BACKGROUND REVIEW DOCUMENT

New Established *In Vitro* Eye Irritation Test Method using Reconstructed Human Corneal Epithelium, LabCyte CORNEA-MODEL24

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LIST OF ACRONYMS AND ABBREVIATIONS

BCOP	Bovine Corneal Opacity and Permeability
BRD	Background Review Document
CAS	Chemical Abstracts Service
CV	Coefficient of variation
D-PBS	Dulbecco's phosphate buffered saline
EBSS	Earle's balanced salt solution
EC	European Commission
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EIT	Eye irritation test
EU	European Union
FBS	Fetal bovine serum
FL	Fluorescein Leakage
GHS	Globally Harmonized System (of Classification and Labeling of Chemicals)
GLP	Good Laboratory Practices
HPLC/UPLC	High performance liquid chromatography/Ultra high performance liquid chromatography
ICE	Isolated Chicken Eye
JaCVAM	Japanese Center for the Validation of Alternative Methods
JSAAE	Japanese Society for Alternatives to Animal Experiments
LabCyte CORNEA-MODEL24 EIT	LabCyte24 EIT
MMAS	Modified maximum average score
MTS	3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
RhCE	Reconstructed human corneal epithelium

SD	Standard deviation
SLS	Sodium Lauryl Sulfate
SOP	Standard operation procedure
STE	Short time exposure assay
TG	Test Guideline
UN	United Nations
VMT	Validation management team
VRM	Validated reference method
WST-1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt
WST-8	Water soluble tetrazolium salt-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
XTT	2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl] -2H-tetrazolium hydroxide

PREFACE

The location, physiological structure and sensitivity of the ocular surface predispose it to exposure from a variety of potentially hazardous environmental conditions and substances on a daily basis. Many different materials and chemicals can result in damage to the cornea that may vary from irritation and inflammation causing mild discomfort to tissue corrosion resulting in irreversible blindness. These include household, industrial, agricultural and military products, cosmetics, toiletries and may even include certain ocular drugs. Eye toxicity tests are therefore required to ensure that the risks associated with products meet suitable safety criteria and are clearly labeled.

For many years, the ocular irritation potential of these substances has mainly been evaluated by the Draize eye test, which was developed in the 1940s (Draize et. al., 1944). The Draize eye test is the eye toxicity test officially accepted in the Organization for Economic Co-operation and Development (OECD) test guidelines (TG) 405 (OECD, 2012a) for regulatory purposes in the classification of eye-irritancy substances. The Draize eye test is mainly based on the scoring of observed macroscopic changes in the rabbit cornea, conjunctiva, and iris. However, it is often criticized for both ethical (animal welfare) and scientific reasons (subjective scoring, low inter-laboratory reproducibility, or sensitivity differences with humans) (Christian et. al., 1996). Therefore, there is a strong need for an *in vitro* evaluation method which can be used to assess eye irritancy.

The public interests in animal alternative tests have increased recently and the development of these tests has become a critical task for the cosmetic industry globally. In addition, the development of alternative methods is accelerating in the world due to new regulations like the banning of cosmetics in animal ocular irritation tests in the EU (Directive 2003/15/EC, 2003). To date, a lot of *ex vivo* or *in vitro* ocular irritancy test methods have been developed as alternative method to the Draize eye test. Regarding those *ex vivo*, the Bovine Corneal Opacity and Permeability (BCOP) test and the Isolated Chicken Eye (ICE) test were found to adequately predict severe irritancy and non-irritancy although they could not identified materials for irritancy, they were adopted as OECD TG 437 and 438 (OECD, 2013a; OECD, 2013b). In addition, concerning those *in vitro*, the fluorescein leakage (FL) test method, short time exposure test using a monolayer culture system, and the eye irritation test (EIT) method using reconstructed human corneal epithelial tissue (RhCE) were adopted as OECD TG 460, 491 and 492 (OECD, 2012b; OECD, 2015c, OECD, 2015a).

The LabCyte CORNEA-MODEL24 EIT (LabCyte24 EIT) that is proposed in this background review document (BRD) is an alternative *in vitro* eye irritation test method using the RhCE tissue

model. The LabCyte CORNEA-MODEL24 displays a high similarity to human corneal epithelium in the histological morphology (Katoh et. al., 2012). A test protocol based on a simplified cytotoxic approach was set up in the same manner as an already the validated skin irritation test method using a reconstructed human epidermal tissue model for OECD TG 439 (OECD, 2010), so as to promptly and easily predict the eye irritation potential of a test substance.

Category classification of ocular irritation by LabCyte24 EIT is determined based on the relative cell viability assessed. A test substance that has a relative viability of 40% or less is categorized as an irritant (the United Nations (UN) Globally Harmonized System (GHS) Category 1, 2A and 2B) and a test substance that has a relative viability greater than 40% is categorized as a non-irritant (UN GHS No Category).

The objective of this BRD is to describe the brief explanation of the feature of the LabCyte 24 EIT, that is the optimization study, several pre-validation studies and also that the current status of the LabCyte24 EIT, including what is known about its accuracy and reliability.

EXECTIVE SUMMARY

This Background Review Document (BRD) reviews available data and information regarding the eye irritation test (EIT) method using LabCyte CORNEA-MODEL24 (LabCyte24 EIT) for identifying ocular irritants. The test method was reviewed for its ability to predict ocular irritant as defined by the United Nations (UN) Globally Harmonized System (GHS) of classification and labeling of chemicals (UN 2003). The objective of this background review document is to describe the brief explanation of the feature of the LabCyte24 EIT, that is the optimization study, several pre-validation studies and also that the current validation status of the LabCyte24 EIT, including what is known about its accuracy and reliability.

Finding *in vitro* eye irritation testing alternatives to animal testing such as the Draize eye test, which uses rabbits, is essential from the standpoint of animal welfare. It has been developed a reconstructed human corneal epithelial (RhCE) tissue model, the LabCyte CORNEA-MODEL24, which has a representative corneal epithelium like structure. Histological analysis of LabCyte CORNEA-MODEL24 shows a complete corneal epithelium containing three major corneal epithelial layers, including a superficial layer, a wing cell layer, and a basal layer. The corneal epithelial marker, Cytokeratin 3, mucins (mucin-1 and mucin-16), cell adhesion molecules (E-cadherin, Claudin-1 and Desmoglein-3) and basement membrane consistent (Laminin 332) were expressed in the appropriate regions, as seen in a human corneal epithelium. In analysis of LabCyte CORNEA-MODEL24 tissue by electron microscope, well developing microvilli was shown in the superficial layer of the LabCyte CORNEA-MODEL24. Therefore the histological structure of the LabCyte CORNEA-MODEL24 is considered that is highly similar to that of a native human corneal epithelium. The LabCyte CORNEA-MODEL24 provides a promising alternative to animal testing, a means to assess corneal irritation.

Protocol optimization was examined in order to establish a new alternative method for eye irritancy evaluation with the LabCyte CORNEA-MODEL24 tissue. A test protocol based on a simplified cytotoxic approach was set up in the same manner as an already validated skin irritation testing method as the Organization for Economic Co-operation and Development (OECD) test guideline (TG) 439 (OECD, 2010), so as to promptly and easily predict the eye-irritation potential of a test chemical. Therefore, to optimize EIT protocol using the LabCyte CORNEA-MODEL24, two important conditions were examined in order to designate exposure periods and post-exposure incubation periods in this optimization study. From the results of the optimization study, the application periods for chemicals were set at 1 minute for Liquid chemicals or 24 hours for Solid chemicals, and the post-exposure incubation periods were set at 24 hours for Liquids or zero for Solids to see below the Section 3-1 in this BRD. Furthermore,

through the ring study (to see below Section 3-2), the study of protocol modification (see below the Section 3-3), the comparison study of between 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Water soluble tetrazolium salt-8 (WST-8; [2-(2-methoxy-4-nitrophenyl) -3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]) assay (see below Section 3-4) and the optimization study of WST-8 assay condition (to see below Section 3-5), the improved LabCyte24 EIT method was finally established.

For the evaluation of predictive performance of the improved LabCyte24 EIT by in-house study, 139 chemicals with wide-range were selected from some published database (ECETOC, 1998; ICCVAM, 2006; OECD, 2015b) and some previous reports (Doucet et. al, 2006; Takahashi et. al., 2008; Takahashi et. al., 2009; Kaluzhny et. al., 2011; Kolle et. al., 2011), and these chemicals were examined to assess the performance of the improved LabCyte COLNEA-MODEL24 EIT. The predictions of the improved LabCyte CORNEA-MODEL24 eye irritation test methods were highly correlated with *in vivo* eye irritation (sensitivity 100%, specificity 73.8%, and accuracy 88.2%).

In order to confirm the availability of LabCyte CORNEA-MODEL24 after shipment to outside of Japan, LabCyte CORNEA-MODEL24 is exported to the laboratory of Konkuk University (Korea) laboratory and the improved LabCyte24 EIT was performed at the laboratory of Konkuk University. From the results of the negative control and the positive control in the laboratory of Konkuk University were adapted to the acceptance criteria in three batches were suggested that the performance of the LabCyte CORNEA-MODEL24 after shipping to outside of Japan is enough usefulness for the eye irritation testing. Furthermore, proficiency chemicals listed in the OECD TG 492 (OECD, 2015a) which was OECD TG for the RhCE EIT method were correctly predicted by improved LabCyte24 EIT in the laboratory of Konkuk University, it was concluded that the improved LabCyte24 EIT is able to perform outside of Japan.

In several developing RhCE EITs, the EIT test method using EpiOcular model has only completed a formal validation study and the EpiOcular EIT was implemented as validated reference method (VRM) into the OECD TG 492 in 2015 (OECD, 2015a). The LabCyte24 EIT procedure is based on the measurement of viable cells and therefore its assay principle is same to that of the EpiOcular EIT which is VRM of OECD TG 492 (OECD, 2015a). The Performance Standard (OECD, 2015b) for the OECD TG 492 is described that cell source, pre-exposure step, and each step of chemical application were appropriately set each condition if necessary. The LabCyte24 EIT has been set each condition of such test component as shown below the section 4. On the other hands, method of cell viability measurement is used MTT assay in the OECD TG 492 (OECD, 2015a), however the LabCyte24 EIT is used WST-8 assay. From MTT assay

and WST-8 assay are commonly one of tetrazolium reduction assay method and therefore it was thought that both assays were functionally similar.

Finally, the validation management team (VMT) of the LabCyte24 EIT judged the improved LabCyte CORNEA-MODEL24 is similar assay method to the RhCE EIT for the OECD TG 492. Therefore, the me-too validation study of LabCyte24 EIT is planned according to the PERFORMANCE STANDARD for the OECD TG 492 in older to assess its reliability (reproducibility within and between laboratories) and its relevance (predictive capacity).

1. INTRODUCTION AND RATIONALE FOR THE PROPOSED USE OF IN VITRO TEST METHODS TO IDENTIFY OCULAR IRRITANTS

1-1. INTRODUCTION

1-1.1. Historical Background of Ocular Irritation Tests and Rationale Their Development

Many chemicals and materials may result in damage to the cornea that may vary from irritation and inflammation causing mild discomfort to tissue corrosion resulting in irreversible blindness. These include household, industrial, agricultural, cosmetics, toiletries products and ocular drugs if incorrectly administered. To reduce the risk of exposure to dangerous substances, all manufactured consumer products and their ingredients should be evaluated their eye irritation potential.

Evaluation methods of eye irritancy are therefore indispensable to ensure that the risks associated with such materials. For many years, the eye irritation potential of these substances has mainly been evaluated by the Draize eye test which has been established by Draize et al (Draize et. al., 1944). The procedure involves the application of 0.1 mL (or 0.1 g Solid) test substance onto the cornea and conjunctival sac of one eye of a conscious rabbit for up to 72 hours while the other eye serves as an untreated control. After assessment of the eye irritation potential according to the Organization for Economic Co-operation and Development (OECD) test guidelines (TG) 405 (OECD, 2012a) which is the TG for the Draize eye test, test substances are classified according to the United Nations (UN) Globally Harmonized System (GHS) (UN, 2009), as Category 1 (severe irritants causing irreversible damage to the eye), Category 2A (moderate irritants having reversible effects on the eyes) or Category 2B (mild ocular irritants). Non-irritating substances are not labelled according to the UN GHS system. The UN GHS is based upon averaged single tissue observations which can account for the reversibility of the observed chemical effects (Eskes et. al., 2005). However, it is often criticized for both ethical (animal welfare) and scientific reasons (subjective scoring, low inter-laboratory reproducibility, or sensitivity differences with humans) (Christian et. al., 1996). Therefore, there is a strong need for alternative method which can be used to assess eye irritancy.

To date, numbers of *ex vivo* or *in vitro* assay methods have been developed as alternative methods to the Draize eye test.

Above all, the Bovine Corneal Opacity and Permeability (BCOP) test and the Isolated Chicken Eye (ICE) test were found to adequately predict severe irritancy although they were not

recommended for the identification of materials not classified for irritation, they were adopted as OECD TG 437 and 438 (OECD, 2009a; OECD, 2009b). Furthermore, their OECD TGs extending the applicability will be revised, which are accepted for the identification of non-irritant chemicals in the field of eye irritation (OECD, 2013a; OECD, 2013b). However, these test methods still have many shortcomings such as use of non-human tissue and unsatisfactory predictive capacity for human ocular irritation, reflecting a considerable demand for novel and more human-like alternative tests.

On the other hands, as *in vitro* eye irritation test, the fluorescein leakage (FL) test method was also adopted as an OECD test guideline (OECD, 2012).

Furthermore, a rabbit corneal epithelial cell line has been made available as a useful alternative when evaluated by a cytotoxicity assay (Hagino et. al.. 2008; Takahashi et. al., 2008; Takahashi et. al. 2009). In such proposed test methods, the short time exposure (STE) assay using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay has completed a formal validation study and the STE assay was implemented into the OECD TG 491 in 2015 (OECD, 2015c).

Although these assays are easy to perform economically, there are some disadvantages, in that it is often not possible to assess accurately any insoluble or volatile test material because they must be dissolved in the test solvent.

To solve their problems, there have been increasing expectations for the development of an eye irritation test (EIT) method using a reconstructed human corneal epithelial (RhCE) tissue model, fabricated with tissue engineering techniques. Since eye irritants affect corneal epithelium directly, the disruption of structural integrity, and decreased viability of the cornea are important indices of eye irritation. Especially, the RhCE models well recapitulate the stratified and differentiated human corneal epithelium and are capable of evaluating neat test materials directly without using any test solvent (Lotz et. al., 2016).

EITs using four RhCE models have been reported up to now; EpiOcular (MatTek, MA, USA) model to reconstruct using normal human epidermal keratinocyte (Stern et al., 1998; Kaluzhny et. al., 2011; Pfannenbecker et. al., 2013), SkinEthic HCE (SkinEthic, France) model to reconstruct using immortalized human corneal epithelial cell line (Van Goethem et. al., 2006, Cotovio et. al., 2010; Alépée et. al., 2013), LabCyte CORNEA-MODEL24 (Japan Tissue Engineering Co., Ltd (J-TEC), Japan) model to reconstruct using normal human corneal epithelial cells (Katoh et. al., 2012; Katoh et. al., 2013) and MCTT-HCE (Modern Cell & Tissue Technologies, Korea) model to reconstruct using normal human corneal epithelial cells which is same to LabCyte CORNEA-MODEL24 tissue (Jung et. al., 2011; Jang et. al., 2015). All EITs using four RhCE models are based on the cell viability measurement as endpoint of eye

irritancy prediction.

In these EITs using RhCE models, the EIT test method using EpiOcular model has only completed a formal validation study and the EpiOcular EIT was implemented as validated reference method (VRM) into the OECD TG 492 in 2015 (OECD, 2015a).

1.1.2. The Proposed EIT Method using LabCyte CORNEA-MODEL24

The LabCyte CORNEA-MODEL24 which is proposed in this background review document (BRD) is generated from corneal epithelial cells originating from normal human corneal epithelial tissue. The culture of normal human corneal epithelial cells on a membrane insert results in three-dimensional RhCE tissue equivalent similar to the *in vivo* human corneal epithelium with a verifiable expression of corneal epithelium marker (Katoh et. al., 2012). The LabCyte CORNEA-MODEL24 tissue construct consists of at least three viable layers which is composed superficial cell layer, wing cell layer and basal cell layer and a no cornified surface, showing a corneal epithelium like structure analogous to that found in human eye (Katoh et. al., 2012; Poumay et. al., 2004).

In order to develop an ocular irritancy test method using an RhCE model, it was made to refer the strategy of a validated skin irritation test method using a reconstructed human epidermal tissue (OECD, 2010). In the LabCyte CORNEA-MODEL24 EIT (LabCyte24 EIT), the test material is applied topically to the RhCE tissue surface and then tissue viability is measured following exposure and a post-exposure incubation periods, that its strategy of its test procedure is equal to that of skin irritation test method for the OECD TG 439 (OECD, 2010).

The RhCE tissue viability in the LabCyte24 EIT is measured by enzymatic conversion of the vital dye Water Soluble Tetrazolium Salt-8 (WST-8; [2-(2-methoxy -4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]) by the viable cells of the tissue into an orange-color WST-8 formazan salt that is quantitatively measured assay solution directly. A cut-off value of 40% viability of the negative control value was considered and used to classify test chemicals as eye irritancy (UN GHS Category 1, 2A and 2B) or eye no irritancy (UN GHS No Category).

Also the assay principles of the LabCyte24 EIT is considered to be similar to the EpiOcular ETI which is VRM of RhCE EIT for OECD TG492 (OECD, 2015a).

1-2. Scientific Basis for the Proposed Test Method

1.2.1. Propose and Mechanism Basis of LabCyte24 EIT

Chemical-induced serious eye damage/eye irritation, manifested *in vivo* mainly by corneal opacity, iritis, conjunctival redness and/or conjunctival chemosis, is the result of a cascade of events beginning with penetration of the chemical through the cornea and/or conjunctiva and production of damage to the cells. Cell damage can occur by several modes of action, including cell membrane lysis, coagulation of macromolecules, saponification of lipids and alkylation or other covalent interactions with macromolecules (Scott et. al., 2010).

A recent report showed the general concept that slight eye irritants affect only the superficial corneal epithelium, the mild and moderate irritants (UN GHS Category 2A and 2B) affect principally the epithelium and superficial stroma, and the severe irritants (UN GHS Category 1) act through to deeper parts of the stroma, potentially as far as full stromal depth (Maurer et. al., 2002). Since the LabCyte CORNEA-MODEL24 reconstructs only the corneal epithelium, it is thought that the toxicity at all layers of the corneal epithelium can be distinguished from toxicity only at the superficial layer of tissue in the LabCyte24 EIT. Therefore, it is suggested that the LabCyte24 EIT might be able to distinguish between no or slight eye irritants (UN GHS No Category) and mild to severe eye irritants (UN GHS Category 1, 2A and 2B).

The relative cell viability of the treated tissues at the end of test chemicals exposure and then post-exposure periods is measured using WST-8 assay. Chemicals not requiring classification and labelling according to UN GHS Category are identified as those that do not decrease tissue viability below a defined threshold (i.e., tissue viability > 40%, for UN GHS No Category).

1.2.2. Similarities and Differences of Modes of Action of Between the LabCyte24 EIT and the Draize Eye Test

1-2.2.1. The Draize Eye Test

For many years, the ocular irritation potential of chemicals mostly has been evaluated by the Draize eye test. This test method involves instillation of the test substance into the lower conjunctival sac of the rabbit eye, and evaluates the cornea, the iris, and the conjunctiva for adverse effects after exposure to the potential irritant.

The cornea is evaluated both for the degree of corneal opacity and the area of the cornea in which opacity is involved.

The iris is assessed for inflammation, iridal folds, congestion, swelling, circumacorneal injection, reaction to light, hemorrhage, and gross destruction.

The conjunctiva is evaluated for the degree of redness, chemosis (swelling), and discharge (Draize et. al. 1944).

1-2.2.2. Comparison of the LabCyte24 EIT with Draize Eye Test

In the LabCyte24 EIT, cytotoxicity is determined by the viability of the RhCE tissues. The tissue viability is measured by WST-8 assay method. While these *in vitro* toxicity measurements using the RhCE tissue are correlated with *in vivo* ocular irritation corneal effects, they represent only one aspect of the overall complex response of the eye to irritants, which involves other tissues such as the iris and conjunctiva.

In contrast, the *in vivo* rabbit eye test involves a qualitative visual evaluation of the severity of adverse effects on the cornea, the iris, and the conjunctiva, as well as the reversibility of any ocular effects detected at selected intervals up to 21 days after exposure. In the LabCyte24 EIT, a test substance is exposed directly to cells for just 1 minute for Liquid chemicals and 24 hours for Solid chemicals, and then rinsed off. And then blotted tissues were post-exposure incubated for 24 hours for only Liquid chemicals. In the *in vivo* rabbit eye test, test substances are applied to the conjunctival sac. Because the rabbit eye can blink and/or tear, exposure of the test substance to the cornea will be affected by these factors in terms of coverage or duration. The production of tear film for the protection of eye tissue is also not present in the LabCyte CORNEA-MODEL24. When compared with an i*n vivo* rabbit eye test, application of a test substance in the absence of this protective barrier might be expected to cause an increase in false positive outcomes. On the other hands, in some test substances (e.g., Solids), blinking can also induce mechanical damage *in vivo*, contributing to a higher degree of irritation. However, this protective mechanism for the eye are absent in the LabCyte24 EIT.

1.2.3. Applicability and Limitations of LabCyte24 EIT

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

required.

Other limitation of this assay method is a possible interference of the test chemical with the WST-8 endpoint. A colored test chemical or one that directly reduces WST-8 (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the WST-8 endpoint. However, these test chemical are a problem only if at the time of the WST-8 test sufficient amounts of the test chemical are still present on (or in) the tissues. In case of this unlikely event, the (true) metabolic WST-8 reduction and the contribution by a colored test material or (false) direct WST-8 reduction by the test material can be quantified using freeze-killed RhCE tissues.

LabCyte24 EIT is applicable to substances and mixtures, and to solids, liquids, semi-solids and waxes as same applicability to OECD TG 492 VRM (OECD, 2015a). The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required.

1-3. Regulatory Rationale and Applicability

1-3.1. Current Regulatory Testing Requirements

In recent years, several regulations and regulatory agencies have contributed to a greater emphasis on alternative animal testing for ocular irritation (7th amendment to the Cosmetic Directive [Directive 2003/15/EC, 2003], Registration Evaluation Authorization and Restriction of Chemicals [REACH]).

For the ocular irritation animal alternative test, it may be unlikely to completely replace the Draize test by a single *in vitro* test because the Draize eye test evaluates a range of criteria for injury and inflammation to the eye. The tiered approach of several *in vitro* assays combined was proposed in order to estimate the irritation potential for a wide range of chemical classes; Hayashi et. al., 2012a; Hayashi et. al, 2012b).

The LabCyte24 EIT is a cytotoxicity test involves exposing to the RhCE tissue. Furthermore, to apply neat testing material on the RhCE tissue directly in spite of physical state of test material, it's applicable at the wide ranges of test chemicals in the LabCyte24 EIT. For these reasons, the LabCyte24 EIT method could be predict the eye irritancy of wide range chemical to consider the bottom-up assay according to the tiered approach to establishing an ocular

irritation animal alternative testing paradigm.

1-3.2. Intended Regulatory Use(s)

The RhCE EIT test methods such as the LabCyte24 EIT have been proposed for identifying of ocular irritancy (e.g., Category 1 or Category 2 per the GHS classification system [UN 2003]) in the accepted the OECD TG 492 (OECD, 2015a).

1-3.3. The Similarities and Differences in the Endpoint Measured in the Proposed Test and Currently Used In Vivo Reference Test

As mentioned in Section 1.1.2, this test method was based on the cytotoxicity of the RhCE tissue because the corneal tissue is one of the main targets during accidental eye exposures, and damage to the corneal tissues can result in visual impairment or loss.

In the LabCyte24 EIT, cytotoxicity is determined by the viability of the RhCE tissues. The viability is measured by WST-8 assay method. While these *in vitro* toxicity measurements using the cultured cell line are correlated with *in vivo* ocular irritation corneal effects, they represent only one aspect of the overall complex response of the eye to irritants, which involves other tissues such as the iris and conjunctiva.

1-3.4. How the proposed test fits into the overall strategy of hazard or safety

As mentioned in Section 1.3.1, for the ocular irritation animal alternative test, it may be unlikely to completely replace the Draize eye test by a single *in vitro* test because the Draize eye test evaluates a range of criteria for injury and inflammation to the eye. The tiered approach combined several *in vitro* assays was proposed in order to estimate the irritation potential for a wide range of chemical classes (Hagino et. al., 2008; McNamee et. al., 2009; Scott et. al., 2010; Hayashi et. al., 2012a; Hayashi et. al, 2012b).

The LabCyte24 EIT is being considered for use in identification ocular not irritancy (UN GHS No category) from ocular irritancy (UN GHS category 1 or category 2). For these reasons, the LabCyte24 EIT could be considered a bottom up assay in the tiered approach to establishing an ocular irritation animal alternative testing paradigm.

1-4. Objection of This Background Review Document

The objective of this BRD is to describe the brief explanation of the feature of the LabCyte24 EIT, that is the optimization study, several pre-validation studies and also that the current status of the LabCyte24 EIT, including what is known about its accuracy and reliability.

2. DESCRIPTION OF LabCyte CORNEA-MODEL24

2-1. Condition of the Functional Reconstructed human Corneal Epithelial Tissue Model, LabCyte CORNEA-MODEL24

LabCyte CORNEA-MODEL24 is a new, commercially available reconstructed human cultured corneal epithelial model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human corneal epithelial cells whose biological origin is human eye. In order to expand the human corneal epithelial cells while maintaining their phenotype, they are cultured with 3T3-J2 cells as a feeder layer (Green, 1978; Rheinwald et. al., 1975). Reconstruction of human cultured corneal epithelial tissue is achieved by cultivating proliferating corneal epithelial cells on an inert filter substrate (surface 0.3 cm^2) at the air-liquid interface for 13 days, with an optimized medium containing 5% fetal bovine serum. The result is a multilayer structure consisting of a fully differentiated corneal epithelium with features of the normal human corneal epithelial tissues, consisting of superficial layer, wing cell layer and basal layer (Fig.2-1). The LabCyte CORNEA-MODEL24 is embedded in an agarose gel containing a nutrient solution and shipped in 24-well plates at around 18°C.

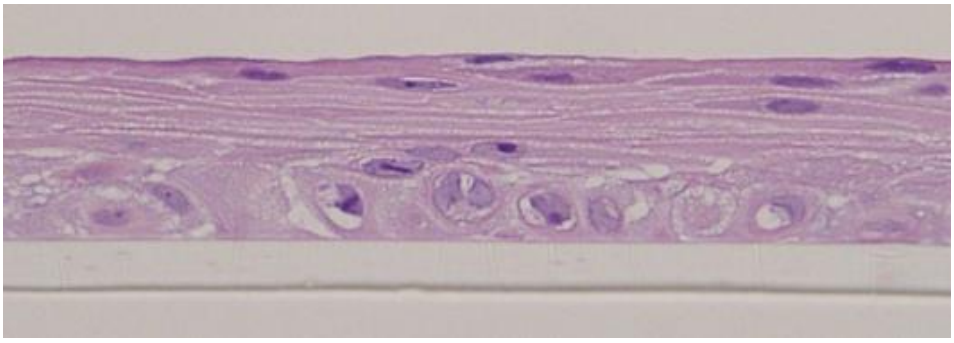


Fig.2-1. Histological cross-sectional views of the LabCyte CORNEA-MODEL24 with H&E staining (original magnification: ×200).

