WLR. An average reproducibility with the validation study test chemicals of 80% (not meeting the 85% VMG reproducibility target) set by the VMG. Actually, only one out of four participating laboratories met this target. The validation study did not fully establish the reasons for the WLR performance figures obtained.
- The conclusion on the BLR is considered reasonable in light of the marginal difference between the lowest BLR (79.2%) and expected performance of 80%. *See above*
- The accuracy values for S/NS classification (76%) and sub-categorization (57%) are lower than those reported earlier (84% (S/NS) and 72% (sub-categorization)) and based on historical data on 100 chemicals (Ashikaga et al. 2010. ATLA 38; 275-284) which were submitted to EURL-ECVAM as part of the test submission. The ESAC believes this may be explained in part by the smaller number of chemicals used for the validation study, some of which were not part of the historical data set. A separate communication from the test developers suggests that the historical data may have contained proportionately fewer difficult chemicals (e.g. the three chemicals consistently wrongly classified in the validation study are not part of the 100 chemical set). Assessment/description of the applicability domain was not the objective of this study. Consequently, the small number of chemicals used in the validation study, which was set to satisfy the primary goal of the study, is not sufficient on its own to draw robust conclusions on the applicability domain.

3) Possible use of the test method, i.e. to identify (also within a strategy) skin sensitizers, and additional recommendations (as required) on the proper scientific use of the test method within such a strategy.

- As yet no applicability domain has been described for this method. Deciding whether or not a chemical falls within the applicability domain of the test will be a challenge with regard to pro-haptens, metal (salts), chemicals with limited solubility/stability in water, volatile compounds and auto-fluorescent compounds.
- Regarding potency class, the data obtained did not support the use of the h-CLAT as a stand-alone assay for potency classification. This is in agreement with the statement of the VMG that the assay should be further evaluated for its capacity to "contribute" to a potency classification.

<u>Recommendations:</u>

- The ESAC considered that the target value for WLR was a realistic and justified one and were therefore concerned that three of the four laboratories failed to meet this target. The ESAC recommends that the sources of variability be identified (e.g. the time course for expression of the cell surface markers; the state of cell differentiation be a source of variability), and that solutions be provided. Poor reproducibility may create difficulties in interpreting data as part of an ITS due to conflicting results. A review of the existing 100 chemical/24 chemical datasets might identify properties of chemicals for which this test is not an appropriate method for investigating skin sensitisation potential.
- The ESAC recommends better explaining, clarifying or defining (1) the predictive capacity, the ability of the cell system and biomarkers to selectively identify skin sensitisation, and the sources of variability; and (2) the applicability domain of the h-CLAT to reduce the frequency to inconsistent results, either through further testing (i.e. prospective

validation) or through retrospective analysis of existing information (retrospective validation: data grouping / meta-analysis).

- For greater efficiency, the SOP could be adapted by eliminating the need for a third evaluation run in case where the first two runs are consistent as a third inconsistent run does not change the outcome.
- Based on the ESAC assessment of the validation study data, the available limited evidence does not support the use of the test method for GHS sub-classification of sensitizers: that was not, however, a primary objective of the validation study. Additional information and evidence are required when further consideration is given to the use of the test method for this purpose (see for example Nukada et al., 2012; 2013). Nukada et al. (2013) reported a data integration strategy including HCLAT, DPRA and DEREK for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Using a tiered system of h-CLAT and DPRA an accuracy of 86% and 73% for the potential and potency prediction was obtained. The tiered system showed a higher sensitivity (from 88 to 96%) compared with h-CLAT alone. Further studies are needed to identify the best integrated testing strategy or strategies necessary to cover the different regulatory goals and risk assessments (hazard identification, classification, potency assessment, etc.).

3. Informative background to the Mandate and Opinion

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 for those intended for topical application (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 currently 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, in particular as regards to cosmetics.

