ME-TOO VALIDATION REPORT

VALIDATION STUDY FOR LabCyte CORNEA-MODEL24 EYE IRRITATION TEST

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Version 2.5

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LIST OF ACRONYMS AND ABBREVIATIONS

BCOP:	Bovine Corneal Opacity and Permeability
BLR:	Between-Laboratory Reproducibility
BRD	Background Review Document
CAS:	Chemical Abstracts Service
D-PBS:	Dulbecco's Phosphate Buffered Saline
EBSS:	Earle's Balanced Salt Solution
EIT:	Eye Irritation Test
FL:	Fluorescein Leakage
GHS:	Globally Harmonized System of Classification and Labeling of Chemicals
GLP:	Good Laboratory Practice
ICE:	Isolated Chicken Eye
JaCVAM:	Japanese Centre for the Validation of Alternative Methods
JSAAE:	Japanese Society for Alternative to Animal Experiments
MTS	3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT:	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD:	Optical Density
OECD:	Organisation for Economic Co-operation and Development
QC:	Quality Control
RhCE:	Reconstructed human Corneal Epithelium
SD:	Standard Deviation
SLS:	Sodium Lauryl Sulfate
SOP:	Standard Operating Procedure
STE	Short Time Exposure
TG:	Test Guideline
UN:	United Nations
VMT:	Validation Management Team
VRM:	Validated Reference Method
WLR:	Within-laboratory reproducibility
WNT:	Working Group of the National Coordinators of the Test Guideline Project
WST-1	Water soluble tetrazolium salt-1 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt

WST-8:	Water soluble tetrazolium salt-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
XTT-1	2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl] -2H-tetrazolium hydroxide

APPENDICES

Appendix 1: Study Plan ver.2.0.

Appendix 2: Datasheet_v2.5.2. (Japanese)

Appendix 3. Material data sheet (Japanese)

Appendix 4. Result of QC for each lot of LabCyte CORNEA-MODEL24

Appendix 5: LabCyte CORNEA-MODEL24 validation study phase 1 and 2 Statistical analysis report.

Appendix 6: LabCyte CORNEA-MODEL24 validation study phase 1 and 2 Statistical analysis report Supplement 1.

Appendix 7: LabCyte CORNEA-MODEL24 EYE IRRITATION TEST OPERATION PROTOCOL Ver. 2.5.6.

1. GOAL STATEMENT

1-1. The Ultimate Goal

The ultimate goal of this test method is partial replacement of the regulatory Draize eye irritation test (OECD TG 405; OECD, 2012) in a bottom-up approach.

1-2. Primary Goal

The primary goal of this me-too validation study is to assess the within- and between-laboratory reproducibility, as well as the predictive capacity, of the LabCyte CORNEA-MODEL24 Eye Irritation Test (LabCyte24 EIT) in accordance with PERFORMANCE STANDARD (OECD, 2015b) for OECD TG 492.

2. OBJECTIVE

The OECD Working group of the National Coordinators of the Test Guideline Project (WNT) accepted OECD TG No. 492 *in vitro* eye irritation test guideline in July, 2015 (OECD, 2015a). OECD TG 492 addresses the human health endpoint “eye irritation.” One validated test method that conforms to this TG and for which pre-validation, optimization, and validation studies have been completed is an *in vitro* test method using a Reconstructed human Corneal Epithelial tissue (RhCE) model. This method is commercially available as EpiOcular and has been designated as Validated Reference Method (VRM).

The LabCyte24 EIT is another *in vitro* test method that employs a new RhCE model known as the LabCyte CORNEA-MODEL24 and for which protocol optimization has been completed. The objective of this me-too validation is to confirm that the LabCyte24 EIT conforms to OECD TG 492 by assessing its within- and between-laboratory reproducibility, as well as its predictive capacity. The study was performed using a set of 30 test chemicals selected to meet PERFORMANCE STANDARD for OECD TG 492. This 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

To date, a number of *in vitro* test methods have been developed as alternatives to the Draize eye test (Draize et. al., 1944). In particular, the following tests have been found to predict ocular irritancy adequately and were adopted as OECD TG.

Bovine Corneal Opacity and Permeability (BCOP) test (TG 437, OECD 2013a)
Isolated Chicken Eye (ICE) test (TG 438, OECD 2013b)
Fluorescein Leakage (FL) test (TG 460, OECD 2012)
Short Time Exposure (STE) test (TG491, OECD 2015c)
RhCE EIT (TG 492, OECD 2015a).

Since the use of a battery of *in vitro* tests is considered a viable means of improving overall accuracy, there is a clear need for the development of additional *in vitro* test methods.

A newly developed RhCE tissue model known as the LabCyte CORNEA-MODEL24 was recently developed using human corneal epithelial cells. The histological structure of the LabCyte CORNEA-MODEL24 is considered highly similar to that of a native human corneal epithelium. The LabCyte CORNEA-MODEL24 EIT (LabCyte24 EIT) provides a promising alternative to animal testing in assessing corneal irritation. Protocol optimization was studied in order to establish a new alternative method for eye irritancy evaluation using the LabCyte CORNEA-MODEL24 tissue. Through protocol optimization, the ring-study for the technical transferability and several pre-validation studies, an improved LabCyte24 EIT was finally established as shown in the Background Review Document (BRD) for the LabCyte24 EIT.

Upon review, the LabCyte24 EIT Validation Management Team (VMT) determined that the improved LabCyte24 EIT was similar to the EpiOcular EIT, which is a VRM of OECD TG 492. Therefore, a me-too validation study of the LabCyte24 EIT was planned in accordance with performance standard for OECD TG 492. On the advantages in terms of cost and animal welfare, LabCyte24 EIT is considered comparably equal to the VRM of OECD TG 492.

4. RECONSTRUCTED HUMAN CORNEAL EPITHELIAL MODEL

4-1. LabCyte CORNEA-MODEL24

The LabCyte CORNEA-MODEL24 is a commercially available RhCE model produced by Japan Tissue Engineering Co. Ltd. It comprises normal human corneal epithelial cells that are derived from a human cornea, which cells are neither cornified nor keratinized. The cells are cultured with 3T3-J2 cells as a feeder layer in order to expand them while maintaining their phenotype (Rheinwald and Green, 1975; Green, 1978). Reconstruction of the human cultured corneal epithelial tissue is achieved by cultivating and proliferating the corneal epithelial cells on an inert filter substrate with a surface area of 0.3 cm^2 at an air-liquid interface for 13 days using an optimized medium containing 5% fetal bovine serum (FBS). This results in the formation of a multilayer and non-cornified structure comprising a fully differentiated corneal epithelium with features mimicking those of a normal human corneal epithelium described in the section 2-1 of the BRD. For delivery, LabCyte CORNEA-MODEL24 tissues are embedded in an agarose gel containing a nutrient solution and shipped.

Even if the lots of some raw materials (e.g. FBS) change over time, quality and performance of each batch of LabCyte CORNEA-MODEL24 is controlled to ensure a highly stable production. Also the timing of FBS lot change was indicated in the QC information of each batch of LabCyte CORNEA-MODEL24, and different FBS lots have not affect QC data of the LabCyte CORNEA-MODEL24, as described in the section 2-2 of the BRD.

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 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. COMPARISON OF ESSENTIAL TEST METHOD COMPONENTS BETWEEN LabCyte24 EIT AND VRM OF THE PERFORMANCE STANDARD FOR THE OECD TG492

5-1. The Performance Standard for the OECD TG 492

The OECD document, series on Testing & Assessment No 216 includes Performance Standard in vitro RhCE test methods for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage, based on the validated reference method EpiOcular EIT described in the OECD TG 492 (OECD, 2015a), has been declassified and published in 2015 (OECD, 2015b). This PERFORMANCE STANDARD consists of; (i) Essential Test Method Components; (ii) Minimum List of Reference Chemicals, and; (iii) Defined Reliability and Accuracy Values that the proposed test method should meet or exceed.

The (i) Essential Test Method Components of the PERFORMANCE STANDARD describes the general and functional conditions of the RhCE model for the EIT method according the OECD TG492 and specifically explains the procedural conditions of the VRM EIT. The general and functional conditions of LabCyte CORNEA-MODEL24 are described in detail in the BRD.

Essential test method components of RhCE EIT for the OECD TG492 and the corresponding LabCyte24 EIT are shown as follows.

5-2. Essential Differences between Test Method Components of LabCyte24 EIT and the OECD TG492 VRM

Difference between test components of LabCyte24 EIT and the OECD TG492 VRM are shown in Table 1.

Table 1 Description of test components for the RhCE EIT (OECD TG 492)

Test component (Required per PERFORMANCE STANDARD for OECD TG 492.)	LabCyte24 EIT		EpiOcular EIT	
Cell source (Relevant human-derived cells)	Human corneal epithelial cells		Human keratinocytes	
Pre-exposure (To select if necessary)	Overnight incubation		Pre-soak incubation	
Tissue replicates (Min. of 2 tissues)	3 tissues		2 tissues	
Application of test chemical	Liquid	Solid	Liquid	Solid
Quantity (Uniformity)	50 µL (167 µL/cm^2)	10 mg (33 mg/cm^2)	50 µL (83 µL/cm^2)	50 mg (83 mg/cm^2)
Negative control (Determine as appropriate)	Dulbecco's Phosphate Buffered Saline (D-PBS)	Does Not apply	Ultrapure H_2O	
Positive control (Determine as appropriate)	Ethanol	Lauric acid	Methyl acetate	
Application period (Determine as appropriate)	1 minute (Optimized[*1])	24 hours (Optimized[*1])	30 minutes	6 hours
Post-exposure soak (Optimize as appropriate)	None (Not required)	None (Not required)	12 minutes	25 minutes
Post-application period (Optimize as appropriate)	24 hours (Optimized[*1])	0 hours (Optimized[*1])	2 hours	18 hours
Cell viability measurement (MTT assay)	WST-8 assay		MTT assay	
Cell viability threshold value (Determine as appropriate.)	40%		60%	
Detection and correction of WST-8/MTT interference	Using killed tissue		Colored: Using living tissue MTT reducer: Using Killed tissue	
Acceptance criteria SD	≤ 20%		2 tissue (difference of viability): ≤ 20% If 3 tissue: ≤ 18%	

*1) Refer to previous report (Katoh, 2012)

Difference between test components of LabCyte24 EIT and OECD TG492 VRM are as follows:

(1) Cell source
(2) Number of RhCE tissue replicates
(3) Chemical application
(4) Negative control and positive control
(5) Chemical application period
(6) Measurement of tissue viability
(7) Detection and correction of WST-8/MTT interference
(8) Acceptance criterion (SD)

5-3. Comparison and Similarity Considerations between each Test Component LabCyte24 EIT and the VRM of the Performance Standard

5-3.1. Cell Source

In the PERFORMANCE STANDARD for the OECD TG 492 (OECD, 2015b), relevant human-derived cells (e.g., human corneal epithelial cells or keratinocytes) should be used in the RhCE tissue, which should be composed of progressively stratified but not cornified cells. LabCyte CORNEA-MODEL24 comprises normal human corneal epithelial cells that are derived from a human cornea, which cells are neither cornified nor keratinized. This structure is a multilayered and non-cornified structure comprising a fully differentiated corneal epithelium with features mimicking those of a normal human corneal epithelium (Fig. 1), as described in the section 2-1 of the BRD.