Cytekeratin-3 is a specific marker of corneal epithelium, and it was expressed in all layers of the LabCyte CORNEA-MODEL24 (Fig. 2-2(A)). Mucin-1 and mucin-16 that composed the transmembrane glycocalyx in the surface of the corneal epithelium were well expressed in the superficial layer of the LabCyte CORNEA-MODEL24 (Fig. 2-2(B)). Cells in the corneal epithelium are connected by desmosomes, tight junctions and adherence junctions. Claudin-1

which is a marker of a tight junction, desmograin-3 which is a marker of desmosome and E-cadherin which is a marker of an adherence junction are localized in the interface between cells at all cell layers, including the superficial layers (Fig. 2-2(C)). Laminin is an important constituent in the basement membrane at the basal corneal epithelium junction. It was expressed continuously in basal cells of the LabCyte CORNEA-MODEL24 basal layer (Fig. 2-2(C)).

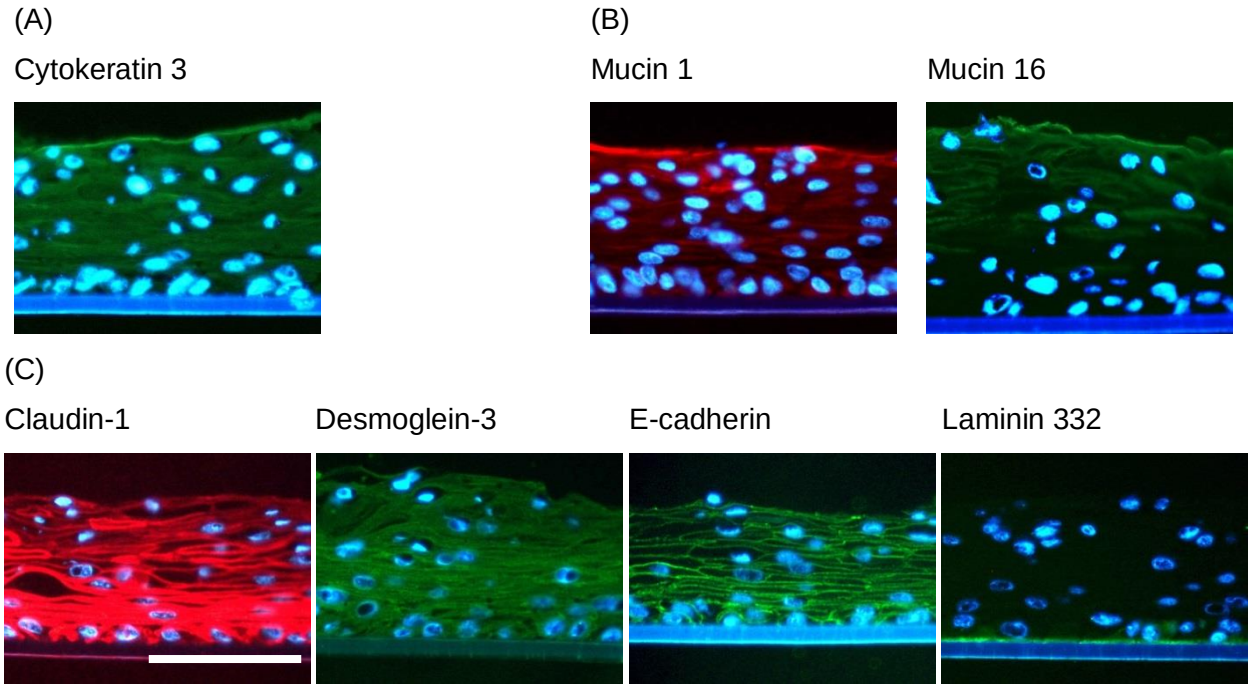


Fig.2-2. Histological cross-sectional views of the LabCyte CORNEA-MODEL24 with immuno-staining:

(a) Immunohistochemical analysis for cytekeratin-3, which is an epithelial marker.

(b) Immunohistochemical analysis for mucin 1 and mucin 16, which are mucous components of superficial cell layers.

(c) Immunohistochemical analysis for Claudin-1, Desmoglein-3, and E-cadherin, which are cell adhesion molecules, and for laminin-332 which is a component of the basement membrane.

(A) Superficial layer

(B) Wing cell layer

(C) Basal layer

Fig.2-3. Transmission electron micrographs of the LabCyte CORNEA-MODEL24.

(A) Superficial cell layer. Note microvilli-like structure (arrows).

(B) Wing cell layer. Note desmosome (dotted circle line).

(C) Basal cell layer. Note hemidesmosome (arrows).

The specific corneal differentiation markers, cell adhesion molecules and basement membrane constituents are expressed in the appropriate regions as seen in a human corneal epithelium.

The basement membrane is smooth like that of human corneal tissues. In the fully developed basement membrane zone, hemidesmosomes are quite numerous along the basal cell layer (Fig. 2-3(A)). The wing cell layer and superficial cell layer of the LabCyte CORNEA-MODEL24 are composed of round and irregular cells connected by highly developed desmosomes (Fig 2-3(B)). Furthermore, the microvilli-like structure is well formed on the surface of the superficial layer in the LabCyte CORNEA-MODEL24 (Fig 2-3(C)). In conclusion, the LabCyte CORNEA-MODEL24, a reconstructed human corneal epithelial model, reproduces many of the characteristics of the native human corneal tissue. Therefore, it provides a morphologically relevant means to assess eye irritation, percutaneous absorption, and other ocular-related research as an alternative to animal testing.

Their histological feature of LabCyte CORNEA-MODEL24 adapts the general condition of the new RhCE for EIT indicated in the OECD TG 492.

Their histological evaluation was already published in our scientific report (Katoh et. al., 2013).

2-2. Quality Control for LabCyte CORNEA-MODEL24

2-2.1. Quality Control Procedures for LabCyte CORNEA-MODEL24

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

The product is released following stringent quality control procedures.

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

1) Tissue viability
2) Barrier function
3) Morphology

2-2.1.1. Tissue Viability

2-2.1.1a. MTT Assay

Three LabCyte CORNEA-MODEL24 tissues are subjected to an MTT assay as follows. Tissues are put in the wells of 24-well plates containing 0.5 mL of MTT medium (0.5 mg/mL; Dojindo Co., Kumamoto, Japan) and are incubated for 3 hours (37°C, 5% CO_2, humidified atmosphere). Formazan produced in the tissues is extracted with isopropanol (300 µL) and the optical density (OD) of the extract (200 µL) is measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.

The mean of the OD values indicates tissue viability for each LabCyte CORNEA-MODEL24 batch.

QC acceptance criteria: $2.5 \geq OD \geq 0.8$

Since the start of the commercial release of the product until December 2015, no batch has been outside the QC acceptance criteria.

2-2.1.1b. WST-8 Assay (Collection as Reference Data not to Control Release Criteria)

Three LabCyte CORNEA-MODEL24 tissues are subjected to a WST-8 assay as follows. Tissues are put in the wells of 24-well plates containing 0.3 mL of WST-8 medium {1:10 dilution

of Cell counting kit-8 (Dojindo Co., Japan): Earle's balanced salt solution (EBSS; Sigma-Aldrich, MO, USA)} and are incubated for 4 hours (37°C, 5% CO_2, humidified atmosphere). The OD of culture supernatant (200 µL) is measured at 450 nm and at 650 nm as a reference absorbance, with WST-8 medium as a blank.

The mean of the OD values indicates tissue viability for each LabCyte CORNEA-MODEL24 batch.

2-2.1.2. Barrier Function: 50% Inhibitory Concentration (IC_{50}) Assay

To evaluate whether the LabCyte CORNEA-MODEL24 tissue resists the rapid penetration of the cytotoxic marker chemical sodium lauryl sulphate (SLS), the viability of the corneal epithelial tissue is estimated in terms of the half maximal inhibitory concentration of 50% (IC50). Various concentrations (0.1, 0.2, 0.3, and 0.4 %(w/v)) of SLS (50 µL) are applied to the LabCyte CORNEA-MODEL24, and cell viability was measured after 1 hour using an MTT assay. All experiments are performed in triplicate. The acceptable range for the LabCyte CORNEA-MODEL24 is shown in Table 2-1.

Table 2-1. QC acceptable range of barrier function.

	Lower limit	Mean	Upper limit
LabCyte CORNEA-MODEL24 IC_{50}(1 hour SLS)	0.10 (w/v)%	0.24 (w/v)%	0.40 (w/v)%

Since the start of the commercial release of the product until December 2015, no batch has been outside the QC acceptance criteria.

2-2.1.3. Morphology

A piece of LabCyte CORNEA-MODEL24 is fixed with 4% parafolmaldehyde and 2% sucrose in Dulbecco's Phosphate Buffered Saline (D-PBS, Invitrogen, CA, USA) for more than three hours and processed for embedding in paraffin. Five-micrometer vertical sections are cut and stained with hematoxylin and eosin for light-microscopic examination.

QC acceptance criteria: Confirmation of the formation of multilayered corneal epithelium-like tissue.

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

2-2.2. Batch Control Information for LabCyte CORNEA-MODEL24

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

Table 2-2. Batch information for LabCyte CORNEA-MODEL24

Year	Month	Tissue viability (Mean OD)		Barrier function
		MTT (A570/650)	WST-8 (A450/650)	Mean (%)
2010	February	1.17		0.28
	March	1.44		0.25
	April	1.23		0.28
	May	1.24		0.28
	June	1.60		0.29
	July	1.48		0.24
	September	1.74		0.25
	October	1.63		0.28
	November	1.52		0.21
	December	1.47		0.25
2011	January	1.40		0.20
	February	1.46		0.28
	March	1.48		0.28
	April	1.44		0.20
	May	1.40		0.22
	June	1.29		0.19

	July		1.24	0.19
	August		1.34	0.19
	September		1.23	0.26
	October		1.20	0.21
	November	FBS lot changed	1.28	0.20
	December		1.25	0.19
2012	January		1.22	0.19
	February		1.17	0.21
	March		1.23	0.25
	April		1.16	0.22
	May		1.18	0.19
	June		1.04	0.22
	July		1.11	0.19
	August		1.14	0.23
	September		1.33	0.27
	October		1.38	0.22
	November		1.48	0.19
	December		1.44	0.26
2013	January		1.47	0.20
	February		1.37	0.24
	March		1.17	0.25
	April		1.18	0.23
	May		1.16	0.21
	June		1.02	0.23
	July		1.29	0.23
	August		1.27	0.18
	September		1.29	0.20
	October		1.23	0.24
	November		1.29	0.19
	December		1.20	0.20
2014	January		1.30	0.25
	February		1.14	0.26
	March		1.35	0.25
	April	FBS lot changed	1.35	0.22

	May	1.25		0.27
	June	1.18		0.26
	July	1.34		0.31
	August	1.22		0.27
	September	1.25		0.25
	October	1.13		0.29
	November	1.11		0.24
	December	1.14		0.25
2015	January	1.12	1.02	0.26
	February	1.18	1.06	0.24
	March	1.15	1.13	0.22
	April	1.07	1.02	0.24
	May	1.16	1.00	0.26
	June	1.06	0.92	0.24
	July	1.06	1.13	0.26
	August	0.99	1.03	0.28
	September	1.02	1.03	0.22
	October	1.23	0.87	0.25
	November	0.92	0.84	0.25
	December	1.04	0.99	0.24
	Mean±SD	1.26±0.16	1.00±0.09	0.24±0.03
	Coefficient Variation	12.7%	9.0%	13.4%

As shown in Table 2-2, the mean±SD of tissue viability (MTT assay / WST-8 assay) and barrier function (IC_{50}) from the evaluation of continuous batches of LabCyte CORNEA-MODEL24 were 1.26 ± 0.16% (MTT assay), 1.00 ± 0.09% (WST-8 assay) and 0.24 ± 0.03%, respectively. The tissue viability and barrier function of LabCyte CORNEA-MODEL24 tissue have remained constant, indicating reproducibility [low coefficient variation (CV): 12.7% (MTT assay), 9.0% (WST-8 assay) and 13.4%, respectively] and monthly consistency is high.

Note

FBS lot used LabCyte CORNEA-MODEL24 cultivation had been changed twice (at November, 2011 and at April, 2014). Performance of FBS lot has been checked before changing lot using

and only passed FBS lot was accepted for production of LabCyte CONREA-MODEL24. Each LabCyte CORNEA-MODEL24 batch between acceptable QC range have been consistently produced using all lot of FBS.

3. OPTIMIZATION AND IMPROVMENT STUDY OF THE LabCyte24 EIT

3-1. Optimization of Suitable Time Periods for Test Chemicals Combining Sample Exposure and Post-exposure Incubation

3-1.1. Purpose of This Study

For the confirmation of suitable LabCyte24 EIT protocol, two important conditions were examined in order to designate exposure periods and post-exposure incubation periods for both Liquid chemicals and Solid chemicals, respectively.

3-1.2. Optimization of Suitable Exposure and Post-exposure Incubation Periods for the Liquid Test Chemicals

3-1.2.1. Study Conditions

This study condition was summarized followings.

Table 3-1. The test chemical set for initially optimization of the LabCyte24 EIT

Name	CAS number[1)]	*In vivo* class[2)]	Physical state
3-Methoxy-1,2-propanediol	623-39-2	NC	Liquid
Polyethylene glycol 400	25322-68-3	NC	Liquid
Glycerol	56-81-5	NC	Liquid
Methyl cyclopentane	96-37-7	NC	Liquid
Tween 20	9005-63-5	NC	Liquid
2-Methyl -1-pentanol	105-30-6	2	Liquid
Triton X-100 (5%)	9002-93-1	2	Liquid
1-Octanol	111-87-5	2	Liquid
2-Ethyl-1-hexanol	103-76-7	2	Liquid
n-Hexanol	111-27-3	2	Liquid

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

2) *In vivo* class was referred from the UN GHS classification.
NC: No category (no eye irritant); 2: Category 2 (eye irritant) 2A: 1: Category 1 (eye corrosion)

In order to determine optimal exposure periods for the Liquid chemicals, tissues that applied

Liquid chemicals (50 µL) were incubated for varying time periods (1, 3, 5, 10 and 20 minutes). After exposure, each tissue was rinsed with D-PBS, using a washing bottle to remove any remaining test chemical from the tissue surface. The blotted tissues were then transferred to new wells on 24-well plates containing 500 µL of fresh assay medium. In order to determine optimal post-exposure incubation periods, the blotted tissues were post-exposure incubated for various time periods (0, 2 and 24 hours) under the standard cultivation conditions.

After the post-exposure incubation periods, blotted tissues were transferred to new wells on 24-well plates containing 300 µL of freshly prepared WST-8 solution (1:10 dilution of Cell Counting Kit-8 with D-PBS) for a WST-8 assay. Tissues were incubated for 5 hours under the standard cultivation conditions. Subsequently, 200 µL of culture supernatant were transferred to a 96-well microtiter plate. The OD value of culture supernatant was measured at 450 nm and at 650 nm as a reference absorbance, with WST-8 solution as a blank.

The cell viability was equal to or lower than 50%, the chemical was judged to be UN GHS category 1 or 2, otherwise it was considered GHS No category.

Ten Liquid test chemicals (initially optimization set) as shown in Table 3-1 was used this optimization study.

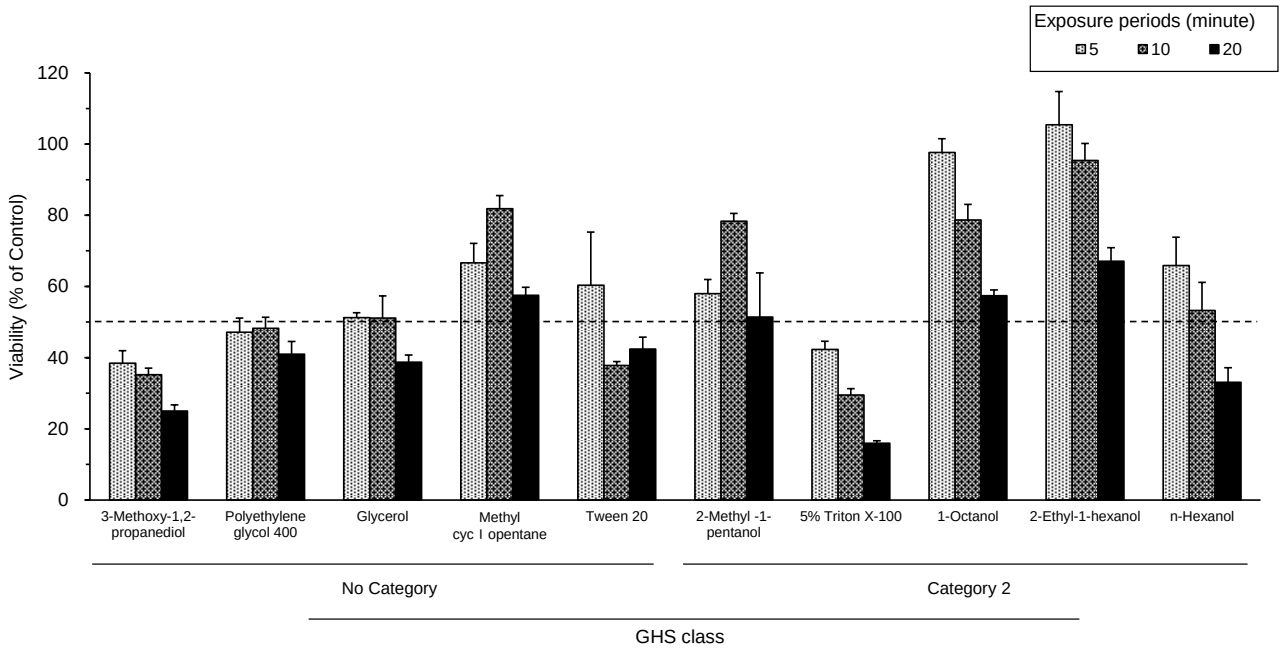
3-1.2.2. Results of Optimization Study for Liquid Chemicals

Ten test chemicals (5 No Category: no irritancy, and 5 Category 2: irritant, in the GHS classification) were selected for the optimization experiments.

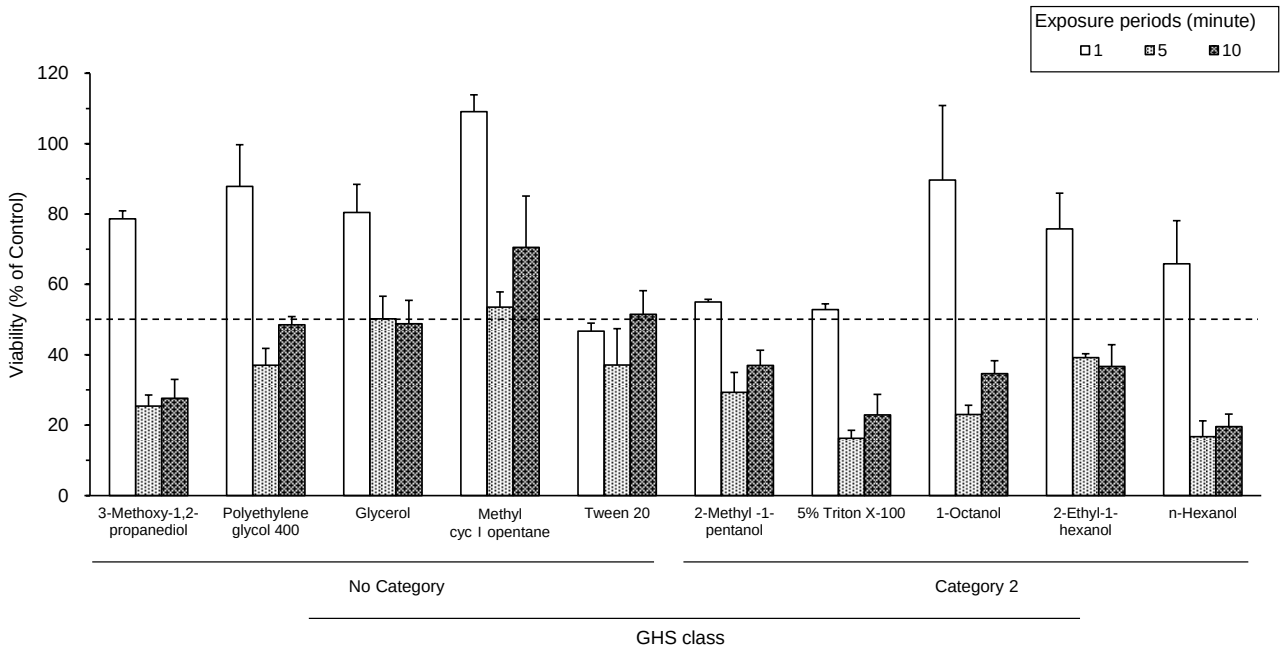
When no post-exposure incubation period was set both false-positive predictions for three chemicals and false-negative predictions for four chemicals were observed in 5 minutes exposure period (Fig. 3-1(A)). Even if exposure periods were gradually prolonged to 20 minutes, the number of chemicals showing a false-positive increased while that of false negative chemicals does not decrease (Fig 3-1(A)), so that, in the end, the correlation with the GHS class was not improved.

When the post-exposure incubation periods were set at 2 hours, four chemicals showing irritant *in vivo* had shown a false negative for a 1 minute exposure period and their sensitivity result was low (Fig 3-1(B)). On the other hand, in the case of exposure periods that were 5 minute or longer, 3 of 5 *in vivo* non-irritant chemicals showed a false-positive, though there was no chemical that showed a false negative (Fig. 3-1(B)), and therefore the correlation with the in vivo class was low during exposure periods.

(A)


Exposure periods (minute)
5
10
20
Viability (% of Control)
120
100
80
60
40
20
0
3-Methoxy-1,2-propanediol
Polyethylene glycol 400
Glycerol
Methyl cyc l opentane
Tween 20
2-Methyl -1-pentanol
5% Triton X-100
1-Octanol
2-Ethyl-1-hexanol
n-Hexanol
No Category
Category 2
GHS class

(B)


Exposure periods (minute)
1
5
10
Viability (% of Control)
120
100
80
60
40
20
0
3-Methoxy-1,2-propanediol
Polyethylene glycol 400
Glycerol
Methyl cyc l opentane
Tween 20
2-Methyl -1-pentanol
5% Triton X-100
1-Octanol
2-Ethyl-1-hexanol
n-Hexanol
No Category
Category 2
GHS class

(Continue)

(C)

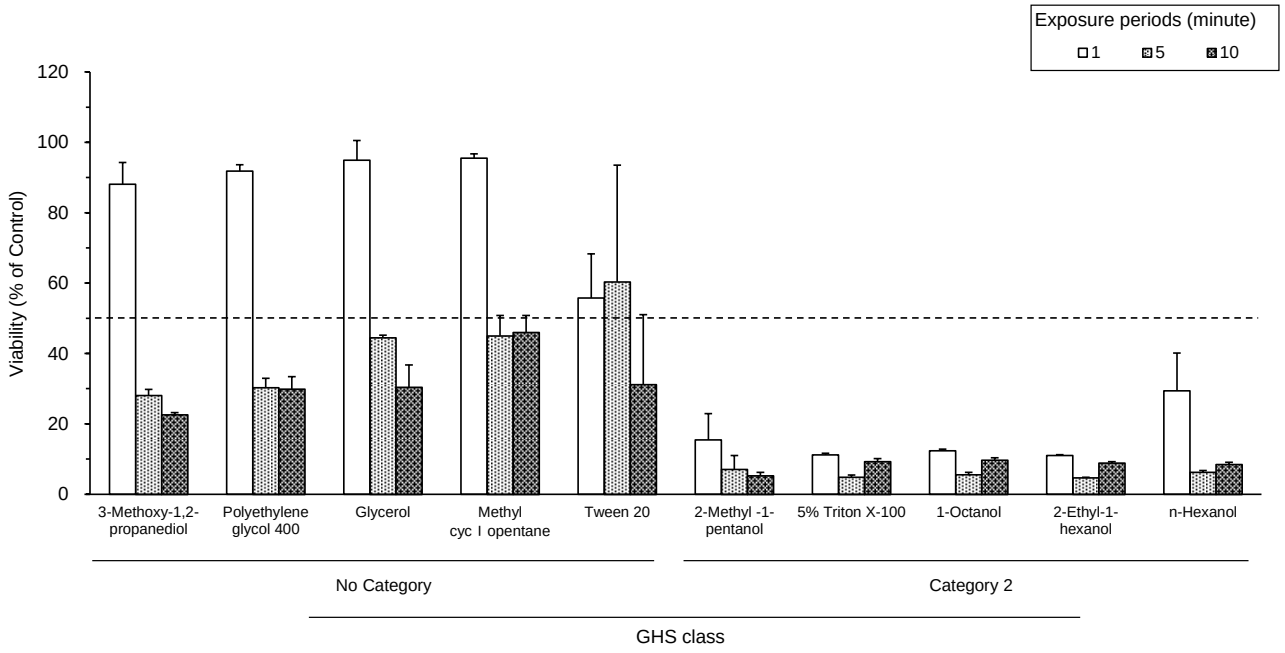


Fig.3-1. Optimization of exposure periods and post-exposure incubation periods for the LabCyte24 EIT for Liquid chemicals.

Post-exposure incubation period was set at 0 hours (A), 2 hours (B) and 24 hours (C), respectively.

When the post-exposure incubation periods were set at 24 hours, all 5 non-irritant chemicals and 5 irritant chemicals were correctly predicted at 1 minute exposure periods, and the prediction results completely correlated to the UN GHS classification (Fig 3-1(C)). The number of chemicals that showed a false-positive increased relatively as the exposure period was extended, while the accuracy of prediction showed a tendency to decrease gradually.

Finally, it was decided that the optimal application conditions for exposure and post-exposure incubation periods for Liquid chemicals should be set at 1 minute and 24 hours, respectively.

3-1.3. Optimization of Suitable Exposure and Post-exposure Incubation Periods for the Solid Test Chemicals

3-1.3.1. Study Conditions

This study condition was summarized followings.

In order to determine optimal exposure periods for the Solid chemicals, tissues that applied Solid chemicals (50 mg) were incubated for varying time periods (1, 4 and 24 hours). After exposure, each tissue was rinsed with D-PBS, using a washing bottle to remove any remaining test chemical from the tissue surface. The blotted tissues were then transferred to new wells on 24-well plates containing 500 µL of fresh assay medium. In order to determine optimal post-exposure incubation periods, the tissues were post-incubated under the same conditions as that of the Liquid chemicals for respective periods (23 hours for an exposure of 1 hour, 20 hours for an exposure of 4 hours and not set for an exposure of 24 hours). After the exposure and post-expose periods, blotted tissues were analyzed for their cell viability through means of a WST-8 assay, in the same manner as for Liquid chemicals.

Table 3-2. The Solid test chemical set for initially optimization of the LabCyte24 EIT

Name	CAS number[1)]	*In vivo* class[2)]	Physical state
4,4'-Methylenebis(2,6-di-tert-butylphenol)	118-82-1	NC	Solid
Silicic acid	7699-41-4	NC	Solid
Sucrose fatty acid ester	-	2	Solid
3,3-Dithiodipropionic Acid	1119-62-6	2	Solid
1-naphtalen acetic acid	86-87-3	2	Solid
Diisopropanolamine	110-97-4	2	Solid
Lauric acid	143-07-7	1	Solid

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

2) *In vivo* class was referred from the GHS classification.

NC: No category (no eye irritant); 2: Category 2 (eye irritant); 1: Category 1 (eye corrosion)

The cell viability was equal to or lower than 50%, the chemical was judged to be UN GHS category 1 or 2, otherwise it was considered UN GHS no category.

Seven Solid test chemicals (initially optimization set) as shown in Table 3-2 was used this optimization study.

3-1.3.2. Results of Optimization Study for Solid Chemicals

Seven test chemicals (two UN GHS No Category: no irritancy, and five UN GHS Category 1 or 2 irritant, in the GHS classification) were selected for the optimization experiments.

