

**Unclassified**

**ENV/JM/MONO(2011)44**

Organisation de Coopération et de Développement Économiques  
Organisation for Economic Co-operation and Development

**07-Dec-2011**

**English - Or. English**

**ENVIRONMENT DIRECTORATE  
JOINT MEETING OF THE CHEMICALS COMMITTEE AND  
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

**PEER REVIEW REPORT OF THE VALIDATION OF THE SKIN IRRITATION TEST USING  
LABCYTE EPI-MODEL 24**

**Series on Testing and Assessment**

**No. 155**

**JT03312996**

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**ENV/JM/MONO(2011)44  
Unclassified**

**English - Or. English**



**OECD Environment, Health and Safety Publications**  
Series on Testing and Assessment

**No. 155**

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Paris 2011

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The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides and Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site ([www.oecd.org/ehs/](http://www.oecd.org/ehs/)).

*This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.*

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

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## FOREWORD

This document presents the Peer Review Report of the validation of the “*Skin Irritation Test using LabCyte EPI-MODEL24*”, as a “*me-too*” development following the Performance Standards of Test Guideline 439: *In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method*, including four annexes. Additional information was generated after the peer review because the first validation study was on-going when OECD experts were discussing draft Performance Standards (PS) for TG 439.

The LabCyte test method has been considered as similar to the Reconstructed Human Epidermis Test Method that is the basis of TG 439, and has therefore been subjected to an additional validation study: a “*me-too*” validation study based on the PS of TG 439. This document also presents:

- Appendix 1: JaCVAM Re-analysis of the initial validation data according to the Performance Standards of TG 439
- Appendix 2: Background Document for a modified skin irritation test using LabCyte EPI-MODEL24
- Appendix 3: Standard Operating Procedure for the Skin irritation test using the LabCyte EPI-MODEL24 for the additional validation study
- Appendix 4: additional validation study: “*me-too*” validation study according to peer review report.

Finally, the report includes the agreement of the Working Group of National Coordinators of the Test Guidelines Programme (WNT) on the follow-up to the peer review.

The project for developing a Test Guideline for the in vitro epidermal model LabCyte EPIMODEL24 to assess skin irritation was proposed by Japan and included in the work plan of the Test Guidelines Programme in 2009. A validation report was published as No. 147 in the Series on Testing and Assessment. An OECD Peer Review was performed in December 2009 and the final Peer Review Panel (PRP) Report was available on 17<sup>th</sup> of March 2010.

This document was endorsed by the WNT at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) agreed to its declassification on 5 October 2011.

This document is published under the responsibility of the Joint Meeting.

**Agreement of the Working Group of National Coordinators of the Test Guidelines Programme on the follow-up to the Peer Review Report**

The peer review report of the validation of the modified skin irritation test method using the LabCyte EPI-Model24 as a TG 439 “me-too” test was submitted to the Working Group of National Coordinators of the Test Guidelines Programme (WNT) at its meeting held on 12-14 April 2011, for endorsement.

Considering

- the issues raised in the peer review report regarding the performance of the assay including within and between laboratory reproducibility, variability between replicates and most notably, the misclassification of the strong irritant reference chemical, 1-bromohexane,
- Additional information provided by Japan to address the issues raised in the peer review report,

The WNT agreed that before adding the “me too test” to TG 439, the Expert Group on Skin Irritation and Corrosion should review all existing data of the LabCyte test method, and submit a recommendation for WNT approval of the test method as a “me-too” test method to TG 439, or for further work if necessary. In case the Expert Group agrees on that the LabCyte test method is validated and should be added to TG 439, this approval would be requested from the WNT by a written procedure.

**SUMMARY REPORT OF THE PEER REVIEW PANEL  
ON LABCYTE EPI-MODEL 24 IN VITRO TEST METHOD  
FOR THE ASSESSMENT OF SKIN IRRITATION POTENTIAL OF CHEMICALS**

**prepared for**

**Environment Directorate  
Organisation for Economic Co-operation and Development Paris**

**March 17, 2010**

## PREAMBLE

This document presents the summary report of the assessment by an independent Peer Review Panel on the LabCyte EPI-MODEL 24 in vitro test method for the assessment of skin irritation potential of chemicals.

Until today, for regulatory purposes the skin irritation potential of chemicals has been investigated by the Draize skin test. In this in vivo experiment, the chemical is applied for usually four hours to the skin of rabbits. Based on a scoring of the two endpoints erythema and oedema formation and the reversibility of such effects, the skin irritation potential is assessed.

However, the advancement of in vitro methods has brought forward reconstructed human epidermis models. These models are based on non-transformed human-derived epidermal keratinocytes. The keratinocytes are cultured to form a multilayered, highly differentiated model of the human *epidermis*. After topical exposure to a neat test chemical, cell viability is measured by the amount MTT [(3-(4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazoliumbromide)] being reduced by dehydrogenase. The relative reduction of viability is then related to the skin irritation potential of the chemical.

After several years of evaluation and optimisation, two models underwent formal validation by the European Centre for the Validation of Alternative Methods (ECVAM) resulting in a statement on the scientific validity of the tests by ECVAM Scientific Advisory Board (ESAC). As a consequence, the Organisation for Economic Co-operation and Development (OECD) is currently in the process of drafting the respective OECD test guideline 'In Vitro Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method'.

Anticipating that in future further reconstructed human epidermis models will be developed, the draft test guideline includes the annex 'Performance standards for assessment of proposed similar or modified in vitro reconstructed human epidermis (RhE) test methods for skin irritation'. These performance standards can be used to assess other analogous test methods (colloquially referred to as "me-too" tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect.

The LabCyte EPI-MODEL 24 in vitro test method is considered to potentially be such a test method, to which the performance standards apply. The method was validated in a study employing several Japanese laboratories. The documentation of this study together with some additional information has been provided to the OECD and constitutes the basis for this peer review.

**Summary Report of the Peer Review Panel  
on the LabCyte EPI-MODEL 24 in vitro test method for the assessment of skin irritation potential of  
chemicals**

**The peer review process**

1. The Peer Review Panel (Panel) was constituted in December 2009, to provide an independent review of the validation of the LabCyte EPI-MODEL 24 in vitro test method for the assessment of skin irritation potential of chemicals. The test method has been considered as a similar Reconstructed Human Epidermis (RhE) Test Method and underwent therefore a validation study referring to the respective performance standards. In this respect it has to be noted that the validation of the LabCyte EPI-MODEL 24 took place while the OECD test guideline 'In Vitro Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method' including the annex on performance standards was still in the drafting process. The work of the Panel was coordinated by a panel chair contracted by the OECD. In addition to experts invited by the Secretariat, potential Panel members were nominated by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) and then approached by the chair. From the originally five assigned panel members, one resigned from the assignment in January 2010, but was immediately replaced. The members of the Panel are listed in Annex 1.

Furthermore, an independent contact person who is familiar with the LabCyte EPI-MODEL 24 was nominated to support the panel in case of open issues needing clarification.

2. The Panel was asked to evaluate the data collected on the test method, and to answer specific charge questions. These questions were proposed by the panel chair and agreed by the (OECD). Panel members were asked to base their review on nine documents, which have been provided by the OECD. These documents contained information relevant to the validation of the LabCyte EPI-MODEL 24.

As background information, they were also provided with the three following documents:

- OECD Guidance Document on the Validation and International Acceptance of New or Updated Methods for Hazard Assessment, Series on Testing and Assessment, Number 34, 2005  
(last access on March 08, 2010, under [http://www.olis.oecd.org/olis/2005doc.nsf/LinkTo/NT00002EAE/\\$FILE/JT00188291.pdf](http://www.olis.oecd.org/olis/2005doc.nsf/LinkTo/NT00002EAE/$FILE/JT00188291.pdf))
- OECD GUIDELINE FOR THE TESTING OF CHEMICALS (DRAFT PROPOSAL FOR A NEW GUIDELINE) - *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* (RhE) Test Method  
(last access on March 08, 2010, under <http://www.oecd.org/dataoecd/1/59/43664841.pdf>)
- European Commission: Explanatory Background Document to the OECD Draft Test Guideline on in vitro Skin Irritation Testing  
(last access on March 08, 2010, under [http://ecvam.jrc.ec.europa.eu/ft\\_doc/ECVAM-BfR-OECDv3.0\\_OECD\\_post\\_ECM2c.pdf](http://ecvam.jrc.ec.europa.eu/ft_doc/ECVAM-BfR-OECDv3.0_OECD_post_ECM2c.pdf))

Two further documents, one describing the content of the review documents, and the other providing a complete version of Table 16 of the file 'D3 LabCyte\_skin\_irritation\_testprotocol\_ver\_7.2 NC', were added by the PRP chair to facilitate the review. All documents, identified by their file names as provided to the PRP, are listed in Annex 2.

The charge to the Panel was to assess to what extent the eight OECD validation criteria set out in the OECD Guidance Document had been met and to evaluate whether the test method complies with the performance standards of the draft OECD Test Guideline on Skin Irritation. It has to be noted that these

two sets of charge questions are not to be considered independent as they address similar aspects. All eleven charge questions are listed in Annex 3. A summary of the Panel's responses to the individual questions is presented in paragraphs 4 to 40 below. For transparency, the individual comments from the Panel members are provided in Annex 4.

3. During the evaluation process, the Panel held two teleconferences which were organised and coordinated by the chair. Subsequently, each Panel member provided written responses on the charge questions to the PRP chair by February 15, 2010. Based on these responses, a draft report was compiled by the chair and provided to the PRP for review and comments (February 22, 2010). In this draft all aspects raised by at least one reviewer were included. Furthermore, it contained some clarifications on specific issues added by the chair. The PRP commented on the draft report until March 02, 2010. Accounting for this feedback and resolving remaining open issues, the final report was drafted by the chair and send to the PRP for approval on March 09, 2010. This report presents the resulting approved responses of the Panel to each of the charge questions.

### **General Panel responses**

4. The Panel stated that the presentation of the review information could have been more structured and focused, e.g. by referencing to the performance standards and/or to the charge questions. Therefore, it proposes to facilitate future reviews by aligning the presentation of information with the review charge questions.

All information in the review documents on IL-1 $\alpha$  has not been considered by the Panel because this endpoint has not been previously declared as formally validated and there are no performance standards available

### **Panel responses to the charge questions - part I: The eight OECD principles and criteria for test method validation**

5. The PRP reached consensus regarding the charge questions relating to the eight OECD principles and criteria for test method validation.

#### ***Charge question 1: A rationale for the test method should be available, including a clear statement of scientific need and regulatory purpose.***

6. The Panel agreed that this criterion had been fully met. The rationale for the test method is clearly stated with regard to the scientific basis and regulatory purpose. In addition, as the LabCyte EPI-MODEL 24 refers to performance standards of presumably similar methods, which are in the process of OECD adoption, the general need and regulatory purpose as defined in the draft guideline applies as well.

#### ***Charge question 2: The relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest should be addressed, describing limitations of the test methods.***

7. The Panel agreed that this criterion had been partly met.

8. In particular, it has not been met with regard to the description of limitations. In the provided SOP this topic is only touched, but not sufficiently covered. For example, substances sticking to the tissue might create problems. Respective information might be deductible from the literature of the validated test methods, e.g. from paragraph 7 of the respective OECD draft test guideline.

9. Although, in general acceptable, the relationship of the method endpoint and the biological effect could be described in more detail. As a potential me-too method this relationship has been described earlier for the already validated methods and does thus apply also to the LabCyte EPI-MODEL 24. However, addition of information as given in paragraph 11 of the OECD Draft guideline (Version 7.6) would be helpful.

***Charge question 3: A detailed protocol for the test method should be available***

10. The Panel agreed that this criterion had been partly met.

11. In particular, a measure of variability between tissue replicates has not been defined.

12. Furthermore, as already addressed under paragraph 8, limitations are not sufficiently covered.

13. Particular protocol changes have been proposed by the panel. The most important suggestion was to explain the prediction model in more detail. Minor suggestions were

- to better define the storage temperature of the MTT solution and the positive control substance
- to better and more objectively define changes considered significant for chemicals that stain the tissue
- to change the instruction ‘...must be repeated three times...’ to ‘...must be performed three times in total...’
- to include instructions for the case, when only two tissues provide measurements, e.g. when one tissue is damaged

***Charge question 4: Within- and between-laboratory reproducibility of the test method should be demonstrated***

14. The Panel agreed that this criterion had been partly met.

15. Most importantly, within- and between-laboratory reproducibility have not been calculated. All required data for the calculation have been provided, but appropriate statistical analysis and documentation is missing and should be included. In particular, these should be addressed by an appropriate measure of variability between runs viabilities, e.g. the standard deviation, and the concordance of classifications based on run. Results should be discussed, especially with regard to less reproducible chemicals

16. Regarding intra-assay reproducibility, a measure of variability between tissues has not been defined, nor calculated (see also paragraph 11.)

***Charge question 5: Demonstration of the test method’s performance should be based on testing of representative, preferably coded reference chemicals***

17. The Panel agreed that this criterion had been met.

18. All chemicals currently proposed by the OECD draft test guideline have been tested, with one acceptable exception, in a coded manner.

***Charge question 6: The performance of test methods should have been evaluated in relation to existing relevant toxicity data as well as information from the relevant target species.***

19. The Panel agreed that this criterion had been met.

20. As only chemicals which have been included in other validation activities leading to the OECD draft test guideline, were tested, the relation to existing toxicity data from the relevant target species was established.

***Charge question 7: All data supporting the assessment of the validity of the test method should be available for expert review***

21. The Panel agreed that this criterion had been partly met.

22. As mentioned before, within- and between-laboratory reproducibility have not been calculated and documented (see paragraph 15).

23. Regarding intra-assay reproducibility, a measure of variability between tissues has not been defined, nor calculated (see also paragraph 11).

***Charge question 8: Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of Good Laboratory Practice (GLP)***

24. The Panel agreed that this criterion had been partly met. It has to be noted that this optional criterion initially led to different individual interpretation by the PRP members.

25. In general, the conduct of the study in the spirit of GLP is acceptable. For example, also in the original validation study, which led to the draft OECD test guideline, not all laboratories were GLP compliant. However, the panel agreed that the adherence to GLP principles could have been described in more detail. If available, documentation on this issue generated in the frame of the validation study of the LabCyte-EPIMODEL 24 should be provided.

#### **Panel responses to the charge questions - part II: Performance Standards of the draft Test Guideline on Skin Irritation**

26. The PRP reached consensus regarding the charge questions relating to the Performance Standards of the draft Test Guideline on Skin Irritation.

***Charge question 9: Adherence to the essential test method components should be demonstrated***

27. The Panel agreed that this criterion had been partly met. This charge question refers to the test method components as specified in the draft OECD test guideline. The respective components will be addressed individually.

28. Functional conditions – Viability: An upper acceptance limit for the negative control tissues, e.g. defined by a maximum optical density (OD), is missing. Although the data base for defining this might be small, it should nevertheless be defined if possible. Furthermore, it has not been defined what is considered as an acceptable variation of viabilities from replicate tissues.

29. Functional conditions – Barrier function: Further data on lipid composition could be added if available.

30. Functional conditions – Morphology: Acceptance criteria required for batch acceptance should be defined.

31. Functional conditions – Reproducibility: A measure of variability between tissues has not been defined, nor calculated (see also paragraph 11).

32. Functional conditions – Quality control (QC): It is not entirely evident how the model producer intends to ensure and demonstrate that each batch of the RhE model used meets defined production release criteria.

***Charge question 10: Reliability and accuracy should be demonstrated by using at least the recommended reference chemicals***

33. The Panel agreed that this criterion had been met.

***Charge question 11: The test should have been assessed based on the defined reliability and accuracy values***

34. The Panel agreed that this criterion had not been met. When addressing this question, the panel agreed to exclude data of ‘Lab e’ from analysis according to data ‘Rule 2’ of paragraph 14 of annex 2 of the OECD draft test guideline, which addresses the performance standards. Therefore, the data of ‘Lab e’ have not been considered.

35. Most importantly, the specific restriction of the performance standards that only two *in vivo* Category 2 substances, 1-decanol and di-n-propyl disulphide, may be misclassified as No Category by more than one participating laboratory, has not been met. In particular, 1-bromohexane, Category 2, has been misclassified as No Category by five of six participating laboratories (excluding ‘Lab e’ data). In addition, but without consequence, butyl methacrylate, Category 2, has been misclassified as No Category in one of six laboratories (excluding ‘Lab e’ data, which also misclassified butyl methacrylate). As a potential solution optimisation of the protocol either regarding the application time (prolongation beyond 30 minutes, with the risk of a decrease in specificity and overall accuracy) or regarding the test substance application (re-spreading of the material during application) has been proposed.

36. The panel agreed that the within- and between laboratory reproducibility has not been assessed with regard to the requirements of the performance standards. A detailed extended evaluation of these reproducibility values addressing the performance standards requirements should be presented (see also paragraph 15).

37. The panel anticipated that one result of a detailed analysis of the within- and between laboratory reproducibility would be that two laboratories might have a within-laboratory reproducibility of lower than 90%. This would not comply with the requirement of paragraph 14 of annex 2 of the OECD draft test guideline that ‘...within-laboratory variability should show a concordance of classifications (GHS Category 2/No Category) obtained in different, independent test runs of the 20 Reference Chemicals within one single laboratory equal or higher ( $\geq$ ) than 90%’.

38. In contrast to the performance standards, the median viability instead of the mean viability, as required in ‘Rule 2’ of paragraph 9 of annex 2 of the OECD draft test guideline, which addresses the performance standards, has been used for deriving a final classification for a complete run sequence of a given laboratory. The panel expects that this will result in only minor differences in results, but suggests to re-analyse the data and document the results accordingly.

39. Regarding the predictive values, the panel agreed that the requirements, i.e. sensitivity  $\geq$  80%, specificity  $\geq$  70% and accuracy  $\geq$  75%, were met, when considering all 25 tested chemicals. It was noted,

however, that the overall sensitivity would be  $\leq 80\%$  when considering only the 19 chemicals tested in the second phase of the study.

## Recommendations

40. The Panel agrees that this report provides a summary of their views on the status of the validation of the LabCyte EPI-MODEL 24 in vitro test method for the assessment of skin irritation potential of chemicals, as detailed in the responses to the questions posed to the Panel and based on the information related to the test method validation provided to the Panel.

41. The report of the Panel, along with the provided review documents on the LabCyte EPI-MODEL 24 should form the basis for decisions on whether the validation meets the OECD principles for validation and the performance standards of the draft OECD Test Guideline '*In Vitro* Skin Irritation: Reconstructed Human *Epidermis* (RhE) Test Method'. The Panel recommends that the OECD considers the Panel report as guidance for recommending additional work required to fully meet all OECD principles and performance standards.

42. Future work should focus especially on the following aspects. Most importantly, the issue of misclassifying 1-bromohexane should be resolved. Furthermore, an extensive analysis of the within- and between reproducibility referring to the performance standards of the draft OECD Test Guideline should be carried out and appropriately documented. It is also recommended to assess variability between replicate tissues and to define a respective acceptance criterion. In order to comply better with the performance standards, analyses using the mean instead of the median for deriving a final classification for a complete run sequence of a given laboratory should be carried out. Finally, appropriate documentation describing and demonstrating the adherence to GLP principles should be provided.

## ANNEX 1

## MEMBERS OF THE PEER REVIEW PANEL

| <b>Panel member</b>   | <b>Affiliation</b>  |
|-----------------------|---|
| Craig Blackstock      | Charles River, Edinburgh, UK  |
| Penny Jones           | Unilever, Bedford, UK   |
| Kristina Kejlová      | National Institute of Public Health, Prague, Czech Republic                                   |
| Albrecht Poth         | Harlan Cytotest Cell Research GmbH, Rossdorf, Germany   |
| Klaus Rudolf Schröder | BioMed- zet Life Science GmbH, Linz, Austria (until 31.12.2009 Henkel AG & Co. KGaA, Germany) |

**ANNEX 2****List of all documents provided to the PRP  
(identified by file name)**

- A1 \_J\_ToxicSci(2009).pdf
- A4 LabCyte\_skin\_irritation\_testprotocol\_ver\_6.01 NC.pdf
- A6 Draft 2nd report LabCyte0907\_\_\_\_.pdf
- Appendix7.pdf
- D1 A suitable exposure time for SIT with LabCyte EPI-MODEL24.pdf
- D2 LabCyte EPI-MODEL background data document.pdf
- D3 LabCyte\_skin\_irritation\_testprotocol\_ver\_7.2 NC.pdf
- LabCyte report 091008.pdf
- D3 Table 16.pdf
- List of J-TEC reference document.pdf
- Content of review documents.docx
- 3rd TG SKIN IRR\_V.7.6\_9 Sept\_09\_Clean.pdf
- GD No.34.pdf
- SKIN IRR BACKGROUND DOC (V.3 0) 14 August 2009.pdf

## ANNEX 3

### Charge questions

#### **PRP Charge questions - part I:**

##### **The eight OECD principles and criteria for test method validation**

1. A rationale for the test method should be available, including a clear statement of scientific need and regulatory purpose.
2. The relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest should be addressed, describing limitations of the test methods.
3. A detailed protocol for the test method should be available.
4. Within- and between-laboratory reproducibility of the test method should be demonstrated.
5. Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals.
6. The performance of test methods should have been evaluated in relation to existing relevant toxicity data as well as information from the relevant target species.
7. All data supporting the assessment of the validity of the test method should be available for expert review.
8. Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of Good Laboratory Practice (GLP)

#### **PRP Charge questions - part II:**

##### **Performance Standards of the draft Test Guideline on Skin Irritation**

9. Adherence to the essential test method components should be demonstrated (*see also question 3*).
10. Reliability and accuracy should be demonstrated by using at least the recommended reference chemicals (*see also question 5*).
11. The test should have been assessed based on the defined reliability and accuracy values (*see also questions 4 and 6*)

## ANNEX 4

**COLLATED COMMENTS FROM THE PEER REVIEW PANEL  
ON THE  
LABCYTE EPI-MODEL24 IN VITRO TEST METHOD  
FOR THE ASSESSMENT OF SKIN IRRITATION POTENTIAL OF CHEMICALS**

| <b>ISSUES</b>                           | <b>COMMENTS AND RECOMMENDATIONS</b>   |
|---|---|
| <b>General comments</b>                 | <p>The overall impression of the Panel is that the LabCyte EPI-MODEL 24 has been properly evaluated in validation activities. Consensus has been reached for all charge questions.</p> <p>Regarding the eight OECD principles and criteria for test method validation, several issues still need to be addressed more thoroughly. The major criticism from all Panel members was that the within-and between-laboratory reproducibility has not been properly analysed and documented. Furthermore, a measurement for variability between identically treated tissues needs still to be defined together with a respective acceptance criterion.</p> <p>Regarding the additional three charge questions referring to the performance standards of the draft Test Guideline on Skin Irritation, the Panel members agreed that the specific restriction that only two <i>in vivo</i> Category 2 substances, 1-decanol and di-n-propyl disulphide, may be misclassified as ‘No Category’ by more than one participating laboratory, has not been met. In particular, 1-bromohexane, Category 2, has been misclassified as ‘No Category’ by five of six participating laboratories.</p> |
| <b>VALIDATION CRITERIA</b>              |   |
| <b>1. Rationale for the test method</b> | <p>The Panel agreed that the rationale for the test method is clearly stated, while the scientific basis and the regulatory purpose are well described. The test is intended to replace the Draize skin irritation test for regulatory purposes as currently defined in OECD Test Guideline 404.</p> <p><b>The Panel agreed that this criterion has been met.</b></p>   |
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| <p><b>2. Relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest</b></p> | <p>The panel agreed that the relevance of using chemically induced cytotoxicity measured by MTT to a human skin model to assess skin irritation potential has been well established already in the evaluation of the models leading to draft OECD Test Guideline. However, it is recommended to describe this more explicitly, e.g. as done in the draft guideline (Version 7.6) in paragraph 11.</p> <p>Furthermore, the panel agreed that the limitations of the model, e.g. regarding certain properties of test chemicals, should be described in more detail as for example done in paragraph 7 the draft guideline (Version 7.6).</p> <p><b>The Panel agreed that this criterion had been partly met.</b></p>   |
| <p><b>3. Availability of a detailed test method protocol</b></p>  | <p>The panel evaluated the provided protocol (version 7.2). Although largely appropriate, the panel recommends to:</p> <ul style="list-style-type: none"> <li>- elaborate on the test method limitations</li> <li>- include a measurement of variability between replicate tissues including a respective acceptance criterion</li> <li>- explain the prediction model in more detail</li> <li>- better define the storage temperature of the MTT solution and the positive control substance</li> <li>- better and more objectively define changes considered significant for chemicals that stain the tissue</li> <li>- change the instruction ‘...must be repeated three times...’ to ‘...must be performed three times in total...’</li> <li>- include instructions for the case, when only two tissues provide measurements, e.g. when one tissue is damaged.</li> </ul> <p><b>The Panel agreed that this criterion had been partly met.</b></p> |
| <p><b>4. Demonstration of within- and between-laboratory reproducibility</b></p>  | <p>The Panel agreed that the presented information on within- and between-laboratory reproducibility is not sufficient. As the generated data would allow a comprehensive evaluation of this aspect, the panel recommends to conduct and document a detailed statistical analysis of within- and between-laboratory reproducibility, including an evaluation of variability between replicate tissues.</p> <p><b>The Panel agreed that this criterion had been partly met.</b></p>  |
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| <p><b>5. Demonstration of the test method's performance based on testing of representative reference chemicals</b></p> | <p>The Panel agreed that the test chemicals, which were tested coded, was representative and appropriate for the demonstration of the test method's performance. In particular, all reference chemicals currently proposed in the draft OECD Test Guideline have been tested with the acceptable exception of tetrachloroethylene.</p> <p><b>The Panel agreed that this criterion has been met.</b></p>  |
| <p><b>6. Test methods evaluation related to existing relevant toxicity data</b></p>                                    | <p>The panel agreed that the data from the test method have been compared with relevant toxicity data, namely with Draize skin irritation test data which it is designed to replace. Furthermore, all chemicals tested have been used previously in validation studies of similar models.</p> <p><b>The Panel agreed that this criterion has been met.</b></p>   |
| <p><b>7. Availability of all relevant data for expert review</b></p>   | <p>The panel assumed that all relevant data has been provided. Furthermore, it noted that the standard protocol is available to the public.</p> <p><b>The Panel agreed that this criterion has been met.</b></p>   |
| <p><b>8. GLP (ideally)</b></p>   | <p>Initially, the optional nature of this criterion led to diverging interpretations. While some reviewers considered the statement that the validation study was carried out in the spirit of GLP as sufficient to fulfil this criterion, other reviewers requested evidence substantiating this statement and considered the criterion not fulfilled. After a discussion of this issue during the review process of this report, the understanding and interpretation of this criterion could be harmonised. As a result, the Panel agreed that the carrying out the validation in the spirit of GLP is acceptable. Nevertheless, the Panel recommended to better document how this was established and to provide respective documentation, if available.</p> <p><b>The Panel agreed that this criterion had been partly met.</b></p> |
| <p><b>PERFORMANCE STANDARDS</b></p>  |  |
| <p><b>9. Test method components</b></p>  | <p>The panel agreed that some detailed information to comprehensively address the test method components was missing.<br/>Regarding 'Functional conditions – Viability', an upper acceptance limit for the negative control tissues, e.g.</p>  |

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|  | <p>defined by a maximum optical density (OD), is missing. Although the data base for defining this might be small, it should nevertheless be defined if possible. Furthermore, it has not been defined what is considered as an acceptable variation of viabilities from replicate tissues.</p> <p>Regarding ‘Functional conditions – Morphology’, an acceptance criteria required for batch acceptance should be defined.</p> <p>Regarding ‘Functional conditions – Reproducibility’: A measure of variability between tissues should be defined.</p> <p>Regarding ‘Functional conditions – Quality control (QC)’, it was not entirely evident to the Panel how the model producer intends to ensure and demonstrate that each batch of the RhE model used meets defined production release criteria.</p> <p><b>The Panel agreed that this criterion had been partly met.</b></p>  |
| <p><b>10. Demonstration of reliability and relevance using reference chemicals</b></p> | <p>After clarification that a lot of review comments to this charge question would be equally well or better suited for charge question 11, the panel agreed that, with one acceptable exception, the currently recommended reference chemicals have been used to demonstrate reliability and relevance of the test method. In addition, six further chemicals, which have been used in the validation of the models leading to the draft OECD Test Guideline, have been tested.</p> <p><b>The Panel agreed that this criterion has been met.</b></p>   |
| <p><b>11. Test assessment based on defined reliability and accuracy values</b></p>     | <p>The panel agreed on several crucial issues. Most importantly and the main reason for failing this criterion, the specific requirement of paragraph 12, Annex 2, of the draft OECD Test guideline (‘...further specific restriction applies to the sensitivity of the proposed <i>in vitro</i> test method inasmuch as only two <i>in vivo</i> Category 2 substances, 1-decanol and di-n-propyl disulphide, may be misclassified as No Category by more than one participating laboratory.’) was not met. The chemical 1-bromohexane, which is an <i>in vivo</i> Category 2 substance, has been classified as ‘negative’ by five out of six laboratories in the validation exercise (excluding laboratory e).</p> <p>Furthermore, within-and between laboratory reproducibility have not been appropriately evaluated. In this regard, the Panel anticipated that, while the between-laboratory reproducibility might fulfil the respective requirements, two laboratories (a and f) might not meet the requirement that the within-laboratory variability should show a concordance of classifications (GHS Category 2/No Category) obtained in different, independent test runs of the 20 Reference Chemicals within one single laboratory equal or higher (<math>\geq</math>) than 90%.</p> <p>Related to this, the Panel recommends additional analyses based on final classifications for a complete run sequence of a given laboratory derived from the mean viabilities (instead of median viabilities) as specified</p> |

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|  | <p>in the performance standards.</p> <p>Regarding the predictive values, the panel agreed that the requirements, i.e. sensitivity <math>\geq 80\%</math>, specificity <math>\geq 70\%</math> and accuracy <math>\geq 75\%</math>, were met, when considering all 25 tested chemicals. It was noted, however, that the overall sensitivity would be <math>\leq 80\%</math> when considering only the 19 chemicals tested in the 2<sup>nd</sup> phase of the study.</p> <p><b>The Panel agreed that this criterion has not been met.</b></p> |
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## Appendix 1

### **JaCVAM Re-Analysis of initial validation data according the Performance Standards of TG 439 August 23, 2010**

During our validation studies, the draft OECD performance standards (PS) that is based on the ECVAM performance standards had been on the table by OECD international experts. We performed our studies using the reference chemical list in the original or revised ECVAM performance standards (ECVAM 2007), in accordance with the validation plan. On the other hand, we did not follow other rules of the performance standard that were not included in the plan. Therefore, we could not calculate within- and between-laboratory reproducibility or analyze our data according to the rules of the performance standard (annex2) in the OECD draft test guideline.

With instructions related to our reanalyzing the data to OECD peer review panel, in this report I have analyzed our data according to the OECD draft performance standards.

With reference to definitions of the rules for reliability and accuracy values, the validation data were set in order as shown below.

1) Invalid data obtained only from Lab a, during run 1 (data not shown). This is because the negative OD detected during run 1 was lower than the acceptable limit. This lab carried out retesting. Therefore, the data Lab a, runs 2-4, were accepted as complete run sequences. All run sequences were completed in all other laboratories.

2) Twenty chemicals from the reference chemical (RC) list in the OECD performance standard (table 1) are used in this report.

3) With the exclusion of the data from Lab e, fully complete data from six laboratories were analyzed with three complete run sequences using all 20 RCs.

Below are our answers to questions regarding data analysis.

1. To questions 3, 4, 7 and 9 in the summary report of the LabCyte peer review panel:

A measure of variability between tissue triplicates was defined in the SOP and calculated in Table 2, and detailed data is presented in the attached files.

2. To questions 4, 7 and 11 in the summary report of the LabCyte peer review panel:

Within- and between-laboratory reproducibility were calculated according to annex 2.

As shown in Tables 3-1 and 3-2, there was no difference in median and mean variability among tissue triplicates.

Within-laboratory reproducibility: equal or higher than 90% in all laboratories as shown in Table 4. 2

Between-laboratory reproducibility: equal or higher than 80% in all laboratories as shown in Table 4. As shown in Tables 5 and 6, accuracy of the required predictive values was sufficient relative to the ECVAM criteria.