The current view therefore is to combine different test methods in order to address different key mechanisms of skin sensitisation: skin bioavailability, haptenation (the protein binding of chemicals which triggers immunological responses), epidermal inflammation, dendritic cell activation and migration, T cell proliferation. Test methods are currently under development which have been specifically designed to address these key mechanistic steps involved in skin sensitisation. Before these test methods can be routinely used, e.g. in ITSs, their capacity to produce reproducible results needs to be demonstrated as a first step. There is ample evidence showing that maturation markers in general, and CD86 and CD54 in specific, are generally up-regulated in response to challenges that cause inflammation and cytotoxicity. There is however a window in which only sensitizers (or the

majority of them) activate dendritic cells (DCs). Cellular stress induced by allergens is different from the one triggered by irritants. Furthermore, hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately against benign antigens, causing inflammatory reactions and tissue damage. Just for clarification, DCs are recognized as important antigen presenting cells in adaptive immunity because of their capacity to stimulate naïve lymphocytes (Banchereau et al., 2000). Langerhans cells (LC) are resident immature DCs in the skin capable to take up and process contact allergens. During this process LC differentiate into mature immunostimulatory cells up-regulating the expression of co-stimulatory molecules such as CD80, CD86 and CD40 and adhesion molecules including CD2, CD11a, CD54, CD58 (Quah and O'Neill, 2005). Activated LC move from the epidermis into the dermis, and into the regional lymphatic system. In the lymph node, LC differentiate into mature dendritic cells and present antigen to specific T lymphocyte using MHC class II molecules to hold the processed antigen in place. Adhesion molecules on both the antigen-presenting cell (i.e. CD86) and the T-cell (i.e. CD28) ensure appropriate contact and co-stimulation. Following appropriate stimulus, a clone of T cells with the ability to react to the antigen, which caused their expansion, is produced. The h-CLAT it is a test method that allows for quantitative analysis of a chemical's potential to induce activation of THP-1 cells (used as a surrogate for human myeloid dendritic cells). This method has been initially proposed by Ashikaga et al. (2002) to identify sensitizers, and Yoshida et al. (2003) reported that naïve THP-1 could respond to sensitizers specifically through augmented expression of co-stimulatory molecules, CD54 and CD86, and considered this as a possible tool to be used as an *in vitro* sensitization test.

4. References

Ashikaga T, Hoya M, Itagaki H, Katsumula Y and Aiba S (2002). Evaluation of CD86 expression and MHC calss II molecule internalization in THP-1 human monocytic cells as predictive endpoints for contact sensitizers. Toxicol. In Vitro 16: 711-716.

Ashikaga T, Sakaguchi H, Sono S, Kosaka N, Ishikawa M, Nukada Y, Miyazawa M,Ito Y, Nishiyama N, Itagaki H. (2010) A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). Altern Lab Anim. Aug;38(4):275-84.

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B and Palucka K (2000). Immunobiology of dendritic cells. Annu. Rev. Immunol. 18: 767-811.

Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP, (2004) Development of a peptide reactivity assay for screening contact allergens. Toxicol Sci. 81; 332-43.

Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP, (2007). Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol Sci. 97, 417-27.

Landsteiner K & Jacobs J (1936) Studies on the sensitisation of animals with simple chemical compounds. Journal of Experimental Medicine 64, 625-639

Nukada Y, Ashikaga T, Miyazawa M, Hirota M, Sakaguchi H, Sasa H, Nishiyama N. (2012) Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency. Toxicol In Vitro. Oct;26(7):1150-60.

Nukada Y, Miyazawa M, Kazutoshi S, Sakaguchi H, Nishiyama N. (2013) Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Toxicol In Vitro. Mar;27(2):609-18.

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

Quah BJC, and O'Neill HC (2005). Maturation of function in dendritic cells for tolerance and immunity. J. Cell. Mol. Med. 9: 643-54.

Takenouchi O, Miyazawa M, Saito K, Ashikaga T and Sakaguchi H (2013). Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients. J. Toxicol. Sci. 38: 599-609.

Yoshida Y, Skaguchi H, Ito Y, Okuda M and Suzuki H (2003). Evaluation of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naive THP-1 cell line. Toxicol. In Vitro 17: 221-228

EURL ECVAM Scientific Advisory Committee

- Prof. Jürgen BORLAK
- Dr. Neil CARMICHAEL
- Dr. Edward CARNEY
- Dr. Harvey CLEWELL
- Prof. Lucio COSTA
- Dr.Kristina KEJLOVÁ
- Dr. David John KIRKLAND
- Prof. Annette KOPP-SCHNEIDER
- Dr. Renate KRÄTKE
- Prof. Claus-Michael LEHR
- Dr. José Maria NAVAS
- Dr. Aldert PIERSMA
- Dr. Jonathan David RICHMOND
- Dr. Erwin L. ROGGEN
- Dr. Dorothea SESARDIC

