ESAC Opinion on the BASF-coordinated Performance Standards-based validation of the LuSens test method for skin sensitisation testing

2016

EURL ECVAM
SCIENTIFIC
ADVISORY
COMMITTEE
(**ESAC**)

ESAC OPINION

on the

BASF-coordinated Performance Standards-based validation of the LuSens test method for skin sensitisation testing

ESAC Opinion No.	2016-04
Relevant ESAC Request No.	2016-04
Date of Opinion	24/06/2016

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Abstract

ESAC, the EURL ECVAM Scientific Advisory Committee, advises EURL ECVAM on scientific issues. Its main role is to conduct independent peer review of validation studies of alternative test methods and to assess their scientific validity for a given purpose. The committee reviews the appropriateness of study design and management, the quality of results obtained and the plausibility of the conclusions drawn. ESAC peer reviews are formally initiated with a EURL ECVAM Request for ESAC Advice, which provides the necessary background for the peer-review and establishes its objectives, timelines and the questions to be addressed. The peer review is normally prepared by specialised ESAC Working Groups. These are typically composed of ESAC members and other external experts relevant to the test method under review. These experts may be nominated by ESAC, EURL ECVAM and partner organisations within the International Cooperation on Alternative Test Methods (ICATM). ESAC ultimately decides on the composition of these Working Groups. ESAC's advice to EURL ECVAM is formally provided as 'ESAC Opinions' and 'Working Group Reports' at the end of the peer review. ESAC may also issue Opinions on other scientific issues of relevance to the work and mission of EURL ECVAM but not directly related to a specific alternative test method.

The ESAC Opinion expressed in this report relates to the peer-review of the BASF-coordinated Performance Standards-based validation of the LuSens test method for skin sensitisation testing.

Ispra, 24 June 2016

ESAC Opinion

In April 2016, the EURL ECVAM Scientific Advisory Committee (ESAC) (Annex 1) received from EURL ECVAM a request for scientific advice on the BASF-coordinated Performance Standards-based validation of the LuSens test method for skin sensitisation testing (Annex 2). ESAC established a working group (WG) (Annex 1) which delivered an ESAC WG report dated 6 June 2016 (Annex 3).

At its 42nd meeting, held on the 9th and 10th June 2016 at EURL ECVAM, Ispra, Italy, the non-Commission members of ESAC unanimously endorsed the following statement which was based on the ESAC WG report:

The replacement of traditional animal-based test methods by alternative ones should ideally be one-to-one replacements. However, the replacement of traditional animal-based test methods for skin sensitisation by animal-free approaches is likely to require an Integrated Approach to Testing and Assessment (IATA). This combines reliable and mechanistically relevant test methods addressing the currently recognised key events in the adverse outcome pathway for skin sensitisation induction (OECD, 2012; OECD, 2016a, b).

Currently, three animal-free test methods have been validated becoming OECD Test Guidelines (TG). The DPRA (TG 442C) provides information about the capacity of the chemical to react with cysteine and/or lysine groups (key event 1). The KeratinoSens™ test method (TG 442D) addresses skin inflammation by assessing the impact of a substance on ARE mediated Nrf2 activation (key event 2). The h-CLAT (TG 442E) addresses key event 3 by measuring the up-regulation of cell membrane markers CD86 and CD54 to predict a skin sensitisation potential.

The test method under evaluation (LuSens) addresses key event 2 by measuring ARE mediated activation of luciferase used as a surrogate for the Nrf2 gene. This test method is therefore very similar to the KeratinoSens™ method.

The validation study of the LuSens method was performed according to the existing guidelines for a formal inter-laboratory study using Performance Standards (OECD, 2005; OECD, 2015). The LuSens test method was apparently easily transferred and showed very good within- and between-laboratory reproducibility (100 % concordance in both cases). In contrast, the LuSens scored both methyl salicylate and eugenol differently compared to KeratinoSens™ and, with a sensitivity of 92 %, a specificity of 75 % and an accuracy of 85 %, it did not comply with one of the Performance Standards requirements: sensitivity ≥ 80 %, specificity ≥ 80 % and accuracy ≥ 80 %.

Careful analysis of the individual run data for methyl salicylate and eugenol, however, showed that both sensitiser and non-sensitiser predictions occurred with either KeratinoSens™ or LuSens (Annex 2, Table 1).

On the basis of 18 out of 20 reference substances being classified correctly, and the two discordant substances being borderline substances (Kolle et al., 2013), ESAC cannot see any scientific reason for choice of one method over the other, suggesting informed test method selection may be based more on price and availability. The LuSens test developers agreed to specify on their web site that the cell line, as well as its quality assessment, are available free of charge.

ESAC concludes that the LuSens test method, like the KeratinoSens™ test method, is ready to be considered for regulatory use in the context of an IATA for skin sensitisation and remains a useful tool for screening and early decision making during product development, within the applicability domain defined in the validation study.

Of note, the ESAC also makes general recommendations on Performance Standards in a separate Opinion (ESAC, 2016), which are relevant to the current OECD Performance Standards on *in vitro* skin sensitisation ARE-Nrf2 Luciferase test methods (OECD Series on Testing and Assessment No. 213; OECD, 2015).

References

- ESAC (2016). ESAC Opinion on the use of Performance Standards to evaluate test methods similar to a Validated Reference Method. ESAC Opinion 2016-06 of 24 June 2016.

- Kolle SA, Basketter DA, Casati S, Stokes WS, Strickland J, van Ravenzwaay B, Vohr H-W, Landsiedel R (2013). Performance standards and alternative assays: Practical insights from skin sensitization. *Regulatory Toxicology and Pharmacology* 65: 278–285.

- OECD (2005). Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. Series on Testing and Assessment No. 34, OECD, Paris.

- OECD (2012). The adverse outcome pathway for skin sensitisation initiated by covalent binding to proteins. Part 1: Scientific evidence. Series on Testing and Assessment No. 168, OECD, Paris.

- OECD (2015). Performance Standards for assessment of proposed similar or modified *in vitro* skin sensitisation ARE-Nrf2 Luciferase test methods. Series on Testing and Assessment No. 213, OECD, Paris.

- OECD (2016a). Guidance Document on the reporting of Defined Approaches to be used within Integrated Approaches to Testing and Assessment. ENV/JM/MONO(2016)28. Series on Testing and Assessment No. 255, OECD, Paris.