When the chemical exposure period has been set to 1 or 4 hours, a sucrose fatty acid ester that was classified in the No Category in the UN GHS classification was wrongly predicted as a non-irritant (Fig. 3-2). On the other hand, all the irritant and non-irritant chemicals in the GHS classification could apparently be predicted between irritating chemical and non-irritating chemicals when the exposure time was set at 24 hours (Fig. 3-2).

Finally, it was decided that the optimal application conditions for exposure and post-exposure incubation periods for Solid chemicals should be set at 24 hours and none, respectively.

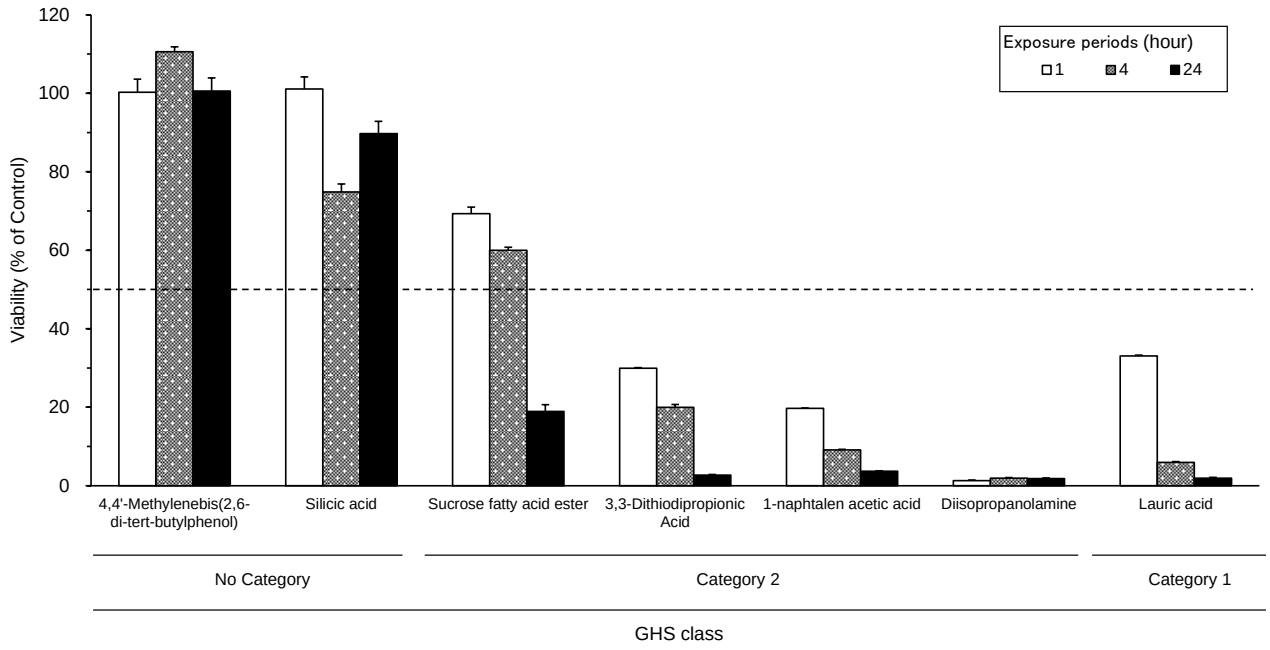


Fig.3-2. Optimization of exposure periods and post-exposure incubation periods for the LabCyte24 EIT for Solid chemicals.

3-1.4. Discussion and Conclusion

In this optimization study, it was decided the optimal application conditions for exposure and post-exposure incubation periods for Liquid and Solid chemicals as shown in the Table 3-3.

Table 3-3. Optimal application period

Periods	Liquid Chemicals	Solid Chemicals
Chemical exposure periods	**1 minute**	**24 hours**
Post-exposure incubation periods	**24 hours**	**Not set**

It is thought that the optimized LabCyte24 EIT protocol for Liquid may reflect the Draize eye test well, as it has very short exposure periods (1 minute) and long recovery periods (24 hours). On the other hand, some Solid chemicals may often adhere to a corneal tissue because their rejection from the eye is not completely accomplished by physiological mechanisms. In the optimized LabCyte24 EIT for Solid chemicals, it was thought that the set-up for long exposure periods (24 hours) could reflect the situation where Solid chemical might often remain in the eye of a test animal in an in vivo Draize test, because of possibly low mobility.

3-1.5. Publication

Detailed results and conclusion of this optimization study were already published in our previous report (Katoh et. al, 2013).

3-2. Ring-Study for the Technical Transferability of the LabCyte24 EIT

3-2.1. Purpose of This Study

In order to assess the technical transferability of the LabCyte24 EIT method, the technical transfer-course for twenty-four laboratories which participated by the general invitation of the planning committee in the Japanese Society for Alternatives to Animal Experiments (JSAAE) has been held and joint research was carried out.

After the technical transfer-course, participating laboratories were performed the LabCyte24 EIT according to technical transfer-course methods with 16 selected test chemicals in order to confirm technical transferability, the reliability and the predictive performance.

3-2.2. Study Plan

3-2.2.1. Participating Laboratories

Twenty-four laboratories were participated in this study (Table 3-4).

Table 3-4. Participating laboratories

Lab No.	Laboratory Name
1	Hatano Research Institute, Food and Drug Safety Center
2	Nihon Kolmar Co.,Ltd
3	ISHIHARA SANGYO KAISHA,LTD
4	Drug Safety Testing Center Co., Ltd
5	FujiFilm Corp.
6	Chemicals Evaluation and Research Institute, Japan
7	TOYO BEAUTY CO.,LTD
8	Safety Research Institute for Chemical Compounds Co., Ltd.
9	Japan Food Research Laboratories
10	ROHTO Pharmaceutical Co., Ltd.
11	OPPEN COSMETICS Co.
12	Taisho Pharmaceutical Co. ,Ltd.
13	Nihon Nohyaku Co., Ltd.
14	IVY COSMETICS Co.

15	Kao Corp.
16	LION CORP.
17	Kobayashi Pharmaceutical Co., Ltd.
18	Mandom Corp.
19	NOEVIR Co.,Ltd.,
20	DRC CO.,LTD
21	BOZO Research Center Inc.
22	Kamakura Techno-Science
23	BioSafety Research Center (BSRC)
24	Japan Tissue Engineering Co., Ltd

3-2.2.2. Selected Test Chemicals

Sixteen test chemicals were selected in this study (Table 3-5).

Table 3-5. Selected test chemicals.

Chemical No.	Test Chemicals	CAS no.	Category	Physical State	GHS class
L0101-L0601	Sucrose fatty acid ester	-	Surfactants (nonionic)	Solid	2
L0102-L0602	4,4'-Methylenebis(2,6-di-tert-butylphenol)	118-82-1	Aromatics	Solid	NC
L0103-L0603	1-Butanol	71-36-3	Alcohols	Liquid	1
L0104-L0604	Sodium hydroxide (1%)	1310-73-2	Inorganic bases	Liquid	2B
L0701-L1201	1-Naphtalen acetic acid	86-87-3	Pesticides	Solid	2
L0702-L1202	Sodium salicylate	54-21-7	Organic salts	Solid	1
L0703-L1203	Tween 20	9005-64-5	Surfactants (nonionic)	Liquid	NC
L0704-L1204	Isopropylalcohol	67-63-0	Alcohols	Liquid	2A
L1301-L1801	2,5-dimethyl-2,5-hexanediol	110-03-2	Alcohols	Solid	1
L1302-L1802	2-Methyl -1-pentanol	105-30-6	Alcohols	Liquid	2B
L1303-L1803	Triton X-100(5%)	57-09-0	Surfactants (nonionic)	Liquid	2
L1304-L1804	Silic acid (Silicon Dioxide n-Hydrate)	7699-41-4	Inorganics	Solid	NC
L1901-L2401	Glycolic acid	79-14-1	Carboxilic acid	Solid	2
L1902-L2402	3,3-Dithiodipropionic Acid	1119-62-6	Sulphur-containing compounds	Solid	2B
L1903-L2403	Benzalkonium chloride (10%)	8001-54-5	Surfactants (cationic)	Liquid	1
L1904-L2404	3-Methoxy-1,2-propanediol	623-39-2	Alcohols	Liquid	NC
Positive control	Ethanol	64-17-5	Alcohols	Liquid	2A
Positive control	Lauric acid	143-07-7	Fatty acids	Solid	1

3-2.2.3. Test Protocol

3-2.2.3a. Protocol for Liquid Chemical

Liquid chemicals (50 µL) were applied to three tissues. In addition, three tissues were treated with 50 µL of D-PBS serving as negative controls and ethanol serving as positive controls. And then tissues applied chemicals were incubated for 1 minute. After exposure, each tissue was rinsed with D-PBS ten times or more, using a washing bottle to remove any remaining test chemical from the tissue surface. The blotted tissues were then transferred to new wells on 24-well plates containing 500 µL of fresh assay medium. And then the tissues were post-exposure incubated for 24 hours under the standard cultivation conditions (at 37°C in a humidified atmosphere of 5% CO_2 in air). After the post-exposure incubation periods, blotted tissues were transferred to new wells on 24-well plates containing 300 µL of freshly prepared WST-8 solution (1:10 dilution of Cell Counting Kit-8 with D-PBS) for a WST-8 assay. Tissues were incubated for 5 hours under the standard cultivation conditions. Subsequently, 200 µL of culture supernatant were transferred to a 96-well microtiter plate. The OD value of culture supernatant was measured at 450 nm and at 650 nm as a reference absorbance, with WST-8 solution as a blank.

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

3-2.2.3b. Protocol for Solid Chemical

Solids (50 mg) in a microtube are applied by decantation on the tissue. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were set apart as non-treated samples and Lauric acid (50 mg) served as positive control. And then tissues were incubated for 24 hours. After exposure, each tissue was rinsed with D-PBS in the same manner as that of Liquid chemicals. In the Solid chemicals, post-exposure incubation was not set. After the rinsing, blotted tissues were analyzed for their cell viability through means of a WST-8 assay, in the same manner as for Liquid chemicals.

3-2.2.3c. Assay Criteria

Only when all three following conditions were satisfied, the test result was accepted. .

Negative control	0.5 ≤ Mean of OD (450nm/650nm) ≤ 2.0
Positive control	Mean of cell viability ≤ 50%

SD	SD of viability of three RhCE tissues (negative control, positive control and test chemical) ≤ 20 %

3-2.2.3d. Prediction Model in This Study

If the mean cell viability was equal to or lower than 50%, the chemical was judged to be an irritant, otherwise, it was considered a non-irritant.

3-2.3. Results

3-2.3.1. Technical Transferability

As shown the Fig.3-3 one run of negative control for Solid did not met the acceptance criteria of OD range (0.5 ≤ Mean OD ≤ 2.0) and then the frequency of invalid test run for the negative control was 0.7% (1/145 test).

On the other hands, all positive control for both Liquid and Solid chemicals met the acceptance criteria of both cell viability (≤ 50%) and SD (≤ 20 %) and then the frequency of invalid test run for the positive control was 0% (0/144 test).

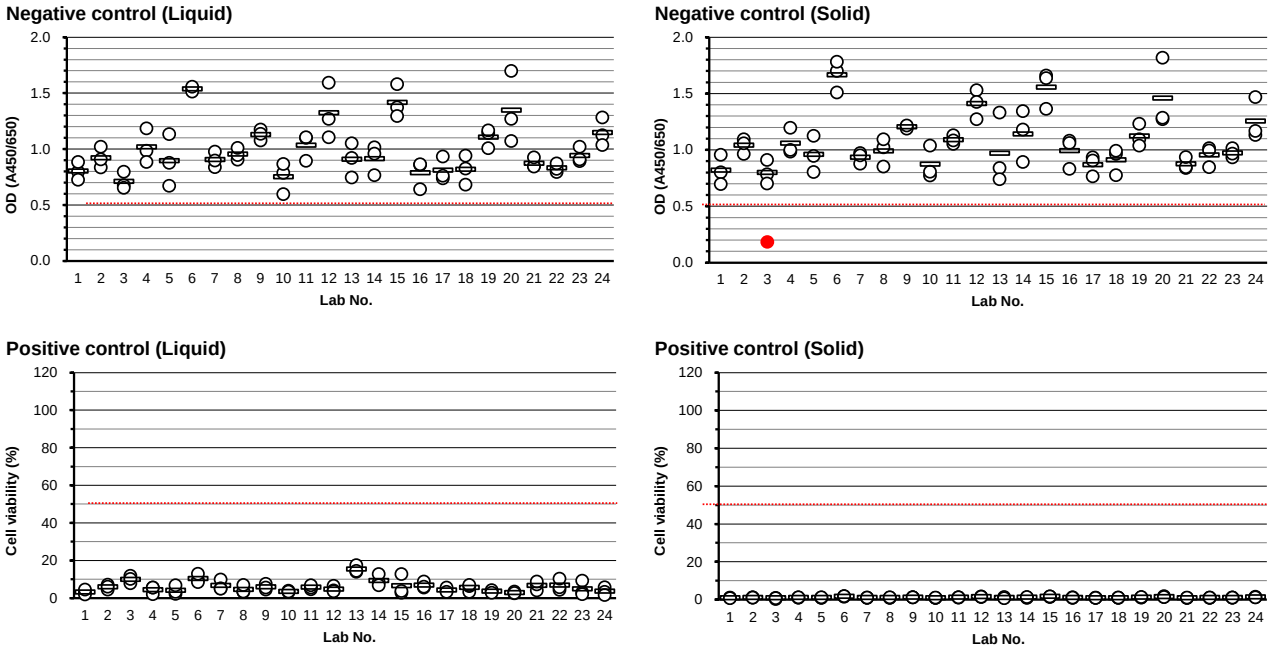


Fig.3-3. Results of negative control and positive control

Table 3-6. Prediction of three independent run

		Cell viability (%)											
		Liquid						Solid					
		1-Butanol		1	Sodium hydroxide (1%)		2B	Sucrose fatty acid ester		2	4,4'-Methylenebis(2,6-di-tert-butylphenol)		NC
Lab No.		Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge
1	R1	4.5	2.4	I	2.6	1.3	I	13.9	2.4	I	97.0	2.7	NI
	R2	6.8	2.3	I	3.8	2.0	I	11.3	3.6	I	77.4	3.9	NI
	R3	7.2	7.4	I	7.6	11.0	I	16.0	1.2	I	108.8	13.3	NI
2	R1	6.2	2.6	I	4.1	0.3	I	13.7	1.0	I	91.4	10.7	NI
	R2	11.4	3.3	I	3.6	0.6	I	15.8	1.7	I	92.3	3.4	NI
	R3	13.3	5.2	I	7.1	3.7	I	24.1	1.9	I	76.7	2.0	NI
3	R1/R2	11.9	2.8	I	2.0	1.1	I	13.6	3.8	I	32.9	4.2	I
	R2/R3	11.3	4.4	I	4.2	1.8	I	16.3	1.1	I	51.0	7.3	NI
	R3/R4	11.6	2.8	I	7.5	0.3	I	9.0	3.1	I	73.4	9.6	NI
4	R1	5.1	3.9	I	1.0	0.3	I	17.2	2.1	I	106.1	6.4	NI
	R2	15.4	9.1	I	2.4	1.6	I	18.3	9.0	I	90.2	15.2	NI
	R3	12.6	4.9	I	1.9	0.6	I	19.2	2.9	I	108.4	2.8	NI
5	R1	6.5	2.8	I	1.6	0.2	I	20.4	5.7	I	110.5	5.6	NI
	R2	4.9	1.2	I	1.3	0.7	I	15.0	4.9	I	110.2	0.9	NI
	R3	9.0	1.0	I	1.2	0.0	I	13.1	4.1	I	89.1	11.1	NI
6	R1	10.6	7.0	I	2.9	1.6	I	14.3	4.6	I	97.2	4.3	NI
	R2	14.7	1.6	I	5.0	3.6	I	21.6	1.8	I	102.3	6.7	NI
	R3	11.8	2.6	I	4.4	3.1	I	10.6	1.6	I	77.7	4.0	NI
		Tween 20		NC	Isopropylalcohol		2A	1-Naphtalen acetic acid		2	Sodium salicylate		1
Lab No.		Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge
7	R1	87.9	0.8	NI	9.0	0.8	I	6.6	0.2	I	4.3	0.0	I
	R2	82.8	0.9	NI	6.3	0.4	I	7.1	0.8	I	5.6	0.7	I
	R3	82.6	2.3	NI	5.6	0.4	I	8.0	0.5	I	6.2	0.5	I
8	R1	4.6	1.1	I	3.9	4.5	I	7.1	9.3	I	0.7	0.1	I
	R2	92.2	2.8	NI	10.8	4.1	I	10.7	5.7	I	0.8	0.1	I
	R3	94.3	4.0	NI	9.2	1.8	I	11.1	6.1	I	0.7	0.1	I
9	R1	86.9	6.3	NI	6.5	0.9	I	1.8	0.5	I	1.3	0.1	I
	R2	115.5	14.7	NI	8.7	3.0	I	2.6	0.3	I	1.5	0.1	I
	R3	111.1	1.9	NI	5.2	2.7	I	2.4	1.0	I	1.0	0.0	I
10	R1	95.5	18.8	NI	3.0	2.2	I	6.2	5.1	I	1.4	0.1	I
	R2	65.2	2.7	NI	6.0	0.8	I	4.2	2.0	I	2.0	0.1	I
	R3	83.5	18.0	NI	8.3	4.3	I	2.7	0.7	I	1.3	0.1	I
11	R1	30.7	10.6	I	7.8	3.9	I	1.5	0.7	I	0.9	0.1	I
	R2	117.4	10.2	NI	4.5	1.1	I	1.3	0.3	I	0.7	0.4	I
	R3	110.3	13.3	NI	11.3	1.4	I	2.7	0.8	I	1.5	0.5	I
12	R1	78.6	62.8	NI	7.1	3.7	I	5.4	2.4	I	0.7	0.0	I
	R2	96.3	22.0	NI	2.7	1.5	I	1.4	0.1	I	0.8	0.1	I
	R3	117.2	9.7	NI	21.9	9.1	I	4.7	1.0	I	0.7	0.0	I

(Continue)

		Cell viability (%)											
		2-Methyl -1-pentanol		2B	Triton X-100(5%)		2	2,5-dimethyl-2,5-hexanediol		1	Silic acid		NC
Lab No.		Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge
13	R1	34.3	3.9	I	4.5	0.2	I	4.7	0.4	I	67.2	1.4	NI
	R2	30.0	5.9	I	4.3	0.7	I	3.2	0.2	I	53.2	1.5	NI
	R3	30.0	7.9	I	4.2	1.3	I	2.7	0.3	I	63.4	13.4	NI
14	R1	28.0	8.9	I	23.5	3.4	I	1.4	0.5	I	35.6	7.6	I
	R2	36.5	6.9	I	21.3	2.7	I	1.0	0.4	I	47.0	3.5	I
	R3	43.8	6.3	I	21.8	2.3	I	0.7	0.0	I	41.5	9.2	I
15	R1	23.8	3.7	I	3.3	1.4	I	4.0	0.2	I	44.0	15.7	I
	R2	8.7	9.3	I	7.4	7.7	I	7.2	6.3	I	35.8	5.1	I
	R3	29.4	5.4	I	2.4	0.3	I	3.5	0.3	I	47.6	3.2	I
16	R1	21.7	4.4	I	1.6	0.5	I	1.4	0.1	I	51.0	8.2	NI
	R2	24.0	10.0	I	1.1	1.1	I	1.7	0.1	I	45.4	8.3	I
	R3	21.0	2.1	I	1.5	0.4	I	1.4	0.1	I	61.4	7.6	NI
17	R1	27.2	9.0	I	1.7	0.1	I	2.4	0.7	I	39.1	6.3	I
	R2	27.9	1.5	I	2.1	0.8	I	2.1	0.2	I	71.0	1.9	NI
	R3	25.1	4.2	I	1.8	0.1	I	1.5	0.0	I	42.0	2.7	I
18	R1	30.0	2.3	I	5.0	2.3	I	1.6	0.2	I	37.6	2.4	I
	R2	17.6	0.9	I	5.0	2.3	I	2.9	0.5	I	42.5	1.4	I
	R3	25.0	3.3	I	11.0	1.2	I	2.4	0.3	I	43.4	4.1	I
		Benzalkonium chloride (10%)		1	3-Methoxy-1,2-propanediol		NC	Glycolic acid		2	3,3-Dithiodipropionic acid		2B
Lab No.		Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge	Mean	SD	Judge
19	R1	-0.8	0.3	I	47.9	6.6	I	-2.4	0.0	I	-0.2	0.7	I
	R2	1.4	0.2	I	40.0	3.0	I	-0.1	0.4	I	11.8	1.4	I
	R3	0.8	0.1	I	48.9	13.5	I	0.3	0.4	I	4.6	3.0	I
20	R1	0.7	0.2	I	54.5	4.0	NI	-0.1	0.5	I	2.6	1.4	I
	R2	1.1	0.5	I	62.1	17.1	NI	0.1	0.2	I	1.1	0.1	I
	R3	1.2	0.3	I	51.6	4.8	NI	0.0	0.2	I	3.5	1.1	I
21	R1	2.2	0.4	I	70.6	5.9	NI	1.0	0.1	I	32.0	5.0	I
	R2	2.5	1.2	I	95.2	3.6	NI	0.7	0.1	I	13.9	0.6	I
	R3	1.8	0.1	I	93.4	4.5	NI	0.6	0.1	I	14.2	2.2	I
22	R1	1.6	0.1	I	76.3	8.7	NI	0.4	0.1	I	5.3	4.8	I
	R2	1.5	0.0	I	81.6	39.4	NI	0.6	0.2	I	4.3	2.8	I
	R3	1.5	0.0	I	62.6	13.4	NI	0.6	0.2	I	13.4	2.1	I
23	R1	1.6	0.2	I	75.4	2.2	NI	1.2	0.7	I	1.8	0.2	I
	R2	0.7	0.1	I	50.1	6.2	NI	0.9	0.1	I	2.3	1.0	I
	R3	1.5	0.3	I	47.7	12.8	I	1.7	0.2	I	2.8	0.7	I
24	R1	0.7	0.2	I	85.7	9.0	NI	2.4	0.8	I	3.4	1.2	I
	R2	1.3	0.3	I	73.0	12.1	NI	2.2	1.6	I	4.1	1.1	I
	R3	0.6	0.2	I	82.0	8.0	NI	0.6	0.2	I	2.9	2.5	I

3-2.3.2. Within-Laboratory Reproducibility

In three independent runs within a single laboratory, the non-concordance of prediction of *in vitro* eye irritation was occurred by chemicals of 6 test (Incidence rate: 6/96 test = 6.3%; Table 3-6). These chemicals belonged to the group classified no-category (no-eye irritant) in the GHS

classification (Incidence rate: 6/24 test= 25%).

3-2.3.3. Between-Laboratory Reproducibility

The results of ocular irritancy prediction of 4 data in 4 laboratories were not in agreement with the GHS classification (Table 3-6).

These chemicals belonged to the group classified no-category (non-eye irritant) in the GHS classification.

3-2.3.4. Predictive Capacity

Specificity of this protocol was not high (53/72 test=73.6%; Table 3-6).

Sensitivity of this protocol was 100% (216/216 test = 100%; Table 3-6).

Overall accuracy of this protocol was 93.4% (269/288 test = 93.4%; Table 3-6).

3-2.4. Discussion and Conclusion

It was thought that technical transferability of this EIT was easy from the results acceptance criterion of positive control and negative control in this ring study.

From these results, it was found that both within- and between-laboratory reproducibility in the LabCyte24 EIT were enough high.

It was also confirmed that the LabCyte24 EIT was the method which chemicals showing false negative did not generate easily.

In spite of having been the test chemical selection which put weight on the negative detection rate, some case showing false positive generated (19/72 test = 26.4%).

Because it was considered that the washing method for Solid or viscous chemicals (Tween 20) caused these results, the opinion that modification of the LabCyte24 EIT protocol should be indispensable was offered from some participating laboratories.

From the above result, although it might be useful method to prediction of a non-eye irritant chemicals, it seemed that the intensity of eye irritation cannot be distinguishable with this EIT.

3-3. Modification of LabCyte24 EIT (Pre-Validation Study Phase 01)

3-3.1. Purpose of This Study

In the ring study (see the section 3.2), it was requested that protocol modification of LabCyte24 EIT was distinguished for decrease variation and false-positive.

Study team was discussed about protocol modification. Variable widely of cell viability were sometime incidence at viscus chemicals or Solid chemicals because they might be remain on the tissue after washing. On the other hands, because false-positive chemicals were often a borderline chemicals, it might be effective to change the cut-off value.

Finally, study team was accepted the modification of LabCyte24 EIT protocol as followings points;

1) The cut-off value of eye irritation: From 50% to 40%.
2) Solid application amount: From 50 mg to 10 mg.
3) Washing method: From mild to more strong.

Then, in order to confirm the technical transferability, within- and between-laboratory reproducibility of this modified EIT protocol, it was carried out the pre-validation study phase 01 by five laboratories. A purpose of this study is to examine whether the result of the modified CORNEA-MODEL24 EIT method can achieve proficiency level of preset criteria. In this study, it was set the assay criteria as follows;

1) SD of negative control, positive control and the test chemicals are ≤ 20%.
2) The dispersion of the cell viability by viscus chemical (Tween 20) is smaller than the ring study (see the section 3.2).
3) The incidence rate of false positive is less than the ring study (see the section 3.2).

3-3.2. Study plan

3-3.2.1. Participating Laboratories

Five laboratories were participated in this study (Table 3-7).

Table 3-7. Participating laboratories.

Lab No.	Laboratory Name
A	Mandom Corp.
B	Drug Safety Testing Center Co., Ltd
C	Fujifirm Corp.
D	Nihon Kolmar Co.,Ltd
E	OPPEN COSMETICS Co.

3-3.2.2. Selected Test Chemicals

Twelve test chemicals were selected in this study (Table 3-8).

Table 3-8. Selected test chemicals.

Test chemical	CAS No.	Category	Physical state	GHS class
1-Butanol	71-36-3	Alcohols	Liquid	1
3-Methoxy-1,2-propanediol	623-39-2	Alcohols	Liquid	NC
2-Methyl-1-pentanol	105-30-6	Alcohols	Liquid	2B
Glycerol	56-81-5	Polyols	Liquid	NC
Tween 20	9005-64-5	Surfactants (nonionic)	Liquid	NC
TritonX-100 (5%)	9002-93-1	Surfactants (nonionic)	Liquid	2
4,4'-Methylenebis(2,6-di-tert-butylphenol)	118-82-1	Aromatics	Solid	NC
Sucrose Fatty Acid Ester	none	Surfactants (nonionic)	Solid	2
Silicic acid	7699-41-4	Inorganics	Solid	NC
Phenothiazine	92-84-2	Amines	Solid	NC
3,3-Dithiodipropionic Acid	1119-62-6	Sulpha-containing compounds	Solid	2B
1-naphtalen acetic acid	86-87-3	Pesticides	Solid	2

3-3.2.3. Test Protocol

3-3.2.3a. Protocol for Liquid Chemical

Liquid chemicals (50 µL) were applied to three tissues. In addition, three tissues were treated with 50 µL of D-PBS serving as negative controls and ethanol serving as positive controls. And then tissues applied chemicals were incubated for 1 minute. After exposure, each tissue was strongly washed with D-PBS ten times or more, using a washing bottle to remove any remaining test chemical from the tissue surface. The blotted tissues were then transferred to new wells on 24-well plates containing 500 µL of fresh assay medium. And then the tissues were

post-exposure incubated for 24 hours under the standard cultivation conditions (at 37°C in a humidified atmosphere of 5% CO_2 in air). After the post-exposure incubation periods, blotted tissues were transferred to new wells on 24-well plates containing 300 µL of freshly prepared WST-8 solution (1:10 dilution of Cell Counting Kit-8 with D-PBS) for a WST-8 assay. Tissues were incubated for 5 hours under the standard cultivation conditions. Subsequently, 200 µL of culture supernatant are transferred to a 96-well microtiter plate. The OD value of culture supernatant was measured at 450 nm and at 650 nm as a reference absorbance, with WST-8 solution as a blank.