ESAC Working Group Skin Sensitisation

- Dr. Erwin ROGGEN (ESAC member, Chair of ESAC WG and rapporteur)
- Dr. Ed CARNEY (ESAC member)
- Prof. A. Wallace HAYES (ESAC member)
- Dr. Maja ALECSIC (external expert)
- Dr. Emanuela CORSINI (external expert)
- Dr. David LOVELL (external expert)
- Dr. Michael WOOLHISER (external expert)
- Prof. Yong HEO (external expert, ICATM nomination)

ESAC / ESAC WG Coordination

Dr. Claudius GRIESINGER

ANNEX 2

ECVAM REQUEST FOR ESAC ADVICE

on

an ECVAM-coordinated study concerning the transferability and reliability of the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing

Title page information	
Abbreviated title of ESAC request	h-CLAT test method for skin sensitisation testing
ESAC REQUEST Nr.	2013-02
Template used for preparing request	EP 2.01
Date of finalising request	3/6/2013
Date of submitting request to ESAC	3/6/2013 for discussion at ESAC38 18/19 June 2013
Request discussed through	ESAC 38
Opinion expected at (date)	Q4 of 2013: ESAC plenary meeting or through written procedure
File name of this request	**ESAC REQUEST 2013-03 h-CLAT-final.doc**

TABLE OF CONTENTS

1. TYPE OF REQUEST

Request Type	Identify request ("YES")
R1 ESAC Peer Review of a Prevalidation Study or Validation Study	**YES**: Validation study addressing mainly reliability
If R1)applies please specify further:	
▶Prevalidation Study	
▶Prospective Validation Study	In the period between 2010 and 2012 EURL ECVAM coordinated a **validation study focusing on an assessment of reliability** of three test methods for skin sensitisation testing: 1) the *Direct Peptide Reactivity Assay (DPRA)*, 2) the *human Cell Line Activation Test (h-CLAT)*, 3) the *Myeloid U937 Skin Sensitisation Test* (MUSST). This request focuses on the h-CLAT test method.
▶Retrospective Validation Study	
▶Validation Study based on Performance Standards	
R2 Scientific Advice on a test method submitted to ECVAM for validation (e.g. the test method's biological relevance etc.)	
R3 Other Scientific Advice (e.g. on test methods, their use; on technical issues such as cell culturing, stem cells, definition of performance standards etc.)	

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

Validation of the reliability of the Human Cell Line Activation Test (h-CLAT)

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 skin allergy, leading to the disease called allergic contact dermatitis (ACD) in humans.

The identification of the skin sensitisation potential represents an important component of the safety assessment of new and existing substances including cosmetic ingredients. Current regulatory predictive tests for skin sensitisation rely on the use of animals. These include: guinea-pig tests (Buehler Test and Guinea-pig Maximisation Test) (TG 406, OECD 1992; TM B06, EC 2008a), the murine Local Lymph Node Assay (LLNA) (TG 429, OECD 2010a; TM B42, EC 2008a) and its non-radio-isotopic variants (TG 422a, OECD 2010b; TG 422b, OECD 2010c).

The key events underlying of the induction of skin sensitisation are well understood and have been recently documented by the OECD in its report on: "The Adverse Outcome Pathway (AOP) for Skin Sensitisation Initiated by Covalent Binding to Proteins" (OECD 2012a; 2012b). These include: 1) the ability of the chemical to penetrate the skin and reach the site of haptenation (skin bioavailability), 2) the covalent binding of the chemical to skin proteins (haptenation), 3) the release of pro-inflammatory signals and the induction of cyto-protective cellular pathways in keratinocytes 4) the activation and maturation of Dendritic cells (DC) the skin immunocompetent cells, 5) the migration of DC from skin to the regional lymph nodes, 6) the presentation by DC of the haptenated protein to T cells and the clonal expansion of memory T cells (lymphocytes capable of being stimulated and activated specifically by the haptenated protein).

Progress has been made in recent years in the development of mechanistically-based alternative methods for hazard identification some of which might also be able to contribute to potency prediction. However, none of these tests is currently regarded to have the potential to function as a stand-alone method to fully replace the animal tests. Instead, it is proposed that a combination of in *in silico*, *in chemico* and *in vitro* tests, addressing the key biological events of skin sensitisation, will be needed to achieve this goal.

Proposals on how to use these methods in Integrated Testing Strategies (ITS)/Integrated Approaches to Testing and Assessment (IATA) for both hazard identification and potency prediction are emerging.

2) The Human Cell Line Activation Test (h-CLAT).