- OECD (2016b). Guidance Document on the reporting of Defined Approaches and individual information sources to be used within Integrated Approaches to Testing and Assessment (IATA) for skin sensitisation. ENV/JM/MONO(2016)29. Series on Testing and Assessment No. 256, OECD, Paris.

Annex 1

COMPOSITION OF ESAC AND ESAC WORKING GROUP

Composition of ESAC and ESAC Working Group

EURL ECVAM Scientific Advisory Committee (ESAC)

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

ESAC Working Group (WG)

- Dr. Erwin L. ROGGEN (ESAC Member, WG Chair)
- Prof. Annette KOPP-SCHNEIDER (ESAC Member)
- Dr. David BASKETTER (EURL ECVAM nomination)
- Dr. Steve ENOCH (EURL ECVAM nomination)
- Prof. Yong HEO (Catholic University of Daegu; ICATM nomination by KoCVAM)
- Dr. Reiko ADACHI (NIHS; ICATM nomination by JaCVAM)

EURL ECVAM (Secretariat)

- Dr. João BARROSO (ESAC Coordinator)
- Dr. Silvia CASATI
- Prof. Maurice WHELAN (Head of Unit)

Annex 2

EURL ECVAM REQUEST FOR ESAC ADVICE

EUROPEAN COMMISSION

DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

ESAC Request 2016-04

EURL ECVAM Scientific Advisory Committee (ESAC)

EURL ECVAM REQUEST FOR ESAC ADVICE

on the

BASF-coordinated Performance Standards-based validation of the LuSens test method for skin sensitisation testing

Title page information	
Abbreviated title of ESAC request	PS-based validation of LuSens
ESAC REQUEST No.	2016-04
Template used for preparing request	EP 3.02
Date of finalising request	31/03/2016
Date of submitting request to ESAC	01/04/2016
Request discussed through	Written procedure previous to ESAC 42
Opinion expected at (date)	ESAC 42 (June 2016)
File name of this request	**ER2016-04_ESAC_REQUEST_LuSens.doc**

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1.   TYPE OF REQUEST

Request Type	Identify request ("YES")	
R1 ESAC Peer Review of a Prevalidation Study or Validation Study	**YES, external validation study (i.e. not coordinated by EURL ECVAM)**	
If R1)applies please specify further:		
►Prevalidation Study	**NO**	
►Prospective Validation Study	NO	
►Retrospective Validation Study	**NO**	
►Validation Study based on Performance Standards	**YES** The validation study is based on the OECD Performance Standards No. 213: "Performance Standards for Assessment of Proposed Similar or Modified In Vitro Skin Sensitisation ARE-NRF2 Luciferase Test Methods (PS) (OECD 2015a)". The validation study was performed by the test method developer.	
R2 Scientific Advice on a test method submitted to EURL ECVAM for validation (e.g. the test method's biological relevance etc.)	**NO**	
R3 Other Scientific Advice (e.g. on test methods, their use; on technical issues such as cell culturing, stem cells, definition of performance standards etc.)	**NO**	

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

BASF PS-based validation of the LuSens assay for skin sensitisation testing

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

1) Background to skin sensitization and current regulatory adopted tests

Skin sensitisation is the toxicological endpoint associated with substances that have the intrinsic ability to cause 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 chemicals including cosmetic ingredients. Traditional regulatory predictive tests for skin sensitisation rely on the use of animals. These include: the guinea-pig tests (Buehler Test and Guinea-pig Maximisation Test) (OECD TG 406), the murine Local Lymph Node Assay (LLNA, OECD TG 429) and its non-radio-isotopic variants (OECD TG 422a and 422b).

In recent years progress has been made in the development and regulatory adoption of alternative methods for skin sensitization hazard identification. Two methods validated and/or peer-reviewed by EURL ECVAM, the direct peptide reactivity assay (DPRA) and the KeratinoSens™ have been adopted by the OECD as Test Guidelines 442C and 442D respectively. A third method, the human Cell Line Activation Test is in the final stages of the OECD adoption process at the time of writing of this request.

Methods developed in the area are addressing key chemical/biological mechanisms leading to the acquisition of skin sensitization. Such mechanisms have been described in the form of an Adverse Outcome Pathway (AOP; OECD 2010a, 2010b). Within the skin sensitisation AOP the molecular initiating event (i.e. the first key event - KE) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (LLNA).

The LuSens is proposed to address KE2 of the AOP. As for the other skin sensitization test methods evaluated by EURL ECVAM and adopted by the OECD, also the LuSens does not have the potential to function as a full-replacement stand-alone method. 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. A defined approach to testing and assessment combining LuSens data with other information has been developed (Urbisch et al., 2015; Ramirez et al, 2014 and Bauch et al., 2012).

2) The LuSens test method

The LuSens Assay is an in vitro reporter gene assay that is used to support the assessment of the sensitizing potential of a substance. It utilizes human skin cells harboring the luciferase reporter gene, to detect the induction of the cytoprotective responses elicited by the genes controlled by antioxidant response element; these can be activated by electrophilic stress by reactivity with

cysteine residues in the respective protein. Keratinocytes are the first cells exposed to a substance by skin penetration. The keap1 protein contains cystein residues which have been shown to be highly susceptible to haptenization by electrophilic sensitizers (see also the DPRA and KeratinoSens[TM] assays). In the presence of a hapten, the transcription factor Nrf2, which lies associated with keap1 in the cytoplasm, dissociates from keap1 and translocates to the nucleus. Nrf2 binds to the ARE response elements and activates the transcription of various downstream protective genes, e.g. glutathione (GSH). The keap1 protein therefore constitutes an intracellular sensor protein for 'electrophilic stress' by cystein reactive substances. The LuSens assay uses this pathway to identify sensitizers which cause electrophilic stress in keratinocytes by coupling the ARE-response element to a luciferase gene. The luciferase activity triggered after 48 h incubation with a test substance is an indicator for the activation of the Keap1/Nrf2/ARE signaling pathway (Ade et al. 2009, Natsch 2011, Natsch & Emter 2008, Vandebriel et al. 2010). The 'activation' of keratinocytes is the key step 2 of the AOP of skin sensitization. The LuSens is considered a similar method to the validated and regulatory adopted KeratinoSens[TM] (TG 442D, OECD 2015b; EURL ECVAM, 2014).