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

3-3.2.3b. Protocol for Solid Chemical

Solids (10 mg) in a microtube were applied by decantation on the tissue. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were set apart as non-treated samples and Lauric acid (10mg) served as positive control. And then tissues are incubated for 24 hours. After exposure, each tissue was rinsed with D-PBS in the same manner as that of Liquid chemicals. In the Solid chemicals, post-exposure incubation was not set. After the rinsing, blotted tissues were analyzed for their cell viability through means of a WST-8 assay, in the same manner as for Liquid chemicals.

3-3.2.3c. Assay Criteria

Only when all three following conditions were satisfied, the test result was accepted. .

Negative control	0.5 ≤ Mean of absorbance (450nm/650nm) ≤ 2.0
Positive control	Mean of cell viability ≤ 40%
SD	SD of viability of three RhCE tissues (negative control, positive control and test chemical) ≤ 20 %

3-3.2.3d. Prediction Model in This Study

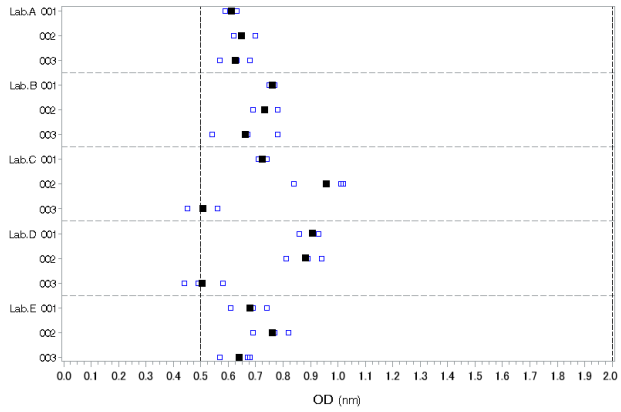
If the mean cell viability was equal to or lower than 40%, the chemical was judged to be an irritant, otherwise, it was considered a non-irritant.

3-3.3. Results

3-3.3.1. Negative control and positive control

Fig. 3-4 shows the absorbance values for the negative control.

Liquid



Solid

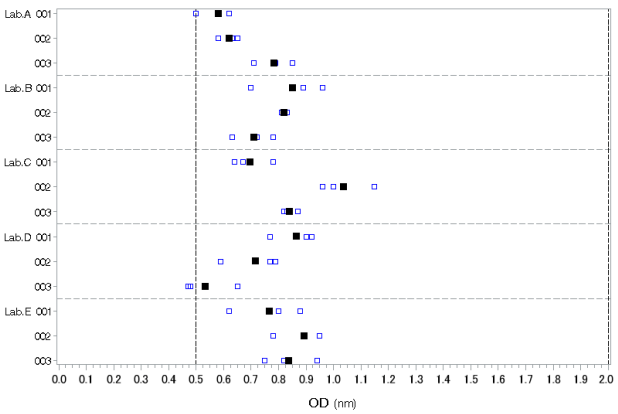
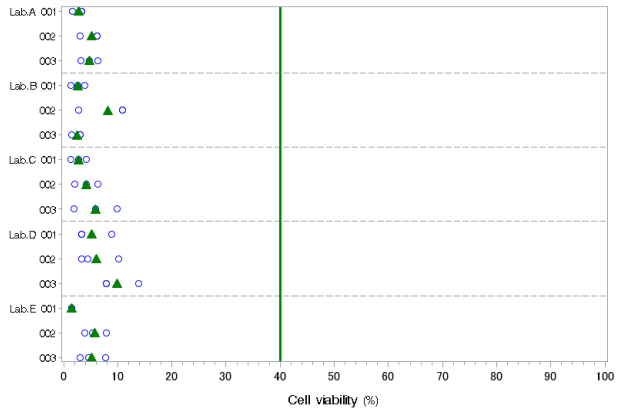


Fig.3-4. Results of negative control

All data for the negative control met the acceptance criteria of both OD (0.5 ≤ OD ≤ 2.0) and SD (≤ 20 %) and then the frequency of invalid test run for the negative control was 0%.

Table 3-5 shows the absorbance values for the positive control.

Liquid



Solid

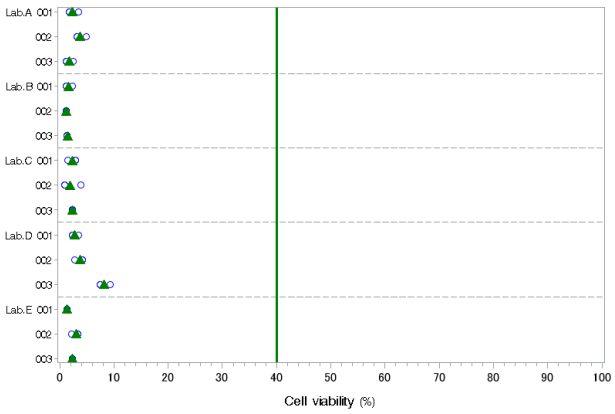


Fig.3-5. Results of positive control.

All data for the positive control met the acceptance criteria of both cell viability (≤ 40%) and SD (≤ 20 %) and then the frequency of invalid test run for the positive control was 0%.

Table 3-9. Prediction of three independent run

Test chemical	GHS Class	Lab	A			B			C			D			E		
		run	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
3-Methoxy-1,2-propanediol	NC	Cell viability	57.4	48.5	55.3	88.6	84.5	120	47.9	41.5	95.4	65.8	62.5	104	40.7	41.2	70.3
		SD	9.8	4.5	9.6	13.8	6.2	5.3	8.1	2.2	3	3.5	8.6	4.6	5.9	6.1	15.1
		Judgement	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Glycerol	NC	Cell viability	47	82	78.2	98.2	107	141	72.8	42.2	73.7	78.7	73.9	114	58.3	57.9	81.3
		SD	2.5	1.5	11.2	5.3	2.8	18.8	16.1	13.4	18.3	12.1	14.9	6.1	13.7	6	5.6
		Judgement	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Tween 20	NC	Cell viability	69.4	90.2	75	107	116	127	85.7	102	57.9	94.9	86.7	109	109	95.2	95.8
		SD	3.8	5	0	5.9	3.6	10	19.6	16.6	15.8	1.9	5.7	2	5.1	8	13
		Judgement	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
4,4'-Methylenebis (2,6-di-tert-butylphenol)	NC	Cell viability	101	76.9	77.9	102	91.1	98.6	100	110	103	88.8	94.4	114	70	52.2	66.5
		SD	2.6	7.3	6.8	8	3.1	9.9	9.6	5.5	6.6	1.8	2.1	6.6	4.6	1.7	3.7
		Judgement	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Silicic acid	NC	Cell viability	77	40.9	48.5	101	90.2	128	80.4	78.5	82.9	71	101	78.1	87.4	56.7	53.4
		SD	2.6	10.4	5.6	13	4.9	7.8	7.6	7.2	6.6	2.9	9.8	17.3	6.9	7.5	2.5
		Judgement	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
Phenothiazine	NC	Cell viability	113	106	57	82	91.5	139	108	97.4	94.4	83	103	96.3	71.7	75	83.7
		SD	9.5	6.7	9.4	8.8	5.3	8.6	6.3	11.7	6.8	6.4	3.5	16.4	4.7	8.9	6.2
		Judgement	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
TritonX-100 (5%)	2	Cell viability	3.3	15.5	2.1	1.3	1.4	1.5	9.7	8.7	2	2.6	6.8	9.9	17.6	24.1	18.2
		SD	0	1.5	0.9	0	0	0	7.7	3.4	0	0.6	5.9	3.4	6.4	6.5	12.1
		Judgement	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Sucrose Fatty Acid Ester	2	Cell viability	20.7	26.3	9.4	16.9	16.7	23	14.4	18.3	14.7	15.8	25.1	38.1	10	11.2	13.9
		SD	1.7	5.2	4.5	5.8	0.7	4.3	2.9	3.5	4.2	2.4	5	5.7	3.3	4	1.4
		Judgement	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
1-naphtalen acetic acid	2	Cell viability	3.4	3.2	2.6	7.8	4.5	0	3.3	3.2	3.2	3.1	2.8	10	1.7	3	2.8
		SD	0	0	0	3.6	1.4	0	0.8	0.6	0.7	0.7	0	1.1	0.8	0.6	1.4
		Judgement	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
2-Methyl-1-pentanol	2B	Cell viability	27.3	21.1	14.9	22.8	33.6	7	27.6	24.7	23.7	14	18.9	21.9	26	19.3	30.2
		SD	2.5	3.6	0.9	5.5	6.1	9.6	0	3.7	6.8	11	2.9	9.9	9.5	3	8.9
		Judgement	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
3,3-Dithiodipropionic Acid	2B	Cell viability	3.4	3.8	2.6	3.9	3.7	1.4	3.3	2.9	2.8	2.7	3.3	14.4	0.4	2.2	0.4
		SD	0	0.9	0	4.8	3.2	0	0.8	1	0.7	0.7	0.8	1.1	0.8	1.1	0.7
		Judgement	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
1-Butanol	1	Cell viability	4.9	2.6	4.3	15.4	16.8	10.6	5.1	1.7	7.2	5.9	18.2	17.9	4.4	4.4	11.5
		SD	1.6	0.9	2.4	2	5.5	10.6	2.9	1.2	4.1	2.3	1.1	6.9	3.9	2	9
		Judgement	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I

3-3.3.2. Within-Laboratory Reproducibility

In three independent runs within a single laboratory, the non-concordance of prediction of *in vitro* eye irritation was not occurred (Table 3-9). Therefore, reproducibility of within-laboratory in this study was 100% at the all participating laboratories.

3-3.3.3. Between-Laboratory Reproducibility

The rate of between-laboratory reproducibility in this study was 100% (Table 3-9). Therefore, all laboratories had high between-laboratory reproducibility.

3-3.3.4. Predictive capacity

In this study, prediction results at all laboratories were able to correctly identify eye irritation all chemicals. Therefore, the sensitivity, specificity, and accuracy of this EIT protocol were all 100% (Table 3-9).

3-3.3.5. Proficiency Level of Preset Criteria

This EIT method achieved the all criteria. Proficiency level was summarized in Table 3-10.

Table 3-10. Prediction of three independent run

(A) Incidence of out of acceptance criteria (SD: > 20%)

	Ring study (Section 4.2)	Pre-validation study 01
Negative control	0.7% (1/145 test)	0% (0/30 test)
Positive control	0% (0/145 test)	0% (0/30 test)
Test chemicals	0.5% (3/576 test)	0% (0/180 test)

(B) The dispersion of the cell viability by Tween 20

	Ring study (Section 4.2)	Pre-validation study 01
False positive	11.1% (2/18 test)	0% (0/30 test)
SD > 20%	11.1% (2/18 test)	0% (0/30 test)

(Continue)

(C) The dispersion of the cell viability by Tween 20

	Ring study (Section 4.2)	Pre-validation study 01
Non concordance results in three dependen test	6.3% (4/96 test)	0% (0/60 test)
False positive	26.4% (19/72 test)	0% (0/90 test)

SD of negative control, positive control and the test chemicals were met assay criteria and incidence of invalid run was smaller than the ring study (Table 3-10),.

The dispersion of the cell viability by Tween 20 was smaller than the ring study results (Table 3-10).

The false positive was less than the ring study results (to see the Section 4.2).

3-3.4. Discussion and Conclusion

From these results, it was confirmed that both within- and between-laboratory reproducibility by this EIT protocol were higher than the ring Study (see the Section 4.2).

Furthermore, it was also confirmed that the predictive performance in this EIT protocol were higher than the ring study (see the Section 4.2).

However, it was confirmed that large dispersion of the cell viability sometimes occurred, especially it had varied widely the absorbance of negative control and then also the cell viability of the test chemicals of GHS no category. As the cause of such large dispersion, it was suggested the possibility that the reaction of WST-8 assay was not stable among inter-test run.

Therefore it was assumed that reconsideration of WST-8 assay condition and comparative examination between the WST-8 assay and the MTT assay might be necessary.

3-4. Comparison study of MTT assay for the LabCyte24 EIT (Pre-Validation Study Phase-02, 03)

3-4.1. Purpose of This Study

In the pre-validation study phase 01 (see the Section 3-3), large dispersion of the cell viability was sometimes occurred, it was pointed out the possibility that WST-8 reaction is unstable as their reason.

Therefore, it was requested that the comparison study of WST-8 assay and MTT assay for the LabCyte24 EIT.

In this comparison study, it was confirmed as follow points;

1) The comparison of OD range of negative control between WST-8 assay and MTT assay.
2) The comparison of the prediction results of test chemicals between WST-8 assay and MTT assay.

3-4.2. Study Plan

3-4.2.1. Participating Laboratories

Three laboratories were participated in this study (Table 3-11).

Table 3-11. Participating laboratories.

Lab No.	Laboratory Name
B	Drug Safety Testing Center Co., Ltd
C	Fujifirm Corp.
D	Nihon Kolmar Co., Ltd

3-4.2.2. Selected Test Chemicals

As chemical shown bolder line or variable widely in the pre-validation study phase 01, four test chemicals were selected in this comparison study (Table 3-12).

Table 3-12. Selected test chemicals.

Test Chemical	CAS no.	Category	Physical state	GHS class
3-Methoxy-1,2-propanediol	623-39-2	Alcohols	Liquid	NC
Glycerol	56-81-5	Polyols	Liquid	NC
Sucrose Fatty Acid Ester	-	Surfactants (nonionic)	Solid	2
Silicic acid	7699-41-4	Inorganics	Solid	NC

3-4.2.3. Test Protocol

3-4.2.3a. Protocol for Liquid Chemical

Liquid chemicals (50 µL) were applied to three tissues. In addition, three tissues were treated with 50 µL of D-PBS serving as negative controls and ethanol serving as positive controls. And then tissues applied chemicals were incubated for 1 minute. After exposure, each tissue was strongly washed with D-PBS ten times or more, using a washing bottle to remove any remaining test chemical from the tissue surface. The blotted tissues were then transferred to new wells on 24-well plates containing 500 µL of fresh assay medium. And then the tissues were post-exposure incubated for 24 hours under the standard cultivation conditions (at 37°C in a humidified atmosphere of 5% CO_2 in air). After the post-exposure incubation periods, the cell viability were determined by WST-8 assay or by MTT assay.

WST-8 assay

Blotted tissues were transferred to new wells on 24-well plates containing 300 µL of freshly prepared WST-8 solution (1:10 dilution of Cell Counting Kit-8 with D-PBS) for a WST-8 assay. Tissues were incubated for 5 hours under the standard cultivation conditions. Subsequently, 200 µL of culture supernatant were transferred to a 96-well microtiter plate. The OD value of culture supernatant are measured at 450 nm and at 650 nm as a reference absorbance, with WST-8 solution as a blank.

MTT assay

Tissues were put in the wells of 24-well plates containing 500 µL of MTT medium (0.5 mg/mL; Dojindo Co., Japan) and were incubated for 3 hours (37°C, 5% CO_2, humidified atmosphere). MTT Formazan produced in the tissues was extracted with isopropanol (300 µL) and the extract (200 µL) was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol

as a blank.

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

3-4.2.3b. Protocol for Solid Chemical

Solids (10 mg) in a microtube were applied by decantation on the tissue. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were set apart as non-treated samples and Lauric acid (10 mg) served as positive control. And then tissues were incubated for 24 hours. After exposure, each tissue was rinsed with D-PBS in the same manner as that of Liquid chemicals. In the Solid chemicals, post-exposure incubation was not set. After the rinsing, blotted tissues were analyzed for their cell viability through means of a WST-8 assay or MTT assay, in the same manner as for Liquid chemicals.

3-4.2.3c. Assay Criteria

Only when all three following conditions were satisfied, the test result was accepted. .

Negative control	0.5 ≤ Mean of absorbance (450nm/650nm) ≤ 2.0
Positive control	Mean of cell viability ≤ 40%
Standard Deviation (SD)	SD of viability of three RhCE tissues (negative control, positive control and test chemical) ≤ 20 %

3-4.2.3d. Prediction Model in This Study

If the mean cell viability was equal to or lower than 40%, the chemical was judged to be an irritant, otherwise, it was considered a non-irritant.

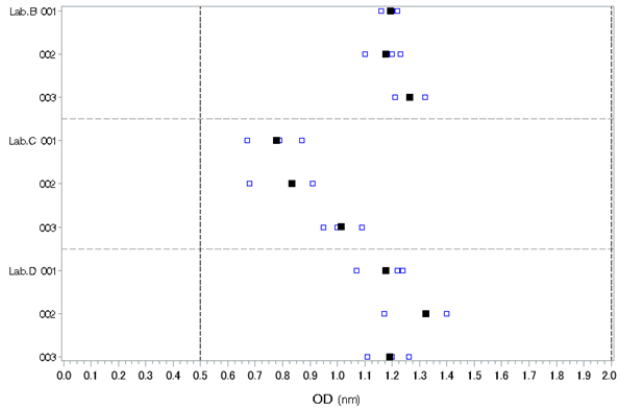
3-4.3. Results

3-4.3.1. Negative Control

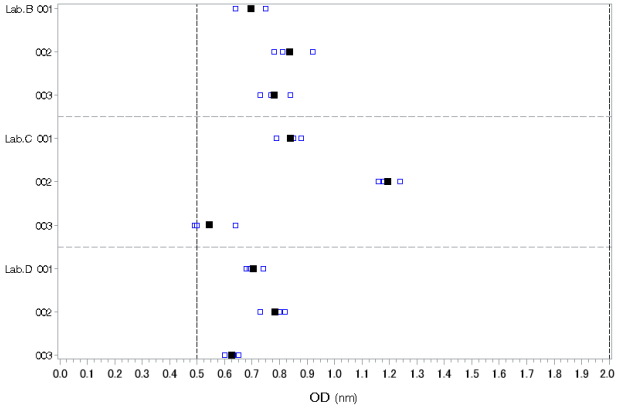
Fig. 3-9 shows the OD for the negative control at three independent test run.

Liquid negative control

WST-8 assay

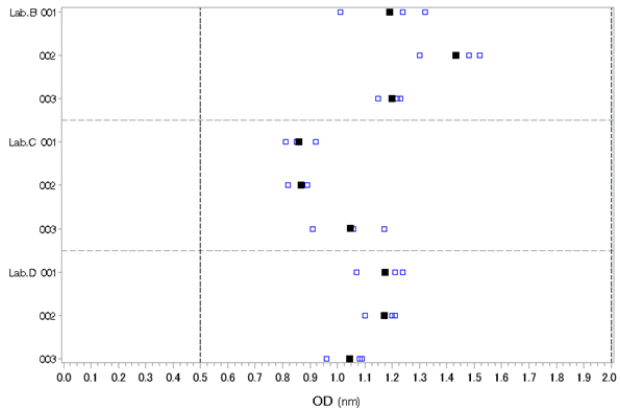


MTT assay



Solid negative control

WST-8 assay



MTT assay

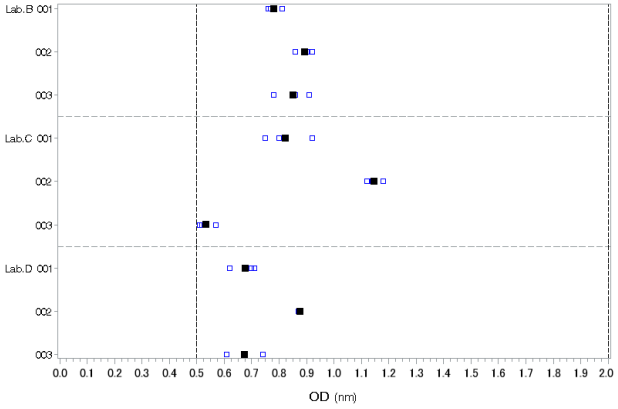


Fig.3-6. Results of negative control

All data for the negative control of both the WST-8 assay and the MTT assay met the acceptance criteria of OD (0.5 ≤ OD ≤ 2.0) and SD (OD ≤ 20%) (Fig. 3-6).

OD range of the negative control of WST-8 assay was between 0.6 and 1.4, on the other hand, that of MTT assay was between 0.5 and 1.2.

From these results, it was not apparent difference of OD value and variably between the WST-8 assay and the MTT assay.

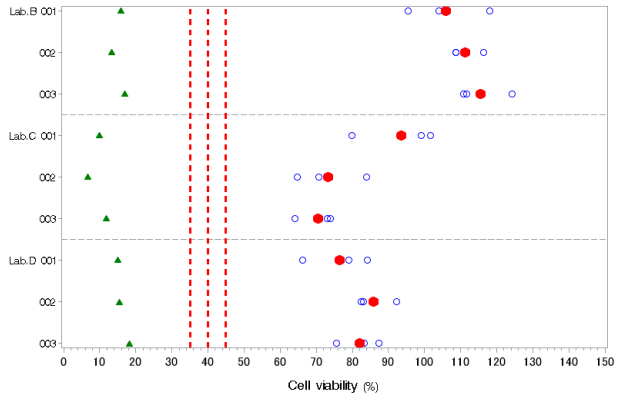
3-4.3.2. Test chemicals

All data for the test chemicals of both the WST-8 assay and the MTT assay met the acceptance criteria of SD (OD ≤ 20%) (Fig. 3-7).

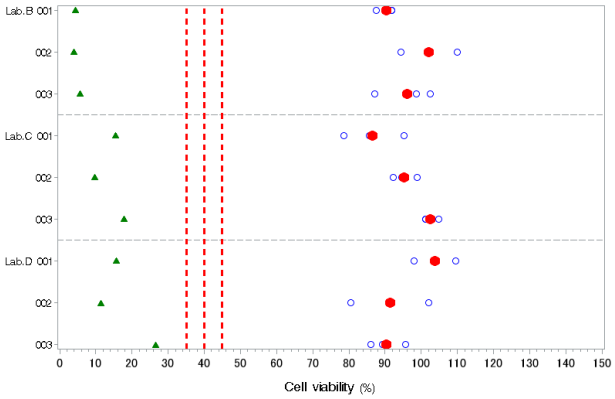
In the MTT assay, Sucrose Fatty Acid Ester which GHS Category 2 chemical was predicted as false negative at all participating laboratories although results of two laboratories were non-concordance between three independent run. Cell viability range of that chemical in the MTT assay are between 28% and 100% and therefore it was resulted varied widely.

3-Methoxy-1,2-propanediol (GHS No category)

WST-8 assay

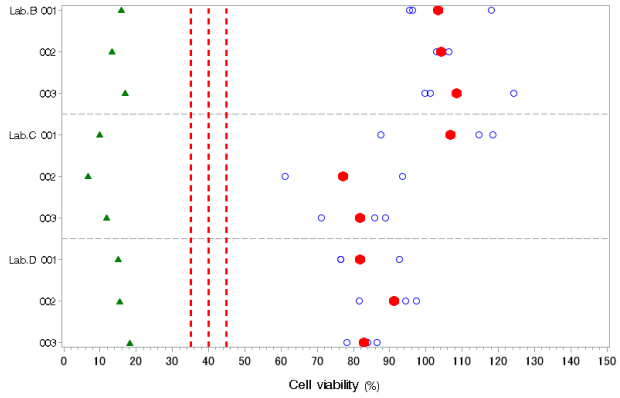


MTT assay

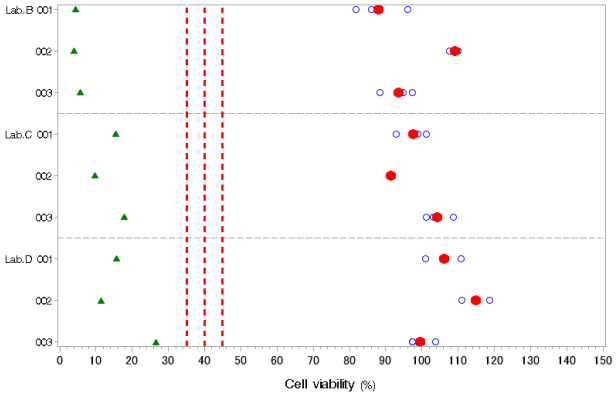


Glycerol (GHS No Category)

WST-8 assay



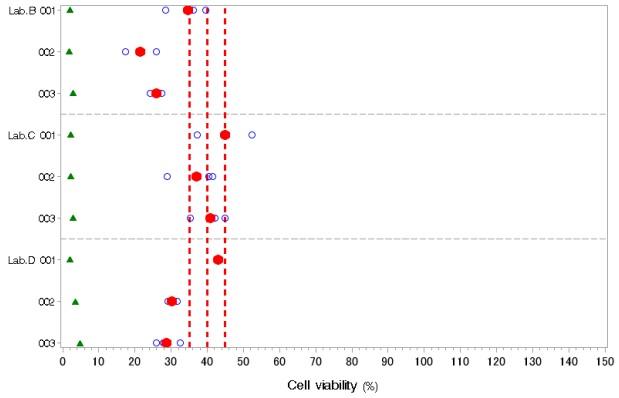
MTT assay



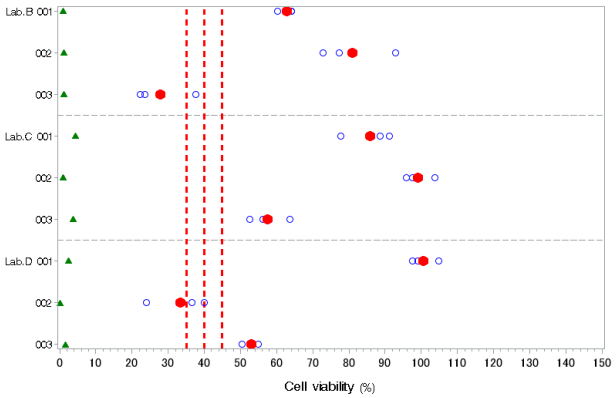
(Continue)

Sucrose Fatty Acid Ester (GHS Category 2)

WST-8 assay

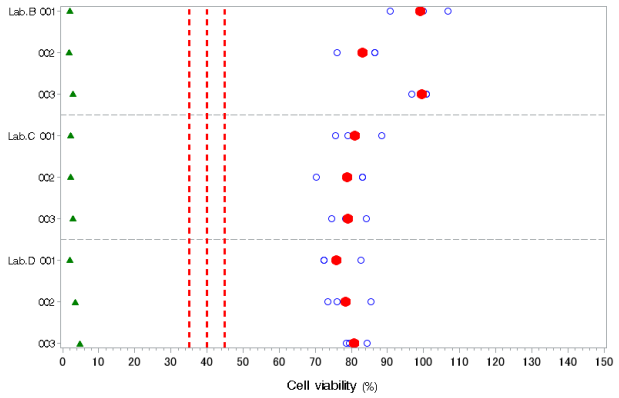


MTT assay



Silicic acid (GHS No category)

WST-8 assay



MTT assay

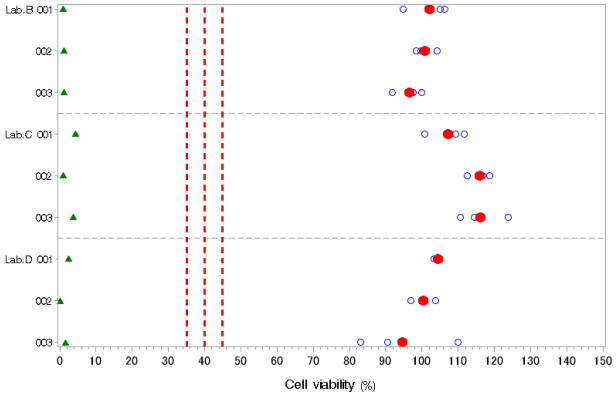


Fig.3-7. Results of Cell viability of test chemicals

Other three GHS No category chemicals (3-Methoxy-1,2-propanediol, Glycerol, and Silicic acid) was not apparent difference of their cell viability between the WST-8 assay and the MTT assay (Fig.3-7).

