Organisation for Economic Co-operation and Development

ENV/CBC/MONO(2026)12

Unclassified

English - Or. English

29 June 2026

ENVIRONMENT DIRECTORATE
CHEMICALS AND BIOTECHNOLOGY COMMITTEE

Validation Report of the Epi2sensA for Skin Sensitisation

OECD Series on Testing and Assessment

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JT03590123

This work was approved and declassified by the Chemicals and Biotechnology Committee on [02/07/2026].

Please cite this publication as:

OECD (2026), *Validation Report of the Epi2sensA for Skin Sensitisation*, OECD Series on Testing and Assessment, No. 434, OECD Environment, Health and Safety, Paris, link to ONE M&P document https://one.oecd.org/document/ENV/CBC/MONO(2026)12/en/pdf

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Foreword

This document contains the Validation report of the EpiDerm-Epidermal Sensitisation Assay (Epi2SensA), a me-too method of the Epidermal Sensitisation Assay (EpiSensA), an in vitro method for identifying the skin sensitisation potential of chemicals. It supports the development of this test method for inclusion in Appendix IC of Test Guideline 442D, for in vitro skin sensitisation assays, which assesses the Adverse Outcome Pathway Key Event on Keratinocyte activation.

The Epi2SensA, proposed by France and Japan, is based on a reconstructed human epidermis model in which gene expression is measured for four markers that reflect the keratinocyte response to the early phase of skin sensitisation. The validation report was peer reviewed by an international Peer Review Panel in 2025. The Peer review report and the Validation report of the Epi2SensA were circulated for comments to the Working Party of the National Coordinators of the Test Guidelines Programme (WNT) in December 2025. The WNT approved the Validation report at its 38th meeting in April 2026. The WNT also approved the Peer review report of the Epi2SensA.

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

EpiDerm™ Epidermal Sensitisation Assay (Epi2SensA) validation study report

Version 2

February 19th 2026

Content

Summary

1. Since the publication of the "The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins" in 2012, several methods based on the modelling of AOP Key events have been validated and integrated into TG 442C, D and E (1). In 2023, JaCVAM has scientifically validated the epidermal sensitisation assay (EpiSensA), an in vitro test method addressing KE2. In this method, gene expressions of four mechanistically-relevant markers, reflecting the keratinocyte response to the early phase of skin sensitisation are measured after exposure of a reconstructed human epidermis (RhE), the LabCyte EPI-MODEL 24 (J-TEC, Japan), to a chemical (2).

2. The aim of this catch-up study was to evaluate in the Epi2SensA me-too method the applicability of another RhE model, the EpiDerm™, as a complement to the Labcyte EPI-MODEL-24 model. Like for other OECD methods, e.g. TG439, the possibility of enabling access to two RhE suppliers offers the option of giving users a choice between two sources and ensuring the possibility of back-up from one supplier in the event of production or delivery problems. The fact that the EpiSensA method was originally developed using the EpiDerm™ model and only later changed to LabCyte EPI-MODEL24 was a factor in the choice of this model for a me-too method (3).

3. The Epi2SensA method, was adapted by Mattek (Ashland, USA) over 2023-2024, using the EpiDerm™ model. A Standard Project Submission Form (SPSF) was submitted and integrated to the OECD work plan under the number 4.172 as « Me-Too validation of the reconstructed human epidermis EpiDerm™ model for the EpiSensA method". This catch-up study was designed to determine if the EpiDerm™ RhE model could be used with a test protocol resembling the one used for the successfully validated EpiSensA with the Labcyte EPIMODEL-24 RhE model.

4. In 2024, the method was transferred to 3 naïve laboratories in the USA, Germany and Japan. The coded phase of the catch-up study was conducted on 2025 in blind situation according to the OECD guidance n°34. At the end of the experimental study, the statistician conducted the data analysis using the original acceptance criteria, resulting in an average WLR of 63.9%, a BLR of 70%, and averages of 66.6% specificity, 90.5% sensitivity, and 83.3% accuracy. As several acceptance criteria were missed, a subsequent analysis incorporated two modifications discussed and agreed by the core VMT. These modifications were a change to the acceptance criteria of cell viability (from 80 to 60%) and an adaptation of the prediction model, requiring two positive genes for at least one concentration (instead of one). These adjustments resulted in an improved method performance. Two of the three laboratories met the WLR acceptance criterion of 80% and the BLR exceeded the acceptance value of 80%. All laboratories achieved acceptable sensitivity and specificity and though one laboratory's accuracy and sensitivity were slightly below the required threshold, the average specificity, sensitivity, and accuracy across all laboratories were 88.9%, 88.1%, and 88.3%, respectively. Validation set analysis identified probable sources of the WLR and sensitivity variability observed.

5. Given the demonstrated performance and in light of some issues identified, e.g. the debatable suitability of using Cetrimide as a performance standard reference substance, the VMT decided that the Epi2SensA is overall promising enough to be submitted to peer review for progressing its acceptance as a me-too method to the EpiSensA.

1 Management of the Study

1.1 Study objectives

6. The objective of this study was to carry out a me-too validation study for a me-too method to the validated EpiSensA method using the MaTtek EpiDerm™ model (EPI-200) as experimental system. The performance standards of the EpiSensA (4) have been be applied to this new method called Epi2SensA, to determine its reliability (within- and between-laboratory reproducibility) and relevance (predictive capacity). This me-too validation is part of the OECD work plan under the number 4.172 as « Me-Too validation of the reconstructed human epidermis EpiDerm™ model for the EpiSensA method ».

1.2 Study Plan

1.2.1 Validation Management Team

7. This validation study has been carried out under the supervision of a validation management team (VMT). The VMT core team comprised 5 members, supported by 9 observers from the participating laboratories. The core VMT managed and scheduled the validation process, trainings, encoding and distribution of test materials, evaluation and statistical verification of test results, and is responsible for this report. The composition and respective role of participants to the VMT is detailed in Table 1.

Table 1: Composition of the validation management team with core members highlighted in grey

Name	Company/institution	Role	Observation
Christian Pellevoisin	Urbilateria, Saint Cyr sur Loire, France	Member	Chair
Hajime Kojima	NIHS, Japan	Member	Co-Chair
Sebastian Hofmann	seh consulting + services, Paderborn, Germany	Member	Biostatistician
Takao Ashikaga	JaCVAM	Member	JaCVAM liaison
Marisa Meloni	VitroScreen, Milano, Italy	Member	Chemical management
Mitch Klausner	MatTek, Ashland, USA	Observer	Lead laboratory
Kalyani Guntur	MatTek, Ashland, USA	Observer	Lead laboratory
Tim Landry	MatTek, Ashland, USA	Observer	Lead laboratory
Kazuto Narita	Food and Drug Safety Center (FDSC), Hadano, Japan	Observer	Participating laboratory 1
Shigehiro Tachibana	Food and Drug Safety Center (FDSC), Hadano, Japan	Observer	Participating laboratory 1
Kojima k	Food and Drug Safety Center (FDSC), Hadano, Japan	Observer	Participating laboratory 1
Helge Gehrke	Eurofins BioPharma Product Testing Munich, Germany	Observer	Participating laboratory 2
Robert Mills-Goodlet	Eurofins BioPharma Product Testing Munich, Germany	Observer	Participating laboratory 2
Vic Johnson	Burleson Research Technologies, Morrisville, USA	Observer	Participating laboratory 3

8. Marisa Meloni oversaw the organisation and service contract of her company VitroScreen for the chemical management of the validation study. She subsequently left the company in September 2024 and did not wish to continue her involvement in the VMT. Sample management was therefore carried out under the existing contract managed directly by VMT members.

1.2.2 Structure of the study

9. The catch-up validation study was conducted in two main phases, in accordance with the study plan established prior to the start of the experimental study (Appendix I). In the phase 1, the test method was transferred to 3 naïve laboratories. After verification that the laboratories mastered the method, the second phase started to assess in blind condition the within-laboratory (WLR) and between-laboratory reproducibility (BLR) and the predictivity of the test method. Based on the statistician's recommendation, it was decided to introduce an early interim data analysis phase 2, after the laboratories had performed two runs to check for acceptable reproducibility. To maintain the blindness of the validation, participating laboratories received a list of 8 coded samples to be tested in this phase. Once approved by the VMT, phase 2 was continued.

   Phase 1: training and transfer to the 3 naive laboratories

   Phase 2: Evaluation of 20 chemicals (44 coded samples per laboratory), 8 in one run and 12 in three repeated runs to assess the BLR, WLR and predictivity of the mee too method (see Table 3). The phase 2 was an intermediate step to evaluate reproducibility between and within laboratory on a subset of 8 samples of the 44 samples to be tested.

10. For phase 2 study, it was decided for feasibility reasons to limit each run to four samples tested. Indeed, with four concentrations tested for each sample, and the controls this represents between 60 and 66 EpiDerm™ tissues per run depending on the number of solvents used. Phases 2 and 3 therefore required 11 independent runs in each laboratory to test the 44 coded samples.

1.2.3 Participating Laboratories

11. Four laboratories participated to the catch-up validation, two in the USA, one in Europe and one in Japan. MatTek, Ashland, USA, was the lead laboratory. It was responsible for preparing the final standard operating procedure (SOP) and for the training of the participating laboratories (Appendix III).

12. Participating laboratories as testing laboratories in the me-too validation study:

    - Lead laboratory: (Study director: Kalyani Guntur) MatTek Headquarters, 200 Homer Ave, Ashland, MA 01721, USA, www.mattek.com
    - Laboratory 1: (Study director: Vic Johnson) Burleson Research Technologies, 120 First Flight Lane, Morrisville, NC 27560, www.brt-labs.com
    - Laboratory 2: (Study director: Robert Mills-Goodlet) Eurofins BioPharma Product Testing, Munich, Germany, https://www.eurofins.de/biopharma-product-testing/
    - Laboratory 3: (Study director Kazuto Narita) Food and Drug Safety Center Hatano Research Institute (FDSC), Safety Testing Department, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan, http://www.fdsc.or.jp/

13. During the discussion on the provisional timetable of the coded phases, the FDSC laboratory indicated that its workload would not allow it to complete the 11 series within the planned timeframe. Regarding the willing of the VMT to keep a Japanese laboratory in the catch-up validation study it has been decided that the FDSC laboratory will only test a subset of 8

chemicals used to evaluate the between laboratory reproducibility (BLR). In parallel, Mattek has been added to the participating laboratories in order to have 3 different laboratories testing the full set of 44 samples covering the 20 chemicals.

1.3 Adherence to OECD guidance and GLP

14. According to the OECD guidance n°34, it is not mandatory, even if preferable, to carry out validation strictly in compliance with GLP principles. Nevertheless, participating laboratories have performed all procedures in accordance with GLP principles, including, but not limited to, the use of standard operating procedures, proper data recording and record keeping.

1.4 Test Chemicals

15. The test chemicals proposed in the Performance Standards for the Assessment of Proposed Similar or Modified In Vitro Epidermal Sensitisation Assay (EpiSensA) Test Methods (OCED, 2024) were used. The 20 recommended reference substances represent the full range of in vivo skin sensitisation effects, which act via various mechanisms, and are representative of different chemical categories based on their functional groups.

Table 2: Minimum List of Reference Substances for Determination of Reproducibility and Predictive Capacity of similar or modified EpiSensA test method (adapted from OECD 2024)

No.	Proficiency substances	CAS No.	Physical state	*in vivo* prediction[1]	LogP	Pre/Pro-hapten	Vehicle[2]	EpiSensA results for each marker gene[2, 3]				VRM
								ATF3	GCLM	DNAJB4	IL-8	prediction
1	2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (GHS Cat.1A)	2,17		AOO	p	p	p	p/n	Positive
2	p-Phenylenediamine	106-50-3	Solid	Sensitiser (GHS Cat.1A)	-0,3	X	AOO	p/n	p	p	p/n	Positive
3	Metol	55-55-0	Solid	Sensitiser (GHS Cat.1A)	0.63	X	DW	p	p	p	p/n	Positive
4	Tetrachlor-osalicylanilide	1154-59-2	Solid	Sensitiser (GHS Cat.1A)	**5,87**		AOO	p	n	p/n	p	Positive
5	Lauryl galate	1166-52-5	Solid	Sensitiser (GHS Cat.1A)	**6,9**	X	AOO	p/n	n	p/n	p/n	Negative
6	Methyl heptine carbonate	111-12-6	Liquid	Sensitiser (GHS Cat.1A)	2,79		AOO	p	p	p	P	Positive
7	Isoeugenol	97-54-1	Liquid	Sensitiser (GHS Cat.1A)	3.04	X	AOO	p/n	p	p	p/n	Positive
8	Glyoxal 40% solution in water	107-22-2	Liquid	Sensitiser (GHS Cat. 1A	-0.08		DW	p/n	p	p/n	p/n	Positive
9	Abietic acid	514-10-3	Solid	Sensitiser (GHS Cat.1B)	**3.92**	X	AOO	p	p	p	p	Positive
10	Dibutyl aniline	613-29-6	Liquid	Sensitiser (GHS Cat.1B)	**4,7**	X	AOO	p/n	n	n	p	Positive
11	Amyl cinnamic aldehyde	122-40-7	Liquid	Sensitiser (GHS Cat.1B)	**3.99**		AOO	p	n	p	p	Positive
12	Benzisothiazolinone	2634-33-5	Solid	Sensitiser (GHS Cat.1B)	0,8		AOO	p/n	p/n	p/n	p/n	Positive[5]
13	Imidazolidinyl urea	39236-46-9	Solid	Sensitiser (GHS Cat.1B)	-0.86		DW	p/n	n	p/n	p	Positive
14	Farnesol	4602-84-0	Liquid	Sensitiser (GHS Cat.1B)	**4,91**		AOO	p	p/n	p	p	Positive
15	Cetrimide	57-09-0	Solid	Non-sensitiser (Not classified)	3,18		50% EtOH	n	n	n	n	Negative
16	Lactic acid[4]	50-21-5	Liquid	Non-sensitiser (Not classified)	-0,72		DW	n	n	n	n	Negative
17	Benzyl butyl phthalate	85-68-7	Liquid	Non-sensitiser (Not classified)	**4,84**		AOO	n	n	n	n	Negative
18	Diethyl phthalate	84-66-2	Liquid	Non-sensitiser (Not classified)	2,44		AOO	p/n	n	p/n	p	Positive

19	Hexane	110-54-3	Liquid	Non-sensitiser (Not classified)	**3,9**		AOO	n	n	n	n	Negative
20	1-Iodehexane	638-45-9	Liquid	Non-sensitiser (Not classified)	**3,99**		AOO	n	p	p	p	Positive

1: The in vivo hazard and potency prediction is based on LLNA data (TG497, SD Annex3) (Urbisch, 2015). The in vivo potency is derived using the criteria based on UN GHS Sub-categorisation.
2: Based on historical results (Mizumachi et al, 2018) (EpiSensA validation report).
3: "p" indicates that the fold-induction of marker gene exceeds the cut-off value with ≥ 80% viability. "n" indicates that the fold-induction of marker gene doesn't exceed the cut-off value with ≥ 80% viability. "p/n" means both "p" and "n" are acceptable because both "p" and "n" results were obtained at the validation study.
4: MTT assay should be performed instead of LDH assay.
5: Reference substance which was not 100% concordant between laboratories.
Grey: A subset of 12 out of the 20 reference substances that should be used for an assessment of within-laboratory reproducibility (WLR).

1.5 Acquisition, coding, and distribution

16. The Chemical management Team was composed of the VMT core members and was leaded by Marisa Meloni until she left the VMT. The chemical management team subcontracted to VitroScreen (Milano, Italy) the acquisition, coding, and distribution of the chemicals.

1.5.1 Grouping approach

17. Like the VRM, the Epi2SensA protocol begins with a first experiment to assess the cytotoxic concentration of the chemical product before performing the main experimental study to perform the gene expression analysis. To this end, the cytotoxicity test should be carried out at least a week before the main study, with up to six concentrations tested in duplicate, i.e. 12 tissues, per test encoded sample. Testing the cytotoxicity of all the 44 encoded samples would require up to 530 EpiDerm™ models per laboratory and a substantial amount of laboratory work.

18. Participants highlighted the significant costs and considerable workload involved in assessing cytotoxicity alone. Yet, measuring cytotoxicity using EpiDerm™ models is the basis for several OECD methods (TG431, 439, 498) for which corresponding validation studies, as well as years of use, have demonstrated the robustness, reproducibility, and transferability of this result. Therefore, out of a concern for controlling costs and the workload of participating laboratories, the VMT consulted the OECD expert group on a proposed strategy that would ensure compliance with OECD 34 principle by maintaining the effectiveness of the coding while reducing the burden on participating laboratories.

19. In the proposed strategy, the dose concentration test (step 2) is performed by each laboratory only for 10 of the 44 encoded samples. These 10 samples comprise six samples from the 12 substances for WLR assessment and 4 samples from the eights substances to be tested only once (informing BLR). The 10 samples will be encoded like the other samples but with an additional label indicating that a concentration-finding study shall be performed. The remaining 34 samples are assigned to three groups, each with a defined range of 4 concentrations to be tested, eliminating the need for concentration-finding studies. The assignment to the groups is based on cytotoxicity testing and results from Mattek's historical data.

20. In summary, with the 3-group approach the 44 samples per laboratory are assigned as follows:

- 10 coded samples to be tested in the concentration finding study
- 20 coded samples in the group 1 to be tested at 100%, 25%, 12.5% and 6.25%
- 9 coded samples in the group 2 to be tested at 3.125%, 1.56%, 078% and 0.39%

- 5 coded samples in the group 3 to be tested at 0.39%, 0.195%, 0.098% and 0.049%

21. The request for opinion from the OECD Expert Group on Skin Sensitisation related to the validation design of the Epi2SensA Me-Too test method took place on November 2024. Based on the positive feedback received by the VMT from the expert group on November 18, the proposed strategy was selected to conduct the coded phases 2 and 3 of the validation process.

1.5.2 Chemical Acquisition, Coding, and Distribution

22. The training and evaluation of transferability phase was performed with non-coded chemicals ordered directly by the participating laboratories. The 5 chemicals used in this phase did not belong to the set of chemicals used for the validation of the me-too method.

23. The interlaboratory phase 2 and 3 to evaluate the within- and the between-laboratory reproducibility, as well as of the predictive capacity were made with using coded chemicals. The acquisition, preparation, coding, sampling and distribution to the 4 laboratories has been subcontracted to VitroScreen. VitroScreen was chosen for its experience in the field of in vitro testing and its involvement in previous validations.

24. VitroScreen's responsibility for chemical acquisition, coding, and distribution were as follows:

- Preparation of N= 44 vials (5g/5mL per vial) for each laboratory + 20 as reserve VitroScreen representing a total of 152 vials coded to be used for the 3 testing. It should be noted that 8 vials were taken from the reserve for the FDSC laboratory.
- Coding randomly done for each laboratory in an Excel file and secured in VitroScreen server. A double-blinded (different codes for different labs) was done.
- Each lab received a safety package, i.e. a sealed envelope containing 20 sealed envelopes (corresponding to the 20 coded test items) for each of the 20 chemicals sent to the lab, identified by code and containing the MSDS for the relevant chemical. Storage conditions were mentioned. According to the MSDS the safety warning (if any) was labelled and introduced in a separate accompanying document for adequate use of PPE.
- Management of shipment follow-up (sample date of reception, sample integrity and document signatures) : all the original documents will be secured and delivered to MatTek at the end of the study if requested.
- Collaboration for decoding of the chemicals with the third party responsible of the statistical analyses of the tests results performed by the laboratories.
- Confirmation that the coding of the chemicals was not broken by the laboratories and returning once the study is completed the MSDS for chemicals disposal by the laboratories.

Table 3: Coding of the 44 samples for the 3 laboratories conducting the entire study and 8 samples of the fourth laboratory

	Phase 2		Phase 3								
	RUN 1	RUN 2	RUN 3	RUN 4	RUN 5	RUN 6	RUN 7	RUN 8	RUN 9	RUN 10	RUN 11
LAB A	A123-0	A274-3	A192-3	A115-0	A301-3	A346-2	A279-3	A313-1	A249-2	A351-3	A127-2
	A186-1	A143-0	A401-0	A174-1	A278-1	A138-1	A366-2	A323-1	A240-1	A305-2	A382-1
	A271-2	A443-0	A198-0	A478-0	A283-1	A497-2	A431-1	A288-1	A329-1	A262-2	A289-1
	A411-2	A426-0	A169-0	A146-0	A263-1	A471-1	A133-1	A223-1	A306-1	A361-1	A398-1
LAB B	B160-0	B224-3	B121-3	B130-0	B317-3	B328-2	B242-3	B391-1	B203-2	B388-3	B141-2
	B163-1	B185-0	B486-0	B104-1	B241-1	B108-1	B365-2	B343-1	B222-1	B375-2	B183-1
	B231-2	B466-0	B111-0	B438-0	B243-1	B479-2	B452-1	B396-1	B208-1	B235-2	B298-1
	B418-2	B427-0	B107-0	B117-0	B311-1	B454-1	B272-1	B202-1	B385-1	B316-1	B347-1
LAB C	C188-0	C265-3	C167-3	C176-0	C390-3	C342-2	C210-3	C318-1	C212-2	C315-3	C189-2
	C166-1	C118-0	C476-0	C194-1	C286-1	C132-1	C359-2	C340-1	C296-1	C369-2	C184-1
	C211-2	C492-0	C165-0	C444-0	C251-1	C468-2	C491-1	C345-1	C244-1	C206-2	C239-1
	C481-2	C439-0	C149-0	C120-0	C380-1	C440-1	C287-1	C275-1	C378-1	C383-1	C335-1

LAB D	RUN 1	RUN 2
	D414-2	D424-0
	D433-0	D410-1
	D457-2	D416-0
	D493-0	D474-1

25. The principle of the coding was a first letter for the laboratory: Lab A: Eurofins, Lab B: Mattek, Lab B: BRT, Lab D: FDSC (indicated below by X). The samples were coded by unique and randomly generated three digits numbers (indicated below by YYY), a dash and a unique number from 0 to 3 for categorisation (range of concentration to be tested):

    - "XYYY-0": to be tested in the concentration finding study
    - "XYYY-1": to be tested at 100%, 25%, 12.5% and 6.25%
    - "XYYY-2": to be tested at 3.125%, 1.56%, 078% and 0.39%
    - "XYYY-"3: to be tested at 0.39%, 0.195%, 0.098% and 0.049%

1.5.3 Handling

26. Each participating laboratory was provided with the essential information about the test chemicals (e.g., their physical state, their sample weight or volume, and their storage instructions) by VitroScreen. Each laboratory was responsible for storing the respective chemicals in accordance with the storage instructions, and separately received the sealed safety information, including Safety Data Sheets (SDSs) describing their hazard identification, their exposure control, and the personal protection required for each chemical. The test chemicals were delivered directly to the study directors of each laboratory. The SDSs were accessed only in the event of accident, and the information were disclosed only to those who had to know.

1.6 Timeline of Epi2SensA catch-up validation study

27. The validation study took place over 14 months, from June 2024 to August 2025.

Table 4: Overview of the main validation steps

Date	Task
21/06/2024	First VMT Meeting
01/07/2024	Circulation of the "Study Plan and SOP for Epi2SensA validation"
05/08/2024	Circulation of planning of the runs for preparation
08/08/2024	2nd VMT meeting

15/08/2024	Circulation to the VMT of the SOPv1 and template for collecting the results
28/08/2024	Circulation to the VMT of the SOP v1.1 and the final version of the EpiSensA performance standard
18/11/2024	Third VMT meeting. Decision for a new run with Eugenol and a negative compound. Organise a technical meeting between Mattek, BRT and Eurofins to check step by step the SOP to identify the potential source of variability in the results.
21/11/2024	Outcome of the request for opinion from the OECD Expert Group on Skin Sensitisation related to the validation design of the Epi2SensA Me-Too test method
30/01/2025	VMT 4 – Based on results of Phase 1 training/transfert decision to go to Phase 2: initial step to evaluate reproducibility between and within laboratory on a subset of the 8 samples to be tested.
12/01/2025	Circulation of SOP v1.2
Feb 2025	Following FDSC's alert that it would be unable to meet the provisional schedule for phases 2 and 3, discussions between VMT members led to Mattek being brought in as the fourth laboratory to test the 44 samples. FDSC will initially conduct only two runs on eight samples.
11/02/2025	Shipping by Vitroscreen of the coded samples to the laboratories
10/03/2025	Circulation of the SOP v2 and Main study template study for data collection to the laboratories.
28/03/2025	VMT5 – Presentation by Sebastian Hoffman of data analysis of Phase 2 with 8 first coded samples and decision to continue the Phase 3 of the validation.
28/05/2025	VMT6 – Integration of the results of FDSC in the analysis of the Phase 2. Discussion about organisation of an independent peer review. Sharing feedback from discussion with Nathalie Delrue on the possibility to call for volunteers from the OECD skin sensitisation expert group.
09/06/2025	Emanuela Corsini accepted to chair the peer review panel
25/06/2025	Call for peer reviewers to the OECD expert group
30/07/2025	VMT7 - Review of files received and closure of the experimental phase. Start of the statistical analysis of the results of phase 2 and 3.

2 Statistical Analysis of Test Data

28. The results in this section are based on the complete statistical analysis report by biostatistician Sebastian Hoffmann, provided as Appendix II.

2.1 Data Analysis

29. All runs were checked for validity. Only valid runs were submitted to the evaluation of test concentration validity. The data of test substances with at least one valid test concentration were analysed according to the prediction model.

2.2 Performances

2.2.1 Analysis according to VRM criteria:

30. This analysis performed by the biostatistician was conducted by applying the acceptance criteria of the performance standards.

Table 5: Comparison of the WLR, BLR and predictive capacity according to the original analysis with green highlighted cells indicating acceptance criteria met

	acceptance criterion	original			
		Mattek	Eurofins	BRT	Average
WLR	≥ 80%	66,7% (8/12)	58,3% (7/12)	66,7% (8/12)	63,9%
BLR	≥ 80%	70%			70%
Specificity	≥ 65%	66,7% (4/6)	83,3% (5/6)	50,0% (3/6)	66,6%
Sensitivity	≥ 85%	92,9% (13/14)	85,7% (12/14)	92,9% (13/14)	90,5%
Accuracy	≥ 85%	85,0% (17/20)	85,0% (17/20)	80,0% (16/20)	83,3%
Balanced accuracy		82.89%	84.5%	71.5%	78.6%

31. This analysis shows that the PS acceptance criteria for WLR and BLR were not met, while the predictive capability acceptance criteria were satisfied, with the exception of BRT specificity and consequently accuracy (Table 5).