The **Human Cell Line Activation Test** addresses the role that Langerhans cell (LC) and dermal dendritic cells (DC) play in the induction of skin sensitization. These cells are important mediators in the skin sensitization process since they are capable of presenting the hapten-protein conjugate to responsive T lymphocytes in the lymph nodes draining the site of exposure (Kimber and Cumberbatch, 1992). The maturation process of LC and DC from antigen processing cells to antigen presenting cells is considered a key event in the acquisition of skin sensitisation. This maturation process involves the modulation of the expression of cell surface phenotypic markers, those most commonly reported being CD54, CD80, CD86 and major histocompatibility complex (MHC) class II (Galvao dos Santos et al., 2009). This knowledge has been exploited in the development of *in vitro* tests based on the use of DC-like immortalized cell-lines to screen the skin sensitization potential of chemicals.

The h-CLAT measures the modulation of CD86 and CD54 protein markers on the surface of THP-1 cells (human monocytic cell line) by flow cytometric analysis, following 24 hour cell exposure to 8 concentrations of a test substance. The concentrations used in the main experiment are selected on the basis of the CV75 value, the estimated concentration of test substance yielding 75% cell viability, previously determined with a propidium iodide viability assay. A chemical is classified as sensitiser if the expression of either the CD86 and/or the CD54 is equal or exceeds a defined threshold in at least 2 of 3 independent evaluations.

The h-CLAT test method was jointly developed by Kao Corporation and Shiseido. Extensive development/optimisation/evaluation work including assessment of the test method's performance in multi-laboratory ring trials was conducted prior submission to ECVAM. The submission to ECVAM reported results for 100 chemicals with an accuracy of 84% for distinguishing sensitisers from non-sensitisers compared to LLNA data.

3) Study objectives and design

The validation of the h-CLAT test method was part of larger validation study involving the assessment of two other test methods, the Direct Peptide Reactivity Assay (DPRA) and the Myeloid U937 Skin Sensitisation Test (MUSST). The validation study was coordinated by ECVAM in the period between 2010 and 2012 with the primary objective of assessing the test methods' transferability and within and between laboratory reproducibility in view of their potential future use in integrated non-animal approaches intended to reduce and replace the currently used animal tests for skin sensitisation hazard identification.

As a secondary goal of the study, the experimental data were used to perform:

a) A preliminary evaluation of the ability of the three tests to reliably discriminate skin sensitising (S) from non-sensitising (NS) chemicals as defined by the Globally Harmonised System (GHS) of classification and labelling of substances (category 1; no category) (UN, 2011) and as implemented in the European Commission Regulation on classification, labelling and packaging (CLP) of substances and mixtures (EC, 2008b).

b) Where possible, a preliminary consideration of the ability of the three tests to contribute to potency categorisation e.g. GHS sub-category 1A (strong sensitisers) and 1B (other sensitisers) as defined in the fourth revised edition of GHS (2011).

24 coded test items were tested by each of the four laboratories participating in the study for the evaluation of the h-CLAT (Kao and Shiseido as the lead laboratories, Biossay and EURL ECVAM as the naïve laboratories) to generate information on the between-laboratory reproducibility. A subset of 15 chemicals was tested two additional times in each laboratory for the evaluation of the within-laboratory reproducibility.

With respect to the ECVAM's modular approach to validation (Hartung et al., 2004) the study generated information on modules 1) test definition, 2) within laboratory reproducibility, 3) transferability and 4) between laboratory reproducibility. In addition, the experimental data contributed to modules 5) predictive capacity and 6) applicability domain. **However, the number of chemicals used in this validation study, which was based on statistical considerations related to the evaluation of the reproducibility only, was not sufficient on its own to conclude on the last two modules.**

4) Study results

The main results for the study's primary goal are summarised in the table below:

Module	Results
Module 2 WLR	Evaluation of the WLR for a subset (n=15) of the validation study chemicals in each laboratory focused on the concordance of predictions (sensitizer versus non-sensitiser) as determined by the results of three independent experiments. Kao Laboratory WLR=86.7% Shiseido Laboratory WLR=80% EURL ECVAM Laboratory WLR=80% Bioassay Laboratory WLR=73.3%
Module 3 Transferability	Both naïve laboratories (EURL ECVAM and Bioassay) succeeded in transferring the protocol to their testing facilities.
Module 4 BLR	Evaluation of the BLR for the 24 chemicals focused on the concordance of the predictions (sensitiser versus non-sensitiser) and was calculated by comparing the two naïve laboratories with each of the two lead laboratories separately. Naïve and Kao BLR=83.3% Naïve and Shiseido BLR=79.2% Overall BLR=79.2%

5) Conclusions of the VMG

The VMG concluded that the information generated in this validation study demonstrates that the h-CLAT is a robust test method that can be easily transferred to properly equipped laboratories sufficiently experienced in cell culture and flow cytometry analysis. In addition the study results support the fact that the h-CLAT is a reproducible test method that can contribute to the determination of the sensitization potential of substances.