**Table 1: Reference test chemicals according to OECD performance standards (PS)**

| No. | Validation code | Chemical  | CAS number | GHS label  | In vivo score (PII) |
|-----|-----------------|---|------------|------------|---------------------|
| 1   | 01              | 1-bromo-4-chlorobutane                            | 6940-78-9  | no         | 0                   |
| 2   | 02              | diethyl phthalate                                 | 84-66-2    | no         | 0                   |
| 3   | 04              | naphtalen acetic acid                             | 86-87-3    | no         | 0                   |
| 4   | 05              | allyl phenoxy-acetate                             | 7493-74-5  | no         | 0.3                 |
| 5   | 06              | isopropanol                                       | 67-63-0    | no         | 0.3                 |
| 6   | 07              | 4-methyl-thio-benzaldehyde                        | 3446-89-7  | no         | 1                   |
| 7   | 08              | methyl stearate                                   | 112-61-8   | no         | 1                   |
| 8   | 10              | heptyl butyrate                                   | 5870-93-9  | no         | 1.7                 |
| 9   | 11              | hexyl salicylate                                  | 6259-76-3  | no         | 2                   |
| 10  | A               | Cinnamaldehyde                                    | 104-55-2   | no         | 2                   |
| 11  | 14              | 1-decanol   | 112-30-1   | Category 2 | 2.3                 |
| 12  | 15              | cyclamen aldehyde                                 | 103-95-7   | Category 2 | 2.3                 |
| 13  | 16              | 1-bromohexane                                     | 111-25-1   | Category 2 | 2.7                 |
| 14  | B               | 2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl | 322-76821  | Category 2 | 2.7                 |
| 15  | C               | Potassium hydroxide (5%aq)                        | 168-21815  | Category 2 | 3                   |
| 16  | 18              | di-n-propyl disulphide                            | 629-19-6   | Category 2 | 3                   |
| 17  | D               | Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl      | 7340-90-1  | Category 2 | 3.3                 |
| 18  | E               | 1-Methyl-3-phenyl-1-piperazine                    | 5271-27-2  | Category 2 | 3.3                 |
| 19  | 20              | heptanal  | 111-71-7   | Category 2 | 4                   |
| 20  | F               | 1,1,1-Torichloroethane                            | 200-02463  | Category 2 | 4                   |

**Table 2: Mean viability at each test for each chemical according to OECD PS**

| No. | Code | Vivo       | Score | Exp. | Lab.  |       |       |       |       |       |
|-----|------|------------|-------|------|-------|-------|-------|-------|-------|-------|
|     |      |            |       |      | a     | b     | c     | d     | f     | g     |
| 01  | 01   | no         | 0     | 1    | 31.0  | 47.1  | 10.6  | 14.3  | 14.3  | 10.6  |
|     |      |            |       | 2    | 11.2  | 10.4  | 20.3  | 9.1   | 11.2  | 10.6  |
|     |      |            |       | 3    | 11.6  | 16.1  | 12.4  | 9.6   | 10.4  | 14.0  |
| 02  | 02   | no         | 0     | 1    | 79.8  | 66.9  | 88.1  | 102.3 | 75.3  | 96.0  |
|     |      |            |       | 2    | 76.5  | 61.7  | 89.7  | 89.8  | 67.2  | 94.8  |
|     |      |            |       | 3    | 65.2  | 88.7  | 85.8  | 67.6  | 75.7  | 103.3 |
| 3   | 04   | no         | 0     | 1    | 106.3 | 94.4  | 97.1  | 106.1 | 100.1 | 104.8 |
|     |      |            |       | 2    | 95.2  | 100.2 | 99.9  | 100.9 | 92.8  | 103.3 |
|     |      |            |       | 3    | 96.5  | 98.6  | 97.8  | 98.4  | 92.7  | 109.8 |
| 4   | 05   | no         | 0.3   | 1    | 78.5  | 61.7  | 91.4  | 79.4  | 71.9  | 96.8  |
|     |      |            |       | 2    | 78.5  | 71.9  | 95.2  | 70.5  | 39.3  | 89.9  |
|     |      |            |       | 3    | 74.1  | 84.5  | 89.2  | 66.1  | 55.1  | 88.4  |
| 5   | 06   | no         | 0.3   | 1    | 92.5  | 77.9  | 81.0  | 91.3  | 87.8  | 87.2  |
|     |      |            |       | 2    | 79.4  | 83.5  | 79.1  | 102.4 | 94.4  | 81.2  |
|     |      |            |       | 3    | 82.4  | 80.5  | 83.6  | 82.7  | 81.1  | 54.1  |
| 6   | 07   | no         | 1     | 1    | 24.1  | 10.8  | 20.8  | 21.7  | 15.8  | 31.5  |
|     |      |            |       | 2    | 12.6  | 12.6  | 16.2  | 13.8  | 31.1  | 22.5  |
|     |      |            |       | 3    | 17.8  | 13.2  | 15.2  | 19.8  | 15.6  | 19.9  |
| 7   | 08   | no         | 1     | 1    | 111.9 | 86.7  | 75.3  | 109.4 | 89.7  | 101.1 |
|     |      |            |       | 2    | 90.2  | 100.6 | 82.3  | 107.5 | 97.8  | 100.9 |
|     |      |            |       | 3    | 95.3  | 104.8 | 77.2  | 103.0 | 96.5  | 109.0 |
| 8   | 10   | no         | 1.7   | 1    | 115.9 | 115.4 | 107.5 | 114.3 | 104.0 | 107.9 |
|     |      |            |       | 2    | 104.1 | 110.1 | 103.6 | 108.2 | 101.2 | 108.4 |
|     |      |            |       | 3    | 86.5  | 111.3 | 103.7 | 105.5 | 101.2 | 113.1 |
| 9   | 11   | no         | 2     | 1    | 113.7 | 105.0 | 101.0 | 102.4 | 103.1 | 102.8 |
|     |      |            |       | 2    | 98.1  | 106.6 | 94.6  | 105.8 | 98.0  | 100.5 |
|     |      |            |       | 3    | 112.6 | 103.7 | 94.1  | 102.7 | 94.6  | 109.0 |
| 10  | A    | No         | 2     | 1    | 13.3  | 11.8  | 13.2  | 13.8  | 11.4  | 13.7  |
|     |      |            |       | 2    | 14.2  | 10.2  | 22.5  | 9.9   | 11.3  | 8.7   |
|     |      |            |       | 3    | 14    | 11.1  | 12.3  | 13.2  | 14.3  | 14.3  |
| 11  | 14   | Category 2 | 2.3   | 1    | 11.1  | 12.1  | 14.7  | 10.7  | 13.1  | 13.5  |
|     |      |            |       | 2    | 6.6   | 8.3   | 9.5   | 11.7  | 16.7  | 12.0  |
|     |      |            |       | 3    | 6.8   | 8.8   | 9.1   | 10.2  | 17.0  | 10.6  |
| 12  | 15   | Category 2 | 2.3   | 1    | 11.1  | 9.3   | 13.1  | 8.0   | 8.6   | 9.2   |
|     |      |            |       | 2    | 7.1   | 10.2  | 19.3  | 8.6   | 5.9   | 24.7  |
|     |      |            |       | 3    | 8.2   | 9.9   | 8.1   | 9.2   | 7.1   | 9.2   |
| 13  | 16   | Category 2 | 2.7   | 1    | 67.9  | 92.0  | 51.5  | 18.1  | 59.6  | 64.9  |
|     |      |            |       | 2    | 32.2  | 54.1  | 86.3  | 79.2  | 50.4  | 79.6  |
|     |      |            |       | 3    | 59.8  | 98.3  | 81.7  | 37.7  | 67.5  | 86.5  |
| 14  | B    | Category 2 | 2.7   | 1    | 1.5   | 2.2   | 2.5   | 4     | 1.7   | 3.9   |
|     |      |            |       | 2    | 3.1   | 2.2   | 2.9   | 3     | 2.6   | 3.7   |
|     |      |            |       | 3    | 1.5   | 2.5   | 3     | 3.9   | 3.2   | 4.7   |
| 15  | C    | Category 2 | 3     | 1    | 0.7   | 0.7   | 0.7   | 6.9   | 0.8   | 1     |
|     |      |            |       | 2    | 1.3   | 1.1   | 1.4   | 2     | 4.8   | 0.4   |
|     |      |            |       | 3    | 0.5   | 0.8   | 1     | 0.8   | 1     | 0.3   |
| 16  | 18   | Category 3 | 3     | 1    | 82.1  | 46.5  | 91.2  | 83.7  | 69.2  | 92.4  |
|     |      |            |       | 2    | 78.3  | 50.6  | 87.3  | 69.9  | 80.6  | 85.9  |
|     |      |            |       | 3    | 25.3  | 100.0 | 87.5  | 59.0  | 71.9  | 94.4  |
| 17  | D    | Category 2 | 3.3   | 1    | 14.5  | 24    | 12.7  | 10.3  | 13.8  | 19.3  |
|     |      |            |       | 2    | 13.6  | 16    | 12.5  | 18.3  | 8.8   | 15.2  |
|     |      |            |       | 3    | 18.6  | 15.5  | 12.6  | 23    | 19.2  | 14.1  |
| 18  | E    | Category 2 | 3.3   | 1    | 3.9   | 3.4   | 3.4   | 8.2   | 3.2   | 4.1   |
|     |      |            |       | 2    | 4.5   | 2.7   | 3.3   | 3.9   | 4.2   | 3.1   |
|     |      |            |       | 3    | 1.8   | 3.5   | 3.5   | 3.7   | 5     | 5.1   |
| 19  | 20   | Category 3 | 4     | 1    | 31.1  | 24.8  | 10.4  | 9.6   | 8.1   | 8.8   |
|     |      |            |       | 2    | 9.3   | 8.0   | 7.6   | 16.9  | 7.8   | 6.7   |
|     |      |            |       | 3    | 29.5  | 9.3   | 7.6   | 30.9  | 8.2   | 8.6   |
| 20  | F    | Category 2 | 4     | 1    | 5.6   | 7.2   | 6.5   | 6.4   | 5.2   | 7.2   |
|     |      |            |       | 2    | 5.7   | 6.1   | 6.8   | 5.4   | 7.4   | 6.8   |
|     |      |            |       | 3    | 5.4   | 4.2   | 6.5   | 5.4   | 5     | 7.6   |

**Table 3-1: Mean viability at three tests for each chemical according to OECD PS**

| NO. | Code | GHS label  | a     | b     | c     | d     | f     | g     |
|-----|------|------------|-------|-------|-------|-------|-------|-------|
| 1   | 01   | no         | 17.9  | 24.5  | 14.4  | 11.0  | 12.0  | 11.7  |
| 2   | 02   | no         | 73.8  | 72.4  | 87.8  | 86.6  | 72.7  | 98.0  |
| 3   | 04   | no         | 99.3  | 97.8  | 98.2  | 101.8 | 95.2  | 105.9 |
| 4   | 05   | no         | 77.0  | 72.7  | 91.9  | 72.0  | 55.4  | 91.7  |
| 5   | 06   | no         | 84.8  | 80.7  | 81.2  | 92.1  | 87.8  | 74.2  |
| 6   | 07   | no         | 18.2  | 12.2  | 17.4  | 18.4  | 20.8  | 24.6  |
| 7   | 08   | no         | 99.1  | 97.4  | 78.3  | 106.6 | 94.7  | 103.7 |
| 8   | 10   | no         | 102.1 | 112.2 | 104.9 | 109.3 | 102.1 | 109.8 |
| 9   | 11   | no         | 108.1 | 105.1 | 96.6  | 103.6 | 98.6  | 104.1 |
| 10  | A    | no         | 13.8  | 11.0  | 16.0  | 12.3  | 12.3  | 12.2  |
| 11  | 14   | Category 2 | 8.2   | 9.7   | 11.1  | 10.9  | 15.6  | 12.0  |
| 12  | 15   | Category 2 | 8.8   | 9.8   | 13.5  | 8.6   | 7.2   | 14.4  |
| 13  | 16   | Category 2 | 53.3  | 81.4  | 73.1  | 45.0  | 59.1  | 77.0  |
| 14  | B    | Category 2 | 2.0   | 2.3   | 2.8   | 3.6   | 2.5   | 4.1   |
| 15  | C    | Category 2 | 0.8   | 0.8   | 1.0   | 3.2   | 2.2   | 0.6   |
| 16  | 18   | Category 2 | 61.9  | 65.7  | 88.7  | 70.9  | 73.9  | 90.9  |
| 17  | D    | Category 2 | 15.6  | 18.5  | 12.6  | 17.2  | 13.9  | 16.2  |
| 18  | E    | Category 2 | 3.4   | 3.2   | 3.4   | 5.3   | 4.2   | 4.1   |
| 19  | 20   | Category 2 | 23.3  | 14.0  | 8.6   | 19.2  | 8.0   | 8.1   |
| 20  | F    | Category 2 | 5.5   | 5.8   | 6.6   | 5.7   | 5.9   | 7.2   |

**Table 3-2: Median viability at three tests for each chemical according to OECD PS**

| NO. | Code | GHS label  | a     | b     | c     | d     | f     | g     |
|-----|------|------------|-------|-------|-------|-------|-------|-------|
| 1   | 01   | no         | 11.6  | 16.1  | 12.4  | 9.6   | 11.2  | 10.6  |
| 2   | 02   | no         | 76.5  | 66.9  | 88.1  | 89.8  | 75.3  | 96.0  |
| 3   | 04   | no         | 96.5  | 98.6  | 97.8  | 100.9 | 92.8  | 104.8 |
| 4   | 05   | no         | 78.5  | 71.9  | 91.4  | 70.5  | 55.1  | 89.9  |
| 5   | 06   | no         | 82.4  | 80.5  | 81.0  | 91.3  | 90.7  | 81.2  |
| 6   | 07   | no         | 17.8  | 12.6  | 16.2  | 19.8  | 21.3  | 22.5  |
| 7   | 08   | no         | 95.3  | 100.6 | 77.2  | 107.5 | 100.9 | 101.1 |
| 8   | 10   | no         | 104.1 | 111.3 | 103.7 | 108.2 | 101.2 | 108.4 |
| 9   | 11   | no         | 112.6 | 105.0 | 94.6  | 102.7 | 98.0  | 102.8 |
| 10  | A    | no         | 14.0  | 11.1  | 13.2  | 13.2  | 11.4  | 13.7  |
| 11  | 14   | Category 2 | 6.8   | 8.8   | 9.5   | 10.7  | 16.7  | 12.0  |
| 12  | 15   | Category 2 | 8.2   | 9.9   | 13.1  | 8.6   | 7.1   | 9.2   |

|    |    |            |      |      |      |      |      |      |
|----|----|------------|------|------|------|------|------|------|
| 13 | 16 | Category 2 | 59.8 | 92.0 | 81.7 | 37.7 | 59.6 | 79.6 |
| 14 | B  | Category 2 | 1.5  | 2.2  | 2.9  | 3.9  | 2.6  | 3.9  |
| 15 | C  | Category 2 | 0.7  | 0.8  | 1.0  | 2.0  | 1.0  | 0.4  |
| 16 | 18 | Category 2 | 78.3 | 50.6 | 87.5 | 69.9 | 71.9 | 92.4 |
| 17 | D  | Category 2 | 14.5 | 16.0 | 12.6 | 18.3 | 13.8 | 15.2 |
| 18 | E  | Category 2 | 3.9  | 3.4  | 3.4  | 3.9  | 4.2  | 4.1  |
| 19 | 20 | Category 2 | 23.3 | 14.0 | 8.6  | 19.2 | 8.0  | 8.1  |
| 20 | F  | Category 2 | 5.6  | 6.1  | 6.5  | 5.4  | 5.2  | 7.2  |

**Table 4: Reproducibility according to OECD PS using mean viabilities**

| Index              | Lab.  |       |       |       |       |       |
|--------------------|-------|-------|-------|-------|-------|-------|
|                    | a     | b     | c     | d     | f     | g     |
| Within-laboratory  | 18/20 | 19/20 | 20/20 | 19/20 | 20/20 | 20/20 |
|                    | 90    | 95    | 100   | 95    | 100   | 100   |
| Between-laboratory | 19/20 |       |       |       |       |       |
|                    | 95.0  |       |       |       |       |       |

**Table 5: Sensitivity, specificity and accuracy on MTT assay vs GHS-EU classification according to OECD PS**

| Index       | Lab.  |       |       |       |       |       |
|-------------|-------|-------|-------|-------|-------|-------|
|             | a     | b     | c     | d     | f     | g     |
| Sensitivity | 8/10  | 8/10  | 8/10  | 9/10  | 8/10  | 8/10  |
|             | 80    | 80    | 80    | 90    | 80    | 80    |
| Specificity | 7/10  | 7/10  | 7/10  | 7/10  | 7/10  | 7/10  |
|             | 70    | 70    | 70    | 70    | 70    | 70    |
| Accuracy    | 15/20 | 15/20 | 15/20 | 16/20 | 15/20 | 15/20 |
|             | 75    | 75    | 75    | 80    | 75    | 75    |

**Table 6: Mean and range of sensitivity, specificity and accuracy on the MTT assay using LabCyte EPI-MODEL vs. GHS-EU classification according to OECD PS**

|                 | N | Mean | Min. | Max. | ECVAM criteria |
|-----------------|---|------|------|------|----------------|
| Sensitivity (%) | 6 | 81.7 | 80.0 | 90.0 | 80.0           |
| Specificity (%) | 6 | 70.0 | 70.0 | 70.0 | 70.0           |
| Accuracy (%)    | 6 | 76.7 | 75.0 | 80.0 | 75.0           |

**Appendix 2****Background document for the modified skin irritation test using LabCyte EPI-MODEL24 (LabCyte EPI-MODEL24 SIT)****Japan Tissue Engineering Co., Ltd. R&D****TABLE OF CONTENTS**

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## **1. Abstract**

A validation study of an *in vitro* skin irritation test method using a reconstructed human epidermal model for replacement of the Draize test was conducted by the European Centre for the Validation of Alternative Methods (ECVAM), and a protocol using EpiSkin™ (SkinEthic, France) was approved as a validated reference method (VRM) in April, 2007. Structural and performance test criteria for skin models are defined in the ECVAM Performance Standards. We have performed several evaluations of our reconstructed human epidermal model LabCyte EPI-MODEL24 (Japan Tissue Engineering Co., Ltd, Japan), and have confirmed that it is suitable for skin irritation tests defined by the ECVAM Performance Standards.

The original study for the development of a test method showed that test chemicals could be classified into non-irritants and irritants using the LabCyte EPI-MODEL24 (ORIGINAL STAGE). The VRM consists of a 15 minute exposure period, followed by a rinsing step and a 42 hour post-incubation period before quantitative measurement of cell viability using an MTT reaction. First, we examined the exposure period when the test chemicals are applied and found that the optimal exposure period is 15 minutes. After that, the performance of the original LabCyte EPI-MODEL24 SIT, which included an optimization study, was confirmed in intra-laboratory study and then evaluated on relevance and ability to meet minimum criteria described in the ECVAM performance standards for phase 1, 2 and 3 validation studies. The results of the validation studies for the original LabCyte EPI-MODEL24 SIT were summarized in JSAAE REPORTS and then these reports were assessed by a peer review panel in OECD, according to the OECD draft test guideline for skin irritation testing, which was presented in 2009.

However, the OECD peer review panel pointed out that the performance of the original LabCyte EPI-MODEL24 SIT did not meet the criteria shown in OECD draft test guideline for skin irritation testing, because the prediction results for the chemical 1-bromohexane, which is an *in vivo* Category 2 substance, were shown as negative in the original LabCyte EPI-MODEL24 SIT. Various modifications of the original LabCyte EPI-MODEL24 SIT have been examined by the kit supplier in order to solve the 1-bromohexane problem, with the result that they were able to solve the problem by modifying the washing protocol for the testing chemicals (the modified LabCyte EPI-MODEL24 SIT). When the predictive potency of the modified LabCyte EPI-MODEL24 SIT was evaluated with the 20 test chemicals which were listed in the new OECD TG 439 for *in vitro* skin irritation testing, adopted in July, 2010, the sensitivity, the specificity and the overall accuracy were 90 %, 70 %, and 80%, respectively.. It was thought that these results met the acceptance criteria described in OECD TG 439. In order to confirm the performance of the modified LabCyte EPI-MODEL24 SIT according to OECD TG 439, a final validation study was executed from August to November, 2010. Results demonstrated high reliability and acceptable accuracy in the MTT assay, for use as a stand-alone assay to distinguish between skin irritants and non-irritants. Details of the results of the validation study will be described in a Japanese Center for the Validation of Alternative Methods (JaCVAM) final report.

## **2. Purpose of this document**

The principle of *in vitro* skin irritation testing using a reconstructed human epidermis (RhE) model as described in the new OECD test guideline 439 (OECD TG 439; [Attachment 1](#)) for *in vitro* skin irritation testing, is to detect the initiating events in the following cascade of skin irritation. Chemical-induced skin irritation, manifested by erythema and oedema, is the result of a cascade of events beginning with penetration of the stratum corneum and damage to the underlying layers of keratinocytes. The dying keratinocytes release mediators that begin the inflammatory cascade which acts on the cells in the dermis, particularly the stromal and endothelial cells. It is the dilation and increased permeability of the endothelial cells that produce the observed erythema and oedema. The RhE-based test methods measure the initiating events in the cascade.

The main purpose of this document is to propose a new *in vitro* skin irritation test method using LabCyte EPI-MODEL24 (Japan Tissue Engineering Co., Ltd, Japan; the LabCyte EPI-MODEL24 SIT) as a “me-too” test based on the validation test method (VRM) for EpiSkin™ (SkinEthic, France) according to OECD TG 439.

This document is composed of the following categories:

1. Description of the LabCyte EPI-MODEL24: It is confirmed that quality control for the LabCyte EPI-MODEL24 quality standards and/or the characteristics thereof meet with the criteria described in OECD TG 439.
2. Optimization of the LabCyte EPI-MODEL24 SIT and assessment of the original SIT (OPTIMIZATION STAGE): The optimal exposure period for the LabCyte EPI-MODEL24 SIT was investigated as a method based on VRM, according to the ECVAM performance standard (1). The reliability performance of the original (optimized) SIT was evaluated based on the OECD draft test guideline for skin irritation testing (2).
3. Modification of the original LabCyte EPI-MODEL24 SIT and assessment of the modified SIT (MODIFICATION STAGE): The original LabCyte EPI-MODEL24 SIT was modified because the original LabCyte EPI-MODEL24 SIT did not meet the criteria shown in the OECD draft test guideline (2). The performance of the modified SIT was evaluated with respect to reliability and accuracy, according to OECD TG 439.

In the end, through the above research stages it was confirmed that the performance of the LabCyte EPI-MODEL24 SIT meets the acceptance criteria shown in OECD TG 439 as a “me-too” test method similar to VRM.

### **3. Introduction**

Trials to replace the Draize skin irritation test on rabbits *in vivo*, carried out according to the OECD TG 404 test guidelines (3), have been underway for many years worldwide, especially in the European Union (EU). These efforts have been accelerated by the enforcement of the 7th Amendment to the Cosmetics Directive and of EU regulations for the registration, evaluation, and authorization of chemicals (REACH). Investigation of various *in vitro*/ex vivo test systems showed that the best results were achieved by a system using a three-dimensional reconstructed human epidermal model (4, 5). The European Centre for the Validation of Alternative Methods (ECVAM) therefore evaluated two reconstructed human epidermal models, EpiSkin™ (SkinEthics, Nice, France) and EpiDerm™ (MatTek, MA, USA), during a prevalidation study of *in vitro* skin irritation tests during 2000–2001 (5, 6). However, since the predictive performance of the two models in the ECVAM prevalidation study did not meet the acceptance criteria set by the Management Team, further investigations were required to improve the test protocols (6). Subsequently, during 2003–2004 a common protocol for the *in vitro* evaluation of skin irritation potential was developed, optimized, and allowed to proceed to a formal ECVAM validation study (7, 8). As a result of the study, an *in vitro* skin irritation test (SIT) using the EpiSkin™ reconstructed human epidermal model (EpiSkin™ test method) has been scientifically validated as a stand-alone method of distinguishing skin irritants from non-irritants according to the EU classification (9). Furthermore, the ECVAM performance standards for applying a new human epidermis model to *in vitro* skin irritation have been documented based on the VRM (1). The ECVAM performance standards can then be used to evaluate the accuracy and reliability of other analogous test methods, also known as “me-too” tests, either based on similar scientific principles and measures or to predict the same biological or toxic effect.

Various other available *in vitro* three-dimensional epidermis equivalents have been developed, such as the LabCyte EPI-MODEL24 (Japan Tissue Engineering Co. Ltd., Japan). The LabCyte EPI-MODEL24 is a new reconstructed human epidermis model, which is grown for 13 days in a chemically defined medium using normal human keratinocytes (10). The tissue model consists of a fully differentiated epidermis, including a basal cell layer, stratum spinosum, stratum granulosum and stratum corneum (10, 11). Since the launch of commercially released kits in 2005, the LabCyte EPI-MODEL24 has mainly been used for skin irritation studies (11-15) or for skin corrosion (16), but it is also commonly used for UV-related experiments (17-19), for DNA microarray (20,21) and for various experiments about skin function (22-26).

In order to develop a test method according to the VRM (EpiSkin™ test methods), which is able to discriminate between non-irritant versus irritant test substances using the LabCyte EPI-MODEL24, the optimal exposure period for LabCyte EPI-MODEL24 was determined before the assessment of its performance. Then, the performance of the original LabCyte EPI-MODEL24 SIT (15 minute exposure) after the optimization study was evaluated for its relevance and its ability to meet ECVAM minimum performance standards criteria.

The validation study of the original LabCyte EPI-MODEL24 SIT was executed from 2008 to 2009. The results for validation studies of the original LabCyte EPI-MODEL24 SIT were summarized in JSAAE REPORTS (Attachment 2, 3).

During the validation study of the original LabCyte EPI-MODEL24 SIT, two important decisions were presented by ECVAM. One of them changed the test chemical exposure period for the modified EpiDerm™ SIT, from 15 minutes to 60 minutes, while the other stated that the SIT using SkinEthic RHE (SkinEthics, Nice, France), which was a new reconstructed human epidermal model, was regarded by ECVAM as sufficiently similar in comparison with the VRM and was admitted as a validation study on the basis of the ECVAM performance standards (27). Furthermore, the ECVAM performance standards

were revised (the ECVAM performance standards (updated)); 28,29) in 2009. It was stated that the *in vivo* classification of test chemicals would be changed from referring to the EU DSD (European Classification System based on the Dangerous Substance Directive) to the UN GHS (United Nations Globally Harmonized System of Classification and Labelling of Chemicals). This change was reflected in the OECD draft test guideline for skin irritation testing (2).

It should be noted that, the JSAAE REPORTS (Attachment 2, 3) about the validation study for the original LabCyte EPI-MODEL24 SIT were assessed by a peer review panel in OECD according to the acceptance criteria as shown in the OECD draft test guideline. Unfortunately, the OECD peer review panel pointed out that the performance of the original LabCyte EPI-MODEL24 SIT did not meet the criteria shown in the OECD draft test guideline for skin irritation testing (2), because the prediction results of the chemical 1-bromohexane, which is an *in vivo* Category 2 substance, was shown as negative in the original LabCyte EPI-MODEL24 SIT by five out of six laboratories in the validation exercise. In order to solve the problem where 1-bromohexane showed a false-negative, various modification trials for LabCyte EPI-MODEL24 SIT were examined by the kit supplier. From the results of their examination, it was found that the problem could be solved by the improvement of the washing protocol (the modified LabCyte EPI-MODEL24 SIT). Using the 20 test chemicals listed in the OECD TG 439 (Attachment 1), which was adopted as the new guideline for the *in vitro* skin irritation testing by OECD in July, 2010, the predictive potency of the modified LabCyte EPI-MODEL24 SIT was evaluated, with the result that the sensitivity, specificity and overall accuracy were 90%, 70% and 80%, respectively. It was thought that these results met the acceptance criteria described in the OECD TG 439. In order to confirm the performance of the modified LabCyte EPI-MODEL24 SIT according to OECD TG 439, the final and formal validation study of the modified LabCyte EPI-MODEL24 SIT was performed from August to November, 2010.

#### 4. Description of LabCyte EPI-MODEL24

##### 4-1. Condition of the functional reconstructed human epidermis, LabCyte EPI-MODEL24

LabCyte EPI-MODEL24 is a new, commercially available reconstructed human cultured epidermal model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand the human keratinocytes while maintaining their phenotype, they are cultured with 3T3-J2 cells as a feeder layer (30,31). Reconstruction of human cultured epidermis is achieved by cultivating proliferating keratinocytes on an inert filter substrate (surface 0.3 cm<sup>2</sup>) at the air-liquid interface for 13 days, with an optimized medium containing 5% fetal bovine serum. The result is a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum (Fig.4-1). The LabCyte EPI-MODEL24 is embedded in an agarose gel containing a nutrient solution and shipped in 24-well plates at around 18°C.

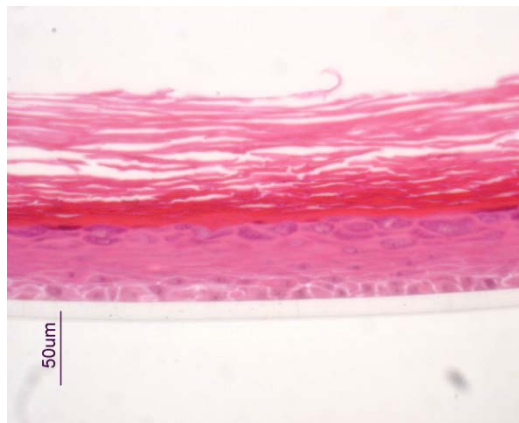


Fig.4-1. Histological cross-sectional views of the LabCyte EPI-MODEL24 with H&E staining (original magnification:  $\times 40$ ).

Histological examination shows a completely stratified epidermis containing all major epidermal layers, including a stratum basal (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC) (Fig.4-1). In addition, specific epidermal differentiation markers and basement membrane constituents are expressed in the appropriate regions, as seen in human skin (Fig.4-2).

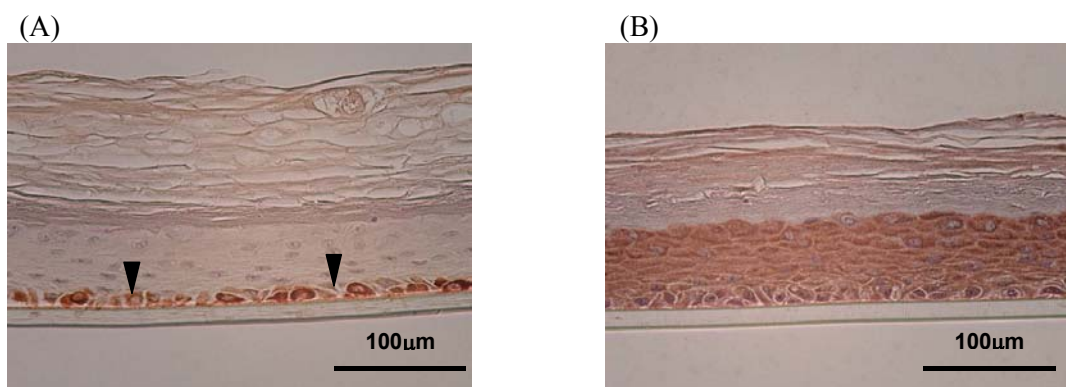


Fig.4-2. Histological cross-sectional views of LabCyte EPI-MODEL24 with

immunohistochemical staining using Laminin (a) and Transglutaminase (b).

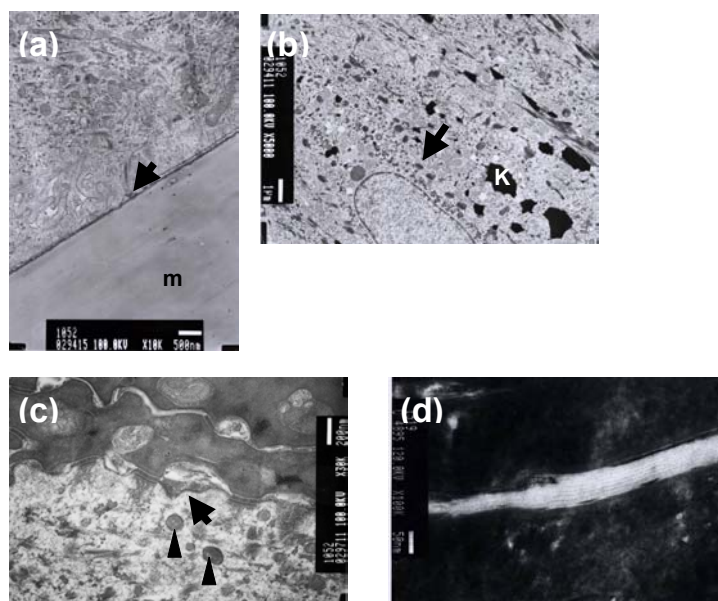


Fig.4-3. Transmission electron micrographs of the LabCyte EPI-MODEL24:

- (a) Basement membrane (original magnification;  $\times 10,000$ ). Note anchoring filament (arrows).
- (b) Keratohyalin granules in SG cells which are connected by desmosome (original magnification;  $\times 5000$ ). Note keratohyalin granules (K), desmosome (arrows).
- (c) Lipid lamella bodies in the interface between SG and SC (original magnification;  $\times 30,000$ ). Note lipid lamellar bodies (arrow head) and their extrusion (arrows).
- (d) Lipid bilayer at SC (original magnification;  $\times 100,000$ ). Additional ruthenium tetroxide fixation was used.

Ultra-structurally, it was possible to observe a fully developed basement membrane zone, consisting of a highly developed lamina densa, lamina lucida, and anchoring filaments. Extrusion of lamellar bodies was observed at the interface between the SG and SC (Fig.4-3). Lipid lamellae, showing a characteristic electron dense and electron lucent pattern, were present. Keratohyalin granules were ubiquitously present in the granular cells at the SG (Fig.4-3).

Their histological evaluation was summarized in a scientific report (10).

The synthesis of specific lipids, including ceramides, which are known to be responsible for the water barrier of the stratum corneum, was detected in the LabCyte EPI-MODEL (22).

#### 4-2. Quality control for LabCyte EPI-MODEL24

##### 4-2a. Quality control procedures for LabCyte EPI-MODEL24

LabCyte EPI-MODEL24 is manufactured according to a defined standard operation procedure (SOP). All batches of the LabCyte EPI-MODEL24 production are checked for their viability, barrier function and morphology.

The product is released following stringent quality control procedures.

The quality of the final product is assessed by the following protocol and decision criteria;

1. Tissue viability: MTT assay

Three LabCyte EPI-MODEL24 tissues were subjected to an MTT assay as follows. Tissues were put in the wells of 24-well plates containing 0.5 ml of MTT medium (0.5 mg/ml; Dojindo Co., Kumamoto, Japan) and were incubated for three hours (37°C, 5% CO<sub>2</sub>, humidified atmosphere). Formazan produced in the tissues was extracted with isopropanol (300 µl) and the extract (200 µl) was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank. The mean of the OD values indicates tissue viability for each LabCyte EPI-MODEL24 batch. QC acceptance criteria: OD ≥ 0.8

2. Barrier function: 50% inhibitory concentration (IC<sub>50</sub>) assay.

To evaluate whether the stratum corneum in the LabCyte EPI-MODEL24 tissue resists the rapid penetration of the cytotoxic marker chemical sodium lauryl sulphate (SLS), the viability of the epidermis tissue was estimated in terms of the half maximal inhibitory concentration (IC<sub>50</sub>). Various concentrations (0.1, 0.2, 0.3, and 0.4%(w/v)) of SLS (25 µl) were applied to the LabCyte EPI-MODEL24, and cell viability was measured after 18 hours using an MTT assay. All experiments were performed in triplicate. The acceptable range for the LabCyte EPI-MODEL24 is shown in Table 4-1.

**Table 4-1: QC acceptable range of barrier function**

|                                 | Lower limit | Mean        | Upper limit |
|---------------------------------|-------------|-------------|-------------|
| LabCyte EPI-MODEL24             | 0.14 (w/v)% | 0.26 (w/v)% | 0.40 (w/v)% |
| IL <sub>50</sub> (18 hours SLS) | (1.4mg/ml)  | (2.6mg/ml)  | (4.0mg/ml)  |

The test protocol for the barrier function of the LabCyte EPI-MODEL24 was established in May, 2008. In order to set an acceptable QC range, data for a barrier function database was collected from June to December, 2008. From June 2008 to October 2010, there was no batch with results outside the QC acceptance criteria.