2.2.2 Analysis with modified criteria

32. Based on observation made during the Phase 2 interim analysis and the data analysis, the core VMT agreed to analyse the validation data with two modifications. First, as many test concentrations missed the acceptance criterion of at least 80%, this value was reduced to 60%. Second, the prediction model was modified by considering a test substance positive if at least two genes (instead of one) exceed the gene-specific Imax threshold at any accepted tested concentration.

33. With the updated criteria for the EpiDerm™ model, a tested concentration is accepted if mean cell viability is at least 60% (rather than 80% with the VRM), and a chemical is considered positive if at least two genes exceed the Imax threshold at that concentration. The scientific rational for the modifications are presented in the Chapter “Compliance with the essential test method components of the VRM”.

34. The analysis of the results with these criteria adapted to the Epi2Sensa showed a substantial improvement in performance (Table 6).

Table 6: Comparison of the WLR, BLR and predictive capacity with adapted criteria to the Epi2SensA. Green highlighted cells indicating met acceptance criteria

	acceptance criterion	modified			
		Mattek	Eurofins	BRT	Average
WLR	≥ 80%	91,7% (11/12)	83,3% (10/12)	75% (9/12)	83,3%
BLR	≥ 80%	85%			85%
Specificity	≥ 65%	100% (6/6)	83.3% (5/6)	83,3% (5/6)	88,9%
Sensitivity	≥ 85%	92.9% (13/14)	78.6% (11/14)	92,9% (13/14)	88.1%
Accuracy	≥ 85%	95,0% (19/20)	80,0% (16/20)	90,0% (18/20)	88,3%
Balanced accuracy		96,5%	81%	88,1%	88.5%

35. Table 6 shows that the acceptance criteria for WLR of Mattek and Eurofins and the BLR were met by the modified analysis. The predictive capacity criteria were fulfilled except for Eurofins’s sensitivity and consequently accuracy. All laboratories failed to correctly identify lauryl gallate as positive, very similar to the VRM EpiSensA . In addition, Eurofins did not correctly identify the two

sensitisers (Cat. 1B) Dibutyl aniline and Benzisothiazolinone. Eurofins and BRT missed criteria for one substance only, which are discussed in the next paragraph.

Discussion of Eurofins Accuracy and BRT's WLR with the modified criteria

36. Different parameters have been considered by the VMT in the interpretation of the results of the me-too validation study. Although not required by the PS, the test sample was provided in a coded manner to the testing laboratories by the independent contractor VitroScreen (Milan, Italy). Additionally, the set of chemical from the PF list contains a significant ratio of borderline chemicals with observed variability in their historical results. In addition, two laboratories, BRT and Eurofins, were new users for RT-PCR, which, even if not justifying, may explain some results.

2.2.3 Sensitivity at Eurofins

37. Eurofins did not meet sensitivity and accuracy criteria due to one missed sensitiser. Scrutiny of historical data for the set of validation, particularly for Benzisothiazolinone, reveals the presence of borderline chemicals and inherent compound variability as major challenges. Benzisothiazolinone has been identified as a difficult-to-test substance with a risk of misclassification upon repeated testing. In the VRM validation study, it was classified as a non-sensitiser by all three participating laboratories in at least one run, with one laboratory ultimately misclassifying it, similarly to the outcome reported by Eurofins. The VRM validation report (12), in Section 5-Test chemicals with results that were false negatives, concluded for Benzisothiazolinone that *"it may be difficult to obtain accurate results consistently, with a strong likelihood of non-concordant outcomes across laboratories."*

38. Additionally, initial misclassifications occurred during the preliminary reproducibility assessment phase 2 (Runs 1 and 2), a period marked by laboratory onboarding and protocol harmonisation meetings. Eurofins used a distinct RNA extraction kit and the technician was also inexperienced with the PCR method, which likely contributed to early errors. Notably, Run 2 omitted the highest required concentration (0.39%). The proper classification of Benzisothiazolinone in Run 5, following technical adjustments and increased familiarity with the protocol, supports the hypothesis that insufficient training and methodological differences participated also to the initial deviation. The combination of the compound's inherent difficulty and variability in the VRM, the fact it was the first substance tested—along with possible limited experience of the technician, may have contributed to its misclassification.

39. Furthermore, Annex 2 (Reference data matrix and comparison) of the Supporting Document to OECD Guideline 497 demonstrates that Benzisothiazolinone would have been accurately classified as a skin sensitiser combining Epi2SensA, rather than Keratinosens, with DPRA and h-Clat in a defined approach (11).

40. When considering Eurofins' sensitivity, it is also important to account for the challenging nature of the other two misclassified chemicals. Lauryl Gallate was a false negative in the VRM performance standard and was misclassified by all three participating laboratories. The VRM validation report (12) suggests for the misclassification of Lauryl Gallate that "*EpiSensA would predict lauryl gallate as negative because of both high lipophilicity (logKow=6.21, suggesting low absorption at higher concentration) and autoxidation*", and this rationale equally applies to the Epi2SensA method.

41. The third chemical misclassified by Eurofins, Dibutyl aniline, has no information on its results variability as it was not included in the validation set of the VRM nor in any proficiency list or

performance standard of OECD TG 442 C, D, or E. However, Like Lauryl Gallate, it has high hydrophobicity (LogKow=5.97) and is a putative pro-hapten requiring metabolic activation (12). Consequently, variability in penetration due to hydrophobicity may affect metabolisation rates, leading to occasional misclassification. Additionally, Dibutyl aniline's high lipophilicity classifies it among chemicals for which the LLNA model gives potential false positive results (14). Its lack of structural alerts and absence of human data highlight the borderline nature of this compound and raise questions about its true classification.

2.2.4 Conclusion on Sensitivity observed at Eurofins:

42. Eurofins' reduced sensitivity mainly stemmed from the misclassification of inherently variable or borderline substances (notably Benzisothiazolinone, Lauryl Gallate, and Dibutyl aniline). Early technical factors and limited experience contributed, but subsequent correct classifications of Benzisothiazolinone indicate that the deviation reflects compound-specific challenges rather than a systematic method deficiency. By omitting the result of just one of these products for the reasons stated above, the sensitivity reaches 85.7% and the accuracy 85% at Eurofins, thus meeting the acceptance criteria.

2.2.5 WLR at BRT

43. At BRT, three chemicals exhibited variability between runs, only one too many to meet the WLR criterion. Analysis of the result show that this underperformance can be balanced by the recognised variability of Cetrimide in the VRM. Moreover, analysis of the data shows that a dilution error is very likely the cause of the misclassification for methyl heptine carbonate.

44. During validation of the VRM, Cetrimide was already frequently misclassified with VRM and led the VMT to conclude in the validation report that "*cetrimide is known to be relatively difficult to identify as a non-sensitiser, since the fold induction of the ATF3 and IL-8 expressions increase near to the respective cut-off values*" (12).

45. The other compound misclassified in one run at BRT was Methyl heptine carbonate. What is noteworthy, when reviewing all runs across the three laboratories, is that this substance consistently produced strong gene induction and clear positive results at all concentrations—except at BRT in Run 6, where no gene induction was detected for any concentration (Table 8). This discrepancy calls into question the validity of that particular run. Methyl heptine carbonate, is a volatile compound and errors in handling or dilution preparation could explain this result. Additionally, this chemical was assigned to group 2 for the grouping approach with a starting concentration of 3.13%. However, preliminary dose-finding with samples A115-0, B130-0 and C176-0 indicated it should have been tested from 100%. While this did not impact classification in other runs, the low concentration tested suggests that even a minor handling error with this volatile chemical could account for the Run 6 misclassification of sample C342-2.

Table 8: Results for the 9 coded samples of Methyl heptane carbonate tested in the validation.

		conc. (in%)	viability (%)	ATF3 (15-fold)	GCLM (2-fold)	DNAJB4 (2-fold)	IL8 (4-fold)	Result
BRT	C342-2 Run 6	3,13	99,35	0,91	1,16	0,90	0,73	NS
		1,56	98,10	0,74	1,04	0,92	0,72	
		0,78	97,19	0,86	1,00	0,86	0,61	
		0,39	99,41	1,10	1,32	0,81	0,82	
	C176-0 Run 4	100,00	100,85	48,04	2,96	25,74	1,06	S
		25,00	91,92	26,83	4,31	22,04	0,96	
		12,50	100,85	29,55	9,86	47,49	0,72	
		6,25	99,32	15,59	14,82	36,98	0,41	
	C206-2 Run 10	3,13	96,54	5,61	11,09	17,06	0,58	S
		1,56	96,43	6,83	10,08	460,37	35,69	
		0,78	97,71	1,43	11,10	5,81	1,52	
		0,39	98,35	1,13	8,00	3,45	1,21	
Eurofins	A115-0 Run 4	**100**	102,99	24,22	5,80	48,50	0,66	S
		50	97,17	19,89	7,29	45,71	0,63	
		25	98,22	11,07	8,84	29,71	0,33	
		12,5	92,17	12,70	8,41	31,19	0,31	
	A346-2 Run 6	**100**	100,07	39,23	7,04	61,53	1,93	S
		50	101,15	38,76	5,77	50,40	1,38	
		25	90,25	30,63	8,26	41,50	1,32	
		12,5	91,10	42,92	7,10	48,49	1,23	
	A262-2 Run 10	3,13	93,89	13,04	10,80	10,07	1,13	S
		1,56	92,65	9,54	10,70	8,08	1,07	
		0,78	100,29	3,40	7,58	3,35	0,94	
		0,39	97,56	2,67	7,96	3,44	1,48	
Mattek	B130-0 Run 4	**100**	88,42	50,90	4,34	21,83	1,10	S
		25	98,64	74,38	2,88	44,68	1,57	
		12,5	99,18	46,88	7,13	49,98	1,35	
		6,25	99,30	34,99	4,81	21,09	1,16	
	B328-2 Run 6	3,125	100,77	8,21	15,14	23,21	0,64	S
		1,56	98,48	7,31	26,08	9,17	0,61	
		0,78	100,04	2,76	81,07	2,93	0,76	
		0,39	99,79	3,13	32,95	3,71	0,99	
	B235-2 Run 10	3,125	100,39	12,56	17,50	29,17	0,52	S
		1,56	99,04	3,64	15,33	11,20	0,37	
		0,78	99,45	2,63	17,88	9,84	0,91	
		0,39	98,08	1,91	12,67	7,00	1,74	

Yellow cells indicating gene with Imax value reached - Grey cells indicating absence of induction of any gene at any concentration.

46. For DNCB, the third compound contributing to variability at BRT, the result observed in one run is difficult to explain because this substance is generally clearly identified as a sensitiser by all validated methods, including VRM. However, it is important to emphasise that out of the nine validation runs performed with this compound, only one failed to classify it as a sensitiser.

2.2.6 Conclusion on WLR observed at BRT:

47. In summary, among the three substances for which variability was observed in three runs at BRT, only one compound, DNCB, cannot be explained. The error with Methyl heptine Carbonate is likely due to a handling or concentration error, particularly relevant for a volatile compound being tested at an unexpectedly low starting concentration. This isolated error, if excluded, BRT's WLR would be 83.3%, thereby meeting the acceptance criteria required by the Performance Standards.

2.3 Conclusion from the core VMT on the performances

48. Using the modified criteria, this me-too validation of Epi2SensA across three laboratories demonstrated excellent average specificity (88.9%) and sensitivity (88.1%). The VMT considers that the missed compound at Eurofins results from the specific challenge posed by Lauryl Gallate, Benzisothiazolinone and Dibutyl aniline in the test set combined with inexperience during the initial runs with Benzisothiazolinone. WLR (within-laboratory reproducibility) and BLR (between-laboratory reproducibility) results reflect the robustness of the method. At BRT, a single WLR deviation was likely due to a handling error when preparing Methyl heptane carbonate dilutions, a volatile compound.

49. The VMT concludes that the overall performance of the method, once initial runs and specific known errors are contextualised, meets both the spirit and the performance standard criteria. Therefore, the acceptance of the validation study as sufficiently robust and in accordance with regulatory expectations is justified.

3 The Epi2SensA test method

3.1 Intended purpose of the test method

50. The Epi2SensA *in vitro* method has the same intended use as the VRM. It will be used in conjunction with other *in chemico* and *in vitro* tests in the OECD TG442 series as part of an IATA in the AOP framework to identify the sensitisation potential of substances for the classification and labelling of skin sensitisation hazards.

3.2 Applicability of the test method

51. The Epi2SensA and the EPiSensA are based on the use of a RhE model to assess induction of 4 genes by potential skin sensitiser. The EpiDerm™ model and the LabCyte EPI-MODEL 24 used in the Epi2SensA and EpiSensA methods, respectively, have both already been validated for skin corrosion (OECD TG439) and skin irritation (OECD TG439) where they share the same applicability domain. It is therefore reasonable to assume that the two methods share the specific characteristics related to the use of a RhE model, such as metabolic activity and physico-chemical properties of the chemicals tested.

52. EpiSensA, the VRM, has shown to be applicable to a broad range of chemicals covering relevant ranges of chemical classes, reaction mechanisms, skin sensitisation potency and physico-chemical properties (Table 9). Since RhE models allow the application of test chemicals directly to the surface of the RhE, a lipophilic vehicle as applied in animal tests can be used. Epi2SensA, as the VRM method have shown to be applicable to lipophilic chemicals (e.g., Log Kow > 3.5).

Table 9: Performance of VRM and Epi2SensA for pre/pro-hapten and lipophilic chemicals in the validation set (PF).

Chemical	CAS	LogP	Pre/Pro Hapten	Classification GHS in vivo	VRM classification	Epi2SensA classification
Lauryl gallate	1166-52-5	**6,21**	**X**	GHS Cat. 1A	Non-sensitiser	Non-sensitiser
Tetrachlorosalicylanilide	1154-59-2	**5,87**		GHS Cat. 1A	Sensitiser	Sensitiser
Farnesol	4602-84-0	**4,91**	**X**	GHS Cat. 1B	Sensitiser	Sensitiser

Dibutyl aniline	613-29-6	**4,7**	**X**	GHS Cat. 1B	Sensitiser	Sensitiser
Abietic acid	514-10-3	**3,92**	**X**	GHS Cat. 1B	Sensitiser	Sensitiser
Amyl cinnamic aldehyde	122-40-7	**3.99**		GHS Cat. 1B	Sensitiser	Sensitiser
Isoeugenol	97-54-1	3,04	**X**	GHS Cat. 1B	Sensitiser	Sensitiser
Metol	55-55-0	0.63	**X**	GHS Cat. 1A	Sensitiser	Sensitiser
Benzyl butyl phthalate	85-68-7	**4,84**		Non-sensitiser	Non-sensitiser	Non-sensitiser
1-Iodehexane	638-45-9	**3.99**		Non-sensitiser	Non-sensitiser	Non-sensitiser
Hexane	110-54-3	**3.9**		Non-sensitiser	Non-sensitiser	Non-sensitiser

53. There is a known limitation in the regulatory approved in vitro sensitisation test methods in terms of applicability domain (i.e., pre/pro-hapten and lipophilic chemicals). The RhE models used in Epi2SensA and EpiSensA methods exhibit metabolic capacity that mimics that of human skin (5,6). Therefore, the test methods have shown to be applicable to pro-haptens (i.e., chemicals requiring enzymatic activation to induce skin sensitisation potential) and pre-haptens (i.e., chemicals that become sensitisers through abiotic transformation) (2,7).

3.3 Compliance with the essential test method components of the VRM

3.3.1 The RhE model: EpiDerm™ (EPI-200)

54. The first publication on the EpiSensA method dates back to 2013 and was based on the use of the EpiDerm™ model (Mattek, USA, Europe, China). The method developer then shifted to the Labcyte EPI-MODEL-24 for finalising the method. During discussions with the developers of the method, it appears that the change in epidermal model for the development of the method was justified by the availability of a locally reconstructed human epidermal model.

55. The EpiDerm™ model (EPI-200) used in the Epi2SensA method, consists of normal human-derived epidermal keratinocytes, which have been cultured to form a 3-dimensional, multilayered, highly differentiated model of the human epidermis (8). It consists of organised basal, spinous and granular layers, and a multilayer stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo.

56. The EpiDerm™ model is part of several methods included in different test guidelines to assess skin corrosion (OECD TG431), skin irritation (OECD TG439) phototoxicity (OECD TG498) of chemicals and skin irritation of Medical Devices (ISO 10993-23). Furthermore, it has been scientifically validated by the European Center for Validation of Alternative Methods (EURL ECVAM) to assess the genotoxicity potential of chemicals in the Reconstructed Skin Micronucleus assay (RSMN).

57. To evaluate the skin sensitisation of chemicals with the Epi2SensA method, Mattek proposes a ready-to-use kit named EPI-200-SENSA KIT, which includes 24 EpiDerm™ tissues (EPI-200), 6 and 24 well plates, culture medium, a 10% Triton-X solution, PBS, and the SOP.

3.3.2 Production and quality control (QC) of the EpiDerm™ model

58. The EpiDerm™ model is manufactured according to defined quality assurance procedures (GMP) in three different sites, MatTek Corporation, Ashland, MA, USA, MatTek IVLSL, Bratislava, Slovakia and Cellex Biotech, Shanghai, China. All biological components of the epidermis and the culture

medium are tested by manufacturer for viral, bacterial, fungal and mycoplasma contamination. The barrier function is verified by determination of the ET-50 value following exposure to Triton X-100 (1%) for up to 10 hours for each EpiDerm™ lot. The ET-50 must fall within a range established based on a historical database of results (Table 10).

Table 10: QC batch release criteria of the EpiDerm™ model

	Lower acceptance limit	Upper acceptance limit
EpiDerm™ SIT (EPI-200) (1% Triton X-100)	ET50 = 4.0 hr	ET50 = 8.7 hr

59. The morphology and thickness of the tissues are also verified by histological analysis of each batch to ensure that they are within acceptable ranges. They attest the appropriate formation of the epidermal barrier, the presence of a functional stratum corneum, a viable basal cell layer, and intermediate spinous and granular layers.

3.3.3 Adaptation to the EpiDerm™ RhE model of the VRM procedure condition

60. During the preliminary testing by the lead laboratory, Mattek (Ashland, USA), modification of the protocol has been performed to adapt the VRM SOP to the EpiDermTM model. It is well recognised that some parameters, volume, length of exposure, are models dependent. Such type of adaptation has been made for other OECD methods with RhE models, e.g., corrosion and skin irritation (TG439, 431).

Volume applied on the tissue

61. Using the EpiDerm™ model, the volume of chemical applied was increased from 5 µL in the VRM protocol to 10 µL. Indeed, the surface area of EpiDerm is twice that of Labcyte and requires a larger volume to keep the same ratio quantity applied to surface. On a practical level, 10 µL is also easier to apply (Table 11).

Table 11: Comparison of the applied volume with the EpiDerm™ and the Labcyte models

Tissue model	Volume applied	Surface of the RHE	Ratio volume /surface
EpiDerm™	10µL	0.63 cm2	15.9µL/cm2
LabCyte	5 µL	0.32 cm2	15.6 µL/cm2

3.3.3.1 Cell viability

62. During preliminary experiments using the EpiDerm™ model with the VRM SOP, unexpected cytotoxicity occurred for some compounds. Investigation of origin of this issue led to some modification of the VRM SOP that have been presented in a poster at the SOT congress in 2024 (9).

3.3.3.1.1 Length of exposure to the chemicals

63. With 6 hours exposure duration, two weak skin sensitisers, eugenol and methyl methacrylate, were misclassified as non-sensitisers because of cytotoxicity at the higher concentrations tested. It was decided to evaluate a shorter duration of exposure, 1 hours instead of 6 hours, to reduce cytotoxicity effects. A 5-hour post-incubation period was maintained to allow analysis of gene expression 6 hours after RhE contact with the chemical. Indeed, from discussion with the method developer (Dr H. Mizumachi, Dr M. Masaaki and Dr S. Sho from Kao Corporation), based on time-kinetics experiments, 6 hours post exposure is the right time to measure gene expression

level in the RhE models. Similar timing is also used in the SENS-IS method, another in vitro method using gene expression with RhE models for skin sensitisation. In the SENS-IS protocole the RT-PCR is performed 6 hours (15 minutes of exposure + 6 post-incubation) after contact with the chemical.

64. This modification of the SOP, allowed to solve the issue for Eugenol but not for another weak sensitiser, Methyl methacrylate.

3.3.3.1.2 Killed control

65. Even with a shorter exposure time to chemicals as described in the previous paragraph, elevated level of cytotoxicity was observed for some products.

66. With the LDH method to assess cell viability in the VRM, the cell viability is calculated by comparison of the LDH released by the tissue to the maximum LDH release measured in a killed control tissue. Any underestimation of the maximum amount of LDH released by the killed control leads to an underestimation of the calculated cell viability of the tested RhE.

67. Based on Mattek internal experience with the EpiDerm™ model, the way used in the VRM to "kill" the tissues was questioned. To investigate this parameter, the kinetic of LDH release by the EpiDerm™ model was evaluated using different condition. In the same tissues, cell viability calculated using the LDH method was compared, with the MTT reduction method used with EpiDerm™ in other OECD guidelines for skin corrosion/irritation. This demonstrated that using the VRM standard operating procedure for killed control with the EpiDerm™ RhE, maximum LDH release was not achieved. Significant residual cell viability is measured with the MTT method for the killed control (Figure 1). More stringent conditions were investigated to ensure complete tissue death. The conditions selected for the EpiDerm™ model to achieve 100% cell death with maximum LDH release are obtained by increasing the volume of Triton-X applied and by adding the Triton-X inside the culture medium rather than topically (Figure 2).

Figure 1: Comparison of LDH release and cell viability of the EpiDerm™ measured by MTT after 6, 24 or 48 hours exposure to 10 µL of 10% Triton-X applied topically.

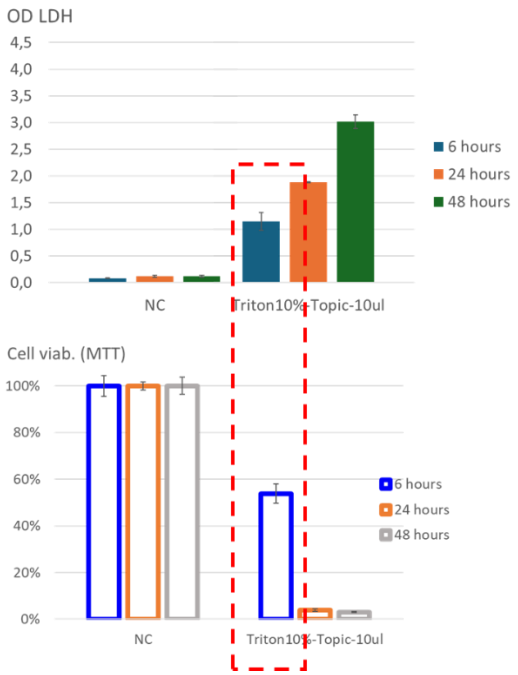
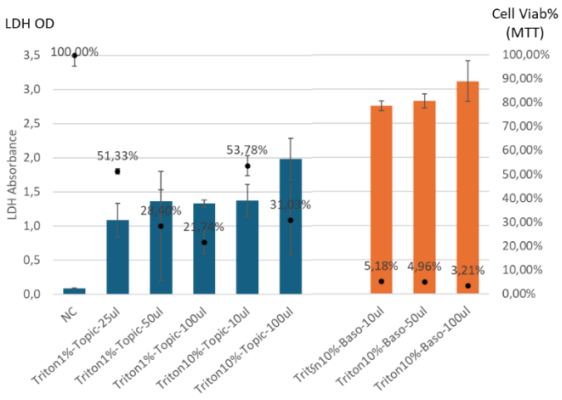


Figure 2: Comparison of topical and baso-lateral application of different quantity of Triton-X on the the LDH relasa and cell viability.

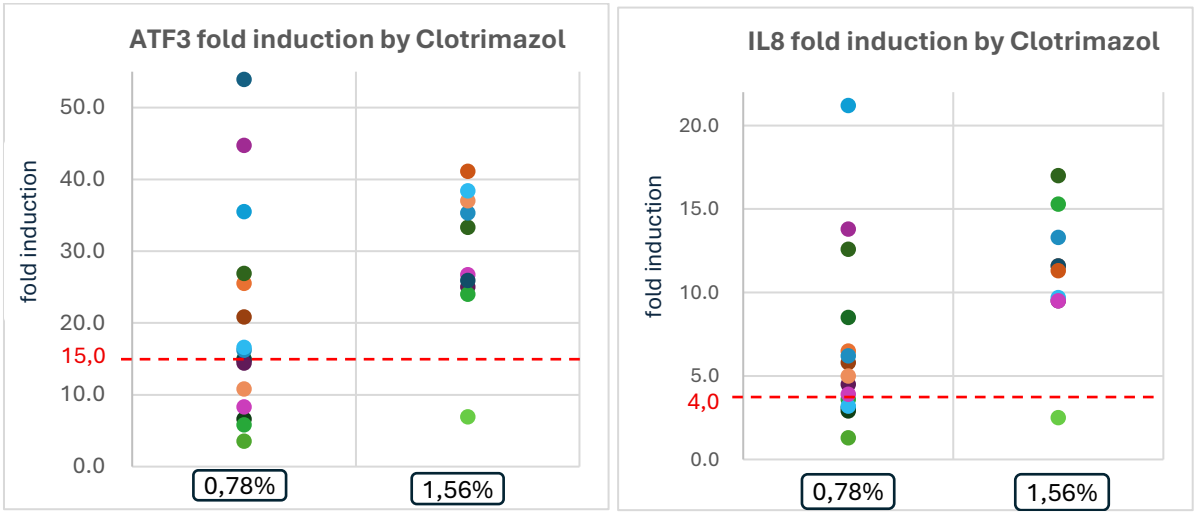


68. Finally, 6 hours exposure was conserved but the exposure to Triton-X was done in the medium of culture rather than topically, and the volume was increased from 10 to 50uL to ensure to completely kill the tissues.