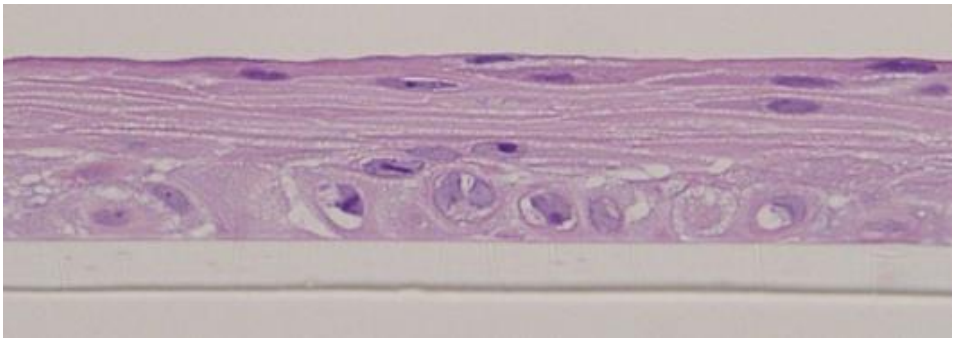

Fig. 1. Structure of LabCyte CORNEA-MODEL24

Concerning the cell source of RhCE tissues, LabCyte CORNEA-MODEL24 tissue is concordance with the PERFORMANCE STANDARD requirement for the OECD TG 492 (OECD, 2015b).

5-3.2. Number of RhCE Tissue replicates

In the PERFORMANCE STANDARD for the OECD TG 492 (OECD, 2015b), at least two tissue replicates should be used for each test chemical and each control substance in each run. In the LabCyte24 EIT, three tissue replicates are used for each chemical and control substance.

Concerning the number of RhCE tissue replicates, the LabCyte24 EIT protocol is in concordance with the PERFORMANCE STANDARD requirement for the OECD TG 492 (OECD, 2015b).

5-3.3. Chemical Application Amount

In the PERFORMANCE STANDARD for the OECD TG 492 (OECD, 2015b), a sufficient amount of the chemical or control substance should be applied to uniformly cover the corneal epithelial surface while avoiding an infinite dose. In the VRM, approximately 80 μL/cm^2 of liquid chemicals, and 80 μL/cm^2 of solid chemicals are applied. On the other hand, in the LabCyte24 EIT, approximately 165 μL/cm^2 of liquid chemicals, and 33 mg/cm^2 of solid chemicals are applied. Application amounts of the liquid and solid chemicals in the LabCyte24 EIT satisfy the condition described in the PERFORMANCE STANDARD for the OECD TG 492 (applied to uniformly cover the corneal epithelial surface while avoiding an infinite dose).

Concerning chemical application amounts, LabCyte24 EIT protocol is in concordance with the PERFORMANCE STANDARD requirement for the OECD TG 492 (OECD, 2015b).

5-3.4. Negative Control and Positive Control

In the PERFORMANCE STANDARD for the OECD TG 492 (OECD, 2015b), separate negative and positive controls are needed for each protocol of the test method. In the VRM, the positive control and the negative control substances used are neat methyl acetate and ultra-pure H_2O, respectively, for both liquid and solid chemicals. In the LabCyte24 EIT, the positive control and the negative control substances used are ethanol and D-PBS for liquid

chemicals, and lauric acid and no treatment for solid chemicals. Concurrently, the negative control should be included in each run to demonstrate that the viability and the sensitivity determined with the positive control of the tissues are within acceptance ranges defined based on historical data, which is described in the section 3-2, 3-3, and 3-4 of the BRD. The concurrent negative control provides the baseline to calculate the relative percent viability of the tissues treated with the test chemicals.

Concerning selection and setting of negative and positive control, LabCyte24 EIT protocol is in concordance with the PERFORMANCE STANDARD requirement for the OECD TG 492 (OECD, 2015b).

5-3.5. Chemical Exposure, Post-exposure Immersion and Post-exposure Incubation Periods

In the PERFORMANCE STANDARD for the OECD TG 492 (OECD, 2015b), it was described that two different treatment protocols may be used for different types of chemicals, for Liquid test chemicals or for Solid test chemicals. If different protocols are used to an EIT, they may differ in terms of their exposure periods, post-exposure incubation immersion periods and post-exposure incubation periods.

In order to establish suitable exposure and post-exposure incubation periods for the new RhCE EIT using the LabCyte CORNEA-MODEL24 tissue, a protocol optimization study was performed as described in the section 3-1 of the BRD. In the study for liquid chemicals, the prediction of LabCyte24 EIT only resulted in high correlation to the *in vivo* classification when the exposure was set to a short period (1 minute), as well as post-exposure incubation was set to a long period (24 hours). In contrast, in the study for solid chemicals, a long exposure period (24 hours) was required to eliminate false-negative predictions in the LabCyte24 EIT. Through the protocol optimization study, the chemical exposure and post-exposure incubation periods for LabCyte24 EIT were set to 1 minute and 24 hours for liquid test chemicals, and 24 hours without post-exposure incubation for solid test chemicals.

Concerning the selection and setting of the chemical exposure pattern, LabCyte24 EIT protocol is in concordance with the PERFORMANCE STANDARD requirement.

5-3.6. Measurement of Tissue Viability

In the RhCE EIT test method for the OECD TG 492, the cell viability is measured as an

endpoint for the prediction of eye irritation. In the OECD TG 492, MTT assay is selected for the measurement of cell viability. MTT assay is a tetrazolium reduction assay. MTT is reduced by cellular dehydrogenase and produces insoluble MTT formazan with blue color. A variety of tetrazolium salts which are reduced by cellular dehydrogenase in the same manner as the MTT assay and change to water soluble formazan charging various colors have been developed. Commonly used tetrazolium salts include MTT (which produces water-insoluble formazan), MTS, XTT, WST-1 and WST-8 (which do not produce water-soluble formazan).

Since cellular hydrogenase is rapidily inactivated by damaged cells, the degree of coloring by formazan dye directly correlates to cell viability. Therefore, the cell vialibity can be quantified through the determination of such coloring degree of formazan dye. Such tetrazolium reduction assay is widely accepted as a simple test method for analysis of cell viability.

While many kinds of tetrazolium salts have been developed, MTT is the one that has been widely used, and is the assay is selected for the EpiOcular EIT for the OECD TG492 VRM.

On the other hand, in the LabCyte24 EIT, WST-8 assay, which produces water soluble formazan, was selected to determine cell viability. The principle of the WST-8 assay is the same as the MTT assay. Furthermore, since the WST-8 assay does not require an extraction step, the procedure is much simpler compared to the MTT assay.

WST-8 was demonstrated to be of value for use as an indicator for cell viability with higher sensitivity than conventional tetrazolium salts, including MTT (Tominaga et. al., 1999).

The outline of MTT assay and WST-8 assay is described in BRD.

Since WST-8 formazan is water soluble, it does not form crystals like MTT. Therefore, after the incubation with the WST-8 solution, OD of reaction medium can be directly analyzed to obtain the number of viable cells. Unlike MTT, a dissolution step is not required in the WST-8 assay. This leads to shorter process periods, which in turn might contribute to more accurate test results compared to the MTT assay.

The reaction principle of WST-8 assay is the same to that of MTT assay, as both are tetrazolium reduction assays that utilize dehydrogenase activity. Furthermore, from the knowledge and highly correlated results between WST-8 assay and MTT assay, described in BRD, it is assumed that the measurement data from assays are almost equal.

Finally, the LabCyte24 EIT VMT determined that the measurement method of the cell viability of the LabCyte24 EIT was similar to that of the OECD TG 492 VRM, and its protocol was in concordance with the PERFORMANCE STANDARD requirement for the OECD TG 492 (OECD, 2015b).

5-3.7. Detection and Correction of MTT/WST-8 Assay Interference

A possible limitation of this EIT might be some test chemicals that directly affect the WST-8/MTT endpoints. Colored chemicals and/or WST-8 reducers may interfere with the MTT/WST-8 assay.

In the LabCyte24 EIT, both coloring chemicals and WST-8 reducers were detected using the same protocol; coloring interference is corrected using killed tissues (see the Section 8.1.3). However, the VRM in the PERFORMANCE STANDARD for the OECD TG 492 detects coloring interference by spectral analysis and this interference is corrected using living tissues without MTT reaction. The reason why LabCyte24 EIT adopted a common protocol for both types of interference is because the WST-8 detection system can sufficiently detect and correct coloring chemicals, as well as WTS-8 reducers. This was determined taking into consideration the fact that the adsorption and residual pathway of both types of chemicals are similar in both living and killed tissues.

Because the principle of detection and correction of coloring interfering chemicals adopted by the LabCyte24 EIT and the VRM of OECD TG492 is considered similar, LabCyte24 EIT protocol is in concordance with the PERFORMANCE STANDARD requirement for the OECD TG 492 (OECD, 2015b) with regards to the detection and correction of WST-8/MTT assay interference chemicals.