3. Morphology.

A piece of LabCyte EPI-MODEL24 was fixed with 4% paraformaldehyde and 2% sucrose in 0.1M phosphate buffered saline (PBS) (pH 7.4) for more than three hours and processed for embedding in paraffin. Five-micrometer vertical sections were cut and stained with hematoxylin and eosin for light-microscopic examination.

QC acceptance criteria: Confirmation of the formation of multilayered epidermis-like tissue containing a stratum corneum.

Since the start of the commercial release of the product until October 2010, no batch has been outside the QC acceptance criteria..

**4-2b. Batch control information for LabCyte EPI-MODEL24**

Quality control data for the tissue viability and barrier function of each LabCyte EPI-MODEL24 batch is shown in Table 4-2.

**Table 4-2: Batch information for LabCyte EPI-MODEL24**  
**Tissue viability: October, 2005 – October, 2010**  
**Barrier function: June, 2008 – October, 2010**

| Year | Month    | Tissue viability |                         | Barrier function |                         |
|------|----------|------------------|-------------------------|------------------|-------------------------|
|      |          | Mean±SD<br>(OD)  | CV <sup>1)</sup><br>(%) | Mean±SD<br>(%)   | CV <sup>1)</sup><br>(%) |
| 2005 | October  | 1.03±0.18        | 17.6                    |                  |                         |
|      | November | 1.21±0.15        | 12.7                    |                  |                         |
|      | December | 1.23±0.12        | 10.0                    |                  |                         |

Table 4-2: Continued

| Year | Month     | Tissue viability |                      | Barrier function |                         |
|------|-----------|------------------|----------------------|------------------|-------------------------|
|      |           | Mean±SD<br>(OD)  | CV <sup>1)</sup> (%) | Mean±SD<br>(%)   | CV <sup>1)</sup><br>(%) |
| 2006 | January   | 1.34±0.06        | 4.3                  |                  |                         |
|      | February  | 1.14±0.16        | 13.7                 |                  |                         |
|      | March     | 1.24±0.26        | 21.3                 |                  |                         |
|      | April     | 1.48±0.13        | 8.7                  |                  |                         |
|      | May       | 1.49±0.18        | 11.9                 |                  |                         |
|      | June      | 1.37±0.17        | 12.4                 |                  |                         |
|      | July      | 1.41±0.15        | 10.6                 |                  |                         |
|      | August    | 1.43±0.07        | 5.2                  |                  |                         |
|      | September | 1.52±0.12        | 8.1                  |                  |                         |
|      | October   | 1.93±0.09        | 4.6                  |                  |                         |
|      | November  | 1.98±0.19        | 9.7                  |                  |                         |
|      | December  | 1.68±0.13        | 7.6                  |                  |                         |
| 2007 | January   | 1.70±0.08        | 4.7                  |                  |                         |
|      | February  | 1.62±0.09        | 5.3                  |                  |                         |
|      | March     | 1.67±0.11        | 6.9                  |                  |                         |
|      | April     | 1.51±0.09        | 6.1                  |                  |                         |
|      | May       | 1.52±0.09        | 6.1                  |                  |                         |
|      | June      | 1.66±0.10        | 6.3                  |                  |                         |
|      | July      | 1.50±0.22        | 15.0                 |                  |                         |
|      | August    | 2.07±0.40        | 19.3                 |                  |                         |
|      | September | 1.48±0.18        | 12.4                 |                  |                         |
|      | October   | 1.70±0.15        | 8.6                  |                  |                         |
|      | November  | 1.92±0.19        | 9.7                  |                  |                         |
|      | December  | 1.70±0.05        | 3.2                  |                  |                         |
| 2008 | January   | 1.74±0.20        | 11.4                 |                  |                         |
|      | February  | 1.37±0.23        | 17.0                 |                  |                         |
|      | March     | 1.35±0.18        | 13.7                 |                  |                         |
|      | April     | 1.42±0.15        | 10.8                 |                  |                         |
|      | May       | 1.33±0.14        | 10.7                 |                  |                         |
|      | June      | 1.28±0.03        | 2.0                  | 0.23±0.02        | 10.1                    |
|      | July      | 1.32±0.03        | 2.3                  | 0.24±0.04        | 17.9                    |
|      | August    | 1.26±0.04        | 3.5                  | 0.27±0.01        | 5.2                     |
|      | September | 1.31±0.06        | 4.6                  | 0.28±0.03        | 9.1                     |
|      | October   | 1.55±0.06        | 3.7                  | 0.26±0.01        | 4.7                     |
|      | November  | 1.54±0.07        | 4.2                  | 0.26±0.00        | 1.1                     |
|      | December  | 1.51±0.09        | 6.1                  | 0.26±0.02        | 6.8                     |
| 2009 | January   | 1.23±0.29        | 23.8                 | 0.25±0.01        | 4.1                     |
|      | February  | 1.41±0.12        | 8.7                  | 0.25±0.01        | 5.0                     |
|      | March     | 1.45±0.09        | 6.1                  | 0.28±0.01        | 4.7                     |
|      | April     | 1.44±0.23        | 15.7                 | 0.23±0.02        | 6.6                     |
|      | May       | 1.60±0.17        | 10.8                 | 0.24±0.01        | 4.2                     |
|      | June      | 1.34±0.10        | 7.4                  | 0.26±0.04        | 15.9                    |
|      | July      | 1.36±0.07        | 4.9                  | 0.25±0.03        | 12.7                    |
|      | August    | 1.47±0.08        | 5.4                  | 0.24±0.02        | 9.0                     |
|      | September | 1.40±0.11        | 8.0                  | 0.24±0.01        | 4.7                     |

|          |           |     |           |      |
|----------|-----------|-----|-----------|------|
| October  | 1.54±0.11 | 6.9 | 0.29±0.04 | 13.6 |
| November | 1.58±0.05 | 3.4 | 0.26±0.02 | 6.1  |
| December | 1.52±0.12 | 8.0 | 0.24±0.02 | 6.8  |

Table 4-2. Continued

| Year             | Month                          | Tissue viability |                      | Barrier function |                         |
|------------------|--------------------------------|------------------|----------------------|------------------|-------------------------|
|                  |                                | Mean±SD<br>(OD)  | CV <sup>1)</sup> (%) | Mean±SD<br>(%)   | CV <sup>1)</sup><br>(%) |
| 2010             | January                        | 1.51±0.15        | 9.9                  | 0.26±0.00        | 1.6                     |
|                  | February                       | 1.60±0.12        | 7.5                  | 0.25±0.01        | 3.4                     |
|                  | March                          | 1.52±0.08        | 5.0                  | 0.28±0.03        | 9.4                     |
|                  | April                          | 1.44±0.13        | 8.9                  | 0.25±0.01        | 4.6                     |
|                  | May                            | 1.63±0.03        | 1.9                  | 0.25±0.01        | 5.1                     |
|                  | June                           | 1.58±0.12        | 7.6                  | 0.24±0.02        | 8.9                     |
|                  | July                           | 1.58±0.08        | 4.8                  | 0.25±0.01        | 4.0                     |
|                  | August                         | 1.49±0.10        | 6.8                  | 0.24±0.01        | 5.2                     |
|                  | September                      | 1.47±0.12        | 8.1                  | 0.25±0.01        | 4.1                     |
|                  | October                        | 1.42±0.08        | 5.5                  | 0.25±0.02        | 8.8                     |
| Tissue viability | October, 2005 to October, 2010 | 1.49±0.23        | 15.5                 | 0.25±0.02        | 9.2                     |
| Barrier function | June, 2008 to October, 2010    |                  |                      |                  |                         |

1) Coefficient Variation.

As shown in Table 4-2, the mean SD of tissue viability and barrier function (IC<sub>50</sub>) from the evaluation of continuous batches of LabCyte EPI-MODEL24 (with tissue viability data taken from October, 2005 to October, 2010 and barrier function data taken from June, 2008 to October, 2010) were 1.49 ± 0.23% and 0.25 ± 0.02%, respectively. The tissue viability and barrier function of LabCyte EPI-MODEL24 tissue have remained constant, indicating reproducibility (low coefficient variation (CV): 15.5% and 9.2%, respectively) and monthly consistency is high.

### 4-3. Shipment of LabCyte EPI-MODEL24 out of Japan

#### 4-3a. Purpose

To examine whether LabCyte EPI-MODEL24 is exportable, it was sent to China and viability and barrier function tests were performed.

#### 4-3b. Quality check of the exported LabCyte EPI-MODEL24

##### 1. Viability

Three LabCyte EPI-MODEL24 tissues were subjected to an MTT assay as follows. Tissues were put in the wells of 24-well plates containing 0.5 ml of MTT medium (0.5 mg/ml) and were incubated for three hours (37°C, 5% CO<sub>2</sub>, humidified atmosphere). Formazan produced in the tissues was extracted with isopropanol (300  $\mu$ l) and the extract (200  $\mu$ l) was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank. The mean OD value indicates the tissue viability for each LabCyte EPI-MODEL24 batch.

##### Acceptance criteria:

The mean OD  $\geq$  0.8

##### 2. Barrier function

The following test is performed using 15 tissue models.

To evaluate whether the stratum corneum in the LabCyte EPI-MODEL24 tissue resists the rapid penetration of the cytotoxic marker chemical sodium lauryl sulphate (SLS), the viability of the epidermis tissue was estimated in terms of the half maximal inhibitory concentration (IC<sub>50</sub>).

Various concentrations (0.1, 0.2, 0.3, and 0.4%(w/v)) of SLS (25 µl) are applied to the LabCyte EPI-MODEL24, and cell viability is measured after 18 hours by an MTT assay. All experiments are performed in triplicate. The MTT assay is performed using three tissue models and their viabilities are determined.

Acceptance criteria:

$$0.14\% \leq IC50 \leq 0.4\%$$

LabCyte EPI-MODEL24 was exported three times and it was confirmed that results were within the acceptance criteria in all batches.

**4-3c. Shipment schedule**

|               |                    |
|---------------|--------------------|
| 1st shipment: | August 24, 2009.   |
| 2nd shipment: | August 31, 2009.   |
| 3rd shipment: | September 14, 2009 |

**4-3d. Test facility**

Shanghai Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China  
 Technical Center For Animal, Plant And Food Inspection and Quarantine Head of Animal and Toxicology Lab. (Shanghai, China)

**4-3e. Results and discussion**

Shipment results are shown in Table 4-3.

**Table 4-3: Results of Shipments to China**

| Lot No.        | Shipping date | Delivery date | Transportation period |
|----------------|---------------|---------------|-----------------------|
| LCE24-090824-A | Aug. 24, 2009 | Aug. 26, 2009 | 2 days                |
| LCE24-090831-A | Aug. 31, 2009 | Sep. 2, 2009  | 2 days                |
| LCE24-090914-A | Sep. 14, 2009 | Sep. 16, 2009 | 2 days                |

The QC results for the exported LabCyte EPI-MODEL24 from the Shanghai Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China are shown in Table 4-4.

**Table 4-4: QC results for LabCyte EPI-MODEL24 exported to China**

| Lot No.        | Starting date for QC testing | Viability            |               |      | Barrier function (IC50(%)) | QC result |
|----------------|------------------------------|----------------------|---------------|------|----------------------------|-----------|
|                |                              | Tissue (570nm/650nm) | Blank (570nm) | Fold |                            |           |
| LCE24-090824-A | Aug. 26, 2009                | 0.871±0.035          | 0.040±0.000   | 21.8 | 0.32                       | Pass      |
| LCE24-090831-A | Sep. 2, 2009                 | 1.011±0.033          | 0.037±0.000   | 27.1 | 0.27                       | Pass      |
| LCE24-090914-A | Sep. 16, 2009                | 1.054±0.034          | 0.038±0.000   | 28.0 | 0.25                       | Pass      |

All three batches of the LabCyte EPI-MODEL24 shipped to China were within the QC acceptance criteria as described in the Test Protocol, suggesting that the quality of the LabCyte EPI-MODEL24 was maintained during the overseas shipment.

## **5. Optimization of the LabCyte EPI-MODEL24 SIT and assessment of the original SIT (OPTIMIZATION STAGE)**

### **5-1. Summary of the OPTIMIZATION STAGE**

The aim of the optimization study was to develop a test method able to discriminate non-irritant versus irritant test chemicals using the LabCyte EPI-MODEL24 as a “me-too“ test based on the VRM (EpiSkin™ test method) according to the ECVAM performance standard (1). The VRM consists of a 15 minute exposure period of test chemicals, followed by a rinsing step and a 42 hour post-incubation period before quantitative measurement of cell viability using an MTT reaction.

First of all, we examined a suitable exposure period for the LabCyte EPI-MODEL24 SIT and the optimized exposure period was set at 15 minutes. The performance of the original LabCyte EPI-MODEL24 SIT, which was reflected in the results of the optimization study, has been confirmed by intra-laboratory study.

After that, the original LabCyte EPI-MODEL24 SIT was evaluated for relevance and the ability to meet minimum criteria described in the ECVAM performance standards in the phase 1, 2 and 3 formal validation studies. The results of validation studies for the original LabCyte EPI-MODEL24 SIT were summarized in JSAAE REPORTS (Attachments 2,3) and then their reports were assessed by a peer review panel in OECD according to the OECD draft test guideline for skin irritation testing which was presented in 2009 (2).

### **5-2. Materials and methods**

#### **5-2a. Test chemicals**

Nineteen test chemicals shown in Table 5-1 were selected among the twenty reference chemicals of the ECVAM performance standard (1). By definition, reference chemicals are used to determine if the performance of a new *in vitro* human skin model system for skin irritation testing is comparable to that of the VRM in the ECVAM performance standard.

Unfortunately, tri-isobutyl phosphate (No.13) was not used in the examination because it was unavailable in Japan.

**Table 5-1: Selected test chemicals for the optimization of a suitable exposure period.**

| No.  | Test chemicals             |                          |                                    |                   |                             |
|------|----------------------------|--------------------------|------------------------------------|-------------------|-----------------------------|
|      | Name                       | CAS number <sup>1)</sup> | <i>In vivo</i> class <sup>2)</sup> | PII <sup>3)</sup> | Supplier                    |
| 1    | 1-bromo-4-chlorobutane     | 6940-78-9                | NI                                 | 0                 | Wako chemical               |
| 2    | diethyl phthalate          | 84-66-2                  | NI                                 | 0                 | Wako chemical               |
| 3    | di-propylene glycol        | 25265-71-8               | NI                                 | 0                 | Wako chemical               |
| 4    | naphthalen acetic acid     | 86-87-3                  | NI                                 | 0                 | Wako chemical               |
| 5    | allyl phenoxyacetate       | 7493-74-5                | NI                                 | 0.3               | Wako chemical               |
| 6    | isopropanol                | 67-63-0                  | NI                                 | 0.3               | Wako chemical               |
| 7    | 4-methyl-thio-benzaldehyde | 3446-89-7                | NI                                 | 1.0               | Wako chemical               |
| 8    | methyl stearate            | 112-61-8                 | NI                                 | 1.0               | Kanto chemical              |
| 9    | allyl heptanoate           | 142-19-8                 | NI                                 | 1.7               | Wako chemical               |
| 10   | heptyl butyrate            | 5870-93-9                | NI                                 | 1.7               | Aldrich                     |
| 11   | hexyl salicylate           | 6259-76-3                | I                                  | 2.0               | Sigma Fluka                 |
| 12   | terpinyl acetate           | 80-26-2                  | I                                  | 2.0               | Alfa Aesar                  |
| (13) | (tri-isobutyl phosphate)   | 126-71-6                 | I                                  | 2.0               | <u>Unavailable in Japan</u> |

|    |                        |          |   |     |                |
|----|------------------------|----------|---|-----|----------------|
| 14 | cyclamen aldehyde      | 103-95-7 | I | 2.3 | Wako chemical  |
| 15 | 1-decanol              | 112-30-1 | I | 2.3 | Wako chemical  |
| 16 | 1-bromohexane          | 111-25-1 | I | 2.7 | Wako chemical  |
| 17 | $\alpha$ -terpineol    | 98-55-5  | I | 2.7 | Kanto chemical |
| 18 | di-n-propyl disulphide | 629-19-6 | I | 3.0 | Wako chemical  |
| 19 | butyl methacrylate     | 97-88-1  | I | 3.0 | Wako chemical  |
| 20 | heptanal               | 111-71-7 | I | 3.3 | Kanto chemical |

1) CAS No.: Chemical abstracts service registry number.

2) I: irritant, NI: non irritant

3) PII: primary irritation index.

### 5-2b. *Experimental protocol for a suitable exposure period for the LabCyte EPI-MODEL24 SIT*

LabCyte EPI-MODEL24 tissues were shipped from the supplier on Mondays and delivered to recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 ml) and incubated overnight (37°C, 5% CO<sub>2</sub>, humidified atmosphere). On the next day, the tissues were topically exposed to the test chemicals. Liquids (25  $\mu$ l) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25  $\mu$ l sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied by using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25  $\mu$ l distilled water, and three tissues serving as positive controls were exposed to 5% SLS. After 10, 15, 20 or 30 minutes of exposure, each tissue was carefully rinsed with PBS (Invitrogen, CA, USA) ten times using a washing bottle to remove any remaining test chemical from the surface. The blotted tissues were then transferred to new wells on 24-well plates containing 1 ml of fresh assay medium.

The treated and control tissues were post-incubated for 42 hours (37°C, 5% CO<sub>2</sub>, humidified atmosphere). When the 42-hour post-incubation period was completed, blotted tissues were transferred to new wells on 24-well plates containing 0.5 ml of freshly prepared MTT medium (0.5 mg/ml) for the MTT assay. Tissues were incubated for three hours (37°C, 5% CO<sub>2</sub>, humidified atmosphere) and were then transferred to microtubes containing 300  $\mu$ l isopropanol, completely immersing the tissue. Formazan extraction was performed at room temperature and the tissues were allowed to stand overnight. Subsequently, 200  $\mu$ l extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.

The tissue viability was calculated as a percentage relative to the viability of negative controls. The mean of the three values from identically treated tissues was used to classify a chemical according to the prediction model.

In this study, the prediction model for skin irritation potential with LabCyte EPI-MODEL24 was set with reference to the conditions for the VRM (EpiSkin<sup>TM</sup> test method) described in the ECVAM performance standards. This prediction model is described in Table 5-2. In the event that the three independent results within an individual batch were not in agreement, the result that occurred twice was used.

**Table 5-2: Prediction models for the LabCyte EPI-MODEL24 SIT**

| Cell viability  | Judgment     |
|-----------------|--------------|
| mean $\leq$ 50% | Irritant     |
| mean $>$ 50%    | Non irritant |

### 5-2c. *Protocol for the original LabCyte EPI-MODEL24 SIT*

The test protocol for the LabCyte EPI-MODEL24 SIT is described in section 5-2b based on the

EpiSkin™ VRM. From the result of the investigation of a suitable exposure period (see section 5-2b and 5-3b), the exposure period in the original LabCyte EPI-MODEL24 SIT was set at 15 min. The overall test protocol is described in the original SOP for the LabCyte EPI-MODEL24 SIT (ver. 4.0).

Three independent tests were performed on different batches of the LabCyte EPI-MODEL24, with three tissues per test chemical. After the 42-hour post-incubation period was completed, conditioned medium was collected for analysis of interleukin-1 alpha (IL-1 $\alpha$ ) release according to the VRM.

The tissue viability was calculated as a percentage relative to the viability of negative controls. The mean of the three values from identically treated tissues was used to classify a chemical according to the prediction model.

The amount of IL-1 $\alpha$  release in the conditioned medium collected after 42 hours was determined using the IL-1 $\alpha$  ELISA kit (Invitrogen, CA, USA), according to the detailed instructions of the manufacturer.

In this study, the prediction model for skin irritation potential with the LabCyte EPI-MODEL24 was set in reference to the conditions for the VRM (EpiSkin™ test method) described in the ECVAM performance standards. This prediction model is described in Table 5-4. In the event that the three independent results within an individual batch were not in agreement, the result that occurred twice was used.

**Table 5-4: Prediction models for the original LabCyte EPI-MODEL24 SIT**

| Cell viability (1st) | IL-1 $\alpha$ ELISA (2nd)                         | Judgment     |
|----------------------|---|--------------|
| mean $\leq$ 50%      |   | Irritant     |
| mean $>$ 50%         | IL-1 $\alpha$ content (mean) $\geq$ 120 pg/tissue |              |
| mean $>$ 50%         | IL-1 $\alpha$ content (mean) $<$ 120 pg/tissue    | Non irritant |

### 5-3. Results

#### 5-3a. Investigation of a suitable exposure period for the LabCyte EPI-MODEL24 SIT

The results of the investigation of a suitable exposure period for the LabCyte EPI-MODEL24 SIT were evaluated by cell viability, as shown in Table 5-5.

**Table 5-5: Results obtained from the experiment with various exposure periods**

| Test chemical              |                                    | Exposure period (minutes) |                                     |                 |                                     |                 |                                     |                 |                                     |
|----------------------------|------------------------------------|---------------------------|-------------------------------------|-----------------|-------------------------------------|-----------------|-------------------------------------|-----------------|-------------------------------------|
|                            |                                    | 10                        |                                     | 15              |                                     | 20              |                                     | 30              |                                     |
| Name                       | <i>In vivo</i> class <sup>1)</sup> | viability (%)             | <i>In vitro</i> class <sup>1)</sup> | viability (%)   | <i>In vitro</i> class <sup>1)</sup> | viability (%)   | <i>In vitro</i> class <sup>1)</sup> | viability (%)   | <i>In vitro</i> class <sup>1)</sup> |
| 1-bromo-4-chlorobutane     | NI                                 | 44.0 $\pm$ 12.8           | I                                   | 49.6 $\pm$ 5.0  | I                                   | 25.3 $\pm$ 7.5  | I                                   | 14.5 $\pm$ 6.5  | I                                   |
| diethyl phthalate          | NI                                 | 93.3 $\pm$ 8.4            | NI                                  | 108.0 $\pm$ 3.9 | NI                                  | 118.5 $\pm$ 1.6 | NI                                  | 92.4 $\pm$ 12.6 | NI                                  |
| di-propylene glycol        | NI                                 | 90.3 $\pm$ 2.8            | NI                                  | 108.1 $\pm$ 6.4 | NI                                  | 95.3 $\pm$ 5.7  | NI                                  | 90.5 $\pm$ 3.3  | NI                                  |
| naphthalen acetic acid     | NI                                 | 92.4 $\pm$ 7.7            | NI                                  | 103.3 $\pm$ 1.8 | NI                                  | 100.8 $\pm$ 7.0 | NI                                  | 84.9 $\pm$ 4.2  | NI                                  |
| allyl phenoxy-acetate      | NI                                 | 87.5 $\pm$ 11.3           | NI                                  | 100.6 $\pm$ 5.4 | NI                                  | 90.7 $\pm$ 6.4  | NI                                  | 91.6 $\pm$ 2.8  | NI                                  |
| isopropanol                | NI                                 | 83.5 $\pm$ 6.3            | NI                                  | 102.1 $\pm$ 1.6 | NI                                  | 68.7 $\pm$ 11.9 | NI                                  | 9.6 $\pm$ 1.1   | I                                   |
| 4-methyl-thio-benzaldehyde | NI                                 | 15.0 $\pm$ 2.7            | I                                   | 17.2 $\pm$ 1.6  | I                                   | 10.9 $\pm$ 2.6  | I                                   | 15.5 $\pm$ 2.6  | I                                   |
| methyl stearate            | NI                                 | 94.4 $\pm$ 2.0            | NI                                  | 113.0 $\pm$ 2.6 | NI                                  | 89.6 $\pm$ 7.0  | NI                                  | 117.2 $\pm$ 4.0 | NI                                  |
| allyl heptanoate           | NI                                 | 92.9 $\pm$ 3.1            | NI                                  | 115.9 $\pm$ 8.6 | NI                                  | 83.3 $\pm$ 3.6  | NI                                  | 117.2 $\pm$ 5.4 | NI                                  |
| heptyl butyrate            | NI                                 | 95.2 $\pm$ 9.5            | NI                                  | 126.3 $\pm$ 1.2 | NI                                  | 90.9 $\pm$ 4.6  | NI                                  | 121.2 $\pm$ 5.3 | NI                                  |
| hexyl salicylate           | I                                  | 97.5 $\pm$ 5.6            | NI                                  | 107.4 $\pm$ 4.8 | NI                                  | 87.6 $\pm$ 2.4  | NI                                  | 113.4 $\pm$ 3.5 | NI                                  |
| terpinyl acetate           | I                                  | 37.7 $\pm$ 11.8           | I                                   | 52.3 $\pm$ 16.3 | NI                                  | 22.4 $\pm$ 3.3  | I                                   | 22.3 $\pm$ 3.7  | I                                   |
| cyclamen aldehyde          | I                                  | 16.9 $\pm$ 0.3            | I                                   | 16.0 $\pm$ 3.2  | I                                   | 13.6 $\pm$ 2.3  | I                                   | 14.6 $\pm$ 1.9  | I                                   |
| 1-decanol                  | I                                  | 16.1 $\pm$ 1.0            | I                                   | 19.2 $\pm$ 1.3  | I                                   | 14.2 $\pm$ 0.9  | I                                   | 15.7 $\pm$ 2.4  | I                                   |

|  |   |          |    |           |    |            |    |           |    |
|--|---|----------|----|-----------|----|------------|----|-----------|----|
| 1-bromohexane                                    | I | 92.2±4.0 | NI | 102.0±5.9 | NI | 103.5±16.3 | NI | 70.8±15.8 | NI |
| α-terpineol                                      | I | 7.9±1.5  | I  | 10.7±0.2  | I  | 10.0±0.7   | I  | 8.5±1.4   | I  |
| 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HC | I | 5.8±0.2  | I  | 5.6±0.3   | I  | 4.3±0.7    | I  | 5.1±0.7   | I  |
| di-n-propyl disulfide                            | I | 98.0±3.5 | NI | 96.7±6.0  | NI | 93.4±8.0   | NI | 90.7±4.0  | NI |
| butyl methacrylate                               | I | 73.2±7.5 | NI | 19.3±3.2  | I  | 14.6±0.6   | I  | 15.3±1.5  | I  |
| Heptanal   | I | 22.2±5.5 | I  | 12.6±4.5  | I  | 11.3±1.8   | I  | 12.1±0.7  | I  |

In the case of a 10 minute exposure period, butyl methacrylate was evaluated as a non-irritant, though it had been classified as an irritant *in vivo* (shown in red). Because the sensitivity of the prediction with a 10 minute exposure period was lower than that for the group with an exposure period of 15 minutes or longer, it was thought that a 10 minute exposure period was not long enough.

With the 30 minute exposure period, on the other hand, isopropanol was indicated as an irritant, though it had been classified as non-irritant *in vivo* (shown in blue). It was decided that the 30 minute exposure period was too long and unsuitable, because the specificity of prediction was lower than that for groups with exposure periods of 20 minutes or less.

In the case of a 15 minute exposure period, 1-bromo-4-chlorobutane (non-irritant *in vivo* class) and terpinyl acetate (irritant *in vivo* class) resulted in cell viabilities of around 50%, while both of them were apparently indicated as an irritant in the 20 minute exposure. *In vivo* skin irritancy of 1-bromo-4-chlorobutane is very low, as its primary irritation index is 0 and it was thought that the 15 minute exposure period was better than the 20 minute exposure period because the cell viability for the 15 minute exposure period was higher. Also, *in vivo* skin irritancy of terpinyl acetate is at the borderline between non-irritant and irritant, and it was thought that the 15 minute exposure period was better than the 20 minute exposure because cell viability was around 50%.

From the above considerations, it was concluded that the suitable exposure period was 15 minutes. The original LabCyte EPI-MODEL24 SIT, which reflected the optimization study for a suitable exposure period, was referred to in the SOP for the ver.4.0 of the LabCyte EPI-MODEL24 SIT.

### 5-3b. Assessment of the original LabCyte EPI-MODEL24 SIT by intra-laboratory study

Nineteen test chemicals were tested using the LabCyte EPI-MODEL24 and the common protocol described in the ECVAM performance standard. Three independent runs were performed on different batches of LabCyte EPI-MODEL24, with three tissues per test chemical (Table 5-1). When only cell viability is used as an indicator, negative predictions for three chemicals, hexyl salicylate (No. 11), 1-bromohexane (no. 16), and di-n-propyl disulphide (no. 18), were not concordant with the results of the *in vivo* classification in the group of nine irritants and also two chemicals, 1-bromo-4-chlorobutane (no. 1) and 4-methyl-thio-benzaldehyde (no. 7), were classified as positive in the group of ten non-irritants, (Table 5-6).

Table 5-6. Results of skin irritation tests by cell viability and IL-1α release evaluation.

| Test chemical | No.                    | Name | Run | The original SIT using LabCyte EPI-MODEL24 |   |      |   |                        |       |      | <i>In vitro</i> prediction by cell viability and IL-1α release <sup>1)</sup> |    |                        |    |
|---------------|------------------------|------|-----|--|---|------|---|------------------------|-------|------|--|----|------------------------|----|
|               |                        |      |     | Cell viability (%)                         |   |      | <i>In vitro</i> prediction by cell viability only <sup>1)</sup> | IL-1α(pg/tissue)       |       |      |  |    |                        |    |
|               |                        |      |     | Mean                                       | ± | SD   |   | Judgment <sup>1)</sup> |       | Mean | ±  | SD | Judgment <sup>1)</sup> |    |
| 1             | 1-bromo-4-chlorobutane | 1    | 1   | 44.8                                       | ± | 6.7  | I   | I                      | 90.5  | ±    | 4.7  | NI | I                      | I  |
|               |                        | 2    | 2   | 34.6                                       | ± | 11.8 | I   |                        | 129.5 | ±    | 13.7   | I  | I                      |    |
|               |                        | 3    | 3   | 25.1                                       | ± | 7.6  | I   |                        | 178.6 | ±    | 17.4   | I  | I                      |    |
| 2             | diethyl phthalate      | 1    | 1   | 95.4                                       | ± | 3.2  | NI  | NI                     | 29.2  | ±    | 33.0   | NI | NI                     | NI |
|               |                        | 2    | 2   | 100.7                                      | ± | 7.7  | NI  |                        | 15.5  | ±    | 5.0  | NI | NI                     |    |
|               |                        | 3    | 3   | 102.5                                      | ± | 2.5  | NI  |                        | 51.7  | ±    | 10.4   | NI | NI                     |    |

