Validation Study of the Vitrigel-EIT method as an alternative to in vivo eye irritation testing

Study Report, Version 2.0

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VITRIGEL-EIT Validation Management Team (VMT)

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100 # Abbreviations

CVM	Collagen Vitrigel Membrane
EIT	Eye Irritancy Test
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
GHS	Globally Harmonized Systems of Classification and Labeling
GLP	Good Laboratory Practice
HCE	Human Corneal Epithelium
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
JaCVAM	Japanese Centre for the Validation of Alternative Methods
NI	Non-irritant
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organization for Economic Co-operation and Development
PET	Polyethylene terephthalate
SOP	Standard operating procedure
STE	Short time exposure
TEER	Transepithelial electrical resistance
UN	United Nations
VMT	Validation management team

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1 Abstract

Collagen vitrigel membrane (CVM) comprises high-density collagen fibrils that are equivalent to in vivo connective tissues and is easily handled with tweezers. Takezawa et al. developed a human corneal epithelium (HCE) model by three-dimensional culturing of HCE-T cells on a CVM scaffold in a chamber that provided an air–liquid interface culture system. They further used their HCE model to establish a new test method, known as the Vitrigel-eye irritancy test (Vitrigel-EIT) method, which can be used to estimate the ocular irritation potential of test chemicals by analyzing relative changes in transepithelial electrical resistance (TEER) over time.

This trial was conducted to validate the reliability and relevance of the Vitrigel-EIT method at three participating laboratories in the spirit of GLP by verifying the within- and between-laboratory reproducibility for 42 test chemicals as well as the capacity for distinguishing non-irritants from irritants in a bottom-up approach.

The results showed 80–100% within-laboratory reproducibility at all three laboratories and an excellent between-laboratory reproducibility of 92%. Unfortunately, the predictive capacity for distinguishing non-irritants from irritants per UN GHS categories in a bottom-up approach was not favorable because of false negative rates as high as 17%. After considerable review of the data, however, it was determined that excluding test chemicals with a pH level of 5 or less as well as solid test chemicals with a logP value of 2.5 or more and a density of less than 0.95 g/ cm^3 or greater than 1.10 g/cm^3 improved the false negative rate to as low as 7%.

These results suggest that, with a carefully defined applicability domain, the Vitrigel-EIT method is a useful alternative to the Draize test for distinguishing test chemicals that are ocular non-irritants from those that are irritants.

2 Introduction

Collagen vitrigel membrane (CVM) comprises high-density collagen fibrils that are equivalent to in vivo connective tissues and is easily handled with tweezers. In addition, it has excellent transparency and permeability of high molecular weight proteins and is now used as a cell culture scaffold in a number of advanced studies (Takezawa et al., 2004, 2007a–c). Takezawa et al. developed a corneal epithelium model utilizing a CVM scaffold that facilitates the maintenance of corneal epithelial phenotype in a monolayer of rabbit corneal epithelial cells (Takezawa et al., 2008). Still, there are significant differences in sensitivity to exogenous chemicals between humans and rabbits, so they also developed a human corneal epithelium (HCE) model by three-dimensional culturing of HCE-T cells on the CVM scaffold in a chamber that provided an air–liquid interface culture system (Takezawa et al., 2011a). Here, HCE-T cells are a SV40-immortalized cell strain established by Araki-Sasaki et al (Araki-Sasaki et al., 1995). The HCE-T cell line is one of the most favored human cornea epithelium-derived cells and frequently used for various cornea epithelium-related studies because it is easy to maintain the stable characteristics of cornea epithelial cells in culture (Kim et al., 2016, Yamasaki et al., 2009). The scaffold was fabricated on a polyethylene terephthalate (PET) membrane of a Millicell chamber suitable for assaying the transepithelial electrical resistance (TEER) of epithelial cells. The TEER assay is considered a suitable method for in vivo evaluation of the integrity of the tight junction of the corneal epithelium (Uematsu et al., 2007). Takezawa et al. then used the HCE model to verify that relative change over time in TEER is a useful indicator for assessing the ocular irritancy of four test chemicals, including mild irritants (Takezawa et al., 2011a). The HCE model, however, is not considered suitable for immuno-histological analyses due to difficulties in preparing frozen sections with a PET membrane. To overcome this inconvenience, they developed a novel chamber that merely accompanies a CVM without the PET membrane as well as established a process for its mass production (Takezawa et al., 2011b, 2012). More recently, they established a new test method for estimating the ocular irritancy of test chemicals by analyzing the relative changes over time in TEER after exposing HCE models reconstructed in CVM chambers to test chemicals. This new test method is called the Vitrigel eye irritancy test (Vitrigel-EIT) method. Thus far, thirty chemicals have been

classified successfully as irritants or non-irritants without false negatives using the Vitrigel-EIT method (Yamaguchi et al., 2013).

In association with the International Collaboration on Alternative Test Methods (ICATM), an international validation management team (VMT) was organized to validate the reliability and relevance of this test method, and a validation study was performed with the cooperation of three Japanese laboratories. Testing was conducted using a protocol developed by Yamaguchi and Takezawa using test chemicals distributed via the Japanese Center for the Validation of Alternative methods (JaCVAM). Descriptive statistics are used to summarize the data obtained from the testing.

The aim of this trial is to validate the capability of the Vitrigel-EIT method as well as to assess transferability and between-laboratory reproducibility in preparation for incorporating this test into the screening of test chemicals for the eye irritation potential in accordance with the United Nations' Globally Harmonized System of Classification and Labelling of Chemicals (GHS) categories (United Nations, 2013). This multi-phase validation study of the Vitrigel-EIT method was undertaken in accordance with:

i) the principles and criteria documented in the Organization for Economic Co-operation and Development (OECD) No. 34 Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (OECD, 2005),

ii) the Modular Approach to Validation (Hartung et al., 2004), and

iii) the concepts discussed in The Principles of Good Laboratory Practice: Application to In Vitro Toxicological Studies (Cooper-Hannan et al., 1999).

Testing performed as part of a validation study should ideally be performed in accordance with GLP (OECD, 1998) and necessarily include, without being limited to, the use of standard operating procedures (SOP) and adequate recording of data as well as suitable reporting of results and archival record keeping.

The "modular approach to validation" is a general conceptual framework for documenting the validation of a test method (Hartung et al., 2004; OECD, 2005). In this approach, the information needed to support the validity of the method is organized into modules, as follows.

- Module 1: Test Definition
- Module 2: Within-laboratory repeatability and reproducibility
- Module 3: Between-laboratory transferability
- Module 4: Between-laboratory reproducibility
- Module 5: Predictive capacity
- Module 6: Applicability domain
- Module 7: Performance standards

The modular approach introduced by Hartung et al. (2004) allows the use of datasets from a variety of sources, and this principle was applied in our assessment of the scientific validity of the Vitrigel-EIT method. As a specific goal, this validation study was designed to clarify whether or not the Vitrigel-EIT test method is a useful alternative to the Draize test method in a bottom-up approach for distinguishing chemical substance.

3 Methods

3.1 Study Plan

3.1.1 Purpose

This validation study is designed to assess the reliability (within- and between-laboratory reproducibility) and relevance (predictive capacity) of the Vitrigel-EIT method using a challenging set of test chemicals for which high quality in vitro and in vivo data are available. The test chemicals are to include each type of UN GHS category as classified by in vivo data and predictive capacity is to be assessed primarily in accordance with UN GHS classification in a bottom-up approach (Scott, 2010).

3.1.2 Organization

Members of the VMT contribute their collective expertise in the underlying science and scientific design, management, and evaluation of validation studies. The management structure for this validation study of the Vitrigel-EIT method is shown in Fig. 1.

The VMT is responsible for overseeing the conduct of the validation study, including signing and dating the approval of all protocols, study plans, reports, and amendments.

The members of the VMT as well as their respective roles and expertise for this validation study of the Vitrigel-EIT method are shown in Table 1 and Fig. 1.

Table 1. The Vitrigel-EIT Validation Management Team

Name	**Role and expertise**	**Affiliation**
Hajime Kojima	Trial coordinator, Chemical management and Quality assurance	Japanese Center for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS)
Toshiaki Takezawa Hiroyuki Yamaguchi	Developer of this assay and expertise underlying science as the lead laboratory	Institute of Agrobiological Sciences (NIAS), National Agriculture and Food Research Organization (NARO)
Takashi Sozu	Data analysis and biostatistics dossier	Tokyo Univ. of Science
Liaison members		
Nicole Kleinstreuer	Validation study expertise	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/ Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), USA
Michael-Wilhelm SCHAEFFER	Validation study expertise	European Union Reference Laboratory for Alternatives to Animal Testing (EIRL ECVAM), Italy
Lim, Chae-Hyung	Validation study expertise	Korean Center for the Validation of Alternative Methods (KoCVAM), Korea

Wannhsin Chen	Validation study expertise	Industrial Technology Research Institute, (ITRI), Taiwan

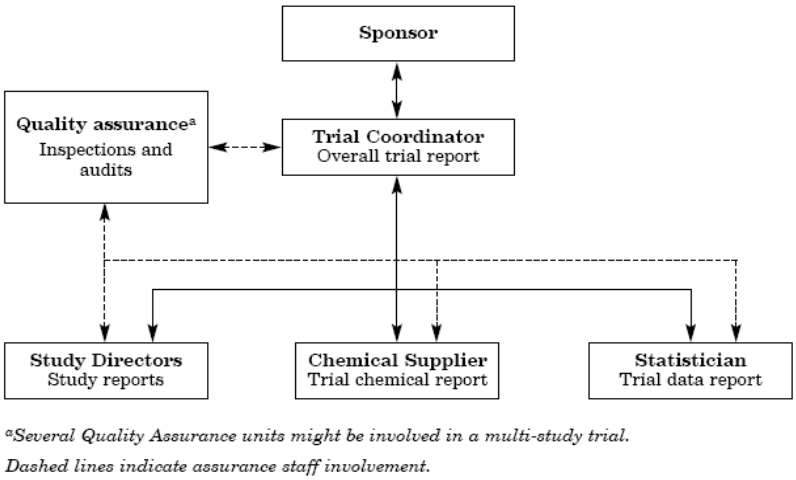


Fig.1. Management Structure for the Vitrigel-EIT validation study

3.1.2.1 Trial coordinator

A trial coordinator was appointed by the VMT to be responsible for preparing draft study plans, a study protocol, and a list of test chemicals as well as to convene ad hoc VMT meetings for review and finalization of the study plan, the study protocol, and the test chemical list. The trial coordinator was also responsible for other administrative duties related to the validation study.

3.1.2.2 Chemical management group

The chemical management group comprised at least one member selected from the VMT and was responsible for preparing a list of test chemicals as well as conferring with the trial coordinator to finalize the list test chemicals to be used in the validation study. It also prepared and distributed non-coded or coded lists of test chemicals to chemical distributors.

3.1.2 3 Data analysis group

The data analysis group comprised at least one member selected from the VMT and was responsible for providing an objective analysis of data obtained in this validation study as well as for performing

statistical processing of the data.

3.1.2.4 Record management group

The record management group comprised at least one member selected from the VMT as well as a representative of the lead laboratory was responsible for preparing the test protocol, the test chemical preparation sheets, blank data sheets, and any other necessary materials as well as for distributing these materials to the participating laboratories. It also collected the completed forms and data sheets after testing, reviewed the records for errors and omissions, and requested correction as necessary.

3.1.2.5 Lead laboratory

The lead laboratory represents the test developers and was responsible for providing the test method protocol as well as test chemical preparation record forms, blank data sheets, and all other necessary documentation. The lead laboratory was also responsible for providing revised versions of the protocol as necessary throughout the entire validation study. The VMT consulted with both the lead laboratory and the other participating laboratories on technical issues.

3.1.2.6 Participating laboratories

The following three laboratories in Japan participated in the testing of substances using the Vitrigel-EIT method. The name of the on-site study director is given in parenthesis.

Lab A: Hatano Research Institute, Food and Drug Safety Center (FDSC), Hatano, Kanagawa (Mika Watanabe)

Lab B: Bozo Research Center (BRC), Tokyo (Takayuki Fukuda)

Lab C: Daicel Corporation (Daicel), Himeji, Hyogo (Kunihiko Yamashita)

All three of these laboratories were naïve and were selected for participation by the VMT after practical training that provided a good indication of the robustness of the test method.

A coordinator from each of these three laboratories participated in VMT activities as observers and was responsible for ensuring that the tests were performed in accordance with the study protocol as well as for filling out and submitting all necessary records and forms upon completion of testing.

3.1.3 Study design

This validation study of the Vitrigel-EIT method was carried out in four phases in accordance with the study plan as described in Appendix 8.1 and summarized in Table 2.

Table 2. Overview of the Vitrigel-EIT validation study

Phase	The number of the test chemicals	The number of the repetitions	Examination
0	5	3	Within- laboratory transferability
I	10	3	Between- laboratory transferability & Within- and between- laboratory reproducibility
II	10	1	Between- laboratory reproducibility
III	36	1	Between- laboratory reproducibility and predictability

3.1.3.1 Training of personnel at the participating laboratories

A technical transfer workshop to explain the principles of and protocol for validation of the Vitrigel-EIT method was held May 22 and 23, 2013, with personnel from all three laboratories in attendance. Instructors from the lead laboratory explained the test method while demonstrating the protocol. All personnel in attendance performed the assay themselves, using saline, ethanol and silicic acid anhydrate. After the workshop, the coordinators from each participating laboratory agreed to purchase the cell line from RIKEN BioResource Center (Tsukuba, Japan) and to sign a memorandum pertaining to borrowing the TEER recorder.

3.1.3.2 Phase 0

Phase 0 was designed to assess between-laboratory transferability by testing five non-coded test chemicals using protocol ver. 1.30e. Each test chemical was determined to be either positive or negative by obtaining consistent results from each of three runs.

3.1.3.2 Phase I

Phase I was designed to assess within and between-laboratory reproducibility by testing ten coded test

chemicals using protocol ver. 1.51e. Each test chemical was determined to be either positive or negative by obtaining consistent results from each of three runs in three different sets.

3.1.3.3 Phase II

The original plan was split into two parts: A and B. Phase IIA was designed to assess the between-laboratory reproducibility of ten coded test chemicals using protocol ver. 1.61e, after which Phase IIB was to validate an additional thirty coded test chemicals using the same protocol. Phase IIB was canceled when the results of Phase IIA led to a decision to undertake a major revision of protocol ver. 1.61e. Consequently, Phase IIA was renamed Phase II, and the planned Phase IIB was incorporated into a newly designed Phase III using the protocol ver. 1.71e.