3) History of submissions
The optimised test method was pre-submitted to EURL ECVAM in 2011. Following the assessment of this submission, EURL ECVAM requested additional information on the test method in order to be able to evaluate whether it qualified to proceed to step 2 of the submission process. This information was provided by the test submitter in January 2012 and reviewed by EURL ECVAM. Following this assessment, the test submitter was invited to proceed to step 2 of the EURL ECVAM submission process. The full submission was received by EURL ECVAM in March 2015. Subsequent to the evaluation of the LuSens TST, EURL ECVAM requested additional information/clarifications to be provided. The final TST was received by EURL ECVAM in December 2015.

The 2015 full submission concerns a PS-based validation study conducted to fulfil the requirements laid down in the OECD Performance Standards (PS) for demonstrating comparable performance to those of the validated KeratinoSens™ (Ramirez et al., 2016) and adherence to the essential test methods components to demonstrate similarity with the validated method (KeratinoSens™). In addition to the data generated during the PS-based validation study, the submission reports historical data for an additional set of chemicals.

The reproducibility (100%) within- and between-laboratories of the LuSens test method exceeded the criteria for reproducibility defined in the OECD PS. The LuSens also exceeded the 80% PS target values for the overall accuracy and sensitivitiy (LuSens, 85% accuracy and 92% sensitivitiy; actual for KeratinoSens™ based on the 20 reference substances 87.0% accuracy, 86.7% sensitivity) but failed to meet the 80% target value for the specificity (LuSens 75%; actual for KeratinoSens™ 87.5%). As requested by the PS the accuracy values have been derived using a weighted calculation.

Test substance	*In vivo* categories (OECD, 2015)	LuSens (Overall No of positive experiments - validation study)	KeratinoSens™ (Overall No of positive experiments - interlaboratory study)
4-methoxyacetophenone	Non-sensitiser	9/9	POS (No not available)
Glycerol	Non-sensitiser	0/11	0/19
Isopropanol	Non-sensitiser	0/11	0/17
Salicylic acid	Non -sensitiser	0/11	0/17
Chlorobenzene	Non-sensitiser	0/3	1/17
Lactic acid	Non-sensitiser	0/3	1/19
Methylsalicylate	Non-sensitiser	3/3	3/17
Sulfanilamide	Non-sensitiser	0/3	1/17
Ethylene glycol dimethacrylate	Sensitiser (weak)	9/9	17/17
Phenyl benzoate	Sensitiser (weak)	0/9	2/19
Citral	Sensitiser (moderate)	9/9	17/17
Isoeugenol	Sensitiser (moderate)	9/9	19/19
Methyldibromo glutaronitrile	Sensitiser (strong)	11/11	17/17
para-phenylenediamine	Sensitiser (strong/extreme)	9/9	17/17
2,4-dinitrochlorobenzene	Sensitiser (extreme)	11/11	17/17
4-nitrobenzylbromide	Sensitiser (extreme)	9/9	17/17
Eugenol	Sensitiser (weak)	3/3	9/19
2-Mercaptobenzothiazole	Sensitiser (moderate)	3/3	19/19
4-methylaminophenol sulfate	Sensitiser (strong)	3/3	17/17
Oxazolone	Sensitiser (extreme)	3/3	19/19

For the same set of PS substances the LuSens and the KeratinoSens™ assays overall correctly predicted 17 of the total of 20 test substances and wrongly predicted 3 test substances. Whereas the LuSens assay led to two false positive predictions (4-methoxy-acetophenone and methyl salicylate) and one false negative (phenyl benzoate), the KeratinoSens™ assay led to one false positive (4-methoxy-acetophenone) and two false negative predictions (eugenol and phenyl benzoate). The only false negative prediction of the LuSens assay is a weak sensitizer.

Conclusions of the Test Submitter on the information submitted to EURL ECVAM

According to the test developer (BASF) the information submitted to EURL ECVAM for entering the peer-review process indicates that the LuSens assay can be transferred to naïve laboratories having experience in cell culture without on-site training. It has demonstrated to provide comparable accuracy to the KeratinoSens™ assay, whereby, showing remarkable reproducibility of 100%. A systematic comparison, using the same set of test substances, among the LuSens and KeratinoSensTM assays has demonstrated good concordance. A concordance of 94% was reached for 52 test substances for which human data were available and 93% for the 61 test substances for which LLNA data were available (Ramirez et al., 2014). A more recent study has shown that from a similar set of 69 test substances, 61 provided the same overall outcome, resulting in an interchangeability of 88%. When comparing the accuracy of the LuSens assay and the KeratinoSensTM assay for thesame 20 PS of the OECD TG 442D lead to same results. Most importantly, the implementation of the LuSens assay in a testing strategy has demonstrated to also lead to comparable predictions as if the KeratinoSens™ assay would have been integrated in the testing strategy (Ramirez et al., 2014). Therefore, the LuSens has fulfilled to the best the requirements specified in the OECD TG 442D for me-too assay.

References
Ade N, Leon F, Pallardy M, Peiffer JL, Kerdine-Romer S, Tissier MH, Bonnet PA, Fabre I, Ourlin JC. (2009). HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: role of the Keap1/Nrf2 pathway. Toxicol Sci. Feb;107(2):451-60.

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. Aug;63(3):489-504.

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Natsch A, Caroline B, Leslie F, Frank G, Kimberly N, Allison H, Heather I, Robert L, Stefan O, Hendrik R, Andreas S, Roger E. (2011). The intra- and inter-laboratory reproducibility and predictivity of the KeratinoSensTM assay to predict skin sensitizers in vitro: results of a ring-study in five laboratories. Toxicol In Vitro. 25, 733-44.

Natsch A, Emter R. (2008). Skin sensitizers induce antioxidant response element dependent genes: application to the in vitro testing of the sensitization potential of chemicals. Toxicol Sci. Mar;102(1):110-9.

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. 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 http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm

OECD 2015a. Performance Standards for assessment of proposed similar or modified in vitro skin sensitisation ARE-NrF2 luciferase test methods. Series on Testing and Assessment No. 213

OECD 2015b. Test Guideline 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method

Ramirez T, Mehling A, Kolle SN, Wruck CJ, Teubner W, Eltze T, Aumann A, Urbisch D, van Ravenzwaay B, Landsiedel R. (2014). LuSens: a keratinocyte based ARE reporter gene assay for use in integrated testing strategies for skin sensitization hazard identification. Toxicol In Vitro. Dec;28(8):1482-97.

Ramirez T, Stein N, Aumann A, Remus T, Edwards A, Norman KG, Ryan C, Bader JE, Fehr M, Burleson F, Foertsch L, Wang X, Gerberick F, Beilstein P, Hoffmann S, Mehling A, van Ravenzwaay B, Landsiedel R. (2016). Intra- and inter-laboratory reproducibility and accuracy of the LuSens assay: A reporter gene-cell line to detect keratinocyte activation by skin sensitizers. Toxicol In Vitro. Apr;32:278-86.