Table 5-6. Continued

| Test chemical |                            | The original SIT using LabCyte EPI-MODEL24 |                    |       |      |                         |   |                           |       |       |                         |  |    |
|---------------|----------------------------|--|--------------------|-------|------|-------------------------|---|---------------------------|-------|-------|-------------------------|--|----|
| No.           | Name                       | Run  | Cell viability (%) |       |      |                         | <i>In vitro</i> prediction by only cell viability <sup>1)</sup> | IL-1 $\alpha$ (pg/tissue) |       |       |                         | <i>In vitro</i> prediction by cell viability and IL-1 $\alpha$ release <sup>1)</sup> |    |
|               |                            |  | Mean               | $\pm$ | SD   | Judgement <sup>1)</sup> |   | Mean                      | $\pm$ | SD    | Judgement <sup>1)</sup> |  |    |
| 3             | di-propylene glycol        | 1  | 95.4               | $\pm$ | 2.8  | NI                      | NI  | 6.5                       | $\pm$ | 3.6   | NI                      | NI   | NI |
|               |                            | 2  | 103.9              | $\pm$ | 8.4  | NI                      |   | 16.7                      | $\pm$ | 6.3   | NI                      |  |    |
|               |                            | 3  | 99.0               | $\pm$ | 5.1  | NI                      |   | 16.0                      | $\pm$ | 12.1  | NI                      |  |    |
| 4             | naphthalen acetic acid     | 1  | 96.8               | $\pm$ | 5.2  | NI                      | NI  | 2.9                       | $\pm$ | 1.5   | NI                      | NI   | NI |
|               |                            | 2  | 97.0               | $\pm$ | 2.0  | NI                      |   | 5.7                       | $\pm$ | 2.9   | NI                      |  |    |
|               |                            | 3  | 101.8              | $\pm$ | 2.5  | NI                      |   | 12.0                      | $\pm$ | 8.2   | NI                      |  |    |
| 5             | allyl phenoxy-acetate      | 1  | 87.7               | $\pm$ | 5.3  | NI                      | NI  | 36.3                      | $\pm$ | 22.8  | NI                      | NI   | NI |
|               |                            | 2  | 75.1               | $\pm$ | 4.8  | NI                      |   | 115.0                     | $\pm$ | 20.3  | NI                      |  |    |
|               |                            | 3  | 99.2               | $\pm$ | 2.3  | NI                      |   | 27.8                      | $\pm$ | 6.8   | NI                      |  |    |
| 6             | isopropanol                | 1  | 86.2               | $\pm$ | 2.1  | NI                      | NI  | 57.7                      | $\pm$ | 9.9   | NI                      | NI   | NI |
|               |                            | 2  | 86.1               | $\pm$ | 1.4  | NI                      |   | 94.7                      | $\pm$ | 8.8   | NI                      |  |    |
|               |                            | 3  | 94.5               | $\pm$ | 2.6  | NI                      |   | 99.8                      | $\pm$ | 19.8  | NI                      |  |    |
| 7             | 4-methyl-thio-benzaldehyde | 1  | 25.7               | $\pm$ | 2.4  | I                       | I   | 93.9                      | $\pm$ | 10.5  | NI                      | I  | I  |
|               |                            | 2  | 22.7               | $\pm$ | 5.4  | I                       |   | 119.3                     | $\pm$ | 29.1  | NI                      |  |    |
|               |                            | 3  | 20.9               | $\pm$ | 1.2  | I                       |   | 102.9                     | $\pm$ | 2.9   | NI                      |  |    |
| 8             | methyl stearate            | 1  | 107.2              | $\pm$ | 3.2  | NI                      | NI  | 6.7                       | $\pm$ | 0.8   | NI                      | NI   | NI |
|               |                            | 2  | 110.4              | $\pm$ | 1.8  | NI                      |   | 7.9                       | $\pm$ | 1.7   | NI                      |  |    |
|               |                            | 3  | 107.1              | $\pm$ | 3.0  | NI                      |   | 5.6                       | $\pm$ | 1.2   | NI                      |  |    |
| 9             | allyl heptanoate           | 1  | 45.9               | $\pm$ | 42.4 | I                       | NI  | 495.8                     | $\pm$ | 257.3 | I                       | I  | NI |
|               |                            | 2  | 93.7               | $\pm$ | 14.4 | NI                      |   | 86.3                      | $\pm$ | 99.4  | NI                      |  |    |
|               |                            | 3  | 118.9              | $\pm$ | 5.8  | NI                      |   | 11.4                      | $\pm$ | 3.3   | NI                      |  |    |
| 10            | heptyl butyrate            | 1  | 97.2               | $\pm$ | 4.1  | NI                      | NI  | 8.8                       | $\pm$ | 3.4   | NI                      | NI   | NI |
|               |                            | 2  | 109.8              | $\pm$ | 2.7  | NI                      |   | 7.6                       | $\pm$ | 1.8   | NI                      |  |    |
|               |                            | 3  | 99.3               | $\pm$ | 3.7  | NI                      |   | 13.2                      | $\pm$ | 3.1   | NI                      |  |    |
| 11            | hexyl salicylate           | 1  | 103.1              | $\pm$ | 4.4  | NI                      | NI  | 10.6                      | $\pm$ | 4.7   | NI                      | NI   | NI |
|               |                            | 2  | 110.8              | $\pm$ | 3.2  | NI                      |   | 4.9                       | $\pm$ | 2.2   | NI                      |  |    |
|               |                            | 3  | 101.1              | $\pm$ | 4.1  | NI                      |   | 13.5                      | $\pm$ | 9.6   | NI                      |  |    |
| 12            | terpinyl acetate           | 1  | 18.0               | $\pm$ | 10.0 | I                       | I   | 293.4                     | $\pm$ | 30.3  | I                       | I  | I  |
|               |                            | 2  | 36.3               | $\pm$ | 6.5  | I                       |   | 233.6                     | $\pm$ | 83.5  | I                       |  |    |
|               |                            | 3  | 39.1               | $\pm$ | 8.1  | I                       |   | 233.6                     | $\pm$ | 83.5  | I                       |  |    |
| 14            | 1-decanol                  | 1  | 15.5               | $\pm$ | 0.8  | I                       | I   | 175.9                     | $\pm$ | 13.8  | I                       | I  | I  |
|               |                            | 2  | 21.6               | $\pm$ | 1.5  | I                       |   | 166.5                     | $\pm$ | 27.2  | I                       |  |    |
|               |                            | 3  | 17.2               | $\pm$ | 1.8  | I                       |   | 164.4                     | $\pm$ | 21.9  | I                       |  |    |
| 15            | cyclamen aldehyde          | 1  | 19.7               | $\pm$ | 1.8  | I                       | I   | 144.2                     | $\pm$ | 5.8   | I                       | I  | I  |
|               |                            | 2  | 21.8               | $\pm$ | 1.4  | I                       |   | 110.4                     | $\pm$ | 13.3  | NI                      |  |    |
|               |                            | 3  | 17.0               | $\pm$ | 3.6  | I                       |   | 173.2                     | $\pm$ | 38.1  | I                       |  |    |
| 16            | 1-bromohexane              | 1  | 16.9               | $\pm$ | 0.9  | I                       | NI  | 309.3                     | $\pm$ | 96.5  | I                       | I  | I  |
|               |                            | 2  | 59.0               | $\pm$ | 10.0 | NI                      |   | 164.8                     | $\pm$ | 33.3  | I                       |  |    |
|               |                            | 3  | 84.1               | $\pm$ | 7.9  | NI                      |   | 39.0                      | $\pm$ | 36.7  | NI                      |  |    |
| 17            | $\alpha$ -terpineol        | 1  | 12.2               | $\pm$ | 1.3  | I                       | I   | 216.8                     | $\pm$ | 20.3  | I                       | I  | I  |
|               |                            | 2  | 14.5               | $\pm$ | 0.3  | I                       |   | 167.7                     | $\pm$ | 4.8   | I                       |  |    |
|               |                            | 3  | 7.3                | $\pm$ | 0.7  | I                       |   | 239.0                     | $\pm$ | 53.4  | I                       |  |    |
| 18            | di-n-propyl disulphide     | 1  | 93.9               | $\pm$ | 10.7 | NI                      | NI  | 19.1                      | $\pm$ | 16.6  | NI                      | NI   | NI |
|               |                            | 2  | 103.3              | $\pm$ | 4.3  | NI                      |   | 6.5                       | $\pm$ | 2.6   | NI                      |  |    |
|               |                            | 3  | 104.2              | $\pm$ | 9.0  | NI                      |   | 21.4                      | $\pm$ | 15.0  | NI                      |  |    |
| 19            | butyl methacrylate         | 1  | 18.1               | $\pm$ | 11.3 | I                       | I   | 130.5                     | $\pm$ | 39.6  | I                       | I  | I  |
|               |                            | 2  | 44.9               | $\pm$ | 2.1  | I                       |   | 112.3                     | $\pm$ | 11.5  | NI                      |  |    |
|               |                            | 3  | 32.7               | $\pm$ | 2.2  | I                       |   | 148.9                     | $\pm$ | 10.0  | I                       |  |    |
| 20            | heptanal                   | 1  | 11.1               | $\pm$ | 2.1  | I                       | I   | 154.6                     | $\pm$ | 10.3  | I                       | I  | I  |
|               |                            | 2  | 14.3               | $\pm$ | 1.6  | I                       |   | 136.6                     | $\pm$ | 3.5   | I                       |  |    |
|               |                            | 3  | 9.8                | $\pm$ | 0.8  | I                       |   | 189.5                     | $\pm$ | 24.8  | I                       |  |    |

1) I: irritant, NI: non irritant

Dermal irritation is the production of reversible damage with inflammation. IL-1 $\alpha$  is a key cytokine released in the inflammation process of skin irritation. In order to evaluate whether the IL-1 $\alpha$  release could be used as an assay to predict the irritation potential of the test chemicals, we measured the IL-1 $\alpha$  content in the conditioned medium from tissues onto which the chemicals were applied. The results of the IL-1 $\alpha$  assay were used as a second endpoint to support the classification based on the MTT assay results. In this assay, 1-bromohexane (no. 16) was classified as an irritant based on the complementary evaluation of IL-1 $\alpha$  release, although it was classified as a non-irritant in the MTT assay (Table 5-6).

Summarizing the above data, the sensitivity and specificity of predictions improved to 77.8% and 80% (Table 5-7). The overall accuracy was 78.9% (Table 5-7).

**Table 5-7: Contingency table for skin irritation tests by cell viability and IL-1 $\alpha$  release evaluation**

|                            |              | <i>In vivo</i> classification |              |       |
|----------------------------|--------------|-------------------------------|--------------|-------|
|                            |              | Irritant                      | Non-irritant | Total |
| <i>In vitro</i> prediction | Irritant     | 7                             | 2            | 9     |
|                            | Non-irritant | 2                             | 8            | 10    |
|                            | Total        | 9                             | 10           | 19    |
| Sensitivity (%)            |              | 77.8                          |              |       |
| Specificity (%)            |              | 80.0                          |              |       |
| Accuracy (%)               |              | 78.9                          |              |       |

#### 5-4. Discussion

The first purpose of the optimization stage is to set a suitable exposure period for the LabCyte EPI-MODEL24 SIT similar to VRM, as a “me-too” test. From the results of examining various exposure periods ranging from 10 to 30 min, it was judged that the exposure period of 15 minutes was the optimal period for the LabCyte EPI-MODEL24 SIT. The original LabCyte EPI-MODEL24 SIT, which reflected an optimization study of a suitable exposure period, was referred to in the SOP for ver.4.0 of the LabCyte EPI-MODEL24 SIT.

The second aim of this study was to evaluate whether the commonly used irritation protocols described in the ECVAM performance standards (1) were applicable to the original LabCyte EPI-MODEL24 SIT. To compare the performances of the original LabCyte EPI-MODEL24 SIT and VRM, we tested with the 19 test chemicals described in the ECVAM performance standards. Only the prediction for 1-bromohexane differed between the original LabCyte EPI-MODEL24 SIT and VRM, when viability was the endpoint used as an indicator for prediction. This chemical was erroneously predicted as a non-irritant by the LabCyte original EPI-MODEL24 SIT, whereas VRM classified it in concordance with the *in vivo* class. As demonstrated in a previous study (32), the IL-1 $\alpha$  assay can improve prediction and support the viability assay. In this study of the original LabCyte EPI-MODEL24 SIT, the prediction for 1-bromohexane was improved, so that it was truly shown as an irritant through the combination of the MTT and IL-1 $\alpha$  assays. As a result of this improvement, the sensitivity and the specificity of this prediction model using the LabCyte EPI-MODEL24 fulfilled both conditions described in the ECVAM performance standards: the sensitivity of a “me-too” test must be equal to or higher than 70% and the specificity equal to or higher than 80%. The ECVAM performance standard must demonstrate similarity and/or equivalence to the original LabCyte EPI-MODEL24 SIT in a formal inter-laboratory study, on the basis of the VRM. From these considerations, the original LabCyte EPI-MODEL24 SIT was evaluated in a validation study under blind conditions and supported by the Japanese Society for Alternatives to Animal Experiments (JSAAE).

The phase 1 (confirmation of technical transfer) and phase 2 (multisite ) validation studies for the original SIT using the LabCyte EPI-MODEL24 were performed according to the ECVAM performance

standards. During the phase 1 and phase 2 validation studies, assay acceptance criteria regarding negative and positive control for the original LabCyte EPI-MODEL24 SIT were set based on the results of the phase 1 validation study. The acceptance criteria are referred to in the SOP for ver.5.0 of the LabCyte EPI-MODEL24 SIT. Details of the results of the validation study are described in the JSAAE report “VALIDATION STUDY OF *IN VITRO* SKIN IRRITATION TEST USING LABCYTE EPI-MODEL24” ([Attachment 2](#)). The conclusion described in the validation report is as follows:

Based on the EU classification, 9 irritants (one skin irritant could not be purchased in Japan) and 10 non-irritants in the ECVAM Performance Standards were tested by the same 7 labs. The assay demonstrated acceptable reliability of the positive control (100%) and accuracy (71% overall accuracy, 64% overall sensitivity, 79% overall specificity) in the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants. In addition, the IL-1 $\alpha$  endpoint was determined to be unnecessary.

Because it was decided that the IL-1 $\alpha$  endpoint was not necessary after the phase 2 validation study, this conclusion was reflected in the SOP for ver.6.01 of the LabCyte EPI-MODEL24 SIT ([Attachment 4](#)).

In the ECVAM performance standards (updated) ([28](#)) that were revised in 2009, the *in vivo* classification of test chemicals was changed from referring to the EU DSD (European Classification System based on the Dangerous Substance Directive) to referring to the UN GHS (United Nations Globally Harmonised System of Classification and Labelling of Chemicals). This change was reflected in the OECD draft test guideline for skin irritation ([2](#)). With the changes for *in vivo* criteria, the set of reference chemicals described in the performance standard (updated) or the OECD draft test guideline was updated, and 6 chemicals were replaced. In line with these changes, the phase 3 validation study of the original SIT using LabCyte EPI-MODEL24 was performed with newly replaced 6 reference chemicals. Details of the results for the phase 3 validation study are described in the JSAAE report “VALIDATION STUDY OF *IN VITRO* SKIN IRRITATION TEST USING LABCYTE EPI-MODEL24 (2ND REPORT)” ([Attachment 3](#)). The content of the conclusion described in the validation report is as follows:

Based on the GHS-EU classification, 5 irritants and 1 non-irritant in the new ECVAM Performance Standards were tested by 6 labs using LabCyte EPI-MODEL24. The assay demonstrated high reliability with and without laboratories in the MTT assay, for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

The validation reports ([Attachment 2,3](#)) were assessed by a peer review panel in OECD. Unfortunately, the OECD peer review panel pointed out that the prediction results of the chemical 1-bromohexane, which is an *in vivo* Category 2 substance, in the original LabCyte EPI-MODEL24 SIT, did not meet the specific requirement of paragraph 12, Annex 2 of the OECD draft test guideline ([2](#)). Miss-classification of 1-bromohexane was not allowed in the OECD draft test guideline. However, the chemical 1-bromohexane was classified as a false-negative by five out of six laboratories in the validation exercise for the original LabCyte EPI-MODEL24 SIT. Therefore, a trial for the improvement of the original LabCyte EPI-MODEL24 SIT to solve this problem became indispensable.

## **6. Modification of the original LabCyte EPI-MODEL24 SIT and assessment of the modified SIT (MODIFICATION STAGE)**

### **6-1. Summary of the MODIFICATION STAGE**

As recommended by the OECD peer review panel, improvement of the original LabCyte EPI-MODEL24 SIT to solve the problem that 1-bromohexane was shown as a false-negative is indispensable in enabling its use as a “me-too” test similar to VRM (see Section 5). It is surmised that one reason 1-bromohexane showed a false negative in the original LabCyte EPI-MODEL24 SIT (SOP ver.6.01) is because 1-bromohexane did not remain in the reconstructed human epidermis tissues at the post-incubation for 42 hours. In order to solve the 1-bromohexane problem, we tried to find a condition where the 1-bromohexane would be retained in the stratum corneum of the LabCyte EPI-MODEL24 tissues at the post incubation. More concretely, the following item were examined.

- a. Applied amount of test chemical.
- b. Incubation temperature at exposure to the test chemicals.
- c. Washing protocol for test chemicals on the tissue surface.

The 1-bromohexane problem was not solved by modifying chemical application, such as changing the amount of test chemicals applied or changing the temperature of incubation at exposure to the test chemicals. Only by modifying the washing protocol, were we able to solve the 1-bromohexane problem. The modified washing protocol that solved the 1-bromohexane problem was reflected in the SOP for ver.8.2 of the LabCyte EPI-MODEL24 SIT.

When the predictive potency of the modified LabCyte EPI-MODEL24 SIT was evaluated with the 20 test chemicals which were listed in the new OECD TG 439 for *in vitro* skin irritation testing, adopted in July, 2010, the sensitivity, the specificity and the overall accuracy were 90 %, 70 %, and 80%, respectively. It was thought that these results met the acceptance criteria described in the OECD TG 439. Furthermore, a catch-up validation study was performed with the modified LabCyte EPI-MODEL24 SIT. Results demonstrated high reliability and acceptable accuracy in the MTT assay, for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

### **6-2. Materials and methods**

#### ***6-2a. Reference chemicals described in OECD TG 439***

Twenty reference test chemicals shown in Table 6-1 were selected from the list in OECD TG 439 ([Attachment 1](#)). Reference chemicals are used to determine the reliability and accuracy of a proposed similar or modified test method, proven to be sufficiently similar to the VRM, both structurally and functionally.

**Table 6-1: Reference chemicals list in the OECD TG 439**

| No. | Name  | Test chemical            |                                    |                   | Supplier       |
|-----|---|--------------------------|------------------------------------|-------------------|----------------|
|     |   | CAS number <sup>1)</sup> | <i>In vivo</i> class <sup>2)</sup> | PII <sup>3)</sup> |                |
| 1   | 1-bromo-4-chlorobutane                            | 6940-78-9                | NI                                 | 0                 | Wako chemical  |
| 2   | Diethyl phthalate                                 | 84-66-2                  | NI                                 | 0                 | Wako chemical  |
| 3   | naphthalen acetic acid                            | 86-87-3                  | NI                                 | 0                 | Wako chemical  |
| 4   | allyl phenoxy-acetate                             | 7493-74-5                | NI                                 | 0.3               | Wako chemical  |
| 5   | isopropanol                                       | 67-63-0                  | NI                                 | 0.3               | Wako chemical  |
| 6   | 4-methyl-thio-benzaldehyde                        | 3446-89-7                | NI                                 | 1.0               | Wako chemical  |
| 7   | methyl stearate                                   | 112-61-8                 | NI                                 | 1.0               | Kanto chemical |
| 8   | heptyl butyrate                                   | 5870-93-9                | NI                                 | 1.7               | Aldrich        |
| 9   | hexyl salicylate                                  | 6259-76-3                | NI                                 | 2.0               | Sigma Fluka    |
| 10  | Cinnamaldehyde                                    | 104-55-2                 | NI                                 | 2.0               | Aldrich        |
| 11  | 1-decanol   | 112-30-1                 | I                                  | 2.3               | Wako chemical  |
| 12  | Cyclamen aldehyde                                 | 103-95-7                 | I                                  | 2.3               | Wako chemical  |
| 13  | 1-bromohexane                                     | 111-25-1                 | I                                  | 2.7               | Wako chemical  |
| 14  | 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl | 86604-75-3               | I                                  | 2.7               | Wako chemical  |
| 15  | di-n-propyl disulphide                            | 629-19-6                 | I                                  | 3.0               | Wako chemical  |
| 16  | Potassium Hydroxide 5%                            | 1310-58-3                | I                                  | 3.0               | Wako chemical  |
| 17  | Benzene thiol 5-(1,1-dimethylethyl)-2-methyl      | 7340-90-1                | I                                  | 3.3               | Tokyo Chemical |
| 18  | 1-methyl-3-phenyl-1-piperazine                    | 5271-27-2                | I                                  | 3.3               | Tokyo Chemical |
| 19  | heptanal  | 111-71-7                 | I                                  | 3.3               | Kanto chemical |
| 20  | 1,1,1 Trichloroethane                             | 71-55-6                  | I                                  | 4.0               | Wako chemical  |

1) CAS No.: Chemical abstracts service registry number.

2) I: irritant, NI: non irritant

3) PII: primary irritation index.

### 6-2b. Wide-range of test chemicals

Fifty-four test chemicals over a wide range of classes were used for evaluation of the Modified LabCyte EPI-MODEL SIT, as shown in Table 6-2. These chemicals were referred to in the report of Kandárová et. Al (33). This selected test set consists of chemicals tested in EpiDerm and EPISKIN optimization studies preceding the ECVAM skin irritation validation study and also includes chemicals from the ECVAM validation study (32). Available information on irritation potential and classification according to the UN GHS systems are provided in Table 6-2.

**Table 6-2: Fifty-four test chemicals over a MTT of classes**

| No. | Test chemicals                            |                          |                                    |                   |                  |
|-----|---|--------------------------|------------------------------------|-------------------|------------------|
|     | Name                                      | CAS number <sup>1)</sup> | <i>In vivo</i> class <sup>2)</sup> | PII <sup>3)</sup> | Supplier         |
| 1   | 1-bromo-4-chlorobutane                    | 6940-78-9                | No Cat.                            | 0                 | Wako chemical    |
| 2   | diethyl phthalate                         | 84-66-2                  | No Cat.                            | 0                 | Wako chemical    |
| 3   | di-propylene glycol                       | 25265-71-8               | No Cat.                            | 0                 | Wako chemical    |
| 4   | naphthalen acetic acid                    | 86-87-3                  | No Cat.                            | 0                 | Wako chemical    |
| 5   | 3-chloronitrobenzene                      | 121-73-3                 | No Cat.                            | 0                 | LGC<br>STANDARDS |
| 6   | 3,3-dithiodipropionic acid                | 1119-62-6                | No Cat.                            | 0                 | Wako chemical    |
| 7   | 4,4-methylenebis(2,6-di-tert-butylphenol) | 118-82-1                 | No Cat.                            | 0                 | Tokyo Chemical   |
| 8   | 4-amino-1,2,4-triazole                    | 584-13-4                 | No Cat.                            | 0                 | Wako chemical    |
| 9   | benzyl benzoate                           | 120-51-4                 | No Cat.                            | 0                 | Wako chemical    |
| 10  | sodium bicarbonate                        | 144-55-8                 | No Cat.                            | 0                 | Kanto chemical   |
| 11  | erucamide                                 | 112-84-5                 | No Cat.                            | 0                 | Wako chemical    |
| 12  | 1,5-hexadiene                             | 592-42-7                 | No Cat.                            | 0                 | Aldrich          |
| 13  | Polyethylene glycol 400                   | 25322-68-3               | No Cat.                            | 0                 | Wako chemical    |
| 14  | glycerol                                  | 56-81-5                  | No Cat.                            | 0                 | Wako chemical    |
| 15  | 3,3-dimethylpentane                       | 562-49-2                 | No Cat.                            | 0                 | Aldrich          |
| 16  | allyl phenoxy-acetate                     | 7493-74-5                | No Cat.                            | 0.3               | Wako chemical    |
| 17  | isopropanol                               | 67-63-0                  | No Cat.                            | 0.3               | Wako chemical    |
| 18  | benzyl salicylate                         | 118-58-1                 | No Cat.                            | 0.3               | Wako chemical    |
| 19  | lauric acid                               | 143-07-7                 | No Cat.                            | 0.3               | Kanto chemical   |
| 20  | 4-methyl-thio-benzaldehyde                | 3446-89-7                | No Cat.                            | 1.0               | Wako chemical    |
| 21  | methyl stearate                           | 112-61-8                 | No Cat.                            | 1.0               | Kanto chemical   |
| 22  | benzyl acetate                            | 140-11-4                 | No Cat.                            | 1.0               | Wako chemical    |
| 23  | hydroxycitronellal                        | 107-75-5                 | No Cat.                            | 1.0               | Wako chemical    |
| 24  | isopropyl myristate                       | 110-27-0                 | No Cat.                            | 1.0               | Aldrich          |
| 25  | isopropyl palmitate                       | 142-91-6                 | No Cat.                            | 1.0               | Wako chemical    |
| 26  | n-butyl propionate                        | 590-01-2                 | No Cat.                            | 1.0               | Wako chemical    |
| 27  | sodium bisulphite                         | 7631-90-5                | No Cat.                            | 1.0               | Kanto chemical   |
| 28  | benzyl alcohol                            | 100-51-6                 | No Cat.                            | 1.3               | Aldrich          |
| 29  | allyl heptanoate                          | 142-19-8                 | No Cat.                            | 1.7               | Wako chemical    |
| 30  | heptyl butyrate                           | 5870-93-9                | No Cat.                            | 1.7               | Aldrich          |

Table 6-2. Continued

| No. | Name   | Test chemicals           |                                    |                   |                |
|-----|--|--------------------------|------------------------------------|-------------------|----------------|
|     |  | CAS number <sup>1)</sup> | <i>In vivo</i> class <sup>2)</sup> | PII <sup>3)</sup> | Supplier       |
| 31  | 2-ethoxy ethyl methacrylate                      | 2370-63-0                | No Cat.                            | 1.7               | Wako chemical  |
| 32  | hexyl salicylate                                 | 6259-76-3                | No Cat.<br>(Cat. 3)                | 2.0               | Sigma Fluka    |
| 33  | linalyl acetate                                  | 111-95-7                 | No Cat.<br>(Cat. 3)                | 2.0               | Wako chemical  |
| 34  | terpinyl acetate                                 | 80-26-2                  | No Cat.<br>(Cat. 3)                | 2.0               | Alfa Aesar     |
| 35  | Linalool   | 78-70-6                  | No Cat.<br>(Cat. 3)                | 2.0               | Kanto chemical |
| 36  | cinnamaldehyde                                   | 104-55-2                 | No Cat.<br>(Cat. 3)                | 2.0               | Aldrich        |
| 37  | eugenol  | 97-53-0                  | No Cat.<br>(Cat. 3)                | 2.0               | Wako chemical  |
| 38  | cyclamen aldehyde                                | 103-95-7                 | Cat. 2                             | 2.3               | Wako chemical  |
| 39  | 1-decanol  | 112-30-1                 | Cat. 2                             | 2.3               | Wako chemical  |
| 40  | 1-bromohexane                                    | 111-25-1                 | Cat. 2                             | 2.7               | Wako chemical  |
| 41  | $\alpha$ -terpineol                              | 98-55-5                  | Cat. 2                             | 2.7               | Kanto chemical |
| 42  | 1-bromopentane                                   | 110-53-2                 | Cat. 2                             | 2.7               | Kanto chemical |
| 43  | 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HC | 86604-75-3               | Cat. 2                             | 2.7               | Wako chemical  |
| 44  | butyl methacrylate                               | 97-88-1                  | Cat. 2                             | 3.0               | Wako chemical  |
| 45  | di-n-propyl disulphide                           | 629-19-6                 | Cat. 2                             | 3.0               | Wako chemical  |
| 46  | potassium hydroxide 5%                           | 1310-58-3                | Cat. 2                             | 3.0               | Wako chemical  |
| 47  | heptanal   | 111-71-7                 | Cat. 2                             | 3.3               | Kanto chemical |
| 48  | benzene thiol, 5-(1,1-dimethylethyl)-2-methyl    | 7340-90-1                | Cat. 2                             | 3.3               | Tokyo Chemical |
| 49  | 1-methyl-3-phenyl-1-piperazine                   | 5271-27-2                | Cat. 2                             | 3.3               | Tokyo Chemical |
| 50  | SLS (20% aq)                                     | 151-21-3                 | Cat. 2                             | 4.0               | Wako chemical  |
| 51  | 1,1,1 trichloroethane                            | 71-55-6                  | Cat. 2                             | 4.0               | Wako chemical  |
| 52  | tetrachloroethylene                              | 127-18-4                 | Cat. 2                             | 4.0               | Wako chemical  |
| 53  | capric acid (decanoic acid)                      | 334-48-5                 | Cat. 2                             | 4.0               | Wako chemical  |
| 54  | SLS (5% aq)                                      | 127-18-4                 | Cat. 2                             |                   | Wako chemical  |

1) CAS No.: Chemical abstracts service registry number.

2) No Cat.: No Category, Cat. 2: Category 2, Cat. 3: Category 3

3) PII: primary irritation index.

#### 6-2c. Test Protocol for modification of the original LabCyte EPI-MODEL24 SIT

The test protocol for modification of the original LabCyte EPI-MODEL24 SIT is described in section 5-2b. From the conclusion that an IL-1 $\alpha$  endpoint was determined to be unnecessary in the validation studies, the content of IL-1 $\alpha$  was not analyzed in this study. Three independent tests were performed on different batches of LabCyte EPI-MODEL24, with three tissues per test chemical. The overall test protocol is described in the original SOP for the LabCyte EPI-MODEL24 SIT (ver. 6.01).

#### 6-2d. Protocol of the modified LabCyte EPI-MODEL24 SIT (SOP ver.8.2)

Prediction of the skin irritation potential of test chemicals by the modified LabCyte EPI-MODEL24 SIT was performed according to the modified protocol described in the SOP for ver.8.2 and described as

follows. LabCyte EPI-MODEL24 tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 ml) and incubated overnight (37°C, 5% CO<sub>2</sub>, humidified atmosphere). On the next day, the tissues were topically exposed to the test chemicals. Liquids (25 µl) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25 µl sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25 µl distilled water, and three tissues serving as positive controls were exposed to 5% SLS. After 15 minutes of exposure, each tissue was carefully rinsed with PBS at least ten times or more with a washing bottle to completely remove remaining test chemical from the surface. Furthermore, the leftover PBS outside the culture insert was gently removed with a sterile cotton bud, but the residue of PBS inside the insert was not touched. The blotted tissues were then transferred to new wells on 24-well plates containing 1 ml of fresh assay medium.

The treated and control tissues were post-incubated for 42 hours (37°C, 5% CO<sub>2</sub>, humidified atmosphere). When the 42-hour post-incubation period was completed, blotted tissues were transferred to new wells on 24-well plates containing 0.5 ml of freshly prepared MTT medium (0.5 mg/ml) for the MTT assay. Tissues were incubated for three hours (37°C, 5% CO<sub>2</sub>, humidified atmosphere) and were then transferred to microtubes containing 300 µl isopropanol, completely immersing the tissue. Formazan extraction was performed at room temperature and the tissues were allowed to stand overnight. Subsequently, 200 µl extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.

Three independent tests were performed on different batches of LabCyte EPI-MODEL24, with three tissues per a test chemical. The prediction model is shown in Table 6-3. In the event that the three independent results within an individual batch were not in agreement, the result that occurred twice was used.

**Table 6-3: The prediction models for the LabCyte EPI-MODEL24 SIT**

| Cell viability | Judgment     |
|----------------|--------------|
| mean ≤ 50%     | Irritant     |
| mean > 50%     | Non irritant |

#### **6-2e. Detecting chemical interference with MTT endpoints and correction procedures**

A possible limitation of this skin irritation protocol described in OECD TG 439 might be due to the effect of a small amount of test chemicals on the MTT endpoints directly. The following two types of test chemicals can interfere with the MTT assay.

- A. Chemicals that stain epidermis tissue.
- B. Chemicals that can directly reduce MTT.

Test chemicals that stain the epidermis tissue could transfer from the epidermis tissue to the extraction solution and affect the optical density (OD) measurements. Test chemicals that can directly reduce MTT can also affect the optical density (OD) measurements, if the test chemical is present in the epidermis tissues when the MTT viability test is performed. The overall test protocol is described in the original SOP for the LabCyte EPI-MODEL24 SIT (ver. 8.2).

- A. Detection of chemicals that stain the tissue

##### Step 1 (preliminary test)

Twenty-five µl (Liquid) or 25mg (Solid) of the test chemical was added to wells on a 24-well assay plate preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as a control. The mixture was incubated in a CO<sub>2</sub> incubator for 15 minutes. After incubation, the

mixture was evaluated for staining of the distilled water macroscopically. If the colour of the solution changes significantly, the test chemical is presumed to have the potential to stain the tissue and a functional check on viable tissues (Step2) should be performed. If the colour of the solution does not change significantly, it is determined that the test chemical has no potential to stain the tissue.

Step 2 (Functional check on viable tissue)

Twenty-five µl (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the distilled water (Step 1), was added onto the surface of the epidermis tissues. Distilled water is used as a negative control. All procedures of the original SIT using LabCyte EPI-MODEL24 described in section 6-2d were performed. However, the tissue was incubated for 3 hours in a culture medium without MTT instead of incubating in a medium containing MTT, in order to evaluate the staining of the epidermis tissues. The ratio of staining by a test chemical compared with the negative control was calculated. If the ratio of staining by a test chemical is <5%, correction of the results is not necessary. If the ratio is between 5% and 30%, the corrected MTT OD is calculated. If the ratio of staining by a test chemical is >30%, the test chemical must be considered incompatible with the test. However, when the cell viability (%) is <50%, the test chemical is determined as an irritant. Therefore, correction of the results or determination of incompatibility of the test chemical is not necessary in this case.

B. Detection of chemicals that directly reduce MTT

Step 3 (preliminary test)

Twenty-five µl (Liquid) or 25mg (Solid) of the test chemical was added to wells on a 24-well assay plate preliminarily filled with 0.5mL of MTT medium. Untreated MTT medium is used as a control. The mixture was incubated in a CO<sub>2</sub> incubator for 1 hour. After incubation, the mixture was evaluated for the staining of the MTT medium macroscopically. If the MTT medium turns significantly blue/purple, the test chemical can reduce MTT and an additional functional check (Step 4) must be performed. When the color of the solution does not change significantly, the test chemical is determined not to have the potential to reduce MTT.

Step 4 (Functional check on viable tissue)

Twenty-five µl (Liquid) or 25mg (Solid) of a test chemical, which clearly changed the color of the MTT medium to blue/purple (Step3), was added onto the surface of the epidermis tissues. Distilled water is used as a negative control. All procedures of the original SIT using LabCyte EPI-MODEL24 described in section 6-2d were performed. However, epidermis tissues that have been freeze-killed at -20 °C or lower for more than 24 hours were used instead of viable epidermis tissues. The ratio of staining by a test chemical compared with the negative control was calculated. When the ratio of staining by a test chemical is <30%, the corrected MTT OD is calculated. When the ratio of staining by a test chemical is >30%, the test chemical must be considered incompatible with the test. However, when the cell viability (%) is <50%, the test chemical is determined as an irritant. Therefore, correction of the results or determination of incompatibility of the test chemical is not necessary in this case.

### 6-3. Results

#### 6-3a. Modification of the original LabCyte EPI-MODEL24 SIT

First of all, in order to promote the penetration of 1-bromohexane to the stratum corneum, an increase in the applied amount of test chemical was examined. The applied amount of test chemical was 25 $\mu$ L in the original LabCyte EPI-MODEL24 SIT (SOP ver.6.01). Unfortunately, when the applied amount of 1-bromohexane was increased from 25 $\mu$ L to 50 $\mu$ L or 100 $\mu$ L, the cell viability of each tissue did not change at all (Fig.6-1). From these results, it was concluded that the 1-bromohexane problem could not be solved by changing the applied amount of test chemical.

For a purpose similar to the experiment with the applied amount, changing the temperature of incubation at exposure of the test chemical was examined. The incubation temperature at chemical exposure was room temperature in the original LabCyte EPI-MODEL24 SIT (SOP ver.6.01). 1-bromohexane was judged as a skin irritant with incubation at 37°C for chemical exposure (Fig.6-2). However, isopropanol, which is a non irritant chemical in UN-GHS, showed a false-positive under the same condition (Fig.6-2). In the trial with the change in incubation temperature to 37°C at exposure to the test chemicals, the 1-bromohexane problem was solved, but test chemicals that showed a false-positive increased, and the specificity of the LabCyte EPI-MODEL24 SIT decreased.

Therefore, it was thought that the 1-bromohexane problem could not be solved by changing the incubation temperature at exposure to the test chemicals.

