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Peer Review Report of the Alpha-Sens® Test Method Validation Study

OECD Series on Testing and Assessment

E-mail: ehscont@oecd.org.

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Contact us

OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

E-mail: ehscont@oecd.org

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The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank, Basel, Rotterdam and Stockholm Conventions and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

Foreword

This document contains the Peer review report of the Foetal Bovine Serum (FBS) free ARE Nrf2 Luciferase α-Sens® test method, a me-too method of the KeratinoSens™ assay, an in vitro method for identifying the skin sensitisation potential of chemicals.

The scientific validity of the α-Sens® test method was assessed by an international Peer Review Panel in 2025. The peer review report supports the development of the corresponding test method for inclusion in Appendix ID of Test Guideline 442D for in vitro skin sensitisation assays, which assesses the Adverse Outcome Pathway Key Event on Keratinocyte activation. The Peer review report and the Validation report of the α-Sens® test method were circulated for comments to the Working Party of the National Coordinators of the Test Guidelines Programme (WNT) in November 2025. The WNT approved the peer review report at its 38th meeting in April 2026. The WNT also approved the Validation report of the α-Sens® test method.

This report is published under the responsibility of the Chemicals and Biotechnology Committee.

α-Sens
Report of the Peer Review Panel

on

a JaCVAM coordinated study program addressing the validation status of the α-Sens for prospective identification of skin sensitizing chemicals

Report completed by the Peer Review Panel on September 19th, 2025.

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Executive Summary

The α-Sens assay has been proposed as a "me-too" in vitro alternative, providing information on key event 2 (KE2) in the adverse outcome pathway for skin sensitisation. The assay has advantages in terms of technical performance and time required compared to existing methods concerning KE2. The peer review panel (PRP) found the Validation Management Team's report presented the necessary information for an independent review. Consequently, the PRP were able to conclude that the α-Sens assay was well defined, with a clear protocol and criteria for data interpretation. Both within and between laboratory reproducibility information were fully satisfactory, and along with the predictive capacity, very similar to, sometimes better than, other validated methods. Adequate performance standards/proficiency chemicals were also detailed. Accordingly, the PRP concluded that the α-Sens assay met all validation criteria for a "me-too" assay and therefore has been successfully validated for the prediction of skin sensitization hazard in the context of a KE2 method.

Peer Review Panel Composition

David A. Basketter, DSc, FRCPath (PRP Chair) – DABMEB Consultancy Ltd, UK
Emily N. Reinke, PhD – Inotiv, Contractor supporting the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), USA
Emanuela Corsini, PhD – Università degli Studi di Milano, Italy
Kunihiko Yamashita, PhD – Osaka University/Daicel Corp., Japan
Atsuko Miyajima, PhD – NIHS, Japan

Support from Takao Ashikaga, PhD and Akiko Ohno, PhD – JaCVAM/NIHS, Japan

Background

Progress in developing New Approach Methods (NAM) for assessing the skin sensitization potential of chemicals continues apace. The mechanistic key events described by the OECD (notably covalent binding to protein, keratinocyte activation, and dendritic cell activation) have each been addressed by a variety of non-animal assays (e.g. Casati et al., 2022; Aleksic et al, 2024). Efforts to address a fourth key event continue (Fritsch et al., 2024). Currently, 10 non-animal test methods are approved in OECD TG 442C, 442D, and 442E (OECD 2018a; 2018b; 2021b). However, each method has some limitations, especially with challenging materials such as highly lipophilic compounds.

Within the key events, the induction of inflammatory responses and changes in gene expression relevant to specific cell signalling pathways, such as antioxidant/electrophile response element (ARE)-dependent pathways in keratinocytes, have been identified as Key Event 2 (KE2). OECD Test Guideline (TG) 442D (*in* vitro Skin Sensitisation ARE-Nrf2 Luciferase Test Method) details this, and currently offers these assays, ARE-Nrf2 luciferase KeratinoSens™, ARE-Nrf2 luciferase LuSens and EpiSensA.

Although KeratinoSens™ and LuSens have been validated, in these assays ARE activity and cytotoxicity are evaluated on separate plates, with extra time for cytotoxicity evaluation being necessary. Furthermore, from an animal welfare point of view, KeratinoSens™ with the Xeno-free medium method using human serum has also been adopted in TG442D, but human serum is difficult to obtain in some countries, and in general there is large lot-to-lot variation. Therefore, use of defined media to replace foetal bovine serum (FBS) is encouraged as this is more likely to be applicable internationally.