Urbisch D, Mehling A, Guth K, Ramirez T, Honarvar N, Kolle S, Landsiedel R, Jaworska J, Kern PS, Gerberick F, Natsch A, Emter R, Ashikaga T, Miyazawa M, Sakaguchi H. (2015). 5Assessing skin sensitization hazard in mice and men using non-animal test methods. Regul Toxicol Pharmacol. Mar;71(2):337-51.

Vandebriel RJ, van Loveren H (2010). Non-animal sensitization testing: state-of-the-art. Crit Rev Toxicol. May;40(5):389-404.

4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does EURL ECVAM require advice on the current issue?*	The opinion of ESAC should conclude on the quality of the submitted Performance Standards-based validation study on LuSens, which addressed the reliability (transferability, within and between laboratory reproducibility) and the predictive capacity of the method. The opinion should conclude on the equivalence of the performance of the LuSens to that of the validated reference method as outlined in the Performance Standards. Overall, the ESAC opinion, based on the review of the submitted study dossier, should conclude on the adequacy of the LuSens to support the regulatory assessment of skin sensitisation.

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 the following parameters in comparison to respective criteria outlined in the Performance Standards: 1. the similarity of the method to the validated reference method 2. the reproducibility of the method within laboratories (WLR) 3. its transferability to other laboratories 4. its reproducibility between laboratories (BLR) 5. whether the predictive capacity of the method considering the validation study results and historical data is acceptable 6. whether the overall performance of the method supports its use for the regulatory assessment of skin sensitisation 7. whether the OECD Performance Standards No. 213 (OECD 2015a) are adequate for the assessment of proposed similar or modified *in vitro* skin sensitisation ARE-NrF2 luciferase test methods

4.3 TIMELINES

Timelines concerning this request *When does EURL ECVAM require the advice?*	Timeline	Indication
	Finalised ESAC Opinion required by:	June 2016
	Request to be presented to ESAC by written procedure (e.g. <u>due to urgency</u>) prior to the next ESAC	YES
	Request to be presented to ESAC at ESAC plenary meeting	NO

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

5.1 EURL ECVAM PROPOSAL REGARDING REQUEST-RELATED STRUCTURES REQUIRED

Specific structures required within ESAC to address the request	Structure(s) required	Required according to EURL ECVAM? (YES/NO)
Does the advice require an ESAC working group, an ESAC rapporteur etc.?	**S1 ESAC Rapporteur**	NO
	S2 ESAC Working Group	**ESAC members** - Erwin Roggen (Chair) - Annette Kopp-Schneider **EURL ECVAM nominations** - David Basketter (DABMEB Consultancy) - Steven Enoch (Liverpool John Moores University) **ICATM nominations** - Joanna Matheson (CPSC; nominated by ICCVAM) - Yong Heo (Catholic University of Daegu; nominated by KoCVAM) - Reiko Adachi (NIHS; nominated by JaCVAM)
	S3 Invited Experts	NO
	Ad S3: If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	
	If other than above (S1-S3):	

5.2   DELIVERABLES AS PROPOSED BY EURL ECVAM

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

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

Count	Description of document	Already available? (YES/NO)	File name
1	Test Guideline 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method	YES	OECD TG442D 2015.pdf
2	Performance Standards for assessment of proposed similar or modified in vitro skin sensitisation ARE-NrF2 luciferase test methods. Series on Testing and Assessment No. 213	YES	OECD STA213 2015.pdf
3	Test Submission on the LuSens	YES	TST LuSens _Final_291215 (obsolete).pdf TST LuSens _Final_270416.pdf
4	BASF reply to the EURL ECVAM comments	YES	Reply to ECVAM concerns on the Test Submission LuSen_291215.pdf
5	Final EURL ECVAM assessment report	YES	TM2011_10_LuSens updated assessment report.pdf
6	LuSens protocol	YES	Attachment 1a_LuSens_Protocol_version 24122015.pdf
7	LuSens INVITTOX protocol	YES	Attachment 1b_INVITTOX_Final_291215.pdf
8	Test items used to develop and optimise the test method	YES	Attachment 2_Test Chemicals_LuSens.pdf
9	Test items used to assess WLR	YES	Attachment 3_Test Chemicals_WLR_LuSens_inter-lab validation.pdf
10	Data used to assess WLR	YES	Attachment 4_WLR_Ramirez_2014.pdf See also attachments 18 (statistical report)
11	Test items used to assess transferability	YES	Attachment 5_REVISED_List of test items_Transferability_Final.pdf
12	Training protocol	YES	Attachment 6_LuSens_Protocol_Final_13.06.13.pdf
13	Transfer protocol	YES	Attachment 7_LuSens Protocol_26.07.13.pdf

14	Transfer report	YES	Attachment 8a_LuSens_Transferability Report.pdf Attachment 8b_LuSens_Transferability Report_non-blinded.pdf
15	Test items used for BLR assessment	YES	Attachment 9_REVISED_Test Chemicals_BLR_LuSens_inter-laboratory validation_Final.pdf
16	Data used for BLR and Predictive Capacity assessment	YES	See attachments 18 (statistical report)
17	Project plans	YES	Attachment 13a_LuSens Study Plan 180613_Final.pdf Attachment 13b_REVISED_LuSens Experimental Design 150325 final.pdf
18	Scientific papers mentioned in the TST	YES	Attachment_17.pdf
19	Statistical reports	YES	Attachment 18a_Statistical Report LuSens catch-up validation labs coded final 150320.pd Attachment 18b_Statistical Report LuSens catch-up validation final 150320.pdf Attachment 18c_Important Note_Phase II testing_230414. Annex A6 to Attachment 18_241215.pdf Attachment 18d_Data analysis_Template_LuSens_unprotected.xlsx

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

The ESAC unanimously agreed by written procedure on the 2nd of March 2016 on the composition of a new ESAC Working Group for the review of test methods in the area of skin sensitisation.

7.2 TITLE OF THE ESAC WORKING GROUP

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

ESAC WG Sensitisation

7.3 MANDATE OF THE ESAC WORKING GROUP

The EWG is requested to conduct a scientific review of the LuSens PS-based validation study. The review needs to address the questions put forward to ESAC by EURL ECVAM.