Note

The VMT further requested that the protocol be updated to change detection and correction protocols for coloring chemicals according to OECD T G492 VRM (Appendix 7).

5-3.8. Acceptance Criterion (SD)

In the OECD TG 492 (OECD, 2015a) it is defined that the SD value between three tissues should not exceed 18%. On the other hand, in the LabCyte24 EIT, the acceptance criterion of SD has been set to ≤ 20%, which is the accepted limit of difference of viability between two tissue replicates in the EpiOcular EIT. VMT considered that a SD of 20% was acceptable because the difference between ≤ 20% and ≤ 18% is little.

However, in this validation report, it is reported that the reliability and the predictive performance of LabCyte24 EIT should be evaluated with the SD set to ≤ 18%, because the VMT determined that the data analysis of the validation study should be in accordance with the acceptance criteria of OECD TG 492.

5-4. Similarity of LabCyte24 EIT to OCED TG 492 VRM

Based on the points described in the section 5-3, the VMT considers the LabCyte24 EIT to be a derivative of the RhCE EIT method described in OECD TG 492, and this validation study for LabCyte24 EIT was planned in accordance with PERFORMANCE STANDARD for OECD TG 492 (OECD, 2015b).

6. VALIDATION MANAGEMENT STRUCTURE

This validation study was funded in part by the Japanese Society for the Alternative to Animal Experiments (JSAAE).

The management structure is shown in Fig. 2.

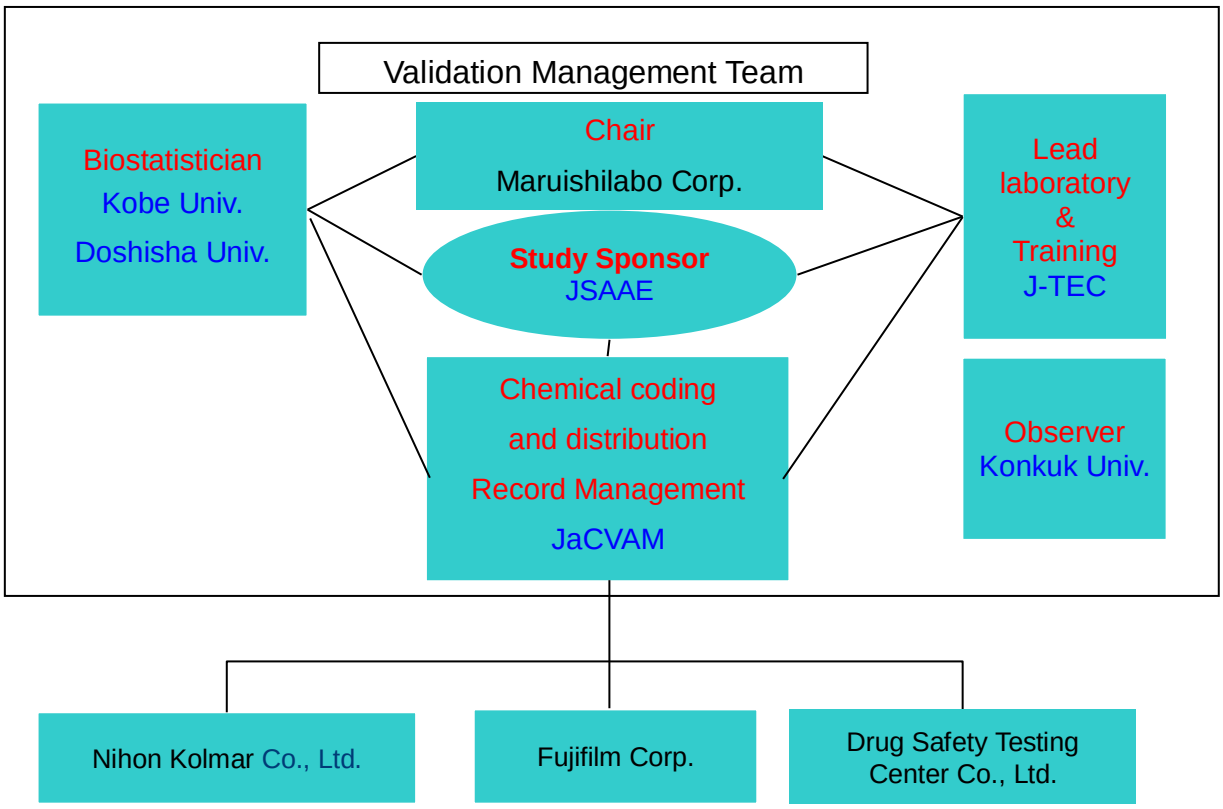

Fig. 2. Management structure for me-too LabCyte24 EIT validation study

6-1. Validation Management Team

A Validation Management Team (VMT) was organized to ensure that the LabCyte24 EIT was validated smoothly and in a scientifically pertinent manner.

The VMT comprised of a trial coordinator, a chemical management group, a data analysis group, a record management group, and the lead laboratory (assay developer). The lead laboratory provided support to the participating laboratories. The VMT was responsible for preparing, reviewing, and finalizing a draft study plan and a study protocol. In addition, the VMT was also responsible for managing the validation study in terms of monitoring its progress,

assuring the quality of study records, and communicating with the participating laboratories.

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

1. Goal statement
2. Project plan including objective
3. Study protocol / amendments
4. Outcome of quality control (QC) 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

6-2. Trial Coordinator (VMT Chairperson)

Trial coordinator was Satoshi Nakahara of Maruishilabo Corp. He declared no conflict of interest associated with this validation study. He was independently responsible for preparing a draft study plan (Appendix 1), study protocol, and test chemical list, as well as for convening ad hoc meetings of the VMT for reviewing and finalizing the content of these documents, and all other aspects of conducting the validation study.

6-3. Chemical Selection, Acquisition, Coding, and Distribution

The chemical management group was chaired by Hajime Kojima of the Japanese Center for the Validation of Alternative Test Method (JaCVAM) and included other JaCVAM staff members. The validation study was conducted in accordance with the PERFORMANCE STANDARD for EpiOcular EIT, which is a VRM of the RhCE EIT VRM, and the chemical management group was not involved in selecting the chemicals.

The test chemicals used for this validation study were encoded and distributed to the participating laboratories by JaCVAM. Thus, the participating laboratories performed the tests without knowing the identity of the test chemicals. The coding procedure used in these studies is not known, and the test chemicals thus coded were used only for this validation study.

6-4. Data Analysis Group

The Data Analysis Group was headed by Takashi Omori of Kobe University and included Mai Endo and Mayuko Satake, both of Doshisha University. They declared no conflict of interest associated with this validation study. The Data Analysis Group performed an independent third-party review of the data obtained in this validation study. They were responsible for independent statistical processing to ensure that measured values were recorded appropriately in the data sheets (Appendix 2).

6-5. Record Management Group

The Record Management Group was headed by Hajime Kojima and included JaCVAM staff members.

The Record Management Group prepared material datasheets (Appendix 3) and distributed them to the participating laboratories. They also collected filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

6-6. Lead Laboratory

J-TEC provides support to the participating laboratories as the lead laboratory.

6-7. VMT Observers

Each participating laboratory sent a representative to observe VMT proceedings. These representatives were responsible for supervising implementation of the testing by laboratory personnel and for creating and submitting all data records and other required documentation to the Record Management after completion of all testing.

Additionally, Jeong Ik Lee of Konkuk University, Korea, was invited to observe VMT proceedings as part of an international validation per the PERFORMANCE STANDARD.

6-8. Participating Laboratories

Validation of the LabCyte24 EIT with thirty test chemicals was performed at the following participating laboratories.

Laboratory A (Lab A): Drug Safety Testing Center Co., Ltd.; Shinsuke Shinoda, Saori Hagiwara
Laboratory B (Lab B): Nihon Kolmar Co., Ltd.; Hidefumi Ikeda, Hideki Nishiura
Laboratory C (Lab C): Fujifilm Corp.; Toshihiko Kasahara, Yusuke Yamamoto

All participants underwent training for the LabCyte24 EIT in the ring-study for the technical transferability described in the section 3-2 of the BRD. Through several pre-validation studies by their laboratories described in the section 3-3 and 3-4 of the BRD, participation of the laboratories on the validation study were permitted by agreement of VMT.

J-TEC, which was lead laboratorie, is a company part of the Fujifilm Corp. group. Fujifilm Corp. participated as an independent laboratory and declared no conflict of interest associated with this validation study.

6-9. Sponsorship

The study was managed and funded jointly by JSAAE, Maruishilabo Corp., JaCVAM, and J-TEC.

1) JSAAE provided funding.
2) Maruishilabo Corp. provided support for:
   - management of the validation study
3) JaCVAM provided funding for:
   - management of the validation study
   - purchase, coding, and distribution of chemicals to the participating laboratories
   - independent QC audit of the data
   - publication of the validation study results
4) J-TEC provided support as lead laboratory for:
   - training the participating laboratories
   - independent QC audit of the LabCyte24 EIT
   - funding the participating laboratories

7. STUDY DESIGN

7-1. Test Chemicals

7-1.1 Chemical Selection

The VMT selected thirty reference chemicals from the PERFORMANCE STANDARD for OECD TG 492 (OECD, 2015b), as shown in Table 3. Reference Chemicals are used to determine if the reproducibility and predictive capacity of a proposed test method are equal to or better than the defined minimum values of an existing VRM, thus demonstrating that the proposed test method is both structurally and functionally similar to an existing VRM.

This validation study was set in two phases (Table 2), because the VMT considered that the testing process of all 30 reference chemicals at once using one batch would exceed the performing capacity of each participating laboratory.

Table 2. Breakdown of substances used for the LabCyte24 EIT validation study

Phase	No. of the substances	No. of the repetitions	Examination
I	18	3	Within and between laboratory reproducibility and predictivity
II	12	3	Within and between laboratory reproducibility and predictivity

Division of thirty test chemicals for each phase was managed by the chemical management group independently, with consideration to proportionally distribute between phases the substances classified by the United Nations (UN) Globally Harmonized System (GHS) as No category, Category 1 and 2.