3.3.3.2 Positive control

69. Gene induction by the positive controls are part of run acceptance criteria. When testing the method with the EpiDerm™ RhE model we experienced some run exclusions because of Clotrimazole which was not reaching the fold induction required for ATF3 (15 fold) and IL8 (4 fold). With Clotrimazole 0.78%, accordingly to VRM for one of the positive control, 40% of runs failed because of low fold induction mainly for ATF3 gene. To overcome this issue 10 runs were performed including Clotrimazole 0.78% and Clotrimazole 1.56%. With this higher concentration of Clotrimazole only 1 run was excluded (10% failure) because of Clotrimazole acceptance criteria (Figure 3). It was then decided to modify the SOP of Epi2SensA with the 1.56% Clotrimazole concentration for positive control.

Figure 3: Repeated fold induction of ATF3 gene induced by Clotrimazole at 0.78% or 1.56%



3.4 Modification of acceptance criteria and predictive model

70. Based on preliminary data at the lead laboratory and results of the interim analysis conducted with two runs for each lab, the core VMT implemented a modification to the acceptance criteria for tested concentrations, lowering the cell viability threshold from 80% to 60%. In addition, the core VMT agreed to assess the impact when changing the prediction model to require two genes to exceed the gene-specific induction thresholds for considering a test substance positive. The significant improvement in performance resulting from the implementation of these modifications enables the method to meet the acceptance criteria for validation.

71. The core VMT therefore decided to implement the following two rules in the EPi2SensA method:

3.4.1.1 Acceptance criteria of a tested concentration:

72. The result of at least one tested concentration that shows ≥ 60% mean cell viability should be used. If the mean cell viability is < 60% for a given tested concentration, the result for that tested

concentration should be excluded for a positive prediction but might be used for a negative prediction.

3.4.1.2 Prediction model

73. An Epi2SensA prediction is considered positive if at least two of the following conditions are met:

    - The Imax for ATF3 is > 15 for at least one tested concentration.
    - The Imax for GCLM is > 2 for at least one tested concentration.
    - The Imax for DNAJB4 is > 2 for at least one tested concentration.
    - The Imax for IL-8 is > 4 for at least one tested concentration.

74. The Epi2SensA prediction is considered negative if the three conditions below are met:

    - The mean fold-induction value of the marker genes exceeds for at most one the respective cut-off values for any of the four genes at the tested concentrations that shows ≥ 60% mean cell viability;
    - At least 3 concentrations were tested;
    - If the highest concentration tested was lower than the highest soluble or stably dispersed concentration, at least one mean cell viability at the tested concentrations is < 60%.

3.4.1.3 Rational for modification of acceptance criteria and predictive model

75. Preliminary studies by the lead laboratory have shown a tendency for the LDH method to overestimate cytotoxicity compared to MTT measurements for the EpiDerm™ model. Although this issue has been addressed by modifying the method used to obtain killed controls, it cannot be completely excluded that this tendency persists. Considering that in validated OECD methods for skin irritation, the cut-off value for a non-irritant chemical is 50%, a lower threshold of cell viability acceptance was considered. Another important parameter was that initially the EpiSensA method with the EpiDerm™ model had an acceptance criterion for viability set at 50% (3). This was later raised to 80% when the method was adapted to the Labcyte model (10). The decision of the core VMT to reduce the viability threshold to 60% clearly enhanced test sensitivity, resulting in consistently positive predictions for the sensitiser benzisothiazolinone across laboratories. It reduced the need for retesting for certain chemicals like p-phenylenediamine, making borderline results more interpretable. The 60% threshold still maintains sufficient tissue integrity while allowing detection of sensitisation responses that occur at concentrations causing moderate cytotoxicity.

76. Regarding the prediction model, the OECD validation frameworks (notably Guidance Document No. 34 and associated Performance Standards) recognise that at the completion of a validation study, there may be situations where the data clearly indicates that the decision criteria need to be refined in order to increase the predictive capacity. This is particularly true when me-too methods are using different tissues models. As an example, in the OECD TG 431 for skin corrosion, different time points (3', 60' and 240' for Episkin™, 3' and 60' only for EpiDerm™, SkinEthic™, epiCS® and LabCyte EPI-MODEL24) and cell viability threshold values (35% for Episkin™, 50%, 25%, 18%, 15% for EpiDerm™, SkinEthic™, epiCS® and LabCyte) are required depending of the tissues. As another example, OECD TG 492 for eye irritation specifies distinct viability cut-off thresholds that define "No Category" chemicals for each accepted cornea-like tissue model: typically >60% viability for EpiOcular™ and SkinEthic™ HCE EITL, >50% for SkinEthic™ HCE EITS, >40% for LabCyte CORNEA-MODEL24 EIT, and > 35% for liquids and > 60% for solids in MCTT HCE™ EIT.

77. Modifications of the criteria for acceptance and for prediction were implemented to address the use of the EpiDerm™ model. They markedly enhanced the method's robustness and predictive performance (Table 11). Observed specificity for Mattek and BRT is increased, without affecting that of Eurofins. Sensitivity remains unchanged for Mattek and BRT but is slightly reduced for Eurofins. Both BLR and WLR are improved for all three laboratories. Despite BRT not meeting the WLR acceptance criteria— most likely induced by handling or concentration error —the study demonstrated that Epi2SensA achieves performance acceptable for a similar method to the validated reference method (VRM).

Table 11: Comparison of the WLR, BLR and predictive capacity according to the original analysis and according to the modified acceptance criteria, with green highlighted cells indicating met acceptance criteria

	acceptance criterion	Mattek		Eurofins		BRT		Average original	Average modified
		original	modified	original	modified	original	modified		
WLR	≥ 80%	66,7% (8/12)	91,7% (11/12)	58,3% (8/12)	83,3% (10/12)	66,7% (8/12)	75,0% (9/12)	63,9%	83,3%
BLR	≥ 80%	original: 70%			modified: 85%			70%	85%
Specificity	≥ 65%	66,7% (4/6)	100% (6/6)	83,3% (5/6)	83,3% (5/6)	50,0% (3/6)	83,3% (5/6)	66,6%	88,9%
Sensitivity	≥ 85%	92,9% (13/14)	92,9% (13/14)	85,7% (12/14)	78,6% (11/14)	92,9% (13/14)	92,9% (13/14)	90,5%	88.1%
Accuracy	≥ 85%	85,0% (17/20)	95,0% (18/20)	85,0% (17/20)	80,0% (16/20)	80,0% (16/20)	90,0% (18/20)	83,3%	88,3%
Balanced accuracy		82.89%	96,5%	84.5%	81%	71.5%	88,1%	78.6%	88.5%

3.5 Protocol

78. The following is a description of the procedures for the Epi2SensA test method, which comprises two steps: concentration-finding study and main study (Gene expression analysis). Depending on the conclusions of the peer review, the Epi2SensA standard operating procedure will be submitted to the Tracking System for Alternative methods towards Regulatory acceptance (TSAR) to ensure the widest possible accessibility.

79. Excepted the adaptation described in the previous paragraph "Adaptation to the EpiDerm™ of the VRM procedure condition", the procedure of the Epi2SensA method is similar to the procedure of the VRM, the EpiSensA method.

3.5.1 Preincubation of RhE model

80. After reception, the EpiDerm tissues, in a sterile hood place the tissues in a 6-well plate with fresh medium of culture (EPI-200-ASY medium) for cultivation into the incubator (37±1°C, 5±1% CO2, 90±10% RH) for 60 ± 5 minutes. At the end of the first 1hr pre-incubation period, carefully remove the medium and replace it with 0.9 ml of fresh medium. Incubate the tissues (37±1°C, 5±1% CO2, 90±10% RH) overnight.

3.5.2 Vehicle selection and test chemical solubility

81. Assessment of solubility is conducted prior to testing. The solubility of each chemical is evaluated and confirmed visually. For this purpose, test chemicals are dissolved or stably

dispersed at a concentration of 50% in acetone: olive oil; 4:1 (AOO, 20% v/v of olive oil in acetone) as a first vehicle option, (e.g. 0.25 g of test chemical is measured, and 0.5 mL of AOO is added), distilled water (DW) as a second vehicle option, or 50% v/v % ethanol in DW (50% EtOH) as a third vehicle option. If the test chemical is not soluble or does not stably disperse (i.e. a colloid or suspension in which the test chemical does not settle or separate from the vehicle into different phases within 10 minutes of preparation at room temperature) at a concentration of 50% in any of the vehicles, the highest soluble concentration should be determined by 2-fold serial dilutions beginning with 50% down to 0.0122%. If the test chemical is not soluble or does not form a stable dispersion at 0.0122%, the chemical is not applicable for testing using Epi2SensA. The appropriate vehicle is defined as the vehicle that dissolves the test chemical or forms a stable dispersion at the highest concentration tested. It should be verified whether the highest concentration determined can be prepared at weight per volume by pipetting the necessary quantity into a glass vial. If the highest soluble or stably dispersed concentration is determined to be 0.0488%, 0.0244%, or 0.0122%, the subsequent concentration-finding study (Section 4.3) can be skipped, and main study should be performed (Section 6). In cases in which a vehicle other than AOO, DW, or 50% EtOH is used, appropriate scientific rationale for use of that vehicle should be provided.

3.5.3 Concentration-finding study

82. A concentration-finding assay is performed to determine the concentrations of test chemical be used for the main study, which looks at gene expression analysis. In the main study, test chemical concentrations that initially show ≥ 80% mean viability, now revised to a mean viability of ≥ 60%, should be used, along with at least one mean viability <80% initially, now revised to < 60%, for negative judgement. Therefore, the lowest test chemical concentration that induces a < 80% tissue viability initially and now revised to < 60%, is determined in concentration-finding study.

83. Test chemicals are prepared on the day of testing and dissolved or stably dispersed in an appropriate vehicle at the highest concentration determined. Starting from the highest concentration, 4-fold serial dilutions are prepared to 0.0122 or 0.0244% (w/v) in the corresponding vehicle. Thus, depending on the starting concentration, a varying number of dilutions are prepared and tested. The corresponding vehicles utilised for the preparation of the test chemicals are used as the vehicle controls. Both vehicle control and killed control are used for calculation of viability. Vehicle control is used to define 100% viability, and killed control is used to define 0% viability. For tested chemicals, duplicate (two EpiDerm RhE) are used for each concentration.

84. Cell viability is measured by a lactate dehydrogenase (LDH) assay utilising formazan as the dye. LDH is a stable cytoplasmic enzyme present in all cell types, and it is released into the cell culture medium as a result of damage to the plasma membrane. The LDH assay measures the amount of formazan dye produced by released LDH.

3.5.3.1 Preparation

85. Prepare the test chemical at the highest concentration in the appropriate vehicle (solid: weigh and dissolve; liquid: start from 100%) in a glass vial, then prepare 4-fold serial dilutions down to 0.024 w/v % or lower.

3.5.3.2 Topical applications (1-hour treatment/rinsing/ 5-hour post-incubation):

86. All procedures should be performed under sterile conditions, starting with pre-warming the assay medium at 37°C for 15 minutes and carefully planning the plate layout, especially separating liquid test chemicals at 100% from controls into individual 6-well plates. Each tissue insert is placed in a well with 0.9mL medium, treated with 10µL of working solution (N=2 tissues per solution), and appropriate controls are prepared; tissues are incubated for 1 hour, rinsed 15 times, then undergo a 5-hour post-exposure incubation, while killed and blank medium controls are processed in parallel according to specific instructions. These steps ensure controlled exposure, proper rinsing, and reliable viability measurement for each experimental and control group.

Figure 1: Priniciple of the dose finding step

3.5.3.3 LDH Cytotoxicity assessment

87. Pipet 2 x 50 µL aliquots of the medium from each tissue into a 96-well plate and perform a lactate dehydrogenase (LDH) assay using an LDH cytotoxicity detection kit according to the manufacturer's instructions. Also, pipet 2 x 50 µL aliquots of the blank medium into the 96 well plate. Prepare the following reaction mix (Solution A + Solution B) for the requisite amount and add an equal volume (50 µL) of the reaction mix to the collected medium in a 96-well plate using a multi-channel pipette. Incubate the plate for 30 minutes at room temperature in the dark. After 30 minutes, stop the reaction by adding 50µL/well of 1M HCl using a multi-channel pipette.

88. Measure the absorbance at 490 or 492 nm and the reference wavelength (≥ 650 nm) using a 96-well plate absorbance reader. The measurement should be performed immediately (< 1hr) after adding HCl.

• Calculate Δabs.for each sample by subtracting the absorbance at reference wavelength from the absorbance at 490 nm.

• Calculate tissue viability using the following equation:

$$cell\ viability\ (\%) = 100 - \frac{\Delta abs.\,of\ test\ chemical\ treatment - \Delta abs.\,of\ vehicle\ control)}{mean\ \Delta abs.\,of\ killed\ control - \Delta abs.\,of\ vehicle\ control} * 100$$

• The lowest concentration showing less than 80% tissue viability initially, now revised to less than 60%, is used for the subsequent main study.

3.5.4 Main study (Gene expression analysis)

3.5.4.1 Preparation of test chemicals and control substances for the main study

3.5.4.1.1 Positive Controls

89. Clotrimazole dissolved to a final concentration of 1.56 w/v% in AOO and 4-nitrobenzyl bromide (4NBB) dissolved in AOO to a final concentration of 0.10 w/v% are used as the positive controls.

For example, 0.0312 g of clotrimazole is measured into a glass vial and 1 mL of AOO is added. After clotrimazole is solubilised (mixed thoroughly with vortexing as needed), it is diluted 2X with AOO in a glass vial – add 0.5 mL of the solubilised clotrimazole to 0.5 mL of AOO. For 4NBB, 0.0100 g of 4NBB is weighed into a glass vial, and 1 mL of AOO is added. Once 4NBB is solubilised (mixed thoroughly; can be vortexed), it is diluted 10X with AOO in a glass vial – add 0.1 mL of the solubilised 4NBB to 0.9 mL of AOO

3.5.4.1.2 Negative Control

90. The vehicle controls in each run are used as the controls.

3.5.4.1.3 Killed Control

91. Triton X-100 dissolved to a final concentration of 10 w/v% in DW is used for the killed control. The killed control is used to determine maximum LDH released from killed corresponding to 0% viability tissue. The 10% Triton-X solution for killed control is supplied by MatTek as part of the EPI-200-SENSA kit.

3.5.4.1.4 Test chemicals

92. Prepare the test chemical at the lowest concentration that caused <80% tissue viability initially, now revised to <60%, in the dose-finding study, then make 2-fold serial dilutions—choosing 3 concentrations if the viability–concentration curve is steep or 5 if it is shallow—adjusting the highest concentration to one that gave >90% viability; if no concentration reduced viability below 80% initially, now revised to 60%, prepare at least 3 serial dilutions from the highest soluble concentration or 100% neat.

3.5.4.2 Topical application: 1 hour exposure followed by 5-hour post-incubation

93. For each test chemical, one run is required to obtain a prediction. Three tissue units for each test chemical concentration, positive control substance and vehicle controls, two tissue units for the killed control, and one tissue unit for the non-treated control are used for the gene expression analysis.

• Apply an aliquot (10 µL) of the sample to the center of each RhE surface using a positive displacement pipette. The tip of the pipette must not touch the surface of the tissue. The surface of EpiDerm™ RhE tissue is hydrophobic, so when the vehicle is distilled water or another polar solvent, mesh (Part #: EPI-MESH) must be used to ensure homogeneous distribution of the test product on the tissue surface. The mesh is not used for samples dissolved in AOO.

• Incubate the treated tissues for 1 hour at 37±1°C, 5±1% CO2, 90±10% RH. After 1 hour, rinse the tissues 15X by pipetting 0.5 mL of DPBS onto the tissue surface. Use a repeater pipette (e.g. Multipette M4, Eppendorf) adjusted for 0.5 mL volume. After the 15 rinses, remove any excess DPBS by gently shaking the insert, blot the insert on sterile blotting paper, and put the insert back into in the same medium for post-exposure of 5h. Return the 6-well plates to the incubator (37±1°C, 5±1% CO2, 90±10% RH) for the 5h ± 5 min post-exposure.

3.5.4.3 RNA Isolation and cDNA preparation

3.5.4.3.1 Lysis of tissues

94. The lysis and RNA extraction protocols presented in the SOP uses RNAqueous Kit (Thermo Fisher) but also mentions that other commercially available kits can also be used.

95. The tissues are separated from the inserts using an RNA clean scalpel and homogenised in the RNA lysis buffer using a motorised electric homogeniser or a battery driven motor and pestle. After all tissue samples are completely homogenised, add an additional 300 µL of RNA lysis

buffer. The lysates are stored at -80°C overnight (minimum) or until ready to perform RNA isolation.

3.5.4.3.2 RNA isolation

96. In summary, samples are thawed, clarified by centrifugation, and the supernatant is mixed with ethanol before being passed through a filter cartridge for RNA binding, followed by sequential washes with kit-provided solutions. Finally, RNA is eluted in two steps using preheated elution buffer, producing purified RNA ready for downstream applications. Total RNA concentration of the sample is measured using a spectral photometer (e.g. NanoDrop).

3.5.4.3.3 cDNA Synthesis

97. The cDNA synthesis method outlined in the Epi2SensA SOP uses the RT2 First Strand Kit (Qiagen – Part # 330404) but other commercially available kits can also be used.

98. Basically, cDNA is synthetised from RNA after a genomic DNA elimination step by adding a reverse transcription mix and performing sequential incubations at 42 °C and 95 °C. Then, the resulting cDNA is diluted in RNase free water, and stored at −20 °C or used directly for RT-PCR.

3.5.4.4 Quantitative RT-PCR

99. After cDNA synthesis, the expression levels of marker genes (i.e. ATF3, GCLM, DNAJB4, and IL-8) and the endogenous control gene (i.e. glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) are analysed using RT-qPCR. Quantitative RT-PCR was performed using the RT2 SYBR Green PCR Mastermix.

3.5.4.5 Data evaluation

100. Relative gene expression levels versus control (fold induction) are calculated using the 2-ΔΔCt method described below:

- Calculate the ΔCt value by subtracting the Ct value of GAPDH from the Ct value of the respective marker genes.
- Calculate the mean ΔCt value of the vehicle control.
- Calculate the ΔΔCt value by subtracting the mean ΔCt value of the vehicle control from the ΔCt value of the test chemical or positive control.
- Calculate the fold induction by 2-ΔΔCt.

3.5.4.6 Acceptance criteria

101. The following acceptance criteria should be met for a run to be considered valid:

• The cell viability of at least two tissue units of the vehicle control should be ≥ 90%. If the cell viability of only one vehicle control is < 90%, the Ct values obtained from the remaining two tissue units should be used.

• The mean cell viability of both positive controls (i.e. 1.56% [w/v] clotrimazole and 0.10% [w/v] 4-nitrobenzyl bromide (4-NBB)) should be ≥ 80%.

• In the 1.56% (w/v) clotrimazole positive control, the mean fold-induction values for ATF3 and IL-8 should exceed the cut-off value (i.e. the ATF3 fold-induction value should be > 15, and the IL-8 fold-induction value should be > 4).

• In the 0.10% (w/v) 4NBB positive control, the mean fold-induction values for GCLM and DNAJB4 should exceed the cut-off value (i.e. the GCLM fold-induction value should be > 2, and the DNAJB4 fold-induction value should be > 2).

102. The following acceptance criteria should be met in order to consider a tested concentration's result valid:

• The result of at least one tested concentration that shows ≥ 60%* mean cell viability should be used. If the mean cell viability is < 60% for a given tested concentration, the result for that tested concentration should be excluded for a positive prediction but might be used for a negative prediction.

(*) value adapted for the EpiDerm™ model in the Epi2SensA method

• When the mean GAPDH Ct value for a given test chemical concentration is within ±1 of the mean GAPDH Ct value of the corresponding vehicle control, the result obtained at that concentration is acceptable

3.5.4.7 Prediction model

103. Each test chemical is evaluated in one run to derive a prediction (positive or negative).

104. An Epi2SensA prediction is considered positive if at least two* of the following conditions is met:

- The Imax for ATF3 is > 15 for at least one tested concentration.
- The Imax for GCLM is > 2 for at least one tested concentration.
- The Imax for DNAJB4 is > 2 for at least one tested concentration.
- The Imax for IL-8 is > 4 for at least one tested concentration.

(*) value adapted for the EpiDerm™ model in the Epi2SensA method

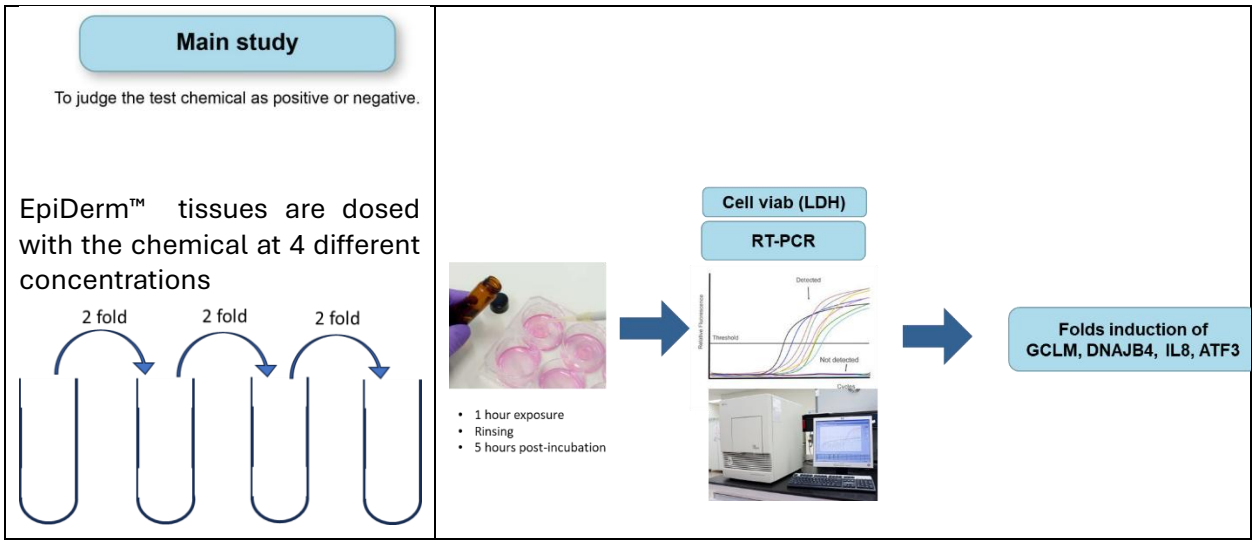
105. The Epi2SensA prediction is considered negative if the three conditions below are met:

• The mean fold-induction value of the marker genes does not exceed the respective cut-off values for any of the four genes at the tested concentrations that shows ≥ 80% mean cell viability;

• At least 3 concentrations were tested;

• If the highest concentration tested was lower than the highest soluble or stably dispersed concentration, at least one mean cell viability at the tested concentrations is < 60%;

Figure 2: Schematic representation of the main study



3.5.5 Test Definition: Conclusions of the Validation Management Team

106. The VMT concluded that the Epi2SensA adaptation using the EpiDerm™ (EPI-200) RhE model represent RhE model-dependent modifications but are compliant with the essential test method components of the VRM.

4 Transferability

4.1 General aspects

4.2 Training and Transfer of the test method to participating laboratories

107. The transfer phase took place from July to December 2024. Mattek Ashland who adapted the EpiSensA method to the EpiDerm™ model was the lead laboratory in charge of the transfer of the method to the 3 naive laboratories. Based on their internal experiences, the 3 laboratories had different needs :

- FDSC participated to the validation of the validated reference method (VRM) and was comfortable in the use of the EpiDerm™ model.
- Eurofins Germany has long experience of the use of EpiDerm™ (EPI-200 model) for other OECD methods. The involved team had no previous experience for RT-PCR and have internal support.
- BRT was new to the use of RhE models such as the EpiDerm™ model. RT-PCR was also a new method to be implemented at the BRT. The study director had experience with RT-PCR from a previous position.

108. The lack of RT-PCR experience at Eurofins and BRT proved to be a source of variability and required numerous alignment steps during both the transfer phase and the intermediate phase 2 reproducibility assessment.

109. The Epi2SensA standard operating procedure (SOP) was shared with the 3 participating laboratories. Several online meetings were organised to discuss the tips and tricks of the SOP and training video have been shared with them:

• MatTek EpiDerm™ Kit Unpackaging and Tissue Equilibration:https://youtu.be/bqtpY5ZxoAY?si=n5mtqgauG04jBKR5

• In vitro skin irritation test using the reconstructed human epidermal model, EpiDerm™ (the OECD TG439 protocol is different from Epi2SensA but it was useful to give an idea on how to handle the tissues even if dosing and rinsing steps are different): https://youtu.be/w2JAnqQCPEM?si=gSdT95xFVW4S9gwl

• Video tutorial of the Epi2SensA steps with the EpiDerm™ tissues: https://drive.google.com/file/d/1no7XfSGq2lWYLe58walQXO1J_xRDHJRm/view?usp=sharing

110. FDSC and Eurofins were considered ready to start the transfer in September 2024 without a specific training at Mattek facilities. For BRT, which has never used RhE models before, it was decided that they will perform a preliminary test with EpiDerm™ models in August to learn how to handle them.

111. In September 2024, the 3 laboratories performed a first experiment with the positive controls only. After verification of the results by the lead laboratory, the laboratories have carried out three successive runs with the to test 5 chemicals, 2 sensitisers and 3 non-sensitisers (Table 9). The chemicals were selected to not belong to the performance standard of the VRM method.

Table 9: After a first run with the positive controls only, the 4 laboratories have tested in 3 runs 2 sensitisers and 3 non-sensitisers.

Test Run	Training RUN 1	Training RUN 2	Training RUN 3
Clotrimazol 1.56%	Clotrimazol 1.56%	Clotrimazol 1.56%	Clotrimazol 1.56%
4-NBB 0.78%	4-NBB 0.78%	4-NBB 0.78%	4-NBB 0.78%
	Glycerol CAS 56-81-5	Salicylic Acid CAS 69-72-7	Glycerol CAS 56-81-5
	Eugenol CAS 97-53-0	2-Aminophenol CAS 95-55-6	Isopronanol CAS 67-63-0

112. The two positive controls, Clotrimazole and 4-NBB, performed correctly by all the laboratories (Table 10). For negatives, Salicylic acid was misclassified positive by 3 laboratories. From Saito et al., 2017, Salicylic acid has a higher induction for ATF3 than expected and is also, affecting the LDH assay (10). This can explain false positive classification. Glycerol was correctly classified as negative excepted by the lead laboratory. This was unexpected because Mattek correctly classified it in previous experiments. Saito et al. and Mizumachi et al. in their respective publications in 2017 and 2018, have also reported some misclassification with Glycerol when tested at high concentrations (7, 8). Isopropanol was correctly classified by the 4 laboratories. The two positive controls, Eugenol and 2-Aminophenol were correctly classified. However, several runs failed because they did not meet the acceptance criteria for GAPDH Ct values and for cell viability.