References

EC 2008a. COUNCIL REGULATION No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal of the European Union No L 142 , p. 1 – 739.

EC 2008b. Regulation No 1272/2008 of 16 December 2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Official Journal of the European Union L 353, p. 1-1355.

Galvao dos Santos G, Reinders J, Ouwehand K, Rustemeyer T, Schepper RJ & Gibbs S (2009) Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound. Toxicology and Applied Toxicology 236(3); 372-382.

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

Kimber I & Cumberbatch M (1992) Dendritic cells and cutaneous immune responses to chemical allergens. Toxicology and Applied Pharmacology 117; 137-46.

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 (2010a) The Local Lymph Node Assay. Guidelines for Testing of Chemicals No. 429, Paris.

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

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

OECD, Organisation for Economic Cooperation and Development (2012a). The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins; Part 1: Scientific Evidence Series on Testing and Assessment No.168 ENV/JM/MONO(2012)10/PART1. Paris.

OECD, Organisation for Economic Cooperation and Development (2012b). The Adverse Outcome

Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins; Part 2: Use of the AOP to Develop Chemical Categories and Integrated Assessment and Testing Approaches Series on Testing and Assessment No.168 ENV/JM/MONO(2012)10/PART2. Paris.

UN, United Nations (2011). Globally Harmonised System of Classification and Labelling of Chemicals (GHS). Part 3: Health and Environmental Hazards. New York, NY, USA, and Geneva, Switzerland.

4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does ECVAM require advice on the current issue?*	The opinion of ESAC should support EURL ECVAM with respect to the development of an EURL ECVAM recommendation on the h-CLAT assay outlining (1) the scientific basis of the assay, (2) its overall performance as assessed during the study and based on other (e.g. published) information, (3) its applicability and limitations. Furthermore, the advice of ESAC should support ECVAM with respect to the analysis of possible data gaps that need to be addressed in view determining the test method's potential use and usefulness within integrated approaches for skin sensitisation hazard and risk assessment.

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 study was conducted appropriately in view of the objective of the study. The study objective was to assess (1) the reproducibility of the h-CLAT method within one laboratory (WLR) (2) its transferability to other laboratories (3) its reproducibility between laboratories (BLR) (4) Furthermore, the study aimed at assessing, in a preliminary manner, the predictive capacity of the test method for distinguishing between sensitisers and non-sensitisers and, where possible, to appraise its potential to contribute to a further sub-categorisation of sensitisers into two subcategories (1A and 1B). When reviewing the design and conduct of the study, the following issues should be addressed in particular: (a) Clarity of the test definition (module 1) (b) Clarity of the definition of the study objective and study management (c) Appropriateness of the study design & execution in view of the study objectives, *inter alia:* ○ Is the **number of tested chemicals (24)** 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? (d) Appropriateness of the **study execution** (e.g. were there pre-defined test acceptance criteria, were these respected? How were exceptions / deviations handled? Were provisions specified for retesting? Was the number of repetitions sufficient? etc.) (e) Appropriateness of the **statistical analysis** used for analysing WLR, transferability, BLR and (preliminary) predictive capacity. 2) **CONCLUSIONS OF STUDY:** The ESAC is requested to assess whether the conclusions, as presented in the Validation Study Report, are substantiated by the information generated in the study and are plausible with respect to existing information and current views (e.g. literature). In particular: (a) Are the conclusions on **reproducibility** (WLR and BLR) as well as transferability justified and plausible? (b) Are the conclusions on preliminary **predictive capacity** justified and plausible with respect to existing information (c) 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)? (d) Do the data generated with this defined set of chemicals **together** with available existing data provide sufficient information on the **applicability and possible limitations** of the test method, in particular in view of its potential use within an ITS for sensitisation? 3) **SUGGESTED USE OF THE TEST METHOD:** The ESAC is requested (a) to evaluate, on the basis of the data summarised in the validation study report, the possible use of the test 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, technical limitations etc.) and (c) to identify possible further information required (i.e. are there gaps) to be able to determine the potential use and usefulness of the test method within integrated approaches.