7-1.2 Coding and Distribution

The coding and distribution of test chemicals was subcontracted by JaCVAM to a reliable independent laboratory. A list of the coded chemicals is shown in Table 2. A single coding was given to each chemical, but not to each repeat chemical.

Table 3. Reference Chemicals list in the OECD TG 492

No.	Test chemical	CAS number	Physical State	In vivo class	Supplier	Chemical Code			Phase
						Lab A	Lab B	Lab C	
1	(Ethylenediaminepropyl)-trimethoxysilane	1760-24-3	Liquid	Cat 1	Sigma-Aldrich	LaA101	LaB106	LaC127	II
2	Methylthioglycolate	2365-48-2	Liquid	Cat 1	Sigma-Aldrich	LaA112	LaB101	LaC130	II
3	Tetraethylene glycol diacrylate	17831-71-9	Liquid	Cat 1	Sigma-Aldrich	LaA113	LaB111	LaC101	I
4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	Solid	Cat 1	WAKO*1	LaA124	LaB115	LaC104	II
5	2,5-Dimethyl-2,5-hexanediol	110-03-2	Solid	Cat 1	Sigma-Aldrich	LaA125	LaB120	LaC107	I
6	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis - (benzenesulphonate)	27344-41-8	Solid	Cat 1	WAKO	LaA102	LaB124	LaC110	I
7	Sodium oxalate	62-76-0	Solid	Cat 1	Sigma-Aldrich	LaA111	LaB102	LaC113	I
8	2,4,11,13-Tetraazatetradecane-diimidami de, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous)	18472-51-0	Liquid	Cat 2A	Sigma-Aldrich	LaA114	LaB107	LaC116	I
9	Gamma-Butyrolactone	96-48-0	Liquid	Cat 2A	Aldrich	LaA123	LaB112	LaC119	I
10	1,5-Naphthalenediol	83-56-7	Solid	Cat 2A	TCI*2	LaA126	LaB116	LaC122	II
11	Sodium benzoate	532-32-1	Solid	Cat 2A	Sigma-Aldrich	LaA103	LaB121	LaC125	I
12	2-Methyl-1-pentanol	105-30-6	Liquid	Cat 2B	Sigma-Aldrich	LaA110	LaB125	LaC128	I
13	Diethyl toluamide	134-62-3	Liquid	Cat 2B	Sigma-Aldrich	LaA115	LaB103	LaC102	II
14	1,4-Dibutoxy benezene	104-36-9	Solid	Cat 2B	TCI	LaA122	LaB108	LaC105	II
15	2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	Solid	Cat 2B	Aldrich	LaA127	LaB117	LaC108	I
16	1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Liquid	No Cat	Sigma-Aldrich	LaA104	LaB122	LaC111	I
17	2-Ethoxyethyl methacrylate	2370-63-0	Liquid	No Cat	Sigma-Aldrich	LaA109	LaB126	LaC114	I
18	3-Phenoxybenzyl alcohol	13826-35-2	Liquid	No Cat	Sigma-Aldrich	LaA116	LaB130	LaC117	II
19	4-(Methylthio)-benzaldehyde	3446-89-7	Liquid	No Cat	Sigma-Aldrich	LaA121	LaB104	LaC120	II
20	Dipropyl disulphide	629-19-6	Liquid	No Cat	Sigma-Aldrich	LaA128	LaB109	LaC123	I
21	Ethyl thioglycolate	623-51-8	Liquid	No Cat	Sigma-Aldrich	LaA105	LaB113	LaC126	II
22	Piperonyl butoxide	51-03-6	Liquid	No Cat	Sigma-Aldrich	LaA108	LaB118	LaC129	I
23	Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Viscous	No Cat	WAKO	LaA117	LaB127	LaC103	I
24	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	Solid	No Cat	Sigma-Aldrich	LaA120	LaB129	LaC106	II
25	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phe nyl]imino]bis-ethanol	3179-89-3	Solid	No Cat	AK Scientific	LaA129	LaB105	LaC118	II
26	2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	Solid	No Cat	Sigma-Aldrich	LaA106	LaB110	LaC112	I
27	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	Solid	No Cat	Sigma-Aldrich	LaA107	LaB114	LaC115	I
28	Cellulose, 2-(2-hydroxy -3-(trimethylammonium) propoxy) ethyl ether chloride (91%)	68610-92-4	Solid	No Cat	Sigma-Aldrich	LaA118	LaB119	LaC109	I
29	Potassium tetrafluoroborate	14075-53-7	Solid	No Cat	Sigma-Aldrich	LaA119	LaB123	LaC121	I
30	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-o xo- 2,5-dihydro-furan-3-yl) phosphate	66170-10-3	Solid	No Cat	Sigma-Aldrich	LaA130	LaB128	LaC124	II

*1) WAKO: Wako Pure Chemical Industries, Ltd.

*2) TCI: Tokyo Chemical Industry Co., Ltd.

7-2. Defined Reliability and Accuracy Value

All thirty of the reference chemicals listed in Table 2 were tested at each of the three participating laboratories, in order to evaluate the reliability and relevance of the LabCyte24 EIT. Each laboratory performed three independent runs for each test chemical with different tissue batches and at sufficient time intervals. Each run comprised three concurrently tested replicates for each test chemical, negative control, and positive control.

7-2.1 Within-Laboratory Reproducibility

The VMT decided that the target value for within-laboratory reproducibility of the LabCyte24 EIT should be equal to or better than a value derived from a VRM of OECD TG 492. Since the results for the EpiOcular EIT were 93%, 93% and 97% at three participating laboratories, the target for this validation study of the LabCyte24 EIT was set to 90% or higher.

7-2.2 Between-Laboratory Reproducibility

The VMT decided that the target value for between-laboratory reproducibility of the LabCyte24 EIT should be equal to or better than a value derived from a VRM of OECD TG 492. Since the results for the EpiOcular EIT was 90% concordance between the three participating laboratories, the target for this validation study of the LabCyte24 EIT was set to 85% or higher.

7-2.3 Predictive Capacity

The VMT decided that the target value for accuracy (sensitivity, specificity, and overall accuracy) of the LabCyte24 EIT should be equal to or better than a value derived from a VRM of OECD TG 492, as well as suitable for the species of interest, as shown in Table 4. Thus, the target value for sensitivity was set to be equal to or higher than 90%, and that for specificity was set to be equal to or higher than 60%. No restrictions to the sensitivity or specificity were applied; non-concordance with the *in vivo* classification of any test chemical was acceptable just as long as the final sensitivity and specificity of the test method achieved the target value, and the overall accuracy was equal to or higher than 75%.

The restriction was added, however, that none of the UN GHS Category 1 reference chemicals were to be under-predicted as no category from valid test results from the

participating laboratories.

Table 4. Required predictive values for sensitivity, specificity and overall accuracy.

Sensitivity	Specificity	Overall Accuracy
≥ 90% (EpiOcular EIT: 93%)	≥ 60% (EpiOcular EIT: 63.0%)	≥ 75% (EpiOcular EIT: 78.2%)

7-2.4 Study Quality Criteria

In the event that a test result is determined to be invalid due to failure to meet acceptance criteria for the test chemical, control chemicals, or any other reason, a maximum of two additional retests per test chemical are permitted to augment the data set. Since retesting requires concurrent testing with a positive control and negative control, a maximum number of two additional runs are permitted for each test chemical.

It is conceivable that, even after retesting, one or more of the participating laboratories will fail to obtain a minimum of three valid runs for each test chemical, which could result in an incomplete data matrix. A dataset is considered valid, however, as long as the following three criteria are all met:

1) There is one complete test sequence for each of the thirty test chemicals at any one of the three participating laboratories.
2) Each of the three participating laboratories must achieve a minimum of 85% complete test sequences. This means that, there are no more than four incomplete test sequences out of 30 test sequences at any one laboratory.
3) At least 90% of all the test sequences at all three laboratories must be complete. This means that, for 30 test chemicals each at three participating laboratories, there are a total of no more than nine incomplete test sequences out of 90 test sequences in total.

In this context, the term “test sequence” refers to the total number of independent tests performed for a single test chemical in a single laboratory, including retests, or between three to five tests. A complete test sequence comprises three valid test results. A test sequence comprising less than three valid test results is considered incomplete.

7-3. Data Collection, Handling, and Analysis

Working in close collaboration with JaCVAM, the independent biostatistician collected and organized the data using custom data collection software, which included decoding the coded chemicals and performing statistical analyses using statistical tools that were approved by the VMT.

7-4. Quality Assurance and GLP

All participating laboratories conducted testing in the spirit of GLP.

Quality assurance of all the data and records was performed by JaCVAM. After completion of all testing, all study documents were submitted to the chairperson of VMT and only data sheets were forwarded by e-mail to the biostatistician. The chairperson reviewed the contents of the study documents and clarified illegible or unclear content by contacting each group by e-mail or telephone.

8. PROTOCOL

8-1. Protocol of the LabCyte24 EIT

Prediction of eye irritation potential of test chemicals using the LabCyte24 EIT was performed according to the protocol described in LabCyte CORNEA-MODEL24 Eye Irritation Test Operation Protocol Ver. 2.5.2. Test Operation Protocol Ver. 2.5.2 was also used to estimate the predictive performance of the LabCyte24 EIT method using 136 test chemicals over a wide range of chemical classes described in BRD.

The LabCyte CORNEA-MODEL24 tissues were shipped to the participating laboratories, and the tissues were aseptically removed from the agarose medium, placed in wells containing 500 µL of assay medium, and then incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air.

