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

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1. RATIONALE AND BACKGROUND

1.1 The LabCyte CORNEA-MODEL24 EYE IRRITATION TEST

The LabCyte CORNEA-MODEL24 eye irritation test (LabCyte24 EIT) is designed to identify test chemicals that cause acute eye irritation by measuring cytotoxic effects using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assay on the reconstructed human corneal epithelial (RhCE) model. The LabCyte24 EIT is not a kit, but LabCyte CORNEA-MODEL24 tissues are commercially available at a minimum of 24 LabCyte CORNEA-MODEL24 tissues per order.

1.2 BACKGROUND OF LabCyte24 EIT

Assessment of ocular irritation is an essential part of early testing procedures for the evaluation and hazard classification of substances. Therefore, it plays an important role in the safety evaluation of general consumer products and materials. Novel substances used in consumer products must undergo comprehensive toxicological evaluation for eye irritation and a variety of other adverse outcomes. To date, the *in vivo* Draize eye test has been the international standard assay for acute ocular toxicity evaluation (irritation and corrosion) and is described (including optimizations and refinements) in OECD Test Guideline 405. However, the use of this test has been questioned and strongly criticized for ethical concerns related to animal welfare, because it is painful to the rabbits. Thus, alternative strategies and tests are urgently required in order to evaluate the eye irritation potential of new chemicals. Corneal epithelial cells on the surface of the eye are to first to be exposed to substances and have been widely studied for links to the biological role of tissue and gene regulation. Three-dimensional RhCE models are useful as a multilayered, standardized tissue that mimics the human corneal epithelium.

The LabCyte24 EIT was developed as a replacement for the Draize eye irritation test. The Draize scoring system is heavily weighted towards corneal damage (80 out of 110 total score), because irreversible damage to the cornea can lead to blindness. Since damage to the cornea is so crucial for human health, corneal tissue can be considered a useful tool for the development of *in vitro* eye irritation testing.

1.3 BASIS OF THE TEST METHOD

Damage induced by eye irritants generally progresses from the corneal epithelium through the stroma and potentially to the endothelium, and the LabCyte24 EIT is able to provide information on the first stages of this progression. Irritants damage cells while penetrating the corneal epithelium layer, and the cytotoxic progression can be estimated by analyzing cell viability in the LabCyte CORNEA-MODEL24 tissue using standardized methods. Although the tissues represent only the corneal epithelium, (very mild responses would also be reflective of some conjunctival irritation), it can be used to estimate deeper damage as far as the stroma by the analyzing cell viability.

The relative viability of the tissue exposed to a test chemical is measured using the WST-8 assay immediately after exposure and again after a post-exposure period. A viability of 40% of the negative control value was used as the cutoff in identifying test chemicals as an irritant (GHS Category 1 or 2) or an non-irritant (GHS No Category). Some culture environments might allow the detection of very small quantities of cytokines secreted by the corneal epithelial tissue in response to topical application of test chemicals.

1.4 LIMITATION OF THE METHOD

One limitation of this assay method is potential 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) could interfere with the WST-8 endpoint. This is only a problem, however, if there are significant residual levels of the test chemical on or in the tissue at the time of the WST-8. Although this is considered unlikely, if it did happen, both the actual metabolic WST-8 reduction and the quasi direct WST-8 reduction by a colored test chemical can be quantified using the procedure described in **Section 3.2 “TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT”**.

1.5 BRIEF BASIC PROCEDURE

On the day of receipt, LabCyte CORNEA-MODEL24 tissues are incubated overnight to release transport–stress-related compounds and debris.

For liquid test chemicals, tissues are topically exposed for 1 minute. Preferably, three tissues are used for each test chemical as well as for the positive and negative controls. After exposure, tissues are thoroughly rinsed and blotted to remove the test chemical, then transferred to fresh medium and post-incubated for 24 hours. For solid test chemicals, tissues are exposed for 24 hours but are not post-incubated.

After post-incubation of tissue exposed to liquid test chemicals or after exposure of tissue exposed to solid test chemicals, the tissues are each transferred to a well containing the WST-8 medium in a 1:10 dilution with Earle balanced salt solution (EBSS). After a four-hour WST-8 incubation, the orange water-soluble formazan salt is formed in the WST-8 medium by cellular mitochondria and optical density (OD) of the WST-8 medium is measured using a spectrophotometer at 450 nm and 650 nm as reference. Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues. Test chemicals that produce a relative cell viability below 40% of the negative control are predicted to be irritants.

1.5.1 LabCyte CORNEA-MODEL24

The LabCyte CORNEA-MODEL24 is a commercially available RhCE model produced by Japan Tissue Engineering Co. Ltd. It comprises normal human corneal epithelial cells that are derived from a human cornea. The cells are cultured with 3T3-J2 cells as a feeder layer in order to expand them while maintaining their phenotype (Rheinwald and Green, 1975; Green, 1978). Reconstruction of the human cultured corneal epithelial tissue is achieved by cultivating and proliferating the corneal epithelial cells on an inert filter substrate with a surface area of 0.3 cm^2 at an air-liquid interface for 13 days using an optimized medium containing 5% fetal bovine serum. This results in the formation of a multilayer structure comprising a fully differentiated corneal epithelium with features mimicking those of a normal human corneal epithelium. For delivery, LabCyte CORNEA-MODEL24 tissues are embedded in an agarose gel containing a nutrient solution and shipped in 24-well plates.