3.1.3.4 Phase III

Phase III was designed to assess the between-laboratory reproducibility and predictive capacity of the Vitrigel-EIT method for thirty-six coded test chemicals using protocol ver. 1.71e. Each test chemical was determined to be either positive or negative based on obtaining consistent results from each of three runs in one set.

3.1.4 Success criteria

Success criteria for within and between-laboratory reproducibility was 80%. The predictive capacity was assessed using thirty-six coded test chemicals. The results of statistical analysis were used to determine the preliminary design for validation study as well as automatization of the test leading to an increased dataset.

Issues related to the applicability domain were discussed by the VMT decision during assessment of between-laboratory reproducibility.

3.2 Summary of protocol

The current test protocol is ver. 1.80e, which was designed per Yamaguchi et al., 2013, 2015 and is

shown in Appendix 8.2. The data sheet format is shown in Appendix 8.3.

3.2.1 Culturing HCE-T cells

An SV40-immortalized HCE cell strain (HCE-T cells, RCB no. 2280) was obtained from RIKEN BioResource Center (Tsukuba, Japan). The cells were maintained in a culture medium comprising a 1:1 mixture of Dulbecco's modified eagle medium and nutrient mixture F-12 supplemented with 5% heat-inactivated fetal bovine serum, 5 μg/mL recombinant human insulin, 10 ng/mL recombinant human epidermal growth factor, 0.5% dimethyl sulfoxide, 100 units/mL penicillin and 100 μg/mL streptomycin (Araki-Sasaki et al., 1995; Yamasaki et al., 2009). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

3.2.2 Preparation of collagen vitrigel membrane chambers

A collagen xerogel membrane chamber (ad-MED Vitrigel™) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The collagen xerogel membrane chamber was set in the well of a 12-well plate. Then, the collagen xerogel membrane was immersed in the culture medium by pouring 1.5 mL outside and 0.5 mL inside the chamber in the well for 10 min to convert the xerogel into vitrigel immediately before use.

3.2.3 Reconstruction of a human corneal epithelium model

The culture medium outside the chamber in the well of a 12-well plate was replaced with 1.5 mL of fresh medium. The medium inside the chamber was removed and 0.5 mL of a cell suspension in a culture medium at a density of 1.2×10^5 cells/mL was poured onto the CVM in the chamber and cultured for 2 days at 37°C. Subsequently, the cells were cultured for 4 days at the air–liquid interface to fabricate a HCE model after removing the inside medium and changing the outside medium outside of the chamber. The medium outside the chamber was changed on the third day of culturing at the air–liquid interface.

3.2.4 Mode of action in vivo

Time-dependent relative changes of TEER values after exposing chemicals to in vitro human corneal epithelial models are considered to be an excellent indicator for extrapolating the destructive activity of the chemicals against the barrier function of human corneal epithelium in vivo. For this reason, the TEER assay is a simple and suitable method for evaluating corneal irritancy and permeability quantitatively and continuously (Uematsu et al., 2007). Therefore, it is important to develop an assay system that can facilitate not only the reconstruction of human corneal epithelial model but also the TEER measurement and the chemical exposure.

Our preliminary results based on the testing of four chemicals demonstrated a correlation between irritancy potential and changes in TEER. We found that non-irritants caused virtually no change in TEER, moderate irritants caused only a gradual decrease of limited magnitude in TEER, and strong irritants caused a rapid decrease of significant magnitude in TEER (Takezawa et al. 2011a). During further testing of 30 chemicals, we consistently observed these three patterns, which we were able to express mathematically using three parameters, namely, time lag, intensity, and plateau (Yamaguchi et al. 2013).

In this study, we aimed to develop such an ideal assay method utilizing HCE-T cells and the collagen vitrigel membrane chamber useful for TEER measurement.

3.2.5 Calculation of TEER values for HCE models

The electrical resistance of a HCE model in a CVM chamber (R_{model}) and of a blank CVM chamber (R_{blank}) were measured using the TEER recorder shown in Fig. 2. The TEER value was calculated as follows:

$$TEER = (R_{model} - R_{blank}) \times \text{effective surface area } (1.0\ cm^2)$$

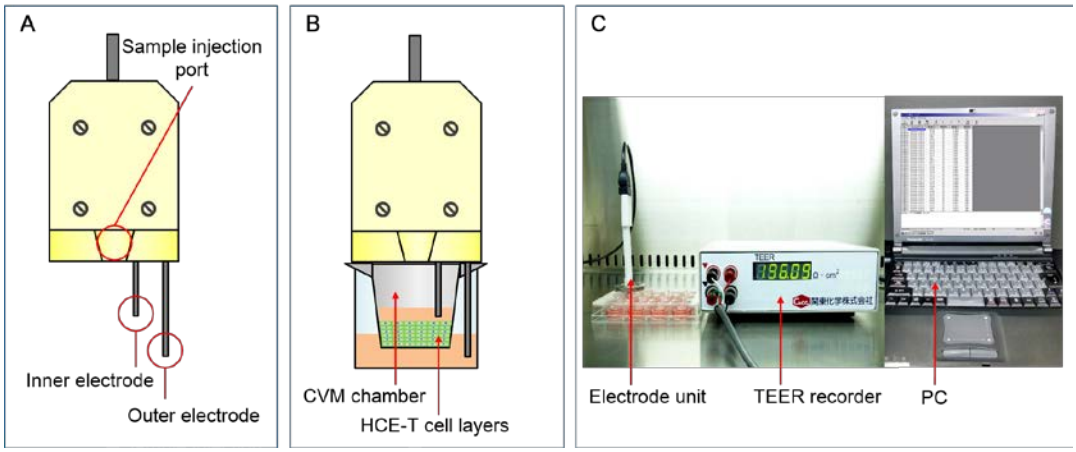


Fig.2. Schematic illustrations on the TEER measurement electrodes for HCE model and gross observation of TEER recorder system.

The electrode unit (A), the electrode unit applied for the culture media via HCE model (B) and the TEER recorder system (C).

3.2.6 Exposure to test chemicals

A solution of test chemical was prepared in a culture medium at a concentration of 2.5% (weight/volume), which is considered appropriate for measuring TEER values without undue influence from the electrical resistance of the test chemical itself. Test chemicals were manually mixed in the medium until the test chemical dissolves or for a maximum of one minute. If the test chemical does not dissolve readily, try using the following techniques in the following order to dissolve it: a) mix mechanically for a maximum of one minute using a vortex mixer, b) sonication for a maximum of 20 minutes, or c) heating to a maximum temperature of 70°C. After trying each technique, the temperature of each test chemical solution was checked. Test chemical solution that is well dissolved or homogeneously dispersed, was moved to the next step. For test chemicals that proved to be insoluble or immiscible using the above technique, a test chemical solution was prepared as a homogeneous suspension by mixing the test chemical in the medium by vortex for up to 1 minute immediately before use (Fig.3). The pH level of each 2.5% test chemical solution was measured using universal pH test paper from ADVANTEC (Tokyo, Japan).

The HCE models were exposed to a test chemical on day 6, as follows: First, 500 μL of culture

medium was poured in the chamber and the TEER recorder was used to obtain a pre-exposure R_{model} value for each model. Next, the medium inside the chamber was replaced with 500 μL of test chemical solution and R_{model} values were measured at intervals of 10 seconds for a period of 3 min after exposure to the test solution. Here, it is essential to obtain the reproducible data that the measurement is started within 2 to 5 seconds after adding the test chemicals. Because the liquid condition around the electrode is often unstable within 2 seconds after exposing the test chemical solution. Also, the HCE model has already been influenced with the test chemicals over 5 seconds. Three runs were made for each test chemical and a new HCE model was used in each. Test chemical exposure was conducted at an ambient temperature of 28±2°C. The ambient temperature of 28±2°C for the HCE model was achieved by regulating the temperature of the 12-well plate using a hot plate, a water bath or an air conditioner. Here, it is important to confirm that the actual temperature of culture medium is 28±2°C.

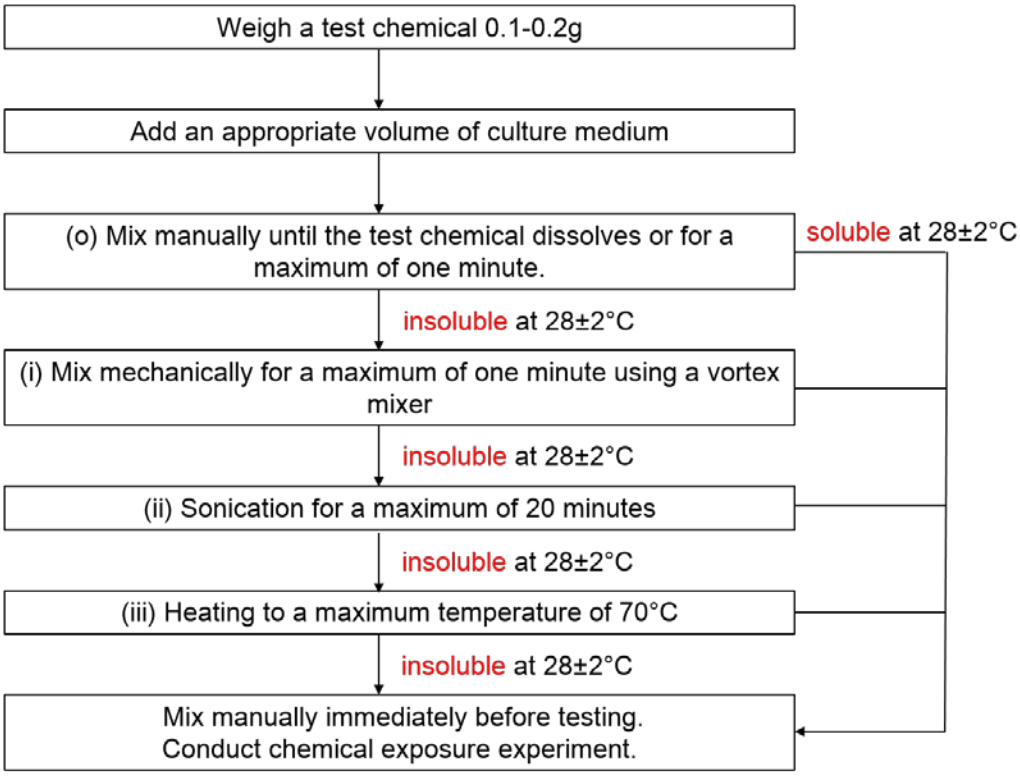


Fig.3. Preparation of test chemical solution per the revised protocol

3.2.7 Calculating eye irritancy of test chemicals

The TEER values for each test chemical were measured during the three runs and then copied to a data sheet, where eye irritancy was calculated automatically. The mean TEER values for all three tests were plotted on a time line and a profile of TEER values (dP/dT) was automatically analyzed for three parameters: time lag (t_1), intensity ($-[P_2 - P_1]/[t_2 - t_1]$), and plateau level ($100 - P_2$). Time lag (t_1) is defined as the maximum time at which a profile was maintained at $0 \geq dP/dT > -0.03\%$/second. The starting time of plateau level (t_2) after the profile was maintained at $dP/dT \leq -0.03\%$/second for a particular period of time was defined as the initial time at which the profile was maintained at $0 \geq dP\ (P_3 - P_2)/dT\ (t_3 - t_2) > -0.03\%$/s. The time ($t_3$) is represented in the equation ($t_3 = t_2 + 30$ seconds) because the plateau level was evaluated by the profile for 30 seconds. P_1, P_2, and P_3 are the percentages against the initial TEER value at t_1, t_2, and t_3 after exposure to the test chemical, as shown in Fig. 4. A score for each index was calculated using the above formula. Subsequently, the eye irritation potential of test chemicals was determined to be either irritant or non-irritant, in accordance with the criteria shown in Table 3.

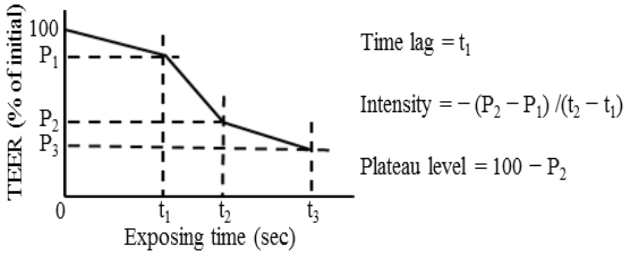


Fig. 4. Schematic illustration showing an analysis of a TEER profile after exposure of a model to a test chemical.

t_1 represents time lag, and t_2 represents the start of the plateau level. t_3 is defined as $t_2 + 30$ s.

P_1, P_2, and P_3 indicated a percentage relative to the initial TEER value at t_1, t_2, and t_3, respectively.

Table 3. Eye irritancy criteria.

Criteria	Prediction
Time lag ≤ 180 or Intensity ≥ 0.05 or Plateau level > 5.0	Irritant (I)
Time lag > 180 and Intensity < 0.05 and Plateau level ≤ 5.0	Non-irritant (NI)

3.2.8 Correlation with the UN GHS classification

The correlation with the UN GHS classification of test chemicals was estimated by calculating sensitivity, specificity, and accuracy, as follows.

Sensitivity (%) = A/(A + B) × 100

Specificity (%) = D/(C + D) × 100

Accuracy (%) = (A + D)/(A + B + C + D) × 100

A is the number of test chemicals classified as irritants by both the traditional UN GHS classification and the Vitrigel-EIT method. B is the number of test chemicals classified as irritants by the traditional UN GHS classification and as non-irritants by the Vitrigel-EIT method. C is the number of test chemicals classified as non-irritants by the traditional UN GHS classification and as irritants by the Vitrigel-EIT method. D is the number of test chemicals classified as non-irritants by both the traditional UN GHS classification and the Vitrigel-EIT method.

3.2.9 Commercial availability and/or intellectual property rights to the test method and its components

All components and reagents using in the test method are commercially available. HCE-T cells can be globally distributed from RIKEN BioResource Center. The Vitrigel-EIT method is available without any restriction by its intellectual property rights. Vitrigel is registered trade mark of National Agriculture and Food Research Organization (Tsukuba, Japan).