8-1.1 LabCyte24 EIT Protocol for Liquid Chemicals

After incubation, the tissue was topically exposed to 50 µL of a liquid test chemical, which was applied with a micropipette. Each test chemical was applied to three tissue replicates. Additionally, three tissue replicates were treated with 50 µL of Dulbecco's Phosphate Buffered Saline (D-PBS, Invitrogen, CA, USA) as a negative control and three more with ethanol as a positive control. The exposed tissue replicates were then incubated for one minute. Next, each tissue was carefully rinsed at least ten times with D-PBS applied from a washing bottle to remove any residual test chemical from the tissue surface. The tissues were then blotted and placed in new wells containing 500 µL of fresh assay medium. The tissues were again incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. After incubation, the tissues were blotted and placed in new wells containing 300 µL of freshly prepared WST-8 solution for a WST-8 assay (Ishiyama et. al., 1997). WST-8 solution comprised a 1:10 dilution of Cell Counting Kit-8 (Dojindo Co., Japan) with Earle's balanced salt solution (EBSS; Sigma-Aldrich, MO, USA). The tissues were again incubated for 4 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. Finally, 200 µL of culture supernatant was placed in a microtiter plate and the optical density (OD) value of the culture supernatant was measured at 450 nm and at 650 nm as a reference absorbance, with WST-8 solution as a blank.

$$\text{Cell Viability (\%)} \frac{\text{OD of the test chemical}}{\text{OD of negative control}} \times 100$$

The cell viability was calculated as the ratio of the OD of the test chemical to OD of negative control. The mean of three replicate tissues was used to predict eye irritation potential of the test chemical according to the prediction model.

8-1.2 LabCyte24 EIT Protocol for Solid Chemicals

After incubation, the tissue replicates were placed in 500 µL of fresh assay medium and topically exposed to 10 mg of a solid test chemical from a microtube. Each test chemical was applied to three replicate tissues. Additionally, three untreated tissue replicates were set apart for use as a negative control and three more were treated with Lauric acid as a positive control. The exposed tissue replicates were incubated for 24 hours and then carefully rinsed with D-PBS just like the liquid chemicals. Solid chemicals were not incubated after exposure to test chemicals. After the rinsing, the tissue replicates were blotted and analyzed for cell viability using the WST-8 assay, just like the liquid chemicals.

8-1.3 Detecting and Correcting Chemical Interference with WST-8 Endpoints

A possible limitation of this EIT might be some test chemicals that will affect the WST-8 endpoints directly. There are two kinds of test chemicals that can interfere with the WST-8 assay.

A. Chemicals that stain epithelial tissues directly.
B. Chemicals that reduce WST-8 directly.

Test chemicals that stain the corneal epithelial tissues could possibility transfer from the corneal epithelial tissue to the WST-8 reaction buffer and affect OD measurements.

Test chemicals that reduce WST-8 directly could possibly affect OD measurements if the test chemical is present in the corneal epithelial tissues when the WST-8 viability test is performed.

A procedure for detecting such test chemicals is described below.

8-1.3.1 PRELIMINARY TEST

WST-8 solution was prepared by making a 1:10 dilution of the Cell Counting Kit-8 with EBSS.

Either 50 µL of a liquid test chemical or 10 mg a solid test chemical are added to the wells of a

24-well assay plate that contains 0.3 mL of WST-8 solution in each well. Untreated WST-8 solution is used as control. Close the lid of 24-well assay plate and incubate the mixture in CO_2 for about 4 hours. After incubation, shake the mixture gently and examine the staining of the diluted WST-8 medium macroscopically.

8-1.3.2 FUNCTIONAL CHECK ON VIABLE TISSUE

For each test chemical that clearly changed the color of the diluted WST-8 solution in the preliminary test above, apply either 50 µL of liquid test chemicals or 10 mg of solid test chemicals to the surface of corneal epithelial tissues. Perform all steps of the LabCyte24 EIT described in sections 7-1.1 to 7-1.2 using corneal epithelial tissues that have been freeze-killed twice at -80°C or lower for 30 minutes instead of viable ones. Then calculate the corrected OD as follows:

Corrected OD = A − (B − C),
where:
A is the OD of viable tissue exposed to a test chemical,
B is the mean OD of freeze-killed tissue exposed to a test chemical, and
C is the mean OD of freeze-killed tissue exposed to the negative control.

If the corrected OD is below 0, the OD is considered to be 0.

When the cell viability is <40%, the test chemical is predicted to be GHS category 1 or 2, and there is no need to calculate a corrected value.

8-2. Prediction Model for the LabCyte24 EIT

The *in vivo* eye irritation classification of test chemicals was based on the UN GHS classification (United Nations, 2003).

According to the UN GHS , an irritant (Category 1 or 2) is predicted if the mean relative tissue viability of three individual tissues exposed to the test chemical falls below 40% of the mean viability of the negative control (Table 5).

Table 5. Prediction model for the LabCyte24 EIT

***In vitro* results**	**Prediction for UN GHS**
Tissue viability is ≤ 40%	Irritant (Category 1 or 2)
Tissue viability is > 40%	Non Irritant (No Category)

8-3. Acceptance Criteria

8-3.1 Negative Control

The absolute OD of the negative control for liquid test chemicals on tissue treated with sterile D-PBS or of the negative control for solid test chemicals on untreated tissue in the WST-8 assay is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures and under specific conditions of use.

The negative control for both liquids and solids has to be tested for each run.

0.5 ≤ Mean OD (A450/650) measured value ≤ 1.3

This acceptance range of negative control OD was established from the historical QC data of tissue viability, described in the section 2-2 (Table 2-2) of BRD, and consideration reflecting both the shipment stress of LabCyte CORNEA-MODEL24 and the stress by all test processes of LabCyte24 EIT.

It has been confirmed that acceptance lower limit (0.5 OD) could provide sufficiently reliable prediction for the LabCyte24 EIT, through the ring-study for the technical transferability and several pre-validation studies described in the section 3 (Fig 3-3, 3-4 and 3-6) of the BRD. This is supported by the assessment for reliability of LabCyte24 EIT in this validation study.

8-3.2 Positive Control

Ethanol is used as the positive control for liquid test chemicals and is tested concurrently with the liquid test chemicals. Lauric acid is used as the positive control for solid test chemicals and is tested concurrently with the solid test chemicals.

Concurrent here means the positive control for both liquids and solids has to be tested for each run.

Mean tissue viability ≤ 40%

8-3.3 Standard Deviation

Since eye irritation potential is predicted from the mean viability of three individual tissues, the variability of tissue replicates must be kept at an acceptably low level.

Standard Deviation (SD) of tissue viability of three identically treated replicates for negative control, positive control, and test chemicals ≤ 20%

Note

Results of the validation study were evaluated under the condition of SD ≤ 18%, as well as SD ≤ 20% by the data analysis group, as reported in the statistical analysis reports (Appendices 5 and 6). In addition, the reliability and the predictive performance of LabCyte24 EIT that were evaluated with the SD ≤ 18% acceptance criteria was reported in this validation report because VMT had finally determined to adopt the OECD TG 492 acceptance criteria, although the present validation study has been performed according to the SD ≤ 20% acceptance criteria defined as described above.

8-4. Applicability Domain and Limitation

One limitation of the RhCE EIT method is that it does not allow discrimination between eye irritation/reversible effects on the eye (UN GHS Category 2) and serious eye damage/irreversible effects on the eye (UN GHS Category 1), nor between eye irritants (UN GHS optional Category 2A) and mild eye irritants (UN GHS optional Category 2B), as defined by UN GHS (UN, 2003). For these purposes, further testing with other suitable test methods is required.

LabCyte24 EIT is applicable to substances and mixtures, and to solids, liquids, semi-solids and waxes with the same applicability as the OECD TG 492 VRM (OECD, 2015a). The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. However, LabCyte24 EIT does not allow the testing of gases and aerosols for the same reasons described in the OECD TG492 VRM (OECD, 2015a).

9. RESULTS

9-1. QC of the Tissue Models

The QC data for the tissue models used in this validation study demonstrated that the tissue viability as measured in the MTT/WST-8 assay, and barrier function as measured IC50 after treatment for one hour with various concentrations of SLS solution, was stable among the different batches provided to each laboratory, as shown in the results of QC for each lot of LabCyte CORNEA-MODEL24 (Appendix 4). Also, all batches of LabCyte CORNEA-MODEL24 showed multilayered corneal epithelium-like tissue (at least three layers) of viable cells and a non-keratinized surface (Appendix 4). Using this data, the VMT was able to confirm the completeness of the epithelial tissue layers used in this validation study. All the batches used for the validation study had passed the manufacturer's model supply criteria for LabCyte CORNEA-MODEL24 (Appendix 4).

9-2. Quality Assurance

Assays and quality assurance were carried out in the spirit of GLP, although not all participating laboratories were GLP certified. The participating laboratories conducted the experiments according to the protocol described in ver. 2.5.2 of the SOP. All raw data and data sheets were reviewed at each laboratory and then checked for errors and omissions by both the VMT data analysis group and the record management group. The results accurately reflect the raw data (Appendices 5 and 6).

9-3. Negative Control

Table 6 shows absorbance values for the negative control.

Table 6. Absorbance and SD of cell viability in negative control

		Phase 1							Phase 2					
		1	2	3	R01	R02	Mean	SD	1	2	3	R01	Mean	SD
Liquid	Lab A	0.79 (2.1%)	0.54 (7.7%)	0.58 (9.7%)			0.64	0.13	0.76 (9.3%)	0.64 (5.4%)	0.61 (10.8%)		0.67	0.08
	Lab B	0.70 (4.6%)	0.64 (5.8%)	0.97 (4.0%)			0.77	0.18	0.72 (11.7%)	0.61 (15.7%)	0.81 (9.7%)		0.71	0.10
	Lab C	0.82 (9.3%)	0.75 (11.0%)	0.61 (1.1%)			0.73	0.11	0.63 (8.5%)	0.61 (2.6%)	1.08 (4.7%)	0.70 (2.4%)	0.76	0.22
Solid	Lab A	0.65 (5.2%)	0.51 (4.5%)	0.57 (12.2%)			0.58	0.07	0.57 (4.4%)	0.63 (12.3%)	0.76 (11.8%)		0.65	0.10
	Lab B	0.85 (11.8%)	0.59 (8.0%)	0.65 (8.7%)			0.7	0.14	0.54 (6.3%)	0.78 (6.1%)	0.62 (5.1%)		0.65	0.12
	Lab C	0.60 (15.6%)	0.60 (8.5%)	0.71 (10.9%)	0.61 (11.8%)	0.52 (13.5%)	0.61	0.07	0.65 (11.1%)	0.53 (15.6%)	0.91 (5.6%)		0.70	0.19

Upper row: OD (Absorbance 450nm/650nm); Lower row: SD of cell viability.