The review should focus on the quality of the submitted PS-based validation study on LuSens, which addressed the reliability (transferability, within and between laboratory reproducibility) and the predictive capacity of the method. The opinion should conclude on the equivalence of the performance of the LuSens to that of the validated reference method as outlined in the PS.

7.4 DELIVERABLES OF THE ESAC WORKING GROUP

The ESAC WG is requested to deliver to the chair of the ESAC and the ESAC Coordinator a detailed **ESAC Working Group Report** outlining its analyses and conclusions and **a draft ESAC Opinion.** 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 WORKING GROUP

Item	Proposed date/time	Action	Deliverable
1	17-19 May 2016	Working Group meeting	Draft ESAC WG report and draft ESAC opinion
2	27 May 2016	Circulation of final WG report and draft ESAC opinion to ESAC	Final draft ESAC WG report and draft ESAC opinion
3	9-10 June 2016	Endorsement of WG report and ESAC opinion at ESAC42 meeting	Final ESAC WG report and ESAC opinion

7.6   QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WORKING GROUP

The ESAC WG is requested to address the **questions posed to the ESAC** which have been broken down further in more **specific questions** by the ESAC chair, the chair of the ESAC WG and the Secretariat (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 EURL 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 Secretariat will provide guidance if necessary.

APPENDIX 1   REPORTING TEMPLATE

The appended ESAC WG template suggests a structure that is in close agreement with the EURL ECVAM information requirements ("modules") for scientific review following validation and allows at the same time for the description of the analysis and conclusions concerning more specific questions.

The template can be used for various types of validation studies (e.g. prospective full studies, retrospective studies, performance-based studies and prevalidation studies). Depending on the study type and the objective of the study, not all sections may be applicable.

However, for reasons of consistency and to clearly identify which information requirements have not been sufficiently addressed by a specific study, this template is uniformly used for the evaluation of validation studies.

The current template is

TEMPLATE_ESAC-WG_REPORT-v6.doc

Annex 3

ESAC WORKING GROUP PEER REVIEW CONSENSUS REPORT

EURL ECVAM
SCIENTIFIC
ADVISORY
COMMITTEE
(**ESAC**)

ESAC Working Group Peer Review Consensus Report

on the

BASF-coordinated Performance Standards-based validation of the LuSens test method for skin sensitisation testing

Title page information	
File name	ESAC_WG_Report_LuSens.doc
Abbreviated title of ESAC request	PS-based validation of LuSens
Relating to ESAC REQUEST No.	2016-04
Request discussed through	Written procedure previous to ESAC 42
Report to be handed over to ESAC Chair and EURL ECVAM Coordinator by	Erwin L. Roggen
Version tracking	

Date	Version	Author(s)	Description
19 May 2016	V1.0	ESAC WG	First draft agreed by the ESAC WG
27 May 2016	V2.0	ESAC WG	Second revised draft after commenting
01 June 2016	V3.0	ESAC WG	Third revised draft after commenting
06 June 2016	V4.0	ESAC WG	Final ESAC WG draft sent to ESAC for endorsement

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ESAC Working Group

This report was prepared by the "ESAC Working Group for Skin Sensitisation" (ESAC WG SS), charged with conducting a detailed scientific peer review of on the BASF-coordinated Performance Standards-based validation of the LuSens test method for skin sensitisation testing.

The ESAC WG had the following members:

ESAC members
- Erwin Roggen (Chair)
- Annette Kopp-Schneider

EURL ECVAM nominations
- David Basketter (DABMEB Consultancy)
- Steven Enoch (Liverpool John Moores University)

ICATM nominations
- Yong Heo (Catholic University of Daegu; ICATM nomination by KoCVAM)
- Reiko Adachi (NIHS; ICATM nomination by JaCVAM)

ESAC Coordination:
- João Barroso (ESAC Coordinator)
- Silvia Casati

Abbreviations used in the document

- **BLR**   Between-laboratory reproducibility
- **CI**   Confidence Interval
- **EURL ECVAM**   European Union Reference Laboratory for Alternatives to Animal Testing
- **ESAC**   EURL ECVAM Scientific Advisory Committee
- **ESAC WG**   ESAC Working Group
- **GLP**   Good Laboratory Practice
- **IATA**   Integrated Approach to Testing and Assessment
- **PS**   Performance Standards
- **SOP**   Standard Operating Procedure (used here as equivalent to 'protocol')
- **VMT**   Validation Management Team
- **WLR**   Within-laboratory reproducibility

1. Study objective and design

1.1 Analysis of the clarity of the study objective's definition

(a) ESAC WG summary of the study objective as outlined in the Test Submission

From the project plan:

Pre-validation of the LuSens test method, in a formal inter-laboratory study according to the OECD guidance document No. 34 (OECD, 2005). The point of reference is the KeratinoSens™ assay.

The primary aim was on its transferability and reproducibility (within and between laboratories) in a multi-laboratory ring trial (goal 1).

The secondary aim was to preliminary evaluate the accuracy of the method in discriminating sensitisers and non-sensitisers (according to UN GHS for classification and labelling of chemicals for skin sensitisation (cat. 1; no cat.) (UN, 2015) and ECR No. 1272/2008 on classification, labelling and packing of substances and mixtures (EC, 2008)) (goal 2).

(b) Appraisal of the clarity of study objective as outlined in the Test Submission

The study objective, as well as both aims, is sufficiently clear to allow persons skilled in the process of validation of cell-based test methods to understand.

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

The LuSens test method is considered to be a potentially important component of the safety evaluation of chemicals. Skin sensitisation assessment represents a standard requirement of chemical legislation (UN GHS for classification and labelling of chemicals for skin sensitisation (cat. 1; no cat.) (UN, 2015) and ECR No. 1272/2008 on classification, labelling and packing of substances and mixtures (EC, 2008)).

The drive behind the development of test method similar to the KeratinoSens™ assay was the need (according to the developers) of a test method free of licensing or any measure of protection. This will be published at the BASF home page.

(a) Analysis of the scientific rationale provided in the Test Submission

The scientific background of the LuSens is identical to the background of the KeratinoSens™ assay, which recently became an OECD test guideline (OECD, 2015a). Both methods exploit the central role of Nrf-2 transcription factor activity in the development of skin sensitisation.

It is well known that cysteine-reactive skin sensitisers activated the Nrf-2/KEAP1 pathway, resulting in the increase in Nrf-2 activity. This activation can be mimicked *in vitro* by employing a reporter gene for luciferase (instead of the Nrf-2 transcription factor) under the control of an antioxidant response element (ARE). The measured endpoint is the upregulation of luciferase activity after 48 hrs of exposure. This sequence of events was outlined by the test submitters in a concise but precise way.