3.3 Test chemicals

3.3.1 Selection and distribution of test chemicals

The test chemicals were selected to ensure that a diverse range of substances were represented, and

aspects such as eye-irritant level per UN GHS categories, physical state, chemical class, and incidence of eye lesions were considered. Preference was given to test chemicals for which high-quality in vivo data is available, especially when the data included results from individual animals. The list includes test chemicals that were previously used in the 3-dimensional corneal model (such as EpiOcular) validation studies by EURL-ECVAM (ECETOC, 1998), the Short Time Exposure test validation study by JaCVAM and independent peer review (ICCVAM, 2010, 2013), and the OptiSafe™ evaluation study by NICEATM.

All the test chemicals selected for this validation study are available commercially, were selected by the chemical management group, and approved by the VMT. All the test chemicals used in Phases I, II, and III were coded, and their names were provided only after completion of the study. A total of 42 substances were tested by all three laboratories.

3.3.2 Test chemicals for Phases 0, I, II, and III

3.3.2.1 Test chemicals for Phase 0

Five test chemicals were selected by the VMT for use in validating between-laboratory transferability during Phase 0, as shown in Table 4. The five non-coded test chemicals were delivered to each participating laboratory by the VMT.

Table 4. List of test chemicals selected for Phase 0

No.	Test chemical	CASRN	State	Density (g/cm^3)	logP	pH	GHS
Positive control	Ethanol	64-17-5	Liquid	-0.31	7	2A	Category 1
0-1	Benzalkonium chloride	8001-54-5	Solid	0.99	1.68	7	Category 1
0-2	2-Propanol	67-63-0	Liquid	0.78	0.05	7	Category 2A
0-3	Glycerol	56-81-5	Liquid	1.26	-1.76	7	No Category
0-4	n-Hexanol	111-27-3	Liquid	0.82	2.03	7	Category 2A
0-5	Silicon dioxide n-hydrate	7699-41-4	Solid	1.58	-	7	No Category

3.3.2.2 Test chemicals for Phase I

Ten test chemicals were selected by the VMT for use in validating within- and between-laboratory

reproducibility during Phase I, as shown in Table 5. The ten test chemicals comprised five irritants and five non-irritants, five of which were solid and five of which were liquid, as shown in Table 5. To assess the within-laboratory reproducibility, the VMT selected ten test chemicals in Phase I. The VMT decided this scale based on our biostatistician's opinion about the statistical validity of the number of test chemicals used for the ECVAM validation study for skin sensitization. A detailed background is addressed at appendix 8-12. The ten test chemicals were coded and delivered in three sets to each participating laboratory by the VMT. Refer to the chemical selection report in Appendix 8.4 for code numbers.

Table 5. List of test chemicals selected for Phase I

No.	Test chemical	CASRN	State	Density (g/cm^3)	logP	pH	GHS
1-1	Imidazole	288-32-4	Solid	1.03	-0.08	9	Category 1
1-2	Cyclohexanol	108-93-0	Liquid	0.96	1.23	7	
1-3	3,3-Dithiodipropionic acid	1119-62-6	Solid	1.45	-0.15	4	Category 2A or 2B
1-4	Acetone	67-64-1	Liquid	0.79	-0.24	7	
1-5	3-Chloropropionitrile	542-76-7	Liquid	1.16	0.18	5	
1-6	Ammonium nitrate	6484-52-2	Solid	1.72	-	8	
1-7	n,n-Dimethylguanidine sulfate	598-65-2	Solid	-	-	7	No Category
1-8	Toluene	108-88-3	Liquid	0.87	2.73	7	
1-9	3-Methoxy-1,2-propanediol	623-39-2	Liquid	1.11	-1.13	7	
1-10	Gluconolactone	90-80-2	Solid	1.61	-2.48	6	

3.3.2.3 Test chemicals for Phase II

Ten test chemicals were selected by the VMT for use in validating between-laboratory reproducibility during Phase II, as shown in Table 6. The ten test chemicals comprised four classified UN GHS Category 1, three classified UN GHS Category 2A or 2B, and three classified UN GHS No Category, five of which were

477 solids and five of which were liquid, as listed in Table 6. The ten test chemicals were
478 coded and delivered in one set to each participating laboratory by the VMT. Refer to
479 the chemical selection report in Appendix 8.4 for code numbers.
480
481 Table 6. List of test chemicals selected for Phase II

No.	Test chemical	CASRN	State	Density (g/cm^3)	logP	pH	GHS
2-1	Imidazole	288-32-4	Solid	1.03	-0.08	9	Category 1
2-2	Cyclohexanol	108-93-0	Liquid	0.96	1.23	7	
2-3	Sodium dodecyl sulfate	151-21-3	Solid	0.40	1.60	7	
2-4	Sodium salicylate	54-21-7	Solid	0.32	0.42	7	
2-5	Cyclopentanol	96-41-3	Liquid	0.95	2.41	7	Category 2A or 2B
2-6	2-Methyl-1-pentanol	105-30-6	Liquid	0.83	1.76	7	
2-7	α-Hexylcinnamaldehyde	101-86-0	Liquid	0.95	5.12	7	
2-8	n,n-Dimethylguanidine sulfate	598-65-2	Solid	-	-	7	No Category
2-9	Toluene	108-88-3	Liquid	0.87	2.73	7	
2-10	Gluconolactone	90-80-2	Solid	1.61	-2.48	6	

482

483 *3.3.2.4 Test chemicals for Phase III*

484 Thirty-six test chemicals were selected by the VMT for use in validating between-laboratory
485 reproducibility and predictive capacity during Phase III, as shown in Table 7. The number of chemicals,
486 total 36 chemicals, was decided in consideration of Kanto Chemical's ability to supply the CVM
487 chambers as well as the participating laboratories' testing capacity. All test chemicals were selected to
488 ensure that a diverse range of substances were represented, and aspects such as eye-irritant level per
489 UN GHS categories, physical state, chemical class, and incidence of eye lesions were considered.
490 Preference was given to test chemicals for which high-quality in vivo data is available, especially
491 when the data included results from individual animals. The number of test chemicals in each GHS
492 classification is shown in Table 8. The number of solid and liquid test chemicals is show in Table 9.
493 The thirty-six test chemicals were coded and delivered in one set to each participating laboratory by

494 the VMT. Refer to the chemical selection report in Appendix 8.4 for code numbers.

495 The chemical master at Lab C revealed the name of test chemical No. 3-16, sodium chloroacetate,

496 which was subsequently eliminated from the list and cyclopentanol was delivered by the VMT as an

497 alternative.

498

Table 7. List of test chemicals selected for Phase III

No.	Test chemical	CASRN	State	Density (g/cm^3)	logP	pH	GHS
3-1	2,5-Dimethyl-2,5-hexanediol	110-03-2	Solid	0.90	1.19	7	Category 1
3-2	2-Benzyl-4-chlorophenol	120-32-1	Solid	1.19	3.60	7	
3-3	2,2-Dimethyl butanoic acid	595-379	Liquid	0.93	1.90	4	
3-4	Captan	133-06-2	Solid	1.74	2.80	7	
3-5	Tetra-n-octylammonium bromide	14866-33-2	Solid	0.94	3.45	7	
3-6	Butanol	71-36-3	Liquid	0.81	0.88	8	
3-7	3-(2-Aminoethylamino) propyl]trimethoxysilane	1760-24-3	Liquid	1.01	-1.00	10	
3-8	Sodium dodecyl sulfate	151-21-3	Solid	0.40	1.60	7	
3-9	m-Phenylenediamine	108-45-2	Solid	1.14	-0.33	8	
3-10	Tetraethylene glycol	17831-71-9	Liquid	1.13	1.26	7	
3-30	Imidazole	288-32-4	Solid	1.03	-0.08	9	
3-32	Sodium salicylate	54-21-7	Solid	0.32	0.42	7	
3-11	gamma-Butyrolactone	96-48-0	Liquid	1.13	-0.64	7	Category 2A or 2B
3-12	Methyl acetate	79-20-9	Liquid	0.93	0.18	7	
3-13	Myristyl alcohol	112-72-1	Solid	0.82	6.03	7	
3-14	2,6-Dichlorobenzoyl chloride	4659-45-4	Liquid	1.47	2.54	3	
3-15	Dibenzyl phosphate	1623-08-1	Solid	1.46	1.71	3	
3-17	1-(2-Propoxy-1-methylethoxy)-2-propanol	29911-27-1	Liquid	0.94	1.14	7	
3-18	Camphene	79-92-5	Solid	0.84	1.94	7	
3-19	Ethyl-2-methylacetoacetate	609-14-3	Liquid	1.00	0.78	7	
3-20	Propylene glycol propyl ether	1569-01-3	Liquid	0.89	0.56	8	
3-31	2-Methyl-1-pentanol	105-30-6	Liquid	0.83	1.76	7	
3-33	α-Hexylcinnamaldehyde	101-86-0	Liquid	0.95	5.12	7	
3-37	Cyclopentanol	96-41-3	Liquid	0.95	2.41	7	
3-21	Methyl amyl ketone	110-43-0	Liquid	0.82	1.98	7	No Category
3-22	2-(n-Dodecylthio)ethanol	1462-55-1	Liquid	0.91	-	7	
3-23	iso-Octylthioglycolate	25103-09-7	Liquid	0.97	4.36	7	
3-24	2,4-Difluoronitrobenzene	446-35-5	Liquid	1.46	-1.18	7	

3-25	tetra-Aminopyrimidine sulfate	5392-28-9	Solid	1.65	0.27	3
3-26	2,4-Pentanediol	625-69-4	Liquid	0.96	0.35	8
3-27	iso-Octyl acrylate	29590-42-9	Liquid	0.88	4.61	7
3-28	Silicon dioxide n-hydrate	7699-41-4	Solid	1.58	-	7
3-29	Potassium tetrafluoroborate	14075-53-7	Solid	2.51	-	7
3-34	n,n-Dimethylguanidine sulfate	598-65-2	Solid	-	-	7
3-35	Toluene	108-88-3	Liquid	0.87	2.73	7
3-36	Gluconolactone	90-80-2	Solid	1.61	-2.48	6

Table 8. Breakdown of test chemicals used in Phase III

GHS				Total
Category 1	Category 2A/2B	Category 2B	No Category	
12	8	4	12	36

Table 9. Breakdown of test chemicals used in Phase III per physical state

Solid	Liquid	Total
16	20	36

3.4 Quality assurance

All testing at the participating laboratories was conducted in accordance with the principles of Good Laboratory Practice (GLP, OECD 1998), and were well documented, including a discussion of any impact on study results. Records were kept of the maintenance of measuring instruments, the production of HCE models, and the preparation and application of test chemicals using a format prepared by the lead laboratory. The data was input using a format developed for this validation study by the lead laboratory and the biostatistician. Personnel at the participating laboratories recorded the necessary information, including the code names of each test chemical, names and date of preparation of solvents, degree of solubility or suspensibility, and concentration of the test solution. These records were sent from the participating laboratories to JaCVAM, where they were checked for validity and accuracy as well as archived.

3.5 Record collection and analysis

Data collection and analysis were performed in close collaboration with biostatisticians and the quality

assurance group. Independent biostatisticians collected and organized data as shown in Appendix 8.5 using custom data collection software, and all records were checked by the quality assurance group. Any concerns at the participating laboratories over record keeping were resolved by the on-site study director and reported at VMT meetings.

At the final VMT meeting, all data was finalized and decoded by the trial coordinator, after which the biostatisticians performed a statistical analysis. Data management procedures and statistical tools were approved by the trial coordinator and the data analysis group. Any deviation found in the analysis was well documented, including a discussion of any impact on study results. Test results were evaluated for correlation with UN GHS classification based on predetermined criteria.

Predictive capacity of the Vitrigel-EIT method was evaluated using data from Phase III. First, an analysis was performed to assess predictive capacity in accordance with UN GHS classification per either a bottom-up or a top-down approach (Scott, 2010). Further analysis was then performed to reduce false negatives by limiting the scope of the applicability domain.

4 Results

All data were analyzed by biostatisticians as shown in Appendix 8.5. The quality assurance group checked all records, following the quality assurance protocol, as summarized in Appendix 8.6.

4.1 Study duration

Phase 0 was conducted from June to December 2013, using protocol ver. 1.30e.

Phase I was conducted from March to April 2014, using protocol ver. 1.51e.

Phase II was conducted from June to September 2014, using protocol ver. 1.61e.

Phase III was conducted from November 2014 to January 2015, using protocol ver. 1.71e.

VMT meetings were held during the intervals between these phases. The minutes of the VMT meetings are show in Appendix 8.7.

4.1.1 Phase 0

Phase 0 was designed to assess between-laboratory transferability by testing five non-coded test

chemicals using protocol ver. 1.30e.

Although the results were generally good, two issues were identified: the results for glycerol obtained at BRC were inconsistent, and those for ethanol (positive control) obtained at Daicel did not meet the success criteria for between-laboratory reproducibility shown in Tables 10 and 11. With the exception of the results for glycerol obtained at BRC, the data was overall highly consistent. The results for two of the three runs of ethanol at Daicel fell below the acceptance criteria for positive control (plateau level: 20 to 30%) in Fig.5. At the 1st VMT meeting, members discussed a proposal to use benzalkonium chloride as the positive control instead of ethanol, in order to ensure clear and consistent results. Ultimately, ethanol was used as a reference control, and its range was modified to 15–30% at plateau level. This exact range was to be finalized based on the results of Phase I.

The VMT requested additional testing at BRC and Daicel using a revised protocol, ver. 1.40e. After confirming the results of the additional testing (data not shown), all VMT members agreed to proceed with Phase I. The following key issues were addressed by revising the protocol to ver. 1.51e prior to the start of Phase I.