113. All the raw data have been shared with the VMT in the Excel file “POOL-PHASE-I.xlsx” and discussed at the fourth VMT meeting. Potential sources of variability in results related to RNA

extraction and cell viability were highlighted, along with ways to reduce this risk in the next phase. Consensus have been raised to close Phase 1 Training/Transfer and to move to next step of the validation.

Table 10: Results of the training/transfert phase 1

		Epi2SensA classification			
Chemical	GHS classification	BRT	Eurofins	FDSC	Mattek
Clotrimazol	Sensitiser	Sensitiser	Sensitiser	Sensitiser	Sensitiser
4-NBB	Sensitiser	Sensitiser	Sensitiser	Sensitiser	Sensitiser
Glycerol	Non sensitiser	Non sensitiser	Non sensitiser	Non sensitiser	Sensitiser
Isopropanol	Non sensitiser	Non sensitiser	Non sensitiser	Non sensitiser	Non sensitiser
Salicylic acid	Non sensitiser	Sensitiser	Sensitiser	Sensitiser	Non sensitiser
Eugenol Run 1	Sensitiser	Sensitiser Failed GAPDH acceptance	Sensitiser	Non sensitiser	Sensitiser
Eugenol Run 2	Sensitiser	Sensitiser Failed Cell Viab acceptance	Sensitiser Failed Cell Viab acceptance	Sensitiser Failed Cell Viab acceptance	Sensitiser Failed Cell Viab acceptance
2-Aminophenol	Sensitiser	Sensitiser Failed Cell Viab acceptance	Sensitiser	Sensitiser	Sensitiser

4.3 Results of technical transfer

114. The method has been considered mastered by the 3 laboratories at the end of the transfer phase. Two technicians have been trained in each laboratory to ensure any necessary back-up.

115. In light of Mattek's preliminary results and those from the training phase, the possibility of modifying certain run acceptance criteria was raised. However, it was decided to continue validation using the existing acceptance criteria and, based on the data generated, to consider a retrospective study to assess the impact of suggested acceptance criteria modifications.

4.4 Modifications made to the SOP

116. The transfer phase took place from July to December 2024. During this phase several technical meetings were organised between by the lead laboratory with the participating laboratories leading to release of different updates of the SOP.

Version	Date	Key Changes
0.3	24/06/2024	• Initial draft. Baseline procedure and acceptance criteria.
1.0	15/08/2024	• Minor wording improvements
1.1	28/08/2024	• Updated table of contents, • Updated Table 1 to include medium control,

		• Added Annex E with contents of EPI-200-SENSA kit.
1.2	08/01/2025	• Updated table of contents, • Updated Table 1 to include medium control • Added Annex E contents of EPI-200-SENSA kit. • Updated "6.5 Lysis of tissues" and "6.6 RNA extraction". • Updated "9.Acceptance criteria" and "10.Prediction model". • Added notes that kits for tissue lysis, RNA extraction, and cDNA synthesis are presented as examples. Other commercially available kits can also be used.
2	10/03/2025	• Update of the cell viability calculation formula in sections 4.3, 6.3, and Annex B.

5 VMT overall conclusions and recommendations

5.1 Overall Conclusions

117. The objective of this study was to perform a "me-too" validation of the Epi2SensA method following the requirements of the Performance Standards for the Assessment of Proposed Similar or Modified In Vitro Epidermal Sensitisation Assay (EpiSensA) Test Methods (OECD GD 396, 2024). To this end, , the adherence to the essential test method components was assessed and the within-laboratory reproducibility (WLR), the between-laboratory reproducibility (BLR) and the predictivity of the Epi2Sensa were evaluated across three independent laboratories (Mattek, Eurofins, BRT), using the 20 reference chemicals listed in the performance standards, supported by a fourth laboratory (FDSC) that tested a few samples.

118. Considering that:

1- Modification of the cell viability threshold from 80% to 60% and refinement of the predictive model to require at least two genes exceeding induction thresholds for a positive result, resulted in substantial improvements in robustness and predictive accuracy.
2- Performance of the Epi2SensA following these adaptations demonstrated acceptable WLR (average 83.3%), BLR (85%), specificity (average 88.9%), sensitivity (average 88.1%), and accuracy (average 88.3%).
3- Deviations in reproducibility at BLR and sensitivity at Eurofins were linked to known challenging chemicals, a potential handling error, and initial technical onboarding.

The core VMT considers that the Epi2SensA method utilising the EpiDerm™ (EPI-200) reconstructed human epidermis model was successfully validated as a "me-too" method to the EpiSensA reference method (VRM), addressing the second key event (KE2) of the skin sensitisation Adverse Outcome Pathway.

5.2 Recommendations

119. The core VMT requested the lead laboratory to expand the number of compounds tested to confirm the positive impact of the modified predictive model on the predictive performance of the Epi2SensA method.

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Appendix I

Study plan for the me-too validation of the Epi2SensA

Version 1.0 (July1st 2024)

Document history			
version	date	Authors	observation
1.1	27/06/2024	Christian Pellevoisin Sebastian Hoffmann	

Table of content

Objective of the study

Since the publication of the “The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins” in 2012, several methods modelling of AOP Key events have been validated and integrated into TG 442C, D and E. In 2023, JaCVAM finalized the scientific validation of the EpiSensA method, which was integrated into TG 442D in 2024. This method is based on the use of a reconstructed human epidermis (RhE), the LabCyte EPI-MODEL24 model from J-TEC as an experimental system.

The objective of this study is to carry out a me-too validation for a similar method using the MaTtek EpiDerm™ model (EPI-200) as experimental system. The draft performance standards of the EpiSensA (available here) will be applied to determine its reliability (within- and between-laboratory reproducibility) and relevance (predictive capacity). The MaTtek EpiDerm™ model is a RhE model already used in the experimental system in the validate test methods of OECD TG431, TG439 and TG498 for corrosion, irritation and phototoxicity, respectively. The fact that the EpiSensA method was originally developed using this model and only later changed to LabCyte EPI-MODEL24 is also an important factor in the choice of this model for the me-too validation (1).

This me-too validation is part of the OECD work plan under the number 4.172 as « Me-Too validation of the reconstructed human epidermis Epiderm model for the EpiSensA method ».

Validation management team

This validation study will be carried out under the supervision of a validation management team (VMT). The VMT will manage and schedule the validation process, trainings, encoding and distribution of test materials, evaluation and statistical verification of test results, and will draw final conclusions. The composition and respective role of participants to the VMT is detailed in Table 1.

Table 1 : Composition of the validation management team

Name	Company/institution	Role	Observation
Christian Pellevoisin	Urbilateria, Saint Cyr sur Loire, France	Member	Chair
Hajime Kojima	NIHS, Japan	Member	Co-Chair
Sebastian Hofmann	seh consulting + services, Paderborn, Germany	Member	Biostatistician
Takao Ashikaga	JaCVAM	Member	JaCVAM liaison
Marisa Meloni	VitroScreen, Milano, Italy	Member	Chemical management
Mitch Klausner	MatTek, Ashland, USA	Observer	Lead laboratory
Kalyani Guntur	MatTek, Ashland, USA	Observer	Lead laboratory
Tim Landry	MatTek, Ashland, USA	Observer	Lead laboratory
Kazuto Narita	Food and Drug Safety Center (FDSC), Hadano, Japan	Observer	Participating laboratory 1

Shigehiro Tachibana	Food and Drug Safety Center (FDSC), Hadano, Japan	Observer	Participating laboratory 1
Kojima k	Food and Drug Safety Center (FDSC), Hadano, Japan	Observer	Participating laboratory 1
Helge Gehrke	Eurofins BioPharma Product Testing Munich, Germany	Observer	Participating laboratory 2
Robert Mills-Goodlet	Eurofins BioPharma Product Testing Munich, Germany	Observer	Participating laboratory 2
Vic Johnson	Burleson Research Technologies, Morrisville, USA	Observer	Participating laboratory 3

Study design

According to the OECD guidance n°34, it is not mandatory, even if preferable, to carry out validation strictly in compliance with GLP principles. Nevertheless, participating laboratories will perform all procedures in accordance with GLP principles, including, but not limited to, the use of standard operating procedures, proper data recording and record keeping.

Participating laboratories

Lead laboratory: MatTek, Ashland, USA, will be the lead laboratory. It will be responsible for preparing the final standard operating procedure (SOP) and for the training of the participating laboratories.

Participating laboratories: The three laboratories below will participate as testing laboratories in the me-too validation study.

- Laboratory 1: Food and Drug Safety Center Hatano Research Institute (FDSC), Safety Testing Department, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan, http://www.fdsc.or.jp/
- Laboratory 2: Eurofins BioPharma Product Testing, Munich, Germany, https://www.eurofins.de/biopharma-product-testing/
- Laboratory 3: Burleson Research Technologies, 120 First Flight Lane, Morrisville, NC 27560, www.brt-labs.com

Study phases

The validation study will be conducted in two phases. In the first phase, the test method is transferred to the 3 naïve laboratories. After verification that the method is mastered by the laboratories, phase 2 will allow to evaluate within-laboratory and between-laboratory reproducibility and predictive capacity of the test method.

Phase I – Transfer and training

The final SOP-v1 will be send to the participating laboratories with a video explaining the experimental steps. In step 1, a first experiment will be performed with the positive controls. After verification of the results by the lead laboratory, the laboratories will have to carry out two successive studies (step 2 and step 3) with the chemicals listed in

Table 3 that are not belonging to the performance standard of the VRM method. In step 2, the positive controls will be tested again to obtain an indication of how well the results from step 1 can be reproduced. In addition, Eugenol, a weak to moderare sensitizer in the LLNA and in humans, and the clear non-sensitizer Glycerol will be tested. In step 3,the laboratory will evaluate another sensitizer, 2-Aminophenol and a non sensitizer., salicylic acide.

Table 3:

Phase I - step 1	Phase I - step 2	Phase I - step 3
Clotrimazol 1.56%	Clotrimazol 1.56%	Clotrimazol 1.56%
4-NBB 0.78%	4-NBB 0.78%	4-NBB 0.78%
	Glycerol CAS 56-81-5	Salicylic Acid CAS 69-72-7
	Eugenol CAS 97-53-0	2-Aminophenol CAS 95-55-6

Phase II – WLR/BLR:

The WLR step, will allow to evaluate the within laboratory reproducibility (WLR). It will be calculated on the basis of the concordance of classifications obtained by each participating laboratory for the subset of 12 reference substances identified in bold italics in Table 2, using three qualified runs.

The BLR step, will allow to evaluate the between laboratory reproducibility (BLR). It will be calculated on the basis of the concordance of classifications obtained by at least three participating laboratories for the 20 reference substances listed in Table 2. For the 12 substances for which each laboratory should generate three classifications (in the WLR step), one single final classification should be derived per laboratory based on the mode of the three predictions obtained.

Reference test substances

The chemicals listed in the EpiSensA draft performance standards will be tested in this study (Table 2).

The test chemicals management such as coding and distribution will be performed by VitroScreen. VitroScreen has participated in several validations and is part of the EU-NETVAL laboratory network that supports EURL ECVAM's validation studies to assess the reliability and relevance of alternative methods.

Table 2 : Minimum List of Reference Substances for Determination of Reproducibility and Predictive Capacity of similar or modified EpiSensA test method (3)

No.	Proficiency substances	CAS No.	Physical state	*in vivo* prediction[1]	Vehicle[2]	EpiSensA results for each marker gene[2, 3]				VRM
						ATF3	GCLM	DNAJB4	IL-8	prediction
1	**2,4-Dinitrochlorobenzene**	97-00-7	Solid	Sensitiser (GHS Cat.1A)	AOO	p	p	p	p/n	Positive

2	**p-Phenylenediamine**	106-50-3	Solid	Sensitiser (GHS Cat.1A)	AOO	p/n	p	p	p/n	Positive
3	Metol	55-55-0	Solid	Sensitiser (GHS Cat.1A)	DW	p	p	p	p/n	Positive
4	Tetrachlor-osalicylanilide	1154-59-2	Solid	Sensitiser (GHS Cat.1A)	AOO	p	n	P p	p	Positive
5	**Lauryl galate**	1166-52-5	Solid	Sensitiser (GHS Cat.1A)	AOO	p/n	n	p/n	p/n	Negative
6	**Methyl heptine carbonate**	111-12-6	Liquid	Sensitiser (GHS Cat.1A)	AOO	p	p	p	P	Positive
7	Isoeugenol	97-54-1	Liquid	Sensitiser (GHS Cat.1A)	AOO	p/n	p	p	p/n	Positive
8	**Glyoxal 40% solution in water**	107-22-2	Liquid	Sensitiser (GHS Cat. 1A	DW	p/n	p	p/n	p/n	Positive
9	**Abietic acid**	514-10-3	Solid	Sensitiser (GHS Cat.1B)	AOO	p	p	p	p	Positive
10	Dibutyl aniline	613-29-6	Liquid	Sensitiser (GHS Cat.1B)	AOO	p/n	n	n	p	Positive
11	Amyl cinnamic aldehyde	122-40-7	Liquid	Sensitiser (GHS Cat.1B)	AOO	p	n	p	p	Positive
12	**Benzisothiazolinone**	2634-33-5	Solid	Sensitiser (GHS Cat.1B)	AOO	p/n	p/n	p/n	p/n	Positive[5]
13	Imidazolidinyl urea	39236-46-9	Solid	Sensitiser (GHS Cat.1B)	DW	p/n	n	p/n	p	Positive
14	**Farnesol**	4602-84-0	Liquid	Sensitiser (GHS Cat.1B)	AOO	p	p/n	p	p	Positive
15	**Cetrimide**	57-09-0	Solid	Non-sensitiser (Not classified)	50%EtOH	n	n	n	n	Negative
16	**Lactic acid**[4]	50-21-5	Liquid	Non-sensitiser (Not classified)	DW	n	n	n	n	Negative
17	Benzyl butyl phthalate	85-68-7	Liquid	Non-sensitiser (Not classified)	AOO	n	n	n	n	Negative
18	**Diethyl phthalate**	84-66-2	Liquid	Non-sensitiser (Not classified)	AOO	p/n	n	p/n	p	Negative
19	**Hexane**	110-54-3	Liquid	Non-sensitiser (Not classified)	AOO	n	n	n	n	Negative
20	1-Iodehexane	638-45-9	Liquid	Non-sensitiser (Not classified)	AOO	n	p	p	p	Positive

Distribution of the reference test substances

All test substances for the Phase II – WLR/BLR will be distributed together with the preparation method by VitroScreen, unprepared and coded, to participating laboratories.

The test substances will be packaged to minimize damage and risk during shipment. Participating laboratories will be notified by VitroScreen when the test substance is shipped. Participating laboratories will inform immediately VitroScreen upon receipt.

Material Safety Data Sheet (MSDS) for all coded chemicals will be provided in a sealed envelope to the participating laboratories for use in emergency. In case of an emergency requiring opening of the envelop, the laboratory musst immediately inform the chair and co-chair of the VMT. After completion of the validation study, the participating laboratory should return unopened MSDS to the VMT chair.

Data management

A protected data recording template will be provided to the laboratories for both experimental phases. Test results data will be collected using the template, named as agreed, quality controlled and approved by the study director of the laboratory and submitted to the biostatistician and the VMT chair. All experiments conducted in Phase 2, incl. those that were considered invalid, have to be reported.

Copies of all raw data and related data should be stored and retained in participating laboratories for a minimum of five years from the time of the completion of the validation study. Electronically stored data should be created by copying or backing up files on a regular basis.

Provisional timetable

The duration of the "me-too" validation is estimated at 17 months, including the key stages listed in table 4.

Table 4: Provisional timetable

Optimization/SOP	3 months	May-July 2024
First meeting VMT		June 21 2024
Second meeting VMT		August 2024
Phase 1 - Transfert/Training	2 months	August-September 2024
Third meeting VMT		October 2024
Phase 2 - WLR	5 months	October 2024 - February 2025
Fourth meeting VMT		January 2025
Phase 3 - BLR	2 months	March - April 2025
Statistical analysis/Report	2 months	May - June 2025
Review	3 months	July - September 2025

Funding

Mattek will cover the cost of the biostatistician and of VitroScreen for the chemical management. Training and technical support for participating laboratories will be provided by Mattek.

The 3 participating laboratories will support the human and consumable costs of the experiments. Mattek will apply a 35% discount on the cost of the EPI-200 models that will be used by participating laboratories for this validation.

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Appendix II

Epi2SensA me-too validation

Statistical report

February 23, 2026

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1. Introduction

The Epidermal Sensitisation Assay (EpiSensA), an in vitro test method that uses the 'LabCyte EPI-MODEL24' reconstructed human epidermis model and measures inflammatory responses and the induction of cytoprotective gene to discriminate skin sensitising substances from non-skin sensitising substances, was added to the OECD TG 442D in 2024.

The Epi^{2}SensA was developed as a so-called 'me-too' test method to the EpiSensA using the Reconstructed human Epidermis (RhE) EpiDermTM from MatTek Corporation (EPI-200). To evaluate its similarity to the EpiSensA, the Epi^{2}SensA was assessed in a validation study according to the 'Performance Standards for the Assessment of Proposed Similar or Modified In Vitro Epidermal Sensitisation Assay (EpiSensA) Test Methods' (OECD 2024), here called PS. Note that at the time, when this study was planned, only a draft of the PS was available, which seems to be very similar to the final version.

A 'me-too' validation study was performed with four participating laboratories Mattek, Eurofins, BRT and FDSC. Details of the participating laboratories can be found in the 'Study plan'.

This report summarises the statistical analysis of the study data and compares them to the requirements of the PS (OECD 2024). Here, the similarity criteria, which are described in the chapter 'Defined reliability and accuracy values', are addressed. Besides the compliance with acceptance criteria, the within-and between laboratory reproducibility and predictive performance were analysed and compared to the target values as defined in PS (OECD 2024). Meeting the PS requirements is one of the prerequisites for a so-called "me-too" test method to gain acceptance for regulatory testing.

2. Validation study design

The validation study was designed according to the requirements of the PS (see paragraph 41), i.e. at least three laboratories test the 20 reference substances (Annex A), 12 of which in three independent runs. Mattek, Eurofins and BRT tested all reference substances. Deviating from the original study plan, FDSC tested eight reference test substance once each due to resource limitations. Although not required by the PS, the test sample was provided in a coded manner to the testing laboratories by the independent contractor VitroScreen (Milan, Italy).

The study was conducted in two phases. In a first phase eight samples were tested in each laboratory. The samples were selected by the core VMT to inform reproducibility, and the laboratories were given the code of the samples to test in the phase. The results were analysed to control if the reproducibility within and between laboratories was acceptable. In the second phase, the remaining sample were tested.

3. Data management

All invalid and valid runs had to be reported using a EXCEL-template. It was initially planned that the files should be send to both the validation study manager C. Pellevoisin and the biostatistician S. Hoffmann. As the laboratories send most files to C. Pellevoisin, it was agreed that he would collect all files and provide them to S. Hoffmann.

From all submitted files working copies were made. From these, data were semi-automatically extracted into a summary data file, in which all further data management was carried out. Approx. 30% of the data transfer and was double-checked against the original files without identifying any errors.

Working copies did not modify any raw data, with two exceptions:

- Runs with one vehicle control tissue cell viability < 95%: For the cases the SOP specifies that 'the Ct values obtained from the remaining two tissue units should be used'. This required a manual change in the files.

- Test concentration with the first tissue being not reported: In these cases, the data template would not return a mean Ct-value due to the formula used. This required to manually change of the formula to calculate the mean from the second and third tissue.

4. Acceptance criteria

The acceptance criteria under chapter 8 'REQUIREMENTS FOR QUALIFIED TESTING' of SOP version 2.0 from March 10, 2025, were used to determine the validity of a run:

- The tissue viability of at least two of the tissues exposed to the vehicle control should be ≥95%. If the viability of only one tissue is < 95%, Ct values from the remaining two tissues should be used.
- Mean tissue viability of both positive controls should be ≥80%.
- For the positive control of 1.56 w/v% clotrimazole, the mean values of fold induction for *ATF3* and *IL-8* should exceed the cut-off value.
- For the positive control of 0.10 w/v% 4NBB, the mean values of fold induction for *GCLM* and *DNAJB4* should exceed the cut-off value.

If these requirements were not met, the run was considered invalid.

The following criteria were applied to determine the validity of each test concentration:

- The result of at least one tested concentration that shows ≥ 80% mean cell viability should be used. If the mean cell viability is < 80% for a given tested concentration, the

result for that tested concentration should be excluded for a positive prediction but might be used for a negative prediction.

- When the mean GAPDH Ct value for a given test chemical concentration is within ±1 of the mean GAPDH Ct value of the corresponding vehicle control, the result obtained at that concentration is acceptable

As there was no criterion on the minimum required number of tissues per test concentration in the SOP, test concentrations with data for only one tissue were also considered invalid.

5. Data analysis

All runs were checked for validity. Only valid runs were then submitted to the evaluation of test concentration validity. The data of test substances with at least one valid test concentration were analysed according to the prediction model:

An Epi2SensA prediction is considered **positive** if at least one of the following conditions is met:

- The Imax for ATF3 is > 15 for at least one tested concentration.
- The Imax for GCLM is > 2 for at least one tested concentration.
- The Imax for DNAJB4 is > 2 for at least one tested concentration.
- The Imax for IL-8 is > 4 for at least one tested concentration.

The Epi2SensA prediction is considered **negative** if the three conditions below are met:

- The mean fold-induction value of the marker genes does not exceed the respective cut-off values for any of the four genes at the tested concentrations that shows ≥ 80% mean cell viability;
- At least 3 concentrations were tested;
- If the highest concentration tested was lower than the highest soluble or stably dispersed concentration, at least one mean cell viability at the tested concentrations is < 80%.

Within-laboratory (WLR) and between-laboratory (BLR) reproducibility were calculated based on the concordance of the predictions. For the BLR, the mode of the three predictions obtained with the substances tested for WLR, i.e. the most frequent prediction, as specified in the EpiSensA Performance Standards.

The analysis up to this point was conducted blind, i.e. with the codes intact. On August 11, 2025, the results were sent to the core VMT for their information. In parallel, they were sent to VitroScreen by e-mail requesting the information to unblind the data analysis. VitroScreen sent the coding information on the same day.

Accuracy, specificity and sensitivity were calculated by comparison of the Epi2SensA results with the reference substance results (see column ‘In vivo predictions’ in Annex A). For the repeatedly testes substances the mode of the three predictions was used according to the PS.

It should be noted that concentration-response, response pattern, e.g. of the genes, replicate variability, borderline ranges and appropriateness of prediction model thresholds were considered beyond scope of this analysis, but have the potential to reveal issues and ways to further improve the Epi2SensA.

6. Results

6.1 Acceptance of runs

Fourty runs were submitted by the four laboratories. Mattek conducted 12 runs, of which one was invalid. Eurofins and BRT submitted 13 runs each, including 1 and 2 invalid runs, respectively. FDSC conducted two runs, both valid. In all cases, invalidity was due to one or both positive controls responses being below the cut-offs. In total, four of 40 runs were invalid, i.e. 10%. A summary of the acceptability of runs is provided in Annex B.

6.2 Acceptance of test sample concentrations

Test concentrations were not acceptable if at least one of the three criteria was missed:

- mean cell viability $\geq$ 80%
- GAPDH Ct value within ±1 of the mean GAPDH Ct value of the corresponding vehicle control
- data available/reported for at least two tissues

Table 1 summarises the acceptance of test concentrations and indicates the frequencies, with which the individual criteria were met. The total acceptance of test concentrations of the three main laboratories, which tested 180 or 188 concentrations each, ranged between 75,0% and 76,7%.

Table 1: Summary of valid test concentrations per laboratory

Lab	no. concentrations tested	No. with valid cell viability	No. with valid GAPDH Ct value	No. with at least two tissues	No. valid test concentrations (total and %)
Mattek	180	168	156	177	138 (76,7%)
Eurofins	180	151	164	176	135 (75,0%)
BRT	180	170	143	178	139 (77,2%)
FDSC	32	30	31	32	30 (93,8%)

Except for FDSC, each laboratory had one test sample, for which all test concentration were not acceptable. These sample were retested in subsequent runs.

Mattek also retested samples B272 and B396, accidently repeats of the same test substance, without any formal need to do so. Therefore, these retests were not considered in the data analysis.

6.3 Reproducibility within laboratories

The three main laboratories tested 12 reference substances in three independent runs according to the PS and as identified in Annex A to evaluate the within-laboratory reproducibility of the Epi2SensA.

The Mattek results with the 12 substances for WLR are summarised in Table 2. For eight substances concordant predictions were obtained, resulting in a WLR of 8/12 =66,7%. The substances with discordant predictions were Lauryl gallate, Benzisothiazolinone, Cetrimide and Diethyl phthalate.