To address these concerns, α-Sens®, an assay for assessing skin sensitisation potential, was developed by Chemical Evaluation and Research Institute, Japan (CERI) as an alternative to *in vivo* testing and existing KE2 methods. The α-Sens® cell line was originally developed as "α-Sens (without ®)", the multi-clone cell system of PHK 16-0b cell line (immortalized human keratinocyte by HPV16 virus genome) stably transfected with two reporter constructs using Lentiviral Vector System, antioxidant response element (ARE) inducible *Firefly* luciferase gene for ARE response and Thymidine Kinase (TK) promoter-driven *Renilla* luciferase gene for cell viability measurement (Maeda et al., 2020). α-Sens was further improved by modifying the reporter gene construct and was established as a single clone cell system, "α-Sens®". α-Sens® aims therefore to offer a level of performance that is equivalent or higher than existing assays whilst offering a simpler operation plus resource saving conditions, and, importantly, the assay can be conducted without any recourse to the use of FBS.

The PRP held its initial face to face meeting on March 10 and 11, 2025 in Tokyo, Japan. and continued to interact through repeated video conferencing during 2025.

Evaluation Criteria

Evaluation Criterion 1: A rationale for the test method should be available, including a description of the human health effect, a clear statement of scientific need, and regulatory application.

The Peer Review Panel (PRP) agreed that when considering α-Sens, these aspects, the rationale, the health effect, scientific need and regulatory application have been sufficiently addressed by the test developer.

In addition, given that this validation was conducted as a "me-too" assay to be included in OECD TG 442D, the rationale and description of the human health effect have been well established.

Addition of new test methods within OECD TG 442D requires justification as to the inclusion of an additional method, with clear advantages as to why it is a potential improvement or advantage beyond those already included. Comments relating to this appear in the subsequent PRP responses.

Evaluation Criterion 2: The toxicological mechanisms and the relationship between the test method endpoint(s) with the biological effect and the toxicity of interest should be addressed, describing the limitations of the test method.

The PRP confirmed that α-Sens addresses key event 2 (KE2) in the skin sensitisation AOP, as described in OECD TG 442D and "The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins,", being based on the ARE-Nrf2 transcriptional activation pathway. Limitations were discussed, notably that the assay is not intended as a standalone method. In addition, as an aqueous assay, the solubility of test substances will be a limitation, however, some effort to accommodate higher logP materials has been made by the test developer. The PRP noted, that in common with other assays, the system lacks robust metabolic capacity.

Evaluation Criterion 3: A detailed test method protocol should be available. Transferability to other labs should be demonstrated.

A comprehensive protocol for α-Sens is available.

The transferability of α-Sens to three naive laboratories has been successfully demonstrated via a three-step training programme involving exchange of trial substances.

A more formalized evaluation of transferability was also conducted under Phase 1 of the validation study plan. This involved nine chemicals (six positive, three negative). The training experience and evaluation of outcomes from Phase 1 led to revisions to the test protocol to ensure clarity. The PRP agreed that these changes were such that would not adversely impact test outcomes.

Evaluation Criterion 4: The within and between laboratory reproducibility of the test method should be demonstrated.

The PRP agreed that α-Sens within (WLR) and between laboratory reproducibility (BLR) has been demonstrated successfully. The WLR averaged 97% (range 92% - 100%), the BLR was 96%.

Evaluation Criterion 5: Demonstration of the test method's performance should be based on testing a diversity of chemicals, preferably coded reference chemicals.

As a "me-too" method, the chemical selection was based on the 20 chemicals detailed in OECD Performance Standard document (OECD TG 213). The Validation Management Team (VMT) added an additional five substances, particularly to test predictivity of logP >3.5 chemicals and to expand the number of skin sensitisation GHS sub-classification 1B chemicals. The PRP finds this to have been acceptable, and appreciated the evaluation of additional chemicals, noting that all were individually coded and distributed by JaCVAM for each laboratory and each repetition.

Evaluation Criterion 6: Predictive capacity should be demonstrated using representative chemicals.

The PRP notes that in the Performance Standards for Assessment of Proposed Similar or Modified In Vitro Skin Sensitisation ARE-Nrf2 Luciferase Test Method, Series on Testing and Assessment 213, the predictive capacity for a me-too assay should be at least 80% for each of sensitivity, specificity and accuracy. For α-Sens, when considering only the 20 Performance Standard substances, the sensitivity was 92% for all three laboratories, specificity was 83% (range 75% - 86%) and accuracy was 88% (range 85% - 90%). When the additional five substances were included, sensitivity was 88% for all three laboratories, specificity was 85% (range 78% - 89%) and accuracy was 87% (range 84% - 88%). It was noted that one laboratory experienced difficulties with a single borderline substance, methyl salicylate, which brought their results down compared to the other two laboratories.

At the recommendation of the PRP, the test method developers also provided an assessment of predictive capacity without methyl salicylate, a borderline chemical. Marginal improvements in performance were noted. Additionally, the test method developers performed a more comprehensive review of performance using the Defined Approaches for Skin Sensitization dataset, for both α-Sens and KeratinoSens™, another KE2 assay. This allowed for an evaluation of 162 chemicals when compared to LLNA reference data, and 112 chemicals when compared to human reference data. As noted in the validation report, the α-Sens had a higher predictive capacity against both reference sets than KeratinoSens™, except for specificity when compared to LLNA (65% vs 68% respectively). The PRP appreciated this additional evaluation to show the robustness of the assay.