- Success criteria for the reference control: Range at plateau level of 10–30%
- Ambient temperature during TEER measurement: 18–30ºC
- Time from start of exposure to start of measurement: within 2 seconds

562 Table 10-1. Data for Phase 0, Trial 1

No.	Test chemical	FDSC				BRC				Daicel			
		Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result
Negative control		190 (NI)	-0.03 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI
Positive control (ethanol)		20 (I)	0.13 (I)	23 (I)	I	0 (I)	0.11 (I)	21 (I)	I	10 (I)	0.09 (I)	18 (NI)	N
0-1	Benzalkonium chloride	0 (I)	0.32 (I)	58 (I)	I	0 (I)	0.30 (I)	54 (I)	I	0 (I)	0.21 (I)	37 (I)	I
0-2	2-Propanol	10 (I)	0.17 (I)	32 (I)	I	0 (I)	0.16 (I)	29 (I)	I	10 (I)	0.13 (I)	24 (I)	I
0-3	Glycerol	0 (I)	0.31 (I)	22 (I)	I	10 (I)	0.12 (I)	4 (NI)	I	0 (I)	0.25 (I)	13 (I)	I
0-4	n-Hexanol	0 (I)	0.21 (I)	38 (I)	I	30 (I)	0.14 (I)	23 (I)	I	10 (I)	0.15 (I)	27 (I)	I
0-5	Silicon dioxide n-hydrate	190 (NI)	-0.02 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI

563

564

565 Table 10-2. Data for Phase 0, Trial 2

No.	Test chemical	FDSC				BRC				Daicel			
		Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result
Negative control		190 (NI)	-0.01 (NI)	0 (NI)	NI	190 (NI)	-0.02 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI
Positive control (ethanol)		10 (I)	0.12 (I)	22 (I)	I	0 (I)	0.12 (I)	21 (I)	I	10 (I)	0.13 (I)	24 (I)	I
0-1	Benzalkonium chloride	0 (I)	0.32 (I)	57 (I)	I	0 (I)	0.30 (I)	54 (I)	I	0 (I)	0.33 (I)	60 (I)	I
0-2	2-Propanol	0 (I)	0.13 (I)	24 (I)	I	0 (I)	0.18 (I)	32 (I)	I	10 (I)	0.13 (I)	24 (I)	I
0-3	Glycerol	0 (I)	0.31 (I)	12 (I)	I	190 (NI)	-0.10 (NI)	2 (NI)	NI	0 (I)	0.21 (I)	12 (I)	I
0-4	n-Hexanol	10 (I)	0.15 (I)	28 (I)	I	0 (I)	0.21 (I)	37 (I)	I	40 (I)	0.11 (I)	19 (I)	I
0-5	Silicon dioxide n-hydrate	190 (NI)	-0.01 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI	190 (NI)	0.00 (NI)	0 (NI)	NI

566

Table 10-3. Data for Phase 0, Trial 3

No.	Test chemical	FDSC				BRC				Daicel			
		Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result
Negative control		190 (NI)	-0.01 (NI)	0 (NI)	NI	190 (NI)	-0.10 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI
Positive control (ethanol)		20 (I)	0.12 (I)	22 (I)	I	0 (I)	0.14 (I)	21 (I)	I	10 (I)	0.10 (I)	19 (NI)	NI
0-1	Benzalkonium chloride	0 (I)	0.34 (I)	62 (I)	I	0 (I)	0.29 (I)	52 (I)	I	0 (I)	0.30 (I)	55 (I)	I
0-2	2-Propanol	10 (I)	0.15 (I)	29 (I)	I	0 (I)	0.16 (I)	29 (I)	I	10 (I)	0.13 (I)	24 (I)	I
0-3	Glycerol	0 (I)	0.30 (I)	18 (I)	I	0 (I)	0.41 (I)	16 (I)	I	0 (I)	0.19 (I)	13 (I)	I
0-4	n-Hexanol	0 (I)	0.22 (I)	39 (I)	I	0 (I)	0.16 (I)	28 (I)	I	20 (I)	0.15 (I)	27 (I)	I
0-5	Silicon dioxide n-hydrate	190 (NI)	-0.01 (NI)	0 (NI)	NI	190 (NI)	-0.04 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI

Table 11. Combined results for Phase 0

No.	Test chemical	FDSC			BRC			Daicel		
		1	2	3	1	2	3	1	2	3
Negative control		Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Positive control (ethanol)		Pass	Pass	Pass	Pass	Pass	Pass	NG	Pass	NG
0-1	Benzalkonium chloride	I	I	I	I	I	I	I	I	I
0-2	2-Propanol	I	I	I	I	I	I	I	I	I
0-3	Glycerol	I	I	I	I	NI	I	I	I	I
0-4	n-Hexanol	I	I	I	I	I	I	I	I	I
0-5	Silicon dioxide n-hydrate	NI	NI	NI	NI	NI	NI	NI	NI	NI

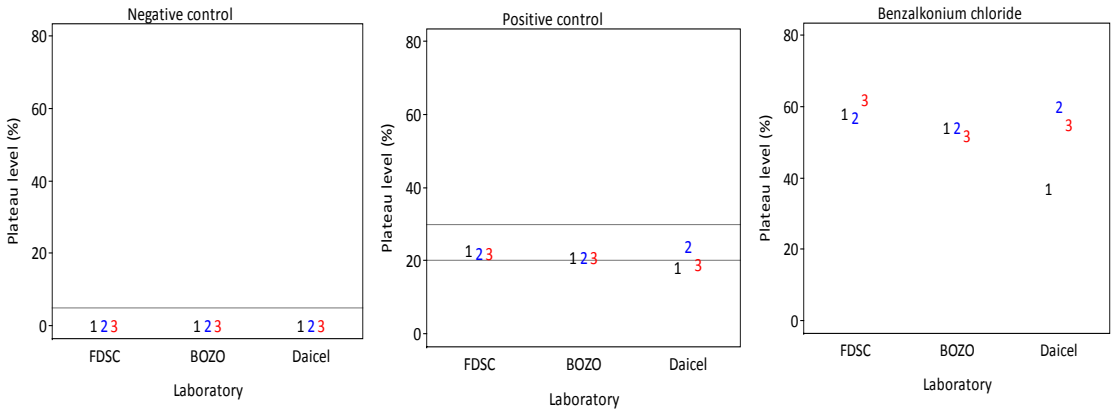


Fig. 5. Distribution of the three trials of Phase 0

4.1.2 Phase I

Phase I was designed to assess within and between-laboratory reproducibility by testing ten coded test chemicals using protocol ver. 1.51e.

The results for two of nine runs of the reference control (ethanol) at FDSC did not initially meet the success criteria, but were successfully retested, as shown in Tables 12 and 13. Analysis of Phase 0 and Phase I results as well as concerns for quality assurance of the HCE models led the VMT to include success criteria for the reference control in the next version of the test protocol. Consequently, the VMT recommended that the range for the reference control should be revised, so expanded success criteria for the positive and reference controls were developed by the lead laboratory. Furthermore, the results for test chemical No. 1-7, n,n-dimethyl guanidine sulfate, and No. 1-10, gluconolactone at FDSC as well as for test chemical No. 1-8, toluene, at Daicel failed to satisfy the success criteria for the within-laboratory reproducibility, as shown in Tables 12 and 14. All results at BRC met the success criteria. Thus, the within-laboratory reproducibility was 80% at FDSC, 90% at Daicel, and 100% at BRC, which was sufficient to satisfy the success criteria of 80% as stated in the study plan. Although the results for No. 1-1, imidazole, and No. 1-8, toluene, were somewhat inconsistent, the data showed a between-laboratory reproducibility of 80%, which met the acceptance criteria of 80% as stated in the study plan. The following key issues were addressed by revising the protocol to ver. 1.61e prior to the start of Phase II.

- Revised the term “room temperature” to read “ambient temperature for the experiment,” because control of ambient temperature is necessary.
- Included success criteria for the reference control and changed the phrase “Plateau level is between 10% and 30%, inclusive” to “Plateau level is between 10% and 40%, inclusive”.
- Change the ambient temperature for TEER measurement from “between 18 and 30°C” to “between 22 and 30°C,” because temperature of the HCE model can affect TEER.
- Changed the description of the procedure for preparing test chemical solutions

  Old: If the test chemical has not been dissolved, try to dissolve it by the mechanical mixture for a maximum 1-minute period using a vortex, by the sonication for a maximum 20-minute period, or by the heating to70°.

  New: If the test chemical does not dissolve readily, try one of the following techniques: a) mix mechanically for a maximum of one minute using a vortex mixer, b) sonication for a maximum of 20 minutes, or c) heating to a maximum temperature of 70°C.

  This was done, because some personnel at the participating laboratories misunderstood the procedure during Phase 1 and thought that all three of these techniques should be performed. Also, the term “vortex” was corrected to “vortex mixer.”
- Added a precaution to seal the 15-mL tube tightly during testing to prevent volatilization of the test chemical solutions, as follows: “To prevent volatilization of test chemical solutions, the 15-mL tube should be sealed tightly after weighing test chemicals, except when adding culture medium and sampling the 2.5% test chemical solution.”
- Added instructions to reject and retest any result in which there is a significant discrepancy between the initial TEER value and the TEER value measured at 0 seconds, which would indicate some technical issue, such as electrical noise or improper use of electrode, as follows: “If there is a discrepancy of 40 $\Omega \cdot cm^2$ or more between the initial TEER value and the TEER value measured at 0 seconds, reject the test results and retest using another HCE model.”

619 Table 12-1. Data for Phase I, Trial 1

No.	Test chemical	FDSC				BRC				Daicel			
		Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result
Negative control		190 (NI)	-0.02 (NI)	0 (NI)	NI	190 (NI)	-0.04 (NI)	0 (NI)	NI	190 (NI)	-0.02 (NI)	0 (NI)	NI
Positive control		0 (I)	0.35 (I)	64 (I)	I	0 (I)	0.39 (I)	51 (I)	I	0 (I)	0.35 (I)	64 (I)	I
Reference control		20 (I)	0.09 (I)	16 (I)	I	0 (I)	0.18 (I)	16 (I)	I	10 (I)	0.14 (I)	26 (I)	I
1-1	Imidazole	190 (NI)	0.00 (NI)	0 (NI)	NI	120 (I)	0.15 (I)	11 (I)	I	130 (I)	0.12 (I)	9 (I)	I
1-2	Cyclohexanol	10 (I)	0.23 (I)	42 (I)	I	0 (I)	021 (I)	37 (I)	I	0 (I)	0.29 (I)	51 (I)	I
1-3	3,3-Dithiodipropionic acid	190 (NI)	-0.12 (NI)	0 (NI)	NI	190 (NI)	-0.07 (NI)	0 (NI)	NI	190 (NI)	-0.06 (NI)	0 (NI)	NI
1-4	Acetone	30 (I)	0.08 (I)	15 (I)	I	10 (I)	0.07 (I)	6 (I)	I	0 (I)	0.16 (I)	28 (I)	I
1-5	3-Chloropropionitrile	10 (I)	0.18 (I)	32 (I)	I	10 (I)	0.12 (I)	22 (I)	I	20 (I)	0.22 (I)	38 (I)	I
1-6	Ammonium nitrate	0 (I)	0.77 (I)	54 (I)	I	0 (I)	1.36 (I)	27 (I)	I	0 (I)	0.79 (I)	48 (I)	I
1-7	n,n-Dimethylguanidine sulfate	0 (I)	0.40 (I)	32 (I)	I	0 (I)	0.37 (I)	7 (I)	I	0 (I)	0.44 (I)	26 (I)	I
1-8	Toluene	190 (NI)	0.01 (NI)	0 (NI)	NI	170 (I)	0.02 (NI)	1 (NI)	I	190 (NI)	0.02 (NI)	1 (NI)	NI
1-9	3-Methoxy-1,2-propanediol	190 (NI)	-0.07 (NI)	0 (NI)	NI	190 (NI)	-0.08 (NI)	0 (NI)	NI	190 (NI)	-0.12 (NI)	0 (NI)	NI
1-10	Gluconolactone	0 (I)	0.21 (I)	11 (I)	I	0 (I)	0.34 (I)	3 (NI)	I	0 (I)	0.22 (I)	9 (I)	I

620

621 Table 12-2. Data for Phase I, Trial 2

No.	Test chemical	FDSC				BRC				Daicel			
		Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result
Negative control		190 (NI)	0.00 (NI)	0 (NI)	NI	190 (NI)	-0.03 (NI)	0 (NI)	NI	190 (NI)	-0.04 (NI)	0 (NI)	NI
Positive control		0 (I)	0.33 (I)	59 (I)	I	0 (I)	0.23 (I)	42 (I)	I	0 (I)	0.42 (I)	75 (I)	I
Reference control		20 (I)	-	3 (NI)	NG	0 (I)	0.12 (I)	21 (I)	I	0 (I)	0.17 (I)	30 (I)	I
1-1	Imidazole	190 (NI)	-0.03 (NI)	0 (NI)	NI	160 (I)	0.13 (I)	4 (NI)	I	140 (I)	0.11 (I)	7 (I)	I
1-2	Cyclohexanol	30 (I)	0.16 (I)	25 (I)	I	0 (I)	0.40 (I)	48 (I)	I	0 (I)	0.34 (I)	51 (I)	I
1-3	3,3-Dithiodipropionic acid	190 (NI)	-0.06 (NI)	0 (NI)	NI	190 (NI)	-0.07 (NI)	0 (NI)	NI	190 (NI)	-0.05 (NI)	0 (NI)	NI
1-4	Acetone	10 (I)	0.04 (NI)	10 (I)	I	0 (I)	0.12 (I)	21 (I)	I	0 (I)	0.17 (I)	30 (I)	I
1-5	3-Chloropropionitrile	90 (I)	0.19 (I)	20 (I)	I	10 (I)	0.20 (I)	36 (I)	I	10 (I)	0.21 (I)	39 (I)	I
1-6	Ammonium nitrate	0 (I)	0.69 (I)	21 (I)	I	0 (I)	0.67 (I)	27 (I)	I	0 (I)	1.05 (I)	52 (I)	I
1-7	n,n-Dimethylguanidine sulfate	190 (NI)	-0.05 (NI)	2 (NI)	NI	0 (I)	0.53 (I)	21 (I)	I	0 (I)	0.54 (I)	27 (I)	I
1-8	Toluene	190 (NI)	-0.01 (NI)	0 (NI)	NI	150 (I)	0.02 (NI)	1 (NI)	I	190 (NI)	0.00 (NI)	0 (NI)	NI
1-9	3-Methoxy-1,2-propanediol	190 (NI)	-0.05 (NI)	0 (NI)	NI	190 (NI)	-0.12 (NI)	0 (NI)	NI	190 (NI)	-0.11 (NI)	0 (NI)	NI
1-10	Gluconolactone	190 (NI)	-0.04 (NI)	0 (NI)	NI	0 (I)	0.28 (I)	8 (I)	I	10 (I)	0.18 (I)	10 (I)	I