Table 2: WLR at Mattek (S: sensitiser; NS: non-sensitiser)

Proficiency substances for WLR	*in vivo* prediction	code	vehicle	No. valid concentrations	Max. test concentration	Epi²SensA result for each marker gene				Epi²SensA prediction	WLR
						ATF3	GCLM	DNAJB4	IL-8		
2,4-Dinitrochlorobenzene	S (1A)	B121-3	AOO	4	0,39	0	1	1	0	Positive	YES
		B242-3	AOO	4	0,39	0	1	1	0	Positive	
		B388-3	AOO	3	0,195	1	1	1	0	Positive	
p-Phenylenediamine	S (1A)	B185-0	EtOH 50%	4	3,125	0	1	1	0	Positive	YES
		B231-2	AOO	1	0,39	0	0	1	0	Positive	
		B375-2	AOO	4	1,25	0	1	1	0	Positive	
Lauryl galate	S (1A)	B111-0	AOO	4	25	0	1	0	0	Positive	NO
		B222-1	AOO	3	12,5	0	0	0	0	Negative	
		B316-1	AOO	4	12,5	0	0	0	1	Positive	
Methyl heptine carbonate	S (1A)	B130-0	AOO	4	100	1	1	1	0	Positive	YES
		B235-2	AOO	4	3,125	0	1	1	0	Positive	
		B328-2	AOO	4	3,125	0	1	1	0	Positive	
Glyoxal 40% solution in water	S (1A)	B108-1	DW	1	6,25	1	1	1	1	Positive	YES
		B243-1	DW	3	25	1	1	1	1	Positive	
		B343-1	DW	2	12,5	1	1	0	0	Positive	
Abietic acid	S (1B)	B104-1	AOO	4	12,5	0	1	1	1	Positive	YES
		B241-1	AOO	4	12,5	0	1	1	1	Positive	
		B391-1	AOO	4	12,5	0	1	1	0	Positive	
Benzisothiazolinone	S (1B)	B160-0	AOO	2	0,0975	0	0	0	0	Negative	NO
		B224-3	AOO	2	0,098	0	0	0	0	Negative	
		B317-3	AOO	2	0,0975	0	1	1	0	Positive	
Farnesol	S (1B)	B117-0	AOO	4	100	1	0	1	1	Positive	YES
		B208-1	AOO	3	100	1	0	1	1	Positive	
		B311-1	AOO	3	100	1	1	1	1	Positive	
Cetrimide	NS (no Cat.)	B365-2	EtOH 50%	4	3,125	0	0	0	0	Negative	NO
		B141-2	EtOH 50%	4	3,125	0	0	0	1	Positive	
		B203-2	EtOH 50%	2	0,39	0	1	1	0	Positive	
Lactic acid	NS (no Cat.)	B183-1	DW*	2	12,5	0	0	0	0	Negative	YES
		B272-1	DW*	3	12,5	0	0	0	0	Negative	
		B396-1	DW	2	12,5	0	0	0	0	Negative	
Diethyl phthalate	NS (no Cat.)	B107-0	AOO	4	100	0	0	0	0	Negative	NO
		B298-1	AOO	4	100	0	0	1	0	Positive	
		B385-1	AOO	3	100	0	0	0	0	Negative	
Hexane	NS (no Cat.)	B136-1	AOO	4	100	0	0	0	0	Negative	YES
		B202-1	AOO	4	100	0	0	0	0	Negative	
		B347-1	AOO	4	100	0	0	0	0	Negative	
									WLR (overall)	**66,7% (8/12)**	

The Eurofins results with the 12 substances for WLR are summarised in Table 3. For seven substances concordant predictions were obtained, resulting in a WLR of 7/12 =58,3%. The substances with discordant predictions were Benzisothiazolinone, farnesol, Cetrimide, Lactic

acid and Diethyl phthalate. It should also be noted that the sensitisers lauryl gallate was consistently predicted as negative.

Table 3: WLR at Eurofins (S: sensitiser; NS: non-sensitiser)

Proficiency substances for WLR	*in vivo* prediction	code	vehicle	No. valid concentrations	Max. test concentration	Epi²SensA result for each marker gene				Epi²SensA prediction	WLR
						ATF3	GCLM	DNAJB4	IL-8		
2,4-Dinitrochlorobenzene	S (1A)	A192-3	AOO	3	0,2	0	1	1	0	Positive	YES
		A279-3	AOO	3	0,2	0	1	1	0	Positive	
		A351-3	AOO*	4	0,4	1	1	1	1	Positive	
p-Phenylenediamine	S (1A)	A271-2*	AOO	2	0,8	0	1	1	0	Positive	YES
		A143-0	AOO	1	0,8	0	1	1	0	Positive	
		A305-2	EtOH	2	0,8	0	1	1	0	Positive	
Lauryl galate	S (1A)	A198-0	AOO	4	50	0	0	0	0	Negative	YES
		A240-1	AOO	4	25	0	0	0	0	Negative	
		A361-1	AOO	4	50	0	0	0	0	Negative	
Methyl heptine carbonate	S (1A)	A115-0	AOO	4	100	1	1	1	0	Positive	YES
		A346-2	AOO	4	100	1	1	1	0	Positive	
		A262-2	AOO*	4	3,1	0	1	1	0	Positive	
Glyoxal 40% solution in water	S (1A)	A283-1	DW	3	50	1	0	1	1	Positive	YES
		A138-1	DW	2	25	1	1	1	1	Positive	
		A323-1	DW	2	13	1	1	1	1	Positive	
Abietic acid	S (1B)	A174-1	AOO	3	6,3	0	1	1	1	Positive	YES
		A278-1	AOO*	4	13	0	1	1	1	Positive	
		A313-1	AOO	4	13	1	1	1	1	Positive	
Benzisothiazolinone	S (1B)	A123-0*	AOO	2	0,1	0	1	0	0	Positive	NO
		A274-3	AOO	3	0,1	0	0	0	0	Negative	
		A301-3	AOO*	3	0,2	0	1	1	0	Positive	
Farnesol	S (1B)	A146-0	AOO	3	100	0	0	0	0	Negative	NO
		A263-1	AOO*	3	50	1	1	1	0	Positive	
		A329-1	AOO	4	100	1	0	1	1	Positive	
Cetrimide	NS (no Cat.)	A366-2	EtOH*	2	0,8	0	0	0	0	Negative	NO
		A249-2	EtOH	2	0,8	0	0	0	0	Negative	
		A127-2	EtOH	3	1,6	0	0	0	1	Positive	
Lactic acid	NS (no Cat.)	A133-1	DW	3	100	0	0	0	0	Negative	NO
		A288-1	DW	2	13	0	0	0	1	Positive	
		A382-1	DW	3	100	0	0	0	0	Negative	
Diethyl phthalate	NS (no Cat.)	A169-0	AOO	4	100	0	0	0	0	Negative	NO
		A306-1	AOO	4	100	0	0	0	0	Negative	
		A289-1	AOO	4	100	0	0	1	0	Positive	
Hexane	NS (no Cat.)	A186-1*	AOO	4	100	0	0	0	0	Negative	YES
		A223-1	AOO	4	100	0	0	0	0	Negative	
		A398-1	AOO	4	100	0	0	0	0	Negative	
WLR (overall)										**58,3% (7/12)**	

The BRT results with the 12 substances for WLR are summarised in Table 4. For eight substances concordant predictions were obtained, resulting in a WLR of 8/12 =66,7%. The

substances with discordant predictions were Lauryl gallate, Methyl heptine carbonate, Cetrimide and Diethyl phthalate.

Table 4: WLR at BRT (S: sensitiser; NS: non-sensitiser)

Proficiency substances for WLR	*in vivo* prediction	code	vehicle	No. valid concentrations	Max. test concentration	Epi²SensA result for each marker gene				Epi²SensA prediction	WLR
						ATF3	GCLM	DNAJB4	IL-8		
2,4-Dinitrochlorobenzene	S (1A)	C167-3	AOO	4	0,4	0	1	1	0	Positive	YES
		C210-3	AOO	3	0,4	1	1	1	0	Positive	
		C315-3	AOO	2	0,4	0	1	1	0	Positive	
p-Phenylenediamine	S (1A)	C211-2	AOO	3	1,6	0	1	1	0	Positive	YES
		C118-0	AOO	3	1,6	0	1	1	1	Positive	
		C369-2	AOO	2	1,6	0	1	1	1	Positive	
Lauryl galate	S (1A)	C165-0	AOO	4	25	0	0	0	0	Negative	NO
		C296-1	AOO	4	13	0	0	0	0	Negative	
		C383-1	AOO	4	25	0	0	1	0	Positive	
Methyl heptine carbonate	S (1A)	C176-0	AOO	4	100	1	1	1	0	Positive	NO
		C342-2	AOO	4	3,1	0	0	0	0	Negative	
		C206-2	AOO	4	3,1	0	1	1	1	Positive	
Glyoxal 40% solution in water	S (1A)	C251-1	DW	3	1	1	0	1	0	Positive	YES
		C132-1	DW	2	25	1	0	0	1	Positive	
		C340-1	DW	3	25	1	1	1	1	Positive	
Abietic acid	S (1B)	C194-1	AOO	4	13	0	1	1	1	Positive	YES
		C286-1	AOO	3	0,1	0	1	1	0	Positive	
		C318-1	AOO	4	6,3	0	1	1	0	Positive	
Benzisothiazolinone	S (1B)	C188-0	AOO	2	0,1	0	1	1	0	Positive	YES
		C265-3	AOO	1	0	0	1	1	0	Positive	
		C390-3	AOO	3	0	0	1	1	0	Positive	
Farnesol	S (1B)	C380-1	AOO	4	1	1	0	1	1	Positive	YES
		C244-1	AOO	4	100	1	0	1	1	Positive	
		C120-0	AOO	4	100	1	0	1	1	Positive	
Cetrimide	NS (no Cat.)	C359-2	EtOH	4	3,1	0	1	1	0	Positive	NO
		C212-2	EtOH	4	3,1	0	0	0	0	Negative	
		C189-2	EtOH	1	3,1	0	0	0	0	Negative	
Lactic acid	NS (no Cat.)	C287-1	DW	3	100	0	0	0	0	Negative	YES
		C345-1	DW	1	100	0	0	0	0	Negative	
		C184-1	DW	3	100	0	0	0	0	Negative	
Diethyl phthalate	NS (no Cat.)	C149-0	AOO	4	100	0	0	1	0	Positive	NO
		C378-1	AOO	1	100	0	0	1	0	Positive	
		C239-1	AOO	3	100	0	0	0	0	Negative	
Hexane	NS (no Cat.)	C166-1	AOO	1	100	0	0	0	0	Negative	YES
		C275-1	AOO	4	100	0	0	0	0	Negative	
		C335-1	AOO	4	100	0	0	0	0	Negative	
									WLR (overall)		**66,7% (8/12)**

In summary, the WLR of the three laboratories was 58,3%, 66,7% and 66,7%, i.e. all below the WLR acceptance criterion of ≥ 80% specified in the PS. Cetrimide and Diethyl phthalate were

not reproducible in all laboratories, while Lauryl gallate and Benzisothializone had discordant results in two laboratories.

6.4 Reproducibility between laboratories

The BLR was assessed based on the data of the four laboratories (Annexes C1, C2, C3, C4). For WLR substances, the mode of the three predictions was used. Of the 20 reference substances, 14 substances were reproducible between laboratories, i.e. 70% (Table 5). Of these, six were tested in four laboratories and eight in three laboratories. Discordant predictions were obtained for Lauryl gallate, Dibutyl aniline, Benzisothiazolinone, Cetrimide, Benzyl butyl phthalate and Diethyl phthalate. For three of these, Mattek obtained a different prediction compared to the other laboratories and for two BRT obtained a different prediction compared to the other laboratories. Diethyl phthalate was predicted positive by two laboratories and negative by the two others. In summary, the BLR of 70% does not meet the BLR acceptance criterion of ≥ 80% specified in the PS.

Table 5: BLR (S: sensitiser; NS: non-sensitiser)

Proficiency substances	*in vivo* prediction	Laboratory				BLR
		Mattek	EUR	BRT	FDSC	
2,4-Dinitrochlorobenzene	S (1A)	Positive	Positive	Positive	nt	YES
p-Phenylenediamine	S (1A)	Positive	Positive	Positive	nt	YES
Metol	S (1A)	Positive	Positive	Positive	Positive	YES
Tetrachlor-osalicylanilide	S (1A)	Positive	Positive	Positive	Positive	YES
Lauryl galate	S (1A)	**Positive**	Negative	Negative	nt	**NO**
Methyl heptine carbonate	S (1A)	Positive	Positive	Positive	nt	YES
Isoeugenol	S (1A)	Positive	Positive	Positive	Positive	YES
Glyoxal 40% solution in water	S (1A)	Positive	Positive	Positive	nt	YES
Abietic acid	S (1B)	Positive	Positive	Positive	nt	YES
Dibutyl aniline	S (1B)	Positive	Negative	Positive	Negative	**NO**
Amyl cinnamic aldehyde	S (1B)	Positive	Positive	Positive	Positive	YES
Benzisothiazolinone	S (1B)	**Negative**	Positive	Positive	nt	**NO**
Imidazolidinyl urea	S (1B)	Positive	Positive	Positive	Positive	YES
Farnesol	S (1B)	Positive	Positive	Positive	nt	YES
Cetrimide	NS (no Cat.)	**Positive**	Negative	Negative	nt	**NO**
Lactic acid	NS (no Cat.)	Negative	Negative	Negative	nt	YES
Benzyl butyl phthalate	NS (no Cat.)	Negative	Negative	**Positive**	Negative	**NO**
Diethyl phthalate	NS (no Cat.)	Negative	Negative	**Positive**	nt	**NO**
Hexane	NS (no Cat.)	Negative	Negative	Negative	nt	YES
1-Iodehexane	NS (no Cat.)	Positive	Positive	Positive	Positive	YES
				BLR (overall)		**70,0% (14/20)**

6.5 Predictive capacity (Accuracy)

The predictive capacity was determined by comparing the Epi2SensA predictions with the in vivo predictions specified in the PS. The results are summarised in Table 6. Focusing on the

three laboratories testing all 20 reference substances, all met the sensitivity acceptance criterion of ≥ 85%. However, the strong sensitiser Lauryl gallate (UN GHS Cat. 1A) was underpredicted by Eurofins and BRT, which is, according to the PS, only acceptable if a clear rationale can be given. Nevertheless, also EpiSensA underpredicted Lauryl gallate. The validation management team suggested the rationale that '… EpiSensA would predict lauryl gallate as negative because of both high lipophilicity (logKow=6.21, suggesting low absorption at higher concentration) and autoxidation', which appears to have convinced the peer review panel (OECD, 2023). Mattek and Eurofins met the specificity criterion of ≥ 65%, and consequently also the accuracy criterion of ≥ 85%. As BRT overpredicted three sensitisers, the specificity (50%) and consequently the accuracy (80%) did not meet the respective acceptance criteria. As FDSC tested only eight reference substances, the results obtained cannot be assessed against the PS acceptance criteria.

Considering that the rationale for the underprediction of Lauryl gallate applies, the predictive capacity acceptance criteria of the PS were met, except for BRT specificity and accuracy due to overpredicting one non-sensitizer too much. In this context it should also be considered that the EpiSensA's specificity for the six non-sensitisers was 66,7% due to positive results for 1-Iodehexane and Diethyl phthalate, which were also overpredicted by Epi2SensA in all and one laboratory, respectively. Averaging the predictivity parameters across the three laboratories resulted in a specificity of 66,7%, a sensitivity of 90,5% and an accuracy of 83,3%, i.e. meeting the acceptance criteria.

Table 6: Predictive capacity (S: sensitiser; NS: non-sensitiser; orange: false negative predictions; yellow: false positive predictions; green: acceptance criterion met)

Proficiency substances	*in vivo* prediction	Laboratory			
		Mattek	EUR	BRT	FDSC
2,4-Dinitrochlorobenzene	S (1A)	Positive	Positive	Positive	nt
p-Phenylenediamine	S (1A)	Positive	Positive	Positive	nt
Metol	S (1A)	Positive	Positive	Positive	Positive
Tetrachlorosalicylanilide	S (1A)	Positive	Positive	Positive	Positive
Lauryl galate	S (1A)	Positive	Negative	Negative	nt
Methyl heptine carbonate	S (1A)	Positive	Positive	Positive	nt
Isoeugenol	S (1A)	Positive	Positive	Positive	Positive
Glyoxal 40% solution in water	S (1A)	Positive	Positive	Positive	nt
Abietic acid	S (1B)	Positive	Positive	Positive	nt
Dibutyl aniline	S (1B)	Positive	Negative	Positive	Negative
Amyl cinnamic aldehyde	S (1B)	Positive	Positive	Positive	Positive
Benzisothiazolinone	S (1B)	Negative	Positive	Positive	nt
Imidazolidinyl urea	S (1B)	Positive	Positive	Positive	Positive
Farnesol	S (1B)	Positive	Positive	Positive	nt
Cetrimide	NS (no Cat.)	Positive	Negative	Negative	nt
Lactic acid	NS (no Cat.)	Negative	Negative	Negative	nt
Benzyl butyl phthalate	NS (no Cat.)	Negative	Negative	Positive	Negative
Diethyl phthalate	NS (no Cat.)	Negative	Negative	Positive	nt
Hexane	NS (no Cat.)	Negative	Negative	Negative	nt
1-Iodehexane	NS (no Cat.)	Positive	Positive	Positive	Positive
Specificity (≥ 65%)		66,7% (4/6)	83,3% (5/6)	50,0% (3/6)	50,0% (1/2)
Sensitivity (≥ 85%)		92,9% (13/14)	85,7% (12/14)	92,9% (13/14)	83,3% (5/6)
Accuracy (≥ 85%)		85,0% (17/20)	85,0% (17/20)	80,0% (16/20)	75,0% (6/8)

In summary, the PS acceptance criteria for WLR and BLR were not met, while the predictive capacity acceptance criteria were fulfilled, with the exception of BRT's specificity and accuracy.

6.6 Discussion of the performance

When interpreting the results obtained for WLR, BLR and predictive capacity it should be considered that coded samples were tested, although not required by the PS.

Furthermore, at the interim analysis conducted with two runs for each laboratory (except FDSC) an impact of the test concentrations viability threshold of 80% was observed.

In addition, the core VMT agreed to assess the impact when changing the prediction model to requiring two genes to exceed the gene-specific induction thresholds for considering a test substance positive.

Therefore, the changes to the viability acceptance criteria and to the prediction model are considered and their impact of the performance evaluated.

- Test concentration viability

Reducing the test concentration viability acceptance criterion of ≥ 80% would result in more acceptable test concentration and likely more positive predictions.

Reducing the criterion to ≥ 70 % would have resulted in four additionally valid test concentrations for Mattek. The first repeat of p-Phenylenediamine (sample B231) would have had two more valid concentrations resulting in a clearer positive response (higher induction of DNABJ4 and induction of GCLM), one more valid concentration for Isoeugenol (sample B418) without any impact on the prediction and it would have made the second repeat of p-Phenylenediamine (sample B185) valid and positive, avoiding a retest.
For Eurofins, ten test concentrations would have become valid. Eight of them would have had no impact on the prediction, but would have substantiated a positive response in some cases. However, the two highest test concentration of the first repeat with Cetrimide (sample A366) would have been acceptable, making it positive (clear induction of IL8).

For BRT, one concentration of the second repeat of Benzisothiazolinone (sample C265) would have become valid, making it clearer positive.

For FDSC, two more test concentrations would have become valid without any impact on the prediction.

Reducing the criterion to ≥ 60 % would have resulted in five additionally valid concentrations at Mattek, 12 at Eurofins, 2 at BRT and none at FDSC. In most cases there would have been no impact on the predictions, but some would have substantiated positive responses. However, Mattek's first and second repeat of Benzisothiazolinone (samples B160 and B224) would have become positive (for GCLM and DNABJ4). In addition, also the second Eurofins' repeat of Benzisothiazolinone (sample A274) would have become positive (for GCLM).

In summary, a reduction of the acceptance criterion for viability could lead to improved prediction and reduction of the number of retests. When applied to the validation data set, a viability acceptance of ≥ 70% would have made Cetrimide false positive at Eurofins, reducing the laboratory's specificity.

A viability acceptance of ≥ 60% would have led to consistently positive predictions within and between laboratories for the sensitiser Benzisothiazolinone. This would lead to an increase of the WLR to 75,0% at Mattek and to 66,7% at Eurofins, a BLR of 75,0% and sensitivity of 100% at Mattek, increasing the accuracy to 90,0%.

- Modified prediction model

Observing a few positive predictions based on a single gene only, the core VMT agreed to explore to modify the PM by requiring two genes exceeding the gene-specific induction threshold for a positive prediction. The modified PM is presented here:

An Epi2SensA prediction is considered **positive** if at least two of the following conditions are met:

- The Imax for ATF3 is > 15 for at least one tested concentration.
- The Imax for GCLM is > 2 for at least one tested concentration.
- The Imax for DNAJB4 is > 2 for at least one tested concentration.
- The Imax for IL-8 is > 4 for at least one tested concentration.

The Epi2SensA prediction is considered **negative** if the three conditions below are met:

- The mean fold-induction value of the marker genes exceeds for at most one the respective cut-off values for any of the four genes at the tested concentrations that shows ≥ 60% mean cell viability;
- At least 3 concentrations were tested;
- If the highest concentration tested was lower than the highest soluble or stably dispersed concentration, at least one mean cell viability at the tested concentrations is < 80%.

Table 7: Summary of changes due to modified acceptance criteria by laboratory and criterion

laboratory	**Changes in prediction due to modification of the viability acceptance criterion and the prediction model**
Mattek	• Benzisothiazolinone samples B160 and B224 become positive (due to viability) • p-phenylenediamine sample B231 becomes positive (viability) and the first test of sample B185 would have become valid and positive (viability) • Cetrimide sample B141 becomes negative (due to PM) • Lauryl gallate samples B111 and B316 become negative (PM) • Diethyl phthalate sample B298 becomes negative (PM) • 1-Iodehexane sample B454 becomes negative (PM)
Eurofins	• Isoeugenol samples A411 becomes positive (viability) • Benzisothiazolinone samples A123 and A274 become negative (PM) • Cetrimide samples A366 and A127 become negative (PM) • Lactid acid sample A288 becomes negative (PM) • Diethyl phthalate sample A289 becomes negative (PM)
BRT	• 2,4-Dinitrochlorobenzene sample C167 becomes negative (PM) • Lauryl gallate sample C383 becomes negative (PM) • Benzyl butyl phthalate C439 becomes negative (PM) • Diethyl phthalate samples C149 and C378 become negative (PM)
FDSC	none

In terms of reproducibility and predictivity, these changes have the following impact.

- WLR

The WLR would improve in all laboratories (Table 8), with Mattek and Eurofins meeting the acceptance criterion of ≥ 80%. BRT would still not meet the acceptance criterion (75,0%). However, if Cetrimide, a substance also leading to inconsistent result in the EpiSensA were excluded, also BRT would meet the WLR acceptance criterion (9/11=81,8%).

- BLR

The BLR would be improved from 70,0% to 85,0%, meeting the acceptance criterion of ≥ 80%.

- Predictivity capacity

The predictive capacity would be affected in both directions, depending on the laboratory (Table 8). Mattek's specificity would be improved to 100% resulting also in a higher accuracy (95%), while the sensitivity would not be impacted. Eurofins' specificity would also not be affected, but the sensitivity would be reduced below the acceptance value. At BRT, the specificity would improve to 83,3%, increasing also the accuracy, so that does meet the acceptance criteria. BRT's sensitivity would remain 92,9%.

Only Eurofins would not have all predictive capacity acceptance criteria, due to one the sensitivity.

Table 8: Comparison of the WLR, BLR and predictive capacity according to the original analysis and according to the modified acceptance criteria, with green highlighted cells indicating met acceptance criteria

	acceptance criterion	Mattek		Eurofins		BRT	
		original	modified	original	modified	original	modified
WLR	≥ 80%	66,7% (8/12)	91,7% (11/12)	58,3% (8/12)	83,3% (10/12)	66,7% (8/12)	75,0% (9/12)
BLR	≥ 80%	original: 70%			modified: 85%		
Specificity	≥ 65%	66,7% (6/6)	100% (6/6)	83,3% (5/6)	83,3% (5/6)	50,0% (3/6)	83,3% (5/6)
Sensitivity	≥ 85%	92,9% (13/14)	92,9% (13/14)	85,7% (12/14)	78,6% (11/14)	92,9% (13/14)	92,9% (13/14)
Accuracy	≥ 85%	85,0% (17/20)	95,0% (18/20)	85,0% (17/20)	80,0% (16/20)	80,0% (16/20)	90,0% (18/20)

In summary, the performance of the Epi2SensA would have improved with the modified acceptance criteria. While the WLR acceptance criterion was not met by one laboratory only, the BLR improved to an acceptable level. The predictive capacity acceptance criteria were fulfilled, except for Eurofins' sensitivity and, as a consequence, also accuracy.

Annex A

Minimum List of Reference Substances for Determination of Reproducibility and Predictive Capacity of similar or modified EpiSensA test method (reproduced from OECD 2024)

No.	Proficiency substances	CAS No.	Physical state	*in vivo* prediction[1]	Vehicle[2]	EpiSensA results for each marker gene[2, 3]				VRM
						ATF3	GCLM	DNAJB4	IL-8	prediction
1	2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (GHS Cat.1A)	AOO	p	p	p	p/n	Positive
2	p-Phenylenediamine	106-50-3	Solid	Sensitiser (GHS Cat.1A)	AOO	p/n	p	p	p/n	Positive
3	Metol	55-55-0	Solid	Sensitiser (GHS Cat.1A)	DW	p	p	p	p/n	Positive
4	Tetrachlor-osalicylanilide	1154-59-2	Solid	Sensitiser (GHS Cat.1A)	AOO	p	n	p/n	p	Positive
5	Lauryl galate	1166-52-5	Solid	Sensitiser (GHS Cat.1A)	AOO	p/n	n	p/n	p/n	Negative
6	Methyl heptine carbonate	111-12-6	Liquid	Sensitiser (GHS Cat.1A)	AOO	p	p	p	P	Positive
7	Isoeugenol	97-54-1	Liquid	Sensitiser (GHS Cat.1A)	AOO	p/n	p	p	p/n	Positive
8	Glyoxal 40% solution in water	107-22-2	Liquid	Sensitiser (GHS Cat. 1A	DW	p/n	p	p/n	p/n	Positive
9	Abietic acid	514-10-3	Solid	Sensitiser (GHS Cat.1B)	AOO	p	p	p	p	Positive
10	Dibutyl aniline	613-29-6	Liquid	Sensitiser (GHS Cat.1B)	AOO	p/n	n	n	p	Positive
11	Amyl cinnamic aldehyde	122-40-7	Liquid	Sensitiser (GHS Cat.1B)	AOO	p	n	p	p	Positive
12	Benzisothiazolinone	2634-33-5	Solid	Sensitiser (GHS Cat.1B)	AOO	p/n	p/n	p/n	p/n	Positive[5]
13	Imidazolidinyl urea	39236-46-9	Solid	Sensitiser (GHS Cat.1B)	DW	p/n	n	p/n	p	Positive
14	Farnesol	4602-84-0	Liquid	Sensitiser (GHS Cat.1B)	AOO	p	p/n	p	p	Positive
15	Cetrimide	57-09-0	Solid	Non-sensitiser (Not classified)	50%EtOH	n	n	n	n	Negative
16	Lactic acid[4]	50-21-5	Liquid	Non-sensitiser (Not classified)	DW	n	n	n	n	Negative
17	Benzyl butyl phthalate	85-68-7	Liquid	Non-sensitiser (Not classified)	AOO	n	n	n	n	Negative
18	Diethyl phthalate	84-66-2	Liquid	Non-sensitiser (Not classified)	AOO	p/n	n	p/n	p	Negative
19	Hexane	110-54-3	Liquid	Non-sensitiser (Not classified)	AOO	n	n	n	n	Negative
20	1-Iodehexane	638-45-9	Liquid	Non-sensitiser (Not classified)	AOO	n	p	p	p	Positive

1: The in vivo hazard and potency prediction is based on LLNA data (TG497, SD Annex3) (Urbisch, 2015). The in vivo potency is derived using the criteria based on UN GHS Sub-categorisation.
2: Based on historical results (Mizumachi et al, 2018) (EpiSensA validation report).
3: "p" indicates that the fold-induction of marker gene exceeds the cut-off value with ≥ 80% viability. "n" indicates that the fold-induction of marker gene doesn't exceed the cut-off value with ≥ 80% viability. "p/n" means both "p" and "n" are acceptable because both "p" and "n" results were obtained at the validation study.
4: MTT assay should be performed instead of LDH assay.
5: Reference substance which was not 100% concordant between laboratories.
Grey: A subset of 12 out of the 20 reference substances that should be used for an assessment of within-laboratory reproducibility (WLR).