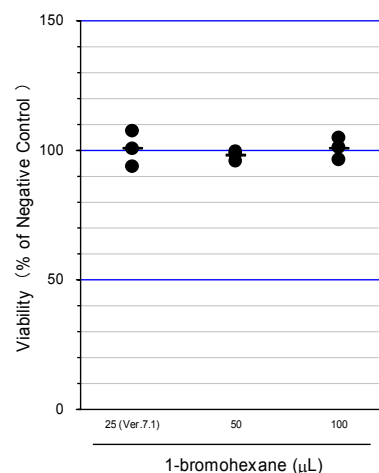


Fig-6-1 Influence of applied amount of chemical on cell viability of LabCyte EPI-MODEL24

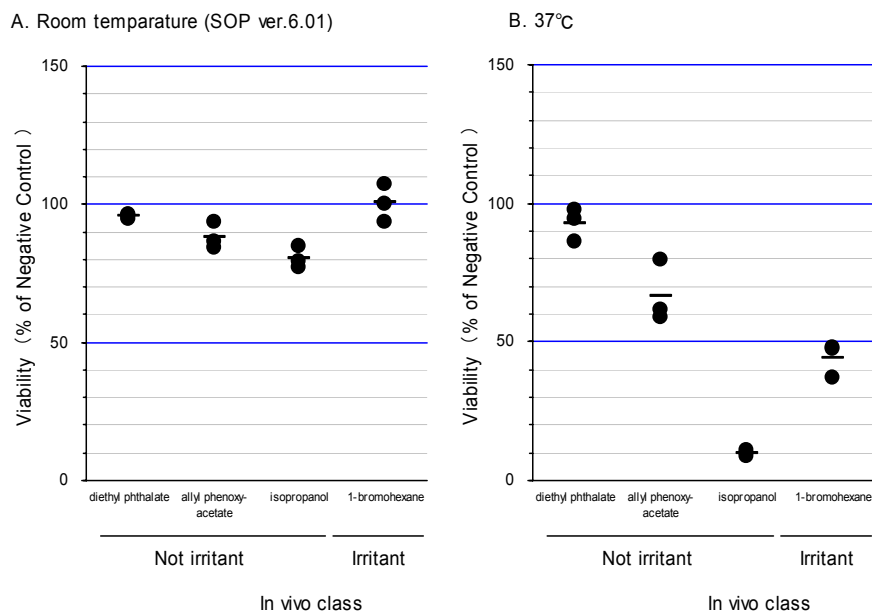


Fig.6-2 Influence of change in incubation temperature on the cell viability of LabCyte EPI-MODEL24

On the other hand, in order to retain chemical penetration into the stratum corneum, we modified the washing protocol as follows:

1. The PBS stream from the washing bottle doesn't hit directly on the tissue surface, but it hits the wall of the cell culture insert and the tissue surface is washed by the PBS current.
2. PBS inside the cell culture insert may be removed by gently tapping only once at the top of the beaker after each rinsing if necessary.
3. When removing the leftover PBS from the culture insert with a cotton bud, the inner side of the cell culture insert is not used, in order to prevent test chemicals that have penetrated the stratum corneum from being extracted by the cotton pad.

With the modification of the washing protocol, 1-bromohexane was then judged as a skin irritant (Fig.6-3). On the other hand, diethyl phthalate, allyl phenoxy-acetate, and isopropanol, which are all non irritant chemicals in the UN-GHS classification, were judged as non irritants under the same conditions (Fig.6-3).

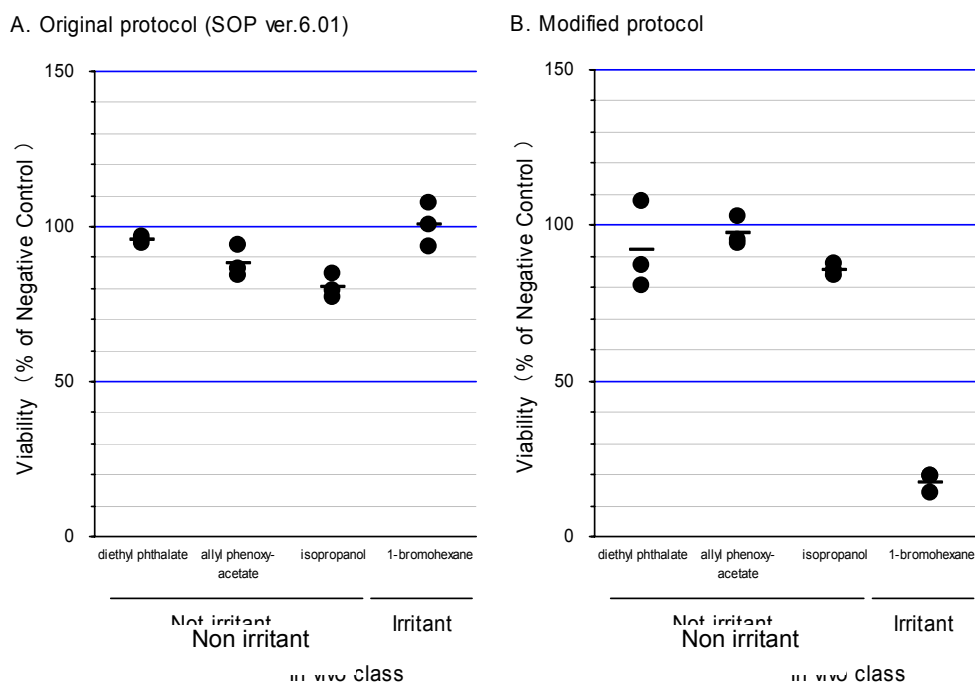


Fig.6-3 Influence of change to washing protocol on cell viability of LabCyte EPI-MODEL24

From these results, it was suggested that the 1-bromo-hexane problem could be solved by modifying the washing protocol.

Therefore, the modified washing protocol that where 1-bromo-hexane could be judged as a skin irritant was reflected in the SOP for ver.8.2 of the LabCyte EPI-MODEL24 SIT ([Attachment 7](#)).

Points in the washing protocol from the SOP for ver.6.01 which were modified in the SOP for ver.8.2 are shown in Table 6-3.

**Table 6-4: Washing protocol modifications from the ver. 6.01 SOP to the ver.8.2 SOP**

| Modifications                             | SOP ver.6.01 (Original SIT)   | SOP ver.8.2 (Modified SIT)   |
|---|---|--|
| 1. The PBS stream from the washing bottle | There were no instructions about the PBS stream.  | It was instructed that the PBS stream must avoid hitting directly on the tissue surface.   |
| 2. Removal of PBS by tapping              | Instructions stated that one must tap as much as possible after each rinsing.                                   | It was instructed to tap only once if necessary  |
| 3. How to use the cotton pad              | Instructions stated that the cotton pad should be used on the inner and outer sides of the cell culture insert. | It was instructed to use the cotton pad only on the outer side of the cell culture insert. |

**6-3b. Assessment of the modified LabCyte EPI-MODEL24 SIT based on the OECD TG 439 for intra-laboratory study**

For 10 irritant chemicals (no.11 to no.20) *in vivo*, the results for di-n-propyl disulphide (no. 15) were not concordant with those of the *in vivo* classification (Table 6-4). 1-bromo-hexane (no.13) was judged as an irritant as shown by the modified LabCyte EPI-MODEL24 SIT (Table 6-4). On the other hand, for

10 non-irritants (from no.1 to no.10), 3 chemicals, 1-bromo-4-chlorobutane (no. 1), 4-methyl-thio-benzaldehyde (no. 6) and cinnamaldehyde (no. 10) were classified as positive by the modified LabCyte EPI-MODEL24 SIT (Table 6-4).

**Table 6-5: Result of the modified LabCyte EPI-MODEL24 SIT with 20 test chemicals listed in OECD TG 439.**

| Test chemical |                            | The modified LabCyte EPI-MODEL24 SIT |                    |   |      |                       |    |
|---------------|----------------------------|--------------------------------------|--------------------|---|------|-----------------------|----|
| no.           | Name                       | Run                                  | Cell viability (%) |   |      | <i>In vitro</i> class |    |
|               |                            |                                      | Mean               | ± | SD   | Decision              |    |
| 1             | 1-bromo-4-chlorobutane     | 1                                    | 17.3               | ± | 4.4  | I                     | I  |
|               |                            | 2                                    | 23.7               | ± | 1.5  | I                     |    |
|               |                            | 3                                    | 18.6               | ± | 2.1  | I                     |    |
| 2             | diethyl phthalate          | 1                                    | 104.3              | ± | 4.4  | NI                    | NI |
|               |                            | 2                                    | 103.1              | ± | 1.5  | NI                    |    |
|               |                            | 3                                    | 55.4               | ± | 13.8 | NI                    |    |
| 3             | naphthalen acetic acid     | 1                                    | 92.7               | ± | 1.2  | NI                    | NI |
|               |                            | 2                                    | 107.8              | ± | 2.0  | NI                    |    |
|               |                            | 3                                    | 98.5               | ± | 3.8  | NI                    |    |
| 4             | allyl phenoxy-acetate      | 1                                    | 94.7               | ± | 1.8  | NI                    | NI |
|               |                            | 2                                    | 96.3               | ± | 3.6  | NI                    |    |
|               |                            | 3                                    | 56.0               | ± | 3.8  | NI                    |    |
| 5             | isopropanol                | 1                                    | 83.5               | ± | 5.6  | NI                    | NI |
|               |                            | 2                                    | 87.7               | ± | 2.8  | NI                    |    |
|               |                            | 3                                    | 82.7               | ± | 8.5  | NI                    |    |
| 6             | 4-methyl-thio-benzaldehyde | 1                                    | 24.6               | ± | 5.8  | I                     | I  |
|               |                            | 2                                    | 21.4               | ± | 1.1  | I                     |    |
|               |                            | 3                                    | 21.8               | ± | 0.8  | I                     |    |
| 7             | methyl stearate            | 1                                    | 98.6               | ± | 4.8  | NI                    | NI |
|               |                            | 2                                    | 113.1              | ± | 4.5  | NI                    |    |
|               |                            | 3                                    | 101.5              | ± | 4.9  | NI                    |    |
| 8             | heptyl butyrate            | 1                                    | 105.9              | ± | 2.0  | NI                    | NI |
|               |                            | 2                                    | 108.8              | ± | 10.4 | NI                    |    |
|               |                            | 3                                    | 109.7              | ± | 1.3  | NI                    |    |
| 9             | hexyl salicylate           | 1                                    | 88.4               | ± | 8.2  | NI                    | NI |
|               |                            | 2                                    | 119.5              | ± | 2.0  | NI                    |    |
|               |                            | 3                                    | 112.2              | ± | 2.2  | NI                    |    |
| 10            | cinnamaldehyde             | 1                                    | 21.4               | ± | 4.1  | I                     | I  |
|               |                            | 2                                    | 30.1               | ± | 5.9  | I                     |    |
|               |                            | 3                                    | 35.6               | ± | 4.6  | I                     |    |
| 11            | 1-decanol                  | 1                                    | 24.9               | ± | 3.8  | I                     | I  |
|               |                            | 2                                    | 29.8               | ± | 6.5  | I                     |    |
|               |                            | 3                                    | 42.1               | ± | 2.9  | I                     |    |
| 12            | cyclamen aldehyde          | 1                                    | 30.1               | ± | 11.2 | I                     | I  |
|               |                            | 2                                    | 24.2               | ± | 7.7  | I                     |    |
|               |                            | 3                                    | 36.0               | ± | 4.5  | I                     |    |
| 13            | 1-bromohexane              | 1                                    | 36.3               | ± | 8.2  | I                     | I  |
|               |                            | 2                                    | 40.3               | ± | 10.8 | I                     |    |
|               |                            | 3                                    | 43.0               | ± | 3.8  | I                     |    |

Table 6-5. Continued

| Test chemical |  | Run | The modified LabCyte EPI-MODEL24 SIT |   |      | Decision | <i>In vitro</i> class |
|---------------|--|-----|--------------------------------------|---|------|----------|-----------------------|
| no.           | Name   |     | Cell viability (%)                   |   |      |          |                       |
|               |  |     | Mean                                 | ± | SD   |          |                       |
| 14            | 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HC | 1   | 10.7                                 | ± | 1.5  | I        | I                     |
|               |  | 2   | 11.0                                 | ± | 1.7  | I        |                       |
|               |  | 3   | 16.4                                 | ± | 2.5  | I        |                       |
| 15            | di-n-propyl disulphide                           | 1   | 74.9                                 | ± | 8.6  | NI       | NI                    |
|               |  | 2   | 77.1                                 | ± | 8.9  | NI       |                       |
|               |  | 3   | 63.2                                 | ± | 7.6  | NI       |                       |
| 16            | Potassium hydroxide (5% aq.)                     | 1   | 3.3                                  | ± | 0.4  | I        | I                     |
|               |  | 2   | 2.5                                  | ± | 0.9  | I        |                       |
|               |  | 3   | 3.4                                  | ± | 1.4  | I        |                       |
| 17            | benzene thiol, 5-(1,1-dimethylethyl)-2-methyl    | 1   | 22.2                                 | ± | 8.3  | I        | I                     |
|               |  | 2   | 23.3                                 | ± | 2.4  | I        |                       |
|               |  | 3   | 37.2                                 | ± | 16.8 | I        |                       |
| 18            | 1-methyl-3-phenyl-1-piperazine                   | 1   | 12.1                                 | ± | 4.5  | I        | I                     |
|               |  | 2   | 20.5                                 | ± | 5.7  | I        |                       |
|               |  | 3   | 13.0                                 | ± | 4.4  | I        |                       |
| 19            | Heptanal   | 1   | 13.7                                 | ± | 9.9  | I        | i                     |
|               |  | 2   | 18.2                                 | ± | 1.8  | I        |                       |
|               |  | 3   | 19.9                                 | ± | 2.5  | I        |                       |
| 20            | 1,1,1-trichloroethane                            | 1   | 13.4                                 | ± | 0.6  | I        | I                     |
|               |  | 2   | 12.0                                 | ± | 1.1  | I        |                       |
|               |  | 3   | 10.1                                 | ± | 4.3  | I        |                       |

Summarizing the data, the sensitivity and specificity of predictions by the modified LabCyte EPI-MODEL24 SIT were 90.0 % and 70.0 %, respectively (Table 6-5). The overall accuracy was 80.0% (Table 6-5). We believe that these results meet the acceptance criteria described in *OECD TG 439* (Attachment 5).

Table 6-6: Prediction model for the modified LabCyte EPI-MODEL24 SIT according to the SOP for ver.8.2.

|                            |              | <i>In vivo</i> classification |              |       |
|----------------------------|--------------|-------------------------------|--------------|-------|
|                            |              | Irritant                      | Non-Irritant | Total |
| <i>In vitro</i> prediction | Irritant     | 9                             | 3            | 12    |
|                            | Non-irritant | 1                             | 7            | 8     |
|                            | Total        | 10                            | 10           | 20    |
| Sensitivity (%)            |              | 90.0                          |              |       |
| Specificity (%)            |              | 70.0                          |              |       |
| Accuracy (%)               |              | 80.0                          |              |       |

### 6-3c. Assessment of the modified LabCyte EPI-MODEL24 SIT using 54 test chemicals over a wide range of classification

First, 54 chemicals were examined to detect interference with MTT endpoints. Of the 54 tested

chemicals, there were no chemicals which directly stained epidermis tissue (data not shown). On the other hand, in the experiment for direct MTT reduction (6-2e, STEP 3), 4-methyl-thio-benzaldehyde (no. 20), benzyl alcohol (no. 28), cinnamaldehyde (no. 37), eugenol (no. 38), cyclamen aldehyde (no. 39), heptanal (no. 48) and benzene thiol 5-(1,1-dimethylethyl)-2-methyl (no. 49) were detected as potential MTT reducers (Fig. 6-4).

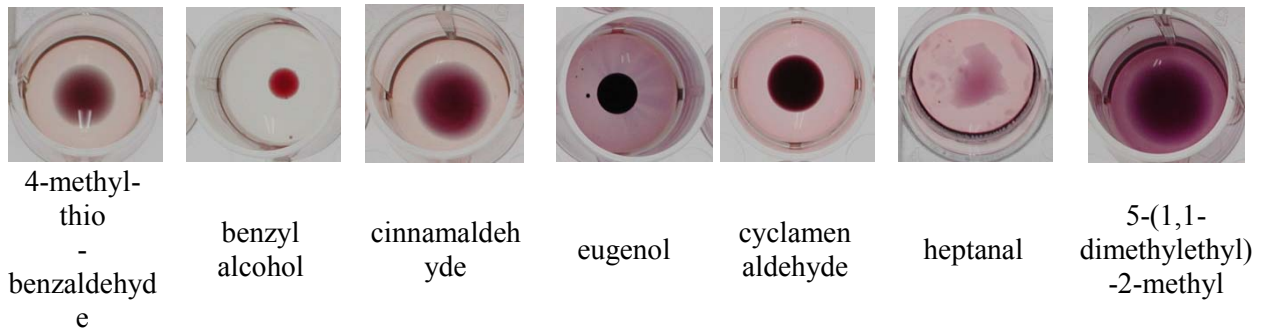


Fig.6-4 Direct MTT reduction ability (STEP 3).

**Table 6-7: Detection of MTT reducer in 54 test chemicals.**

| Positive chemicals in step 3                 | Cell viability (%: not corrected) | Staining Ratio (% /NC <sup>1</sup> ) | Corrected viability (%) |
|--|-----------------------------------|--------------------------------------|-------------------------|
| 4-methyl-thio-benzaldehyde                   | 21                                | 13                                   | 20                      |
| benzyl alcohol                               | 13                                | 14                                   | 12                      |
| cinnamaldehyde                               | 23                                | 80                                   | incompatible (16)       |
| eugenol                                      | 34                                | 145                                  | incompatible (21)       |
| cyclamen aldehyde                            | 26                                | 90                                   | incompatible (19)       |
| heptanal                                     | 26                                | 16                                   | 25                      |
| benzene thiol 5-(1,1-dimethylethyl)-2-methyl | 33                                | 56                                   | incompatible (28)       |

Therefore, we examined whether these substances reduced MTT using freeze-killed tissue instead of living tissue. As a result, the staining ratio (compared with a negative control) for 4-methyl-thio-benzaldehyde, benzyl alcohol, and heptanal were between 5% and 30 % (Table 6-7). Therefore corrected cell viabilities for these test chemicals had to be calculated (Table 6-7). However, the staining ratios for cinnamaldehyde, eugenol, cyclamen aldehyde, and benzene thiol 5-(1,1-dimethylethyl)-2-methyl were higher than 30 % and the cell viabilities of all potential MTT inducers were lower than 50%, so these chemicals were finally determined as irritants.

Next, the performance of the modified LabCyte EPI-MODEL24 SIT was evaluated with the 54 test chemicals. Results obtained with each chemical are given in 6-8. Sodium bisulphite (no. 27) and 2-ethoxy ethyl methacrylate (no. 31) showed different predictions in three independent test runs. Concordance with classifications obtained within-laboratory had a sufficient level of reproducibility for the modified LabCyte EPI-MODEL24 SIT at 96% (52/54).

As for 17 irritants (no.39 to no.54), the results for di-n-propyl disulphide (no. 46) were not concordant with those of the *in vivo* classification (Table 6-8). With regard to 37, non-irritants (from no.1 to no.37), 11 chemicals, 1-bromo-4-chlorobutane (no. 1), 4-methyl-thio-benzaldehyde (no. 20), benzyl acetate (no.

22), hydroxycitronellal (no. 23), n-butyl propionate (no. 26), benzyl alcohol (no. 28), 2-ethoxy ethyl methacrylate (no. 31), terpinyl acetate (no. 34), linalol (no.35), cinnamaldehyde (no. 36) and eugenol (no. 37) were classified as positive by the modified LabCyte EPI-MODEL24 SIT (Table 6-8).

In summary, based on the mean of the three independent runs (Table 6-8), 26 out of 37 non-irritants and 16 out of 17 irritants in the GHS classification were classified correctly by the modified LabCyte EPI-MODEL24 SIT. The statistical parameters describing assay performance are displayed in Table 6-9. Sensitivity and specificity of predictions by the modified LabCyte EPI-MODEL24 SIT were 94.1% and 70.3%, respectively. Overall accuracy was 77.8% (Table 6-9).

**Table 6-8: Results for the modified LabCyte EPI-MODEL24 SIT tested with 54 chemicals.**

| Test chemical |   | The modified LabCyte EPI-MODEL24 SIT (SOP ver.8.2) |                    |   |      |          | In vitro class | VRM  | EpiDerm™ SIT |
|---------------|---|--|--------------------|---|------|----------|----------------|------|--------------|
| No.           | Name  | Run  | Cell viability (%) |   |      | Judgment |                |      |              |
|               |   |  | Mean               | ± | SD   |          |                |      |              |
| 1             | 1-bromo-4-chlorobutane                        | 1  | 17.3               | ± | 4.4  | I        | I              | I    |              |
|               |   | 2  | 23.7               | ± | 1.5  | I        |                |      |              |
|               |   | 3  | 18.6               | ± | 2.1  | I        |                |      |              |
| 2             | diethyl phthalate                             | 1  | 104.3              | ± | 4.4  | NI       | NI             | NI   |              |
|               |   | 2  | 103.1              | ± | 1.5  | NI       |                |      |              |
|               |   | 3  | 55.4               | ± | 13.8 | NI       |                |      |              |
| 3             | di-propylene glycol                           | 1  | 109.9              | ± | 2.0  | NI       | NI             | NI   |              |
|               |   | 2  | 100.2              | ± | 10.2 | NI       |                |      |              |
|               |   | 3  | 92.9               | ± | 12.2 | NI       |                |      |              |
| 4             | naphthalen acetic acid                        | 1  | 92.7               | ± | 1.2  | NI       | NI             | NI   |              |
|               |   | 2  | 107.8              | ± | 2.0  | NI       |                |      |              |
|               |   | 3  | 98.5               | ± | 3.8  | NI       |                |      |              |
| 5             | 3-chloronitrobenzene                          | 1  | 104.3              | ± | 4.4  | NI       | NI             | NI   |              |
|               |   | 2  | 98.0               | ± | 2.2  | NI       |                |      |              |
|               |   | 3  | 95.2               | ± | 6.9  | NI       |                |      |              |
| 6             | 3,3-dithiodipropionic acid                    | 1  | 100.0              | ± | 5.6  | NI       | NI             | NI   |              |
|               |   | 2  | 98.9               | ± | 2.5  | NI       |                |      |              |
|               |   | 3  | 89.9               | ± | 10.1 | NI       |                |      |              |
| 7             | 4,4-methylenebis<br>(2,6-di-tert-butylphenol) | 1  | 103.9              | ± | 10.6 | NI       | NI             | NI   |              |
|               |   | 2  | 100.0              | ± | 3.5  | NI       |                |      |              |
|               |   | 3  | 100.0              | ± | 9.9  | NI       |                |      |              |
| 8             | 4-amino-1,2,4-triazole                        | 1  | 101.0              | ± | 6.9  | NI       | NI             | NI   |              |
|               |   | 2  | 97.4               | ± | 6.7  | NI       |                |      |              |
|               |   | 3  | 98.8               | ± | 8.4  | NI       |                |      |              |
| 9             | benzyl benzoate                               | 1  | 105.7              | ± | 2.0  | NI       | NI             | NI   |              |
|               |   | 2  | 99.6               | ± | 3.2  | NI       |                |      |              |
|               |   | 3  | 100.3              | ± | 10.1 | NI       |                |      |              |
| 10            | sodium bicarbonate                            | 1  | 99.6               | ± | 3.7  | NI       | NI             | NI   |              |
|               |   | 2  | 100.0              | ± | 5.5  | NI       |                |      |              |
|               |   | 3  | 100.3              | ± | 12.4 | NI       |                |      |              |
| 11            | Erucamide                                     | 1  | 102.5              | ± | 2.2  | NI       | NI             | NI   |              |
|               |   | 2  | 92.7               | ± | 2.8  | NI       |                |      |              |
|               |   | 3  | 90.0               | ± | 11.0 | NI       |                |      |              |
| 12            | 1,5-hexadiene                                 | 1  | 85.6               | ± | 12.2 | NI       | NI             | NI   |              |
|               |   | 2  | 92.5               | ± | 7.2  | NI       |                |      |              |
|               |   | 3  | 95.6               | ± | 3.8  | NI       |                |      |              |
| 13            | polyethylene glycol 400                       | 1  | 106.6              | ± | 8.7  | NI       | NI             | NI   |              |
|               |   | 2  | 98.2               | ± | 5.4  | NI       |                |      |              |
|               |   | 3  | 103.2              | ± | 4.3  | NI       |                |      |              |
| 14            | Glycerol                                      | 1  | 125.7              | ± | 9.0  | NI       | NI             | NI   |              |
|               |   | 2  | 98.1               | ± | 6.5  | NI       |                |      |              |
|               |   | 3  | 103.3              | ± | 6.5  | NI       |                |      |              |
| 15            | 3,3-dimethylpentane                           | 1  | 72.3               | ± | 3.2  | NI       | NI             | NI   |              |
|               |   | 2  | 76.3               | ± | 9.7  | NI       |                |      |              |
|               |   | 3  | 90.8               | ± | 1.9  | NI       |                |      |              |
| 16            | allyl phenoxy-acetate                         | 1  | 94.7               | ± | 1.8  | NI       | NI             | NI   |              |
|               |   | 2  | 96.3               | ± | 3.6  | NI       |                |      |              |
|               |   | 3  | 56.0               | ± | 3.8  | NI       |                |      |              |
| 17            | isopropanol                                   | 1  | 83.5               | ± | 5.6  | NI       | NI             | NI   |              |
|               |   | 2  | 87.7               | ± | 2.8  | NI       |                |      |              |
|               |   | 3  | 82.7               | ± | 8.5  | NI       |                |      |              |
| 18            | benzyl salicylate                             | 1  | 93.6               | ± | 16.4 | NI       | NI             | NI   |              |
|               |   | 2  | 98.2               | ± | 3.6  | NI       |                |      |              |
|               |   | 3  | 99.9               | ± | 9.7  | NI       |                |      |              |
| 19            | lauric acid                                   | 1  | 94.0               | ± | 4.0  | NI       | NI             | I    |              |
|               |   | 2  | 109.2              | ± | 7.4  | NI       |                |      |              |
|               |   | 3  | 110.0              | ± | 6.3  | NI       |                |      |              |
| 20            | 4-methyl-thio-benzaldehyde                    | 1  | 24.6               | ± | 5.8  | I        | I              | NI/I |              |
|               |   | 2  | 21.4               | ± | 1.1  | I        |                | I    |              |
|               |   | 3  | 21.8               | ± | 0.8  | I        |                |      |              |
| 21            | methyl stearate                               | 1  | 98.6               | ± | 4.8  | NI       | NI             | NI   |              |
|               |   | 2  | 113.1              | ± | 4.5  | NI       |                |      |              |
|               |   | 3  | 101.5              | ± | 4.9  | NI       |                |      |              |
| 22            | benzyl acetate                                | 1  | 11.3               | ± | 2.7  | I        | I              | I    |              |
|               |   | 2  | 29.8               | ± | 4.7  | I        |                |      |              |
|               |   | 3  | 37.4               | ± | 8.0  | I        |                |      |              |
| 23            | hydroxycitronellal                            | 1  | 19.8               | ± | 11.2 | I        | I              | NI   |              |
|               |   | 2  | 20.4               | ± | 1.8  | I        |                | I    |              |
|               |   | 3  | 32.3               | ± | 6.0  | I        |                |      |              |
| 24            | isopropyl myristate                           | 1  | 107.9              | ± | 3.7  | NI       | NI             | NI   |              |
|               |   | 2  | 97.3               | ± | 5.2  | NI       |                |      |              |
|               |   | 3  | 97.2               | ± | 12.4 | NI       |                |      |              |
| 25            | isopropyl palmitate                           | 1  | 104.1              | ± | 10.6 | NI       | NI             | NI   |              |
|               |   | 2  | 102.5              | ± | 1.3  | NI       |                |      |              |
|               |   | 3  | 115.9              | ± | 2.2  | NI       |                |      |              |

Table 6-8. Continued

| no. | Test chemical<br>Name                                | The modified LabCyte EPI-MODEL24 SIT (SOP for<br>ver.8.2) |                    |   |      |          | In vitro<br>class | VRM  | EpiDerm™<br>SIT |
|-----|--|---|--------------------|---|------|----------|-------------------|------|-----------------|
|     |  | Run   | Cell viability (%) |   |      | Judgment |                   |      |                 |
|     |  |   | Mean               | ± | SD   |          |                   |      |                 |
| 26  | n-buthyl propionate                                  | 1   | 23.1               | ± | 4.7  |          |                   |      |                 |
|     |  | 2   | 41.2               | ± | 6.4  |          |                   |      |                 |
|     |  | 3   | 45.9               | ± | 6.4  |          |                   |      |                 |
| 27  | sodium bisulphite                                    | 1   | 11.1               | ± | 5.5  |          | NI                | NI   |                 |
|     |  | 2   | 61.2               | ± | 6.8  | NI       | NI                | NI   |                 |
|     |  | 3   | 74.7               | ± | 9.7  | NI       | NI                | NI   |                 |
| 28  | benzyl alcohol                                       | 1   | 5.6                | ± | 1.3  |          |                   | NI   |                 |
|     |  | 2   | 17.5               | ± | 4.9  |          |                   |      |                 |
|     |  | 3   | 17.8               | ± | 2.0  |          |                   |      |                 |
| 29  | allyl heptanoate                                     | 1   | 95.6               | ± | 5.7  | NI       | NI                | NI   |                 |
|     |  | 2   | 108.9              | ± | 10.7 | NI       | NI                | NI   |                 |
|     |  | 3   | 104.6              | ± | 3.3  | NI       | NI                | NI   |                 |
| 30  | heptyl butyrate                                      | 1   | 105.9              | ± | 2.0  | NI       | NI                | NI   |                 |
|     |  | 2   | 108.8              | ± | 10.4 | NI       | NI                | NI   |                 |
|     |  | 3   | 109.7              | ± | 1.3  | NI       | NI                | NI   |                 |
| 31  | 2-ethoxy ethyl methacrylate                          | 1   | 29.3               | ± | 5.2  |          |                   | NI/I |                 |
|     |  | 2   | 43.1               | ± | 5.9  |          |                   |      |                 |
|     |  | 3   | 74.4               | ± | 3.6  | NI       | NI                | NI   |                 |
| 32  | hexyl salicylate                                     | 1   | 88.4               | ± | 8.2  | NI       | NI                | NI   |                 |
|     |  | 2   | 119.5              | ± | 2.0  | NI       | NI                | NI   |                 |
|     |  | 3   | 112.2              | ± | 2.2  | NI       | NI                | NI   |                 |
| 33  | linalyl acetate                                      | 1   | 101.2              | ± | 1.1  | NI       | NI                | NI/I |                 |
|     |  | 2   | 86.8               | ± | 6.7  | NI       | NI                | NI   |                 |
|     |  | 3   | 92.6               | ± | 12.6 | NI       | NI                | NI   |                 |
| 34  | terpinyl acetate                                     | 1   | 26.2               | ± | 4.1  |          |                   | NI/I |                 |
|     |  | 2   | 33.6               | ± | 3.3  |          |                   |      |                 |
|     |  | 3   | 36.3               | ± | 10.9 |          |                   |      |                 |
| 35  | Linalool   | 1   | 7.9                | ± | 0.7  |          |                   |      |                 |
|     |  | 2   | 16.6               | ± | 1.9  |          |                   |      |                 |
|     |  | 3   | 25.4               | ± | 4.0  |          |                   |      |                 |
| 36  | cinnamaldehyde                                       | 1   | 21.4               | ± | 4.1  |          |                   |      |                 |
|     |  | 2   | 30.1               | ± | 5.9  |          |                   |      |                 |
|     |  | 3   | 35.6               | ± | 4.6  |          |                   |      |                 |
| 37  | Eugenol  | 1   | 18.5               | ± | 2.2  |          |                   |      |                 |
|     |  | 2   | 30.4               | ± | 4.4  |          |                   |      |                 |
|     |  | 3   | 32.2               | ± | 7.1  |          |                   |      |                 |
| 38  | cyclamen aldehyde                                    | 1   | 30.1               | ± | 11.2 |          |                   |      |                 |
|     |  | 2   | 24.2               | ± | 7.7  |          |                   |      |                 |
|     |  | 3   | 36.0               | ± | 4.5  |          |                   |      |                 |
| 39  | 1-decanol  | 1   | 24.9               | ± | 3.8  |          |                   |      |                 |
|     |  | 2   | 29.8               | ± | 6.5  |          |                   |      |                 |
|     |  | 3   | 42.1               | ± | 2.9  |          |                   |      |                 |
| 40  | 1-bromohexane  | 1   | 36.3               | ± | 8.2  |          |                   |      |                 |
|     |  | 2   | 40.3               | ± | 10.8 |          |                   |      |                 |
|     |  | 3   | 43.0               | ± | 3.8  |          |                   |      |                 |
| 41  | α-terpineol  | 1   | 13.6               | ± | 1.3  |          |                   |      |                 |
|     |  | 2   | 17.4               | ± | 1.6  |          |                   |      |                 |
|     |  | 3   | 33.0               | ± | 5.8  |          |                   |      |                 |
| 42  | 1-bromopentane                                       | 1   | 24.3               | ± | 15.1 |          |                   |      |                 |
|     |  | 2   | 17.7               | ± | 3.9  |          |                   |      |                 |
|     |  | 3   | 23.7               | ± | 5.7  |          |                   |      |                 |
| 43  | 2-chloromethyl-3,5-dimethyl<br>-4-methoxypyridine HC | 1   | 10.7               | ± | 1.5  |          |                   |      |                 |
|     |  | 2   | 11.0               | ± | 1.7  |          |                   |      |                 |
|     |  | 3   | 16.4               | ± | 2.5  |          |                   |      |                 |
| 44  | butyl methacrylate                                   | 1   | 28.6               | ± | 6.1  |          |                   |      |                 |
|     |  | 2   | 24.5               | ± | 4.1  |          |                   |      |                 |
|     |  | 3   | 33.6               | ± | 11.8 |          |                   |      |                 |
| 45  | di-n-propyl disulphide                               | 1   | 74.9               | ± | 8.6  | NI       | NI                | I/NI |                 |
|     |  | 2   | 77.1               | ± | 8.9  | NI       | NI                | NI   |                 |
|     |  | 3   | 63.2               | ± | 7.6  | NI       | NI                | NI   |                 |
| 46  | potassium hydroxide 5%                               | 1   | 3.3                | ± | 0.4  |          |                   | I/NI |                 |
|     |  | 2   | 2.5                | ± | 0.9  |          |                   |      |                 |
|     |  | 3   | 3.4                | ± | 1.4  |          |                   |      |                 |
| 47  | heptanal   | 1   | 13.7               | ± | 9.9  |          |                   | I/NI |                 |
|     |  | 2   | 18.2               | ± | 1.8  |          |                   |      |                 |
|     |  | 3   | 19.9               | ± | 2.5  |          |                   |      |                 |
| 48  | benzene thiol, 5-(1,1-dimethylethyl)-2-<br>methyl    | 1   | 22.2               | ± | 8.3  |          |                   |      |                 |
|     |  | 2   | 23.3               | ± | 2.4  |          |                   |      |                 |
|     |  | 3   | 37.2               | ± | 16.8 |          |                   |      |                 |
| 49  | 1-methyl-3-phenyl<br>-1-piperazine                   | 1   | 12.1               | ± | 4.5  |          |                   |      |                 |
|     |  | 2   | 20.5               | ± | 5.7  |          |                   |      |                 |
|     |  | 3   | 13.0               | ± | 4.4  |          |                   |      |                 |

Table 6-8. Continued

| Test chemical |                             | The modified LabCyte EPI-MODEL24 SIT (SOP ver.8.2) |                    |   |          |                | VRM | EpiDerm™ SIT |
|---------------|-----------------------------|--|--------------------|---|----------|----------------|-----|--------------|
| no.           | Name                        | Run  | Cell viability (%) |   | Judgment | In vitro class |     |              |
|               |                             |  | Mean               | ± | SD       |                |     |              |
| 50            | SLS (20% aq)                | 1  | 9.4                | ± | 0.9      |                | NI  |              |
|               |                             | 2  | 13.2               | ± | 1.3      |                |     |              |
|               |                             | 3  | 13.4               | ± | 4.2      |                |     |              |
| 51            | 1,1,1 trichloroethane       | 1  | 13.4               | ± | 0.6      |                |     |              |
|               |                             | 2  | 12.0               | ± | 1.1      |                |     |              |
|               |                             | 3  | 10.1               | ± | 4.3      |                |     |              |
| 52            | tetrachloroethylene         | 1  | 11.1               | ± | 1.3      |                |     |              |
|               |                             | 2  | 17.1               | ± | 0.9      |                |     |              |
|               |                             | 3  | 22.6               | ± | 2.1      |                |     |              |
| 53            | capric acid (decanoic acid) | 1  | 6.1                | ± | 2.8      |                |     |              |
|               |                             | 2  | 9.8                | ± | 0.4      |                |     |              |
|               |                             | 3  | 17.6               | ± | 3.4      |                |     |              |
| 54            | SLS (5% aq)                 | 1  | 14.5               | ± | 1.9      |                |     |              |
|               |                             | 2  | 11.4               | ± | 1.0      |                |     |              |
|               |                             | 3  | 14.4               | ± | 0.9      |                |     |              |

Source of VRM and EpiDerm™ SIT data (33)

**Table 6-9. Prediction model for skin irritation test by cell viability evaluation with 54 test chemicals and based on the SOP for ver.8.2.**

|                            |              | <i>In vivo</i> classification |              |       |
|----------------------------|--------------|-------------------------------|--------------|-------|
|                            |              | Irritant                      | Non-Irritant | Total |
| <i>In vitro</i> prediction | Irritant     | 16                            | 11           | 27    |
|                            | Non-irritant | 1                             | 26           | 27    |
|                            | Total        | 17                            | 37           | 54    |
| Sensitivity (%)            |              | 94.1                          |              |       |
| Specificity (%)            |              | 70.3                          |              |       |
| Accuracy (%)               |              | 77.8                          |              |       |

#### 6-4. Discussion

As recommended by the OECD peer review panel, the original LabCyte EPI-MODEL24 SIT had to be improved in order to solve the problem where 1-bromohexane was being shown as a false-negative. We hypothesized that it was important to retain 1-bromohexane in the LabCyte EPI-MODEL24 tissue at the post incubation. Therefore, we modified the conditions for the application of test chemicals, such as the applied amount of test chemical and the temperature at exposure to test chemicals. However, unfortunately, the 1-bromohexane problem was not solved by changing conditions under which test chemicals were applied. In the end, it was found that the problem concerning 1-bromohexane could be solved by changing the washing protocol. By changing the washing protocol, 1-bromohexane penetration into the stratum corneum could be sufficiently retained. The judgments for the other 19 reference chemicals listed in OECD TG 439 excluding 1-bromohexane did not change between the original LabCyte EPI-MODEL24 SIT and the modified LabCyte EPI-MODEL24 SIT. With regard to the predictive potency of the modified LabCyte EPI-MODEL24 SIT with the 20 test chemicals listed in the OECD TG 439, the sensitivity, specificity and overall accuracy were 90 %, 70 %, and 80%, respectively. It was thought that these results met the acceptance criteria described in the OECD TG 439.