622 Table 12-3. Data for Phase I, Trial 3

No.	Test chemical	FDSC				BRC				Daicel			
		Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result
Negative control		190 (NI)	-0.02 (NI)	0 (NI)	NI	190 (NI)	-0.02 (NI)	0 (NI)	NI	190 (NI)	-0.04 (NI)	0 (NI)	NI
Positive control		0 (I)	0.36 (I)	65 (I)	I	0 (I)	0.31 (I)	55 (I)	I	0 (I)	0.37 (I)	66 (I)	I
Reference control		0 (I)	0.18 (I)	33 (I)	NG	0 (I)	0.12 (I)	22 (I)	I	10 (I)	0.15 (I)	27 (I)	I
1-1	Imidazole	190 (NI)	-0.01 (NI)	0 (NI)	NI	110 (I)	0.17 (I)	13 (I)	I	60 (I)	0.14 (I)	20 (I)	I
1-2	Cyclohexanol	0 (I)	0.26 (I)	46 (I)	I	0 (I)	0.27 (I)	48 (I)	I	0 (I)	0.33 (I)	59 (I)	I
1-3	3,3-Dithiodipropionic acid	190 (NI)	-0.05 (NI)	0 (NI)	NI	190 (NI)	-0.08 (NI)	0 (NI)	NI	190 (NI)	-0.0 (NI)	0 (NI)	NI
1-4	Acetone	10 (I)	0.10 (I)	19 (I)	I	0 (I)	0.20 (I)	36 (I)	I	0 (I)	0.18 (I)	32 (I)	I
1-5	3-Chloropropionitrile	10 (I)	0.22 (I)	39 (I)	I	20 (I)	0.12 (I)	22 (I)	I	10 (I)	0.25 (I)	44 (I)	I
1-6	Ammonium nitrate	0 (I)	0.62 (I)	37 (I)	I	0 (I)	0.25 (I)	50 (I)	I	0 (I)	0.94 (I)	47 (I)	I
1-7	n,n-Dimethylguanidine sulfate	0 (I)	0.36 (I)	25 (I)	I	0 (I)	0.45 (I)	23 (I)	I	0 (I)	0.46 (I)	27 (I)	I
1-8	Toluene	190 (NI)	-0.03 (NI)	0 (NI)	NI	80 (I)	0.05 (I)	8 (I)	I	130 (I)	0.03 (NI)	2 (I)	I
1-9	3-Methoxy-1,2-propanediol	190 (NI)	-0.09 (NI)	0 (NI)	NI	190 (NI)	-0.12 (NI)	0 (NI)	NI	190 (NI)	-0.11 (NI)	0 (NI)	NI
1-10	Gluconolactone	10 (I)	0.10 (I)	5 (I)	I	0 (I)	0.30 (I)	9 (I)	I	10 (I)	0.18 (I)	10 (I)	I
Reference control (2)		10 (I)	0.12 (I)	22 (I)	I	-	-	-	-	-	-	-	-

623

Table 13. Combined results for Phase I control chemicals

	FDSC			BRC			Daicel		
	1	2	3	1	2	3	1	2	3
Negative control	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Positive control	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Reference	Pass	NG	NG	Pass	Pass	Pass	Pass	Pass	Pass
Reference (2)			Pass						

Table 14. Combined results for Phase I test chemicals

GHS	No.	Test chemical	FDSC			BRC			Daicel		
			1	2	3	1	2	3	1	2	3
Cat.1	1-1	Imidazole	NI	NI	NI	I	I	I	I	I	I
	1-2	Cyclohexanol	I	I	I	I	I	I	I	I	I
Cat. 2A & 2B	1-3	3,3-Dithiodipropionic acid	NI	NI	NI	NI	NI	NI	NI	NI	NI
	1-4	Acetone	I	I	I	I	I	I	I	I	I
	1-5	3-Chloropropionitrile	I	I	I	I	I	I	I	I	I
	1-6	Ammonium nitrate	I	I	I	I	I	I	I	I	I
No Category (NC)	1-7	n,n-Dimethylguanidine sulfate	I	NI	I	I	I	I	I	I	I
	1-8	Toluene	NI	NI	NI	I	I	I	NI	NI	I
	1-9	3-Methoxy-1,2-propanediol	NI	NI	NI	NI	NI	NI	NI	NI	NI
	1-10	Gluconolactone	I	NI	I	I	I	I	I	I	I

4.1.3 Phase II

Phase II was designed to assess the between-laboratory reproducibility of ten coded test chemicals using protocol ver. 1.61e.

Results for two of the ten test chemicals failed to satisfy the success criteria for between-laboratory reproducibility: No. 2-1, imidazole, and No. 2-9, toluene, as shown in Tables 15, 16, and 17. Although the concordance was 80% between the three laboratories, which was sufficient to satisfy the success criteria, the VMT was concerned over the failure to properly identify No. 2-1, imidazole, which is a UN GHS category 1 irritant. Therefore, the VMT was unanimous in recognizing the need to clarify the reason for this failure.

During a VMT teleconference to discuss the results of Phase II, the lead laboratory suggested that it might be necessary to control the ambient temperature at which tests were conducted. The lead laboratory had obtained data at the relatively high ambient temperature of 28°C. In addition, the time dependent TEER profile after exposing imidazole was affected by the temperature. In case the temperature below 22°C, imidazole was classified as non-irritant. All laboratories performed additional testing of No. 2-1, imidazole, under the modified parameters given in Fig.6 and as shown in Table 18. All laboratories correctly identified No. 2-1, imidazole, as an irritant, which suggested the need for rigorous control of the ambient temperature, and led to a major revision of the protocol prior to Phase III.

Due to this revision, the VMT recognized that Phase II data should not be combined with Phase III data to assess predictive capacity and decided to undertake validation of between-laboratory reproducibility and predictive capacity in Phase III using revised protocol ver. 1.71e. In consideration of the capacity of the participating laboratories, the number of test chemicals for Phase III was reduced from 40 in Phases IIA and IIB of the original study plan to just 36. Thus, a total of four chemicals (two from UN GHS category 1, 1 from UN GHS category 2, and 1 No Category) were removed from the original list of test chemicals.

The following key issues were addressed by revising the protocol to ver. 1.71e prior to the start of Phase III.

- Having recognized the need to control ambient temperature, we replaced the instruction “Let stand for 10 minutes (within 2 hours) at the ambient temperature for the experiment” to “Adjust the temperature of the model to 28±2°C.”
- Replaced all instances of the phrase “ambient temperature for the experiment” to “between 22 and 30°C.”
- Changed the success criteria for the reference control from “Plateau level is between 10% and 30%, inclusive” to “Plateau level is 10% or more.” The upper limit for this success criterion will be determined after reviewing the results of Phase III.

667 Table 15. Data for Phase II

No.	Test chemical	FDSC				BRC				Daicel			
		Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result	Time lag	Intensity	Plateau level	Result
Negative control		190 (NI)	-0.04 (NI)	0 (NI)	NI	190 (NI)	-0.08 (NI)	0 (NI)	NI	190 (NI)	-0.02 (NI)	0 (NI)	NI
Positive control		0 (I)	0.41 (I)	74 (I)	I	0 (I)	0.33 (I)	59 (I)	I	0 (I)	0.30 (I)	54 (I)	I
Reference control		0 (I)	0.24 (I)	31 (I)	I	0 (I)	0.14 (I)	25 (I)	I	0 (I)	0.16 (I)	24 (I)	I
2-1	Imidazole	190 (NI)	0.00 (NI)	3 (NI)	NI	140 (I)	0.15 (I)	8 (I)	I	190 (NI)	0.00 (NI)	0 (NI)	NI
2-2	Cyclohexanol	0 (I)	0.51 (I)	51 (I)	I	0 (I)	0.32 (I)	48 (I)	I	0 (I)	0.25 (I)	38 (I)	I
2-3	Sodium dodecyl sulfate	0 (I)	0.41 (I)	74 (I)	I	0 (I)	0.32 (I)	58 (I)	I	0 (I)	0.31 (I)	56 (I)	I
2-4	Sodium salicylate	0 (I)	0.80 (I)	48 (I)	I	0 (I)	0.41 (I)	33 (I)	I	0 (I)	0.54 (I)	33 (I)	I
2-5	Cyclopentanol	0 (I)	0.28 (I)	39 (I)	I	0 (I)	0.22 (I)	40 (I)	I	0 (I)	0.17 (I)	30 (I)	I
2-6	2-Methyl-1-pentanol	0 (I)	0.30 (I)	54 (I)	I	0 (I)	0.23 (I)	35 (I)	I	10 (I)	0.14 (I)	26 (I)	I
2-7	α-Hexylcinnamaldehyde	190 (NI)	-0.03 (I)	0 (NI)	NI	190 (NI)	-0.03 (NI)	0 (NI)	NI	190 (NI)	-0.01 (NI)	0 (NI)	NI
2-8	n,n-Dimethylguanidine sulfate	0 (I)	0.83 (I)	42 (NI)	I	0 (I)	0.37 (I)	26 (I)	I	0 (I)	0.54 (I)	27 (I)	I
2-9	Toluene	60 (I)	0.06 (I)	9 (I)	I	190 (NI)	0.00 (NI)	0 (NI)	NI	190 (NI)	-0.02 (NI)	0 (NI)	NI
2-10	Gluconolactone	0 (I)	0.48 (I)	19 (I)	I	0 (I)	0.30 (I)	12 (I)	I	0 (I)	0.23 (I)	9 (I)	I

668

669 Table 16. Results for Phase II control chemicals

	FDSC	BRC	Daicel
Negative control	Pass	Pass	Pass
Positive control	Pass	Pass	Pass
Reference	Pass	Pass	Pass

670

671

672 Table 17. Results for Phase II test chemicals

GHS	No.	Test chemical	FDSC	BRC	Daicel
Cat. 1	2-1	Imidazole	NI	I	NI
	2-2	Cyclohexanol	I	I	I
	2-3	Sodium dodecyl sulfate	I	I	I
	2-4	Sodium salicylate	I	I	I
Cat. 2A & 2B	2-5	Cyclopentanol	I	I	I
	2-6	2-Methyl-1-pentanol	I	I	I
	2-7	α-Hexylcinnamaldehyde	NI	NI	NI
No Category	2-8	n,n-Dimethylguanidine sulfate	I	I	I
	2-9	Toluene	I	NI	NI
	2-10	Gluconolactone	I	I	I

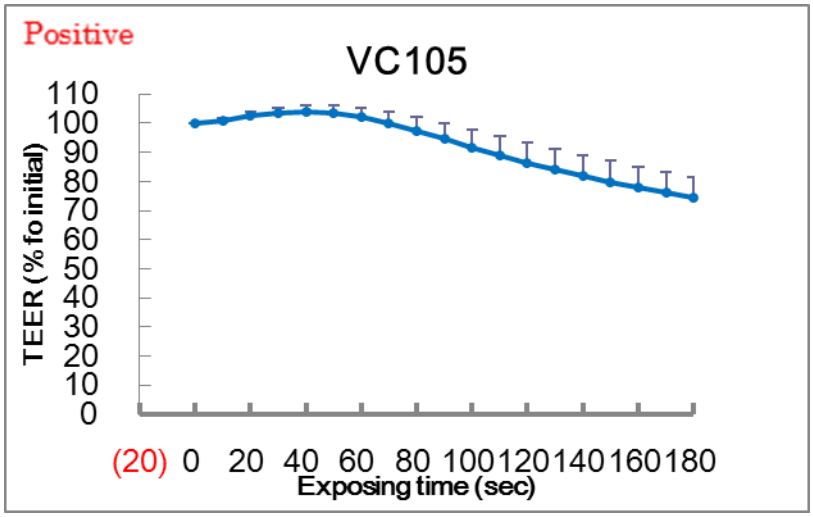
673

674

675 Table 18. List of test conditions at each lab.

	Phase II study			**Additional**		
	Circumstances	Part measured	Temp. (℃)	Circumstances	Part measured	Temp. (℃)
FDSC	Room temp.	Room temp.	24-26	On hot plate	Medium at a well	27-28
BRC	Room temp.	Room temp.	22-25	In Water bath	Medium at a well	27.4-28.6
Daicel	Room temp.	Room temp.	22	Room temp.	Room temp.	28±2
Lead Lab	Room temp.	Medium at a well	28			

676



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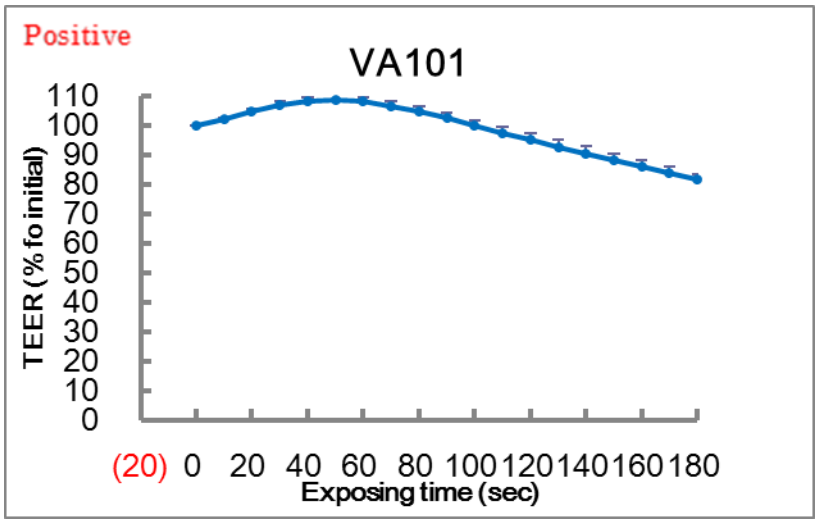

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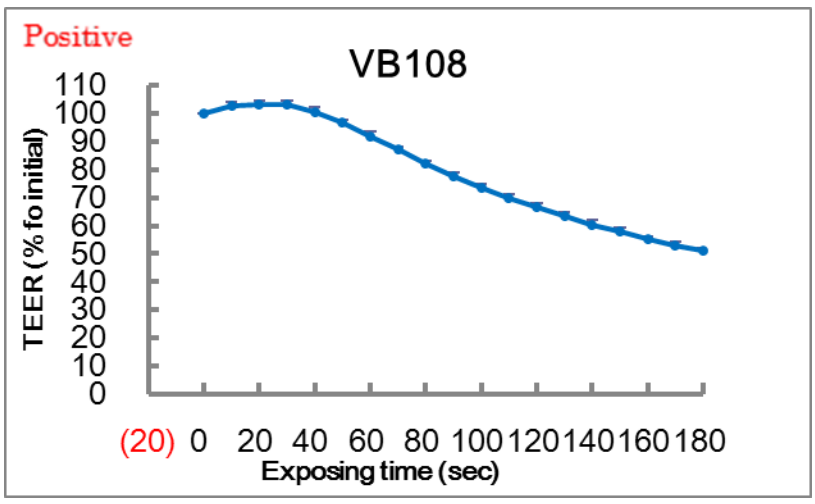

679

680 Fig.6. Additional data of Imidazole on Vitrigel-EIT phase II study

681

4.1.4 Phase III

During Phase III, the VMT received a question about test chemical No. 3-16, sodium chloroacetate, from the on-site study director at Daicel, who opened the MSDS due to concerns over legal compliance in handling deleterious substances. After considering the possibility of using this chemical, the VMT decided instead to delete it from the list of test chemicals, and in its place, distributed to all laboratories a new test chemical: No. 3-37, cyclopentanol. This test chemical is a UN GHS category 2B substance, just like No. 3-16, sodium chloroacetate.