All data (19 test runs of liquid and 20 test runs for solid protocol) for the negative control met acceptance criteria for both the OD range (0.5 ≤ Mean OD ≤ 1.3) and SD (≤ 18%). The frequency of invalid test runs for the negative control was 0%.

9-4. Positive Control

Table 7 shows absorbance values for the positive control. All data for the positive control met all acceptance criteria.

Table 7. Cell viability and its SD of positive control

		Phase 1							Phase 2					
		1	2	3	R01	R02	Mean	SD	1	2	3	R01	Mean	SD
Liquid	Lab A	7.0 (0.9)	19.3 (5.8)	17.9 (3.7)			14.7	6.7	6.4 (3.2)	6.9 (1.7)	11.6 (4.1)		8.3	2.9
	Lab B	25.9 (3.7)	30.3 (8.9)	10.5 (3.3)			22.2	10.4	22.0 (5.0)	34.3 (3.4)	24.3 (9.0)		26.9	6.5
	Lab C	14.8 (11.2)	16.1 (1.2)	21.0 (6.5)			17.3	3.3	13.7 (1.1)	9.6 (1.6)	10.3 (1.1)	9.0 (0.5)	10.7	2.1
Solid	Lab A	0.8 (0.3)	2.1 (0.9)	1.9 (1.4)			1.6	0.7	0.9 (0.6)	0.7 (0.5)	0.9 (1.1)		0.8	0.1
	Lab B	7.7 (2.5)	4.5 (1.4)	5.5 (1.9)			5.9	1.6	9.1 (2.6)	4.9 (1.2)	4.1 (1.7)		6.0	2.7
	Lab C	1.7 (0.3)	0.1 (0.2)	0.0 (0.0)	0.6 (0.4)	1.9 (0.5)	0.9	0.9	1.0 (0.0)	1.8 (0.6)	0.3 (0.1)		1.0	0.6

Upper row: Cell viability (%); Lower row: SD of cell viability (%).

All data (19 test runs of liquid and 20 test runs for solid protocol) for the positive control met acceptance criteria for both cell viability (≤ 40%) and SD (≤ 18%). The frequency of invalid test runs for the positive control was 0%.

Table 8. Mean cell viability of each qualified test run for 30 reference chemicals and concordance of prediction within-laboratory for LabCyte24 EIT

No.	UN GHS *in vivo* Cat.	Lab A					Lab B					Lab C						
		WST-8 interference	1	2	3	WLR	WST-8 interference	1	2	3	WLR	WST-8 interference	1	2	3	R1	R2	WLR
1	Cat. 1	WST-8 interference	6.6 (1.4)	5.8 (1.8)	15.6 (8.7)	C	WST-8 interference	8.3 (5.2)	25.6 (2.0)	16.1 (2.9)	C	WST-8 interference	0.0 (0.0)	4.9 (1.3)	5.1 (1.6)			C
2	Cat. 1	WST-8 interference	0.8 (0.0)	3.9 (0.0)	1.2 (0.0)	C	WST-8 interference	0.4 (0.7)	0.7 (1.3)	0.0 (0.0)	C	WST-8 interference	8.0 (10.5)	30.5 (45.4)	0.0 (0.0)	0.0 (0.0)		C
3	Cat. 1		18.0 (1.6)	27.4 (3.7)	26.1 (3.2)	C		19.1 (3.9)	20.7 (0.8)	27.7 (11.2)	C		17.2 (0.9)	12.5 (2.1)	21.3 (3.4)			C
4	Cat. 1		7.0 (1.0)	8.8 (3.9)	6.4 (0.5)	C		14.0 (3.9)	12.1 (0.3)	13.0 (1.1)	C		4.9 (0.7)	10.1 (1.7)	7.9 (1.0)			C
5	Cat. 1		0.6 (0.3)	1.8 (0.8)	1.6 (0.3)	C		6.1 (2.1)	4.8 (1.4)	6.4 (1.6)	C		2.4 (0.8)	1.3 (0.5)	0.0 (0.0)			C
6	Cat. 1		0.0 (0.0)	0.1 (0.2)	0.0 (0.0)	C		6.4 (2.1)	6.4 (1.0)	5.9 (3.7)	C		0.8 (0.3)	0.6 (0.3)	0.0 (0.0)			C
7	Cat. 1		2.3 (0.7)	4.0 (0.6)	2.4 (0.6)	C		4.0 (0.8)	6.0 (0.9)	6.0 (2.0)	C		3.0 (1.0)	3.6 (0.3)	1.6 (0.5)			C
8	Cat. 2A		0.0 (0.0)	1.2 (1.0)	0.2 (0.2)	C		1.0 (0.7)	0.3 (0.3)	0.9 (0.7)	C		0.0 (0.0)	0.0 (0.0)	0.0 (0.0)			C
9	Cat. 2A		10.5 (4.9)	15.3 (4.8)	17.7 (11.2)	C		39.7 (10.0)	25.4 (7.2)	29.4 (4.9)	C		9.0 (6.0)	15.2 (2.5)	22.7 (3.0)			C
10	Cat. 2A	WST-8 interference	1.9 (3.3)	0.0 (0.0)	4.8 (8.4)	C	WST-8 interference	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	C	WST-8 interference	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)			C
11	Cat. 2A		0.4 (0.5)	1.6 (0.5)	1.9 (0.1)	C		4.3 (1.2)	6.6 (2.5)	6.8 (3.0)	C		1.8 (0.7)	1.7 (0.6)	1.3 (0.2)			C
12	Cat. 2B		18.6 (4.5)	39.0 (5.3)	31.9 (5.9)	C		29.5 (8.4)	37.9 (1.4)	27.5 (2.1)	C		23.4 (3.7)	30.0 (2.0)	32.5 (6.9)			C
13	Cat. 2B		29.9 (11.9)	23.1 (3.6)	29.4 (6.0)	C		34.0 (6.9)	59.8 (7.4)	35.5 (7.4)	NC		29.1 (2.4)	25.2 (6.5)	25.5 (2.0)			C
14	Cat. 2B		35.4 (7.4)	35.3 (5.5)	25.7 (3.8)	C		47.8 (9.0)	27.8 (3.3)	46.9 (0.6)	NC		30.7 (8.6)	37.1 (6.8)	33.3 (2.4)			C
15	Cat. 2B		0.3 (0.3)	1.0 (0.2)	0.2 (0.2)	C		4.0 (0.8)	8.8 (9.0)	2.8 (0.2)	C		1.6 (0.3)	1.0 (0.3)	0.0 (0.0)			C

Upper row: viability in %, Lower row (in bracket): SD in %.

Yellow cells indicate an invalid test run.

(Continued)

No.	UN GHS *in vivo* Cat.	Lab A					Lab B					Lab C						
		WST-8 interference	1	2	3	WLR	WST-8 interference	1	2	3	WLR	WST-8 interference	1	2	3	R1	R2	WLR
16	No Cat.		40.5 (8.2)	55.2 (7.5)	51.9 (5.5)	C		57.7 (4.4)	57.4 (8.8)	53.6 (3.4)	C		42.9 (5.4)	39.9 (6.9)	32.6 (8.1)			NC
17	No Cat.		37.9 (5.8)	33.0 (6.1)	25.9 (5.9)	C		44.9 (9.8)	51.6 (7.9)	41.6 (3.3)	C		46.4 (11.8)	40.5 (2.3)	50.1 (19.7)			C
18	No Cat.		52.3 (5.2)	57.0 (9.4)	55.9 (4.7)	C		52.4 (7.5)	52.7 (9.1)	68.5 (10.8)	C		35.8 (22.9)	48.8 (7.3)	54.4 (11.0)	74.6 (12.8)		C
19	No Cat.		63.8 (2.5)	69.4 (3.2)	81.4 (11.0)	C		75.2 (7.6)	91.3 (2.2)	79.7 (13.0)	C		77.0 (12.5)	72.0 (0.7)	61.0 (10.0)			C
20	No Cat.		103.3 (12.3)	78.7 (2.9)	141.0 (15.0)	C		69.8 (4.6)	101.1 (10.6)	89.9 (7.3)	C		96.3 (5.6)	85.0 (7.5)	121.3 (3.6)			C
21	No Cat.	WST-8 interference	2.5 (4.3)	0.0 (0.0)	3.2 (5.6)	C	WST-8 interference	12.4 (7.2)	0.0 (0.0)	0.0 (0.0)	C	WST-8 interference	3.6 (6.2)	17.2 (26.0)	0.0 (0.0)	0.0 (0.0)		C
22	No Cat.		76.5 (4.5)	104.9 (14.1)	89.7 (6.6)	C		64.3 (10.6)	105.9 (7.8)	85.0 (3.7)	C		121.0 (3.7)	101.4 (8.6)	112.1 (17.5)			C
23	No Cat.		48.0 (4.8)	56.3 (8.6)	84.8 (13.2)	C		52.0 (10.9)	55.0 (1.9)	45.9 (5.9)	C		91.3 (16.4)	57.5 (8.4)	72.9 (12.3)			C
24	No Cat.		68.9 (3.9)	76.3 (2.0)	73.6 (8.2)	C		73.0 (1.9)	91.5 (8.1)	100.1 (2.9)	C		59.6 (8.5)	82.6 (10.8)	74.5 (4.4)			C
25	No Cat.	WST-8 interference	0.5 (0.2)	3.5 (1.8)	1.3 (0.6)	C	WST-8 interference	0.0 (0.0)	5.8 (0.9)	2.9 (0.6)	C	WST-8 interference	0.0 (0.0)	0.1 (0.2)	1.6 (0.1)			C
26	No Cat.		98.9 (6.6)	83.9 (6.4)	96.7 (18.4)	C		74.0 (2.0)	82.1 (12.2)	125.8 (8.8)	C		108.9 (20.0)	98.0 (19.6)	90.0 (22.8)	64.3 (10.2)	88.3 (12.3)	C
27	No Cat.		102.9 (2.0)	80.5 (5.6)	88.8 (8.9)	C		95.0 (7.3)	93.5 (9.9)	97.7 (7.6)	C		89.6 (11.2)	106.6 (6.9)	104.8 (5.3)			C
28	No Cat.		9.1 (3.4)	23.8 (3.7)	9.2 (2.9)	C		14.1 (2.0)	19.0 (3.5)	20.7 (1.5)	C		11.1 (3.7)	11.8 (0.3)	8.5 (3.8)			C
29	No Cat.		60.7 (3.4)	56.5 (3.7)	82.3 (9.8)	C		50.8 (4.7)	91.4 (11.3)	58.2 (5.4)	C		77.5 (11.0)	51.7 (8.1)	70.0 (5.1)			C
30	No Cat.	WST-8 interference	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	C	WST-8 interference	0.0 (0.0)	13.5 (1.7)	20.6 (8.5)	C	WST-8 interference	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)			C

Upper row: viability in %, Lower row: SD in %.