3-4.4. Discussion and Conclusion

In this comparison study between MTT assay and WST-8 assay, the LabCyte24 EIT using MTT assay was not decreased large dispersion of the cell viability to compare with WST-8

assay.

On the other hands, the cell viability of Sucrose Fatty Acid Ester in the MTT assay were resulted viability widely than WST-8 assay and produced false-negative prediction. Furthermore, it was pointed out from participating laboratories that the operation of MTT assay was more complex than WST-8 assay.

From these results, it was concluded that it was distinguished the optimization study of WST-8 assay condition but not changing MTT assay

3-5. Optimization Study for WST-8 Assay Condition

3-5.1. Purpose of This Study

In the WST-8 assay in the LabCyte24 EIT, the OD of negative control has been sometimes occulated variable widely or low OD (see below the Sections 3-2, 3-3 and 3-4). In order to solve above these problems, the optimization of WST-8 assay condition was indispensable.

In the study 1, pH of several buffers or assay medium as the candidate of WST-8 assay solution was evaluated after incubation at the standard cultivation condition.

In the study 2, WST-8 assay were performed using several buffers or assay medium in order to select of optimal buffer for the WST-8 assay.

In the Study 3, WST-8 assay periods was examined by time course experiments using selected buffer in the Study 2.

3-5.2. Study plan

3-5.2.1. Study 1

In order to prefer optimizing WST-8 reaction condition, it was used D-PBS, Hanks' Balanced Salt Solution (HBSS), HBSS without calcium and magnesium (HBSS(-)), EBSS, and EBSS without calcium and magnesium (EBSS(-)) as the buffer of WST-8 reaction. All buffers were not contained phenol red. Each buffer was incubated at 37°C in a humidified atmosphere with or without 5% CO_2 in air for 1 hour. After the incubation periods, pH of each buffer was measured after incubation.

3-5.2.2. Study 2

WST-8 reaction buffer was prepared using each buffer (D-PBS, HBSS, HBSS (-), EBSS, and EBSS (-)). Each WST-8 solution was incubated under the cultivation conditions at 37°C in a humidified atmosphere and 5% CO_2 in air for 5 hours. After the incubation, the OD of each WST-8 reaction solution was measured. Levels and variabilities of OD of between buffers were evaluated.

3-5.2.3. Study 3

WST-8 reaction buffer was prepared using each buffer (HBSS and EBSS). Both WST-8 solutions were incubated under the cultivation conditions at 37°C in a humidified atmosphere and no or 5% CO_2 in air for 1, 3 or 5 hours. After the incubation, the OD of each WST-8 reaction solution was measured. Levels and variabilities of OD of between buffers were compared.

3-5.3. Results

3-5.3.1. Study 1

From various buffers, it was selected five buffers (D-PBS, HBSS, HBSS(-), EBSS and EBSS(-)) often used for the cell washing and short incubation, it was measured pH of buffers made in Gibco, Sigma and Wako. In addition, pH of various buffers were measured after 0.5 or 1 hour incubation under 37°C in a humidified atmosphere with or without 5% CO_2 in air. In HBSS and HBSS(-), pH of all makers were around 7.5 at room temperature and when they were incubated for 1 hour under 37°C, pH of these rose to around 8.2. While, when they were incubated for 1 hour under 37°C with 5% CO_2, pH of these fell to around 7.0.

Table 3-13. PH value of various buffers after the cultivation under 37°C in a humidified atmosphere with or without 5% CO_2

Culture condition		D-PBS(-)			HBSS			HBSS(-)			EBSS	EBSS(-)	Assay medium
	Period (hour)	Gibco	Sigma	Wako	Gibco	Sigma	Wako	Gibco	Sigma	Wako	Sigma	Gibco	J-TEC
RT	0	7.11[2)]	7.14	7.42	7.53	7.21	7.43	7.74	7.48	7.51	7.41	7.84	---
37℃ (ha[1)])	0.5	7.12	7.18	7.37	7.97	7.96	8.09	8.27	8.02	8.07	8.30	8.52	8.22
	1	7.12	7.13	7.37	8.14	8.23	8.30	8.31	8.43	8.25	8.61	8.84	8.62
37℃ (ha) 5%CO_2	0.5	6.79	6.83	6.96	7.08	6.97	7.09	7.14	7.12	7.16	7.66	7.86	7.80
	1	6.70	6.71	6.80	6.96	6.90	7.00	7.11	7.01	7.04	7.69	7.85	7.84

1) ha: humidified atmosphere 2) pH

In D-PBS, pH of all makers were 7.1 to 7.4 at room temperature and when they were incubated for 1 hour under 37°C, pH of these were almost no change. While, when they were incubated for 1 hour under 37°C with 5% CO_2, pH of these degreased about 0.4.

PH of EBSS and EBSS(-) were 7.4 and 7.8 at room temperature and when they were incubated for 1 hour under 37°C, both rose to about 8.6 and 8.8. While, when they were

incubated for 1 hour under 37°C with 5% CO_2, pH of these hardly changed in 7.7 and 7.8. When LabCyte assay medium was incubated for 1 hour under 37°C with 5% CO_2, pH of this medium was around 7.8 and was nearly the same as these of EBSS and EBSS(-) under 37°C with 5% CO_2. There was little maker difference in pH of various buffers, but we decided to use products of Gibco with precedence.

3-5.3.2. Study 2

Each OD values of D-PBS (-) and HBSS were around 0.8 after WST-8 reaction for 5 hours under 37°C in a humidified atmosphere with 5% CO_2. OD values of EBSS and EBSS(-) were about 1.3 and 1.2 respectively, and OD values of EBSS had a slightly higher absorbance than that of EBSS(-) in a humidified atmosphere. However, both buffers caused a large increase in OD value as compared with D-PBS and HBSS. While, OD value of assay medium was about 1.3, and was almost same as EBSS.

Table 3-14. Effect of various buffer on OD value after WST-8 reaction for 5 hours under 37°C in a humidified atmosphere with 5% CO_2

No.	Buffer or medium	Blank OD (450-650)nm	OD after blank correction (450nm-650nm)					
			well 1	well 2	well 3	Mean	SD (%)	CV(%)
1	D-PBS	0.0310	0.8176	0.8989	0.7598	0.8254	6.99	8.47
2	HBSS	0.0370	0.8290	0.8936	0.8813	0.8680	3.43	3.95
3	EBSS	0.0604	1.3859	1.2367	1.3130	1.3119	7.46	5.69
4	EBSS(-)	0.0774	1.2375	1.1720	1.2412	1.2169	3.89	3.20
5	Medium	0.3763	1.3037	1.2035	1.4097	1.3056	10.31	7.90

Medium: Assay medium

3-5.3.3. Study 3

Because the difference between EBSS and EBSS (-) in component was only the thing whether or not calcium and magnesium were included, and the values of pH and OD of WST-8 in two buffers was almost same in Study1 and 2, we predicted that the similar value will go out whichever buffer is selected in Study 3. So there is no decisive factor, we selected EBSS with hesitancy because EBSS had an absorbance a slightly higher that of EBSS(-).

In Study 3, we focused on two buffers of HBSS and EBSS and measured OD with

time-dependent manner in reaction (1, 3, 5 hours). Moreover, we measured about LabCyte assay medium in the same manner.

OD of HBSS increased with time-dependent manner in WST-8 reaction under 37°C in a humidified atmosphere with 5% CO_2 and was about 0.8 at 5 hours reaction. The results were recognized reproducibility with Study 2. OD of EBSS increased with time-dependent manner in WST-8 reaction under 37°C in a humidified atmosphere with 5% CO_2 and OD was about 1.4 at 5 hours reaction, the results was recognized reproducibility with Study 2. OD of EBSS greatly increased than that of HBSS. OD of the LabCyte assay medium was about 1.3.

Table 3-15. Change of OD values with time-dependent manner in the WST-8 reaction under 37°C in a humidified atmosphere with 5% CO_2

No.	Buffer or medium	WST-8 incubation time (hours)	Blank OD (450-650)nm	OD after blank correction (450-650)nm					
				well 1	Well 2	well 3	Mean	SD (%)	CV(%)
1-1	HBSS	1	0.031	0.097	0.118	0.132	0.116	1.76	15.23
1-2		3	0.035	0.455	0.550	0.451	0.485	5.60	11.55
1-3		5	0.034	0.806	0.889	0.814	0.836	4.58	5.47
2-1	EBSS	1	0.043	0.164	0.174	0.175	0.171	0.61	3.56
2-2		3	0.056	0.814	0.888	0.882	0.861	4.11	4.77
2-3		5	0.067	1.383	1.386	1.389	1.386	0.30	0.22
3-1	Assay medium	1	0.306	0.119	0.108	0.114	0.114	0.55	4.85
3-2		3	0.392	0.621	0.710	0.703	0.678	4.95	7.30
3-3		5	0.476	1.224	1.280	1.264	1.256	2.88	2.30

It was thought that Incubation period of WST-8 assay were suitable 4 hours which OD might reach around 1.0, because OD of EBSS was 0.86 for ~~3~~ hours incubation and OD was 1.39 for 5 hours incubation.

3-5.4. Discussion and Conclusion

Reaction buffer of WST-8 assay were usually used suitable medium. However phenol-red or

other factor contained in the assay medium might be affected blank OD value to rise, D-PBS were selected as WST-8 reaction buffer initially.

After the incubation of assay medium at 37°C with 5% CO_2 for 1 hour, pH of assay medium was around 7.8, which was similar to that of EBSS at same incubation condition. $NaHCO_3$ concentration of EBSS is almost the same to assay medium and then it was contributed to stabilization of pH of EBSS under 37°C with 5% CO_2. On the other hands, pH of HBSS after the incubation at 37°C with 5% CO_2 for 1 hour was around 7.0, which was lower than that of EBSS or assay medium.

It was thought that it was resulted to lower pH value by CO_2 because concentration of $NaHCO_3$ of HBSS was low with about 1/8 of that of EBSS.

Also, under 37°C with 5% CO_2, pH of D-PBS was around 6.7 to 6.8, which was very lower to compare with that of the assay medium. From the results, the lower OD level and its wide variably were sometimes happened in cultivation with D-PBS. Therefore it was thought that D-PBS might be not appropriate as a solvent of WST-8 assay, not the most suitable cultivation condition from these results.

On the other hands, because pH of EBSS was stable around 7.7 to similar to that of assay medium, WST-8 reaction were linearly progressed in the good condition for RhCE tissue and therefore level of OD value were increased similar level for that of assay medium.

Finally it was confirmed that EBSS is suitable buffer solution of WST-8 assay for viable condition of the RhCE tissue and reaction period of WST-8 assay was set 4 hours from results of study 3.

4. DISCRIPTION OF THE IMPROVED LabCyte24 EIT

4-1. Overview of LabCyte the Improved CORNEA-MODEL24 EIT Procedure

Prediction of the eye irritation potential of test chemicals by the improved LabCyte24 EIT was performed according to the improved protocol described in the SOP for ver.2.5.2 (Appendix 1).

The LabCyte CORNEA-MODEL24 tissues is shipped from the supplier on Mondays and delivered to recipients on Tuesdays or Wednesdays. Upon receipt, the tissues are aseptically removed from the transport agarose medium, transfer into wells on 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 mL) and pre-incubated overnight at 37°C in a humidified atmosphere of 5 % CO_2 in air (standard cultivation conditions).

4-1.1. The Improved LabCyte24 EIT Procedure for Liquid Chemicals

After the pre-incubation, the tissue is topically exposed to the Liquid test chemicals. Liquids (50 µL) are applied with a micropipette. Viscous Liquids are applied using a positive displacement-type tip with a micropipette, such as the MICROMAN® (Gilson inc., France). Each test chemical is applied to three tissues. In addition, three tissues are treated with 50 µL of D-PBS serving as negative controls and ethanol serving as positive controls. And then tissues applied chemicals are incubated for 1 minute. After exposure, each tissue is rinsed with D-PBS ten times or more, using a washing bottle to remove any remaining test chemical from the tissue surface. The blotted tissues are then transferred to new wells on 24-well plates containing 500 µL of fresh assay medium. And then the tissues are post-incubated for 24 hours under the standard cultivation conditions (at 37°C in a humidified atmosphere of 5% CO_2 in air). After the post-exposure incubation periods, blotted tissues are transferred to new wells on 24-well plates containing 300 µL of freshly prepared WST-8 solution for a WST-8 assay (Ishiyama et. al., 1997; Tominaga et. al., 1999). WST-8 solution is prepared by 1:10 dilution of Cell Counting Kit-8 (Dojindo Co., Japan) with EBSS (Sigma-Aldrich, MO, USA). Tissues are incubated for 4 hours under the standard cultivation conditions (at 37°C in a humidified atmosphere of 5% CO_2 in air). Subsequently, 200 µL of culture supernatant are transferred to a 96-well microtiter plate. The OD value of culture supernatant is measured at 450 nm and at 650 nm as a reference absorbance, with WST-8 solution as a blank.

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

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

4-1.2. The Improved LabCyte24 EIT Procedure for Solid Chemicals

After pre-incubation, the medium is changed to 500 µL of fresh assay medium and then the tissues are topically exposed to the Solid test chemicals. Solids (10 mg) in a microtube are applied by decantation. Each test chemical is applied to three tissues. In addition, three tissues serving as negative controls are set apart as non-treated samples and Lauric acid (10mg) served as positive control. And then tissues are incubated for 24 hours. After exposure, each tissue is rinsed with D-PBS in the same manner as that of Liquid chemicals. In the Solid chemicals, post-exposure incubation is not set. After the rinsing, blotted tissues are analyzed for their cell viability through means of a WST-8 assay, in the same manner as for liquid chemicals.

4-1.3. Prediction Model of the Improved LabCyte24 EIT

As a reference for the *in vivo* eye irritancy classification of each chemical, we used the UN GHS classification (United Nations, 2003).

According to the UN GHS classification (No Category, Category 2 or Category 1), an irritant (Category 1 or 2) is predicted if the mean relative tissue viability of three individual tissues exposed to the test chemical is reduced below 40% of the mean viability of the negative controls (Table 4-1).

Table 4-1. Prediction model of LabCyte24 EIT

***In vitro* results**	**Prediction of eye irritation (UN GHS classification)**
Tissue viability is ≤ 40%	Category 1 or 2
Tissue viability is > 40%	No category

4-2. Description and Rationale for the Test Components of the Improved LabCyte24 EIT

4-2.1. Pre-Exposure Incubation

In the LabCyte24 EIT, the tissue is incubated for 15-30 hours before test chemical exposure.

On the other hand, in the OECD TG 492 (OECD, 2015a), before exposure to test chemicals, the tissue surface of the VRM is pretreated with 20 µL of D-PBS and incubated in the dark at standard culture conditions for 30 minutes to mimic the wetting conditions of human eye (pre-wetting treatment). The LabCyte24 EIT is not set such as pre-wetting treatment for not to be necessary, because the surface of LabCyte CORNEA-MODEL24 tissue has already been wetting condition for represent of corneal surface as shown below to the Section 2.2.

4-2.2. Duration of Chemical Exposure

4-2.2.1. Amount of Chemical Exposure

Exposure volume for Liquid chemical is set 50 µL and on the other hand the amount of Solid chemical is set 10 mg (Table 4-2).

Table 4-2. Amount of chemical application

Liquid chemical	Solid chemicals
50 µL	10 mg

LabCyte24 EIT, application amounts of both Liquid chemicals and Solid chemicals have been set up as sufficient amounts to cover the entire surface of LabCyte CORNEA-MODEL24 tissue.

4-2.2.2. Exposure Periods of Chemicals and Post-exposure incubation Period

Exposure period of test chemicals and post-exposure incubation period for Liquid test chemicals are set 1 minute and 24 hours, respectively (Table 4-3). On the other hands, exposure period for Solid test chemicals is 24 hours and post-exposure incubation periods is not

set (Table 4-3). These have been set throughout optimization study of LabCyte24 EIT as described below in the Section 3-1 and also the previous publication report (Katoh et. al, 2013).

Table 4-3. Exposure Periods of Chemicals and Post-exposure incubation Period

Process of exposure	Physical state of chemicals	
	Liquid	Solid
Chemical exposure periods	1 minuets	24 hours
Post-exposure incubation periods	24 hours	Not set

4-2.3. RhCE Tissue Replication

In the LabCyte24 EIT, the tissue is used each test chemical and each control substance in each run, respectively.

4-2.4. Endpoint(s)

As described below in Section 1, the LabCyte24 EIT was based on the cytotoxicity of RhCE tissue because the corneal epithelial tissues is one of the main target during accidental eye exposures, and damage to the corneal epithelial tissue can results in visual impairment or loss. In the LabCyte24 EIT, cytotoxicity is determined by the viability of the RhCE tissue. The viability of tissues is conventionally measured by WST-8 assay that is one of the tetrazolium reduction assay.

4-2.5. Appropriate Positive Control and Negative Control

4-2.5.1. Negative Control

Negative control is set for Liquid chemicals and Solid chemicals, respectively, because they are different assay condition between Liquid and Solid.

D-PBS is used as negative control for Liquid chemical. D-PBS is widely-used as negative control for the many cytotoxicity assays (Table 4-4).

Table 4-4. Negative control

Liquid	Solid
D-PBS	No application

For Solid chemicals, none treatment is set as negative control. None treatment is also set as negative control for the many cytotoxicity assays (Table 4-4).

4-2.5.2. Positive Control

Positive control is set for Liquid chemicals and Solid chemicals, respectively, because their assay conditions are different between Liquid and Solid.

Positive control for Liquid chemicals is used ethanol (Table 4-5).

Table 4-5. Chemical of positive control

Liquid	Solid
Ethanol (CAS no.: 64-17-5) GHS: Cat 2A	Lauric acid (CAS no.: 143-07-7) GHS: Cat 1

Positive control for Solid chemicals is Lauric acid (Table 4-5).

4-2.6. Acceptance Criteria

4-2.6.1. Negative Control

In the OECD TG 492, it is described that the acceptability range (upper and lower limit) for the negative control OD values (in the test method conditions) should be established by the RhCE tissue construct developer/supplier. As an example, the acceptability ranges for the negative control OD values for the LabCyte24 EIT given Table 4-6.

Table 4-6. Acceptability ranges for negative control

Lower acceptance limit	Upper acceptance limit
0.5 <	< 1.3

4-2.6.2. Positive Control

In the OECD TG 492, it is described that the acceptability range for the positive control cell viability should be established by the RhCE tissue construct developer/supplier. As an example, the acceptability ranges for the negative control OD values for the LabCyte24 EIT given Table 4-7

Table 4-7. Acceptability ranges for positive control

Liquid chemical	Solid chemicals
< 40%	

4-2.6.3. Cell Variability

Cell variability in the LabCyte24 EIT has been evaluated by calculated the mean±SD of the viability values with triplicate tissue performed is less than 20% as shown Table 4-8. If the SD of cell viabilities is more than 20%, an additional assay should be performed.

Table 4-8. Acceptability SD limit

Negative control	Positive control	Test chemicals
< 20%		

4-2.7. Applicability Domain and Limitation

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

Other limitation of this assay method is a possible interference of the test chemical with the WST-8 endpoint. A colored test chemical or one that directly reduces WST-8 (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the WST-8 endpoint. However, these test chemical are a problem only if at the time of the WST-8 test sufficient amounts of the test chemical are still present on (or in) the tissues. In case of this unlikely event, the (true) metabolic WST-8 reduction and the contribution by a colored test material or (false) direct WST-8 reduction by the test material can be quantified using freeze-killed RhCE tissues.

LabCyte24 EIT is applicable to substances and mixtures, and to solids, liquids, semi-solids and waxes as same applicability to OECD TG 492 VRM (OECD, 2015a). The liquids may be aqueous or non-aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be ground to a fine powder before application; no other pre-treatment of the sample is required. Gases and aerosols have not been assessed in a validation study of VRM of OECD TG 492. While it is conceivable that these can be tested using RhCE technology, the OECD TG 492 does not allow testing of gases and aerosols.

5. EVALUATION OF THE PREDICTIVE PERFORMANCE OF THE IMPROVED LabCyte24 EIT USING WIDE-RANGE OF TEST CHEMICALS

5-1. Purpose

In this study, we aimed to estimate the predictive performance of the improved LabCyte24 EIT method using 139 test chemicals over a wide range of chemical classes.

5-2. Materials and Methods

5-2.1. Chemical Selection of Wide-Range Classes

139 test chemicals over a wide range of classes were used for evaluation of the improved LabCyte24 EIT, as shown in Table 5-1. These chemicals were referred to in the following reports.

ECETOC database. (ECETOC, 1998)
Publicly-available documents (ICCVAM, 2006; OECD, 2015b)
Published report (Doucet et. al, 2006; Takahashi et. al, 2008; Takahashi et. al, 2009; Kaluzhny et. al, 2011; Kolle et. al, 2011)

Available information on irritation potential and classification according to the UN GHS systems are provided in Table 5-1, Table 5-2 and Table 5-3.

Table 5-1. Wide range of the test chemicals for evaluation of the LabCyte CORNEA-MODEL24 SIT

Chemical					
No	Name	CAS number[1)]	Category	Physical state	GHS class
1	Hydroxyethyl acrylate	818-61-1	Acrylate; Alcohol	Liquid	1
2	Tetraethylene glycol diacrylate	17831-71-9	Acrylate; Ether	Liquid	1
3	1-Butanol	71-36-3	Alcohol	Liquid	1
4	Cyclohexanol	108-93-0	Alcohol	Liquid	1
5	Benzyl alcohol	100-51-6	Alcohol	Liquid	1
6	(Ethylenediamine-propyl)-trimethoxysilane	1760-24-3	Aliphatic amine, primary; Aliphatic amine, secondary; Alkoxysilane	Liquid	1
7	Monoethanolamine	141-43-5	AlkanolAmine	Liquid	1
8	Diethylethanolamine	100-37-8	Amine	Liquid	1
9	Lactic acid	50-21-5	Carboxilic acid	Liquid	1
10	Acetic acid	64-19-7	Carboxilic acid	Liquid	1
11	Methylthioglycolate	2365-48-2	Carboxylic acid ester; Thioalcohol	Liquid	1
12	Methoxyethyl acrylate	3121-61-7	Ester	Liquid	1
13	Imidazole(20%)	288-32-4	Hydrocarbone	Liquid	1
14	Pyridine	110-86-1	N-containing heterocycle	Liquid	1
15	Di(2-ethylhexyl) sodium sulfosuccinate （10%）	577-11-7	Surfactant (anionic)	Liquid	1
16	Potassium laurate （10%）	10124-65-9	Surfactant (anionic)	Liquid	1
17	Cethylpyridinium bromide (6%)	140-72-7	Surfactant (cationic)	Liquid	1
18	Benzalkonium chloride (10%)	8001-54-5	Surfactant (cationic)	Liquid	1
19	Domiphen bromide （10%）	538-71-6	Surfactant (cationic)	Liquid	1
20	Cethylpyridinium chloride （10%）	6004-24-6	Surfactant (cationic)	Liquid	1
21	Stearyltrimethylammonium chloride （10%）	112-03-8	Surfactant (cationic)	Liquid	1
22	Cethyltrimethylammonium bromide （10%）	57-09-0	Surfactant (cationic)	Liquid	1
23	2,5-Dimethyl-2,5-hexanediol	110-03-2	Alcohol	Solid	1
24	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulphonate)	27344-41-8	Alkene; Biphenyl; Sulfonic acid	Solid	1
25	m-Phenylene diamine	108-45-2	Amine	Solid	1
26	Quinacrine	69-05-6	Amine	Solid	1
27	Chlorhexidine	55-56-1	Amine	Solid	1
28	1,3-Diiminobenz[f]isoindoline	65558-69-2	Amine	Solid	1

(Continue)

Chemical					
No	Name	CAS number[1)]	Category	Physical state	GHS class
29	1,2-Benzisothiazol-3(2H)-one	2634-33-5	Benzo-thiazolinone; Benzo-isothiazolinone	Solid	1
30	Paraformaldehyde	30525-89-4	Ether	Solid	1
31	Lauric acid	143-07-7	Fatty acid	Solid	1
32	Imidazole	288-32-4	Hydrocarbone	Solid	1
33	Tetraoctylammonium bromide	14866-33-2	Hydrocarbone	Solid	1
34	Captan 90-concentrate	133-06-2	Imide	Solid	1
35	Dibenzoyl-L-tartaric acid	2743-38-6	Organic acid	Solid	1
36	2,4-Dihydroxybenzoic acid	89-86-1	Organic acid	Solid	1
37	Sodium salicylate	54-21-7	Organic salt	Solid	1
38	Promethazine hydrochloride	58-33-3	Organic salt	Solid	1
39	Sodium oxalate	62-76-0	Oxocarboxylic acid	Solid	1
40	1-naphtalen acetic acid	86-87-3	Pesticide	Solid	1
41	p-t-Butylphenol	98-54-4	Phenol	Solid	1
42	Benzalkonium chloride (1%)	8001-54-5	Surfactant (cationic)	Liquid	2
43	Diisopropanolamine	110-97-4	AlkanolAmine	Solid	2
44	Glycolic acid	79-14-1	Carboxilic acid	Solid	2
45	Sodium Perborate Tetrahydrate	10486-00-7	Inorganic salt	Solid	2
46	Calcium thioglycollate	814-71-1	Organic salt	Solid	2
47	Ethanol	64-17-5	Alcohol	Liquid	2A
48	Isopropylalcohol	67-63-0	Alcohol	Liquid	2A
49	2-Benzyloxyethanol	622-08-2	Alcohol	Liquid	2A
50	1-Octanol	111-87-5	Alcohol	Liquid	2A
51	2-Ethyl-1-hexanol	104-76-7	Alcohol	Liquid	2A
52	n-Hexanol	111-27-3	Alcohol	Liquid	2A
53	Isobutanol	78-83-1	Alcohol	Liquid	2A
54	2,4,11,13-Tetraazatetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous)	18472-51-0	Aromatic heterocyclic halide; Aryl halide; Dihydroxyl group; Guanidine	Liquid	2A
55	Acetone	67-64-1	Ketone	Liquid	2A
56	Methyl ethyl ketone	78-93-3	Ketone	Liquid	2A

(Continue)

Chemical					
No	Name	CAS number[1)]	Category	Physical state	GHS class
57	gamma-Butyrolactone	21645-51-2	Lactone; Oxolane; Saturated heterocyclic fragment	Liquid	2A
58	Sodium dodecyl sulfate(15%)	151-21-3	Surfactant (anionic)	Liquid	2A
59	Cethylpyridinium bromide (1%)	140-72-7	Surfactant (cationic)	Liquid	2A
60	Triton X-100 (5%)	9002-93-1	Surfactant (nonionic)	Liquid	2A
61	Sodium benzoate	532-32-1	Aryl; Carboxylic acid	Solid	2A
62	1,5-Naphthalenediol	83-56-7	Fused carbocyclic aromatic; Naphthalene; Phenol	Solid	2A
63	p-Formylbenzoic acid	619-66-9	Organic acid	Solid	2A
64	2-Methyl-1-pentanol	105-30-6	Alcohol; Alkane, branched with tertiary carbon	Liquid	2B
65	Diethyl toluamide	134-62-3	Benzamide	Liquid	2B
66	Methyl acetate	79-20-9	Ester	Liquid	2B
67	Sodium hydroxide (1%)	1310-73-2	Inorganic base	Liquid	2B
68	2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	79-92-5	Alkane, branched with tertiary carbon; Alkene; Bicycloheptane	Solid	2B
69	1,4-Dibutoxy benzene	104-36-9	Alkoxy; Aryl; Ether	Solid	2B
70	2,6-Dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate	96568-04-6	Amine	Solid	2B
71	Maneb	12427-38-2	Amine	Solid	2B
72	Ammonium Nitrate	6484-52-2	Inorganic salt	Solid	2B
73	m-Dinitrobenzene	99-65-0	Nitro compound	Solid	2B
74	p-Nitrobenzoic acid	62-23-7	Organic acid	Solid	2B
75	Sodium chloroacetate	3926-62-3	Organic salt	Solid	2B
76	3,3-Dithiodipropionic Acid	1119-62-6	Sulphur-containing compound	Solid	2B
77	Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Acylal; Alcohol; Allyl; Ether	Liquid (Viscous)	NC
78	Propylene glycol	57-55-6	Alcohol	Liquid	NC
79	3-Methoxy-1,2-propanediol	623-39-2	Alcohol	Liquid	NC
80	Ethanol (10%)	64-17-5	Alcohol	Liquid	NC
81	3-Phenoxybenzyl alcohol	13826-35-2	Alcohol; Benzyl; Ether	Liquid	NC
82	4-(Methylthio)-benzaldehyde	3446-89-7	Aldehyde; Aryl; Sulfide	Liquid	NC
83	3,3-Dimethylpentane	562-49-2	Alkane	Liquid	NC
84	Triethanolamine	102-71-6	AlkanolAmine	Liquid	NC