4.3 TIMELINES

Timelines concerning this request *When does ECVAM require the advice?*	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	4Q 2013 (probably through written procedure)
	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	YES Final request presented at ESAC 38, 18/19 June 2013

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. However, no WG needs to be established, as EURL ECVAM has taken the decision to employ the existing *ESAC WG "Sensitisation"* (set up in 2011) also for the h-CLAT review. The WG has already prepared detailed reviews/draft opinions on the DPRA and the Keratinosens test methods. This will add consistency to the review of these three sensitisation test methods and expedite progress as, at the time of issuing this request (June 2013), the VSR is already available and the WG can therefore commence with the review work. **Present ESAC WG:** • Dr. Erwin ROGGEN (ESAC member, Chair of ESAC WG and rapporteur; *3Rs Management and Consultancy, Denmark*) • Prof. A. Wallace HAYES (external expert; *Harvard University, USA*) • Dr. Maja ALECSIC (external expert, *Unilever, UK*) • Dr. Emanuela CORSINI (external expert;

		Dipartimento di scienze farmacologiche e biomoleculari, Università Degli Studi di Milano, Italy) • Dr. David LOVELL (external expert; *University of Surrey, UK*) • Dr. Michael WOOLHISER (external expert; *Dow Chemical Company, USA*) • Prof. Yong HEO (external expert, ICATM nomination (KoCVAM)*; College of Natural Sciences, Catholic University of Deagu, South Korea)*
	S3 Invited Experts	
	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):	

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	
	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	Already available? (YES/NO)	File name
0	EURL ECVAM Validation Study Report	YES	h-CLAT Validation Study Report.pdf
2	Appendices 1-15 to EURL ECVAM Validation Study Report	YES	h-CLAT appendices to VSR.pdf
3	EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification	YES	EURL ECVAM strategy .pdf
4	OECD Report: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1	YES	OECD AOP-part1.pdf
5	Publication: Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound	YES	dos Santos 2009.pdf
6	Publication: A Comparative Evaluation of In Vitro Skin Sensitisation Tests: The Human Cell-line Activation Test (h-CLAT) versus the Local Lymph Node Assay (LLNA)	YES	Ashikaga 2010.pdf
7	Publication: Predicting skin sensitization potential and inter-laboratory reproducibility of a human Cell Line Activation Test (h-CLAT) in the European Cosmetics Association (COLIPA) ring trials	YES	Sakaguchi 2010.pdf
8	Publication Predictive performance for human skin sensitizing potential of the human cell line activation test (h-CLAT)	YES	Nukada 2011
9	Publication: Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency	YES	Nukada 2012a.pdf
10	Publication: Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals	YES	Nukada 2012b.pdf
11	Publication: Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients	NO Will be made available as soon as possible	Takenouchi et al.- Submitted for publication

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 38th meeting on 18/19 June 2013 the ESAC plenary decided to employ the ESAC Working Group "Sensitisation" for preparing a detailed scientific review of the study on the h-CLAT test method for skin sensitisation testing.

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 WG is requested to conduct a scientific review of the ECVAM-coordinated validation study focusing on an assessment of reliability of the h-CLAT test method. 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) 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 ESAC Chair and the ESAC Coordinator 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	***July 2013***	1. Discussion of the mandate and first appraisal of the VSR. 2. Agreement on further timelines and possible work distribution	
2	***Friday 20. September 2013***	Forwarding of initial	

		observations (within ESAC WG template) to ECVAM	
3	***1 & 2 October 2013***	ESAC WG meeting at JRC campus in Ispra, Italy	Draft ESAC WG report
4	***End of November/mid December 2013***	Forwarding final report to ESAC Chair and ESAC Coordinator	Final report, adopted by WG

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WG

The ESAC WG is requested to address the **questions posed to the ESAC** which have been broken down further in more **specific questions** (see section 4.2).

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. The template allows moreover for addressing the issues specific studies outlined in section 4.2. The Coordinator will provide guidance if necessary.

European Commission
EUR 27022 EN – Joint Research Centre – Institute for Health and Consumer Protection

Title: **EURL ECVAM Recommendation on the human Cell Line Activation Test (h-CLAT) for skin sensitisation testing**

Author(s): European Union Reference Laboratory for Alternatives to Animal Testing

Luxembourg: Publications Office of the European Union

2015 – 40 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424 (online)

ISBN 978-92-79-44706-8 (PDF)

doi:10.2788/29986

LB-NA-27022-EN-N

JRC Mission

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Serving society
Stimulating innovation
Supporting legislation

doi:10.2788/29986

ISBN 978-92-79-44706-8

Publications Office