Yellow cells indicate an invalid test run.

Blue cells indicate non irritancy prediction (Mean cell viability >40%).

Red cells indicate irritancy prediction (Mean cell viability ≤40%).

WLR: Within-laboratory reproducibility; C: Concordance, NC: Non-concordance.

9-5. Predicting Eye Irritation Potential of the 30 Test Chemicals

The results of chemical interference detection with WST-8 endpoints are shown in the Table 8. (Ethylenediaminepropyl)-trimethoxysilane (No 1), Methylthioglycolate (No. 2), 1,5-Naphthalenediol (No. 10), Ethyl thioglycolate (No. 21), 2,2'-[[3-Methyl-4- [(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (No. 25) and Trisodium mono-(5-(1,2 -dihydroxyethyl)-4-oxido-2-oxo- 2,5-dihydro-furan-3-yl) phosphate (No. 30) were detected as interfering chemicals in all participating laboratories. WST-8 endpoint ODs of the chemicals were corrected using freeze-killed tissues in each participating laboratories.

Table 8 also shows the mean viability of the test chemicals. All data from Lab B for the 30 test chemicals met the acceptance criteria of SD (≤ 18%) and the frequency of invalid test run for test chemicals was 0% (0/90).

On the other hand, one data point at Lab A and seven data points at Lab C showed a SD of > 18%, which fails to meet the acceptance criteria. One test run of 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (No. 26) at Lab A failed to meet the acceptance criteria (Table 8). Also one test run each of Methylthioglycolate (No. 2), 2-Ethoxyethyl methacrylate (No.17), 3-Phenoxybenzyl alcohol (No. 18), and Ethyl thioglycolate (No. 21) as well as three test runs of 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (No. 26) at Lab C failed to meet the acceptance criteria (Table 8). Three chemicals (No. 2, No.18 and No21) at Lab C were submitted as complete data matrices by re-testing up to two times. On the other hand, chemical (No. 26) at Lab C was tested for five times and resulted three times in non-qualified results, and therefore only two qualified tests were available for this chemical. Also, chemical (No. 26) at Lab A and chemical (No. 17) at Lab C resulted in only two qualified test because re-testing was not performed. The chemicals with only two qualified tests available produced three incomplete run sequences. The frequencies of invalid test runs at Lab A and Lab C were 1% (1/90) and 7% (7/95), respectively.

Table 9. Mean cell viability of 3 independent runs for 30 reference chemicals and concordance of prediction between the laboratories.

No.	UN GHS *in vivo* Cat.	Lab A			Lab B			Lab C			BLR
		Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge	
1	Cat. 1	9.3	5.4	I	16.7	8.7	I	3.3	2.9	I	C
2	Cat. 1	2.0	1.7	I	0.4	0.4	I	2.7	4.6	I	C
3	Cat. 1	23.8	5.1	I	22.5	4.6	I	17.0	4.4	I	C
4	Cat. 1	7.4	1.2	I	13.0	1.0	I	7.6	2.6	I	C
5	Cat. 1	1.3	0.6	I	5.8	0.9	I	1.2	1.2	I	C
6	Cat. 1	0.0	0.1	I	6.2	0.3	I	0.5	0.4	I	C
7	Cat. 1	2.9	1.0	I	5.3	1.2	I	2.7	1.0	I	C
8	Cat. 2A	0.5	0.6	I	0.7	0.4	I	0.0	0.0	I	C
9	Cat. 2A	14.5	3.7	I	31.5	7.4	I	15.6	6.9	I	C
10	Cat. 2A	2.2	2.4	I	0.0	0.0	I	0.0	0.0	I	C
11	Cat. 2A	1.3	0.8	I	5.9	1.4	I	1.6	0.3	I	C
12	Cat. 2B	29.8	10.4	I	31.6	5.5	I	28.6	4.7	I	C
13	Cat. 2B	27.5	3.8	I	43.1	14.5	NI	26.6	2.2	I	NC
14	Cat. 2B	32.1	5.6	I	40.8	11.3	NI	33.7	3.2	I	NC
15	Cat. 2B	0.5	0.4	I	5.2	3.2	I	0.9	0.8	I	C
16	No Cat.	49.2	7.7	NI	56.2	2.3	NI	38.5	5.3	I	NC
17	No Cat.	32.3	6.0	I	46.0	5.1	NI	43.5*		NI	NC
18	No Cat.	55.1	2.5	NI	57.9	9.2	NI	59.3	13.6	NI	C
19	No Cat.	71.5	9.0	NI	82.1	8.3	NI	70.0	8.2	NI	C
20	No Cat.	107.7	31.4	NI	86.9	15.9	NI	100.9	18.6	NI	C
21	No Cat.	1.9	1.7	I	4.1	7.2	I	1.2	2.1	I	C
22	No Cat.	90.4	14.2	NI	85.1	20.8	NI	111.5	9.8	NI	C
23	No Cat.	63.0	19.3	NI	51.0	4.6	NI	73.9	16.9	NI	C
24	No Cat.	72.9	3.7	NI	88.2	13.8	NI	72.2	11.7	NI	C
25	No Cat.	1.8	1.5	I	2.9	2.9	I	0.6	0.9	I	C
26	No Cat.	91.4*		NI	94.0	27.9	NI	76.3*		NI	C
27	No Cat.	90.7	11.3	NI	95.4	2.1	NI	100.3	9.3	NI	C
28	No Cat.	14.0	8.5	I	17.9	3.4	I	10.5	1.7	I	C
29	No Cat.	66.5	13.8	NI	66.8	21.6	NI	66.4	13.3	NI	C
30	No Cat.	0.0	0.0	NI	11.4	10.5	NI	0.0	0.0	NI	C

I: Irritant; NI: Non irritant; BLR: Between-Laboratory Reproducibility,

C: Concordance, NC: Non-concordance. *: Mean of two independent valid test run.

9-6. Reliability

9-6.1 Within-Laboratory Reproducibility

OECD TG 492 PERFORMANCE STANDARD (OECD, 2015b) describes that within-laboratory reproducibility should be calculated based on concordance of classifications using only qualified tests obtained with Reference Chemicals for which at least two qualified tests are available. All participating laboratories produced two or three qualified tests from all reference chemicals in this validation study (Table 8).

An assessment of within-laboratory reproducibility was based on concordance of predictions (UN GHS Category 1/2 and No Category) obtained from replicate test runs of 30 test chemicals at each of the participating laboratories. As shown in Table 8, Lab A consistently predicted the 30 test chemicals in three independent experiments and had a within-laboratory reproducibility rate of 100%. Lab B had non-concordant predictions twice (No. 13 and No. 14) and had a within-laboratory reproducibility of 93% (28/30, Table 8). Lab C had one non-concordant prediction (No. 16) and a within-laboratory reproducibility of 97% (29/30, Table 8). Thus, the ≥ 90% target for within-laboratory reproducibility was achieved at each laboratory.

9-6.2 Between-Laboratory Reproducibility

OECD TG 492 PERFORMANCE STANDARD (OECD, 2015b) describes that for the calculation of between-laboratory reproducibility (BLR) the final classification for each Reference Chemical in each participating laboratory should be obtained by using the arithmetic mean value of viability over the different qualified tests performed. It also describes that between-laboratory reproducibility should be calculated based on concordance of classifications using only qualified tests from Reference Chemicals for which at least one qualified test per laboratory is available. All participating laboratories produce two or three qualified tests from all reference chemicals in this validation study (Table 9).

An assessment of between-laboratory reproducibility was based on concordance of predictions (UN GHS Category 1/2 and No Category) obtained from replicate test runs of 30 test chemicals at all three participating laboratories. As shown in Table 9, between-laboratory reproducibility was 87% (26/30) because there were four non-concordant predictions fourth (No.13, No.14, No16 and No.17). Thus, the ≥ 85% target for between-laboratory reproducibility was achieved.

9-7. Predictive Capacity

An assessment of predictive capacity of the LabCyte24 EIT was based on the concordance between the predictions made with data obtained during this validation study with the *in vivo* categories specified in the PERFORMANCE STANDARD for OECD TG 492 (OECD, 2015b). The 30 test chemicals chosen from the TG 492 Reference Chemicals included 15 UN GHS Category 1/2 chemicals and 15 UN GHS No Category chemicals. OECD TG 492 PERFORMANCE STANDARD (OECD, 2015b) describes that calculation of predictive capacity (i.e., sensitivity, false negatives, specificity, false positives and accuracy) should be done using all qualified tests obtained for each Reference Chemical from at least three laboratories and also describes that the calculations should be based on the individual predictions of each qualified test for each Reference Chemical in each laboratory and neither on the arithmetic mean values of viability over the different qualified tests performed nor on the mode of all predictions obtained (or any other procedure used to summarize the multiple test results obtained into a single prediction per Reference Chemical). All participating laboratories produced two or three qualified tests from all reference chemicals in this validation study (Table 8).

Lab A and Lab C predicted correctly 15 UN GHS Category 1/2 chemicals. On the other hand, Lab B predicted correctly 13 of 15 UN GHS Category 1/2 chemicals, however one valid run of Diethyl toluamide (No. 13) and two valid runs of 1,4-Dibutoxy benzene (No. 14) were predicted as false-negatives in Lab B. (Table 8). Furthermore, all three participating laboratories accurately predicted the seven UN GHS Category 1 test chemicals. Sensitivity was 100% at Lab A, 93.3% at Lab B, and 100% at Lab C, while a cumulative two-by-two table for all three participating laboratories gave a sensitivity of 97.8% (Table 10, 11).