Overall, the PRP agreed that the predictive capacity of α-Sens has been demonstrated successfully.

Evaluation Criterion 7: All data should adequately support the assessment of the validity of the test methods for peer review.

Each test laboratory provided JaCVAM with comprehensive data sheets and all other relevant information on the α-Sens validation study, including every piece of raw data. The PRP accepted that any individual study could be reconstructed from this body of information and thus confirmed that its assessment of α-Sens validation was fully supported.

Evaluation Criterion 8: All data from the validation study supporting the validity of a test method should be obtained in accordance with the principles of Good Laboratory Practice (GLP).

The α-Sens validation studies were carried out in two GLP laboratories and one non-GLP laboratory, but always in accordance with the principles of GLP, with appropriate quality controls in place. Accordingly, the PRP confirmed that this criterion was satisfied.

In addition, the PRP noted that the VMT created data reporting templates and record sheets and distributed them to the participating laboratories. All the records (data sheets and record sheets) from the participating laboratories were carefully checked by QC experts at JaCVAM. All the records were available on the JaCVAM website for review by the PRP and will become widely available when the validation review process is complete.

Evaluation Criterion 9: Applicability domain of the test method should be defined.

As with most in vitro assays, the applicability domain of any test method is not easily defined, however, the VMT did provide evidence of an expanded LogP domain, with LogP >3.5 chemicals included in the evaluation. As α-Sens is a "me-too" variation for ARE-Nrf2 transcriptional activation test methods, it does have a similar applicability domain regarding solubility and metabolic capacity. However, the PRP noted that the updated protocol allows for better evaluation for chemicals with high luminescence or volatility. The PRP considers the applicability domain to have been well defined for this method category.

Evaluation Criterion 10: Proficiency chemicals should be set up in the proposed protocol.

A practical list of 10 reliable proficiency chemicals was provided in the validation report for use in the protocol, with justification for the inclusion of each chemical. The PRP considers this list to be a suitable set of chemicals, spanning a range of potencies, a reasonable number of negatives, and inclusive of both volatile chemicals, chemicals with high luminescence, and chemicals with high LogP.

Evaluation Criterion 11: Performance standards should be set up with the proposed protocol.

The criteria for determining the acceptability of individual test runs is available for the test method and have been fully described in the protocol. This includes cell viability acceptance cut-offs, minimum performance criteria for positive control, and CV cut-offs for solvent control. The PRP considers this acceptable, and appreciates the value of the flow charts giving clarity on determining a successful run, including careful explanations of the cytotoxicity requirements for the assay.

Evaluation Criterion 12: Advantages in terms of time, cost, and animal welfare should be described.

The PRP concluded that the α-Sens provides distinct advantages in the performance of the method. Primarily, the method does not require foetal bovine serum supplementation in media at any time, inclusive of the pre-culture period. Furthermore, α-Sens has a shorter incubation time with more rapid readouts, and requires fewer plates per chemical as compared to other ARE-Nrf2 transcriptional activation-based methods. In part, this is due to the use of dual-luciferase reporter platform, combining both the ARE-Nrf2

activation and cell viability assessments within the same well. This allows for the incorporation of cell viability into the calculation of the method output parameter, normalized ARE activity (nAA).

Taken together, these modifications deliver the potential for notable savings in both consumable costs and personnel time requirements.

Evaluation Criterion 13: Completeness of all data and documents supporting the assessment of the validity of the test method.

The PRP agrees that the data and supporting documentation to assess the validity of the test method were complete and available for PRP review.

Evaluation Criterion 14: Validation Study should be managed and conducted adequately.

The PRP agrees that the validation study was well managed and conducted to a proper standard. The PRP is appreciative that the VMT incorporated labs from multiple countries (Japan and India) to demonstrate transferability and reproducibility of α-Sens across international borders.

Recommendations and Further Considerations

The validation report indicated that the α-Sens cell line has been deposited at the Japanese Collection of Research Bioresources Cell Bank. Consequently, the cell line will be available internationally following TG 442D adoption, requiring a signed MTA and at a reasonable cost. This falls under the FRAND declaration already submitted to the OECD.

Conclusion

The PRP concluded that the α-Sens assay met all validation criteria for a "me-too" assay and therefore has been successfully validated for the prediction of skin sensitization hazard in the context of a KE2 method.

Acknowledgments

The PRP is grateful to VMT members for their hard work and excellent responsiveness to questions. Thanks are also due to JaCVAM for its support in setting up and hosting our meeting in Japan, as well as for setting up video conferences. Finally, the PRP offered a special thank you to Tamaki Yoshikawa for outstanding administrative support.

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