The provided scientific background profiles the LuSens as a test method covering key event 2 of the OECD Adverse Outcome Pathway for Skin Sensitisation (OECD, 2012).

(b) Analysis of the regulatory rationale provided in the Test Submission

The provided regulatory rationale builds largely on the Cosmetics Regulation, and the emergence and application of OECD test guidelines in the context of REACH.

Skin sensitisation assessment represents a standard requirement of chemical legislation (UN GHS for classification and labelling of chemicals for skin sensitisation (cat. 1; no cat.) (UN, 2015) and ECR No. 1272/2008 on classification, labelling and packing of substances and mixtures (EC, 2008)).

1.3 Appraisal of the appropriateness of the study design

Pre-validation of the LuSens test method, in a formal inter-laboratory study according to the OECD guidance document No. 34 (OECD, 2005) and is based on Performance Standards (OECD, 2015b). The point of reference is the KeratinoSens™ assay.

Four laboratories were involved in the study, instead of the required minimum of three.

In Phase I (transferability) 8 non-coded substances were tested. This phase contained the relevant steps: SOP assessment, regular meetings, training.

Moving forward to the next phase the participating laboratories had to comply with two criteria:

1. An 'acceptable' number of valid repetitions, set to be > 70 %;
2. 6/8 substances correctly predicted.

In Phase II (reproducibility) 20 coded reference substances were tested. All the measures required for proper assessment of test method reproducibility were taken: independent test substance distributor (BioTesys) and independent biostatistician (S. Hoffmann), substances of Phase II were provided with an independently generated code by the biostatistician, no communication between the laboratories.

A safety officer was appointed in each laboratory and provided with an envelope containing the data sheets for the blinded substances.

All participating laboratories were GLP compliant. The testing was performed under GLP-like conditions, but without a specific quality assurance officer for each of the laboratories for confirming the quality of the data. This task was performed by the biostatistician who was not GLP accredited.

1.4 Appropriateness of the statistical evaluation

The data were collected to the lead laboratory, transferred into a predefined data analysis template and analysed by S. Hoffmann.

The compliance by the laboratories with the assay and result acceptance criteria was assessed by the biostatistician before analysis of reproducibility and transferability.

The statistical approaches applied were appropriate for the purpose of this Performance Standards-based type of study.

2. Collection of existing data

2.1 Existing data used as <u>reference</u> data

Performance standards of the OECD TG 442D (OECD, 2015a) were employed according to the OECD Series on Testing and Assessment No. 213 – Performance Standards for assessment of proposed similar or modified *in vitro* skin sensitisation ARE-Nrf2 luciferase test methods (OECD, 2015b).

2.2 Existing data used as <u>testing</u> data

See Section 2.1.

2.3 Search strategy for retrieving existing data

See Section 2.1.

2.4 Selection criteria applied to existing data

See Section 2.1.

3. Quality aspects relating to data generated during the study

3.1 Quality assurance systems used when generating the data

See section 1.3.

3.2 Quality check of the generated data prior to analysis

The compliance by the laboratories with the assay and result acceptance criteria was assessed by the biostatistician before analysis of transferability and reproducibility.

4. Quality of data used for the purpose of the study (existing and newly generated)

4.1 Overall quality of the evaluated testing data (newly generated or existing)

Overall, application of the quality and acceptance criteria to the test system seems to assure good quality data.

4.2 Quality of the reference data for evaluating relevance[1]

Performance standards of the OECD TG 442D (OECD, 2015a) were employed according to the OECD Series on Testing and Assessment No. 213 – Performance Standards for assessment of proposed similar or modified *in vitro* skin sensitisation ARE-Nrf2 luciferase test methods (OECD, 2015b).

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

Performance standards of the OECD TG 442D (OECD, 2015a) were employed according to the OECD Series on Testing and Assessment No. 213 – Performance Standards for assessment of proposed similar or modified *in vitro* skin sensitisation ARE-Nrf2 luciferase test methods (OECD, 2015b).

The overall quality of the data is sufficient to draw conclusions on the transferability and reproducibility (within as well as between laboratories).

5. Test definition (Module 1)

The test method is adequately described, and covers the parameters that are relevant for good test performance.

1. The test description specifies the cell line used for the test.

2. The relevance of the endpoints (cell viability and luciferase activity) measured is explained against the background of the available scientific knowledge.

3. Quality and acceptance criteria for untreated cells, as well as positive and negative controls, are described.

4. Acceptance criteria for the results are outlined extensively.

5. The prediction model is clearly formulated.

6. The occurrence of non-qualified tests was calculated to be between 0 % and 22 %. Failure seems often to be related to high cytotoxicity.

6. Test materials

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

See Section 2.1.

6.2 Representativeness of the test items with respect to applicability

See Section 2.1.

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[1] OECD guidance document No. 34 on validation defines relevance as follows: "Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy (concordance) of a test method."

7. Within-laboratory reproducibility (WLR) (Module 2)

7.1 Assessment of repeatability and reproducibility in the same laboratory

- 12 test substances from the Performance Standards for the ARE-Nrf2 luciferase test methods were evaluated coded (OECD, 2015b).

- WLR was based on the concordance of classification of three independent experiments, each consisting of at least two independent repetitions, performed by 3 laboratories.

- The WLR was very good as far as the overall binary classification (Y/N) is concerned (100 %).

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

Overall, the WLR is very good.

8. Transferability (Module 3)

8.1 Quality of design and analysis of the transfer phase

Eight test substances were selected for the transfer phase (Phase I). Four laboratories were involved, of which two had been part of the KeratinoSens™ validation study. One experiment was performed.

The training programme protocol provided was adequate.

For moving forward to Phase II the participating laboratories had to comply with two criteria:

1. An 'acceptable' number of valid repetitions, set to be > 70 %;

2. 6/8 substances correctly predicted.

8.2 Conclusion on transferability to a naïve laboratory / naïve laboratories as assessed by the study

Based on the transferability report and the data of the BLR assessment, the transfer of the test method to the participating laboratories can be considered successful.

9. Between-laboratory reproducibility (BLR) (Module 4)

9.1 Assessment of reproducibility in different laboratories

The BLR was assessed based on twenty reference chemicals recommended in the OECD Performance Standards for assessment of proposed similar or modified *in vitro* skin sensitisation ARE-Nrf2 luciferase test methods (OECD, 2015b).