(Continue)

Chemical					
No	Name	CAS number[1)]	Category	Physical state	GHS class
85	1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	Alkoxy; Ammonium salt; Aryl; Imidazole; Sulphate	Liquid	NC
86	Piperonyl butoxide	51-03-6	Alkoxy; Benzodioxole; Benzyl; Ether	Liquid	NC
87	n-Octyl Ether	629-82-3	Alkoxy; Ether	Liquid	NC
88	2-Ethoxyethyl methacrylate	2370-63-0	Alkoxy; Ether; Methacrylate	Liquid	NC
89	Xylene	1330-20-7	Aromatic	Liquid	NC
90	1,6-Dibromohexane	629-03-8	Brominated Derivative	Liquid	NC
91	1-bromo-4chlorobutane	6940-78-9	Brominated Derivative	Liquid	NC
92	Ethyl thioglycolate	623-51-8	Carboxylic acid ester; Thioalcohol	Liquid	NC
93	Methyl cycropentane	96-37-7	Cycloalkane	Liquid	NC
94	Dipropyl disulphide	629-19-6	Disulfide	Liquid	NC
95	Isopropyl myristate	110-27-0	Ester	Liquid	NC
96	Ethyl acetate	141-78-6	Ester	Liquid	NC
97	Toluene	108-88-3	Hydrocarbone	Liquid	NC
98	2-Methylpentane	107-83-5	Hydrocarbone	Liquid	NC
99	n-Octyl bromide	111-83-1	Hydrocarbone	Liquid	NC
100	cis-Cyclooctene	931-87-3	Hydrocarbone	Liquid	NC
101	Dodecane	112-40-3	Hydrocarbone	Liquid	NC
102	Styrene	100-42-5	Hydrocarbone	Liquid	NC
103	Methyl isobutyl ketone	108-10-1	Ketone	Liquid	NC
104	2-Heptanone(Methyl amylketone)	110-43-0	Ketone	Liquid	NC
105	2-Ethylhexyl p-dimethylamino benzoate	21245-02-3	PABA derivatives	Liquid	NC
106	Glycerol	56-81-5	Polyol	Liquid	NC
107	Polyethylene glycol 400	25322-68-3	Polyol	Liquid	NC
108	Sodium N-lauroyl sarcosinate (30%)	137-16-6	Surfactant (anionic)	Liquid	NC
109	Sodium dodecyl sulfate(1%)	151-21-3	Surfactant (anionic)	Liquid	NC
110	Benzalkonium chloride (0.01%)	8001-54-5	Surfactant (cationic)	Liquid	NC
111	Cethylpyridinium bromide (0.1%)	140-72-7	Surfactant (cationic)	Liquid	NC
112	Benzalkonium chloride (0.1%)	8001-54-5	Surfactant (cationic)	Liquid	NC

(Continue)

Chemical					
No	Name	CAS number[1)]	Category	Physical state	GHS class
113	Tween20	9005-64-5	Surfactant (nonionic)	Liquid	NC
114	Tween80	9005-65-6	Surfactant (nonionic)	Liquid	NC
115	Polyethylene glycol monolaurate (10E.O.)	31943-11-0	Surfactant (nonionic)	Liquid	NC
116	Tween80(10%)	9005-65-6	Surfactant (nonionic)	Liquid	NC
117	3,4-dimethoxy benzaldehyde	120-14-9	Aldehyde; Aryl; Ether	Solid	NC
118	Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	68610-92-4	Alcohol; Ammonium salt; Ether	Solid	NC
119	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol	3179-89-3	Alcohol; Aromatic amine; Azo; Nitrobenzene	Solid	NC
120	2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	Alkane branched with quaternary carbon	Solid	NC
121	2-Mercaptopyrimidine	1450-85-7	Amine	Solid	NC
122	Tetraaminopyrimidine sulfate	5392-28-9	Amine	Solid	NC
123	Ethylenediamine-tetraacetic acid dipotassium salt dihydrate	25102-12-9	Amine	Solid	NC
124	Phenothiazine	92-84-2	Amine	Solid	NC
125	Het Anhydride	115-27-5	Anhydryde	Solid	NC
126	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	Aromatic heterocyclic halide; Aryl halide; Urea derivatives	Solid	NC
127	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	118-82-1	Benzyl; Phenol; tert-Butyl	Solid	NC
128	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	66170-10-3	Dihydroxyl group; Enol; Furanone; Furanondione; Phosphate ester	Solid	NC
129	D(+)-Glucono-1,5-lactone	90-80-2	Ester	Solid	NC
130	Propyl p-hydroxybenzoate	94-13-3	Ester	Solid	NC
131	Anthracene	120-12-7	Hydrocarbone	Solid	NC
132	2,2'-Iminodibenzyl	494-19-9	Hydrocarbone	Solid	NC
133	Silicic acid	7699-41-4	Inorganic	Solid	NC
134	Aluminium hydroxide, 50-57%	21645-51-2	Inorganic base	Solid	NC
135	Potassium tetrafluoroborate	14075-53-7	Inorganic Salt	Solid	NC
136	Magnesium carbonate hydroxide pentahydrate	56378-72-4	Inorganic salt	Solid	NC
137	2,3-Dimethyl-2,3-dinitrobutane	3964-18-9	Nitro compound	Solid	NC
138	2,4-Dichloro-5-sulfamoylbenzoic Acid	2736-23-4	Organic acid	Solid	NC
139	N, N-Dimethylguanidine sulfate	598-65-2	Organic salt	Solid	NC

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

2) *In vivo* class was referred from the GHS classification.

NC: No category (non-eye irritant); 2: Category 2 (eye irritant); 1: Category 1 (eye corrosion)

Table 5-2. Category of the test chemicals for evaluation of the LabCyte24 EIT

Category	Number	Category	Number
Acrylate	2	Fatty acids	1
Alcohols	19	Hydrocarbons	11
Aldehydes	2	Imide	1
Aliphatic amine	1	Inorganic	1
Alkane	3	Inorganic base	2
Alkanol amine	3	Inorganic salts	4
Alkene	1	Ketones/Lactones	5
Alkoxy	5	N-containing heterocycle	1
Amines	11	Nitro compound	2
Anhydryde	1	Organic acid	5
Aromatic	3	Organic salts	5
Benzamide	1	Oxocarboxylic acid	1
Benzo-thiazolinone	1	PAPA derivatives	1
Benzyl	1	Pesticide	1
Brominated derivative	2	Phenol	1
Carboxylic acids	6	Polyol	2
Cycloalkane	1	Sulphur-containing compound	1
Dihydroxyl group	1	Surfactants (anionic)	5
Disulphides	1	Surfactants(cationic)	11
Esters	7	Surfactants(nonionic)	5
Fused carbocyclic aromatic	1		

Table 5-3. Number by physical state and UN GHS classification of the wide range chemicals

UN GHS classification	Liquid Chemicals	Solid Chemicals	Total
Category 1 or 2	41	35	76
No category	40	23	63
Total	81	58	139

5-2.2. Protocol of the improved LabCyte24 EIT

Prediction of the eye irritation potential of test chemicals by the improved LabCyte24 EIT was performed according to the improved protocol described in the SOP for ver.2.5.2 (Appendix 1) and described as follows.

The LabCyte CORNEA-MODEL24 tissues is shipped from to recipients, the tissues are aseptically removed from the transport agarose medium, transfer into wells on 24-well plates with the assay medium (500 µL) and pre-incubated overnight at 37°C in a humidified atmosphere of 5 % CO_2 in air.

5-2.2.1. The Improved LabCyte24 EIT Procedure for Liquid Chemicals

After the pre-incubation, the tissue was topically exposed to the Liquid test chemicals. Liquids (50 µL) were applied with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues were treated with 50 µL of D-PBS serving as negative controls and ethanol serving as positive controls. And then tissues applied chemicals were incubated for 1 minute. After exposure, each tissue was rinsed with D-PBS ten times or more, using a washing bottle to remove any remaining test chemical from the tissue surface. The blotted tissues were then transferred to new wells on 24-well plates containing 500 µL of fresh assay medium. And then the tissues were post-incubated for 24 hours under the standard cultivation conditions (at 37°C in a humidified atmosphere of 5% CO_2 in air). After the post-exposure incubation periods, blotted tissues were transferred to new wells on 24-well plates containing 300 µL of freshly prepared WST-8 solution for a WST-8 assay (Ishiyama et. al., 1997; Tominaga et. al., 1999). WST-8 solution was prepared by 1:10 dilution of Cell Counting Kit-8 (Dojindo Co., Japan) with EBSS (Sigma-Aldrich, MO, USA). Tissues were incubated for 4 hours under the standard cultivation conditions (at 37°C in a humidified atmosphere of 5% CO_2 in air). Subsequently, 200 µL of culture supernatant were transferred to a 96-well microtiter plate. The OD value of culture supernatant was measured at 450 nm and at 650 nm as a reference absorbance, with WST-8 solution as a blank.

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

The tissue viability was calculated as a percentage relative to the viability of negative controls.

The mean of the three values from identically treated tissues was used to classify a chemical according to the prediction model.

5-2.2.2. The Improved LabCyte24 EIT Procedure for Solid Chemicals

After pre-incubation, the medium was changed to 500 μL of fresh assay medium and then the tissues were topically exposed to the Solid test chemicals. Solids (10 mg) in a microtube were applied by decantation. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were set apart as non-treated samples and Lauric acid (10mg) served as positive control. And then tissues were incubated for 24 hours. After exposure, each tissue was rinsed with D-PBS in the same manner as that of Liquid chemicals. In the Solid chemicals, post-exposure incubation was not set. After the rinsing, blotted tissues were analyzed for their cell viability through means of a WST-8 assay, in the same manner as for Liquid chemicals.

5-2.2.3. Prediction Model of the Improved LabCyte24 EIT

As a reference for the *in vivo* eye irritancy classification of each chemical, we used the United Nations globally harmonized system (GHS) classification (United Nations, 2003).

According to the GHS classification (No Category, Category 2 or Category 1), an irritant (Category 1 or 2) was predicted if the mean relative tissue viability of three individual tissues exposed to the test chemical is reduced below 40% of the mean viability of the negative controls (Table 5-4).

Table 5-4. Prediction model of the improved LabCyte24 EIT

***In vitro* results**	**Prediction of eye irritation (UN-GHS classification)**
Tissue viability is ≤ 40%	Category 1 or 2
Tissue viability is > 40%	No category

5-2.2.4. Detecting chemical interference with WST-8 endpoints and correction procedures

A possible limitation of this eye irritation protocol describe in the Section 3-3 might be due to the effect of a small amount of test chemicals on the WST-8 endpoints directly. The following two types of test chemicals can interfere with the WST-8 assay.

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

Test chemical that stains the corneal epithelial tissues has a possibility to transfer from the corneal epithelial tissues to the WST-8 reaction buffer and to affect the OD value measurements.

Test chemical that is able to directly reduce WST-8 can affect the OD value measurements, if the test chemical is present in the corneal epithelial tissues when the WST-8 viability test is performed.

Detection procedure of these test chemicals is described below.

5-2.2.4a. STEP 1 (PRELIMINARY TEST)

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

Fifty-µL (Liquid) or 10mg (Solid) of the test chemical was added into wells of 24-well assay plate pre-filled with 0.3mL of WST-8 solution. Untreated the WST-8 solution was used as control. Close the lid of 24-well assay plate was closed and the mixture is incubated in CO_2 incubator for about 4 hours. After incubation, the mixture was gently shaken and the staining of the diluted WST-8 medium was evaluated macroscopically.

5-2.2.4b. STEP 2 (FUNCTIONAL CHECK ON VIABLE TISSUE)

Fifty-µL (Liquid) or 10mg (Solid) of the test chemical, which clearly changed the color of the diluted WST-8 solution at preliminary test, was added onto the surface of the corneal epithelial tissues. All procedures of the LabCyte24 EIT described in section from 5-2.2.1 to 5-2.2.3 was performed, although the corneal epithelial tissues that have been freeze-killed at -80 °C or lower for 30 minutes twice instead of viable corneal epithelial tissues was used. The tissue corrected

OD was calculated as following;

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

If each tissue corrected OD is below 0, its tissue OD was regarded as 0. When the cell viability (%), which was calculated according to the procedures described in Section 5-2.2.3, is <40%, the test chemical is determined as GHS category 1 or 2 and therefore correction of the results is not necessary.

5-3. Results

5-3.1. Detecting chemical interference with WST-8 endpoints and correction

First, 139 chemicals were examined to detect interference with WST-8 endpoints. Of the 139 tested chemicals, in the experiment for direct WST-8 reduction (5-2.2.4a, STEP 1).

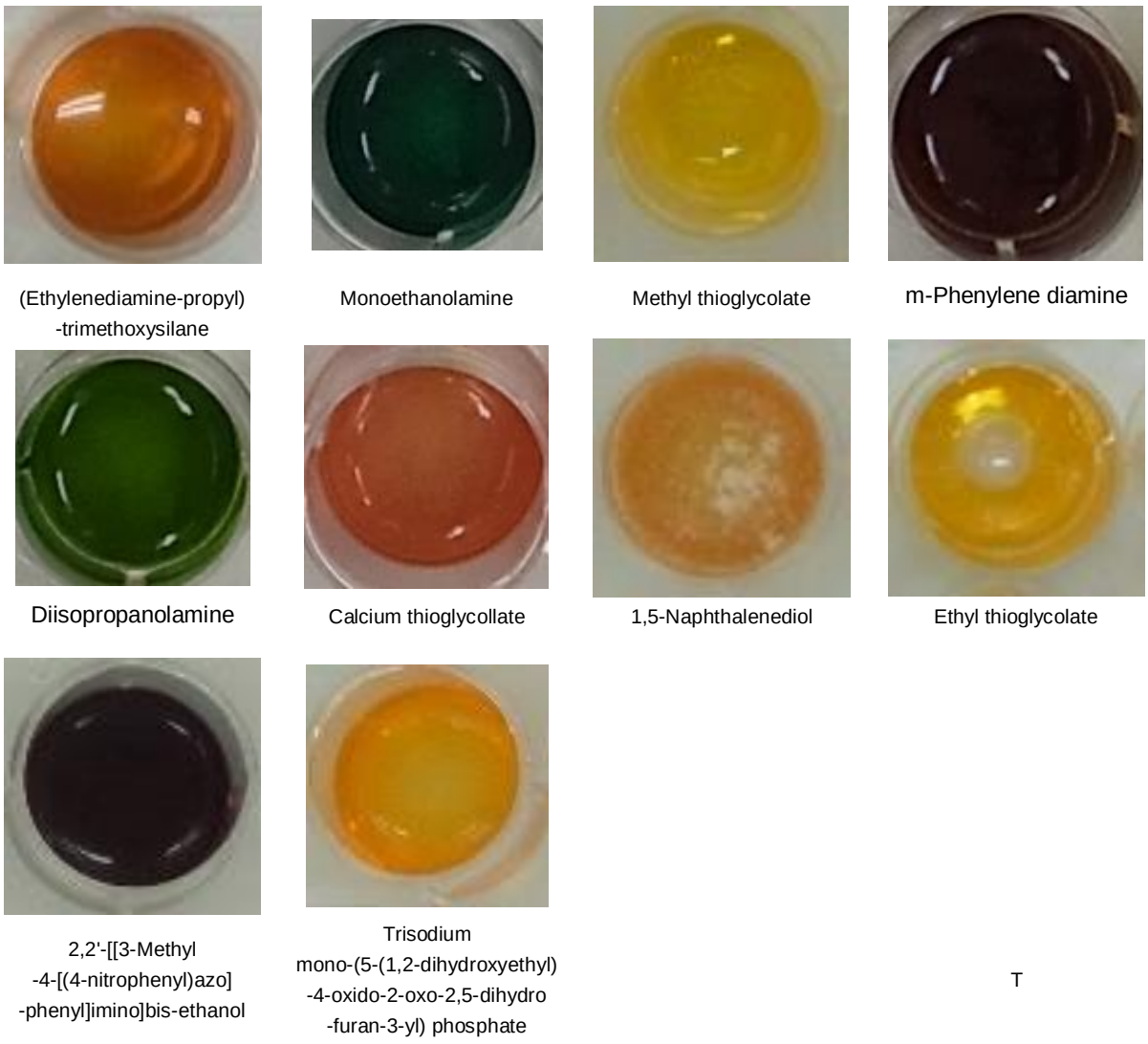


Fig.5-1. Direct WST-8 reduction ability (STEP 1).

As shown in Fig.5-1, (Ethylenediamine-propyl)-trimethoxysilane (no.6), Monoethanolamine (no.7), Methyl thioglycolate (no.11), m-Phenylene diamine (no.25), Diisopropanolamine (no.43), Calcium thioglycollate (no.46), 1,5-Naphthalenediol (no.62), Ethyl thioglycolate (no.92),

2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (no.119), Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate (no.128) were detected as potential WST-8 interference.

Table 5-5. Detection of WST-8 reducer in 139 chemicals

	Positive chemicals in step 1	Cell viability (%: not corrected)	WST-8 interfere (%)	Corrected viability (%)
6	(Ethylenediamine-propyl)-trimethoxysilane	9	3	4
7	Monoethanolamine	6	3	1
11	Methyl thioglycolate	279	360	0
25	m-Phenylene diamine	24	16	1
43	Diisopropanolamine	6	4	1
46	Calcium thioglycollate	19	2	16
62	1,5-Naphthalenediol	14	183	0
92	Ethyl thioglycolate	177	188	14
119	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol	0	0	0
128	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	1	2	0

It was examined whether these chemical reduced WST-8 using freeze-killed tissue instead of living tissue. As a result (Table 5-5), Methyl thioglycolate (no.11) and Ethyl tioglycolate (no.92) were strongly reduced WST-8 and therefore stained over 100%. Even if such chemicals with strong interference WST-8, the cell viabilities of Methyl thioglycolate and Ethyl tioglycolate after corrected showed 0% and 1.4%, respectively. Prediction results of both chemicals were eye irritant and parallel with a prediction result of EpiOcular EIT (the OECD TG492 VRM).

Other 8 chemicals shown WST-8 assay interference, (Ethylenediamine-propyl)-trimethoxysilane (no.6), Monoethanolamine (no.7), m-Phenylene diamine (no.25), Diisopropanolamine (no.43), Calcium thioglycollate (no.46),

1,5-Naphthalenediol (no.62), 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (no.119), Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate (no.128), were all shown lower than 40%, so these chemicals were finally determined as irritants (Table 5-4).

5-3.2. Predictivity of the Improved LabCyte24 EIT

The performance of the improved LabCyte24 EIT was evaluated with the 139 test chemicals. Results obtained with each chemical are given in 5-5.

2-Ethoxyethyl methacrylate (no 88), 1-bromo-4chlorobutane (no. 91), Toluene (no. 97) and 2-Heptanonee (no. 104) showed different predictions in three independent test runs. Concordance with classifications obtained within-laboratory had a sufficient level of reproducibility for the improved LabCyte24 EIT at 97% (135/139).

In the group of 63 test chemicals (no.77 to no.139) that were classified No Category (not classified for irritation) in the GHS classification, positive predictions for 17 test chemicals, 2-Ethoxyethyl methacrylate (no.88), Ethyl thioglycolate (no.92), Ethyl acetate (no.96), Toluene (no.97), Styrene (no.102), Methyl isobutyl ketone (no.103), 2-Heptanone (no.104), 3,4-dimethoxy benzaldehyde (no.117), Cellulose, 2-(2-hydroxy-3-(trimethylammonium) propoxy)ethyl ether chloride (91%; no.118), 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol (no.119), Ethylenediamine-tetraacetic acid dipotassium salt dehydrate (no.123), Het Anhydride (no.125), Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate (no.128), D(+)-Glucono-1,5-lactone (no.129), Propyl p-hydroxybenzoate (no.130), 2,4-Dichloro-5-sulfamoylbenzoic Acid (no.138), and N, N-Dimethylguanidine sulfate (no.139) were not concordant with the results of the *in vivo* classification (Table 5-5).

In the group of 76 test chemicals (no.1 to no.75) that were classified Category 1 or 2 (irritant) in GHS classification, no chemicals were wrongly predicted as non-irritant by the LabCyte24 EIT (Table 5-6).

Table 5-6. Wide range of the test chemicals for evaluation of the LabCyte24 EIT

Chemical			LabCyte CORNEA-MODEL EIT							
			R1		R2		R3		Mean	
No	Name	GHS class	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge
1	Hydroxyethyl acrylate	1	3.9 ± 2.0	Cat2/1	5.6 ± 1.9	Cat2/1	5.7 ± 2.4	Cat2/1	5.0 ± 1.0	Cat2/1
2	Tetraethylene glycol diacrylate	1	28.6 ± 8.6	Cat2/1	22.8 ± 4.5	Cat2/1	24.9 ± 4.4	Cat2/1	25.4 ± 2.9	Cat2/1
3	1-Butanol	1	4.9 ± 0.9	Cat2/1	14.9 ± 2.5	Cat2/1	18.0 ± 2.0	Cat2/1	12.6 ± 6.8	Cat2/1
4	Cyclohexanol	1	16.8 ± 0.8	Cat2/1	14.2 ± 5.7	Cat2/1	16.3 ± 1.9	Cat2/1	15.8 ± 1.4	Cat2/1
5	Benzyl alcohol	1	2.8 ± 0.5	Cat2/1	9.6 ± 1.3	Cat2/1	13.2 ± 1.1	Cat2/1	8.5 ± 5.3	Cat2/1
6	(Ethylenediamine-propyl)-trimethoxysilane	1	8.5 ± 3.8	Cat2/1	5.2 ± 1.2	Cat2/1	5.2 ± 2.5	Cat2/1	6.3 ± 1.9	Cat2/1
7	Monoethanolamine	1	2.9 ± 0.2	Cat2/1	2.5 ± 1.0	Cat2/1	3.0 ± 0.3	Cat2/1	2.8 ± 0.2	Cat2/1
8	Diethylethanolamine	1	2.7 ± 0.2	Cat2/1	5.8 ± 0.6	Cat2/1	2.6 ± 0.3	Cat2/1	3.7 ± 1.9	Cat2/1
9	Lactic acid	1	2.9 ± 0.4	Cat2/1	3.2 ± 0.5	Cat2/1	5.5 ± 0.1	Cat2/1	3.9 ± 1.4	Cat2/1
10	Acetic acid	1	4.2 ± 0.3	Cat2/1	2.8 ± 0.4	Cat2/1	5.3 ± 0.1	Cat2/1	4.1 ± 1.3	Cat2/1
11	Methylthioglycolate	1	0.0 ± 0.0	Cat2/1	3.0 ± 5.3	Cat2/1	0.0 ± 0.0	Cat2/1	1.0 ± 1.8	Cat2/1
12	Methoxyethyl acrylate	1	2.5 ± 0.9	Cat2/1	8.6 ± 1.1	Cat2/1	6.7 ± 0.8	Cat2/1	6.0 ± 3.1	Cat2/1
13	Imidazole(20%)	1	12.4 ± 0.6	Cat2/1	23.1 ± 4.7	Cat2/1	24.7 ± 3.3	Cat2/1	20.0 ± 6.7	Cat2/1
14	Pyridine	1	2.1 ± 0.3	Cat2/1	1.2 ± 0.7	Cat2/1	2.3 ± 0.2	Cat2/1	1.9 ± 0.6	Cat2/1
15	Di(2-ethylhexyl) sodium sulfosuccinate（10%）	1	4.1 ± 0.7	Cat2/1	14.0 ± 4.8	Cat2/1	15.4 ± 0.8	Cat2/1	11.2 ± 6.2	Cat2/1
16	Potassium laurate（10%）	1	32.7 ± 1.5	Cat2/1	8.6 ± 6.7	Cat2/1	20.2 ± 12.1	Cat2/1	20.5 ± 12.1	Cat2/1
17	Cethylpyridinium bromide (6%)	1	15.6 ± 8.0	Cat2/1	15.5 ± 1.2	Cat2/1	11.7 ± 4.0	Cat2/1	14.3 ± 2.2	Cat2/1
18	Benzalkonium chloride (10%)	1	0.7 ± 0.8	Cat2/1	2.7 ± 1.0	Cat2/1	1.3 ± 1.1	Cat2/1	1.6 ± 1.0	Cat2/1
19	Domiphen bromide（10%）	1	0.8 ± 0.1	Cat2/1	0.9 ± 0.0	Cat2/1	0.4 ± 0.4	Cat2/1	0.7 ± 0.2	Cat2/1
20	Cethylpyridinium chloride（10%）	1	2.9 ± 0.4	Cat2/1	7.2 ± 1.3	Cat2/1	22.8 ± 0.4	Cat2/1	11.0 ± 10.5	Cat2/1
21	Stearyltrimethylammonium chloride（10%）	1	9.0 ± 0.7	Cat2/1	16.7 ± 1.0	Cat2/1	37.1 ± 0.4	Cat2/1	20.9 ± 14.5	Cat2/1
22	Cethyltrimethylammonium bromide（10%）	1	7.0 ± 2.3	Cat2/1	12.1 ± 10.8	Cat2/1	29.7 ± 0.4	Cat2/1	16.3 ± 11.9	Cat2/1
23	2,5-Dimethyl-2,5-hexanediol	1	3.1 ± 1.5	Cat2/1	2.3 ± 0.6	Cat2/1	0.8 ± 0.4	Cat2/1	2.1 ± 1.2	Cat2/1
24	Disodium 2,2'-([1,1'-biphenyl]-4,4'-diyldivinylene)bis-(benzenesulphonate)	1	3.8 ± 0.5	Cat2/1	4.1 ± 0.3	Cat2/1	1.7 ± 0.4	Cat2/1	3.2 ± 1.3	Cat2/1
25	m-Phenylene diamine	1	8.9 ± 0.4	Cat2/1	2.0 ± 0.9	Cat2/1	1.4 ± 0.5	Cat2/1	4.1 ± 4.2	Cat2/1
26	Quinacrine	1	3.7 ± 4.1	Cat2/1	10.1 ± 6.6	Cat2/1	6.0 ± 0.7	Cat2/1	6.6 ± 3.3	Cat2/1
27	Chlorhexidine	1	6.9 ± 2.6	Cat2/1	2.6 ± 2.6	Cat2/1	0.7 ± 0.4	Cat2/1	3.4 ± 3.2	Cat2/1
28	1,3-Diiminobenz[f]isoindoline	1	1.5 ± 0.2	Cat2/1	3.2 ± 1.2	Cat2/1	0.7 ± 0.2	Cat2/1	1.8 ± 1.3	Cat2/1
29	1,2-Benzisothiazol-3(2H)-one	1	19.6 ± 1.9	Cat2/1	16.1 ± 9.7	Cat2/1	16.4 ± 11.0	Cat2/1	17.4 ± 1.9	Cat2/1
30	Paraformaldehyde	1	0.9 ± 0.3	Cat2/1	8.9 ± 1.1	Cat2/1	3.5 ± 4.7	Cat2/1	4.4 ± 4.1	Cat2/1