There were some discrepancies in the Phase III results that can be attributed to differences the techniques used to dissolve the test chemicals. To resolve this issue, the protocol was revised to ver. 1.80e by limiting the techniques to be used to dissolve test chemicals.

Also, an additional procedure was included, which calls for the pH level of each 2.5% test chemical solution to be measured using universal pH test paper to ensure that the test chemical falls within the applicability domain.

Other procedural inconsistencies that require further study to determine whether or not standardization is necessary include the following.

a. The time interval from the start of exposure to a test chemical until the start of TEER measurement: 4 s at FDSC, 3 s at BRC, and 2 s at Daicel
b. Temperature of the models: 27.0–28.7°C in culture medium at FDSC, 26.4–28.0°C in a water bath at BRC, and 26.9–28.4°C in culture medium at Daicel
c. Number of insoluble test chemicals: Of the 21 test chemical solutions prepared at FDSC, four exhibited sediment and two exhibited supernatants (Nos. 212, 216); of the 19 test chemicals prepared at BRC, 10 exhibited sediment and seven exhibited supernatants (Nos. 213, 221, 222, 223, 232, 234, and 236); and of the 17 test chemicals prepared at Daicel, seven exhibited sediment and 10 exhibited supernatant (Nos. 202, 210, 218, 219, 220, 224, 230, 231, 233, and 235).
d. Other issues:

At Daicel, different batches of the frozen cell lines were used.

At FDSC, test chemical No. 216 was tested twice, but the data was not approved due to and inappropriate procedure.

All of the aforementioned issues were reported to the VMT, which unanimously agreed that these were minor issues that did not impact data analysis.

In Tables 19, 20, and 21, the between-laboratory reproducibility was 92% (33/36), which met the acceptance criteria of 80%. The results of a few insoluble test chemicals were inconsistent between the laboratories, including No. 3-5, tetra-N-octylammonium bromide, No. 3-14, 2,6-dichlorobenzyl chloride, and No. 3-18, camphene, and the VMT discussed the difficulties inherent in assessing these substances due to low solubility in the culture medium.

The following key issues were addressed by revising the protocol to ver. 1.80e after completion of Phase III.

- Added the term “Universal pH test paper (ADVANTEC, 07011030)” to section 3.
- Added a description of the applicability domain, which was determined per the results for 93 test chemicals.
- Changed the description of the procedure for preparing test chemical solution as follows.

  Old: If the test chemical has not been dissolved, try to dissolve it by selecting an appropriate technique(s) from the following; mechanical mixture for a maximum 1-minute period using a vortex mixer, sonication for a maximum 20-minute period, or heating to maximum 70°C.

  New: If the test chemical does not dissolve readily, try using the following techniques in the following order to dissolve it: a) mix mechanically for a maximum of one minute using a vortex mixer, b) sonication for a maximum of 20 minutes, or c) heating to a maximum temperature of 70°C. After trying each technique, adjust the temperature of each test chemical solution to 28±2°C and check solubility. Move to the next step of the procedure once the test chemical solution is well dissolved or homogeneously dispersed.
- Added a precaution that techniques for dissolving test chemicals are to be set according to

736 the physiochemical properties of the test chemicals.

737

738 The Vitrigel-EIT method was developed primarily to identify ocular non-irritants in a bottom-up
739 approach. As shown in Tables 22, the Vitrigel-EIT method demonstrated an accuracy of between 64
740 and 69% (23 to 25/36), a sensitivity of between 75 and 83% (18 to 20/24), and a specificity of 42%
741 (5/12). These figures are lower than those of in house data obtained by the lead lab and there are too
742 many false negatives for this test method to be useful in a bottom-up approach. Substances that yielded
743 either false negative or false positive results are listed in Table 23.

744

745 Table 19-1. Results for Phase III control chemicals

Set	Test chemical	FDSC					BRC					Daicel				
		Temp. (°C)*	Time lag	Intensity	Plateau level	Result	Temp. (°C)*	Time lag	Intensity	Plateau level	Result	Temp. (°C)*	Time lag	Intensity	Plateau level	Result
1	Negative control	28.0	190 (NI)	-0.03 (NI)	0 (NI)	NI	26.4	190 (NI)	0.00 (NI)	0 (NI)	NI	28.4	190 (NI)	-0.02 (NI)	0 (NI)	NI
	Positive control	28.2	0 (I)	0.45 (I)	82 (I)	I	27.5	0 (I)	0.41 (I)	75 (I)	I	28.4	0 (I)	0.52 (I)	67 (I)	I
	Reference control	27.4	0 (I)	0.20 (I)	28 (I)	I	27.5	0 (I)	0.19 (I)	34 (I)	I	28.4	0 (I)	0.24 (I)	29 (I)	I
2	Negative control	27.2	190 (NI)	-0.03 (NI)	0 (NI)	NI	27.1	190 (NI)	-0.04 (NI)	0 (NI)	NI	27.9	190 (NI)	-0.03 (NI)	0 (NI)	NI
	Positive control	27.3	0 (I)	0.44 (I)	79 (I)	I	27.2	0 (I)	0.40 (I)	73 (I)	I	27.9	0 (I)	0.40 (I)	72 (I)	I
	Reference control	27.2	0 (I)	0.19 (I)	29 (I)	I	27.3	0 (I)	0.19 (I)	35 (I)	I	27.9	0 (I)	0.14 (I)	26 (I)	I
3	Negative control	27.6	190 (NI)	-0.05 (NI)	0 (NI)	NI	27.6	190 (NI)	-0.04 (NI)	0 (NI)	NI					
	Positive control	27.5	0 (I)	0.36 (I)	65 (I)	I	27.7	0 (I)	050 (I)	90 (I)	I					
	Reference control	27.2	0 (I)	0.17 (I)	31 (I)	I	27.7	0 (I)	0.27 (I)	48 (I)	I					

746 * Temperature of the model at the time of exposure to the test chemical solution

747

748 Table 19-2. Results for Phase III test chemicals

No.	Test chemical	FDSC					BRC					Daicel				
		Temp. (°C)*	Time lag	Intensity	Plateau level	Result	Temp. (°C)*	Time lag	Intensity	Plateau level	Result	Temp. (°C)*	Time lag	Intensity	Plateau level	Result
3-1	2,5-Dimethyl-2,5-hexanediol	27.1	10 (I)	0.14 (I)	26 (I)	I	27.6	10 (I)	0.14 (I)	26 (I)	I	27.7	40 (I)	0.18 (I)	27 (I)	I
3-2	2-Benzyl-4-chlorophenol	28.5	0 (I)	0.40 (I)	72 (I)	I	28.0	0 (I)	0.40 (I)	72 (I)	I	27.7	0 (I)	0.40 (I)	73 (I)	I
3-3	2,2-Dimethyl butanoic acid	27.6	0 (I)	0.50 (I)	60 (I)	I	27.5	0 (I)	0.50 (I)	60 (I)	I	27.7	50 (I)	0.23 (I)	31 (I)	I
3-4	Captan	27.0	190 (NI)	-0.05 (NI)	0 (NI)	NI	27.5	190 (NI)	-0.05 (NI)	0 (NI)	NI	27.9	190 (NI)	-0.01 (NI)	0 (NI)	NI

3-5	Tetra-n-octylammonium bromide	27.5	60 (I)	0.17 (I)	23 (I)	I	27.5	60 (I)	0.12 (I)	17 (I)	I	27.6	190 (NI)	0.01 (NI)	0 (NI)	NI
3-6	Butanol	27.6	0 (I)	0.27 (I)	49 (I)	I	27.6	0 (I)	0.27 (I)	49 (I)	I	27.6	0 (I)	0.33 (I)	59 (I)	I
3-7	3- (2-Aminoethylamino)propyl] trimethoxysilane	28.1	0 (I)	0.33 (I)	60 (I)	I	27.6	0 (I)	0.33 (I)	60 (I)	I	27.6	0 (I)	0.41 (I)	73 (I)	I
3-8	Sodium dodecyl sulfate	28.4	0 (I)	0.45 (I)	81 (I)	I	27.6	0 (I)	0.45 (I)	81 (I)	I	27.6	0 (I)	0.46 (I)	82 (I)	I
3-9	m-Phenylenediamine	27.0	0 (I)	0.39 (I)	70 (I)	I	27.6	0 (I)	0.39 (I)	70 (I)	I	26.9	10 (I)	0.42 (I)	74 (I)	I
3-10	Tetraethylene glycol	27.8	0 (I)	0.24 (I)	43 (I)	I	27.7	0 (I)	0.24 (I)	43 (I)	I	26.9	20 (I)	0.20 (I)	35 (I)	I
3-30	Imidazole	28.1	90 (I)	0.24 (I)	23 (I)	I	27.8	90 (I)	0.24 (I)	23 (I)	I	27.7	80 (I)	0.31 (I)	33 (I)	I
3-32	Sodium salicylate	27.7	0 (I)	0.54 (I)	43 (I)	I	27.5	0 (I)	0.54 (I)	43 (I)	I	28.0	0 (I)	0.35 (I)	38 (I)	I
3-11	gamma-Butyrolactone	27.5	0 (I)	0.22 (I)	40 (I)	I	27.7	10 (I)	0.23 (I)	42 (I)	I	26.9	0 (I)	0.21 (I)	37 (I)	I
3-12	Methyl acetate	28,4	0 (I)	0.20 (I)	36 (I)	I	27.6	0 (I)	0.18 (I)	32 (I)	I	26.9	0 (I)	0.18 (I)	32 (I)	I
3-13	Myristyl alcohol	27.1	190 (NI)	-0.02 (NI)	0 (NI)	NI	27.3	190 (NI)	-0.05 (NI)	0 (NI)	NI	28.2	190 (NI)	-0.03 (NI)	0 (NI)	NI
3-14	2,6-Dichlorobenzoyl chloride	28.0	190 (NI)	0.00 (NI)	21 (NI)	NI	27.3	110 (I)	0.46 (I)	33 (I)	I	28.2	190 (NI)	-0.07 (NI)	0 (NI)	NI
3-15	Dibenzyl phosphate	28.1	0 (I)	0.51 (I)	71 (I)	I	27.3	0 (I)	0.39 (I)	59 (I)	I	28.2	0 (I)	0.32 (I)	57 (I)	I
3-17	1- (2-Propoxy-1-methylethoxy)-2-propanol	27.8	0 (I)	1.65 (I)	37 (I)	I	27.4	0 (I)	151 (I)	30 (I)	I	28.1	0 (I)	1.57 (I)	31 (I)	I
3-18	Camphene	27.7	160 (I)	0.08 (I)	4 (NI)	I	27.3	190 (NI)	-0.03 (NI)	0 (NI)	NI	28.4	190 (NI)	-0.01 (NI)	0 (NI)	NI
3-19	Ethyl-2-methylacetoacetate	27.2	0 (I)	0.25 (I)	46 (I)	I	27.5	10 (I)	0.19 (I)	34 (I)	I	28.3	0 (I)	0.23 (I)	42 (I)	I
3-20	Propylene glycol propyl ether	28.1	0 (I)	0.23 (I)	42 (I)	I	27.5	0 (I)	0.23 (I)	42 (I)	I	28.3	10 (I)	0.20 (I)	36 (I)	I
3-31	2-Methyl-1-pentanol	27.9	0 (I)	0.42 (I)	75 (I)	I	27.5	0 (I)	0.42 (I)	75 (I)	I	28.0	0 (I)	0.26 (I)	48 (I)	I
3-33	α-Hexylcinnamaldehyde	27.4	190 (NI)	-0.04 (NI)	0 (NI)	NI	28.0	190 (NI)	-0.04 (NI)	0 (NI)	NI	27.7	190 (NI)	-0.03 (NI)	0 (NI)	NI
3-37	Cyclopentanol	27.4	0 (I)	0.28 (I)	51 (I)	I	28.0	0 (I)	0.28 (I)	51 (I)	I	28.0	0 (I)	0.30 (I)	55 (I)	I
3-21	Methyl amyl ketone	27.4	10 (I)	0.10 (I)	20 (I)	I	27.2	10 (I)	0.10 (I)	20 (I)	I	28.3	30 (I)	0.16 (I)	26 (I)	I