Annex B: Overview of run validity

Lab	Run	run date	operator	AOO: replicates			DW: replicates			50% EtOH: replicates			PC1 viability		PC2 viability		PC1 validity				PC2 validity			
																	ATF3		IL8		GCLM		DNAJB4	
				1	2	3	1	2	3	1	2	3	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
MAT	1	05.03.25	CBR	100,2	99,4	100,4	na	na	na	na	na	na	99,9	0,8	99,0	1,2	45,5	39,9	6,7	5,2	3,0	1,2	7,5	2,6
MAT	2	12.03.25	CBR	99,6	100,3	100,2	na	na	na	97,7	102,0	100,2	96,1	1,4	96,1	1,9	14,1	2,1	3,9	0,5	5,5	1,7	5,8	2,1
MAT	2b	23.04.25	KG	98,7	100,4	100,9	na	na	na	100,3	101,1	98,6	100,3	1,3	98,2	4,1	44,9	12,0	23,0	8,4	5,4	1,2	6,2	1,6
MAT	3	na	na	100,4	101,6	98,0	100,5	99,9	99,6	na	na	na	100,7	1,0	100,5	1,0	17,0	8,9	23,3	14,1	3,4	1,8	6,3	4,7
MAT	4	16.04.25	CBR	96,9	103,0	100,1	na	na	na	na	na	na	101,8	0,6	103,3	0,2	62,9	39,5	65,9	80,4	5,8	0,5	3,5	0,7
MAT	5	07.05.25	CBR	99,9	100,7	99,4	101,0	98,2	100,9	na	na	na	100,2	0,7	98,7	1,9	32,2	14,2	9,6	3,0	7,1	1,9	9,7	0,8
MAT	6	14.05.25	CBR	101,6	101,6	96,8	100,2	101,1	98,6	na	na	na	99,8	1,6	99,3	1,1	62,7	27,8	30,3	13,5	8,6	3,9	6,6	3,1
MAT	7	21.05.25	CBR	99,0	99,9	101,0	102,4	108,7	88,8	102,3	96,7	101,0	100,8	2,3	98,3	1,7	17,7	14,4	7,3	6,2	5,3	0,4	5,8	0,9
MAT	8	28.05.25	CBR	100,7	100,3	99,0	98,6	101,0	100,4	na	na	na	95,7	2,5	95,8	2,8	38,4	11,4	17,4	4,0	4,4	0,5	4,1	0,1
MAT	9	11.06.25	CBR	100,6	102,7	96,7	na	na	na	99,2	101,2	99,6	99,6	1,8	99,6	1,7	42,1	9,5	10,9	1,1	2,3	1,8	2,2	1,9
MAT	10	18.06.25	CBR	101,8	99,6	98,6	na	na	na	na	na	na	98,8	1,4	97,0	1,4	55,2	14,6	46,7	7,7	9,2	0,7	10,0	1,9
MAT	11	02.07.25	CBR	100,3	100,1	99,7	88,8	104,5	106,7	99,6	99,6	100,8	98,3	0,9	98,9	0,8	44,2	12,7	15,3	2,1	6,4	0,5	8,6	1,4
EUR	1b	06.03.25	pni	102,9	104,3	92,8	na	na	na	na	na	na	90,6	5,7	98,9	3,6	49,7	28,4	17,3	9,4	4,4	0,4	3,6	0,4
EUR	2	12.03.25	pni	96,7	106,5	96,8	na	na	na	na	na	na	91,8	3,4	102,2	3,4	30,8	5,4	13,9	3,9	3,3	0,3	4,5	1,1
EUR	3	30.04.25	pni	98,5	103,6	97,9	84,4	106,3	109,3	na	na	na	96,4	1,8	100,8	2,2	31,5	10,9	20,1	8,8	2,1	0,3	2,5	0,6
EUR	4	07.05.25	pni	99,5	97,0	103,4	na	na	na	na	na	na	95,7	2,2	100,2	3,7	40,0	13,5	8,2	3,1	2,8	0,3	5,2	1,1
EUR	5	07.05.25	pni	102,5	89,0	108,5	na	na	na	na	na	na	106,9	4,3	112,5	1,8	23,9	0,8	12,8	4,4	5,4	0,9	5,4	1,5
EUR	6	11.06.25	pni	100,6	103,0	96,4	100,4	100,0	99,6	na	na	na	98,5	1,8	99,2	5,1	34,1	16,3	16,9	4,7	3,2	0,4	5,5	1,0
EUR	7	18.06.25	pni/lbu	98,0	99,6	102,4	100,1	102,1	97,8	104,0	93,3	102,7	95,0	7,4	88,9	8,1	39,6	25,2	16,5	10,0	3,7	0,3	5,1	0,8
EUR	8	25.06.25	pni	98,7	101,8	99,6	na	na	na	na	na	na	100,4	2,9	97,1	4,9	13,7	2,7	2,6	0,7	1,0	0,4	6,4	4,9
EUR	8b	23.07.25	pni/lbu	101,1	102,6	96,3	98,1	101,7	100,2	na	na	na	100,5	1,0	97,8	3,0	83,6	36,0	62,4	24,1	5,4	2,2	7,0	0,1
EUR	9	02.07.25	pni/lbu	100,7	100,4	98,9	na	na	na	102,2	101,3	96,5	96,7	3,9	98,5	4,3	77,5	27,1	54,9	39,9	3,4	0,5	4,4	0,2
EUR	10	09.07.25	pni/lbu	93,6	103,1	103,4	na	na	na	101,4	99,6	99,0	96,7	0,9	101,7	1,8	103,2	9,1	82,5	9,7	4,6	0,7	5,3	1,2
EUR	10b	23.07.25	pni/lbu	101,3	102,7	96,0	na	na	na	na	na	na	100,1	1,3	97,4	3,4	83,6	36,0	62,4	24,1	5,4	2,2	7,0	0,1
EUR	11	16.07.25	pni/lbu	97,7	103,2	99,1	100,9	99,5	99,5	100,4	97,0	102,6	99,9	1,9	101,9	2,2	83,8	82,8	44,4	49,5	3,9	0,7	7,1	1,7
BRT	1	05.03.25	Ryan Hunt	101,6	100,3	98,0	na	na	na	na	na	na	99,8	1,0	96,7	3,3	60,6	35,9	40,9	25,3	4,6	1,7	9,4	2,1
BRT	2	12.03.25	Ryan Hunt	102,0	100,3	97,7	na	na	na	na	na	na	100,0	1,0	98,9	0,2	56,2	32,7	41,0	27,0	0,9	0,6	0,8	0,1
BRT	2b	16.04.25	Ryan Hunt	97,2	102,3	100,5	na	na	na	na	na	na	100,7	1,5	100,8	2,2	29,9	10,3	27,2	2,6	11,4	2,6	31,0	10,7
BRT	3	09.04.25	Ryan Hunt	101,0	99,4	99,5	na	na	na	na	na	na	100,4	2,2	100,7	1,5	1,3	0,4	2,7	0,4	3,2	1,1	9,7	3,7
BRT	3b	30.04.25	Ryan Hunt	99,2	100,5	100,3	101,2	98,0	100,8	na	na	na	99,0	0,6	101,6	0,4	18,0	9,2	12,3	5,6	2,1	0,8	6,7	0,9
BRT	4	07.05.25	Ryan Hunt	100,4	99,1	100,4	na	na	na	na	na	na	99,2	0,6	99,1	2,1	36,5	14,5	15,1	3,7	4,3	0,3	6,9	2,1
BRT	5	14.05.25	Ryan Hunt	99,6	100,6	99,8	100,1	100,4	99,5	na	na	na	97,3	0,5	99,1	2,0	17,0	13,3	8,1	6,4	3,8	0,5	5,8	0,7
BRT	6	21.05.25	Ryan Hunt	100,9	100,5	98,6	99,9	100,3	99,8	na	na	na	99,5	1,1	98,7	1,4	25,3	30,3	61,9	38,9	3,6	1,4	6,2	0,8
BRT	7	11.06.25	Ryan Hunt	102,7	95,1	102,2	99,8	100,3	99,9	100,6	101,6	97,8	100,5	0,7	101,0	2,9	52,8	10,6	23,2	8,7	8,3	1,8	8,4	5,7

BRT	8	25.06.25	Ryan Hunt	98,8	101,8	99,4	101,0	98,7	100,2	na	na	na	100,0	0,8	100,8	1,7	34,2	2,5	18,1	2,5	5,7	0,8	10,6	1,3
BRT	9	02.07.25	Ryan Hunt	100,7	98,2	101,1	na	na	na	100,5	100,7	98,9	99,0	2,6	100,6	1,1	55,1	16,1	19,3	6,7	3,7	0,6	6,8	0,6
BRT	10	18.06.25	Ryan Hunt	101,9	100,4	97,7	na	na	na	na	na	na	98,9	1,4	98,6	1,2	30,4	28,2	15,3	14,0	5,0	0,6	7,9	1,1
BRT	11	09.07.25	Ryan Hunt	102,0	101,7	96,3	100,4	101,1	98,5	99,6	100,6	99,8	99,7	0,6	100,1	1,1	64,2	29,7	45,4	13,1	15,3	8,8	20,1	18,9
FDS	1	25.03.25	Narita/Sato	100,6	99,3	100,1	101,0	98,2	100,7	na	na	na	100,0	0,7	99,9	1,3	49,5	15,4	5,6	1,9	4,9	0,8	7,4	2,9
FDS	2	13.05.25	Narita/Sato	99,9	99,9	100,2	99,3	99,2	101,5	na	na	na	98,8	2,2	98,7	2,4	55,7	45,9	48,7	36,2	4,0	0,5	7,7	0,8

orange: invalid run; red: positive control (PC) invalid; yellow: viability of negative control (NC) tissues < 95%; PC1: Clotrimazole; PC2: 4-NBB; AOO: Acetone olive oil; DW: distilled water

Annex C1: Mattek summary data

RS name	RS result	run	code	VC	no. valid concs.	max. valid conc.	ATF3	GCLM	DNYJB4	IL8	prediction
2,4-Dinitrochloro-benzene	S (1A)	3	B121-3	AOO	4	0,39	0	1	1	0	1
		7	B242-3	AOO	4	0,39	0	1	1	0	1
		10	B388-3	AOO	3	0,195	1	1	1	0	1
p-phenylene-diamine	S (1A)	7	B185-0	EtOH	4	3,125	0	1	1	0	1
		1	B231-2	AOO	1	0,39	0	0	1	0	1
		10	B375-2	AOO	4	1,25	0	1	1	0	1
Metol	S (1A)	3	B486-0	DW	3	3,12	1	1	0	0	1
Tetrachloro-salicylanilide	S(1A)	6	B479-2	AOO	2	0,78	1	1	0	1	1
Lauryl galate	S(1A)	3	B111-0	AOO	4	25	0	1	0	0	1
		9	B222-1	AOO	3	12,5	0	0	0	0	0
		10	B316-1	AOO	4	12,5	0	0	0	1	1
Methyl heptine carbonate	S (1A)	4	B130-0	AOO	4	100	1	1	1	0	1
		10	B235-2	AOO	4	3,125	0	1	1	0	1
		6	B328-2	AOO	4	3,125	0	1	1	0	1
Isoeugenol	S (1A)	1	B418-2	AOO	2	0,78	1	0	1	0	1
Glyoxal 40% solution in water	S (1A)	6	B108-1	DW	1	6,25	1	1	1	1	1
		5	B243-1	DW	3	25	1	1	1	1	1
		8	B343-1	DW	2	12,5	1	1	0	0	1
Abietic acid	S (1B)	4	B104-1	AOO	4	12,5	0	1	1	1	1
		5	B241-1	AOO	4	12,5	0	1	1	1	1
		8	B391-1	AOO	4	12,5	0	1	1	0	1
Dibutyl aniline	S (1B)	4	B438-0	AOO	4	100	0	1	1	0	1
Amyl cinnamic aldehyde	S (1B)	2	B466-0	AOO	4	100	1	1	1	1	1
Benziso-thiazolinone	S (1B)	1	B160-0	AOO	2	0,098	0	0	0	0	0
		2	B224-3	AOO	2	0,098	0	0	0	0	0
		5	B317-3	AOO	2	0,098	0	1	1	0	1
Imidazolidinyl urea	S (1B)	7	B452-1	DW*	3	25	1	0	1	1	1
Farnesol	S (1B)	4	B117-0	AOO	4	100	1	0	1	1	1
		9	B208-1	AOO	3	100	1	0	1	1	1
		5	B311-1	AOO	3	100	1	1	1	1	1
Cetrimide	NS	7	B365-	EtOH	4	3,125	0	0	0	0	0
		11	B141-2	EtOH	4	3,125	0	0	0	1	1
		9	B203-2	EtOH	2	0,39	0	1	1	0	1
Lactic acid	NS	11	B183-1	DW*	2	12,5	0	0	0	0	0
		7	B272-1	DW*	3	100	0	0	0	0	0
		8	B396-1	DW	2	12,5	0	0	0	0	0
Benzyl butyl phthalate	NS	2	B427-0	AOO	3	100	0	0	0	0	0
Diethyl phthalate	NS	3	B107-0	AOO	4	100	0	0	0	0	0
		11	B298-1	AOO	4	100	0	0	1	0	1
		9	B385-1	AOO	3	100	0	0	0	0	0
Hexane	NS	1	B136-1	AOO	4	100	0	0	0	0	0
		8	B202-1	AOO	4	100	0	0	0	0	0
		11	B347-1	AOO	4	100	0	0	0	0	0
1-Iodehexane	NS	6	B454-1	AOO	1	6,25	0	1	0	0	1

RS: reference substances; VC: vehicle control: S: sensitiser; NS: non-sensitiser; * changed due to viability (>60%); ** only one gene positive

Annex C2: Eurofins summary data

RS name	RS result	run	code	VC	no. valid concs.	max. valid conc.	ATF3	GCLM	DNYJB4	IL8	prediction
2,4-Dinitrochloro-benzene	S (1A)	3	A192-3	AOO	3	0,195	0	1	1	0	1
		7	A279-3	AOO	3	0,195	0	1	1	0	1
		10	A351-3	AOO	4	0,39	1	1	1	1	1
p-phenylene-diamine	S (1A)	1	A271-2	AOO	2	0,78	0	1	1	0	1
		2	A143-0	AOO	1	0,781	0	1	1	0	1
		10	A305-2	EtOH	2	0,78	0	1	1	0	1
Metol	S (1A)	3	A401-0	DW*	2	0,78	1	1	1	1	1
Tetrachloro-salicylanilide	S(1A)	6	A497-2	AOO	1	0,39	1	0	0	1	1
Lauryl galate	S(1A)	3	A198-0	AOO	4	50	0	0	0	0	0
		9	A240-1	AOO	4	25	0	0	0	0	0
		10	A361-1	AOO	4	50	0	0	0	0	0
Methyl heptine carbonate	S (1A)	4	A115-0	AOO	4	100	1	1	1	0	1
		6	A346-2	AOO	4	100	1	1	1	0	1
		10	A262-2	AOO	4	3,125	0	1	1	0	1
Isoeugenol	S (1A)	1	A411-2	AOO	1	0,39	0	1	0	0	1
Glyoxal 40% solution in water	S (1A)	5	A283-1	DW	3	50	1	0	1	1	1
		6	A138-1	DW	2	25	1	1	1	1	1
		8	A323-1	DW	2	12,5	1	1	1	1	1
Abietic acid	S (1B)	4	A174-1	AOO	3	6,25	0	1	1	1	1
		5	A278-1	AOO	4	12,5	0	1	1	1	1
		8	A313-1	AOO	4	12,5	1	1	1	1	1
Dibutyl aniline	S (1B)	4	A478-0	AOO	4	100	0	0	0	0	0
Amyl cinnamic aldehyde	S (1B)	2	A443-0	AOO	4	100	0	0	1	1	1
Benziso-thiazolinone	S (1B)	1	A123-0	AOO	2	0,098	0	1	0	0	1
		2	A274-3	AOO	3	0,098	0	0	0	0	0
		5	A301-3	AOO	3	0,195	0	1	1	0	1
Imidazolidinyl urea	S (1B)	7	A431-1	EtOH	4	50	1	0	1	1	1
Farnesol	S (1B)	4	A146-0	AOO	3	100	0	0	0	0	0
		5	A263-1	AOO	3	50	1	1	1	0	1
		9	A329-1	AOO	4	100	1	0	1	1	1
Cetrimide	NS	7	A366-2	EtOH	2	0,78	0	0	0	0	0
		9	A249-2	EtOH	2	0,78	0	0	0	0	0
		11	A127-2	EtOH	3	1,56	0	0	0	1	1
Lactic acid	NS	7	A133-1	DW	3	100	0	0	0	0	0
		8	A288-1	DW	2	12,5	0	0	0	1	1
		11	A382-1	DW	3	100	0	0	0	0	0
Benzyl butyl phthalate	NS	2	A426-0	AOO	4	100	0	0	0	0	0
Diethyl phthalate	NS	3	A169-0	AOO	4	100	0	0	0	0	0
		9	A306-1	AOO	4	100	0	0	0	0	0
		11	A289-1	AOO	4	100	0	0	1	0	1
Hexane	NS	1	A186-1	AOO	4	100	0	0	0	0	0
		8	A223-1	AOO	4	100	0	0	0	0	0
		11	A398-1	AOO	4	100	0	0	0	0	0
1-Iodehexane	NS	6	A471-1	AOO	1	50	0	1	1	0	1

RS: reference substances; VC: vehicle control: S: sensitiser; NS: non-sensitiser

Annex C3: BRT summary data

RS name	RS result	run	code	VC	no. valid concs.	max. valid conc.	ATF3	GCLM	DNYJB4	IL8	prediction
2,4-Dinitrochloro-benzene	S (1A)	3	C167-3	AOO	4	0,39	0	1	1	0	1
		7	C210-3	AOO	3	0,39	1	1	1	0	1
		10	C315-3	AOO	2	0,39	0	1	1	0	1
p-phenylene-diamine	S (1A)	1	C211-2	AOO	3	1,56	0	1	1	0	1
		2	C118-0	AOO	3	1,56	0	1	1	1	1
		10	C369-2	AOO	2	1,56	0	1	1	1	1
Metol	S (1A)	3	C476-0	DW	3	3,13	1	1	1	0	1
Tetrachloro-salicylanilide	S(1A)	6	C468-2	AOO	4	3,125	1	1	1	1	1
Lauryl galate	S(1A)	3	C165-0	AOO	4	25	0	0	0	0	0
		9	C296-1	AOO	4	12,5	0	0	0	0	0
		10	C383-1	AOO	4	25	0	0	1	0	1
Methyl heptine carbonate	S (1A)	4	C176-0	AOO	4	100	1	1	1	0	1
		6	C342-2	AOO	4	3,125	0	0	0	0	0
		10	C206-2	AOO	4	3,13	0	1	1	1	1
Isoeugenol	S (1A)	1	C481-2	AOO	3	1,56	1	1	1	0	1
Glyoxal 40% solution in water	S (1A)	5	C251-1	DW	3	0,25	1	0	1	0	1
		6	C132-1	DW	2	25	1	0	0	1	1
		8	C340-1	DW	3	25	1	1	1	1	1
Abietic acid	S (1B)	4	C194-1	AOO	4	12,5	0	1	1	1	1
		5	C286-1	AOO	2	0,125	0	1	1	0	1
		8	C318-1	AOO	4	6,25	0	1	1	0	1
Dibutyl aniline	S (1B)	4	C444-0	AOO	4	100	0	1	1	0	1
Amyl cinnamic aldehyde	S (1B)	2	C492-0	AOO	4	100	1	0	1	1	1
Benziso-thiazolinone	S (1B)	1	C188-0	AOO	2	0,098	0	1	1	0	1
		2	C265-3	AOO	1	0,049	0	1	1	0	1
		5	C390-3	AOO	3	0,004	0	1	1	0	1
Imidazolidinyl urea	S (1B)	7	C491-1	DW	2	12,5	0	0	1	1	1
Farnesol	S (1B)	5	C380-1	AOO	4	1	1	0	1	1	1
		9	C244-1	AOO	4	100	1	0	1	1	1
		10	C120-0	AOO	4	100	1	0	1	1	1
Cetrimide	NS	7	C359-2	EtOH	4	3,13	0	1	1	0	1
		9	C212-2	EtOH	4	3,13	0	0	0	0	0
		11	C189-2	EtOH	1	3,13	0	0	0	0	0
Lactic acid	NS	7	C287-1	DW	3	100	0	0	0	0	0
		8	C345-1	DW	1	100	0	0	0	0	0
		11	C184-1	DW	3	100	0	0	0	0	0
Benzyl butyl phthalate	NS	2	C439-0	AOO	4	100	0	0	0	1	1
Diethyl phthalate	NS	3	C149-0	AOO	4	100	0	0	1	0	1
		9	C378-1	AOO	1	100	0	0	1	0	1
		11	C239-1	AOO	3	100	0	0	0	0	0
Hexane	NS	1	C166-1	AOO	1	100	0	0	0	0	0
		8	C275-1	AOO	4	100	0	0	0	0	0
		11	C335-1	AOO	4	100	0	0	0	0	0
1-Iodehexane	NS	6	C440-1	AOO	4	100	1	1	1	1	1

RS: reference substances; VC: vehicle control: S: sensitiser; NS: non-sensitiser

Annex C4: FDSC summary data

RS name	RS result	run	code	VC	no. valid concs.	max. valid conc.	ATF3	GCLM	DNYJB4	IL8	prediction
2,4-Dinitrochloro-benzene	S (1A)										
p-phenylene-diamine	S (1A)										
Metol	S (1A)	1	D433-0	DW	3	1,56	1	1	1	0	1
Tetrachloro-salicylanilide	S(1A)	1	D414-2	AOO	4	3,125	1	0	1	1	1
Lauryl galate	S(1A)										
Methyl heptine carbonate	S (1A)										
Isoeugenol	S (1A)	1	D457-2	AOO	3	1,56	1	1	1	0	1
Glyoxal 40% solution in water	S (1A)										
Abietic acid	S (1B)										
Dibutyl aniline	S (1B)	1	D493-0	AOO	4	100	0	0	0	0	0
Amyl cinnamic aldehyde	S (1B)	2	D424-0	AOO	4	100	0	0	1	1	1
Benziso-thiazolinone	S (1B)										
Imidazolidinyl urea	S (1B)	2	D410-1	DW	4	50	1	0	1	1	1
Farnesol	S (1B)										
Cetrimide	NS										
Lactic acid	NS										
Benzyl butyl phthalate	NS	2	D416-0	AOO	4	100	0	0	0	0	0
Diethyl phthalate	NS										
Hexane	NS										
1-Iodehexane	NS	2	D474-1	AOO	4	100	0	1	1	1	1

RS: reference substances; VC: vehicle control: S: sensitiser; NS: non-sensitiser

Appendix III

MATTEK
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	STANDARD OPERATING PROCEDURE	

Epi2SensA

EpiDerm Skin Sensitization Assay

MatTek Corporation

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Table of contents

1. Abbreviations and Definitions

AOO: Acetone: olive oil = 4:1 v/v

AOP: Adverse outcome pathway

ATF3: Activating transcription factor 3

Ct: Cycle threshold

CXCL8: Chemokine (C-X-C motif) ligand 8

DNAJB4: DnaJ (Hsp40) homolog, subfamily B, member 4

DW: Distilled water

Epi2SensA: EpiDerm™ Epidermal Sensitization Assay

50% EtOH: 50 v/v% ethanol in DW

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GCLM: Glutamate-cysteine ligase, modifier subunit

IL-8: Interleukin-8

I_{max}: Maximal fold induction

K_{ow}: Octanol-water partition coefficient

LDH: Lactate dehydrogenase

4NBB: 4-Nitrobenzyl bromide

PCR: Polymerase chain reaction

RhE: Reconstructed human Epidermis

TC: Test chemical

2. Abstract

The Epi2SensA is an *in vitro* method similar to the EpiSensA method for measuring the expression of four marker genes (*ATF3*, encoding Activating transcription factor 3; *GCLM*, encoding Glutamate-cysteine ligase, modifier subunit; *DNAJB4*, encoding dnaJ (Hsp40) homolog, subfamily B, member 4; *IL-8*, encoding Interleukin-8) in the reconstructed human *epidermis* (RhE), EpiDerm (EPI-200, MatTek Corporation) following controlled exposure of test chemicals (Saito et al., 2013, 2017). The two main important keratinocyte responses that occur in the skin sensitization adverse outcome pathway are Inflammatory response and induction of cytoprotective gene pathways (OECD, 2012). This assay is based on the induction of multiple marker genes related to these two keratinocyte responses in the induction of skin sensitization. In addition, Epi2SensA, like the original EpiSensA method, is applicable for not only hydrophilic chemicals but also lipophilic ones (e.g. $LogK_{ow}$>3.5) because lipophilic vehicle which is used in animal test can be used (Saito et al., 2017). Moreover, because RhE models exhibit metabolic capability similar to that of human skin (Hewitt et al., 2013, Tokudome et al., 2015), these methods can correctly detect pre/pro-haptens.