In the evaluation of the performance of the modified LabCyte EPI-MODEL24 SIT with the wide-range of 54 test chemicals, sensitivity, specificity and overall accuracy were 94.1 %, 70.3 %, and 77.8%,

respectively. Prediction potency was thought to be almost equal to the VRM (EpiSkin™ test method) or EpiDerm™ SIT (33).

OECD TG 439 requires demonstration of similarity and/or equivalence to the SIT using the new reconstructed human epidermal model in a formal inter-laboratory study, based on the VRM. It was considered that the performance of the modified LabCyte EPI-MODEL24 SIT needed to be evaluated by a formal validation study under blind conditions, as stipulated by OECD TG 439. Therefore, a catch-up validation study of the modified LabCyte EPI-MODEL24 SIT was performed from September to November, 2010, supported by the Japanese Center for the Validation of Alternative Methods (JaCVAM). Results demonstrated high reliability and acceptable accuracy on the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants. Details of the results of the validation study will be described in a JaCVAM final report.

## **7. Conclusion**

Through a final, formal validation study based on OECD TG 439, this document demonstrates that the modified LabCyte EPI-MODEL24 SIT is a useful *in vitro* methodology for classification of skin irritation potential.

## **8. Acknowledgement**

We would like to thank Ms. Lu Qiu at the Shanghai Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China for her technical support with the QC experiments for LabCyte EPI-MODEL24 after the export of shipments of LabCyte EPI-MODEL24 out of Japan. We would also like to thank Mr. Yoshihiro Yamaguchi and Ms. Maki Nakamura in Kobayashi Pharmaceuticals Co., Ltd. for their gracious support of this work.

**9. Abbreviations**

|               |   |
|---------------|---|
| ECVAM         | European Centre for the Validation of Alternative Methods   |
| EU DSD        | European Classification System based on the Dangerous Substance Directive (DSD)   |
| GHS           | Globally harmonised system on the classification and labelling of chemicals   |
| IL-1 $\alpha$ | Interleukin-1 alpha   |
| JaCVAM        | Japanese Center for the Validation of Alternative Methods   |
| JSAAE         | Japanese Society for Alternatives to Animal Experiments   |
| J-TEC         | Japan Tissue Engineering Co. Ltd.   |
| MTT           | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. A vital dye used to assess cell viability via a colorimetric assay. Cell viability is used as a proxy for predicting the skin irritancy potential of xenobiotic substances in human reconstructed epidermis models. |
| OECD          | Organisation for Economic Cooperation and Development   |
| QC            | Quality control   |
| PBS           | Phosphate buffer saline   |
| RhE           | Reconstructed human epidermis   |
| SD            | Standard deviation  |
| SIT           | Skin irritation test(ing)   |
| SLS           | Sodium lauryl sulphate  |
| TG            | Test guideline  |
| UN GHS        | The GHS system for skin irritation as applicable to all authorities, i.e. using one irritant category.  |
| VRM           | Validated reference method  |

**10. Attachments** (not in this OECD Report)

- Attachment 1. OECD TG 439: OECD guideline for the testing of chemicals. *In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method. (2010)  
Website: <http://browse.oecdbookshop.org/oecd/pdfs/browseit/9743901E.PDF>
- Attachment 2. LABCYTE VALIDATION MANAGEMENT TEAM: JSAAE DRAFT REPORT: VALIDATION STUDY OF *IN VITRO* SKIN IRRITATION TEST USING LABCYTE EPI-MODEL24. APRIL 15, 2009.
- Attachment 3. LABCYTE VALIDATION MANAGEMENT TEAM: JSAAE REPORT: VALIDATION STUDY OF *IN VITRO* SKIN IRRITATION TEST USING LABCYTE EPI-MODEL24 (2ND REPORT). JULY 22, 2009
- Attachment 4. Skin irritation test protocol using the reconstructed human model “LabCyte EPI-MODEL24” Ver.6.01
- Attachment 5. Skin irritation test protocol using the reconstructed human model “LabCyte EPI-MODEL24” Ver.8.2

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**Appendix 3**

**-JSAAE Skin Irritation Test Validation Study-**

SKIN IRRITATION TEST  
USING THE RECONSTRUCTED HUMAN MODEL “LABCYTE EPI-MODEL 24”  
**Ver. 8.2**

LabCyte EPI-MODEL24 SKIN IRRITATION TEST<sup>42 HOURS</sup>

S.O.P.

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## **1. RATIONAL AND BACKGROUND**

### **1.1 SKIN IRRITATION TEST using LabCyte EPI-MODEL 24 (SIT using LabCyte EPI-MODEL 24)**

The SIT using LabCyte EPI-MODEL24 is designed for the prediction of acute skin irritation of chemicals by measurement of its cytotoxic effect, as reflected in the MTT assay, on the Reconstructed Human Epidermis (RHE) model. The SIT using LabCyte EPI-MODEL24 is not a kit; LabCyte EPI-MODEL24 tissues are commercially available per tissues item (with a minimum of 24 LabCyte EPI-MODEL24 tissues per order).

### **1.2 BACKGROUND OF SIT using LabCyte EPI-MODEL24**

Performance standards for applying human skin models to in vitro skin irritation testing were also defined based on the validated test EpiSkin™ test method (ECVAM SIVS, 2007). These performance standards can be then used to evaluate the accuracy and reliability of other analogous test methods (also referred to as “me-too” tests) that are based on similar scientific principles and measure or predict the same biological or toxic effect.

Based on the GHS-EU classification, 12 irritants and 13 non-irritants in the draft performance standards (ECVAM 2007) and the statement by ESAC (ESAC2009) were performed the validation study through the 7 labs SIT using LabCyte EPI-MODEL24. Results were summarized at JSAAE 1st report and 2nd report on this validation study.

### **1.3 BASIS OF THE METHOD**

Chemical-induced skin irritation, manifested by erythema and oedema, is the results of a cascade of events beginning with penetration of the stratum corneum and damage to the underlying layers of keratinocytes. The dying keratinocytes release mediators that begin the inflammatory cascade which acts on the cells in the dermis, particularly the stromal and endothelial cells. It is the dilation and increased permeability of the endothelial cells that produce the observed erythema and oedema. The RhE-based test methods measure the initiating events in the cascade.

The relative viability of the treated tissues was measured at the end of the treatment exposure (15 minutes) followed by a post-exposure period (42 hours) using MTT [(3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazoliumbromide] assay. A cutoff value of 50% viability of the negative control value was considered and used to classify test substances as irritant (I) or non irritant (NI). The culture environment might allow the detection of very small quantities of cytokines secreted by the epidermis in response to topical application of test substances.

#### **1.3.1 TEST SYSTEM DESCRIPTION**

LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, they were cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978). Reconstruction of human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface 0.3 cm<sup>2</sup>) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. It constructs a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18°C.

### 1.3.1.1 Quality control of the test system

The LabCyte EPI-MODEL24 is manufactured according to defined quality assurance procedures. Each batch production was provided with quality controls such as storage conditions, RHE instructions for use, lot number and origin, histology (demonstration of human epidermis-like structure with multilayered stratum corneum), cell viability, barrier function integrity ( $0.14 \leq IC50 \leq 0.4$ ).

### 1.3.1.2 Precautions

The epidermal cells are taken from healthy donor negative to HIV, and Hepatitis. Nevertheless, handling procedures for biological materials should be followed:

- a) It is recommended to wear gloves during handling with the skin and kit components.
- b) After use, the epidermis, the material and all media in contact with it should be decontaminated prior to disposal (e.g. using special containers or autoclaving).

## 1.3.2 ASSAY QUALITY CONTROL

### 1.3.2.1 Assay Acceptance Criterion 1: Negative Control

The absolute OD of the negative control (NC) tissues (treated with sterile DPBS) in the MTT assay is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures and under specific conditions of use.

$$0.7 \leq \text{Mean OD (A570/650) measured value} \leq 2.5$$

### 1.3.2.2 Assay Acceptance Criterion 2: Positive Control

A 5% SDS (in H<sub>2</sub>O) solution (see 7.6.3) is used as positive control (PC) and tested concurrently with the test chemicals. Concurrent means here the PC has to be tested in each assay, but not more than one PC is required per testing day. Viability of positive control should be within  $95 \pm 1$  % confidence interval of the historical data.

$$\text{Mean tissue viability} \leq 40\%$$

### 1.3.2.3 Assay Acceptance Criterion 3: Standard Deviation (SD)

Since in each test skin irritancy potential is predicted from the mean viability determined on 3 single tissues, the variability of tissue replicates should be acceptably low.

$$\text{Standard Deviation (SD) of tissue viability of 3 identically treated replicates for negative control and positive control} \leq 18 \%$$

## 1.4 LIMITATION OF THE METHOD

One limitation of this assay method is a possible interference of the test substance with the MTT endpoint. A colored test substance or one that directly reduces MTT (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the MTT endpoint. However, these test substance are a problem only if at the time of the MTT test (i.e. 42 hours after test substance exposure) sufficient amounts of the test substance are still present on (or in) the tissues. In case of this unlikely event, the (true) metabolic MTT reduction and the contribution by a colored test material or (false) direct MTT reduction by the test material can be quantified by a procedure described in Section 3.2.

The method is not designed for testing of highly volatile test substances, gases and aerosols.

## 1.5 BRIEF BASIC PROCEDURE

On the day of receipt, LabCyte EPI-MODEL24 tissues are conditioned by incubation to release transportstress related compounds and debris overnight. After pre-incubation, tissues are topically exposed to the test chemicals for 15 minutes. Preferably, three tissues are used per test chemical (TC) and for the positive control (PC) and negative control (NC). Tissues are then thoroughly rinsed, blotted to remove the test substances, and transferred to fresh medium. After 42 hr incubation period, the MTT assay is performed by transferring the tissues to the well containing MTT medium (0.5 mg/ml). After 3 hr MTT incubation, the blue formazan salt formed by cellular mitochondria is extracted with 0.3 mL/tissue of isopropanol and the optical density of the extracted formazan is determined using a spectrophotometer at 570 nm and 650 nm as reference. Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues. Skin irritation potential of the test material is predicted if the remaining relative cell viability is below 50%.

## 1.6 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

According to the GHS classification (Category 2 or no label), an irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls.

| <b>In vitro results</b>        | <b>In vivo prediction</b> |
|--------------------------------|---------------------------|
| Tissue viability is $\leq$ 50% | Irritant                  |
| Tissue viability is $>$ 50%    | Non Irritant              |

## 2. MATERIALS

### 2.1 LabCyte EPI-MODEL 24

#### 2.1.1 LabCyte EPI-MODEL 24 KIT COMPONENTS

LabCyte EPI-MODEL 24 kit components are shown in Table 1.

Table 1 - LabCyte EPI-MODEL24 Kit Components

| Component                  | Qty      | Description   |
|----------------------------|----------|---|
| LabCyte EPI-MODEL 24 plate | 1 plate  | Contains 24 culture inserts with tissues fixed in nutritive agar medium for transport (usable area: 0.3cm <sup>2</sup> ). |
| Assay Medium               | 1 bottle | Basic medium for incubation (30mL). Store at refrigeration temperature.   |
| 24-well plate              | 1 plate  | Blank plate for use in assay. Store at room-temperature.  |

#### 2.1.2 SHIPMENT OF LabCyte EPI-MODEL24

LabCyte EPI-MODEL 24 is packed in a special container (Icompo/NIPPON EXPRESS CO., LTD) and delivered by NIPPON EXPRESS CO., LTD. After the Icompo is delivered, examine the contents and make sure that all kit components (LabCyte EPI-MODEL24 plate, assay medium, and 24-well assay plate) are included in the package. Confirm lot numbers and expiration dates also. Record details in [the Methods Documentation Sheet \(MDS\) 1](#).

NIPPON EXPRESS will pick up the Icompo at a later date (generally, the day after the date of delivery), and we ask that you return it with a slip documenting receipt, as well as the insulating materials.

#### 2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL 24

Begin incubating all of the culture inserts after opening the package. Do not store the culture inserts again after opening.

The human epidermis cells used in LabCyte EPI-MODEL 24 originate from a normal donor and are HIV-, HBV-, HCV-, and HPV-negative. However, handle them with enough care and in accordance with the laboratory biosafety guidelines since they contain raw materials of human origin.

## 2.2 TEST CHEMICALS

Coded test chemicals are delivered to each laboratory.

## 2.3 CONSUMABLES

The following consumables are required.

\* The described quantities are necessary so that 1 to 6 samples can be assayed once.

- Assay Medium, 100mL (J-TEC: 402250) 1 bottle
- MTT, 25mg (J-TEC: 403026) 1 bottle
- Wide orifice cell saver tips for micro-pipettes (sterile) 96 tips 1 box
- 24-well assay plate (Becton,Dickinson and Company: 353047) 7 plates
- 96-well plate (Becton,Dickinson and Company: 353072) 1 plates
- Phosphate buffered saline (PBS) 500mL (Invitrogen: 14190-144) 2 bottles
- Isopropanol 500mL (Wako Pure Chemical Industries: 164-08335) 1 bottle
- SLS 25g (SIGMA:L4390) 1 bottle
- Sterile distilled water 20mL (Otsuka Pharmaceutical: 36A1X00001) 1 bottles
- Sterile cotton buds (JAPAN COTTON BUDS: 10A754D) 1 box

## 2.4 OTHERS

### 2.4.1 EQUIPMENT / INSTRUMENTS

- Safety cabinet (or clean bench)
- Water bath (37 °C)
- CO<sub>2</sub> incubator (37 °C, 5%CO<sub>2</sub>, capable of maintaining high humidity)
- Autoclave
- 96-well multi-plate reader (required filters: 450nm, 570nm, 650nm)
- Precision balance (0.1mg)
- Aspirator
- Stop-watches
- Adjustable micro-pipette (10-200µL, 200-1000µL)
- Sharp-edged forceps (sterile)
- Micro spatula (sterile)
- Beaker (1~2L: sterile)
- Sterilizable poly wash bottle (500~1000mL: sterile)

### 2.4.2 CONSUMMABLE ITEMS

- Micro-pipette tips (sterile: 10~200µL, 200~1000µL)
- Microtubes (1.5mL)
- Scalpel (KEISEI MEDICAL INDUSTRIAL: Keisei Scalpel 11A)

## 3. TEST METHOD

\*Perform operations in Section 3.1.1~3.1.4 and Section 3.3.1~3.3.2 aseptically in a safety cabinet (or clean bench).

\*Operations other than above do not need to be performed with an aseptic technique. For these operations, refer to **Section 2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL 24**

## 3.1 PREPARATIONS

### 3.1.1 MTT SOLUTION

- (1) Dissolve MTT in the assay medium to prepare the MTT medium (final concentration: 0.5mg/mL)  
Use ultrasonic cleaning equipment or a vortex mixer as necessary in order to completely dissolve the MTT.

\*Store in a dark, cold place and use it within 24 hours.

- (2) Record details of step (1) above in the MDS 4.

### 3.1.2 POSITIVE CONTROL SUBSTANCE

- (1) Weigh 500mg of SLS precisely.
- (2) To prepare a positive control solution, put the SLS into a graduated cylinder or measuring flask and dilute to 10mL with distilled water (final concentration: 5% w/v)]

\* Store in a dark, cold place and use it within 24 hours.

- (3) Record details of steps (1) and (2) above in the MDS 3.

### 3.1.3 NEGATIVE CONTROL SUBSTANCE

- (1) Use distilled water.

### 3.1.4 POLY WASH BOTTLE FOR PBS

- (1) Sterilize poly wash bottle using an autoclave.
- (2) Fill the sterilized poly wash bottle aseptically with sterile PBS.

### 3.2 TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH MTT ENDPOINT

There are two kinds of test chemicals that interfere with the MTT assay as follows.

- (a) Chemical that stains epidermis tissues.
- (b) Chemical that is able to directly reduce MTT.

Test chemical that stains the epidermis tissues has a possibility to transfer from the epidermis tissues to the extraction solution and to affect the optical density (OD) measurements.

Test chemical that is able to directly reduce MTT can affect the optical density (OD) measurements, if the test chemical is present in the epidermis tissues when the MTT viability test is performed. Detection procedure of these test chemicals is described below.

#### 3.2.1 DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE

##### 3.2.1.1 STEP1 (PRELIMINARY TEST)

- (1) Add 25 $\mu$ L (Liquid) or 25mg (Solid) of the test chemical into wells of 24-well assay plate preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as control.
- (2) Close the lid of 24-well assay plate and incubate the mixture in CO<sub>2</sub> incubator for 15 minutes.
- (3) After incubation, shake the mixture gently and evaluate the staining of the distilled water macroscopically.
- (4) When the color of the solution changes significantly, the test chemical is presumed to have the potential to stain the tissue and a functional check on viable tissues (Step2) should be performed. When the color of the solution does not change significantly, the test chemical is determined not to have a potential to stain the tissue.

##### 3.2.1.2 STEP2 (FUNCTIONAL CHECK ON VIABLE TISSUE)

- (1) Add 25 $\mu$ L (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the distilled water (Step1), onto the surface of the epidermis tissues. Distilled water is used as negative control.
- (2) Follow all procedures described in this SOP Section 3.3 EXECUTION OF THE TEST. However, incubate the tissue for 3 hours in culture media without MTT instead of incubating in media containing MTT to evaluate the staining of the epidermis tissues.
- (3) Calculate ratio of staining by test chemical from the following formula.

$$\text{Ratio of staining by test chemical (\%)} = \frac{\text{OD test chemical} - \text{OD negative control}}{\text{OD negative control}} \times 100$$

- (4) When the ratio of staining by test chemical is <5%, correction of the results is not necessary. When the ratio is between 5% and 30%, the corrected MTT OD is calculated using the following formula.test chemical

$$\text{Corrected MTT OD} = \frac{\text{OD stained tissue (MTT assay)} - \text{OD stained tissue (no MTT assay)}}{\text{OD negative control}}$$

When the ratio of staining by test chemical is >30%, the test chemical must be considered incompatible with the test. However, when the Cell viability (%), which is calculated according to the procedures described in this SOP Section 3.3.5.2, is <50%, the test chemical is determined as irritant. Therefore correction of the results or determination of incompatibility of the test chemical is not necessary.

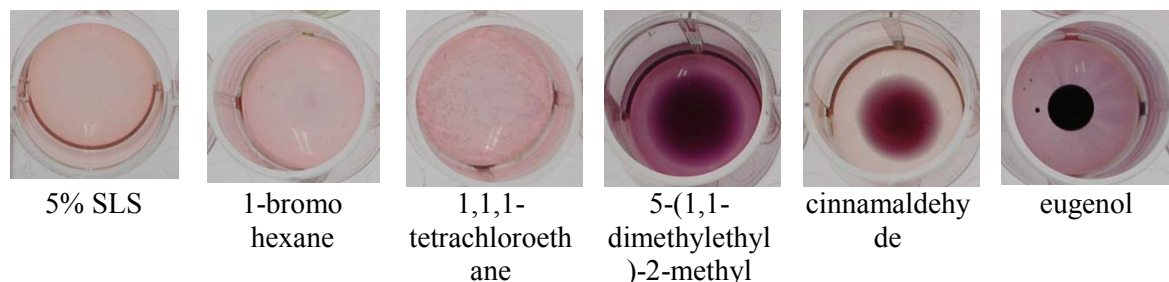
#### 3.2.2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE MTT

##### 3.2.2.1 STEP3 (PRELIMINAY TEST)

- (1) Add 25 $\mu$ L (Liquid) or 25mg (Solid) of the test chemical into wells of 24-well assay plate

preliminarily filled with 0.5mL of MTT medium. Untreated MTT medium is used as control.

- (2) Close the lid of 24-well assay plate and incubate the mixture in CO<sub>2</sub> incubator for 1 hour.
- (3) After incubation, shake the mixture gently and evaluate the staining of the MTT medium macroscopically.
- (4) When the MTT medium turns blue/purple significantly, the test chemical can reduce MTT and additional functional check (Step4) must be performed.



**Photo 1** – Example of test for direct MTT reduction ability (STEP 3). Test substances 5-(1,1-dimethylethyl)-2-methyl, cinnamaldehyde and eugenol and have directly reduced MTT. In these cases, Step 4 must be performed.

#### 3.2.2.2 STEP4 (FUNCTIONAL CHECK ON VIABLE TISSUE)

- (1) Add 25µL (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the MTT medium into blue/purple (Step3), onto the surface of the epidermis tissues. Distilled water is used as negative control.
- (2) Follow all procedures described in this SOP Section 3.3 EXECUTION OF THE TEST. However, use the epidermis tissues that has been freeze-killed at -20 °C or lower for more than 24 hours instead of viable epidermis tissues.
- (3) Calculate ratio of staining by test chemical from the following formula.

$$\text{Ratio of staining by test chemical (\%)} = \frac{\text{OD test chemical} - \text{OD negative control}}{\text{OD negative control}} \times 100$$

- (4) When the ratio of staining by test chemical is <30%, correct OD data using the following formula.

$$\text{Corrected OD} = \frac{\text{OD (viable tissue) test chemical} - [\text{OD (freeze-killed tissue) test chemical} - \text{OD (freeze-killed tissue) negative control}]}{\text{OD (viable tissue) negative control}}$$

When the ratio of staining by test chemical is >30%, the test chemical must be considered incompatible with the test. However, When the Cell viability (%), which is calculated according to the procedures described in this SOP Section 3.3.5.2, is <50%, the test chemical is determined as irritant. Therefore correction of the results or determination of incompatibility of the test chemical is not necessary.

### 3.3 EXECUTION OF THE TEST

#### 3.3.1 PREPARATION OF LabCyte EPI-MODEL 24 (DAY -1)

- (1) Pre-warm the assay medium for 30 minutes to 37 °C using a water bath.
- (2) Fill 3 wells of the 1<sup>st</sup> row of each 24-well assay plate with the pre-warmed assay medium (0.5mL/well).

→ Figure 1

- (3) Open the LabCyte EPI-MODEL 24 aluminum package.
- (4) Open the LabCyte EPI-MODEL 24 plate lid and pick up the culture inserts using sterile forceps.

\*Do not touch the epidermis surface of culture inserts.

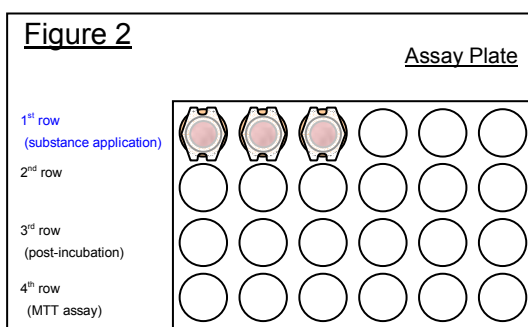
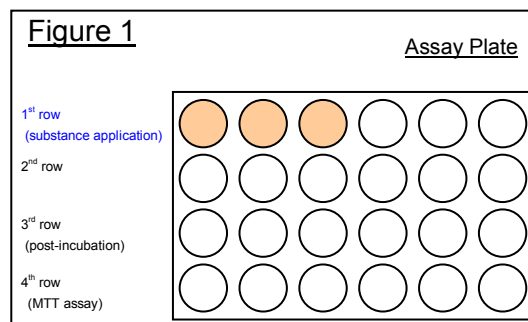
\*Use forceps to remove agar medium sticking to the outside of the culture inserts.

- (5) Transfer the culture inserts into assay medium filled wells of the 1<sup>st</sup> row using sterile forceps.

→ Figure 2

\*Avoid air bubble formation under the tissue inserts.

- (6) Place the plate (lid on) in a CO<sub>2</sub> incubator.
- (7) Incubate overnight (15~30 hours) until Section 3.2.2 “APPLICATION OF TEST CHEMICALS AND RINSING.”
- (8) Record details of steps (1) - (7) above in the MDS 2.



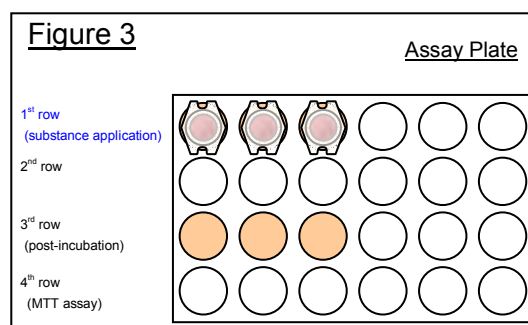
#### 3.3.2 APPLICATION OF TEST CHEMICALS AND RINSING (DAY 0)

##### 3.3.2.1 PREPARATION OF WELLS FOR POST-INCUBATION (3<sup>RD</sup> ROW)

- (1) Pre-warm the assay medium for 30 minutes to 37 °C using a water bath.
- (2) Remove the assay plate from the CO<sub>2</sub> incubator.
- (3) Open the lid of the assay plate, and fill 3 wells of the 3<sup>rd</sup> row with the pre-warmed assay medium (1.0mL/well) using a micropipette.

→ Figure 3

- (4) Place the plate (lid on) in a CO<sub>2</sub> incubator.
- (5) Incubate until application of test chemicals (0~12 hours).
- (6) Record details of steps (1) – (5) above in the MDS 3.



##### 3.3.2.2 APPLICATION OF TEST CHEMICALS

- (1) Remove the assay plate from the CO<sub>2</sub> incubator.
- (2) Apply test chemicals onto the surface of epidermis tissues in the 1<sup>st</sup> row of the assay plate. Use 3 wells per test chemical (N=3).

*FOR LIQUIDS:* Carefully apply 25µL of the test chemical onto the central part of each epidermis using a micropipette. After applied, close the lid of the assay plate and tap the side of the plate outside the safe cabinet (or clean bench) in order for the liquid to spread out over the entire epidermis surface. If necessary, use a micro spatula to coat the unapplied surface with liquids. Do not push the epidermis

surface too hard with the spatula.

\*Use wide orifice cell saver tips for viscous liquids.

→ [Photo 2](#)

Use a pipette, etc. to familiarize yourself with the nature of the test chemicals in advance.

**FOR SOLIDS:** Weigh out 25mg (±1mg) of the solid chemical with a precision balance in advance. Apply first 25µL of distilled water and then the weighed test chemical onto the epidermis surface. Use a micro spatula if necessary to gently spread the test chemical.

→ [Photo 3](#)

\*One 24-well assay plate should be used to assay only one test chemical.

→ [Figure 4](#)

(1 samples x 3(n) = 3 (culture inserts))

(3) Apply test chemical onto each well at 1~3-minute intervals.

(4) Incubate each well for 15 minutes in the cabinet (lid on between the intervals).

\*Close the lid of the assay plate at all times except when applying samples. It might affect the amount of test sample if the lid is kept open, due to air circulation in the safe cabinet (or clean bench).

(5) Record details of steps (1) - (4) above in [the MDS 3](#).

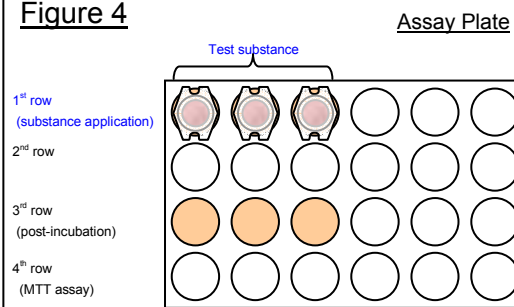
**Photo 2 - Pipette tips for viscous liquids**



**Photo 3 – Applying a solid substance**



**Figure 4**



### 3.3.2.3 REMOVAL OF THE TEST CHEMICALS

(1) 15 minutes (±30 seconds) after applying a chemical, open the assay plate and pick up a culture insert with sterile forceps.

(2) Fill the culture insert with PBS using a PBS filled poly wash bottle. Hit the PBS stream from the washing bottle on the side-wall of the culture insert and wash on the tissue surface by the PBS current.

→ [Photo 4](#)

Attention: Must not to hit the PBS stream on the tissue surface directly. Be careful not to damage the tissue surface.

(3) Discard the PBS into a beaker by tilting the insert. If necessary, remove the PBS inside the culture insert by tapping it above the beaker only once.

→ [Photo 5](#)

(4) Repeat steps (2) and (3) at least 10 times or more as much as possible, and remove all residual test chemical on the tissue surface almost completely.

(5) Gently remove the leftover PBS outside the culture insert with a sterile cotton bud. But don't touch inside the culture insert by a cotton bud.

**Photo 4 - Rinse 1**



**Photo 5 - Rinse 2**

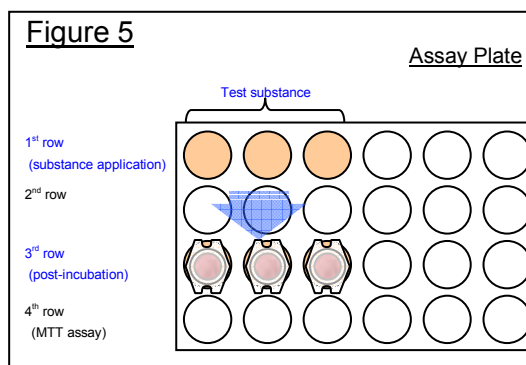


→ Photo 6

Attention: Even if residues of washing PBS remain on the tissue surface, don't do at all because it is not necessary to remove them.



- (6) If test material remains on the epidermis surface, repeat steps (2) ~ (5) again.
- (7) Transfer the blotted culture insert to a well in the 3<sup>rd</sup> row of the same column (for post-incubation).  
→ Figure 5  
\*Avoid air bubble formation under the culture inserts.
- (8) Repeat steps (1) ~ (7) for all the culture inserts at 1~3-minute intervals.
- (9) Record details of steps (1) – (8) above in the MDS 3.



### 3.3.3 POST TREATMENT INCUBATION (DAY 0~2)

- (1) Close the lid of the assay plate and place it in a CO<sub>2</sub> incubator.
- (2) Incubate for 42 hours.

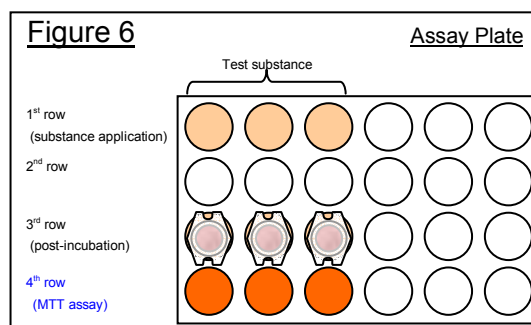
### 3.3.4 MTT ASSAY (DAY 2)

#### 3.3.4.1 PREPARATION OF WELLS FOR MTT ASSAY

- (1) Pre-warm MTT medium for 30 minutes to 37°C using a water bath.
- (2) Remove the assay plate from the CO<sub>2</sub> incubator.
- (3) Open the lid of the assay plate, and fill each well of the 4<sup>th</sup> row with the pre-warmed MTT medium (0.5mL/well) using a micropipette.