- 12 test substances were evaluated by 3 laboratories (lead, L1 and L2) in the context of the WLR assessment, in three independent experiments, and eight were tested in 3 laboratories (lead, L3 and L4) in the context of the BLR assessment, in one experiment. L3 and L4 tested also five of the twelve WLR test substances.

- The data revealed a BLR of 100 %.

9.2 Conclusion on between-laboratory reproducibility as assessed by the study

The BLR is very good as far as the overall binary classification (Y/N) is concerned is concerned.

10. Predictive capacity and overall relevance (Module 5)

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

The predictive capacity of LuSens was assessed on the basis of the inter-laboratory validation study (N = 20 reference substances).

Performance Standards based study

The laboratories tested different number of substances (lead: 20, L1: 12, L2:12, L3: 13, L4: 13)

Predictive capacity against all data (KeratinoSens™ data in brackets)

- o Sensitivity: 92 %, 88 %, 88 %, 100 %, 100 % (83.3 %)
- o Specificity: 75 %, 75 %, 75 %, 86 %, 86 % (87.5 %)
- o Accuracy: 85 %, 83 %, 83 %, 92 %, 92 % (85 %)

The differences in predictive performance between the laboratories, in spite of 100 % WLR and BLR, is due to the fact that the laboratories tested different subsets of the 20 reference substances. The increase in sensitivity and specificity was explained by lack of testing of the false negative (phenyl benzoate) and false positive (4-methoxy-acetophenone by laboratories) by L3 and L4.

Additional information on 74 chemicals from an internal BASF study showed a sensitivity of 74 %, specificity of 74 % and accuracy of 74 % compared to the LLNA.

A comparative study performed by EURL ECVAM using 49 chemicals revealed a sensitivity of 64 %, specificity of 69 % and accuracy of 65 % for LuSens, and a similar sensitivity of 73 %, specificity of 69 % and accuracy of 71 % for KeratinoSens™.

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

In terms of specificity the LuSens does not fulfil the Performance Standards requirements (≥ 80 %).

However, eugenol is an 'incorrect' prediction as compared to KeratinoSens™, but a correct prediction as compared to the LLNA. This results in a higher sensitivity of LuSens compared to KeratinoSens™, and well above the required 80 %.

Overall, the accuracy of both test methods at 85 % is the same for the 20 reference substances. On larger sets of substances the performance values drop markedly for both test methods.

Research has shown that borderline substances like methyl salicylate and eugenol may be classified as both sensitisers and non-sensitisers in repeated tests using the same assay (Kolle et al., 2013). Indeed, careful analysis of the individual run data for methyl salicylate and eugenol showed that both sensitiser and non-sensitiser predictions occurred with either KeratinoSens™ or LuSens (Table 1).

Table 1: Overview of the individual runs obtained with LuSens and Keratinosens™ for the 20 reference substances.

Performance Standards Reference Substances		KeratinoSens™					LuSens				
		Lead Lab	Lab 1	Lab 2	Lab 3	Lab 4	Lead Lab	Lab 1	Lab 2	Lab 3	Lab 4
4-Methoxy-acetophenone	**Non-sensiser**	(P-P-P-P)					(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)		
Glycerol	**Non-sensiser**	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(N-N) (N-N) (N-N)	(N-N) (N-N) (N-N)	(N-N) (N-N) (N-N)	(N-N)	(N-N)
Isopropanol	**Non-sensiser**	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(N-N) (*N-P-N*) (N-N)	(N-N) (N-N) (N-N)	(N-N) (N-N) (N-N)	(N-N)	(N-N)
Salicylic acid	**Non-sensiser**	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(N-N) (N-N) (N-N)	(N-N) (N-N) (N-N)	(N-N) (N-N) (N-N)	(N-N)	(N-N)
Chlorobenzene	Non-sensiser	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(*P-N-N*)	(*P-N-N*)			(N-N)	(N-N)
Lactic acid	Non-sensiser	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(*P-N-N*)			(N-N)	(N-N)
Methyl salicylate	Non-sensiser	(N-N-N)	(*P-N-N*)	(N-N-N)	(*P-N-N*)	(*P-N-N*)	(*N-P-P*)			(P-P)	(P-P)
Sulphanilamide	Non-sensiser	(N-N-N)	(N-N-N)	(N-N-N)	(*P-N-N*)	(N-N-N)	(N-N)			(N-N)	(N) (N)
Ethylene glycol dimethacrylate	**Skin sensitiser (weak)**	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)		
Phenyl benzoate	**Skin sensitiser (weak)**	(N-N-N)	(N-N-N)	(N-N-N)	(N-N-N)	(*N-N-P*)	(N-N) (N-N) (N-N)	(N-N-N) (*P-N-N*) (N-N)	(*P-N-N*) (N-N) (N-N)		
Citral	**Skin sensitiser (moderate)**	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)		
Isoeugenol	**Skin sensitiser (moderate)**	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)		
Methyldibromo glutaronitrile	**Skin sensitiser (strong)**	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P)	(P-P)
para-Phenylenediamine	**Skin sensitiser (strong/extreme)**	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)		
2,4-Dinitrochlorobenzene	**Skin sensitiser (extreme)**	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P)	(P-P)
4-Nitrobenzylbromide	**Skin sensitiser (extreme)**	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)	(P-P) (P-P) (P-P)		
Eugenol	Skin sensitiser (weak)	(*P-N-N*)	(*P-N-N*)	(P-P-P)	(P-P-P)	(*P-N-N*)	(*N-P-P*)			(P-P)	(P-P)
2-Mercaptobenzothiazole	Skin sensitiser (moderate)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P)			(P-P)	(P) (P)
4-Methylaminophenol sulphate	Skin sensitiser (strong)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P)			(*P-N-P*)	(P-P)
Oxazolone	Skin sensitiser (extreme)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P-P)	(P-P)			(P-P)	(P-P)

P = Positive run

N = Negative run

( ) = Tested in addition to what is requested in the PS

( ) brackets correspond to an experiment, e.g., for experiment (N-P-P) the final conclusion is P.

In bold face are the 12 reference substances for which, according to the PS, WLR information needs to be generated. BLR information in turn, is required for all 20 reference substances.