3-22	2- (n-Dodecylthio)ethanol	28.7	190 (NI)	-0.05 (NI)	0 (NI)	NI	27.4	190 (NI)	-0.05 (NI)	0 (NI)	NI	28.3	190 (NI)	0.00 (NI)	0 (NI)	NI
3-23	iso-Octylthioglycolate	27.3	190 (NI)	0.00 (NI)	2 (NI)	NI	27.4	190 (NI)	0.00 (NI)	2 (NI)	NI	27.4	190 (NI)	-0.02 (NI)	0 (NI)	NI
3-24	2,4-Difluoronitrobenzene	27.8	30 (I)	0.13 (I)	20 (I)	I	27.4	30 (I)	0.13 (I)	20 (I)	I	27.4	40 (I)	0.11 (I)	18 (I)	I
3-25	tetra-Aminopyrimidine sulfate	28.7	190 (NI)	-0.09 (NI)	0 (NI)	NI	27.2	190 (NI)	-0.09 (NI)	0 (NI)	NI	27.4	190 (NI)	-0.09 (NI)	0 (NI)	NI
3-26	2,4-Pentanediol	27.4	120 (I)	0.08 (I)	7 (I)	I	27.6	120 (I)	0.08 (I)	7 (I)	I	27.4	130 (I)	0.12 (I)	8 (I)	I
3-27	iso-Octyl acrylate	27.9	190 (NI)	0.00 (NI)	0 (NI)	NI	27.7	190 (NI)	0.00 (NI)	0 (NI)	NI	27.7	190 (NI)	-0.01 (NI)	0 (NI)	NI
3-28	Silicon dioxide n-hydrate	27.3	190 (NI)	-0.04 (NI)	0 (NI)	NI	27.8	190 (NI)	-0.04 (NI)	0 (NI)	NI	27.7	190 (NI)	-0.04 (NI)	0 (NI)	NI
3-29	Potassium tetrafluoroborate	27.8	0 (I)	0.45 (I)	13 (I)	I	27.7	0 (I)	0.45 (I)	13 (I)	I	27.7	0 (I)	0.47 (I)	14 (I)	I
3-34	n,n-Dimethylguanidine sulfate	27.8	0 (I)	0.40 (I)	32 (I)	I	27.7	0 (I)	0.40 (I)	32 (I)	I	28.0	0 (I)	0.84 (I)	25 (I)	I
3-35	Toluene	28.0	80 (I)	0.16 (I)	19 (I)	I	27.7	80 (I)	0.16 (I)	19 (I)	I	28.0	30 (I)	0.12 (I)	20 (I)	I
3-36	Gluconolactone	27.2	0 (I)	0.26 (I)	10 (I)	I	27.5	0 (I)	0.26 (I)	10 (I)	I	28.0	0 (I)	0.31 (I)	9 (I)	I

749 * Temperature of the model at the time of exposure to the test chemical solution

750

751 Table 20. Results for Phase III control chemicals

Chemical	FDSC			BRC			Daicel		
	1	2	3	1	2	3	1	2	3
Negative control	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	-
Positive control	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	-
Reference	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	-

752

753 Table 21. Results for Phase III test chemicals

GHS	No.	Test chemical	FDSC	BRC	Daicel	Lead Lab
Cat. 1	3-1	2,5-Dimethyl-2,5-hexanediol	I	I	I	I
	3-2	2-Benzyl-4-chlorophenol	I	I	I	I
	3-3	2,2-Dimethyl butanoic acid	I	I	I	I
	3-4	Captan	NI	NI	NI	NI
	3-5	Tetra-n-octylammonium bromide	I	I	NI	I
	3-6	Butanol	I	I	I	I
	3-7	3- (2-Aminoethylamino)propyl] trimethoxysilane	I	I	I	I
	3-8	Sodium dodecyl sulfate	I	I	I	I
	3-9	m-Phenylenediamine	I	I	I	I
	3-10	Tetraethylene glycol	I	I	I	I
	3-30	Imidazole	I	I	I	I
	3-32	Sodium salicylate	I	I	I	I
Cat. 2A & 2B	3-11	gamma-Butyrolactone	I	I	I	I
	3-12	Methyl acetate	I	I	I	I
	3-13	Myristyl alcohol	NI	NI	NI	NI
	3-14	2,6-Dichlorobenzoyl chloride	NI	I	NI	I
	3-15	Dibenzyl phosphate	I	I	I	I
	3-17	1-(2-Propoxy-1-methylethoxy)-2-propanol	I	I	I	I
	3-18	Camphene	I	NI	NI	I
	3-19	Ethyl-2-methylacetoacetate	I	I	I	I
	3-20	Propylene glycol propyl ether	I	I	I	I
	3-31	2-Methyl-1-pentanol	I	I	I	I
	3-33	α-Hexylcinnamaldehyde	NI	NI	NI	I
	3-37	Cyclopentanol	I	I	I	I

No Category	3-21	Methyl amyl ketone	I	I	I	I
	3-22	2-(n-Dodecylthio)ethanol	NI	NI	NI	NI
	3-23	iso-Octylthioglycolate	NI	NI	NI	NI
	3-24	2,4-Difluoronitrobenzene	I	I	I	I
	3-25	tetra-Aminopyrimidine sulfate	NI	NI	NI	NI
	3-26	2,4-Pentanediol	I	I	I	I
	3-27	iso-Octyl acrylate	NI	NI	NI	NI
	3-28	Silicon dioxide n-hydrate	NI	NI	NI	NI
	3-29	Potassium tetrafluorobroate	I	I	I	I
	3-34	n,n-Dimethylguanidine sulfate	I	I	I	I
	3-35	Toluene	I	I	I	I
	3-36	Gluconolactone	I	I	I	NI

*In-house data from the lead lab was obtained from non-coded chemicals.

Table 22-1. Phase III contingency table used at FDSC and BRC in a bottom-up approach

		Vitrigel-EIT		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	20	4	24
	No Category	7	5	12
Total		27	9	36

Sensitivity: 83% (20/24)

Specificity: 42% (5/12)

Accuracy: 69% (25/36)

Table 22-2. Phase III contingency table used at Daicel in a bottom-up approach

		Vitrigel-EIT		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	18	6	24
	No Category	7	5	12
Total		25	11	36

Sensitivity: 75% (18/24)

Specificity: 42% (5/12)

Accuracy: 64% (23/36)

759

760 Table 22-3. Phase III contingency tables used at the lead lab in bottom-up approach

		Vitrigel-EIT		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	22	2	24
	No Category	6	6	12
Total		28	8	36

Sensitivity: 92% (22/24)
Specificity: 50% (6/12)
Accuracy: 78% (28/36)

761

762 Table 23. Limitations on applicability at a bottom-up approach in phase III

No.	Test chemicals	Rank	Applicability limitation
3-4	Captan	False negatives	Insoluble after 5 m.
3-5	Tetra-n-octylammonium bromide		Insoluble after 5 m.
3-13	Myristyl alcohol		Insoluble after 5 m.
3-14	2,6-Dichlorobenzoyl chloride		pH of 2.5% solution < 5.0
3-18	Camphene		Protocol revised
3-33	α-Hexylcinnamaldehyde		
3-21	Methyl amyl ketone	False positive	
3-24	2,4-Difluoronitrobenzene		
3-26	2,4-Pentanediol		
3-29	Potassium tetrafluoroborate		
3-34	n,n-Dimethylguanidine sulfate		
3-35	Toluene		
3-36	Gluconolactone		pH of 2.5% solution < 5.0 after 10 m.

763

764 **4.2 Quality assurance**

765 All the records (data sheets and record sheets) from the participating laboratories were checked by
766 JaCVAM, As a result, six record sheets were uncompleted. They were the record sheets on the
767 maintenance of measuring instruments, the culture of HCE models, and the preparation and
768 application of test chemicals at phase I and the preparation and application of test chemicals at phase
769 II in BRC, and application of test chemicals at phase I and phase III in Daicel. Although there are
770 these defectiveness records, JaCVAM considered these records had less effects on quality of data in the
771 validation study.

5 Discussion

5.1 Purpose of the Validation

The validation study was conducted to assess the reliability (within- and between-laboratory reproducibility) and relevance (predictive capacity) of the Vitrigel-EIT method with a challenging set of test chemicals for which high quality in vitro and in vivo data are available. Preference should be given the selection of test chemicals that were classified under UN GHS using individual animal. Unfortunately, the VMT is unable to establish a correlation between results obtained using the Vitrigel-EIT method and EPA categories due to a lack of individual animal data. Therefore, results obtained using the Vitrigel-EIT method are correlated with UN GHS categories only. The Vitrigel-EIT method was developed primarily to identify ocular non-irritants in a bottom-up approach. The VMT also undertook an analysis of a top-down approach to identifying UN GHS Category 1 ocular irritants for comparison with the results from a bottom-up approach.

5.2 Transferability

All test chemicals were successfully identified during Phase 0 in conformance with the results from the lead laboratory, and the protocol was then revised from ver. 1.30e to ver. 1.51e. Further revisions were made to eliminate inconsistencies that were identified during Phase I and Phase II testing. The VMT confirmed that these inconsistencies had been resolved, thereby validating transferability of the test method. A history of revisions made to the Vitrigel-EIT protocol during this process is shown in Fig.7. Significant milestones during this process include:

- Changed the positive control from ethanol to benzalkonium chloride
- Adopted ethanol as reference control for checking the quality of the HCE models
- Defined a procedure for dissolving test chemicals in the culture medium (Fig.3)
- Defined a standard ambient temperature for the experiment
- Revised other minor points in the protocol

In order to check of transferability for regulatory use, a representative set of proficiency chemicals address for regulatory acceptance in appendix 8.9.

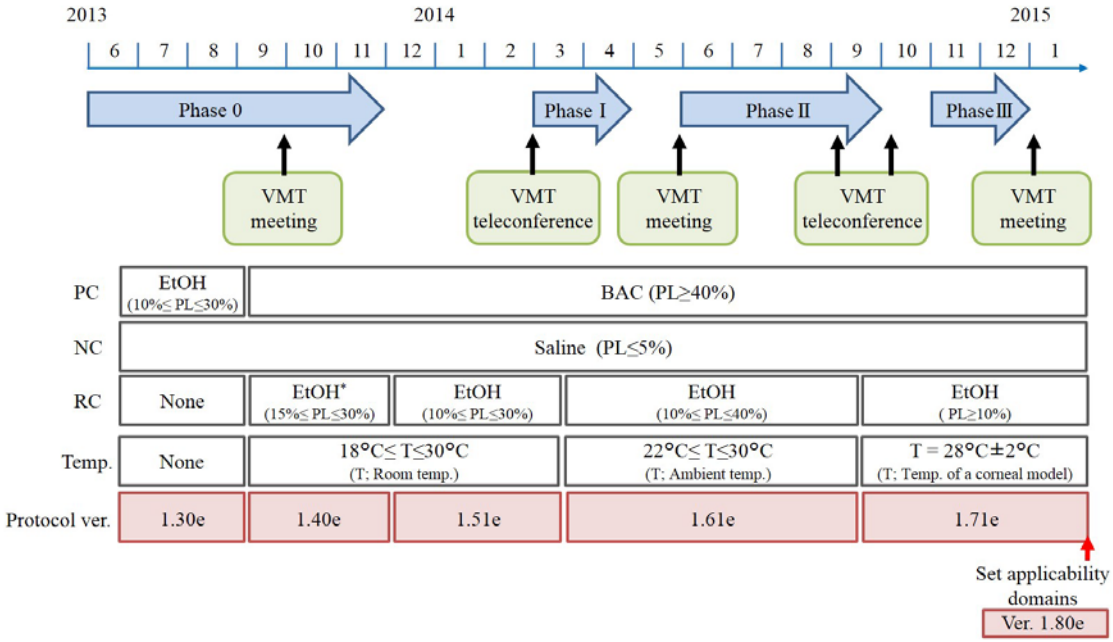

Fig. 7 Vitrigel-EIT protocol revision history

5.3 Within- and between-laboratory reproducibility

The results of Phase I showed that within-laboratory reproducibility was 80% at FDSC, 90% at Daicel, and 100% at BRC, which was sufficient to satisfy the success criteria of 80% as stated in the study plan. The results of Phase II, however, were problematic and not accepted by the VMT, because irrespective of the fact that the results satisfied success criteria for between-laboratory reproducibility, all three participating laboratories obtained a false-negative result for imidazole, a GHS Category 1 irritant. The results of Phase III showed that imidazole was identified correctly by all laboratories and that overall between-laboratory reproducibility was 90%, which was sufficient to satisfy the success criteria of 80% as stated in the study plan. Thus, the VMT concluded that through the process of revising the test protocol, the Vitrigel-EIT method attained an elevated level of between-laboratory reproducibility.

On the other hand, there were nine test chemicals that were used in both Phases II and III. Although there was a significant difference between Phases II and III in the temperature at which measurements were made, results of 7 of these 9 test substances were concordant. Only imidazol and toluene were not concordant between Phases II and III. In order to predict imidazole correctly as an irritant, the

temperature at which measurements were made was revised in the protocol prior to Phase III. Regarding the inconsistencies for toluene in Phase II, Daicel and BRC performed the test at 22 to 25℃ and predicted it to be a non-irritant, although FDSC performed the test at a relatively high 24 to 26℃ and predicted it to be an irritant (Table 18). However, in Phase III, all three laboratories tested at 28±2℃ and predicted toluene to be an irritant. These results suggest that the temperature at which measurements are made is important for achieving reproducible results. Therefore, this data also indicates a high between-laboratory reproducibility for this test method.

5.4 Predictive capacity and relevance

The results obtained from thirty-six test chemicals during Phase III were analyzed to assess their correlation with both existing in vitro and in vivo data and thereby evaluate predictive capacity. The Vitrigel-EIT method was developed primarily to identify ocular non-irritants in a bottom-up approach. Therefore, the test chemicals included UN GHS category 1, 2, 2A and 2B ocular irritants for which in vivo data was available. The Vitrigel-EIT method demonstrated an accuracy of between 64 and 69% (23 to 25/36), a sensitivity of between 75 and 83% (18 to 20/24), and a specificity of 42% (5/12). Sensitivity was low due to six false negatives and specificity (predictive capacity for identifying non-irritants) was low due to seven false positives, as shown in Table 23. The VMT requested the further analysis to determine whether or not predictive capacity could be improved by defining the applicability domain. Ultimately, it was determined that although the results of the validation confirmed an elevated level of reproducibility for this assay, the sample size was insufficient either to evaluate predictive capacity or define a proper applicability domain. Therefore, the VMT recommended that data obtained at the lead laboratory should be used to define an applicability domain suitable for use in a regulatory context.