→ Figure 6

- (4) Close the lid of the assay plate and place it in the CO<sub>2</sub> incubator.
- (5) Incubate until starting MTT assay (about 0 ~ 12 hours).
- (6) Record details of steps (1) – (5) above in the MDS 4.

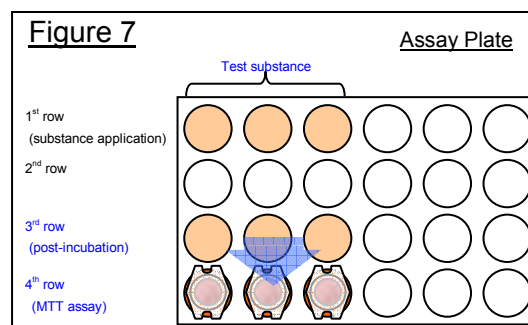


#### 3.3.4.2 MTT ASSAY

- (1) Remove the assay plate from the CO<sub>2</sub> incubator after 42 hours (±1 hour) of post-incubation.
- (2) Transfer each culture insert from the 3<sup>rd</sup> row to the 4<sup>th</sup> row of the corresponding column.

→ Figure 7

- \*Avoid dripping from the base end surface of the culture insert into other wells.  
\*Avoid air bubble formation under the culture inserts.
- (3) Close the lid of the assay plate and place it in the CO<sub>2</sub> incubator.
  - (4) Incubate for 3 hours.
  - (5) Record details of steps (1) – (4) above in the MDS 4.



### 3.3.5 FORMAZAN EXTRACTION AND MEASUREMENT (DAY 2~3)

#### 3.3.5.1 FORMAZAN EXTRACTION

- (1) Remove the assay plate(s) from the CO<sub>2</sub> incubator 3 hours ( $\pm 5$  minutes) after the MTT assay.
- (2) Open the lid of the assay plate and pinch the cultured epidermis from each culture insert of the 4<sup>th</sup> row with forceps.

→ Photo 7

\*Use a micro spatula to scratch up the epidermis or a scalpel to cut the membrane filter on the base of the culture insert if the cultured epidermis cannot be pinched due to damage from a test chemical.

- (3) Transfer the epidermis tissue into a 1.5mL micro tube.
- (4) Add 300 $\mu$ L of isopropanol to the micro tubes and soak the entire epidermis tissue in the isopropanol.
- (5) Incubate the micro tubes in a dark cold place (or refrigerator) overnight (more than 15 hours) in order to completely extract pigments.

\*Tighten the micro tube seal.

\*Periodically shaking the micro tubes will contribute to a more efficient extraction.

- (6) Shake the micro tubes to mix the solution.

\*If split epidermis tissues are suspended, wait until they sink or gently centrifuge them (if a centrifuge is available).

- (7) Transfer 200 $\mu$ L of the solution in each micro tube into each well on a 96-well plate.

\*One well of 200 $\mu$ L of isopropanol should be set as a blank.

\*Figure 8 shows an example of allocation in a 96-well plate.

**Photo 7 - Detachment of epidermis**



**Figure 8 – Allocation for a 96-well plate**

|   | 1        | 2          | 3          | 4          | 5          | 6           | 7           | 8           | 9           | 10          | 11          | 12 |
|---|----------|------------|------------|------------|------------|-------------|-------------|-------------|-------------|-------------|-------------|----|
| A | Blank    |            |            |            |            |             |             |             |             |             |             |    |
| B | DW-1     | Sample 1-1 | Sample 3-1 | Sample 5-1 | Sample 7-1 | Sample 9-1  | Sample 11-1 | Sample 13-1 | Sample 15-1 | Sample 17-1 | Sample 19-1 |    |
| C | DW-2     | Sample 1-2 | Sample 3-2 | Sample 5-2 | Sample 7-2 | Sample 9-2  | Sample 11-2 | Sample 13-2 | Sample 15-2 | Sample 17-2 | Sample 19-2 |    |
| D | DW-3     | Sample 1-3 | Sample 3-3 | Sample 5-3 | Sample 7-3 | Sample 9-3  | Sample 11-3 | Sample 13-3 | Sample 15-3 | Sample 17-3 | Sample 19-3 |    |
| E | 5% SLS-1 | Sample 2-1 | Sample 4-1 | Sample 6-1 | Sample 8-1 | Sample 10-1 | Sample 12-1 | Sample 14-1 | Sample 16-1 | Sample 18-1 | Sample 20-1 |    |
| F | 5% SLS-2 | Sample 2-2 | Sample 4-2 | Sample 6-2 | Sample 8-2 | Sample 10-2 | Sample 12-2 | Sample 14-2 | Sample 16-2 | Sample 18-2 | Sample 20-2 |    |
| G | 5% SLS-3 | Sample 2-3 | Sample 4-3 | Sample 6-3 | Sample 8-3 | Sample 10-3 | Sample 12-3 | Sample 14-3 | Sample 16-3 | Sample 18-3 | Sample 20-3 |    |
| H |          |            |            |            |            |             |             |             |             |             |             |    |

- (8) Record details of steps (1) – (7) above in the MDS 5.

#### 3.3.5.2 OPTICAL DENSITY MEASUREMENTS OF THE EXTRACTS

- (1) Using a 96-well plate reader, measure optical densities (OD) at 570nm and 650nm and determine the measured OD by subtracting the 570nm OD from the 650nm OD.

The equation is shown below:

$$\text{Measured OD} = [570\text{nm OD}_{\text{sample}} - 570\text{nm OD}_{\text{blank}}] - [650\text{nm OD}_{\text{sample}} - 650\text{nm OD}_{\text{blank}}]$$

\*Set the plate reader-calculated value as the measured OD if the 96-well plate reader performs automatic calculations.

- (2) Calculate the cell viability of a sample using the equation below. Furthermore, calculate the variability (SD) of tissue replicates.
- (3) Record details of steps (1) and (2) above in the MDS 5.

$$\text{Cell Viability (\%)} = \frac{\text{Measured OD}_{\text{sample}}}{\text{Mean Measured OD}_{\text{NC}}} \times 100$$

#### 4. ASSESSMENT

##### 4.1 CONDITIONS FOR A SUCCESSFUL STUDY

The skin irritation test should be considered successful if both of the following criteria have been met.

- Tissue viability:  $0.7 \leq \text{mean OD (A570/650) measured value for negative control} \leq 2.5$ .
- Positive control: mean tissue viability for 5% SLS (positive control)  $\leq 40\%$ .
- SD: SD (negative control and positive control) of tissue viability of 3 identically treated replicates  $\leq 18\%$

##### 4.2 ASSAY CRITERIA

The criteria for in vitro interpretation are shown below.

The test must be performed 3 times per a sample in total. Sort the tissue viabilities obtained from the repeated tests in ascending order, and classify the irritancy based on the median of those tissue viabilities.

| Tissue Viability (primary)      | Classification |
|---------------------------------|----------------|
| Tissue viability is $\leq 50\%$ | Irritant       |
| Tissue viability is $> 50\%$    | Non Irritant   |

#### [FLOWCHART] ASSESSMENT FLOWCHART

(1) Tissue viability in negative control → (either criterion is not met) → Assay Failure

$0.7 \leq \text{Mean OD measured value} \leq 2.5$

Positive control (5% SLS) result should be “irritant”

Mean tissue viability  $\leq 40\%$

SD”

SD (negative control and positive control) of tissue viability of 3 identically treated replicates  $\leq 18\%$

↓

(All criteria are met)

↓

(2) Assessment of test samples (3-time repeated tests: all tests satisfy (1))

The median of the 3 tissue viabilities (%)  $\leq 50\%$  → (Yes) → Classified as irritant

↓

(No)

↓

Classified as non irritant

**MDS 1:**  
**RECEIPT OF LABCYTE EPI-MODEL 24**

---

Laboratory name: \_\_\_\_\_ Test name: \_\_\_\_\_ Test No. : \_\_\_\_\_

1. LabCyte EPI-MODEL 24

Date received : \_\_\_\_\_

Lot No. : \_\_\_\_\_

Exouration date : \_\_\_\_\_  
 (MM/DD/YYYY)

Accessories : Assay medium, 30mL  (Lot No. : \_\_ Expiration date : \_\_\_\_\_)  
 (MM/DD/YYYY)  
 24 well assay plate

Note

2. Assay medium

Date received : \_\_\_\_\_

Lot No. : \_\_\_\_\_

Expiration date : \_\_\_\_\_  
 (MM/DD/YYYY)

Note

Date: \_\_\_\_\_ Operator: \_\_\_\_\_ Check date: \_\_\_\_\_ Study director: \_\_\_\_\_  
 (MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date: \_\_\_\_\_ Name: \_\_\_\_\_  
 (MM/DD/YYYY)

**MDS 2:**

**PRE-INCUBATION OF LABCYTE EPI-MODEL 24 (Section 3.3.1)**

---

Laboratory name: \_\_\_\_\_ Test name: \_\_\_\_\_ Test No. : \_\_\_\_\_

1. Warm up the assay medium and add 0.5mL of the assay medium to the wells of the 1st row on the 24-well assay plate.

Assay medium : (Lot No. : \_\_\_\_\_ Expiration date : \_\_\_\_\_)  
(MM/DD/YYYY)

Warm for 30 min.

Add 0.5mL of assay medium to each well

Number of plates : \_\_\_\_\_

2. Transfer culture inserts to wells in the 1st row on the 24-well assay plate.

LabCyte EPI-MODEL 24 : (Lot No. : \_\_\_\_\_ Expiration date : \_\_\_\_\_)  
(MM/DD/YYYY)

Time/date executed : \_\_\_\_\_  
(MM/DD/YYYY HH:MM)

Confirm that there are no bubbles under the cell culture insert.

3. LabCyte EPI-MODEL 24 is cultured in CO<sub>2</sub> incubator overnight.

Time/date of culture start : \_\_\_\_\_  
(MM/DD/YYYY HH:MM)

Planned time/date of exposure to test chemical : \_\_\_\_\_  
(MM/DD/YYYY HH:MM)

Note

Date: \_\_\_\_\_ Operator: \_\_\_\_\_ Check date: \_\_\_\_\_ Study director: \_\_\_\_\_  
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date: \_\_\_\_\_ Name: \_\_\_\_\_  
(MM/DD/YYYY)

**MDS 3-1:****APPLICATION OF TEST CHEMICALS, RINSING AND POST-INCUBATION (Section 3.1.2, 3.3.2 ~ 3.3.3)**

Laboratory name: \_\_\_\_\_ Test name: \_\_\_\_\_ Test No.: \_\_\_\_\_

## 1. Preparation of positive control.

Weight of SLS \_\_\_\_\_ mg Preparation vol. \_\_\_\_\_ mL Operation date : \_\_\_\_\_  
(MM/DD/YYYY HH/MM)

## 2. Warm up the assay medium and add 1.0mL of the assay medium to the wells of the 3rd row on the 24-well assay plate.

Assay medium : (Lot No. : \_\_\_\_\_ Expiration date : \_\_\_\_\_)  
(MM/DD/YYYY)Warm for 30 min.  Add 1.0mL of assay medium. Time/date executed : \_\_\_\_\_  
(MM/DD/YYYY HH/MM)

## 3. Apply test chemicals to the LabCyte EPI-MODEL 24.

Time/date execution started : \_\_\_\_\_ Time/date completed : \_\_\_\_\_  
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

## 4. After exposure to test chemical for 15 min., wash out the LabCyte EPI-MODEL 24 and transfer the culture inserts to the 3rd row on the 24-well assay plate.

PBS : (Lot No. : \_\_\_\_\_ Expiration date : \_\_\_\_\_)  
(MM/DD/YYYY)Time/date execution started : \_\_\_\_\_ Time/date completed : \_\_\_\_\_  
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)Confirm that there are no bubbles under the cell culture insert. 

## 5. Test chemical information

| Test chemical code No.                | Lot No. | Physical state               | Test chemical vol.(weight)<br>(measured weight) | Time of application | Exposure time<br>(15min.) |
|---------------------------------------|---------|------------------------------|---|---------------------|---------------------------|
| Distilled Water<br>(Negative control) |         | Liquid                       | 25µL  | :                   | <input type="checkbox"/>  |
| 5%SLS<br>(Positive control)           |         | Liquid                       | 25µL  | :                   | <input type="checkbox"/>  |
|                                       |         | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg)                          | :                   | <input type="checkbox"/>  |
|                                       |         | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg)                          | :                   | <input type="checkbox"/>  |
|                                       |         | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg)                          | :                   | <input type="checkbox"/>  |
|                                       |         | Liquid,<br>viscous,          | 25µL, ( mg, mg,<br>mg)                          | :                   | <input type="checkbox"/>  |

|  |  |                              |                        |   |                          |
|--|--|------------------------------|------------------------|---|--------------------------|
|  |  | solid                        |                        |   |                          |
|  |  | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg) | : | <input type="checkbox"/> |
|  |  | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg) | : | <input type="checkbox"/> |
|  |  | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg) | : | <input type="checkbox"/> |
|  |  | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg) | : | <input type="checkbox"/> |
|  |  | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg) | : | <input type="checkbox"/> |
|  |  | Liquid,<br>viscous,<br>solid | 25µL, ( mg, mg,<br>mg) | : | <input type="checkbox"/> |

Date: \_\_\_\_\_ Operator: \_\_\_\_\_ Check date: \_\_\_\_\_ Study director: \_\_\_\_\_  
 (MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date: \_\_\_\_\_ Name: \_\_\_\_\_  
 (MM/DD/YYYY)

**MDS 3-2:****APPLICATION OF TEST CHEMICALS, RINSING AND POST-INCUBATION**  
(Section 3.3.2~3.3.3)

Laboratory name: \_\_\_\_\_ Test name: \_\_\_\_\_ Test No. : \_\_\_\_\_

## 5. Test chemical information (continued)

| Test chemical code No. | Lot No. | Physical state         | Test chemical vol.(weight) (measured weight) | Time of application | Exposure time (15min.)   |
|------------------------|---------|------------------------|--|---------------------|--------------------------|
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |
|                        |         | Liquid, viscous, solid | 25µL, ( mg, mg, mg)                          | :                   | <input type="checkbox"/> |

6. Culture LabCyte EPI-MODEL 24 in CO<sub>2</sub> incubator for 42 hrs.Time/date post-incubation started : \_\_\_\_\_  
(MM/DD/YYYY HH:MM)Time/date post-incubation completed : \_\_\_\_\_  
(MM/DD/YYYY HH:MM)

|      |  |
|------|--|
| Note |  |
|------|--|

Date: \_\_\_\_\_ Operator: \_\_\_\_\_ Check date: \_\_\_\_\_ Study director: \_\_\_\_\_  
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date: \_\_\_\_\_ Name: \_\_\_\_\_

(MM/DD/YYYY)

**MDS 4:****MTT ASSAY (Section 3.3.4)**

---

Laboratory name: \_\_\_\_\_ Test name: \_\_\_\_\_ Test No. : \_\_\_\_\_

## 1. Preparation of MTT medium

Preparation vol. \_\_\_\_\_ mL Lot No. \_\_\_\_\_ Time/date executed : \_\_\_\_\_  
(MM/DD/YYYY HH/MM)

## 2. Warm up the MTT medium and add 0.5mL of the MTT medium to the wells in the 4th row on the 24-well assay plate.

MTT medium. : (Lot No. : \_\_\_\_\_ Expiration date : \_\_\_\_\_)  
(MM/DD/YYYY)Warm for 30 min.  Add 0.5mL of the MTT medium.  Time/date executed : \_\_\_\_\_  
(MM/DD/YYYY HH/MM)

## 3. After post-incubation, the LabCyte EPI-MODEL24 transfer to wells of 4th row of 24-well assay plate.

Time/date started : \_\_\_\_\_ Time/date completed : \_\_\_\_\_  
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)Confirm that there are no bubbles under the cell culture insert. 4. Store LabCyte EPI-MODEL 24 culture overnight in CO<sub>2</sub> incubator for 42 hrs.

Information on MTT reaction time

| Test chemical code No.             | Lot No. | MTT reaction start time | Time when MTT reaction ends | Test chemical code No.. | Lot No. | MTT reaction start time | Time when MTT reaction ends |
|------------------------------------|---------|-------------------------|-----------------------------|-------------------------|---------|-------------------------|-----------------------------|
| Distilled Water (Negative control) |         | :                       | :                           |                         |         | :                       | :                           |
| 5%SLS (Positive control)           |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |
|                                    |         | :                       | :                           |                         |         | :                       | :                           |

Note

Date: \_\_\_\_\_ Operator: \_\_\_\_\_ Check date: \_\_\_\_\_ Study director: \_\_\_\_\_  
 (MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date: \_\_\_\_\_ Name: \_\_\_\_\_  
 (MM/DD/YYYY)

**MDS 5:**  
**FORMAZAN EXTRACTION AND MEASUREMENT (Section 3.3.5)**

Laboratory name: \_\_\_\_\_ Test name: \_\_\_\_\_ Test No. : \_\_\_\_\_

- After MTT reaction, use forceps to pick up the cultured epidermis from the cell culture insert and put it in a 1.5mL microtube.

Did you use a scalpel to cut out the cultured epidermis?

Date of execution : \_\_\_\_\_  
 (MM/DD/YYYY)

- Add isopropanol (300µL) to microtube so that the cultured epidermis is completely immersed in isopropanol.

Isopropanol Lot No.\_\_\_\_ To add isopropanol (300µL)

Immersion of the cultured epidermis in isopropanol.

Date of execution : \_\_\_\_\_  
 (MM/DD/YYYY)

- For MTT formazan extraction, allow micro tube to stand in a cold and dark space.  
 Place micro tube in a cold and dark space.

- Extract solution (200mL) is transferred to each well on the 96-well plate.

Transfer to the 96-well plate.

Time/date executed : \_\_\_\_\_  
 (MM/DD/YYYY HH/MM)

Sample location on 96-well plate.

|   | 1                 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------------------|---|---|---|---|---|---|---|---|----|----|----|
| A | blank             |   |   |   |   |   |   |   |   |    |    |    |
| B | Distilled Water-1 |   |   |   |   |   |   |   |   |    |    |    |
| C | Distilled Water-2 |   |   |   |   |   |   |   |   |    |    |    |
| D | Distilled Water-3 |   |   |   |   |   |   |   |   |    |    |    |
| E | 5% SLS-1          |   |   |   |   |   |   |   |   |    |    |    |
| F | 5% SLS-2          |   |   |   |   |   |   |   |   |    |    |    |
| G | 5% SLS-3          |   |   |   |   |   |   |   |   |    |    |    |
| H |                   |   |   |   |   |   |   |   |   |    |    |    |

5. Analyze extract OD at 570nm and 650nm, and calculate the OD(570nm-650nm).

Analyze OD at 570nm and 650nm.

Calculate the OD(570nm-650nm).

Calculate cell viability and SD.

Cell viability and SD are recorded on a separate data sheet.

The data sheet is attached to the back of this sheet.

Check for input errors.

Time/date executed : \_\_\_\_\_

(MM/DD/YYYY HH/MM)

Note

Date: \_\_\_\_\_ Operator: \_\_\_\_\_ Check date: \_\_\_\_\_ Study director: \_\_\_\_\_  
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date: \_\_\_\_\_ Name: \_\_\_\_\_  
(MM/DD/YYYY)

## REVISION HISTORY

| Rev.    | Content  | Date Revised |
|---------|--|--------------|
| Ver.1   | 1) First version   | 27/02/2008   |
| Ver.2   | 1) Revised clerical error.   | 28/02/2008   |
| Ver.3   | 1) Revised the post-incubation time and assessment criteria in compliance with the EpiSkin method described in “Performance Standards for Applying Human Skin Models to in vitro Skin Irritation Testing”<br>2) Added photos and figures for instruction.  | 17/03/2008   |
| Ver.4   | 1) Added MDS 1~6.<br>2) Added instruction and operational steps regarding the IL-1 $\alpha$ ELISA kit.<br>3) Added subsections “Delivery of LabCyte EPI-MODEL24” and “Instruction For Use of LabCyte EPI-MODEL24” to Section 2.<br>4) Added the description regarding test chemicals to Section 2.<br>5) To Section 2, added the description of materials provided by J-TEC separately from other materials.<br>6) Stated the specific calculation procedures in Section 3.2.5.2 “OPTICAL DENSITY MEASUREMENTS OF EXTRACTS”.   | 15/05/2008   |
| Ver.4.1 | 1) Moved scalpel from Section 2.4 “MATERIALS PROVIDED BY J-TEC” to Section 2.5 “MATERIALS NOT PROVIDED WITH THE J-TEC KITS”.<br>2) Removed the description regarding how to execute procedures alone.<br>3) Moved IL-1 $\alpha$ ELISA reagents from Section 3.1 “PREPARATIONS” to Section 3.2 “TEST METHOD”.<br>4) Added a flowchart for the IL-1 $\alpha$ ELISA procedures.<br>5) Changed from “in a cold dark place” to “in a cold dark place (or refrigerator)” regarding formazan extraction.<br>6) Added the description of “ultrasonic cleaning equipment or vortex mixer” as an example of an MTT dissolution method.<br>7) Changed the exposure time column from entering actual time to checkboxes on the MDS 3.  | 21/05/2008   |
| Ver.5.0 | 1) Corrected typing errors in the section number for IL-1 $\alpha$ ELISA reagents.<br>2) Removed the space for SLS lot numbers on the MDS 3.<br>3) Removed the space for PBS lot numbers on the MDS 3.<br>4) Added the space for isopropanol lot numbers on the MDS 5.<br>5) Added a checkbox about using a scalpel when removing tissues in the MDS 5.<br>6) Added the space for IL-1 $\alpha$ ELISA kit lot numbers on the MDS 6.<br>7) Changed the applicable parts of product codes and kit components in Section 2.2, with the change of IL-1 $\alpha$ ELISA kit types to a 96 well test only.<br>8) Decreased the volume by half to 10mL and changed the storage condition from within 1 month to within 24 hours in Section 3.1.2 “POSITIVE CONTROL SUBSTANCE”.<br>9) Added the manufacturers and product codes of the 24-well plate and 96-well plate in Section 2.4 “MATERIALS PROVIDED BY J- | 27/08/2008   |

|           |   |            |
|-----------|---|------------|
|           | <p>TEC”.</p> <p>10) Added specific time frames for incubation or culturing.</p> <p>11) Added the conditions for a successful study in Section 4 “ASSESSMENT”</p> <p>12) Changed the specific method of applying liquids in Section 3.2.2.2 “APPLICATION OF TEST CHEMICALS”.</p> <p>13) Added descriptions in English on the MDS Sheets.</p> <p>14) Changed the application time interval from 1 minute to 1~3minute(s).</p> <p>15) Numbered figures and flowcharts.</p> <p>16) Increased the size of spaces for lot numbers on the MDS Sheets.</p> <p>17) Changed spaces for dates from MM/DD to MM/DD/YYYY.</p> <p>18) Added director check date, study director, secretariat check date and name at the end of each MDS.</p> <p>19) Changed the size of matrixes for sample allocation to a 96-well plate in the MDS 5 &amp; 6.</p> <p>20) Changed the test chemical name to test chemical code in the MDS 3 &amp; 4.</p> <p>21) Divided the MDS 3 into MDS 3-1 and 3-2, and added spaces for date, operator, check date, study director at the end of the MDS 3-1, and spaces for laboratory name, test name and test no. at the beginning of the MDS 3-2.</p> |            |
| Ver. 6.0  | <p>1) Removed the descriptions regarding the measurement of IL-1<math>\alpha</math> production, since the validation committee decided to use cell viabilities only as an index for the skin irritancy test at the meeting in 2009.</p> <p>2) Revised the expression “the materials provided by J-TEC” for the validation study to that for a standard skin irritancy test preparation.</p> <p>3) Clearly stated the cell viability equation to use the mean of measured values.</p> <p>4) Clearly stated to use the median of cell viabilities from the three-time repeated tests as assay criteria.</p>   | 27/02/2009 |
| Ver. 6.01 | <p>1) In order to avoid the possible influence of volatile test chemicals on the results of other test chemicals, the types of test chemicals per plate was changed from 2 chemicals to just 1 chemical.</p>  | 23/03/2009 |
| Ver. 7.01 | <p>1) Test for detecting chemicals that interfere with MTT endpoint was added to Section 3.2.</p>   | 03/07/2009 |
| Ver. 7.2  | <p>1) Revised clerical error.</p>   | 30/09/2009 |
| Ver. 8.1  | <p>1) Added the description about rational and background as following chapters in Section 1.</p> <p>LabCyte EPI-MODEL 24 SKIN IRRITATION TEST (SIT using LabCyte EPI-MODEL24)</p> <p>BACKGROUND OF SIT using LabCyte EPI-MODEL24</p> <p>BASIS OF THE METHOD</p> <p>LIMITATION OF THE METHOD</p> <p>BRIEF BASIC PROCEDURE</p> <p>DATA INTERPRETATION PROCEDURE (PREDICTION</p>  | 30/06/2010 |

|         |  |            |
|---------|--|------------|
|         | <p>MODEL)</p> <p>2) Added photo about chemicals that directly reduce MTT in Section 3.</p> <p>3) Added the washing protocol more detail in Section 3.</p> <p>4) Added assessment about SD.</p> |            |
| Ver.8.2 | <p>1) Changed description about the washing protocol in Section 3.</p> <p>2) Changed unit of consumable reagents and vessels from per a validation study to per a test.</p>                    | 17/08/2010 |

**Appendix 4**

JaCVAM Report:

Me-too Validation Study of *in vitro* Skin Irritation Test using LabCyte EPI-MODEL24  
(LabCyte EPI-MODEL24 SIT)

February 28, 2011

LabCyte Validation Management Team

Members of LabCyte Validation Management Team

Mr. Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods  
Mr. Masakazu Katoh, Japan Tissue Engineering Co. Ltd.  
Mr. Toshiro Yokouchi, Japan Tissue Engineering Co. Ltd.

**Biostatistian group**

Mr. Takashi Omori, Doshisya Univ.

**Participant laboratories**

KOBAYASHI Pharmaceutical Co., Ltd. (Mr. Yoshihiro Yamaguchi and Ms. Maki Nakamura)  
Fancl Corp. (Ms. Tamie Suzuki and Ms. Runa Izumi)  
Drug Safety Testing Center Co., Ltd. (Mr. Shinsuke Shinoda and Ms. Saori Hagiwara)

### **Abbreviations**

CAS: Chemical Abstracts Service  
ECVAM: European Centre for the Validation of Alternative Methods  
ESAC: ECVAM Scientific Advisory Committee  
GHS: Globally Harmonised System  
GLP: Good Laboratory Practice  
ISO: International Organization for Standardization  
JaCVAM: Japanese Centre for the Validation of Alternative Methods  
JSAAE: Japanese Society for Alternatives to Animal Experiments  
J-TEC: Japan Tissue Engineering Co. Ltd.  
NIHS: National Institute of Health Sciences  
OECD: Organisation for Economic Co-operation and Development  
QC: Quality control  
PBS: Phosphate buffered saline  
PS: Performance Standard  
RhE: Reconstructed human epidermis  
SD: Standard deviation  
SLS: Sodium lauryl sulphate  
SPSF: Standard Project Submission Form  
TG: Test Guideline  
UN: United Nations  
VMT: Validation management team  
VRM: Validated reference method  
WNT: National Coordinators of the Test Guideline Project

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### **13. References**

**Appendices:**

1. Validation study of in vitro skin irritation test using LabCyte EPI-MODEL24 (FINAL report)
2. Summary report of the peer review panel on LabCyte EPI-MODEL24 in vitro test method for the assessment of skin irritation potential of chemicals
3. Additional validation of the RhE tests: LabCyte EPI-MODEL24 acute skin irritation prediction
4. Skin Irritation test protocol using the reconstructed human model “LabCyte EPI-MODEL24” (ver.8.2)
5. Original data in the catch-up validation study
6. Test records and data sheet in KOBAYASHI Pharmaceutical Co., Ltd. (example: in Japanese)
7. Result of QC for each lot of LabCyte EPI-MODL24
8. Detailed review documents on the “LabCyte EPI-MODEL24”
9. Masakazu Katoh, Fumiyasu Hamajima, Takahiro Ogasawara, and Ken-ichiro Hata (2009) Assessment of the Human Epidermal Model LabCyte EPI-MODEL for *In Vitro* Skin Irritation Testing According to the ECVAM-Validated Protocol , Journal of Toxicological Science, 34(3) 327-334.
10. OECD TG 439, *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method

## 1. Goal statement

- The ultimate goal of the test strategy is to replace the regulatory Draize skin irritation test to meet the OECD (Organisation for Economic Co-operation and Development) TG (Test Guideline) 404 (OECD, 2002).
- The primary goal of this catch-up validation study is to evaluate the ability of *in vitro* tests to reliably discriminate skin irritant (I) from non-irritant (NI) chemicals, as defined by the OECD and UN (United Nations) proposal for GHS (Globally Harmonised System) for the classification and labelling of skin irritation (category 1/category 2; no category; Anon., 2003).

## 2. Objective

The OECD Working group of the National Coordinators of the Test Guideline Project (WNT) accepted the TG No.439: *in vitro* skin irritation test guideline in March 2010. This TG addresses the human health endpoint of skin irritation. Three validated test methods currently adhere to this TG. Prevalidation, optimization and validation studies have been completed for an *in vitro* test method that uses a Reconstructed human epidermis (RhE) model. This method is commercially available as EpiSkin™ and has been designated as the Validated Reference Method (VRM). Two other commercially available *in vitro* skin irritation RhE test methods, namely the EpiDerm™ SIT (EPI-200) and SkinEthic™ RHE test methods, have shown similar results to the VRM according to Performance Standard (PS) - based validation.

On the other hand, another *in vitro* test system that employs a RhE model (LabCyte EPI-MODEL24) has progressed through protocol optimization as a skin irritation test. A multi-laboratory assessment of this system was performed according to several ECVAM (European Centre for the Validation of Alternative Methods) performance standards (ESAC: ECVAM Scientific Advisory Committee statement, 2007, 2008, 2009). The present objective added the Japanese RhE and other similar models to adhere to the OECD TG 439. A me-too validation study was conducted to assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of this test system. The study included a challenging set of 20 test chemicals that would meet the performance standard set forth in the TG No.439. The validation study was undertaken in accordance with the principles and criteria documented in the OECD *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (No. 34, OECD, 2005) and according to the Modular Approach to validation (Hartung *et al.* 2004).

## 3. Background

Researchers in Japan aimed to include the Japanese RhE (LabCyte EPI-MODEL24; LabCyte EPI-MODEL24 SIT) *in vitro* skin irritation test in the TG as an addition to other similar models in the SPSF (Standard Project Submission Form) that were submitted to OECD WNT by EU delegate in April 2008. The validation study described herein was performed between April 2008 and January 2009 by the Validation Management Team (VMT), with financial support from the Japanese Society for Alternative to Animal Experiments (JSAAE).

The study conducted by VMT referenced the original ECVAM performance standard (ECVAM 2007), in which a range of appropriate models was described as one of the acceptance criteria. After completion of the first phase of the study in August 2008, the VMT discussed the criteria for the Labcyte EPI MODEL24 SIT. The VMT decided that the criteria were not set because there was not enough data to define this kind of range at that time. Furthermore, the pre-specification was considered to have too narrow a range and the draft OECD TG came under review at the time of the discussions. As a result, the reliability of the model was considered to be high. Therefore, the VMT decided that criteria for the range may not be needed for this model, while the check for variation should be done.

Based on validation results of the Labcyte EPI-MODEL24 SIT, a member of the Japanese WNT submitted an SPSF of it to OECD in January 2009 and the OECD WNT accepted this assay in its working

plan in May 2009. The VMT submitted the first validation report to the OECD secretary in July 2009. On the other hand, we confirmed an additional validation study in reference to the new ECVAM performance standards (ESAC statement, 2009) to be revised for the TECD TG between April and May of 2009 and submitted the second validation report and Background Review Documents to the OECD secretary in August 2009.

Using these documents, OECD performed a peer review and we received the peer review report from the OECD secretary in March 2010. The OECD peer review on the LabCyte EPI-MODEL24 SIT *in vitro* test method for the assessment of skin irritation potential of chemicals was performed with the validation report and background review documents. In the summary report, the peer review panel indicated that the issue of misclassifying 1-bromo-hexane should be resolved.

To resolve this issue, the protocol was revised by Japan Tissue Engineering (J-TEC). To confirm general versatility with the revised protocol, we planned an additional validation study according to the OECD performance standard.

## **4. Test methods**

### **4-1. Reconstructed human cultured epidermal model**

LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by J-TEC. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, the cells are cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978). Reconstruction of a human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface area 0.3 cm<sup>2</sup>) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. The process generates a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18 °C (Kato, 2009: Appendix 4).

### **4-2. MODEL supplier**

According to OECD Good Laboratory Practice (GLP) Consensus Document No.5 “*Compliance of Laboratory Suppliers with GLP Principles*”, responsibility for the quality and fitness for use of equipment and materials rests entirely with the management of the test facility (OECD, 1999).

The acceptability of equipment and materials in laboratories complying with GLP must therefore be guaranteed to any regulatory authority to which studies are submitted. In some countries where GLP has been implemented, suppliers belong to national regulatory or voluntary accreditation schemes (for laboratory animals) that can provide users with additional documentation proving that they are using a test system of defined quality.

Audits performed during the study focused on procedures established to guarantee a defined quality of the tissue models.

## **5. Validation management structure**

The management structure of the study is shown in Figure 1.

### **5-1. Validation management team**

The VMT played a central role in overseeing the conduct of the validation study, including implementation of the following aspects of the study:

1) Goal statement

- 2) Project plan including objective
- 3) Study protocol / amendments
- 4) Outcome of QC (Quality Control) audits
- 5) Test chemicals
- 6) Data management procedures
- 7) Timeline / study progression
- 8) Data collection and analysis
- 9) Study interpretation and conclusions
- 10) Reports and publications

The VMT made the final decision on which laboratories would participate in the validation study.