3. Description of Assay

3.1 Biological Endpoint and Endpoint Measurement

Modulation of the expression of four marker genes (ATF3, GCLM, DNAJB4, and IL-8) and one endogenous control gene (GAPDH, encoding the housekeeping protein Glyceraldehyde 3-phosphate dehydrogenase): quantified by real-time PCR analysis following exposure of test chemicals on the surface of *epidermis*.

3.2 Endpoint Value

Fold induction of four marker genes (*ATF3*, *GCLM*, *DNAJB4* and *IL-8*).

3.3 Experimental System

The test system in this SOP is the Reconstructed human Epidermis (RhE) EpiDerm™ from MatTek Corporation (EPI-200). The part # EPI-200-SENSA has been created to include the required culture plates, culture medium, and positive control for the assay (10% Triton X-100). Contents of the EPI-200-SENSA kit are given in ANNEX E.

4. Materials and Preparations

4.1 Test Systems

The Epi2SensA uses the reconstructed human epidermal model, EpiDerm™ (EPI-200-SENSA, MatTek Corporation, Ashland, MA, USA and MatTek IVLSL, Bratislava, Slovakia). The EPI-200 RhE consists of normal human-derived epidermal keratinocytes, which have been cultured to form a 3-dimensional, multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayer stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo*.

4.2 Solubility and Dose Finding

Vehicle selection and assessment of test chemical solubility

Assessment of solubility is conducted prior to testing. The solubility of each chemical is evaluated and confirmed visually. For this purpose, test chemicals are dissolved or stably dispersed at a concentration of 50% in acetone: olive oil; 4:1 (AOO, 20% v/v of olive oil in acetone) as a first vehicle option, (e.g. 0.5 g of test chemical is measured, and 0.5 mL of AOO is added), distilled water (DW) as a second vehicle option, or 50% v/v % ethanol in DW (50% EtOH) as a third vehicle option. If the test chemical is not soluble or does not stably disperse (i.e. a colloid or suspension in which the test chemical does not settle or separate from the vehicle into different phases within 10 minutes of preparation at room temperature) at a concentration of 50% in any of the vehicles, the highest soluble concentration should be determined by 2-fold serial dilutions beginning with 50% down to 0.0122%. If the test chemical is not soluble or does not form a stable dispersion at 0.0122%, the chemical is not applicable for testing using Epi2SensA. The appropriate vehicle is defined as the vehicle that dissolves the test chemical or forms a stable dispersion at the highest concentration tested. It should be verified whether the highest concentration determined can be prepared at weight per volume by pipetting the necessary quantity into a glass vial. If the highest soluble or stably dispersed concentration is determined to be 0.0488%, 0.0244%, or 0.0122%, the subsequent concentration-finding study (Section 4.3) can be skipped, and main study should be performed (Section 6). In cases in which a vehicle other than AOO, DW, or 50% EtOH is used, appropriate scientific rationale for use of that vehicle should be provided.

4.3 Concentration-finding study

A concentration-finding assay is performed to determine the concentrations of test chemical be used for the main study, which looks at gene expression analysis. In the main study, test chemical concentrations that show ≥ 60% mean viability should be used, along with at least one mean viability <60% for negative judgement. Therefore, the lowest test chemical concentration that induces a < 60% tissue viability is determined in concentration-finding study.

Test chemicals are prepared on the day of testing and dissolved or stably dispersed in an appropriate vehicle at the highest concentration determined as specified Section 4.2. Starting from the highest concentration, 4-fold serial dilutions are prepared to 0.0122 or 0.0244% (w/v) in the corresponding vehicle. Thus, depending on the starting concentration, a varying number of dilutions are prepared and tested.

The corresponding vehicles utilized for the preparation of the test chemicals are used as the vehicle controls. Both non-treated control and killed control are used for calculation of viability. Non-treated control is used to define 100% viability, and killed control is used to define 0% viability. For tested chemicals, duplicate (two EpiDerm RhE) are used for each concentration.

Note:

- If the cytotoxicity of the test chemical is unclear, it is recommended that at least 9 working solutions at 2-fold serial dilutions from the highest soluble concentration are tested in one main study. If the mean tissue viability at each of the tested concentrations is <60%, an additional study needs to be performed at 2-fold serial dilutions from the lowest concentration in the first study.
- It is possible to start the experiments for the main study by skipping the dose finding study. However, the results of tested concentrations that show a mean tissue viability of less than 60% should not be considered. The results of at least one tested concentration that shows equal to or greater than 60% mean tissue viability should be used to judge test chemicals as positive or negative.

5. PREPARATIONS

5.1 Assay Medium

Ready-to-use assay medium (EPI-200-ASY) is included with the EPI-200-SENSA kit. The medium should be refrigerated (2-8°C) and used within one month. The assay medium should be pre-warmed for 15 minutes in a 37±1°C water bath just before use.

5.2 Positive Controls

Clotrimazole dissolved to a final concentration of 1.56 w/v% in AOO and 4-nitrobenzyl bromide (4NBB) dissolved in AOO to a final concentration of 0.10 w/v% are used as the positive controls. For example, 0.0312 g of clotrimazole is measured into a glass vial and 1 mL of AOO is added. After clotrimazole is solubilized (mixed thoroughly with vortexing as needed), it is diluted 2X with AOO in a glass vial – add 0.5 mL of the solubilized clotrimazole to 0.5 mL of AOO. For 4NBB, 0.0100 g of 4NBB is weighed into a glass vial, and 1 mL of AOO is added. Once 4NBB is solubilized (mixed thoroughly; can be vortexed), it is diluted 10X with AOO in a glass vial – add 0.1 mL of the solubilized 4NBB to 0.9 mL of AOO

5.3 Negative Control

The vehicle controls in each run are used as the controls.

5.4 Killed Control

Triton X-100 dissolved to a final concentration of 10 w/v% in DW is used for the killed control. The killed control is used to determine maximum LDH released from killed, 0% viability tissue. The killed control is supplied by MatTek as part of the EpiDerm tissue model (Part #: EPI-200-SENSA).

6. Method – Main Study

6.1 DAY 0: Preparation and Pre-incubation

(a) Pipette 0.9 mL of the assay medium, EPI-200-ASY (provided with EPI-200-SENSA) into each well of sterile 6-well plates. Allow the plates to sit in the hood for 5-10 minutes so that the medium warms to room temperature.

(b) Under sterile conditions, open the plastic bag containing the 24-well plate with epidermal

tissues. In a sterile hood, remove the sterile gauze and using sterile forceps carefully remove each insert. Remove any remaining agarose that adheres to the outer sides of the insert by gentle blotting on sterile filter paper or sterile gauze and place the tissues in the 6-well plates previously filled with 0.9 mL of EPI-200-ASY medium.
(c) Perform visual inspection of the inserts within the next 5 minutes. Record any tissue defects and excess moisture on the surface. Do not use tissues with defects or tissues with excessive moisture on the surface. Note: Droplets may condense on the inside of the 24-well during shipping and drop onto the surface of the tissue. Remove these droplets as per the next step.
(d) Dry the surface of the tissues with a sterile cotton tip swab. Place the plates into the incubator (37±1°C, 5±1% CO_2, 90±10% RH) for 60 ± 5 minutes.
(e) At the end of the first 1hr pre-incubation period, carefully remove the medium and replace it with 0.9 ml of fresh medium. Incubate the tissues (37±1°C, 5±1% CO_2, 90±10% RH) overnight for 18-24 hour.

6.2 DAY 1: Topical application

1 hour exposure followed by 5-hour post-incubation

- Pre-warm the supplied assay medium (EPI-200-ASY) in a water bath at 37±1°C for 15 minutes.
- Remove the 6-well plates containing EPI-200 tissues from the incubator approximately 5 minutes before exposure to the test chemical (TC).
- Remove any moisture from the tissue surface using sterile cotton swab. Before exposing the tissues to the TC, replace the medium with fresh 0.9 mL fresh medium.

Figure 1 Example of plate layouts – dosing in 6-well plates

Plate #1

1. Non-treated	2. Killed Ctrl.	3. Killed Ctrl.
4. AOO	5. AOO	6. AOO

Plate #2

7. Clotrimazole 1.56 w/v%	8. Clotrimazole 1.56 w/v%	9. Clotrimazole 1.56 w/v%
10. 4NBB 0.10 w/v%	11. 4NBB 0.10 w/v%	12. 4NBB 0.10 w/v%

Plate #3

13. TC1 0,195%	14. TC1 0,195%	15. TC1 0,195%
16. TC1 0,39%	17. TC1 0,39%	18. TA1 0,39%

Plate #4

19. TC1 0,78%	20. TC1 0,78%	21. TC1 0,78%
22. TC1 1,56%	23. TC1 1,56%	24. TC1 1,56%

Plate #5

Assay medium only (no tissue)	Empty	Empty
Assay medium only (no tissue)	Empty	Empty

- Apply an aliquot (10 μL) of the TC to the center of each RhE surface using a positive

displacement pipette. The tip of the pipette must not touch the surface of the tissue. The surface of EpiDerm RhE tissue is hydrophobic, so when the vehicle is distilled water or another polar solvent, mesh (Part #: EPI-MESH) must be used to ensure homogeneous distribution of the test product on the tissue surface. The mesh is not used for TA's dissolved in AOO.

- Utilize one tissue for the non-treated control and two tissues for the killed control.
- Incubate the treated tissues for 1 hour at 37±1°C, 5±1% CO_2, 90±10% RH. After 1 hour, rinse the tissues 15X by pipetting 0.5 mL of DPBS onto the tissue surface. Use a repeater pipette (e.g. Multipette M4, Eppendorf) adjusted for 0.5 mL volume. After the 15 rinses, remove any excess DPBS by gently shaking the insert, blot the insert on sterile blotting paper, and put the insert back into in the same medium for post-exposure of 5h. Return the 6-well plates to the incubator (37±1°C, 5±1% CO_2, 90±10% RH) for the 5h ± 5 min post-exposure.

Procedure for killed controls:
Before adding Triton X-100 to the basal compartment, remove 50 µL of the medium. Pipette 50µL of 10% Triton-X 100 into the medium of the kill control tissues. Mix the medium by gently shaking the plate. Incubate these tissues for 6hr at 37±1°C, 5±1% CO_2, 90±10% RH. The killed control is not applied topically.

Blank medium wells: Fill 2 wells of a 6-well plate with 0.9 mL of EPI-200-ASY and incubate the plate for 6hr at 37±1°C, 5±1% CO_2, 90±10% RH. This will serve as the blank medium control for the LDH assay.

6.3 LDH Assay

- Pipet 2 x 50 µL aliquots of the medium from each tissue into a 96-well plate and perform a lactate dehydrogenase (LDH) assay using an LDH cytotoxicity detection kit according to the manufacturer's instructions. Also, pipet 2 x 50 µL aliquots of the blank medium into the 96 well plate. Prepare the following reaction mix (Solution A + Solution B) for the requisite amount and add an equal volume (50 µL) of the reaction mix to the collected medium in a 96-well plate using a multi-channel pipette. Incubate the plate for 30 minutes at room temperature in the dark. After 30 minutes, stop the reaction by adding 50µL/well of 1M HCl using a multi-channel pipette.

Figure 2: Example 96-well plate layout for LDH assay:

1	1	9	9	17	17						
2	2	10	10	18	18						
3	3	11	11	19	19						
4	4	12	12	20	20						
5	5	13	13	21	21						
6	6	14	14	22	22						
7	7	15	15	23	23						
8	8	16	16	24	24					blk	blk

- Each number corresponds to the number shown in the upper Figure 1 (6-well plate format).
- Measure the absorbance at 490 or 492 nm and the reference wavelength (≥ 650 nm) using a 96-well plate absorbance reader. The measurement should be performed immediately (< 1hr) after adding HCl.
- Calculate Δabs. for each sample by subtracting the absorbance at reference wavelength from the absorbance of each sample at 490 nm.

- Calculate tissue viability using the following equation.

$$cell\ viability\ (\%) = 100 - \frac{\Delta abs.of\ test\ chemical\ treatment - \Delta abs.of\ vehicle\ control)}{mean\ \Delta abs.of\ killed\ control - \Delta abs.of\ vehicle\ control} * 100$$

- The lowest concentration showing less than 60% tissue viability is used for the subsequent main study.

Note:

- It is possible to start the experiments for the main study by skipping the dose finding study. However, the results of tested concentrations that show a mean tissue viability of less than 60% should not be considered. The results of at least one tested concentration that shows equal to or greater than 60% mean tissue viability should be used to judge test chemicals as positive or negative.

6.4 Preparation of test chemicals

- Prepare the test chemical solution at the lowest concentration showing less than 60% tissue viability in the dose finding study using a glass vial.
- Prepare working solutions of each test chemical as 2-fold serial dilutions from the above concentration. It is recommended to dilute the working solution up to the highest concentration showing >90% tissue viability in the dose finding study. Depending on the slope of tissue viability vs concentration curve, use 3 working solutions if the slope is steep and 5 working solutions if the slope is shallow.
- When the tissue viability is not less than 80% in the dose finding study, use at least 3 working solutions at 2-fold serial dilutions from the highest soluble concentration or 100% (neat).
- Prepare the positive controls as per Section 5.2.

Caution:

- If liquid test chemicals (potentially volatile) or their solutions are applied to the skin tissues, the vapor of the chemicals may cross-contaminate other tissues and effect gene expression, thereby leading to erroneous assay results. Avoid cross-contamination by using a separate 6-well plate for each test chemical.
- The solution should preferably avoid reaching the outer edge of the tissue because the test chemical or vehicle can more easily penetrate the tissue there, which can lead to overestimation of tissue toxicity or gene expression. Therefore, the TA should be pipetted into the center of the tissue.
- When two or more EPI-200 plates are used simultaneously from the same lot, it is only necessary to put the non-treated control, killed control, positive control, and vehicle control in the 1st plate.
- Each test chemical solution and vehicle cannot be re-used and should be prepared fresh for each experiment.

6.5 Lysis of tissues

Note: The lysis protocol presented below uses RNAqueous Kit (Thermo Fisher). Other commercially available kits can also be used.

- Using an RNA clean scalpel (sprayed and cleaned with RNAZap), cut the tissue along with the membrane out of the plastic insert housing. Using an RNase free forceps, transfer the tissues and the membrane to RNase free 1.5 mL microcentrifuge tube containing 300 μL of RNA lysis buffer (supplied with the RNAqueous Kit, Part Number AM1912, Thermo Fisher Scientific). When putting the tissue into tube, make sure that lysis buffer (300 uL) is already present in the tube, and not the other way around. These tubes need to be placed in an ice bucket. Use one tube per tissue sample.

- Homogenize the tissue samples in the RNA lysis buffer using a motorized electric homogenizer or a battery driven motor and pestle. Lyse/ homogenize each tissue by moving the pestle up and down in the tube at least 50 times (while the pestle is spinning). Keep the tubes with the lysis buffer/tissue lysates on ice while the other samples are being homogenized.
- Ensure complete lysis of each tissue sample, the lysis buffer should appear cloudy at the end of lysis. After all tissue samples are completely homogenized, add an additional 300 μL of RNA lysis buffer (supplied in the RNAqueous kit) to each tube, secure the lid on the tube, and invert to tubes few times to mix the homogenates.
- Store the lysates at -80°C overnight or until ready to perform RNA isolation. Note: it is important to store the lysates at -80ºC at least overnight.

6.6. RNA extraction

Note: RNA extraction using the RNAqueous Kit (ThermoFisher Scientific) is presented below. Other commercially available kits can also be used.

- Heat an aliquot of Elution Solution (typically 50-200 μL per prep) in an RNase-free microcentrifuge tube in a heat block set to 70-80ºC.
- Add the recommended volume of 100% ethanol to the 64% Ethanol bottle (provided in the kit). Also add the recommended volume of 100% ethanol to the wash solution #2/3 (provided in the kit).
- Remove the frozen lysates from the -80°C freezer and place them in an ice bucket. While on ice, allow the lysates to thaw for 30-45 minutes. Centrifuge the lysates at 13,000 rpm for 5 minutes at room temperature.
- Transfer the supernatant (about 600 μL) to another labeled, RNA clean 1.5-mL microtube, being careful to not dislodge the pellet/tissue debris. The supernatant should be at room temperature from this step onwards.
- Add an equal volume (600 μL) of 64% Ethanol to the supernatant, secure the lid on the tubes and mix the samples by inverting the tubes several times.
- Transfer half of the lysate/ethanol mixture (600 μL), to a filter cartridge placed in a 1.5 mL collection tube (supplied with RNAqueous kit). Centrifuge for 1 minute at 13,000 rpm at room temperature. Discard the flow-through.
- Repeat this step with the remainder of the lysate/ethanol mixture.
- Add 700 µL of wash Solution #1 (supplied with RNAqueous kit) to the filter cartridge. Centrifuge for 1 min. at 13,000 rpm to wash the filter membrane. Discard the flow-through.
- Add 500 µL of Wash Solution #2/3 (supplied with RNAqueous kit) to the filter cartridge. Centrifuge for 1 minute at 13,000 rpm at room temperature to wash the spin column membrane. Discard the flow-through.
- Add a second aliquot of 500 µL of Wash Solution #2/3 to the filter cartridge and centrifuge for 1 minute at 13,000 rpm at room temperature to wash the spin column membrane. Discard the flow-through.
- Centrifuge at 13,000 rpm for 2 minutes at room temperature to eliminate any possible carryover of wash buffer, or if residual flow-through remains on the outside of the filter cartridge.
- Place the filter cartridge in a new 1.5 mL collection tube (supplied). Add 30 µL preheated (70-80ºC) Elution Solution (supplied) directly to the center of the filter cartridge. Centrifuge for 30 seconds at 13,000 rpm at room temperature to elute the RNA.
- Repeat the elution step with 20 µL preheated Elution Solution (supplied with RNAqueous kit) directly to the center of the filter cartridge. Centrifuge for 30 seconds at 13,000 rpm at room temperature to elute the RNA.
- Discard the filter cartridge.

Measure the total RNA concentration using a spectral photometer (e.g. NanoDrop). The eluted RNA samples must be stored at -80°C.

Note:

- When the mean tissue viability at the tested concentration is high (e.g. >90%) but the mean total RNA concentration is less than 50% of vehicle control, the test chemical may lead to the false estimate of viability by inhibiting the reaction of LDH.

6.7 cDNA synthesis: Using the RT^2 First Strand Kit (Qiagen – Part # 330404)

Note: The cDNA synthesis method outlined below uses the RT2 First Strand Kit (Qiagen – Part # 330404). Other commercially available kits can also be used.

Thaw all components on ice and briefly centrifuge before use.

- Adjust the total RNA concentration to 500 ng/8µL with RNAse free water (supplied) in an RNase free tube or a 96 well PCR plate and transfer 8 µL of each sample into each well of a new 96 well PCR plate (on ice).

Genomic DNA Elimination Step:

- Add 2 µL of Buffer GE to each well containing the 8 µL of RNA.
- Seal the PCR plate with a plate sealer 'C' and briefly centrifuge the plate.
- Incubate the plate for 5min at 42ºC (using a thermocycler), then place immediately on ice for at least 1 min.

cDNA Synthesis:

- Prepare the following Reverse-transcription mix (cDNA synthesis mix) in an RNase free 1.5mL tube

Component	For 1 sample	For 10 samples
5X Buffer BC3	4 µL	40 Ml
Control P@	1 µL	10 µL
RNase-free water	3 µL	30 µL
RE3 Reverse Transcriptase Mix	2 µL	20 µL

- Add 10 µL of reverse-transcription mix to each sample containing genomic DNA elimination mix in the 96 well plate. Seal the plate with a plate sealer "C' and collect by brief centrifugation.
- Incubate the 96 well plate at 42ºC (using a thermocycler) for exactly 15min, then immediately stop the reaction by incubating at 95ºC for 5min (using a thermocycler). These two steps can be programed on the thermocycler with the two temperatures running one after the other.
- Add 91 µL of RNase free water to each reaction. Mix by pipetting up and down several times. This is the concentrated cDNA plate. This plate can be saved at -20ºC at this step or continued with the next step.
- Prepare a **diluted cDNA** plate. To each well of a new 96 well plate, add 100 µL of RNase free water and then add 20 µL of each cDNA. Mix by pipetting up and down several times. Use this diluted cDNA for RT-PCR. The diluted cDNA can be saved at -20ºC at this step or continued with the next step.

6.8 Real-time PCR

Quantitative RT-PCR is performed using the RT2 SYBR Green PCR Mastermix.

- Prepare the following real-time PCR mix for the requisite amount in a 1.5 mL tube for the respective 5 genes.

Component of real-time PCR mix	For 1 sample	For 10 samples
RT2 qPCR Primer Assay	1 µL	10 µL
RNase free water	5.5 µL	55 µL
RT2 SYBR Green master mix	12.5 µL	125 µL

- Mix the contents of each tube by inverting several times and collect the contents by brief centrifugation.
- Add 19 µL of real-time PCR mix to each well of a PCR reaction plate. Use a repeat pipettor for this step. Plate layout is given below.

- Transfer 6 µL of diluted cDNA to the wells containing the real-time PCR mix. Use a multi-channel pipette for this step. Seal the plate (using plate sealer 'B') and collect the sample by brief centrifugation, using a plate centrifuge.

- Measure the cycle threshold (Ct) values of four skin sensitization marker genes (*ATF3*, *GCLM*, *DNAJB4*, and *IL-8*) and one endogenous control gene (*GAPDH*) using either Applied Biosystems, Bio-Rad, Stratagene or Eppendorf cyclers. The PCR program is shown below in Figure 3. The calculation of baseline and threshold should be performed by automatic baseline and threshold determination software.

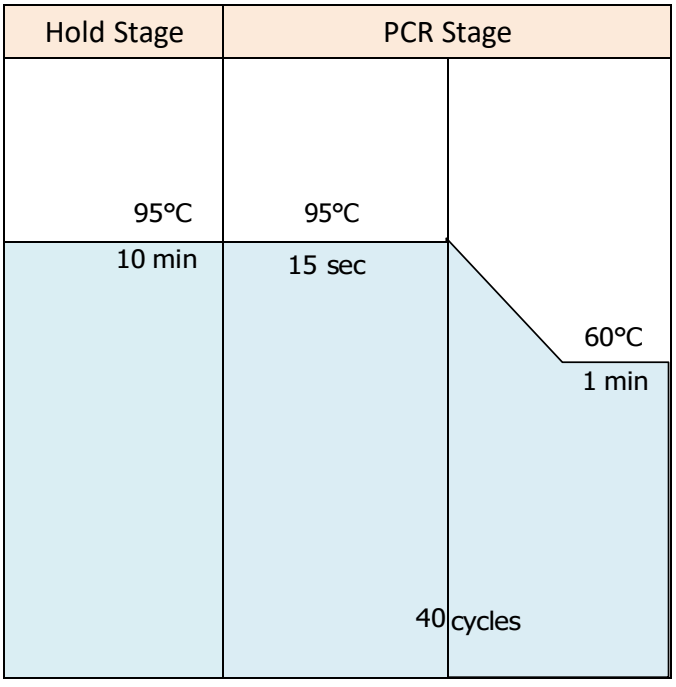

Figure 3: PCR program for the Epi2SensA assay.

Notes:

- Do not separate the cDNA samples for measurement of one gene into different real-time PCR plates. All cDNA samples should be measured on the same plate for each gene.
- All the PCR plates of one experiment should be prepared at the same time in order to reduce the error deriving from micropipette dispensing.
- The prepared PCR plate should be measured within 24 hours if stored on ice in the dark.

Examples of 96-well real-time PCR plate layout.
(Note: The tissues samples 2-3 that were used for kill control are not included in the RT-PCR plate. cDNA is not synthesized from these samples).