Statistical analysis of the agreement between LuSens and KeratinoSens™ based on seven concordant final calls for non-sensitisers, eleven concordant final calls for sensitisers, and two discordant substances reveals a Fleiss' Kappa value of 0.794 (95 %-CI from 0.528 to 1). This Kappa value suggests that both assays show substantial (0.61 ≤ κ ≤ 0.80) if not almost perfect (0.81 ≤ κ ≤ 1.00) agreement.

As already published (Urbisch et al., 2015), LuSens and KeratinoSens™ can be regarded as interchangeable in an integrated/defined approach to testing and assessment. On the basis of 18 out of 20 reference substances being classified correctly, and the two discordant substances being borderline substances (Kolle et al., 2013), the ESAC WG cannot see any scientific reason for choice of one method over the other, suggesting informed test method selection may be based more on price

and availability. The LuSens test developers agreed with the ESAC WG to specify on their web site that the cell line, as well as its quality assessment, are available free of charge.

The ESAC WG concludes that the LuSens test method, like the KeratinoSens™ test method, is ready to be considered for regulatory use in the context of an Integrated Approach to Testing and Assessment (IATA) for skin sensitisation and remains a useful tool for screening and early decision making during product development, within the applicability domain defined in the validation study.

11. Applicability domain (Module 6)

11.1 Appropriateness of study design to conclude on applicability domain, limitations and exclusions

The primary goal of the study was to assess transferability and reproducibility (WLR and BLR) of the test method.

Nevertheless, some potential information on the applicability domain of the test method emerged from the studies discussed in Section 10. Furthermore, LuSens is bound to have the similar strengths and limitations as KeratinoSens™.

11.2 Quality of the description of applicability domain, limitations, exclusions

The proposed applicability domain includes Michael acceptors, Schiff base formers and nucleophilic substitutions.

The ESAC WG disagrees with the inclusion of Schiff base formers into the applicability domain of the method on the basis that this requires an amine-group and not a thiol group. After consultation with the test submitter, it was agreed to define the proposed applicability domain in terms of chemical classes applicable to skin sensitisation, rather than specific mechanistic domains.

Specific limitations:

1. Highly cytotoxic compounds may give rise to non-qualified tests.
2. Low solubility/stability in aqueous solutions.
3. Chemicals interfering with the MTT.
4. Low accuracy with acylating agents.

12. Performance standards (Module 7)

12.1 Adequacy of the proposed Essential Test Method Components

Not applicable.

12.2 Adequacy of proposed Reference Chemicals

Not applicable.

12.3 Adequacy of proposed performance target values

The Performance Standards target values were set to be ≥ 80 % for sensitivity, specificity and accuracy, based on existing KeratinoSens™ performance data.

The WLR (~86 % in one laboratory) and BLR (85 % - 91 % in five laboratories) of the KeratinoSens™ was considered very good (ESAC opinion on a Givaudan-coordinated study on the transferability and reliability of the KeratinoSens™ assay for skin sensitisation testing (2012)). However, it was also highlighted that this test method performs poorly on weak sensitisers: 41 % of the weak and 86 % of the very weak sensitisers were missed. Other research has shown that borderline substances like methyl salicylate and eugenol may be misclassified by the same test method (Kolle et al., 2013).

To eliminate uncertainties concerning the prediction of borderline substances, the ESAC WG recommends that in PS-based assessments, the equivalence of a new method with the reference method(s) is evaluated side by side on the basis of the predictions for the individual reference chemicals instead of comparing sensitivity, specificity and accuracy to pre-specified threshold values.

13. Readiness for standardised use

13.1 Assessment of the readiness for regulatory purposes

The provided data (good WLR, BLR and predictive capacity) suggest that the LuSens test method may be a useful component of a strategy for the regulatory testing and assessment of the skin sensitising potential of chemicals belonging to the applicability domain defined in the validation study.

13.2 Assessment of the readiness for other uses

The provided data (good WLR, BLR and predictive capacity) suggest that the LuSens test method can be used for screening and early decision making during product development.

13.3 Critical aspects impacting on standardised use

Proper training is required.

13.4 Gap analysis

None identified.

14. Other considerations

None.

15. Conclusions on the study

15.1 ESAC WG summary of the results and conclusions of the study

The study was performed according to the existing guidelines for a formal inter-laboratory study using Performance Standards (OECD, 2005; OECD, 2015b). The LuSens test method was easily transferred and showed a very good WLR (100 % concordance) and BLR (100 % concordance).

In terms of specificity, LuSens does not fulfil the Performance Standards requirement (≥ 80 %).

15.2 Extent to which study conclusions are justified by the study results alone

The ESAC WG agrees with the conclusion by the VMT. However, Eugenol is an 'incorrect' prediction as compared to the KeratinoSens™, but a correct prediction as compared to the LLNA. This results in a higher sensitivity of the LuSens compared to KeratinoSens™, well above the required 80 %. Overall, the accuracy of both test methods is the same (85 %) for the 20 reference substances.

15.3 Extent to which conclusions are plausible in the context of existing information

Research has shown that borderline substances like methyl salicylate and eugenol may be misclassified in repeated evaluations using the same test (Kolle et al., 2013). Therefore, on the basis of the data from the 20 reference substances, the ESAC WG concludes that LuSens is relevant for the detection of skin sensitisers, but the low specificity would require its incorporation into a testing strategy, rather than its use as a standalone method.

As already published (Urbisch et al., 2015), LuSens and KeratinoSens™ can be regarded as interchangeable in an integrated/defined approach to testing and assessment. Consequently, the ESAC WG cannot see any scientific reason for choice of one method over the other, suggesting that price and availability are the governing factors. The LuSens test developers agreed with the ESAC WG to specify on their web site that the cell line, as well as its quality assessment, are available free of charge.

The provided data supported by the additional information suggest that the LuSens test method may be useful as a component of an IATA for skin sensitisation or as a tool for screening and early decision making during product development, for the assessment of chemicals belonging to the applicability domain defined in the validation study.

It is recommended that in PS-based assessments, the equivalence of a new method with the reference method(s) is evaluated side by side on the basis of the predictions for the individual reference chemicals instead of comparing sensitivity, specificity and accuracy to pre-specified threshold values.

16. Recommendations

16.1 General recommendations

It is recommended that in PS-based assessments, the equivalence of a new method with the reference method(s) is evaluated side by side on the basis of the predictions for the individual reference chemicals instead of comparing sensitivity, specificity and accuracy to pre-specified threshold values. This would facilitate a direct assessment of the impact on the individual performance values of differences between similar test methods when borderline substances are tested.

16.2 Specific recommendations (e.g. concerning improvement of SOPs)

None.

17. References

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