Responsible VMT members:

Chair (Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods)

The sponsor representative, LabCyte EPI-MODEL 24 suppliers and lead lab (Masakazu Katoh: J-TEC)

## **5-2. Chemical selection, acquisition, coding, and distribution**

- 1) Definition of selection criteria
- 2) Chemical selection
- 3) Liaise with suppliers
- 4) Final check of chemicals provided
- 5) Acquisition
- 6) Coding
- 7) Distribution

Responsible VMT member: Hajime Kojima, JaCVAM

## **5-3 . Independent biostatisticians**

- 1) Approve spreadsheets  
Responsible VMT member: Takashi Omori: Doshisya Univ.

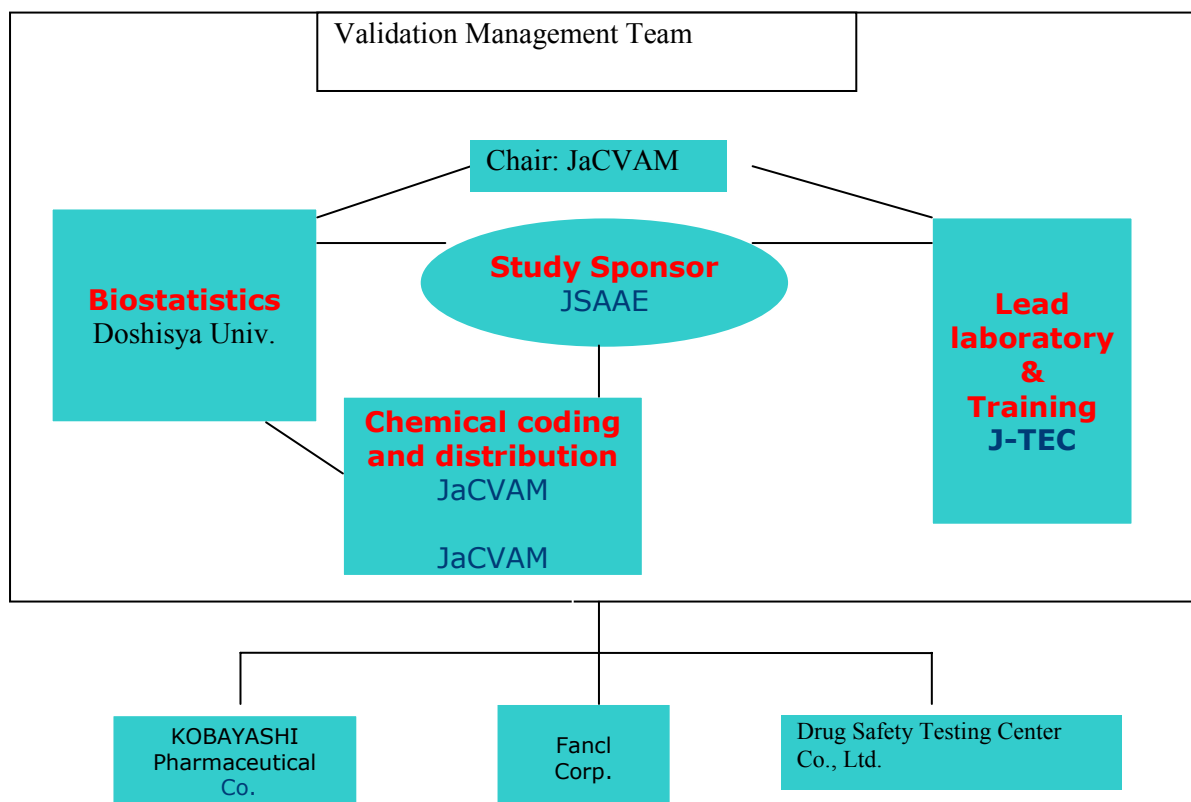


Fig. 1. Management structure of me-too validation study on the LabCyte EPI-MODEL24 SIT

#### 5-4. Participating laboratories

The laboratories participating in the study are shown in **Fig. 1**.

The following three laboratories participated in the validation study for the evaluation of the LabCyte EPI-MODEL 24 assays:

- Laboratory a — KOBAYASHI Pharmaceutical Co., Ltd. (Yoshihiro Yamaguchi and Maki Nakamura)
- Laboratory b — Fancl Corp. (Tamie Suzuki and Runa Izumi)
- Laboratory c — Drug Safety Testing Center Co., Ltd. (Shinsuke Shinoda and Saori Hagiwara)

A lead laboratory was also identified as J-TEC (Mr. Masakazu Kato and Mr Toshihiro Yokouchi). This laboratory did not participate in the validation study.

Each laboratory was responsible for complying with GLP principles and specifying QC aspects of the study.

#### 5-5. Sponsorship

The study was managed and financed by JaCAM and J-TEC.

1) JaCVAM financially supported the following activities:

- management of the study (VMT meetings)
- provision of independent statistical support (VMT meetings)
- purchase, coding, and distribution of chemicals to the laboratories

- independent QC audit of the data
  - publication of the study results
- 2) J-TEC supported the following aspects of the study:
- the lead laboratories for the test method
  - training of the participating laboratories
  - independent QC audit of the LabCyte EPI-MODEL24 SIT
  - financial assistance of the participated laboratories

## 6. Study design and test period

Before initiation of the validation study, J-TEC delegates conducted a training course on using LabCyte EPI-MODEL24 SIT with a revised protocol (ver.8.1) at the National Institute of Health Sciences (NIHS) on July 27, 2010. All technicians from each laboratory participated in this training course. Furthermore, all laboratories participated in preliminary testing. After this preliminary test, the protocol was revised to ver.8.2 to include detailed descriptions of the washing protocol. Three laboratories attended an additional validation study after one laboratory was not able to obtain a positive test result with 1-bromohexane.

The preliminary test was conducted by three laboratories between August and September of 2010. The duration of validation study was between September and November of 2010.

## 7. Test chemicals

### 7-1. Chemical selection

To meet the OECD performance standard, the VMT selected 20 chemicals for testing (Table 1). In the reference chemicals of OECD Test guideline No. 439, tetrachloroethylene was not selected. Instead of it, No.20, 1,1,1-trichloroethane was tested. Because tetrachloroethylene was changed from 1,1,1-trichloroethane at the final step before approving as the test guideline, the VMT escaped the attention of conclusive confirmation. The class of their chemicals is at the same level and the VMT judged not to become a serious problem in this validation study. The final approval of the chemicals proposed by JaCVAM was the responsibility of the VMT. To avoid any potential for bias in the final selection, laboratory representatives on the VMT did not participate in these discussions, nor were they made aware of the chemicals finally approved for testing in the validation study.

**Table 1. Minimum List of Reference Chemicals for Determination of Accuracy and Reliability Values for Similar or Modified RhE Skin Irritation Test Methods and Codes**

| No. | Name | CAS number | UN GHS<br><i>in vivo</i> Cat. | Storage | Chemical code |       |       |
|-----|------|------------|-------------------------------|---------|---------------|-------|-------|
|     |      |            |                               |         | Lab a         | Lab b | Lab c |

|    |   |            |   |      |       |       |       |
|----|---|------------|---|------|-------|-------|-------|
| 1  | 1-bromo-4-chlorobutane                            | 6940-78-9  | No Cat.                                   | RT   | B-261 | D-281 | G-301 |
| 2  | diethyl phthalate                                 | 84-66-2    | No Cat.                                   | RT   | B-262 | D-282 | G-302 |
| 3  | naphthalene acetic acid                           | 86-87-3    | No Cat.                                   | RT   | B-263 | D-283 | G-303 |
| 4  | allyl phenoxy-acetate                             | 7493-74-5  | No Cat.                                   | RT   | B-264 | D-284 | G-304 |
| 5  | isopropanol                                       | 67-63-0    | No Cat.                                   | RT   | B-265 | D-285 | G-305 |
| 6  | 4-methylthio-benzaldehyde                         | 3446-89-7  | No Cat.                                   | RT   | B-266 | D-286 | G-306 |
| 7  | methyl stearate                                   | 112-61-8   | No Cat.                                   | RT   | B-267 | D-287 | G-307 |
| 8  | heptyl butyrate                                   | 5870-93-9  | No Cat.<br>( <i>Optional<br/>Cat. 3</i> ) | RT   | B-268 | D-288 | G-308 |
| 9  | hexyl salicylate                                  | 6259-76-3  | No Cat.<br>( <i>Optional<br/>Cat. 3</i> ) | RT   | B-269 | D-289 | G-309 |
| 10 | cinnamaldehyde                                    | 104-55-2   | No Cat.<br>( <i>Optional<br/>Cat. 3</i> ) | 2-8C | B-270 | D-290 | G-310 |
| 11 | 1-decanol   | 112-30-1   | Cat.2                                     | RT   | B-271 | D-291 | G-311 |
| 12 | cyclamen aldehyde                                 | 103-95-7   | Cat.2                                     | RT   | B-272 | D-292 | G-312 |
| 13 | 1-bromohexane                                     | 11-25-1    | Cat.2                                     | RT   | B-273 | D-293 | G-313 |
| 14 | 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl | 86604-75-3 | Cat.2                                     | RT   | B-274 | D-294 | G-314 |
| 15 | di-n-propyl disulphide                            | 629-19-6   | Cat.2                                     | RT   | B-275 | D-295 | G-315 |
| 16 | potassium hydroxide 5%                            | 1310-58-3  | Cat.2                                     | RT   | B-276 | D-296 | G-316 |
| 17 | benzylthiol,5-(1,1-dimethylethyl)-2-methyl        | 7340-90-1  | Cat.2                                     | RT   | B-277 | D-297 | G-317 |
| 18 | 1-methyl-3-phenyl-1-piperazine                    | 5271-27-2  | Cat.2                                     | RT   | B-278 | D-298 | G-318 |
| 19 | heptanal  | 111-71-7   | Cat.2                                     | RT   | B-279 | D-299 | G-319 |
| 20 | 1,1,1-trichloroethane                             | 71-55-6    | Cat.2                                     | RT   | B-280 | D-300 | G-320 |

1) CAS No.: Chemical abstracts service registry number.

## 7-2. Chemical coding and distribution

Independent coding and distribution of chemicals were contracted out by JaCVAM to an independent laboratory. The undeciphered information on this code was confirmed by the VMT after the validation study because this chemical code list was too easy and simple. The certification of chemicals was according to ISO (International Organization for Standardization) 9001 and GLP, and has proven experience of reliable services. The codes were provided by JaCVAM.

## 8. Protocol

### 8-1. Protocol of the skin irritation test with LabCyte EPI-MODEL24 SIT

According to the suggestion of the OECD peer review panel, J-TEC resolved the false-negative issues of 1-bromohexane. The SOP (Standard Operating Procedure: ver.8.1) included a modified washing protocol in a revision dated June 30, 2010. Modifications to the washing protocol are shown in Table 2 (Detailed process described in Appendix 7). Using the revised SOP, the validation study was performed to show clear data and addressed comments of the OECD peer review panel.

**Table 2. Modification points of washing protocol between SOP ver.7.1 and SOP ver.8.2.**

| Modification points                            | SOP ver.7.1         | SOP ver.8.2  |
|--|---------------------|--|
| 1. Handling the PBS stream from washing bottle | It was not defined. | The revision specified to avoid hitting the tissue surface directly with the PBS stream. |
| 2. Removal of PBS by tapping                   | It was not defined. | It was briefly defined.  |
| 3. Correct use of the cotton pad               | It was not defined. | It was defined to avoid touching the tissue surface directly with the cotton pad.        |

LabCyte EPI-MODEL 24 tissues were shipped from the supplier on Mondays and delivered to recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 mL), and incubated overnight (37°C, 5% CO<sub>2</sub> humidified atmosphere). On the following day, the tissues were topically exposed to the test chemicals. Liquids (25 µL) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25-µL sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25-µL distilled water, and three tissues serving as positive controls were exposed to 5% SLS (sodium lauryl sulphate). After a 15-minute exposure, each tissue was carefully washed with PBS (phosphate buffered saline, Invitrogen, CA, USA) 10 times using a washing bottle to remove any remaining test chemical from the surface. The blotted tissues were then transferred to new 24-well plates containing 1 mL of fresh assay medium.

The treated and control tissues were incubated for 42 hours (37 °C, 5% CO<sub>2</sub> humidified atmosphere). When the 42-hour incubation period was complete, blotted tissues were transferred to new 24-well plates containing 0.5 mL of freshly prepared MTT medium (1 mg/mL; Dojindo Co., Kumamoto, Japan) for the MTT assay. Tissues were incubated for 3 hours (37 °C, 5% CO<sub>2</sub> humidified atmosphere) and then transferred to microtubes containing 0.3 mL isopropanol, which completely immersed the tissue. Formazan extraction was performed at room temperature, and the tissues were allowed to stand overnight.

Subsequently, 200- $\mu$ L extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and 650 nm as a reference absorbance, with isopropanol as a blank. The tissue viability was calculated as a percentage relative to the viability of the negative controls. The median of three values from identically treated tissues was used to classify a chemical according to the prediction model.

## 8-2. Prediction model of skin irritation

In this study, the prediction model (acceptability criteria and positive criteria) of skin irritation potential with LabCyte EPI-MODEL24 SIT was set to refer to the conditions for the OECD TG 439 and its Performance Standards.

### 8-2-1) Acceptance criteria on the RhE test method components

According to paragraph 27; acceptability criteria in the OECD TG 439, tissues treated with the negative controls and positive controls, *i.e.* 5% aqueous SLS, should reflect their ability to respond to an irritant chemical under the conditions of the test method. Associated and appropriate measures of variability between tissue replicates should be defined.

- 1)  $OD_{NC}$  of the negative control is greater than 0.7.
- 2) The viability of the positive control (5% aqueous SLS) is less than 40%.
- 3) If standard deviations (SDs) are used they should be within the one-sided 95% tolerance interval calculated from historical data; for the VRM  $SD < 18\%$ .

### 8-2-2) Positive criteria

The OD values obtained with each test sample can be used to calculate the percentage of viability normalized to the No Category, which is set to 100%. The cut-off value for percentage of cell viability distinguishing irritant from non-classified test chemicals and the statistical procedure(s) used to evaluate the results and identify irritant chemicals, should be clearly defined, documented, and proven to be appropriate. The cut-off values for the prediction of irritation are given below:

The test chemical is considered to be an irritant to skin in accordance with GHS category 2 if the tissue viability after exposure and post-treatment incubation is less than or equal ( $\leq$ ) to 50%.

Depending on the country and regional regulatory requirements, the test chemical may be considered as a No Category if the tissue viability after exposure and post-treatment incubation is more than ( $>$ ) 50%.

### 8-2-3) Study acceptance criteria

It is possible that one or several tests with one or more test chemicals do not meet test acceptance criteria for the test and control chemicals or are not acceptable for other reasons. To complement missing data, a maximum number of two additional tests for each test chemical is admissible ("retesting"). Because retesting requires concurrent testing with a positive control and negative control, a maximum number of two additional runs may be conducted for each test chemical.

It is conceivable that even after retesting, the minimum number of three valid runs required for each tested chemical is not obtained for every Reference Chemical in every participating laboratory, leading to an incomplete data matrix. In such cases the following three criteria should all be met in order to consider the datasets acceptable:

1. All 20 Reference Chemicals should have at least one complete run sequence.
2. In each of at least three participating laboratories, a minimum of 85% of the run sequences need to be complete (for 20 chemicals, three invalid run sequences are allowed in a single laboratory).
3. A minimum of 90% of all possible run sequences from at least three laboratories need to be complete (for 20 chemicals tested in three laboratories, a total of six invalid run sequences are allowed).

### 8-2-4) Rules

The calculation of the reliability and accuracy values of the proposed test method should be done considering all four criteria below, ensuring that values for reliability and relevance are calculated in a predefined and consistent manner:

1. Only data of runs from complete run sequences qualify for calculation of within- and between-laboratory variability and predictive capacity (accuracy) of the test method.
2. The final classification for each Reference Chemical in each participating laboratory should be obtained by using the mean value of viability over the different runs of a complete run sequence.
3. Only data obtained for chemicals that have complete run sequences in all participating laboratories qualify for calculation of between-laboratory variability of the test method.
4. Calculation of the accuracy values should be done on the basis of individual laboratory predictions

obtained for the 20 Reference Chemicals by the different participating laboratories.

In this context, a **run sequence** consists of three independent runs from one laboratory for one test chemical. A **complete run sequence** is a run sequence from one laboratory for one test chemical where all three runs are valid. This means that any single invalid run invalidates an entire run sequence of three runs.

### Within-laboratory reproducibility

An assessment of within-laboratory reproducibility should show that the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of 20 Reference Chemicals within one single laboratory is equal to or higher than ( $\geq$ ) 90%.

### Between-laboratory reproducibility

An assessment of between-laboratory reproducibility is not essential if the proposed test method is to be used in a single laboratory only. For methods to be transferred between laboratories, the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of 20 Reference Chemicals between preferentially a minimum of 3 laboratories should be equal or higher than ( $\geq$ ) 80%.

### Predictive capacity (accuracy)

The accuracy (sensitivity, specificity and overall accuracy) of the proposed similar or modified test method should be comparable or better to that of the VRM, taking into consideration information relating the species of interest (Table 3). The sensitivity should be equal to or higher than ( $\geq$ ) 80%. However, an additional restriction applies to the sensitivity of the proposed *in vitro* test method; only two *in vivo* Category 2 chemicals, *1-decanol* and *di-n-propyl disulphide*, may be misclassified as a No Category by more than one participating laboratory. The specificity should be equal to or higher than ( $\geq$ ) 70%. No restrictions with regard to specificity of the proposed *in vitro* test method were applied; any participating laboratory may misclassify any *in vivo* No Category chemical as long as the final specificity of the test method is within the acceptable range. The overall accuracy should be equal to or higher than ( $\geq$ ) 75%. Although the sensitivity of the VRM calculated for the 20 Reference Chemicals listed in Table 1 is equal to 90%, the defined minimum sensitivity value required for any similar or modified test method to be considered valid is set at 80% because both *1-decanol* (a borderline chemical) and *di-n-propyl disulphide* (a false negative of the VRM) are known to be non-irritant chemicals in humans, although they have been identified as irritants in the rabbit test. Since RhE models are based on cells of human origin, they may predict these chemicals as non-irritant (UN GHS No Category).

**Table3: Required predictive values for sensitivity, specificity and overall accuracy for any similar or modified test method to be considered valid**

| Sensitivity | Specificity | Overall Accuracy |
|-------------|-------------|------------------|
| $\geq 80\%$ | $\geq 70\%$ | $\geq 75\%$      |

### 8-3. Data collection, handling, and analysis

The independent biostatistician for the study collected and organized the data using specific data collection software (Ddatasheet5.0:20090430.xls). They worked in close collaboration with JaCVAM (Hajime Kojima). After decoding the data, JaCVAM performed statistical analyses. The data management procedures and statistical tools applied were approved by the VMT.

### 8-4. Quality assurance, GLP Laboratories

All participating laboratories conducted research following OECD GLP-like principles.

### QC aspects

JaCVAM (Hajime Kojima) assured the quality of all the data and records. After the validation study, all study documents were submitted to the chairperson of VMT and only data sheets were forwarded by e-mail to the biostatistician. All data sheets from one participating laboratory, KOBAYASHI Pharmaceutical Co., Ltd. are provided as an example in Appendix 6. The chairperson reviewed the contents of the study documents and clarified illegible or unclear content by contacting each group by e-mail or telephone.

## 9. Results

### 9-1 Comments in the datasheets

A few comments from each laboratory are listed in Table 4. Application of potassium hydroxide (5%aq) (B276, B296, and B288) caused the model's layers to be desquamated. Upon application of B301, B304, B306, and B310, the cups were discoloured and crystallized. The VMT judged that these occurrences had no effect on the results of the study.

**Table 4 Comments on the datasheets (Viability)**

| Lab | Exp.No. | Lot            | Date    | Comments   |
|-----|---------|----------------|---------|--|
| a   | Main-1  | LCE24-100906-A | 9/9/10  | The model's layers treated by B-276 were desquamated         |
| b   | Main-1  | LCE24-100906-A | 9/8/10  | The model's layers treated by B-296 were desquamated         |
| b   | Main-2  | LCE24-100913-A | 9/15/10 | The model's layers treated by B-288 & B-296 were desquamated |
| c   | Main-1  | LCE24-100830-A | 9/6/10  | Cups treated by B-301, 304, 306 and 310 were discoloured.    |
| c   | Main-2  | LCE24-100913-A | 9/20/10 | Cups treated by B-301, 304, 306 and 310 were discoloured.    |
| c   | Main-3  | LCE24-100920-A | 9/27/10 | Cups treated by B-301, 304, 306 and 310 were discoloured.    |

### 9-2 Negative control

Table 5 shows the absorbance values for the negative control. All data for the negative control met the acceptance criteria.

**Table 5 Viability of negative control**

| Laboratory | Average OD/<br>triplicate tissues | Average, SD at<br>all OD |
|------------|-----------------------------------|--------------------------|
| Lab a      | 0.88                              | 0.91±0.05                |
|            | 0.87                              |                          |
|            | 0.92                              |                          |
|            | 0.98                              |                          |
| Lab b      | 1.03                              | 1.03±0.06                |
|            | 1.02                              |                          |
|            | 1.13                              |                          |
|            | 0.98                              |                          |
|            | 0.98                              |                          |
| Lab c      | 1.09                              | 1.07±0.09                |
|            | 0.98                              |                          |
|            | 1.01                              |                          |
|            | 1.06                              |                          |
|            | 1.20                              |                          |

### **9-3 Positive control**

Table 6 shows the absorbance values for the positive control. All data for the positive control met the acceptance criteria.

**Table 6 Viability of the positive control**

| Laboratory | Average OD/<br>triplicate tissues | Average, SD at<br>all OD |
|------------|-----------------------------------|--------------------------|
| Lab a      | 2.29                              | 2.65±0.68                |
|            | 3.62                              |                          |
|            | 2.59                              |                          |
|            | 2.10                              |                          |
| Lab b      | 4.98                              | 3.87±0.84                |
|            | 3.22                              |                          |
|            | 4.50                              |                          |
|            | 3.02                              |                          |
|            | 3.62                              |                          |
| Lab c      | 2.87                              | 2.69±0.49                |
|            | 3.37                              |                          |
|            | 2.64                              |                          |
|            | 2.55                              |                          |
|            | 2.02                              |                          |

#### 9-4 Viability of chemicals

Table 7 shows the mean viability of testing chemicals at each tissue. Two data points at Lab a, eight data points at Lab b, and four data points at Lab c showed a SD > 18% and did not meet the acceptance criteria. Instead of generating insufficient data, each laboratory re-tested up to two additional runs. At Lab b, No. 15 resulted in a single invalid run, thereby invalidating an entire run sequence of three runs. In addition, the VMT did not accept all data from the fourth or fifth runs. The original data are shown in Appendix 5.

All study acceptance criteria were met as shown below.

1. All 20 Reference Chemicals had at least one complete run sequence at each laboratory.
2. In each of three participating laboratories, at least 95% of the run sequences were complete (One invalid run sequence was allowed in Lab b).
3. 99.4% of all possible run sequences from the three laboratories were complete (for 20 chemicals tested in three laboratories, a total of one invalid run sequence is allowed).

These experiments confirmed the feasibility of the LabCyte EPI-MODEL24 SIT test method.

**Table 7. Mean viability of chemicals at each laboratory**

| Chem. | Lab | Exp.  |       |       |      |      |
|-------|-----|-------|-------|-------|------|------|
|       |     | 1     | 2     | 3     | 4    | 5    |
| 01    | a   | 12.4  | 11.3  | 19.0  |      |      |
|       | b   | 16.5  | 10.7  | 10.6  |      |      |
|       | c   | 9.0   | 9.8   | 9.8   |      |      |
| 02    | a   | 91.7  | 81.5  | 69.6  | 80.1 |      |
|       | b   | 60.9  | 57.5  | 65.5  | 69.5 |      |
|       | c   | 90.5  | 77.4  | 102.0 | 93.0 | 88.7 |
| 03    | a   | 108.0 | 113.0 | 105.0 |      |      |
|       | b   | 96.5  | 96.7  | 90.2  |      |      |
|       | c   | 89.4  | 90.8  | 106.0 | 98.9 | 96.0 |
| 04    | a   | 19.1  | 43.4  | 65.1  | 59.3 |      |
|       | b   | 66.6  | 70.6  | 48.1  | 66.2 |      |
|       | c   | 90.1  | 93.0  | 93.2  |      |      |
| 05    | a   | 89.6  | 77.0  | 67.6  |      |      |
|       | b   | 75.9  | 57.5  | 74.8  | 77.1 |      |
|       | c   | 68.5  | 86.6  | 66.4  | 67.2 | 74.4 |
| 06    | a   | 16.2  | 15.9  | 17.0  |      |      |
|       | b   | 17.3  | 13.5  | 11.4  |      |      |
|       | c   | 15.5  | 16.1  | 12.0  |      |      |
| 07    | a   | 110.0 | 110.0 | 104.0 |      |      |
|       | b   | 98.8  | 93.1  | 76.3  |      |      |
|       | c   | 91.2  | 102.0 | 108.0 |      |      |
| 08    | a   | 109.0 | 122.0 | 111.0 |      |      |
|       | b   | 93.1  | 106.0 | 86.6  |      |      |
|       | c   | 95.5  | 106.0 | 119.0 |      |      |
| 09    | a   | 105.0 | 111.0 | 102.0 |      |      |
|       | b   | 98.0  | 95.7  | 83.5  |      |      |
|       | c   | 99.6  | 100.0 | 113.0 |      |      |
| 10    | a   | 15.7  | 20.3  | 16.0  |      |      |
|       | b   | 11.5  | 15.9  | 11.4  |      |      |
|       | c   | 17.3  | 14.1  | 14.9  |      |      |
| 11    | a   | 14.2  | 16.5  | 9.4   |      |      |
|       | b   | 12.4  | 17.3  | 16.2  |      |      |
|       | c   | 22.1  | 15.1  | 14.1  |      |      |
| 12    | a   | 8.9   | 15.9  | 10.0  |      |      |
|       | b   | 11.0  | 7.8   | 9.0   |      |      |
|       | c   | 6.0   | 7.4   | 5.7   |      |      |
| 13    | a   | 48.0  | 16.2  | 16.1  | 15.5 |      |
|       | b   | 39.5  | 6.6   | 49.6  | 17.2 | 19.0 |
|       | c   | 17.5  | 17.0  | 16.2  |      |      |
| 14    | a   | 2.1   | 4.3   | 4.1   |      |      |
|       | b   | 4.9   | 5.2   | 9.1   |      |      |
|       | c   | 2.8   | 3.4   | 3.2   |      |      |
| 15    | a   | 19.9  | 95.9  | 83.5  |      |      |
|       | b   | 39.1  | 28.0  | 52.7  | 17.5 | 18.5 |
|       | c   | 81.1  | 83.2  | 86.3  |      |      |
| 16    | a   | 0.9   | 1.7   | 1.6   |      |      |
|       | b   | 4.6   | 2.0   | 3.3   |      |      |
|       | c   | 0.9   | 3.1   | 11.6  |      |      |
| 17    | a   | 6.9   | 46.6  | 1.0   |      |      |
|       | b   | 10.6  | 21.0  | 11.6  |      |      |
|       | c   | 6.3   | 5.0   | 6.6   |      |      |
| 18    | a   | 6.7   | 4.5   | 3.6   |      |      |
|       | b   | 9.8   | 10.9  | 11.0  |      |      |
|       | c   | 1.3   | 1.8   | 2.2   |      |      |
| 19    | a   | 9.4   | 10.3  | 10.4  |      |      |
|       | b   | 9.5   | 7.0   | 9.5   |      |      |
|       | c   | 11.9  | 10.2  | 10.9  |      |      |
| 20    | a   | 8.7   | 12.0  | 7.8   |      |      |
|       | b   | 9.1   | 7.9   | 37.6  | 17.4 |      |
|       | c   | 7.6   | 7.0   | 6.8   |      |      |

**Red block**VRM SD > 18%, Not  
accepted data**Light block**

Not accepted for 4th dat

### 9-5. Classification of three independent viabilities at each laboratory

The classifications from individual viabilities and the **mean** of three independent viabilities are shown in Table 8. Lab a misclassified two data points (No. 4 and 15 at the first test), Lab b misclassified two data points (No. 15 at the first and second tests), and Lab c missed no classifications. As previously discussed, the third data point of the test with No. 15 at Lab b induced a single invalid run, thereby invalidating the entire run sequence of three runs. Therefore, the VMT judged “not detected” in the classification of No.15.

**Table 8: Classification using three independent viabilities**

P: Positive, N: Negative, F: Final determination by median, ND: Not detected

| No | UN<br>GHS in<br>vivo Cat. | Lab a |   |   |   | Lab b |   |    |    | Lab c |   |   |   |
|----|---------------------------|-------|---|---|---|-------|---|----|----|-------|---|---|---|
|    |                           | 1     | 2 | 3 | F | 1     | 2 | 3  | F  | 1     | 2 | 3 | F |
| 1  | No                        | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 2  | No                        | N     | N | N | N | N     | N | N  | N  | N     | N | N | N |
| 3  | No                        | N     | N | N | N | N     | N | N  | N  | N     | N | N | N |
| 4  | No                        | P     | N | N | P | N     | N | N  | N  | N     | N | N | N |
| 5  | No                        | N     | N | N | N | N     | N | N  | N  | N     | N | N | N |
| 6  | No                        | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 7  | No                        | N     | N | N | N | N     | N | N  | N  | N     | N | N | N |
| 8  | No                        | N     | N | N | N | N     | N | N  | N  | N     | N | N | N |
| 9  | No                        | N     | N | N | N | N     | N | N  | N  | N     | N | N | N |
| 10 | No                        | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 11 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 12 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 13 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 14 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 15 | Cat.2                     | P     | N | N | N | P     | P | ND | ND | N     | N | N | N |
| 16 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 17 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 18 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 19 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |
| 20 | Cat.2                     | P     | P | P | P | P     | P | P  | P  | P     | P | P | P |

## 10. Discussion

### 10-1. Reliability

#### Within-laboratory reproducibility

An assessment of within-laboratory reproducibility should show a concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of the 20 Reference Chemicals at each laboratory. As shown in Table 8 above, Lab a missed two classifications (No. 4 and 15) and the rate of within-laboratory reproducibility was 90.0% (18/20). Lab b missed one data point (No. 15) and the rate of reproducibility was 95.0% (19/20). Lab c missed no classifications and had a reproducibility rate of 100%. Therefore, results of all laboratories were sufficient, having a reproducibility rate equal to or higher than ( $\geq$ ) 90%.

#### Between-laboratory reproducibility

For methods to be transferred between laboratories, the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of the 20 Reference Chemicals between three laboratories was evaluated. As shown in Table 8, all laboratories missed more than four classifications and the rate of between-laboratory reproducibility was 95.0% (19/20). Therefore, all laboratories had a sufficient between-laboratory reproducibility that was equal to or higher than ( $\geq$ ) 80%.

### 10-2. Predictivity

The accuracy (sensitivity, specificity, and overall accuracy) of the LabCyte EPI-MODEL24 SIT skin irritation test was evaluated by cell viabilities (MTT) as an indicator, and the UN-GHS classifications are shown in Table 9. The sensitivity, specificity, and accuracy of this prediction model at each laboratory were 90–100%, 60–70%, and 75–84.2%, respectively. Some deviations from the OECD Performance standard (sensitivity of 80%, specificity of 70%, and accuracy of 75%; shown in Table 3) were specific adaptations for the LabCyte EPI-MODEL excluding 60% of specificity at Lab a. Unfortunately, one of three laboratories is insufficient with acceptance criteria at the OECD Performance standard. Two of three laboratories, however, are sufficient with acceptance criteria at the OECD Performance standard and the VMT considered that this assay had acceptable reliability of accuracy.

**Table 9. 2x2 tables**

| Lab a                      | <i>In vivo</i> classification |          |              |       |
|----------------------------|-------------------------------|----------|--------------|-------|
|                            |                               | Irritant | Non-Irritant | Total |
| <i>In vitro</i> prediction | Irritant                      | 9        | 4            | 13    |
|                            | Non-irritant                  | 1        | 6            | 7     |
|                            | Total                         | 10       | 10           | 20    |
| Sensitivity (%)            |                               | 90.0     |              |       |
| Specificity (%)            |                               | 60.0     |              |       |
| Accuracy (%)               |                               | 75.0     |              |       |

| Lab b                      |              | <i>In vivo</i> classification |              |       |
|----------------------------|--------------|-------------------------------|--------------|-------|
|                            |              | Irritant                      | Non-Irritant | Total |
| <i>In vitro</i> prediction | Irritant     | 9                             | 3            | 12    |
|                            | Non-irritant | 0                             | 7            | 7     |
|                            | Total        | 9                             | 10           | 19    |
| Sensitivity (%)            |              | 100.0                         |              |       |
| Specificity (%)            |              | 70.0                          |              |       |
| Accuracy (%)               |              | 84.2                          |              |       |

| Lab c                      |              | <i>In vivo</i> classification |              |       |
|----------------------------|--------------|-------------------------------|--------------|-------|
|                            |              | Irritant                      | Non-Irritant | Total |
| <i>In vitro</i> prediction | Irritant     | 9                             | 3            | 12    |
|                            | Non-irritant | 1                             | 7            | 8     |
|                            | Total        | 10                            | 10           | 20    |

|                 |  |      |  |  |
|-----------------|--|------|--|--|
| Sensitivity (%) |  | 90.0 |  |  |
| Specificity (%) |  | 70.0 |  |  |
| Accuracy (%)    |  | 80.0 |  |  |

## 11. Conclusions

Based on the reference list in the OECD Performance Standards, a catch-up validation of the LabCyte EPI-MODEL24 SIT by three labs was performed. The assay demonstrated high reliability within and between laboratories, and acceptable reliability of accuracy (75–84.2% overall accuracy, 90–100% overall sensitivity, and 60-70% overall specificity) on the MTT assay excluding 60% of specificity at one laboratory. Two of three laboratories are sufficient with acceptance criteria at the OECD Performance standard and the VMT considered that this assay had acceptable reliability of accuracy for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

## 12. Acknowledgement

This validation study was supported by the Health and Labour Sciences Research Grant, Japan.

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