Plate #1

1 *ATF3*	4 *ATF3*	5 *ATF3*	6 *ATF3*	7 *ATF3*	8 *ATF3*	9 *ATF3*	10 *ATF3*	11 *ATF3*	12 *ATF3*	13 *ATF3*	14 *ATF3*
15 *ATF3*	16 *ATF3*	17 *ATF3*	18 *ATF3*	19 *ATF3*	20 *ATF3*	21 *ATF3*	22 *ATF3*	23 *ATF3*	24 *ATF3*	25 *ATF3*	26 *ATF3*
27 *ATF3*	28 *ATF3*	29 *ATF3*	30 *ATF3*	31 *ATF3*	32 *ATF3*	33 *ATF3*	34 *ATF3*	35 *ATF3*	36 *ATF3*	37 *ATF3*	38 *ATF3*
39 *ATF3*	40 *ATF3*	41 *ATF3*	42 *ATF3*	43 *ATF3*	44 *ATF3*	45 *ATF3*	46 *ATF3*	47 *ATF3*	48 *ATF3*	NTC *ATF3*	
1 *GCLM*	4 *GCLM*	5 *GCLM*	6 *GCLM*	7 *GCLM*	8 *GCLM*	9 *GCLM*	10 *GCLM*	11 *GCLM*	12 *GCLM*	13 *GCLM*	14 *GCLM*
15 *GCLM*	16 *GCLM*	17 *GCLM*	18 *GCLM*	19 *GCLM*	20 *GCLM*	21 *GCLM*	22 *GCLM*	23 *GCLM*	24 *GCLM*	25 *GCLM*	26 *GCLM*
27 *GCLM*	28 *GCLM*	29 *GCLM*	30 *GCLM*	31 *GCLM*	32 *GCLM*	33 *GCLM*	34 *GCLM*	35 *GCLM*	36 *GCLM*	37 *GCLM*	38 *GCLM*
39 *GCLM*	40 *GCLM*	41 *GCLM*	42 *GCLM*	43 *GCLM*	44 *GCLM*	45 *GCLM*	46 *GCLM*	47 *GCLM*	48 *GCLM*	NTC *GCLM*	

Plate #2

1 *DNAJB4*	4 *DNAJB4*	5 *DNAJB4*	6 *DNAJB4*	7 *DNAJB4*	8 *DNAJB4*	9 *DNAJB4*	10 *DNAJB4*	11 *DNAJB4*	12 *DNAJB4*	13 *DNAJB4*	14 *DNAJB4*
15 *DNAJB4*	16 *DNAJB4*	17 *DNAJB4*	18 *DNAJB4*	19 *DNAJB4*	20 *DNAJB4*	21 *DNAJB4*	22 *DNAJB4*	23 *DNAJB4*	24 *DNAJB4*	25 *DNAJB4*	26 *DNAJB4*
27 *DNAJB4*	28 *DNAJB4*	29 *DNAJB4*	30 *DNAJB4*	31 *DNAJB4*	32 *DNAJB4*	33 *DNAJB4*	34 *DNAJB4*	35 *DNAJB4*	36 *DNAJB4*	37 *DNAJB4*	38 *DNAJB4*
39 *DNAJB4*	40 *DNAJB4*	41 *DNAJB4*	42 *DNAJB4*	43 *DNAJB4*	44 *DNAJB4*	45 *DNAJB4*	46 *DNAJB4*	47 *DNAJB4*	48 *DNAJB4*	NTC *DNAJB4*	
1 *IL-8*	4 *IL-8*	5 *IL-8*	6 *IL-8*	7 *IL-8*	8 *IL-8*	9 *IL-8*	10 *IL-8*	11 *IL-8*	12 *IL-8*	13 *IL-8*	14 *IL-8*
15 *IL-8*	16 *IL-8*	17 *IL-8*	18 *IL-8*	19 *IL-8*	20 *IL-8*	21 *IL-8*	22 *IL-8*	23 *IL-8*	24 *IL-8*	25 *IL-8*	26 *IL-8*
27 *IL-8*	28 *IL-8*	29 *IL-8*	30 *IL-8*	31 *IL-8*	32 *IL-8*	33 *IL-8*	34 *IL-8*	35 *IL-8*	36 *IL-8*	37 *IL-8*	38 *IL-8*
39 *IL-8*	40 *IL-8*	41 *IL-8*	42 *IL-8*	43 *IL-8*	44 *IL-8*	45 *IL-8*	46 *IL-8*	47 *IL-8*	48 *IL-8*	NTC *IL-8*	

Plate #3

1 *GAPDH*	4 *GAPDH*	5 *GAPDH*	6 *GAPDH*	7 *GAPDH*	8 *GAPDH*	9 *GAPDH*	10 *GAPDH*	11 *GAPDH*	12 *GAPDH*	13 *GAPDH*	14 *GAPDH*
15 *GAPDH*	16 *GAPDH*	17 *GAPDH*	18 *GAPDH*	19 *GAPDH*	20 *GAPDH*	21 *GAPDH*	22 *GAPDH*	23 *GAPDH*	24 *GAPDH*	25 *GAPDH*	26 *GAPDH*
27 *GAPDH*	28 *GAPDH*	29 *GAPDH*	30 *GAPDH*	31 *GAPDH*	32 *GAPDH*	33 *GAPDH*	34 *GAPDH*	35 *GAPDH*	36 *GAPDH*	37 *GAPDH*	38 *GAPDH*
39 *GAPDH*	40 *GAPDH*	41 *GAPDH*	42 *GAPDH*	43 *GAPDH*	44 *GAPDH*	45 *GAPDH*	46 *GAPDH*	47 *GAPDH*	48 *GAPDH*	NTC *GAPDH*	

Figure 4: PCR plate layout (assumes PCR will be performed on 48 tissues of the 60 used in the experiment). Each tissue number corresponds to the number shown in the example plate layout (Section 6.2, Figure 1).

7. Data Analysis

For data analysis use the file “Epi2SensA_Template.xls”.

CALCULATION OF FOLD INDUCTION

Relative gene expression levels versus control (fold induction) are calculated using the $2^{-\Delta\Delta Ct}$ method described below.

- Calculate the ΔCt value by subtracting the Ct value of *GAPDH* from the Ct value of the respective marker genes.
- Calculate the mean ΔCt value of the vehicle control.

- Calculate the ΔΔCt value by subtracting the mean ΔCt value of the vehicle control from the ΔCt value of the test chemical or positive control.
- Calculate the fold induction by $2^{-\Delta\Delta Ct}$.

Examples of the fold induction calculation focusing on one marker gene (*ATF3*)

Sample	Conc. (w/v%)	Ct value		ΔCt value	ΔΔCt value	Fold induction
		ATF3	*GAPDH*	*ATF3*	*ATF3*	*ATF3*
AOO (vecicle of clotrimazole)		31.24	18.87	12.37		
		30.96	18.92	12.03		
		31.33	19.18	12.15		
		Mean ΔCt value of vehicle control ⇒		12.18		
Clotrimazole	0.78	25.59	19.59	6.00	-6.19	72.8
		24.50	19.22	5.27	-6.91	120.1
		25.21	19.21	5.99	-6.19	73.0

- Examples of a fold induction calculation sheet (all marker genes)

Sample	Conc. (w/v%)	Ct value					ΔCt value				ΔΔCt value				Fold induction			
		ATF3	*GCLM*	*DNAJB4*	*IL8*	*GAPDH*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*
AOO		31.24	25.79	26.71	27.58	18.87	12.37	6.92	7.83	8.70								
		30.96	25.97	26.69	27.73	18.92	12.03	7.04	7.77	8.81								
		31.33	26.23	26.97	28.12	19.18	12.15	7.05	7.80	8.95								
		Mean ΔCt value of vehicle control ⇒					12.18	7.00	7.80	8.82								
Clotrimazole	0.78	25.59	26.12	26.14	21.83	19.59	6.00	6.53	6.54	2.24	-6.19	-0.47	-1.26	-6.58	72.8	1.4	2.4	95.8
		24.50	26.29	24.31	22.15	19.22	5.27	7.07	5.08	2.92	-6.91	0.06	-2.72	-5.90	120.1	1.0	6.6	59.6
		25.21	26.23	25.50	23.54	19.21	5.99	7.02	6.29	4.33	-6.19	0.01	-1.51	-4.49	73.0	1.0	2.9	22.5
4NBB	0.10	24.90	23.80	24.32	24.82	18.93	5.97	4.87	5.39	5.89	-6.22	-2.14	-2.41	-2.93	74.4	4.4	5.3	7.6
		23.72	24.78	24.93	24.14	19.24	4.48	5.54	5.69	4.90	-7.70	-1.46	-2.11	-3.92	208.2	2.8	4.3	15.1
		25.60	24.42	25.36	24.45	19.35	6.25	5.06	6.01	5.09	-5.94	-1.94	-1.79	-3.73	61.2	3.8	3.5	13.2
Vehicle of sample A		31.92	27.11	27.02	27.22	18.05	13.88	9.07	8.98	9.17								
		32.06	26.86	27.22	27.43	17.96	14.10	8.90	9.26	9.47								
		32.43	27.26	27.36	27.51	18.08	14.34	9.17	9.28	9.42								
		Mean ΔCt value of vehicle control ⇒					14.11	9.05	9.17	9.36								
Sample A	0.78	32.61	27.30	27.55	28.19	18.59	14.02	8.71	8.96	9.59	-0.09	-0.33	-0.21	0.24	1.1	1.3	1.2	0.8
		31.49	27.05	27.22	27.16	18.05	13.43	9.00	9.17	9.10	-0.67	-0.05	0.00	-0.25	1.6	1.0	1.0	1.2
		31.01	26.91	27.07	26.37	17.97	13.04	8.93	9.10	8.40	-1.07	-0.11	-0.07	-0.96	2.1	1.1	1.0	1.9
	1.56	31.75	26.83	27.14	27.28	18.14	13.61	8.69	9.00	9.15	-0.50	-0.36	-0.17	-0.21	1.4	1.3	1.1	1.2
		32.24	26.83	27.12	27.10	18.18	14.06	8.64	8.93	8.91	-0.05	-0.40	-0.24	-0.44	1.0	1.3	1.2	1.4
		31.67	26.40	26.88	26.77	17.96	13.71	8.44	8.92	8.81	-0.40	-0.60	-0.25	-0.54	1.3	1.5	1.2	1.5
	3.13	27.27	27.40	26.96	26.07	18.23	9.04	9.16	8.73	7.83	-5.06	0.12	-0.44	-1.52	33.4	0.9	1.4	2.9
		28.04	27.42	27.01	26.41	18.15	9.89	9.27	8.86	8.26	-4.21	0.22	-0.31	-1.10	18.5	0.9	1.2	2.1
		28.74	27.57	27.02	26.87	18.48	10.26	9.09	8.54	8.39	-3.84	0.04	-0.63	-0.97	14.3	1.0	1.5	2.0
	6.25	25.94	24.88	23.38	23.16	18.49	7.45	6.39	4.89	4.67	-6.66	-2.66	-4.28	-4.69	100.9	6.3	19.5	25.8
		25.53	24.91	23.04	22.03	18.51	7.02	6.40	4.53	3.52	-7.08	-2.64	-4.64	-5.84	135.6	6.2	25.0	57.2
		25.33	24.98	22.78	22.28	18.70	6.64	6.28	4.08	3.58	-7.47	-2.76	-5.09	-5.77	177.3	6.8	34.0	54.8
Sample	Conc. (w/v%)	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*GAPDH*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*

of *epidermis* corresponds to the number shown in the example 24-well *epidermis* plate layout of the main study.

Red highlight indicates the fold induction that exceeds the respective cut-off value for the marker genes.

8. Acceptance criteria

The following acceptance criteria should be met for a run to be considered valid:

- The tissue viability of at least two tissue units of the vehicle controls should be ≥ 90%. If the tissue viability of only one vehicle control is < 90%, the Ct values obtained from the remaining two tissue units should be used.
- The mean tissue viability of both positive controls (i.e. 1.56% [w/v] clotrimazole and 0.10% [w/v] 4-nitrobenzyl bromide (4-NBB)) should be ≥ 80%.
- In the 1.56% (w/v) clotrimazole positive control, the mean fold-induction values for ATF3 and IL-8 should exceed the cut-off value (i.e. the ATF3 fold-induction value should be > 15, and the IL-8 fold-induction value should be > 4).
- In the 0.10% (w/v) 4NBB positive control, the mean fold-induction values for GCLM and DNAJB4 should exceed the cut-off value (i.e. the GCLM fold-induction value should be > 2, and the DNAJB4 fold-induction value should be > 2).

The following acceptance criteria should be met in order to consider a tested concentration's result valid:

- The result of at least one tested concentration that shows ≥ 60% mean tissue viability should be used. If the mean tissue viability is < 60% for a given tested concentration, the result for that tested concentration should be excluded for a positive prediction but can be used for a negative prediction.
- When the mean GAPDH Ct value for a given test chemical concentration is within ±1 of the mean GAPDH Ct value of the corresponding vehicle control, the result obtained at that concentration is acceptable.

9. Prediction Model

Each test chemical is evaluated in one run to derive a prediction (positive or negative).

An Epi2SensA prediction is considered positive if at least two of the following conditions is met:

- The Imax for ATF3 is > 15 for at least one tested concentration.
- The Imax for GCLM is > 2 for at least one tested concentration.
- The Imax for DNAJB4 is > 2 for at least one tested concentration.
- The Imax for IL-8 is > 4 for at least one tested concentration.

The Epi2SensA prediction is considered negative if the three conditions below are met:

- The mean fold-induction value of the marker genes does not exceed the respective cut-off values for any of the four genes at the tested concentrations that shows ≥ 60% mean tissue viability;
- At least 3 concentrations were tested;
- If the highest concentration tested was lower than the highest soluble or stably dispersed concentration, at least one mean tissue viability at the tested concentrations is < 60%.

Note:

- In rare cases, there are some test chemicals that show steep or greatly fluctuating dose response curves for tissue viability and/or fold induction, and the fold induction exceeds the cut-off value just at the lowest concentration with less than 60% mean tissue viability. Such test chemicals should be retested with a narrower dose-response analysis using a lower dilution factor (e.g. $\sqrt{2}$ (=1.41) fold dilution), to determine whether induction has occurred at non-cytotoxic levels (mean tissue viability ≥60%).

- If the GAPDH Ct value does not meet the acceptance criteria at the highest concentration with ≥60% mean tissue viability and the fold induction does not exceed respective cut-off values at the lower concentrations, the test chemical should be retested.

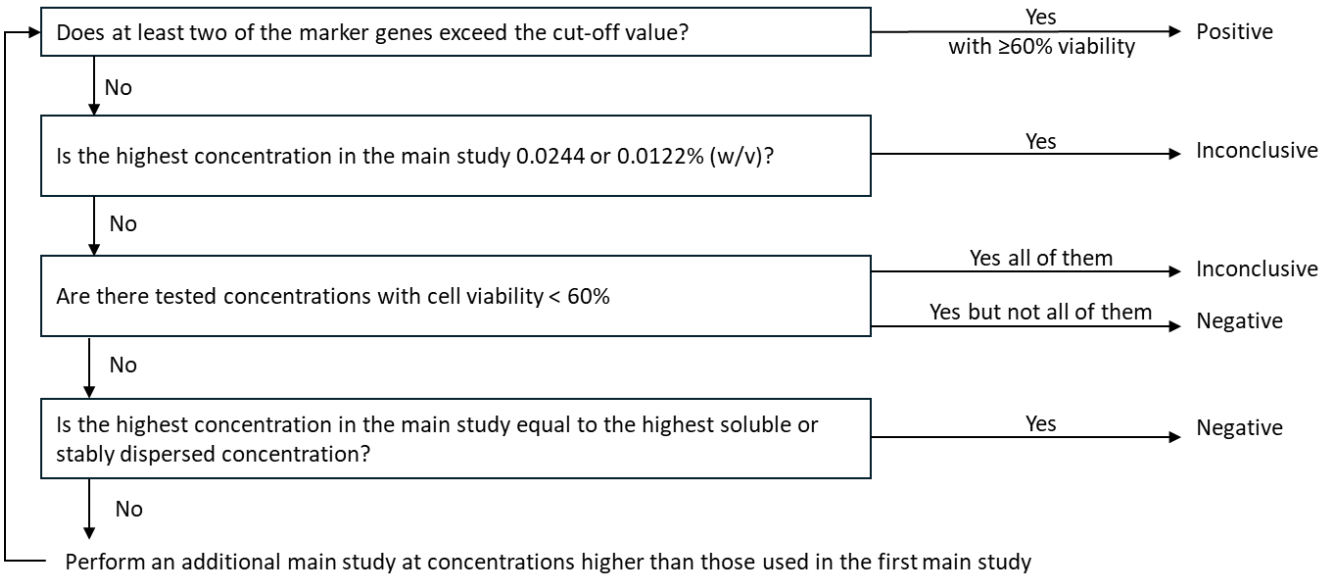

Figure 5. Flow-chart of the Epi^{2}SensA prediction model.

ANNEX A – PROCEDURE OUTLINE

Note: This outline assumes that 60 tissues will be used for the assay. This allows testing of one VC, 2 PCs, the KC, and 4 TAs (tested at 4 concentrations, n=3 tissues/concentration).

Tuesday

1. Unpackage EpiDerm tissues (Part # EPI-200-SENSA) in a sterile hood. Utilize sterilize technique throughout until the tissues are placed in RNAlater.
2. Place tissues in 0.9 mL room temperature assay medium (EPI-200-ASY) in 6-well plates (1 tissue per well).
3. Incubate tissues at 37±1°C/5±1%CO_2/90±10%RH for 1 hour.
4. At the end of 1hr incubation, aspirate medium and replace with 0.9 mL of EPI-200-ASY pre-warmed (for 15 min in a 37°C water batch). Pre-incubate tissues at 37±1°C/5±1%CO_2/90±10%RH for 18-24 hours.

Wednesday

5. Prepare Vehicles, Positive Controls in AOO and test chemicals in respective vehicles. Prepare test chemicals at 4 concentrations.
6. At the end of 18-24 hours pre-incubation, replace medium with 0.9 mL pre-warmed (37°C water bath) assay medium. Pipette 0.9 mL pre-warmed medium into 2 wells of an empty 6-well plate and label the plate as Blank.
7. Label the tissue containing plates with the treatment conditions. Assigning n=1 tissue for untreated control, n=2 tissues for killed control and n=3 tissues for positive controls and each test chemical treatment.
8. Remove 50 µL of culture medium from each well designated for the kill control tissues.
9. Pipette 50µL of 10% Triton-X 100 into the medium of the kill control tissues. Mix the medium by gently shaking the plate. Incubate these tissues for 6hr at 37±1°C/5±1%CO_2/90±10%RH. Incubate the Blank media plate for 6hr as well.
10. Dose tissues (for example at 9:30 AM) with 10µL of vehicle control, positive control and each concentration of the test materials at an interval of 1 minute between each tissue.
11. Once 30 tissues are dosed (10:00 AM), quickly place the 6-well plate containing the tissues into the incubator at 37±1°C/5±1%CO_2/90±10%RH for remainder of 1hr (i.e., 30 minutes)
12. Continue dosing (10:01:AM) the remainder of the 30 tissues similarly at 1-minute intervals. Incubate the tissues at 37±1°C/5±1%CO_2/90±10%RH for remainder of 1hr (i.e., 30 minutes).
13. Immediately (10:30 AM) start rinsing the first batch of the 30 tissues with 0.5mL DPBS, rinsing the tissues 15 times using a repeat pipettor. After the last rinse, blot dry the bottom of the insert on a sterile gauze and place the tissue back in the same previous well of the 6-well plate. Once all 30 tissues are rinsed, place the tissues at 37±1°C/5±1%CO_2/90±10%RH and incubate (11:00 AM) for remainder of 6 hrs (i.e., 4.5hr)
14. Immediately (11:01 AM) start rinsing the second set of 30 tissues with 0.5mL DPBS, rinsing the tissues 15 times using a repeat pipettor. After the last rinse, blot dry the bottom of the insert on a sterile gauze and place the tissue back in the same previous well of the 6-well plate. Once all 30 tissues are rinsed, place the tissues at 37±1°C/5±1%CO_2/90±10%RH and incubate (11:30 AM) for remainder of 6 hrs (i.e., 4.0hr)
15. Prior to the end of the 5 hr post-incubation, fill the wells of 2.5 x 24-well plates with 0.5 mL of RNAlater.
16. At the end of a total of 6hr (3:30 PM) for the first tissues dosed, take the first set of 30 tissues out of the incubator, remove the inserts from the 6-well plate, and place them into the wells of a 24-well plate. After the tissues are transferred, add another 0.5 mL of RNAlater into the tissues inserts. DO NOT DISCARD the medium in the 6-well plates. It is needed for the LDH assay.
17. Seal the plate 24-well plate with parafilm and place in 2-8°C refrigerator.
18. Once the first set of 30 tissues are harvested, take the second set of 30 tissues out of the

incubator (4:00 PM) and repeat the procedure outlined in above 2 steps.

19. Once all the tissues have been harvested, store the 24-well plates overnight at 2-8ºC. Tissues can be stored for up to 1 week in RNAlater at 2-8ºC. If longer storage times are necessary prior to lysis of the tissue and RNA isolation (step 24), then move the plates to a -20±5ºC freezer.
20. Pipet duplicate 50µL aliquots of the spent culture media (and Blank media) from each well of the 6-well plates into a 96 well plate following the plate layout provided in Figure 2 of this protocol. These media aliquots will be used for LDH assay.
21. Seal the 96-well plate with Parafilm and save overnight at 2-8ºC.

Thursday

22. Remove the 96-well media plate (step 21) from the refrigerator and place on a work bench for 15-30 minutes.
23. Follow the procedure outlined for the LDH Assay (Section 6.3).
24. Remove the 24-well containing the tissues in RNAlater from the refrigerator and continue with the following (steps likely to occur over a multi-day period):
    - lysis of epidermis (Section 6.5)
    - RNA isolation (Section 6.6)_
    - RNA concentration measurement (Section 6.6)_
    - genomic DNA elimination (Section 6.7)
    - cDNA synthesis (Section 6.7)
    - Real Time PCR (Section 6.8)

ANNEX B – DOSE FINDING STUDY

- **Preparation:**

- Prepare the test chemical solution at the highest concentration in the appropriate vehicle in a small glass vial (e.g. when preparing 1 mL of 12.5 w/v% solution, add 0.875 mL of the vehicle to 0.125 g of test chemical).
- Prepare working solutions of each test chemical as 4-fold serial dilutions from the highest concentration down to concentrations of 0.024 w/v% or below in the appropriate vehicle (e.g. 6.25, 1.56, 0.39, 0.098, 0.024 w/v%).
- If the test chemical is a liquid, perform the serial dilution from 100% (i.e. 100%, 25, 6.25, 1.56, 0.39, 0.098, 0.024 w/v%).

- Topical applications (1-hour treatment/rinsing/ 5-hour post-incubation):

- These steps should be performed under sterile conditions.
- For 15 minutes, pre-warm the supplied assay medium in a water bath at 37°C.
- Make a plate layout as "Examples of plate layout". If a test chemical is liquid at 100% (neat) at room temperature, separate the tissue units which are used for the liquid test chemical from other test chemicals and controls (e.g. positive controls and vehicle controls) into individual 6-well plates.
- Fill each well of a new 6-well plate with 0.9 mL of the medium and transfer one pre-cultured tissue insert into each of the medium-filled wells using sterile forceps.
- Apply an aliquot (10 µL) of working solution to the center of each tissue using a positive displacement pipette. Use N=2 tissues for each working solution.
- Prepare one tissue unit for the non-treated control and two tissue units for the killed control (treated with 50 µL of 10 w/v% Triton X-100) for the tissue viability measurement.
- Incubate the treated tissues for 1 hour at 37±1°C, 5±1% CO_2, 90±10% RH. After 1 hour, rinse the tissues 15X by pipetting 0.5 mL of DPBS onto the tissue surface. Use a repeater pipette adjusted for 0.5 mL volume. After the 15 rinses, remove any excess DPBS by gently shaking the insert, blot the insert on sterile blotting paper, and put the insert back into in the same medium for post-exposure of 5h. Return the 6-well plates to the incubator (37±1°C, 5±1% CO2, 90±10% RH) for the 5h ± 5 min post-exposure.

Caution:

- Cross-contamination by volatile chemicals should be avoided by using separate 6-well plates for each test chemical.

ANNEX C - FIXED EQUIPMENT

- Sterile hood for cell culture work
- CO_2 incubator (37±1°C, 5±1% CO2, 90±10% humidity)
- Micro-pipettes and tips
- Multi-step pipette and combitips (e.g. Multipette M4, eppendorf)
- Positive displacement pipette and tips for volumes of 10 µL, preferred model is the Microman M10 (Gilson)
- Precision tweezers
- Vortex mixer
- 96-well plate absorbance reader equipped for reading at 490 (or 492) nm and ≥600 nm
- Microtube centrifuge
- Compact centrifuge (applicable to 1.5 mL tube and 0.2 mL PCR tube)
- Plate centrifuge (applicable to 96-well PCR plate)
- Spectral photometer for measurement of RNA concentration, preferred model is the NanoDrop (Thermo Scientific)
- Thermal cycler for cDNA synthesis
- Real-time PCR system, preferred model is the 7500 Fast Real-Time PCR System (Applied Biosystems)
- Other PCR systems that have been used successfully with this protocol:
  CFX Connect Real-Time PCR Detection System (Bio-Rad laboratories)
  ABI PRISM 7900HT machine (Applied Biosystems)
  QuantStudio 3/5 Real-Time PCR System (Applied Biosystems)

ANNEX D: FIXED REAGENTS, CHEMICALS, MATERIALS

	Product	**Company**	**Catalog Number**
Probe, Primer	RT² qPCR Primer Assay	Qiagen	ATF3: PPH00408C-200 GCLM: PPH02099A-200 DNAJB4: PPH01196E-200 CXCL8 (IL-8): PPH00568A-200 GAPDH: PPH00150F-200
PCR master mix	RT² SYBR Green qPCR Mastermix (12)	Qiagen	330502

ANNEX E: CONTENTS OF EPI-200-SENSA KIT (MATTEK CORPORATION)

Item	**Quantity**
EPI-200 Tissue Plate	1
6 WELL PLATE	5
24 WELL PLATE	1
PBS, BOTTLE 125 ML	2
EPI-100-ASY, ASSAY MEDIUM, BOTTLE 100 ML	1
10% TRITON X-100, CONICAL 1 ML	1
EPI2SENSA PROTOCOL	1

ANNEX F: REAGENTS, CHEMICALS, MATERIALS AND OTHER ITEMS

Below are listed the reagents, chemicals, materials and other items used for the routine testing. For most, products from other manufactures will work equally.

	Product	Company	Catalog Number
AOO	Acetone : Olive oil = 4 : 1 v/v Acetone Olive oil	 Sigma	 270725 O1514
DW	Distilled water or 18 MOhm water	Otsuka Pharma Co. Sigma	 6442-85
EtOH	Ethanol, >99.5%	Kanto Chemical Co. Millipore Sigma	14033-00 **459844**
LDH assay kit	Cytotoxicity Detection Kit (LDH)	Sigma	11644793001
1 mol/L HCl	1 mol/L Hydrochloric acid (1N)	Kanto Chemical Co. Millipore Sigma	18591-08 1090571000
Hand-held battery-operated homogenizer	VWR Pellet Mixer	VWR	North American Cat.No. 47747-370 European Article No. 431-0100
Disposable Pestle	VWR Disposable Pestle	VWR	North American Cat. # 47747-358 European Cat # 431-0094
RNA extraction kit	RNAqueous® Total RNA Isolation Kit	ThermoFisher Scientific	AM1912
cDNA synthesis kit	RT2 First Strand Kit (50)	Qiagen	330404
RNAlater Solution	RNAlater Solution	Invitrogen	AM7021
96-well plate	Multi-well culture plate, 96- well, flat bottom, non-treated	BD Falcon	351172
1.5 mL tube	Safe-Lock Tubes	Eppendorf	022363212
PCR plate	Hard-Shell PCR Plates 96-well, thin-well	BIO-RAD	HSP9601
Plate sealer	Microseal ‘C’ Film (for media plate, genomic DNA elimination and cDNA synthesis)	BIO-RAD	MSC-1001
	Microseal ‘B’ seal (for RT-PCR)	BIO-RAD	MSB1001
Positive controls	Clotrimazole, >98.0% CAS No. 23593-75-1 4-Nitrobenzyl bromide, >98.0% CAS No. 100-11-8	Sigma-Aldrich Sigma-Aldrich	C6019 N13054
Nylon Mesh	25 pieces, Nylon mesh circles, 8 mm diameter, 200 µm pores, used for spreading test chemicals dissolved in water	MatTek Corporation	EPI-MESH