Total 132 test chemicals were tested at the lead laboratory and were composed of 118 test chemicals (Appendix 8.10 and Appendix 8.11) including 22 used during Phase III and additional 14 chemicals during Phase III. According to the latest version of the protocol, however, the available data limited at lead laboratory were 57 chemicals tested at 28±2°C in 96 chemicals subtracted 36 chemicals for

844 Phase III from the total 132 chemicals. Hence, the predictive capacity was evaluated by the 93
845 results comprise the data for 36 chemicals during Phase III shown in Table 21 and for 57 chemicals
846 obtained at the lead laboratory shown in Appendix 8.8. The test chemicals were selected to ensure that
847 a diverse range of substances were represented, and aspects such as eye-irritant level per UN GHS
848 categories, physical state, chemical class. The 93 test chemicals are composed of 56 liquids and 37
849 solids. Also, their contents are 28 Category 1 chemicals, 32 Category 2, 2A, 2B chemicals, and 33 No
850 Category chemicals by UN GHS classification. There were 36 coded chemicals tested for Phase III
851 and 57 non-coded chemicals were tested at the lead laboratory. These 93 test chemicals were examined
852 by the Vitrigel-EIT method in accordance with the protocol versions described in Chapter 3.1.3.4 and
853 Appendix 8.8. However, the temperature at which all measurements were made during the chemical
854 exposure experiments was strictly controlled at 28±2°C (Table 19 and Appendix 8.8). Thus we
855 consider this data sufficient for assessing the suitability of the Vitrigel-EIT method for use in a bottom-
856 up approach for identifying ocular non-irritants and in a top-down approach for identifying UN GHS
857 Category 1 ocular irritants. In a bottom-up approach, 60 of the test chemicals were classified as irritant
858 and the other 33 as non-irritant, with results for 73 of the 93 test chemicals matching their UN GHS
859 categories. In contrast, 10 of the 60 test chemicals classified as irritants by in vivo data were identified
860 as non-irritants, a false-negative rate of 17%. Additionally, 10 of the 33 test chemicals classified as
861 non-irritants under UN GHS were identified as irritants, a false-positive rate of 30%. Thus, the
862 Vitrigel-EIT method achieved a sensitivity of 83%, a specificity of 70%, and an accuracy of 78%, as
863 shown in Table 24-1. Data from the lead laboratory also demonstrated that predictive capacity could
864 be improved by expanding the sample size. For example, the specificity achieved in Phase III of this
865 validation study was lower than that obtained from the data of 33 non-irritants resulted in a higher
866 specificity. The list of test chemicals that were either false negative or false positives is shown in Table
867 25.
868 On the other hand, analysis per a top-down approach for identifying UN GHS Category 1 ocular
869 irritants was also performed as a part of this validation study, as shown in Tables 24-2. Regarding
870 identifying test chemicals classified as UN GHS Category 1 in a top-down approach, the Vitrigel-EIT

method demonstrated a sensitivity of 89% (25/28), a specificity of 46% (30/65), and an accuracy of 59% (55/93). Specificity is an important criterion in a top-down approach, which means that Vitrigel-EIT method is not well suited for use in a top-down approach to identifying UN GHS Category 1 ocular irritants.

Table 24-1. Contingency table used for 93 test chemicals in a bottom-up approach

		Vitrigel-EIT		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	50	10	60
	No Category	10	23	33
Total		60	33	93

Sensitivity: 83% (50/60)

Specificity: 70% (23/33)

Accuracy: 78% (73/93)

Table 24-2. Contingency table used for 93 test chemicals in a top-down approach

		Vitrigel-EIT		Total
		I	NI	
UN GHS	Cat.1	25	3	28
	Cat.2A, 2B, No Category	35	30	65
Total		60	33	93

Sensitivity: 89% (25/28)

Specificity: 46% (30/65)

Accuracy: 59% (55/93)

889 Table 25. False test chemicals in a bottom-up approach for 93 test chemicals

No.*	Test chemicals	Rank	Applicability limitation
3-4	Captan	False negatives	Insoluble after 5 min.
3-13	Myristyl alcohol		Insoluble after 5 min.
3-14	2,6-Dichlorobenzoyl chloride		pH of 2.5% solution ≤ 5.0
3-18	Camphene		Protocol revised
3-33	α-Hexylcinnamaldehyde		
19	2-Methylbutanoic acid		pH of 2.5% solution ≤ 5.0
24	3,3'-Dithiodipropionic acid		pH of 2.5% solution ≤ 5.0
26	Ethyl 2,6-dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate		pH of 2.5% solution ≤ 5.0
39	6-Methylpurine		
40	Lactic acid		pH of 2.5% solution ≤ 5.0
3-21	Methyl amyl ketone	False positive	
3-24	2,4-Difuroronitrobenzene		
3-26	2,4-Pentanediol		
3-29	Potassium tetrafluoroborate		
3-34	n,n-Dimethylguanidine sulfate		
3-35	Toluene		
3-36	Gluconolactone		pH of 2.5% solution < 5.0 after 10 min.
8	Methyl isobutyl ketone		
28	Triethanolamine		
37	Cyclohexanone		

890 *Each number corresponds to the number in Table 21 and Appendix 8.8.

5.7 Applicability domain

Analysis of the false-negative reactions shows that five of the ten false-negative chemicals were acidic, and the 2.5% solutions used for exposure had a pH level lower than 5, as shown in Table 25. The TEER values of the HCE models after exposures to each of these five acidic test chemicals that yielded false-negatives increased from their initial values. Interestingly, it was reported that isolated rabbit esophageal mucosal epithelium and normal human bronchial epithelial cell layers in culture displayed increased TEER values when exposed to weak acidic solutions (Farré et al., 2008; Oshima et al., 2012). On the other hand, two of the five non-acidic false-negative chemicals were water-insoluble solids that were easily separated from the culture medium at room temperature, as shown in Table 25. Therefore, the lead laboratory added two restrictions to the applicability domain in consideration of above scientific rationales:

- Exclude all test chemicals that have a pH level of 5 or less in solution (affected 11 tested chemicals).
- Exclude all solids that have both a logP value of 2.5 or more and a density of either less than 0.95 g/cm^3 or over 1.10 g/cm^3 (affected 6 test chemicals).

Under this applicability domain, 17 of the original 93 test chemicals were excluded, as shown in Tables 26, which improve sensitivity from 83 to 93%, specificity from 70 to 69%, and accuracy from 78 to 83%, as shown in Table 27.

Of the 44 irritants, one other that yielded a false-negative was 6-methylpurine, a non-acidic, water-soluble powder. The reason for the false-negative judgment is currently under investigation. The classification of the test chemical in vivo was identified as “Study Criteria Not Met” because the study was terminated before 21 days without full reversibility (scores equal to 0) of all endpoints in all animals, in the absence of any other effects driving a Cat 1 classification (Barroso et al, 2017).

Eight of the 36 test chemicals in Phase III are excluded under the new applicability domain:

No. 3-2 2-Benzyl-4-chlorophenol (insoluble)

No. 3-3 2,2-Dimethyl butanoic acid (pH ≤ 5)

No. 3-4 Captan (insoluble)

No. 3-5 tetra-n-Octylammonium bromide (insoluble)

No. 3-13 Myristyl alcohol (insoluble)

No. 3-14 2,6-Dichlorobenzoyl chloride (pH ≤ 5)

No. 3-15 Dibenzyl phosphate (pH ≤ 5)

No. 3-25 tetra-aminopyrimidine sulfate (pH ≤ 5)

After excluding these eight test chemicals, sensitivity improved from between 75 and 83% to between 88 and 94% (15 to 16/17), specificity changed from 42% to 36% (4/11), and accuracy improved from between 64 and 69% to between 68 and 71% (19 to 20/28).

Of the 17 irritants, two others that yielded false-negatives were No. 3-18, camphene, and No. 3-33, alpha-hexylcinnamaldehyde. Camphene is a waxy, water-insoluble solid, and the false-negative was due to the technique used for dissolving, as described in section 4.1.4 Phase III. Alpha-hexylcinnamaldehyde is a water-immiscible liquid and was identified as an irritant by the lead laboratory (Yamaguchi, 2016). The reason for the discordance of the judgment is currently under investigation, although the classification of alpha-hexylcinnamaldehyde in several studies in vivo was reported as NC and 2A or higher (Barroso et al, 2017). In consideration of the Draize eye test Reference Database (DRD; Barroso et al, 2017), additional testing was performed in the lead laboratory using 114 test chemicals selected from the list of DRD (Appendix 8.13, 8.14).

Table 26-1. Limitations on applicability (pH level 5 or less in 2.5% solution) in a bottom-up approach

No.*	Test chemical	GHS category	Vitrigel-EIT results	pH
3-3	2,2-Dimethyl butanoic acid	1		4
3-14	2,6-Dichlorobenzoyl chloride	2A	False negative	3
3-15	Dibenzyl phosphate	2A		3
3-25	tetra-Aminopyrimidine sulfate	NC		3
19	2-Methylbutanoic acid	1	False negative	4
24	3,3'-Dithiodipropionic acid	2B	False negative	4
26	Ethyl 2,6-dichoro-5-fluoro-beta-oxo-3-pyridinepropanoate	2B	False negative	5

27	3-Chloropropionitrile	2B		5
40	Lactic acid	1	False negative	3
49	Citric acid	2A		3
52	Glycolic acid	2		4

*Each number corresponds to the number in Table 21 and Appendix 8.8.

Table 26-2. Limitations on applicability (solid chemicals with a logP value of 2.5 or more and a density under 0.95 g/cm^3 or over 1.10 g/cm^3 in a bottom-up approach.

No.*	Test chemical	GHS category	Vitrigel-EIT results	LogP	Density (g/cm^3)
3-2	2-Benzyl-4-chlorophenol	1		3.60	1.19
3-4	Captan	1	False negative	2.80	1.74
3-5	Tetra-n-octylammonium bromide	1		3.45	0.94
3-13	Myristyl alcohol	2A	False negative	6.03	0.82
22	Acid red 92	2		7.13	2.16
35	Potassium laurate	1		4.57	1.12

*Each number corresponds to the number in Table 21 and Appendix 8.8

Table 27. Contingency tables used for 76 test chemicals within the applicability domain in bottom-up approach.

		Vitrigel-EIT		Total
		I	NI	
UN GHS	Cat.1, 2A, 2B	41	3	44
	Not Classified	10	22	32
Total		51	25	76

Sensitivity: 93% (41/44)
Specificity: 69% (22/32)
Accuracy: 83% (63/76)

5.6 Other analysis

The VMT discussed the use of an area over the curve (or weighted area under the curve: wAUC) of the TEER measurement to obtain high predictive capacity and requested that the biostatisticians develop new prediction algorithm. As a result, a new statistical algorithm was designed and proposed to improve the predictive capacity, particularly in the area of specificity.

The proposed algorithm involved evaluating the eye irritancy of a test chemical using two parameters: (1) the TEER value measured at the final time point (180 seconds) and (2) the decrease in TEER value across the 180-second measurement period. A suitable cut-off value was determined for these two parameters based on the results of Phase III and in reference to the Youden index. The sensitivity, specificity, and accuracy obtained with the proposed algorithm were then compared with those obtained with the original algorithm. Finally, the validity of the proposed algorithm was confirmed using the results obtained from 118 test chemicals by the lead laboratory (Yamaguchi et al., 2016).

Using a cut-off value of 0.15 for the decrease in TEER value across the measurement period yielded a sensitivity of 67%, a specificity of 92%, and an accuracy of 75%. Based on these results, the VMT decided not to accept the new prediction algorithm to analyze data from this validation study.

5.7 Comparison with other alternative to ocular irritation assay

The Vitrigel-EIT method was developed by measuring relative changes in TEER for a period of 180 second after exposure to 30 test chemicals as previously reported (Yamaguchi et al., 2013). It is generally accepted that at least 100 substances should be tested to assess the predictive capacity of a new test method, and to this end, the developers tested a total of 118 test chemicals of various physical and chemical properties (Yamaguchi et al., 2016). The results of this testing showed that the Vitrigel-EIT test method had a predictive capacity that was comparable to other test methods for which OECD test guidelines are currently being developed. For example, the EpiOcular-EIT method demonstrated a sensitivity of 98%, a specificity of 73%, and an accuracy of 85% (Kaluzhny et al., 2011). Used in a bottom–up approach, the short time exposure (STE) test demonstrated a sensitivity of 88%, a specificity of 80%, and an accuracy of 85% (ICCVAM, 2013) and the predictive capacity of the Vitrigel-EIT method is similar with ones of the other methods (the sensitivity of 93%, a specificity of 69%, and an accuracy of 83%) under the applicability domain.

In addition, the vitrigel-EIT method has some advantages in required time, practicality and cost shown in Table 28. Each of these test methods, however, yields some false-negatives or false-positives. Thus, it is important to clarify the mechanism that results in these false-negatives and false-positives,

977 particularly when developing an in vitro test method suitable for use as an alternative to in vivo testing.
978 The VMT has confirmed the applicability domain proposed by the lead laboratory. Meanwhile,
979 scientists at the lead laboratory consider immuno-histology to be a powerful tool for clarifying the
980 mechanism of false-positive reactions, because the culture model can be easily utilized as frozen
981 sections after completing the Vitrigel-EIT.
982
983 Table 28. Comparative table between the Vitrigel-EIT method and other test methods.

Test method	Vitrigel-EIT	STE (TG491)	EpiOcular-EIT (TG492)
Required time (for 24 test)	**6days** for preparing HCE models **2hours** for chemicals exposure experiment	**4days** for preparing SIRC cell monolayer **3hours** for chemical exposure experiment	**1day** for preparing HCE models **9hours** (liquid) or **30hours** (solid) for chemical exposure experiment
Practicality	Easy	Easy	Difficult to remove test chemicals from HCE models
Cost	¥84,000 for ad-MED Vitrigel	Relatively low	¥144,000 for HCE models
Mechanistic relevance	Epithelial barrier function	Cell viability	Cell viability
Limitation of test chemicals	Exclude acidic chemicals and easily separable water-insoluble solids	Exclude highly volatile substances and all solid chemicals other than Surfactants	Colored sample (need additional procedure)

984
985
986

6 Conclusion

This study was performed in the spirit of GLP at three participating laboratories using a total of 42 test chemicals to validate the Vitrigel-EIT method for within- and between-laboratory reproducibility as well as for the capacity to distinguish non-irritants from irritants in a bottom up approach.

The results showed good within-laboratory reproducibility between 80 and 100% as well as an excellent between-laboratory reproducibility of 92% (33/36). Unfortunately, predictive capacity for distinguishing non-irritants from irritants per UN GHS categories in a bottom-up approach was not favorable because of a high incidence of false negatives as high as 17% (10/60). After considerable review of the data, the applicability domain was revised to exclude test chemicals that have a pH level of 5 or less in solution as well as those that are solids and have both a logP value 2.5 or more and a density of either less than 0.95 g/cm^3 or a density of over 1.10 g/cm^3, which improved the false negative rate to 7% (3/44).

From the above described results, the VMT concluded that the Vitrigel-EIT method demonstrated excellent within- and between-laboratory replicability and that, with a carefully defined applicability domain, it is a useful alternative to the Draize test for distinguishing test chemicals that are ocular non-irritants from those that are irritants.

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