(Continue)

Chemical			LabCyte CORNEA-MODEL EIT							
			R1		R2		R3		Mean	
No	Name	GHS class	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge
29	1,2-Benzisothiazol-3(2H)-one	1	19.6 ± 1.9	Cat2/1	16.1 ± 9.7	Cat2/1	16.4 ± 11.0	Cat2/1	17.4 ± 1.9	Cat2/1
30	Paraformaldehyde	1	0.9 ± 0.3	Cat2/1	8.9 ± 1.1	Cat2/1	3.5 ± 4.7	Cat2/1	4.4 ± 4.1	Cat2/1
31	Lauric acid	1	2.1 ± 0.8	Cat2/1	9.3 ± 0.2	Cat2/1	4.4 ± 4.3	Cat2/1	5.3 ± 3.7	Cat2/1
32	Imidazole	1	1.1 ± 1.1	Cat2/1	8.5 ± 0.1	Cat2/1	3.5 ± 4.4	Cat2/1	4.4 ± 3.8	Cat2/1
33	Tetraoctylammonium bromide	1	13.3 ± 4.5	Cat2/1	16.7 ± 2.5	Cat2/1	14.4 ± 2.0	Cat2/1	14.8 ± 1.7	Cat2/1
34	Captan 90-concentrate	1	12.8 ± 0.8	Cat2/1	5.3 ± 2.7	Cat2/1	3.2 ± 0.7	Cat2/1	7.1 ± 5.0	Cat2/1
35	Dibenzoyl-L-tartaric acid	1	8.9 ± 1.4	Cat2/1	0.6 ± 0.2	Cat2/1	0.2 ± 0.1	Cat2/1	3.2 ± 4.9	Cat2/1
36	2,4-Dihydroxybenzoic acid	1	8.5 ± 0.5	Cat2/1	0.0 ± 0.1	Cat2/1	0.8 ± 0.6	Cat2/1	3.1 ± 4.7	Cat2/1
37	Sodium salicylate	1	9.6 ± 0.9	Cat2/1	1.6 ± 0.3	Cat2/1	2.8 ± 0.4	Cat2/1	4.7 ± 4.4	Cat2/1
38	Promethazine hydrochloride	1	11.8 ± 2.2	Cat2/1	5.0 ± 0.6	Cat2/1	5.4 ± 2.3	Cat2/1	7.4 ± 3.8	Cat2/1
39	Sodium oxalate	1	2.3 ± 1.9	Cat2/1	4.8 ± 0.7	Cat2/1	4.2 ± 2.5	Cat2/1	3.8 ± 1.3	Cat2/1
40	1-naphtalen acetic acid	1	0.1 ± 0.1	Cat2/1	9.4 ± 1.6	Cat2/1	1.8 ± 0.1	Cat2/1	3.8 ± 5.0	Cat2/1
41	p-t-Butylphenol	1	8.5 ± 0.6	Cat2/1	0.1 ± 0.1	Cat2/1	1.3 ± 0.1	Cat2/1	3.3 ± 4.5	Cat2/1
42	Benzalkonium chloride (1%)	2	3.0 ± 1.1	Cat2/1	7.3 ± 4.4	Cat2/1	6.3 ± 4.1	Cat2/1	5.5 ± 2.3	Cat2/1
43	Diisopropanolamine	2	1.4 ± 0.1	Cat2/1	0.8 ± 0.1	Cat2/1	0.5 ± 0.4	Cat2/1	0.9 ± 0.5	Cat2/1
44	Glycolic acid	2	1.1 ± 0.6	Cat2/1	8.3 ± 0.3	Cat2/1	4.1 ± 3.7	Cat2/1	4.5 ± 3.6	Cat2/1
45	Sodium Perborate Tetrahydrate	2	9.3 ± 0.6	Cat2/1	1.4 ± 0.0	Cat2/1	3.1 ± 0.2	Cat2/1	4.6 ± 4.2	Cat2/1
46	Calcium thioglycollate	2	14.4 ± 6.6	Cat2/1	17.9 ± 3.0	Cat2/1	27.8 ± 1.2	Cat2/1	20.1 ± 7.0	Cat2/1
47	Ethanol	2A	13.8 ± 2.3	Cat2/1	12.3 ± 2.5	Cat2/1	13.5 ± 5.0	Cat2/1	13.2 ± 0.8	Cat2/1
48	Isopropylalcohol	2A	7.2 ± 2.9	Cat2/1	6.6 ± 5.0	Cat2/1	8.0 ± 2.2	Cat2/1	7.3 ± 0.7	Cat2/1
49	2-Benzyloxyethanol	2A	5.3 ± 1.1	Cat2/1	33.1 ± 7.4	Cat2/1	12.6 ± 1.4	Cat2/1	17.0 ± 14.4	Cat2/1
50	1-Octanol	2A	3.2 ± 2.1	Cat2/1	4.7 ± 1.1	Cat2/1	4.1 ± 0.4	Cat2/1	4.0 ± 0.7	Cat2/1
51	2-Ethyl-1-hexanol	2A	2.2 ± 0.8	Cat2/1	4.8 ± 0.7	Cat2/1	3.0 ± 0.9	Cat2/1	3.4 ± 1.3	Cat2/1
52	n-Hexanol	2A	25.3 ± 5.5	Cat2/1	38.0 ± 1.3	Cat2/1	19.0 ± 1.4	Cat2/1	27.4 ± 9.7	Cat2/1
53	Isobutanol	2A	10.1 ± 3.9	Cat2/1	23.4 ± 4.9	Cat2/1	15.3 ± 1.4	Cat2/1	16.3 ± 6.7	Cat2/1
54	2,4,11,13-Tetraazatetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous)	2A	1.1 ± 0.2	Cat2/1	18.0 ± 2.5	Cat2/1	20.2 ± 2.9	Cat2/1	13.1 ± 10.5	Cat2/1
55	Acetone	2A	23.9 ± 7.8	Cat2/1	9.3 ± 0.2	Cat2/1	5.7 ± 2.5	Cat2/1	12.9 ± 9.7	Cat2/1
56	Methyl ethyl ketone	2A	7.1 ± 1.6	Cat2/1	14.3 ± 4.8	Cat2/1	13.9 ± 0.8	Cat2/1	11.8 ± 4.1	Cat2/1

(Continue)

Chemical			LabCyte CORNEA-MODEL EIT							
			R1		R2		R3		Mean	
No	Name	GHS class	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge
57	gamma-Butyrolactone	2A	7.2 ± 2.3	Cat2/1	9.2 ± 2.7	Cat2/1	6.3 ± 2.5	Cat2/1	7.5 ± 1.5	Cat2/1
58	Sodium dodecyl sulfate(15%)	2A	34.9 ± 5.6	Cat2/1	25.8 ± 10.5	Cat2/1	28.6 ± 7.9	Cat2/1	29.8 ± 4.7	Cat2/1
59	Cethylpyridinium bromide (1%)	2A	11.6 ± 0.6	Cat2/1	22.4 ± 0.4	Cat2/1	28.6 ± 0.9	Cat2/1	20.9 ± 8.6	Cat2/1
60	Triton X-100 (5%)	2A	0.9 ± 0.4	Cat2/1	17.8 ± 1.3	Cat2/1	19.3 ± 5.4	Cat2/1	12.7 ± 10.2	Cat2/1
61	Sodium benzoate	2A	2.1 ± 1.2	Cat2/1	3.5 ± 0.4	Cat2/1	1.7 ± 0.3	Cat2/1	2.4 ± 0.9	Cat2/1
62	1,5-Naphthalenediol	2A	6.7 ± 5.4	Cat2/1	19.8 ± 6.9	Cat2/1	9.7 ± 4.9	Cat2/1	12.1 ± 6.9	Cat2/1
63	p-Formylbenzoic acid	2A	12.2 ± 0.9	Cat2/1	3.3 ± 1.1	Cat2/1	7.1 ± 0.3	Cat2/1	7.5 ± 4.4	Cat2/1
64	2-Methyl-1-pentanol	2B	14.7 ± 4.7	Cat2/1	37.7 ± 7.6	Cat2/1	19.5 ± 3.5	Cat2/1	24.0 ± 12.1	Cat2/1
65	Diethyl toluamide	2B	13.3 ± 5.9	Cat2/1	39.5 ± 5.9	Cat2/1	28.6 ± 7.4	Cat2/1	27.1 ± 13.2	Cat2/1
66	Methyl acetate	2B	13.1 ± 1.5	Cat2/1	15.5 ± 6.1	Cat2/1	13.3 ± 1.7	Cat2/1	14.0 ± 1.3	Cat2/1
67	Sodium hydroxide (1%)	2B	0.9 ± 0.4	Cat2/1	17.5 ± 1.1	Cat2/1	9.9 ± 1.7	Cat2/1	9.4 ± 8.3	Cat2/1
68	2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	2B	2.2 ± 0.6	Cat2/1	4.3 ± 3.4	Cat2/1	5.1 ± 6.5	Cat2/1	3.9 ± 1.5	Cat2/1
69	1,4-Dibutoxy benzene	2B	29.3 ± 11.8	Cat2/1	34.5 ± 11.2	Cat2/1	26.4 ± 4.6	Cat2/1	30.1 ± 4.1	Cat2/1
70	2,6-Dichloro-5-fluoro-beta-oxo-3-pyridinepropanoate	2B	1.3 ± 0.2	Cat2/1	1.5 ± 0.3	Cat2/1	1.2 ± 0.3	Cat2/1	1.3 ± 0.1	Cat2/1
71	Maneb	2B	7.4 ± 0.6	Cat2/1	2.8 ± 0.3	Cat2/1	0.0 ± 0.0	Cat2/1	3.4 ± 3.8	Cat2/1
72	Ammonium Nitrate	2B	9.8 ± 0.2	Cat2/1	1.6 ± 0.2	Cat2/1	3.0 ± 0.2	Cat2/1	4.8 ± 4.4	Cat2/1
73	m-Dinitrobenzene	2B	9.6 ± 0.1	Cat2/1	3.2 ± 0.1	Cat2/1	2.6 ± 0.7	Cat2/1	5.1 ± 3.9	Cat2/1
74	p-Nitrobenzoic acid	2B	9.0 ± 0.3	Cat2/1	2.0 ± 2.1	Cat2/1	1.1 ± 0.3	Cat2/1	4.1 ± 4.3	Cat2/1
75	Sodium chloroacetate	2B	9.1 ± 0.8	Cat2/1	1.1 ± 0.1	Cat2/1	0.8 ± 0.1	Cat2/1	3.7 ± 4.7	Cat2/1
76	3,3-Dithiodipropionic Acid	2B	11.0 ± 0.2	Cat2/1	3.4 ± 0.3	Cat2/1	5.1 ± 0.6	Cat2/1	6.5 ± 4.0	Cat2/1
77	Polyethylene glycol (PEG-40) hydrogenated castor oil	NC	52.7 ± 8.3	NC	107.9 ± 3.9	NC	74.1 ± 6.8	NC	78.2 ± 27.8	NC
78	Propylene glycol	NC	80.8 ± 10.3	NC	77.6 ± 10.7	NC	87.6 ± 6.5	NC	82.0 ± 5.1	NC
79	3-Methoxy-1,2-propanediol	NC	68.9 ± 11.5	NC	76.5 ± 7.1	NC	70.9 ± 6.3	NC	72.1 ± 3.9	NC
80	Ethanol (10%)	NC	117.9 ± 1.9	NC	115.1 ± 16.9	NC	89.1 ± 18.7	NC	107.4 ± 1.9	NC
81	3-Phenoxybenzyl alcohol	NC	50.3 ± 8.0	NC	60.4 ± 16.0	NC	57.8 ± 11.8	NC	56.2 ± 5.3	NC
82	4-(Methylthio)-benzaldehyde	NC	49.3 ± 8.2	NC	58.9 ± 4.5	NC	43.4 ± 5.4	NC	50.5 ± 7.8	NC
83	3,3-Dimethylpentane	NC	90.4 ± 18.3	NC	88.5 ± 0.7	NC	104.4 ± 4.6	NC	94.4 ± 8.7	NC
84	Triethanolamine	NC	80.9 ± 16.2	NC	75.0 ± 12.2	NC	82.9 ± 6.2	NC	79.6 ± 4.1	NC

(Continue)

Chemical			LabCyte CORNEA-MODEL EIT							
			R1		R2		R3		Mean	
No	Name	GHS class	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge
85	1-Ethyl-3-methylimidazolium ethylsulphate	NC	53.2 ± 19.4	NC	42.6 ± 3.7	NC	49.0 ± 19.8	NC	48.3 ± 5.3	NC
86	Piperonyl butoxide	NC	93.8 ± 3.1	NC	92.5 ± 10.6	NC	85.8 ± 5.4	NC	90.7 ± 4.3	NC
87	n-Octyl Ether	NC	98.5 ± 6.1	NC	89.5 ± 3.8	NC	90.1 ± 9.2	NC	92.7 ± 5.0	NC
88	2-Ethoxyethyl methacrylate	NC	34.9 ± 9.8	Cat2/1	41.2 ± 1.4	NC	37.4 ± 7.8	Cat2/1	37.8 ± 3.2	Cat2/1
89	Xylene	NC	87.0 ± 2.6	NC	71.4 ± 8.8	NC	60.0 ± 13.4	NC	72.8 ± 2.6	NC
90	1,6-Dibromohexane	NC	74.6 ± 17.7	NC	60.6 ± 3.3	NC	53.7 ± 3.1	NC	63.0 ± 10.6	NC
91	1-bromo-4chlorobutane	NC	37.9 ± 6.5	Cat2/1	49.8 ± 10.8	NC	50.2 ± 1.6	NC	46.0 ± 7.0	NC
92	Ethyl thioglycolate	NC	9.9 ± 11.4	Cat2/1	0.0 ± 0	Cat2/1	0.6 ± 1.0	Cat2/1	3.5 ± 5.6	Cat2/1
93	Methyl cycropentane	NC	94.5 ± 4.3	NC	107.7 ± 17.2	NC	84.7 ± 11.2	NC	95.6 ± 11.6	NC
94	Dipropyl disulphide	NC	79.8 ± 7.0	NC	102.6 ± 7.6	NC	100.0 ± 7.4	NC	94.1 ± 12.5	NC
95	Isopropyl myristate	NC	85.4 ± 16.2	NC	85.4 ± 18.3	NC	106.5 ± 0.8	NC	92.4 ± 12.2	NC
96	Ethyl acetate	NC	16.5 ± 3.0	Cat2/1	20.4 ± 3.2	Cat2/1	20.7 ± 4.4	Cat2/1	19.2 ± 2.3	Cat2/1
97	Toluene	NC	31.6 ± 1.1	Cat2/1	25.4 ± 1.0	Cat2/1	47.8 ± 4.6	NC	35.0 ± 11.6	Cat2/1
98	2-Methylpentane	NC	89.9 ± 4.3	NC	128.8 ± 3.7	NC	90.6 ± 7.1	NC	103.1 ± 4.3	NC
99	n-Octyl bromide	NC	84.1 ± 5.3	NC	126.5 ± 11.2	NC	97.3 ± 13.6	NC	102.6 ± 5.3	NC
100	cis-Cyclooctene	NC	70.3 ± 2.0	NC	120.0 ± 1.8	NC	92.0 ± 3.5	NC	94.1 ± 2.0	NC
101	Dodecane	NC	84.0 ± 1.2	NC	101.4 ± 2.5	NC	108.5 ± 1.9	NC	84.0 ± 12.6	NC
102	Styrene	NC	28.8 ± 5.0	Cat2/1	27.8 ± 8.4	Cat2/1	25.8 ± 13.5	Cat2/1	27.5 ± 1.5	Cat2/1
103	Methyl isobutyl ketone	NC	32.6 ± 5.5	Cat2/1	31.1 ± 6.1	Cat2/1	33.2 ± 3.8	Cat2/1	32.3 ± 1.1	Cat2/1
104	2-Heptanone(Methyl amylketone)	NC	25.1 ± 6.9	Cat2/1	34.7 ± 1.8	Cat2/1	47.9 ± 6.5	NC	35.9 ± 11.5	Cat2/1
105	2-Ethylhexyl p-dimethylamino benzoate	NC	91.9 ± 10.4	NC	81.3 ± 6.4	NC	100.8 ± 2.0	NC	91.3 ± 9.8	NC
106	Glycerol	NC	87.3 ± 5.8	NC	79.8 ± 11.8	NC	66.3 ± 4.9	NC	77.8 ± 10.6	NC
107	Polyethylene glycol 400	NC	97.0 ± 8.3	NC	100.2 ± 4.9	NC	76.6 ± 4.4	NC	91.3 ± 12.8	NC
108	Sodium N-lauroyl sarcosinate (30%)	NC	85.1 ± 15.2	NC	93.9 ± 16.6	NC	87.0 ± 13.1	NC	88.6 ± 4.6	NC
109	Sodium dodecyl sulfate(1%)	NC	79.3 ± 3.8	NC	96.4 ± 7.5	NC	97.4 ± 1.0	NC	91.0 ± 10.1	NC
110	Benzalkonium chloride (0.01%)	NC	69.7 ± 15.8	NC	111.9 ± 4.5	NC	109.5 ± 5.3	NC	97.1 ± 23.7	NC
111	Cethylpyridinium bromide (0.1%)	NC	52.5 ± 6.8	NC	87.2 ± 5.1	NC	84.0 ± 5.5	NC	74.6 ± 19.2	NC
112	Benzalkonium chloride (0.1%)	NC	47.9 ± 4.0	NC	67.9 ± 7.2	NC	72.2 ± 5.1	NC	62.7 ± 13.0	NC

(Continue)

Chemical			LabCyte CORNEA-MODEL EIT							
			R1		R2		R3		Mean	
No	Name	GHS class	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge	Viability (%)	Judge
113	Tween20	NC	94.5 ± 5.5	NC	104.5 ± 7.7	NC	83.6 ± 9.6	NC	94.2 ± 10.5	NC
114	Tween80	NC	81.4 ± 14.5	NC	80.0 ± 11.2	NC	104.5 ± 8.5	NC	88.6 ± 13.8	NC
115	Polyethylene glycol monolaurate (10E.O.)	NC	83.8 ± 2.1	NC	77.0 ± 6.1	NC	90.7 ± 10.8	NC	83.8 ± 6.9	NC
116	Tween80(10%)	NC	71.4 ± 13.0	NC	106.9 ± 2.1	NC	88.2 ± 12.8	NC	88.8 ± 17.8	NC
117	3,4-dimethoxy benzaldehyde	NC	3.1 ± 0.0	Cat2/1	2.3 ± 0.1	Cat2/1	2.6 ± 0.0	Cat2/1	2.7 ± 0.4	Cat2/1
118	Cellulose, 2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride (91%)	NC	14.3 ± 7.0	Cat2/1	7.9 ± 5.5	Cat2/1	6.6 ± 3.1	Cat2/1	9.6 ± 4.1	Cat2/1
119	2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol	NC	1.6 ± 0.2	Cat2/1	1.0 ± 0.6	Cat2/1	1.6 ± 0.3	Cat2/1	1.4 ± 0.4	Cat2/1
120	2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	NC	91.7 ± 8.4	NC	115.2 ± 8.7	NC	94.2 ± 9.8	NC	100.4 ± 12.9	NC
121	2-Mercaptopyrimidine	NC	103.1 ± 4.4	NC	107.1 ± 5.8	NC	77.5 ± 4.9	NC	95.9 ± 16.0	NC
122	Tetraaminopyrimidine sulfate	NC	118.1 ± 24.7	NC	109.8 ± 5.4	NC	81.7 ± 5.2	NC	103.2 ± 19.1	NC
123	Ethylenediamine-tetraacetic acid dipotassium salt dihydrate	NC	5.4 ± 0.5	Cat2/1	5.5 ± 0.8	Cat2/1	3.4 ± 1.3	Cat2/1	4.8 ± 1.2	Cat2/1
124	Phenothiazine	NC	64.6 ± 9.6	NC	89.8 ± 4.5	NC	66.0 ± 3.9	NC	73.5 ± 14.2	NC
125	Het Anhydride	NC	2.4 ± 0.5	Cat2/1	0.0 ± 0.1	Cat2/1	0.0 ± 0.0	Cat2/1	0.8 ± 1.4	Cat2/1
126	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	NC	61.8 ± 5.7	NC	72.8 ± 5.9	NC	73.4 ± 10.6	NC	69.3 ± 6.5	NC
127	4,4'-Methylene bis-(2,6-di-tert-butylphenol)	NC	102.8 ± 3.4	NC	72.5 ± 14.3	NC	86.4 ± 11.3	NC	87.2 ± 15.1	NC
128	Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate	NC	4.9 ± 0.8	Cat2/1	9.1 ± 2.9	Cat2/1	5.2 ± 1.4	Cat2/1	6.4 ± 2.3	Cat2/1
129	D(+)-Glucono-1,5-lactone	NC	3.1 ± 0.5	Cat2/1	1.9 ± 0.1	Cat2/1	1.8 ± 0.2	Cat2/1	2.3 ± 0.7	Cat2/1
130	Propyl p-hydroxybenzoate	NC	3.5 ± 2.4	Cat2/1	0.8 ± 0.8	Cat2/1	1.9 ± 1.8	Cat2/1	2.1 ± 1.3	Cat2/1
131	Anthracene	NC	91.5 ± 3.8	NC	114.8 ± 9.8	NC	71.8 ± 6.9	NC	92.7 ± 21.5	NC
132	2,2'-Iminodibenzyl	NC	80.8 ± 4.2	NC	81.9 ± 11.3	NC	81.1 ± 2.1	NC	81.3 ± 0.6	NC
133	Silicic acid	NC	48.3 ± 3.9	NC	96.4 ± 3.0	NC	71.1 ± 5.3	NC	71.9 ± 24.1	NC
134	Aluminium hydroxide, 50-57%	NC	84.1 ± 3.1	NC	105.1 ± 12.1	NC	77.5 ± 8.0	NC	88.9 ± 14.4	NC
135	Potassium tetrafluoroborate	NC	90.4 ± 7.7	NC	111.1 ± 4.3	NC	105.8 ± 12.1	NC	102.4 ± 10.7	NC
136	Magnesium carbonate hydroxide pentahydrate	NC	102.0 ± 7.3	NC	104.8 ± 1.3	NC	81.6 ± 16.8	NC	96.1 ± 12.7	NC
137	2,3-Dimethyl-2,3-dinitrobutane	NC	74.0 ± 1.1	NC	86.7 ± 8.6	NC	70.9 ± 1.6	NC	77.2 ± 8.4	NC
138	2,4-Dichloro-5-sulfamoylbenzoic Acid	NC	16.6 ± 0.5	Cat2/1	13.5 ± 3.3	Cat2/1	2.6 ± 1.7	Cat2/1	10.9 ± 7.3	Cat2/1
139	N, N-Dimethylguanidine sulfate	NC	1.6 ± 0.2	Cat2/1	1.3 ± 0.7	Cat2/1	0.6 ± 0.2	Cat2/1	1.2 ± 0.5	Cat2/1

In summary, based on the mean of the three independent runs (Table 5-7), 45 out of 61 category and all out of 75 Category 1 or 2 in the GHS classification were classified correctly by the improved LabCyte24 EIT. The statistical parameters describing assay performance are displayed in Table 5-7.

Table 5-7. Wide range of the test chemicals for evaluation of the LabCyte24 EIT

		In vivo classification		
		Cat 1 or 2	No category	Total
In vitro prediction	Irritant	76	17	93
	Non-irritant	0	46	46
	Total	76	63	139

Sensitivity (%)	100
Specificity (%)	73.0
Accuracy (%)	87.8

Sensitivity and specificity of predictions by the improved LabCyte24 EIT were 100% and 73.0%, respectively. Overall accuracy was 87.8% (Table 5-6).

5-4. Discussion and Conclusion

In the OECD TG 492 description, the formazan solution may be quantified using either a standard absorbance OD measurement or a high performance liquid chromatography/an ultra high performance liquid chromatography (HPLC/UPLC) spectrophotometry procedure. Especially, it is pointed out that the HPLC/UPLC may be useful to detection correct colored formazan after the colored chemical application because it might be a possibility to distinguish a peak of formazan color from a peak of material color. In the improved LabCyte24 EIT, the formazan solution was quantified using OD measurement, because ocular irritancy of all chemicals could be predicted from measured ODs. But, HPLC/UPLC measurement may be useful about unknown strong-colored chemicals.

When compared with the UN GHS classification, the LabCyte24 EIT classified 17 No Category chemicals (chemicals not classified for irritation), 2-Ethoxyethyl methacrylate, Ethyl

thioglycolate, Ethyl acetate, Toluene, Styrene, Methyl isobutyl ketone, 2-Heptanone, 3,4-dimethoxy benzaldehyde, Cellulose, 2-(2-hydroxy-3-(trimethylammonium) propoxy)ethyl ether chloride (91%), 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol, Ethylenediamine-tetraacetic acid dipotassium salt dehydrate, Het Anhydride, Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate, D(+)-Glucono-1,5-lactone, Propyl p-hydroxybenzoate, 2,4-Dichloro-5-sulfamoylbenzoic Acid, and N, N-Dimethylguanidine sulfate as irritants.

Interestingly, while 11 of 17 chemicals, 2-Ethoxyethyl methacrylate, Ethyl thioglycolate, Ethyl acetate, Toluene, Methyl isobutyl ketone, 2-Heptanone, 3,4-dimethoxy benzaldehyde, 2,2'-[[3-Methyl-4-[(4-nitrophenyl)azo]-phenyl]imino]bis-ethanol, Ethylenediamine-tetraacetic acid dipotassium salt dehydrate, Trisodium mono-(5-(1,2-dihydroxyethyl)-4-oxido-2-oxo-2,5-dihydro-furan-3-yl) phosphate, D(+)-Glucono-1,5-lactone, and N, N-Dimethylguanidine sulfate as irritants were classified as No Category by the UN GHS classification, alternative eye irritation models such as the OECD TG492 VRM define them as irritants (Van Goethem et. al., 2006; Kaluzhny et. al., 2011; OECD, 2015b). Human study data also supports that these chemicals might be eye-irritants (Van Goethem et. al., 2006), indicating that the performance of the improved LabCyte CORNEA-MODEL24 might simulate the response of a human eye to chemical irritants very well.

Even if the 17 above-mentioned test chemicals were false positives, the specificity of the improved LabCyte24 EIT showed 73.0 % and it was enough correlation with the UN GHS classification. Furthermore, all chemicals classified in Category 1 or 2 (eye irritant) in the UN GHS classification were correctly predicted as irritants in the improved LabCyte24 EIT. The sensitivity of this EIT showed 100% correlation with the GHS classification. Finally, an overall accuracy of 87.8 % was obtained. The present results strongly suggest that the improved LabCyte24 EIT could be distinguished No Category from Category 1 or 2 in the UN GHS classification according to Bottom-up strategy and therefore it could be a useful alternative method to the rabbit Draize eye test.

6. THE AVAILABILITY OF LabCyte24 EIT OUTSIDE OF JAPAN: EXPORT OF LabCyte CORNA-MODEL TO KOREA AND CONFIRMATION OF ITS PERFORMANCE

6-1. Purpose

For the LabCyte24 EIT to be included as one of the validated method in the OECD TG492 (OECD, 2015a), it is necessary to demonstrate that this testing method is available internationally.

In order to confirm the availability of LabCyte CORNEA-MODEL24 after shipment to outside of Japan, LabCyte CORNEA-MODEL24 is exported to a Korean laboratory and the EIT using LabCyte CORNEA-MODEL24 was performed at the Korean laboratory

6-2. Export to Korea and Test Protocol

6-2.1. Shipment Schedule

The LabCyte CORNEA-MODEL24 was exported to Korean laboratory as following schedule.