Lab A and Lab C predicted 10 of 15 UN GHS No Category chemicals, while Lab B predicted 11 of 15 UN GHS No Category chemicals. False positives were obtained by all three participating laboratories for four UN GHS No Category chemicals: Ethyl thioglycolate (No. 21), 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (No. 25), Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%) (No. 28), and Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate (No. 30). Lab C obtained a false positive for two valid runs of the UN GHS No Category chemical 1-Ethyl-3-methylimidazolium ethylsulphate (No. 16). Lab A obtained a false positive for UN GHS No Category chemical 2-Ethoxyethyl methacrylate (No. 17). Thus, specificity was 65.9% at Lab A, 73.3% at Lab B, and 67.4% at Lab C, while a cumulative two-by-two table for all three participating laboratories gave a specificity of 68.9% (Table 10, 11).

Overall accuracy was 83.1% at Lab A, 83.3% at Lab B, and 84.1% at Lab C, while a cumulative two-by-two table for all three participating laboratories gave an overall accuracy of 83.5% (Table 10, 11).

Table 10. 2 × 2 tables for the each participating laboratory

Lab A		*In vivo* classification (GHS classification)		
		Cat 1/2	No Category	Total
In vitro prediction	Cat 1/2	45	15	60
	No Cat	0	29	29
	Total	45	44	89
Sensitivity (%)		100		
Specificity (%)		65.9		
Accuracy (%)		83.1		

Lab B		*In vivo* classification		
		Cat 1/2	No Category	Total
In vitro prediction	Cat 1/2	42	12	54
	No Cat	3	33	36
	Total	45	45	90
Sensitivity (%)		93.3		
Specificity (%)		73.3		
Accuracy (%)		83.3		

Lab C		*In vivo classification*		
		Cat 1/2	No Category	Total
In vitro prediction	Cat 1/2	45	14	59
	No Cat	0	29	29
	Total	45	43	88
Sensitivity (%)		100		
Specificity (%)		67.4		
Accuracy (%)		84.1		

Table 11. 2 × 2 tables for cumulative of the all participating laboratories

		In vivo classification		
		Cat 1/2	No Category	Total
In vitro prediction	Cat 1/2	132	41	173
	No Cat	3	91	94
	Total	135	132	267
Sensitivity (%)		97.8		
Specificity (%)		68.9		
Accuracy (%)		83.5		

9-8. Study Quality Criteria

In this validation study, the LabCyte24 EIT was performed on 30 test chemicals at three participating laboratories. One invalid run at Lab A and seven invalid runs at Lab C occurred with SD >18%, however retesting augmented the incomplete run sequences for the acceptance criteria SD ≤ 20% (Table 8), and then three incomplete run sequences (Chemical No17 at Lab C, Chemical No. 26 at Lab A and Chemical No. 26 at Lab C) were produced in the case of acceptance criteria SD ≤ 18% but not SD ≤ 20%.

The dataset had at least one or more than one complete test sequence for each of the thirty test chemicals at all three participating laboratories. Thus, the target (one complete test sequence for each of the thirty test chemicals at any one of the three participating laboratories) for the study quality criteria was achieved.

Each Lab A, Lab B and Lab C achieved 97% (29/30), 100% (30/30) and 93% (28/30) complete test sequence, respectively. Therefore the target (each ≥85%) for the study quality criteria was achieved.

Also all three laboratories achieve 97% (87/90) complete test sequence. Therefore this study quality criteria (≥90%) was achieved.

10. DISCUSSION

10-1. In Consideration of Invalid Runs

All test runs for negative and positive controls met the acceptance criteria. The VMT concluded that the results of the positive and negative control were highly reproducible over time (phase I and phase II) in this validation study (Table 6 and Table 7).

One test run each for Methylthioglycolate (No. 2), 2-Ethoxyethyl methacrylate (No.17), 3-Phenoxybenzyl alcohol (No. 18), and Ethyl thioglycolate (No. 21) at Lab C, as well as one test run at Lab A and three test run at Lab C for 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis -ethanol (No. 26) had a SD > 18%, which failed to meet the acceptance criteria, so these test runs were invalid. Methylthioglycolate (No. 2) and Ethyl thioglycolate (No. 21) are strong WST-8 reducers and the VMT felt that residual test chemicals after the washing process seem to have caused some variability in cell viability. 3-Phenoxybenzyl alcohol (No. 18) and 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo] -phenyl]imino]bis-ethanol (No. 26) were difficult to remove completely during the washing process, because they adhere to the cell culture insert, and thus the VMT felt that residual test chemicals in the cell culture inserts after the washing process might have caused some variability in cell viability.

Finally the run sequences for three chemicals (No. 1, No.18 and No.21) were augmented by retesting with an improved washing procedure. The VMT proposed an improved and standardized washing process as a means to decrease variability in cell viability and recommended that a suitable video presentation of the washing procedure be prepared as an effective means to train naïve laboratories planning to perform the LabCyte24 EIT. The VMT further requested that the protocol be updated to include precautions in the description of the washing process (Appendix 7).

On the other hand, although three incomplete run sequences (chemical No. 17 at Lab C and Chemical No 26 at Lab A and Lab C) were produced, the data quality of this validation study was fully achieved according to the study quality criteria for OECD TG 492 PERFORMANCE STANDARD (OECD, 2015b).

10-2. Reliability

Within-laboratory reproducibility was 100% at Lab A, 93% at Lab B, and 97% at Lab C, thus

achieving the ≥ 90% requirement specified in the TG 492 PERFORMANCE STANDARD (OECD, 2015b).

Also, between-laboratory reproducibility for all three participating laboratories was 87%, thus achieving the ≥ 85% requirement specified in the TG 492 PERFORMANCE STANDARD.

This result demonstrates that the robustness and reliability of the LabCyte24 EIT method is sufficient to meet the PERFORMANCE STANDARD for OECD TG 492 (OECD, 2015b).

10-3. Predictive Capacity

The test results correctly predicted the eye irritation potential for almost all UN GHS Category 1 or 2 chemicals, with only false negative at Lab B: one valid run of Category 2B chemical Diethyl toluamide (No. 13) and two valid runs of UN GHS Category 2B chemical 1,4-Dibutoxy benzene (No. 14). Sensitivity was 93.3 to 100% at each of the participating, as well as 97.8% at all three laboratories collectively, thus meeting the PERFORMANCE STANDARD for OECD TG 492 (OECD, 2015b).

In contrast, there were four false positives among the GHS No Category chemicals: Ethyl thioglycolate (no. 21), 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (No. 25), Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%) (No. 28), and Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate (No. 30). Of the No Category chemicals, Nos. 21, 25, 30 were also false positives in the EpiOcular EIT. Moreover, although not a false positive per se in the EpiOcular EIT, the results for No. 28 in that test were near the cut off line. Given that 26 of 30 test chemicals were predicted correctly, the VMT feels that the LabCyte24 EIT has a predictive capacity similar to that of the EpiOcular EIT.

Sensitivity at all participating laboratories individually, as well as collectively, was from 97.8%, specificity was from 68.9%, and accuracy was from 83.5%. Some deviation from the OECD PERFORMANCE STANDARD—which specify 90% sensitivity, 60% specificity, 75% accuracy—were made for the LabCyte24 EIT. Predictive capacity at all laboratories was sufficient to meet the acceptance criteria of the OECD PERFORMANCE STANDARD (OECD, 2015b).

10-4. Similarity with the OECD TG 492 VRM

As previously described the section 5, the VMT considers LabCyte24 EIT to be functionally similar to the EpiOcular EIT, which is a RhCE EIT VRM for OECD.

Using 30 Reference Chemicals from TG 492 as test chemicals, LabCyte24 EIT and the EpiOcular EIT both gave four false positives for GHS No Category chemicals.

The LabCyte24 EIT yielded a false positive for Cellulose, 2-(2-hydroxy-3-(trimethylammonium) propoxy) ethyl ether chloride (91%) (No. 28), even though the EpiOcular EIT predicted it correctly. Also, the EpiOcular EIT yielded a false negative for 1,4-Dibutoxy benzene (No. 14), even though the LabCyte24 EIT predicted it correctly. The EpiOcular EIT also yielded false positives for 2-Ethoxyethyl methacrylate (No. 17) and 3-Phenoxybenzyl alcohol (No. 18), even though the LabCyte24 EIT predicted it correctly. Predictions of the other 26 chemicals were concordant between the LabCyte24 EIT and the EpiOcular EIT.

These results suggest that LabCyte24 EIT has a predictive capacity similar to or higher than that of the EpiOcular EIT.

11. CONCLUSION

This validation study was intended to demonstrate that the LabCyte24 EIT is capable of fulfilling the PERFORMANCE STANDARD stipulated in OECD TG 492 for similar or modified *in vitro* RhCE EIT methods based on the EpiOcular EIT. The study was designed both to provide the information necessary to validating the test method as well as to minimize the burden placed on the three participating laboratories. Assessment of reliability and accuracy of the method was performed using 30 test chemicals selected from the Reference Chemicals listed in the PERFORMANCE STANDARD of TG 492.

Having achieved within-laboratory reproducibility from 93% to 100% at each of the three participating laboratories as well as a between-laboratory reproducibility of 87% for all three participating laboratories combined, the LabCyte24 EIT clearly meets the PERFORMANCE STANDARD for TG 492.

The LabCyte24 EIT also demonstrated good predictive capacity with an overall accuracy from 83.5%, an overall sensitivity from 97.8%, and an overall specificity from 68.9%, thereby meeting the acceptance criteria stipulated in the OECD PERFORMANCE STANDARD of 75% for accuracy, 90% for sensitivity, and 60% for specificity.

These results suggest that the LabCyte24 EIT is a robust and reliable method for predicting eye irritation potential. Most importantly, the test data provides information useful to proposing the LabCyte24 EIT as me-too method for inclusion in OECD TG 492.

12. ACKNOWLEDGEMENT

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