1st export:	February 15, 2016.	Lot no. LCC24-160215-A
2nd export:	February 29, 2016.	Lot no. LCC24-160229-A
3rd export:	April 18, 2016.	Lot no. LCC24-160418-A

6-2.2. Test Facility

The LabCyte CORNEA-MODEL24 after shipment was evaluated at following laboratory in Korea.

Laboratory name

Konkuk University;

Center for Stem Cell Research, Dept. of Biomedical Science & Technology, Institute of Biomedical Science & Technology (IBST)

120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Republic of Korea.

Laboratory member

Dr. Jeong Ik Lee; Associate Professor.

Dr. Yuna Han; Research Professor

Dr. Lee, Soo Jung; Research Professor

6-2.3. Evaluation Procedure of LabCyte CORNEA-MODEL24 after Shipment

Using LabCyte CORNEA-MODEL24 kit after shipment, the LabCyte24 EIT was performed at the Korean Laboratory according to the improved protocol, which described in the SOP ver.2.5.2 (Appendix 1) for confirmed followings objection.

(1) To confirm that OD and SD of the negative control (Liquid and Solid chemical protocol) are adapted in the acceptance criteria in all independent 3 batches after shipment.

(2) To confirm that the cell viability (%) and SD of the positive control (Liquid and Solid chemical protocol) are adapted in the acceptance criteria in all independent 3 batches after shipment.

(3) To confirm that be able to perform LabCyte24 EIT in according to the improved protocol which described in the SOP ver.2.5.2 (Appendix 1) using proficiency test chemicals as listed in the OECD TG492 (OECD, 2015a).

6-3. Results

6-3.1. Export to Korean Laboratory

Shipment schedules to Korean laboratory are shown in Table 6-1.

Table 6-1. Shipments to Korean laboratory.

Lot No.	Shipping date	Delivery date	Transportation period
LCC24-160215-A	Feb. 15, 2016	Feb. 17, 2016	2 days
LCC24-160229-A	Feb. 29, 2016	Mar. 4, 2016	4 days
LCC24-160418-A	Apr.18 , 2016	Apr. 20, 2016	2 days

Transportation periods of the LabCyte CORNEA-MODEL24 were taken between two and four days from shipping from the supplier (J-TEC, Japan) to arrive to Korean laboratory (KonKuk University, Korea).

6-3.2. Results of Negative Control in the LabCyte24 EIT after Shipment

Using three batches of the LabCyte CORNEA-MODEL24 after shipment, the LabCyte24 EIT was performed according to the improved protocol which was described in the SOP ver.2.5.2 (Appendix 1) at the Korean laboratory (Konkuk University). Results of the negative control (both Liquid and Solid chemical protocol) of three independent batches were shown in Table 6-2.

Table 6-2. OD and SD of negative controls in the EIT using LabCyte CORNEA-MODEL24 after shipment to Korean laboratory.

Batch No.	Physical state of Negative Control	Tissue Viability			
		OD (450nm/650nm)	Judge	SD (%)	Judge
LCC24-160215-A	Liquid (D-PBS)	0.811	Adapt	14.0	Adapt
	Solid (Not treat)	0.810	Adapt	6.0	Adapt
LCC24-160229-A	Liquid (D-PBS)	0.779	Adapt	17.9	Adapt
	Solid (Not treat)	0.629	Adapt	8.4	Adapt
LCC24-160418-A	Liquid (D-PBS)	1.070	Adapt	10.6	Adapt
	Solid (Not treat)	1.022	Adapt	9.9	Adapt

OD of the cell viability of the negative control in three independent batches of LabCyte CORNEA-MODEL24 after shipment are all adapted to the acceptance criteria ($0.5 < OD < 1.3$) of LabCyte24 EIT (Table 6-2).

Also, SD (%) of the cell viability of negative control in three independent batches of LabCyte CORNEA-MODEL24 after shipment are all adapted to the acceptance criteria ($SD < 20\%$) of LabCyte24 EIT (Table 6-2).

From these results on the negative control, it was suggested that LabCyte CORNEA-MODEL24 after shipment to outside of Japan might be able to be useful for the LabCyte24 EIT.

6-3.3. Results of Positive Control in LabCyte24 EIT after Shipment

Using three batches of LabCyte CORNEA-MODEL24 after shipment, the LabCyte24 EIT was performed according to the improved protocol which was described in the SOP ver.2.5.2 (Appendix 1) at the Korean laboratory (Konkuk University). Results of positive control (both Liquid and Solid chemical protocol) of 3 independent batches were shown in Table 6-3

Table 6-3. Cell viability and SD of positive control for EIT using LabCyte CORNEA-MODEL24 exported to Korean laboratory.

Lot No.	Physical state of Positive Control	Cell viability			
		Cell Viability (%)	Judge	SD (%)	Judge
LCC24-160215-A	Liquid (Ethanol)	12.9	Adapt	4.6	Adapt
	Solid (Lauric acid)	13.3	Adapt	10.2	Adapt
LCC24-160229-A	Liquid (Ethanol)	15.4	Adapt	6.8	Adapt
	Solid (Lauric acid)	0.9	Adapt	0.5	Adapt
LCC24-160418-A	Liquid (Ethanol)	4.5	Adapt	4.6	Adapt
	Solid (Lauric acid)	1.2	Adapt	0.2	Adapt

The cell viability (%) of positive control in three independent batches of LabCyte CORNEA-MODEL24 after shipment are all adapted to the acceptance criteria (Cell Viability < 40%) of LabCyte24 EIT (Table 6-3).

Also, SD (%) of the cell viability of positive Control in three independent batches of LabCyte CORNEA-MODEL24 after shipment are all adapted to the acceptance criteria (SD < 20%) of LabCyte24 EIT (Table 6-3).

From these results on the positive control, it was suggested that LabCyte CORNEA-MODEL24 after shipment to outside of Japan might be able to be use for the LabCyte24 EIT.

6-3.4. Results of Proficiency Chemicals in LabCyte24 EIT

In older to confirm whether it is able to use the EIT with LabCyte CORNEA-MODEL24 after shipping, its test was performed in Korean laboratory according to the improved protocol which

described in the SOP ver.2.5.2 (Appendix 1) using proficiency test chemicals (6 GHS Cat 1/2 chemicals and 7 GHS No Cat Chemicals) listed in the OECD TG492.

Table 6-4. Prediction results of proficiency chemicals in the OECD TG492 by EIT using LabCyte CORNEA-MODEL24 exported to Korean laboratory.

Proficiency chemical	Physical state	*In vivo* GHS Cat	LabCyte24 EIT in Konkuk Univ.			EpiOcular EIT[*1] (the OECD TG492 VRM)	
			Viability (%)		Judge	Viability (%)	Judge
			mean	SD			
Tetraethylene glycol diacrylate	**Liquid**	**Cat.1**	**17.9**	**1.9**	**Cat.1/2**	34.9	Cat.1/2
2,5-Dimethyl-2,5-hexanediol	**Solid**	**Cat.1**	**5.3**	**0.3**	**Cat.1/2**	2.3	Cat.1/2
Sodium oxalate	**Solid**	**Cat.1**	**7.1**	**1.2**	**Cat.1/2**	29.0	Cat.1/2
2,4,11,13-Tetraazatetradecanediimidamide,N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-Dgluconate (20%, aqueous)	**Liquid**	**Cat 2A**	**4.4**	**0.3**	**Cat.1/2**	4.0	Cat.1/2
Diethyl toluamide	**Liquid**	**Cat 2B**	**31.3**	**0.4**	**Cat.1/2**	15.6	Cat.1/2
2,2-Dimethyl-3-methylenebicyclo [2.2.1] heptane	**Solid**	**Cat 2B**	**6.7**	**2.5**	**Cat.1/2**	4.7	Cat.1/2
1-Ethyl-3-methylimidazolium ethylsulphate	**Liquid**	**No Cat**	**51.4**	**11.0**	**No Cat**	79.9	No Cat
Dipropyl disulphide	**Liquid**	**No Cat**	**92.7**	**4.1**	**No Cat**	81.7	No Cat
Piperonyl butoxide	**Liquid**	**No Cat**	**76.0**	**15.0**	**No Cat**	104.2	No Cat
Polyethylene glycol (PEG-40) hydrogenated castor oil	**Viscus**	**No Cat**	**61.8**	**3.6**	**No Cat**	77.6	No Cat
1-(4-Chlorophenyl)-3 (3,4-dichlorophenyl) urea	**Solid**	**No Cat**	**75.3**	**7.1**	**No Cat**	106.7	No Cat
2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	**Solid**	**No Cat**	**83.4**	**5.1**	**No Cat**	102.7	No Cat
Potassium tetrafluoroborate	**Solid**	**No Cat**	**54.1**	**3.4**	**No Cat**	88.6	No Cat

*1: To refer th description of the OECD TG492 (OECD, 2015a).

The prediction of the eye irritation of a proficiency test chemical in the LabCyte24 EIT was parallel with *in vivo* GHS classification perfectly in the eye irritation test conducted in a Korean laboratory.

From these results, it was concluded that LabCyte24 EIT was able to be performed outside of Japan.

6-4. Discussion and Conclusion

Using three dependent batch of the LabCyte CORNEA-MODEL24 after the shipment from Japan to Korean laboratory, *in vitro* eye irritation test was performed in the Korean laboratory and the results of the negative control and the positive control were adapted in the acceptance criteria in three batches. From such results are suggested that the performance of the LabCyte CORNEA-MODEL24 after shipping to outside of Japan is enough usefulness for the eye irritation testing.

Furthermore, from that the prediction of eye irritancy of the proficiency chemical is correctly predicted by LabCyte24 EIT, it is concluded that the LabCyte24 EIT is be able to perform outside of Japan.

7. VALIDATION STUDY OF LabCyte24 EIT ACCORDING TO THE PERFORMANCE STANDARD FOR THE OECD TG492

7-1. The Performance Standard for the OECD TG 492

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

In the (i) Essential Test Method Components of the PERFORMANCE STANDARD, it was described about the general conditions and the functional conditions of RhCE model for the EIT method according the OECD TG492 condition and it was also explained specifically procedural conditions of the VRM EIT. In the general condition and functional conditions of LabCyte CORNEA-MODEL24 are detail described in the section 2.2.

In the next section, needed essential assay component of RhCE EIT for the OECD TG492 and the corresponding LabCyte CORENA-MODEL EIT are shown.

7-2. Difference between LabCyte24 EIT and the OECD TG492 VRM Procedural Conditions

In the Table 7-1, it was described about each needed assay component between the RhCE EIT method in the OECD TG492 and LabCyte24 EIT.

Table 7-1. Test component of LabCyte24 EIT

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

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

Difference of test component of Between the OECD TG492 VRM and the LabCyte24 EIT are followings;

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

7-3. Comparison and Similarity Consideration about Each Test Component of Between the VRM in the Performance Standard and LabCyte24 EIT

7-3.1. Cell Source

In the PERFORMANCE STANDARD for the OECD TG 492 (OECD, 2015b), relevant human-derived cells (e.g., human corneal epithelial cells or keratinocytes) should be used to the RhCE tissue, tissue, which should be composed of progressively stratified but not cornified cells. LabCyte CORNEA-MODEL24 is used human corneal epithelial cells, which should be composed of progressively stratified but not cornified cells.

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

7-3.2. Replication of RhCE Tissues

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

About replication of RhCE tissues, the LabCyte24 EIT protocol is concordance with the PERFORMANCE STANDARD requirement for the OECD TG 492 (OECD, 2015b).

7-3.3. Amount of Chemical Application

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

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

7-3.4. Negative Control and Positive Control

In the PERFORMANCE STANDARD for the OECD TG 492, separate the negative control and the positive control are needed for each protocol of the test method. In the VRM, the positive control and the negative control substances used neat methyl acetate and ultra-pure H_2O for both Liquid chemicals and Solid chemicals. In the LabCyte24 EIT, the positive control and the negative control substances used ethanol and D-PBS for Liquid chemicals and lauric acid and no treatment for Solid chemicals. Concurrent the negative control and the positive controls should be included in each run demonstrate that the viability and the sensitivity determined with the positive control of the tissues are within acceptance ranges defined based on historical data. The concurrent negative control provides the baseline to calculate the relative percent viability of the tissues treated with the test chemicals.

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

7-3.5. Chemical Exposure, Post-exposure Immersion and Post-exposure Incubation Periods

In the PERFORMANCE STANDARD for the OECD TG 492, it was described that two different treatment protocols may be used for different types of chemicals, for Liquid test chemicals or for

Solid test chemicals. If different protocols are used to an EIT, they may differ in terms of their exposure periods, post-exposure incubation immersion periods and post-exposure incubation periods.

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

About selection and setting of the chemical exposure pattern, LabCyte24 EIT protocol was concordance with the PERFORMANCE STANDARD requirement.

7-3.6. Measurement of Tissue Viability

7-3.6.1. Cell Viability Measurement with Tetrazolium-Salt

In the RhCE EIT test method for the OECD TG 492, the cell viability is measured as an endpoint for the prediction of eye irritation. In the OECD TG 492, MTT assay is selected by measurement of cell viability. MTT assay is one of tetrazolium reduction assay. MTT is reduced by cellular dehydrogenase and produced insoluble MTT formazan with blue color. A variety of tetrazolium salts which are reduced by cellular dehydrogenase as same manner of MTT assay and changed to water soluble formazan charging various colors have been developed. Commonly used tetrazolium salts are included MTT, MTS, XTT, WST-1 and WST-8 (Table 7-2).

Table 7-2. Commonly used tetrazolium salts

Abbreviation	Chemical name	Water solubility of producing formazan
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide	Insoluble
MTS	3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-s	soluble

	ulfophenyl)-2H-tetrazolium	
XTT	2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide	Soluble
WST-1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt	Soluble
WST-8	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfoph enyl)-2H tetrazolium, monosodium salt	Soluble

If cellular dehydrogenase are rapidly inactivated by damaging cells, degree of coloring by formazan dye is correlate reduced because amount of cellular dehydrogenase is related the cell viability. Therefore, it can be quantified the cell viability by the determination of such coloring degree of formazan dye. Such tetrazolium reduction assay is widely accepted as simple test method for analysis of cell viability.

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

On the other hands, in the LabCyte24 EIT, WST-8 assay which produced water soluble formazan is selected as the determination of the cell viability. WST-8 was demonstrated to be of value for use as indicator for cell viability with higher sensitivity than those of conventional tetrazolium salts including MTT (Tominaga et. al., 1999).

The outline of a MTT assay and WST-8 assay is described to next section and it is mentioned about the similarity of the both method.

7-3.6.1a. MTT Assay

A MTT assay is widely selected a tetrazolium reduction assay in worldwide. MTT assay is selected in skin irritation test method using the reconstructed human epidermal tissue for the OECD TG 439.

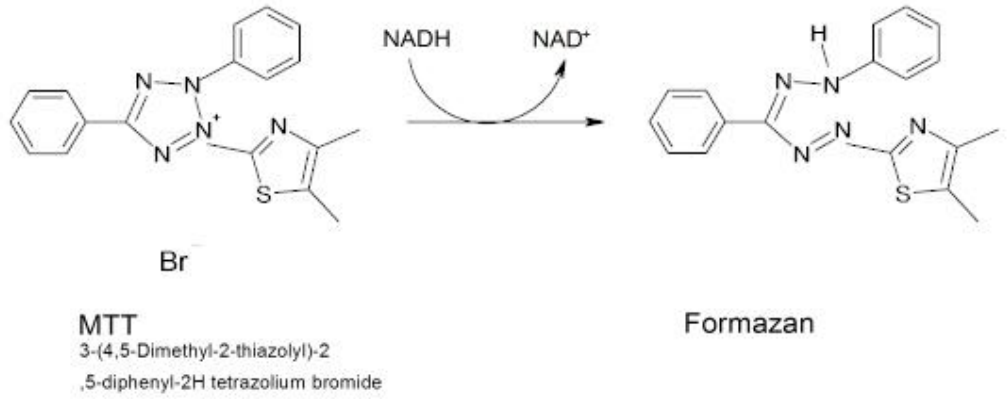


Fig.7-1. MTT assay: Reaction mechanism.

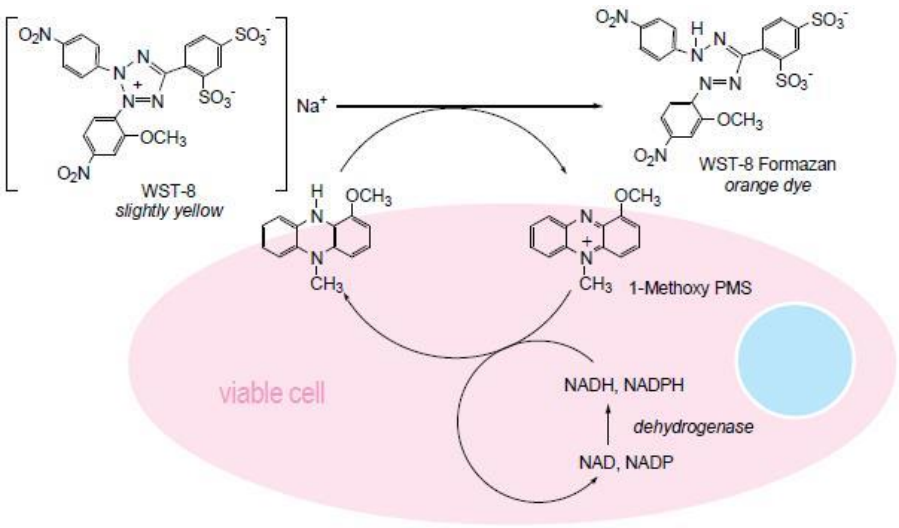
MTT penetrates through cell membrane and concentrate into the mitochondria in the cytosol. MTT is reduced by dehydrogenase through NADH and NADPH which are a coenzyme and then produced blue MTT formazan in the cytosol (Fig.7-1). For MTT formazan is water-insoluble, it is precipitated as crystals of needle-like structure in the cytosol. MTT formazan is solubilized with appropriate organic solvent such as isopropanol and then it is determined the OD (absorbance around 570nm) of solubilized solution.

On the other hand, because MTT formazan precipitate as an insoluble crystal in the viable cell, it induces highly cell toxicity and then their phenomenon might cause a decline of the sensitivity of assay. For the solubilizing process of MTT formazan is indispensable, assay variability might increase by such process.

In order to solve these problem, several tetrazolium salts to produce water-soluble formazan, such as MTS, XTT, WST-8 and WST-8 has been developed (Table 7-2). Among them, WST-8 has developed as an useful tetrazolium salt produces WST-8 formazan with highly water-soluble potency (Ishiyama et. al., 1997).

7-3.6.1b. WST-8 Assay

WST-8 assay is one of tetrazolium reduction assay as same manner of MTT assay and it widely used recently.



http://www.dojindo.com/store/p/456-Cell-Counting-Kit-8.html

Fig.7-2. WST-8 assay: Reaction mechanism.

WST-8 is reduced to an orange-colored WST-8 formazan through electron mediator, 1-Methoxy PMS by NADH and NADPH activities which are generated by cellular dehydrogenase, as indicated in the Fig. 7-2. The amount of WST-8 formazan is dependent on the activity of cellular dehydrogenase, so WST-8/1-Methoxy PMS system can be used to determine the number of living cells and cell viability. For WST-8 formazan is highly water-soluble, it can be directly determined OD (absorbance around 450 nm) of its culture medium without an extraction step. Cell Counting Kit-8, which is a one-bottle solution of the combination both WST-8 and 1-methoxy PMS, is commercially available in worldwide.

Because WST-8 assay is not required both extraction step of MTT formazan and preliminary preparation step by the use of Cell Counting Kit-8, its assay periods is reduced substantially

7-3.6.2. Similarity between MTT Assay and WST-8 Assay

The reaction principle of both WST-8 assay and MTT assay are same, because both test methods are commonly one of the tetrazolium reduction assay which determine the cellular dehydrogenase activity.

7-3.6.2a. Additional Data: Comparison of the LabCyte CORNEA-MODEL24 QC data

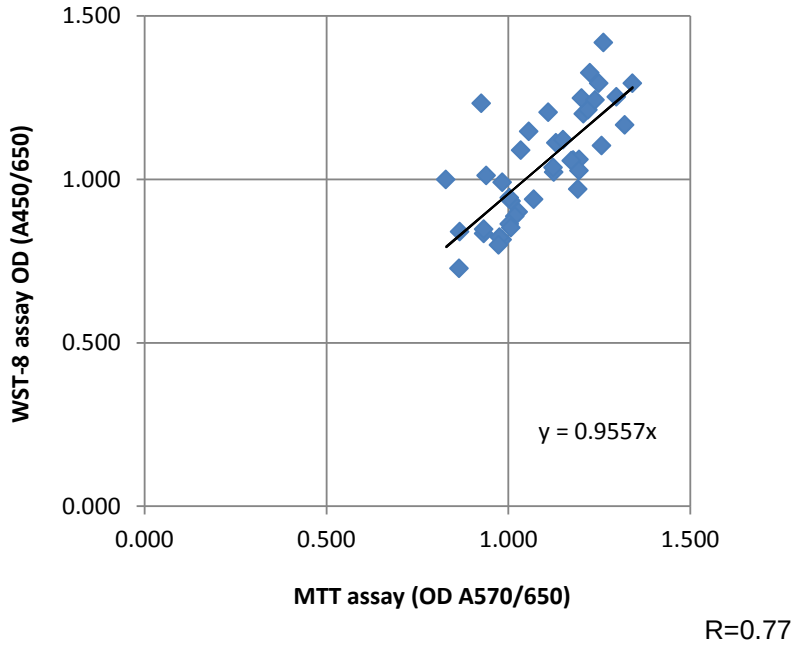


Fig.7-3. WST-8 assay and MTT assay in LabCyte CORNEA-MODEL24 QC results

In the quality control of LabCyte CORNEA-MODEL24, it is performing both WST-8 assay and MTT assay as cell viability test against every batch. The coefficient of correlation (R) between WST-8 assay and MTT assay indicated about 0.77 (Fig. 7-3) and it is shown that the results of both tetrazolium assay are highly correlate.

7-3.6.2b. Additional Data: Time Course Experiment

In order to confirm linearity about both MTT reaction and WST-8 reaction, the time course experiment of WST-8 reaction and MTT reaction are examined in the LabCyte24 EIT at Mandom Co. Ltd. The results showed in Fig. 7-4.

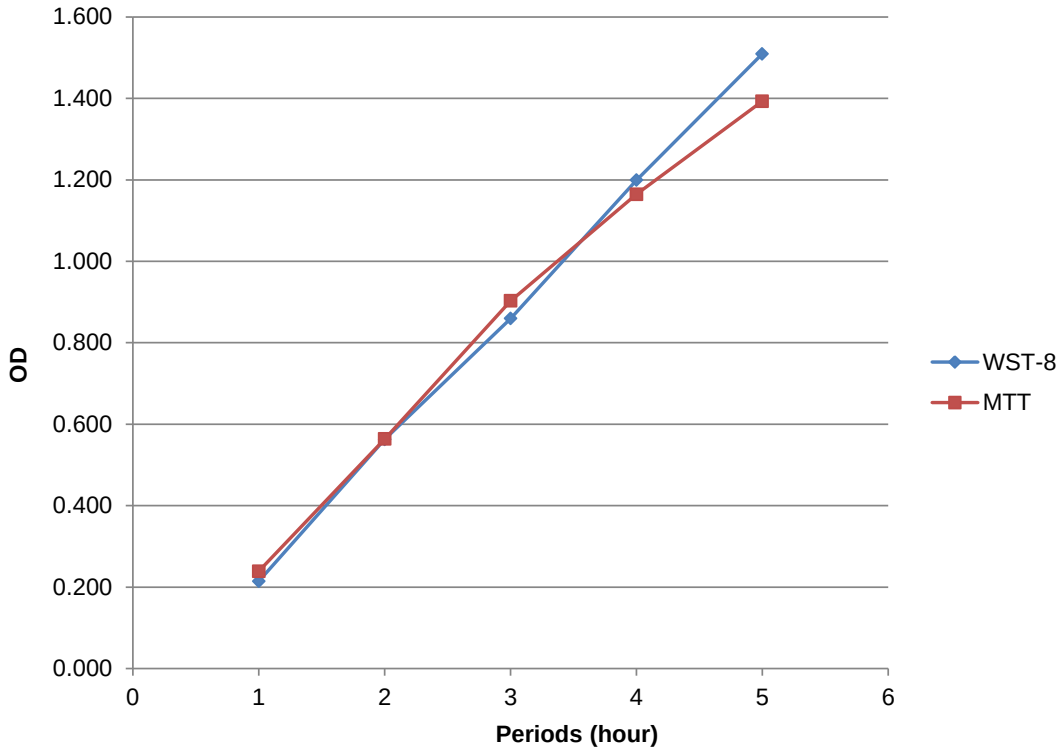


Fig.7-4. Time Courses of WST-8 reaction and MTT reaction in LabCyte24 EIT.

ODs of reaction medium of both WST-8 assay and MTT assay are lineally increased according to reaction periods.

From above results and reaction principles of tetrazolium reduction assay, it was supposed that both reactions reflect of the same cellular dehydrogenase activity.

7-3.6.3. Advantage of WST-8 Assay to MTT Assay

Since WST-8 formazan is water soluble, it does not form crystals like MTT. Therefore, after the incubation with the WST-8 solution, OD of reaction medium can be directly analyzed OD the number of viable cells.

From dissolution step such as MTT formazan are not required in the WST-8 assay, the holding periods of WST-8 assay is shorter to compare with MTT assay (CCK-8 catalog; Attachment 2). Less handling time for WST-8 assay might contribute to more accurate test results.

7-3.6.4. Similarity of LabCyte24 EIT to the OECD TG492 VRM

Reaction principle of WST-8 assay is same to that of MTT assay which is the tetrazolium reduction assay to utilize dehydrogenase activity, and then it is concluded that special quality of WST-8 assay can be mostly covered one of MTT assay.

Furthermore, from the knowledge and the additional data confirmed so far, it is judged the measurement data from WST-8 assay is almost equal to a MTT assay.

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

7-3.7. Detection and Correction of MTT/WST-8 Assay Interference

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

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

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

7-3.8. Acceptance Criterion SD

In the OECD TG 492 (OECD, 2015a), it was defined that SD value between three tissues should not exceed 18%. On the other hand, acceptance criterion of SD has been set in the LabCyte24 EIT, that was referred accept limit of difference of viability between two tissue replicates in the EpiOcular EIT was ≤ 20%. VMT has judged that 20% of SD acceptance criteria was permitted because the difference between ≤ 20% and ≤ 18% might be little.

8. CONCLUSION

Through the optimization study and several pre-validation studies described in below the section 3, it has been developed the improved LabCyte24 EIT is a robust and reliable method to address the initiating event of eye irritation.

Using wide ranged chemicals (139 chemicals), the LabCyte24 EIT demonstrated high predictive performance (88.2% overall accuracy, 100% overall sensitivity, and 73.8% overall specificity).

From the results of LabCyte24 EIT in the Korean laboratory, the performance of the LabCyte CORNEA-MODEL24 after shipping to outside of Japan is enough usefulness for the eye irritation testing and the LabCyte24 EIT is able to perform outside Japan.

Finally, the VMT of the LabCyte24 EIT judged the improved LabCyte CORNEA-MODEL24 is similar assay method to the RhCE EIT VRM for the OECD TG 492 (OECD, 2015a). Therefore, the me-too validation study of LabCyte24 EIT is planned according to the PERFORMANCE STANDARD for the OECD TG 492 in older to assess its reliability (reproducibility within and between laboratories) and its relevance (predictive capacity).

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11. APENDECS

Appendix 1. Eye irritation test protocol using the reconstructed human model “LabCyte CORNEA-MODEL24” Ver.2.5.2

Appendix 2. Cell counting kit-8 catalog.