1.5.1.1 QUALITY CONTROL OF THE TEST SYSTEMS

LabCyte CORNEA-MODEL24 tissue is manufactured in accordance with well-defined quality

assurance procedures. Each production batch was comes with quality control documentation that identifies storage conditions, RhCE instructions for use, lot number and origin, histology (demonstration of human multilayered corneal epithelial-like structure), cell viability, and barrier function integrity ($0.1 \leq IC_{50} \leq 0.4$).

1.5.1.2 PRECAUTIONS

Corneal epithelial cells are taken from healthy donors who are free of HIV or hepatitis. Nevertheless, always adhere to the following procedures for the handling of biological materials:

(a) Always wear gloves during handling of the eye and kit components.

(b) All corneal epithelial tissue as well as all material and media that came in contact with it should be decontaminated prior to disposal using special containers or autoclaving.

1.5.2 ASSAY QUALITY CONTROL

1.5.2.1 ASSAY ACCEPTANCE CRITERION 1: NEGATIVE CONTROL

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

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

1.5.2.2 ASSAY ACCEPTANCE CRITERION 2: POSITIVE CONTROL

Ethanol is used as the positive control (PC) for liquid test chemicals (PC-L) and is tested concurrently with the liquid test chemicals. Lauric acid is used as the PC for solid test chemicals (PC-S) and is tested concurrently with the solid test chemicals.

Concurrent here means that the PC-L and the PC-S are to be tested for each run.

Mean tissue viability ≤ 40%

1.5.2.3 ASSAY ACCEPTANCE CRITERION 3: STANDARD DEVIATION (SD)

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

Standard Deviation (SD) of tissue viability of three identically treated replicates for the negative control, positive control, and test chemicals ≤ 18%

1.6 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

The United Nations Globally Harmonized System (GHS) (United Nations, 2003) was used as a reference for the in vivo eye irritation classification of test chemicals.

For the purpose of this EIT, an eye irritant is defined as a substance that induces reversible ocular lesions after administration to rabbits.

According to GHS classifications, a substance is an irritant (Category 1 or 2) if the mean relative viability of three individual tissues exposed to the test chemical is falls below 40% of the mean viability of the negative control. **(Refer to Table 1.)**

Table 1 Prediction model of LabCyte24 EIT

In vitro results	Prediction
Tissue viability is ≤ 40%	Category 1 or 2
Tissue viability is > 40%	No Category

2. MATERIALS

2.1 LabCyte CORNEA-MODEL24

2.1.1 LabCyte CORNEA-MODEL24 KIT COMPONENTS

LabCyte CORNEA-MODEL24 kit components are shown in **Table 2**.

Table 2 LabCyte CORNEA-MODEL24 Kit Components

Component	Qty	Description
LabCyte CORNEA-MODEL24 plate	1 plate	Contains 24 culture inserts with tissues fixed in nutritive agar medium for transport (usable area: 0.3 cm^2).
Assay Medium	1 bottle	Basic medium for incubation (30 mL). Keep refrigerated.
24-well plate	1 plate	Blank plate for use in assay. Store at room temperature.

2.1.2 SHIPMENT OF LabCyte CORNEA-MODEL24

LabCyte CORNEA-MODEL24 is packed in a special Icompo container available from and delivered by NIPPON EXPRESS CO., LTD. After the kit is delivered, examine the contents and make sure that all components (LabCyte CORNEA-MODEL24 plate, assay medium, and 24-well assay plate) are included in the package. Also confirm lot numbers and expiration dates. Record details in Methods Documentation Sheet (MDS) 1. (See **MSD-1**).

NIPPON EXPRESS will pick up the Icompo container at a later date (generally, the day after delivery), and it should be returned together with an shipping invoice and the insulating materials.

2.1.3 INSTRUCTIONS FOR USE OF LabCyte CORNEA-MODEL24

Always incubate all of the culture inserts after opening the package. Do not store the culture inserts after opening.

The human corneal epithelial tissue cells used in the LabCyte CORNEA-MODEL24 originate from a normal human donor and are HIV-, HBV-, HCV-, and HPV-negative. They are to be handled, nevertheless, with due care and in accordance with laboratory biosafety guidelines for handling human-derived materials.

2.2 CONSUMABLES

The following consumables are required.

*The following quantities are necessary to assay between one and six 6 test chemicals at a time.

- Assay Medium, 100 mL (J-TEC: 402250) 1
- Cell Counting Kit-8, 500 test (Dojindo: CK04) 4
- 24-well assay plate (Becton, Dickinson and Company: 353047) 7-8
- 96-well plate (Becton, Dickinson and Company: 353072 or equivalents) 1
- PBS, 500 mL (Invitrogen: 14190-144 or equivalents) 2 or 3 bottle
- Earle balanced salt solution (EBSS), 500 mL (SIGMA-ALDRICH: E3024) 1 bottle
- Sterile cotton buds (JAPAN COTTON BUDS: 10A754D or equivalents) 1 box
- Micro-pipette tips (sterile: 10~200µL, 200‐1000µL)
- Microtubes (1.5mL)
- Dish (10cm)
- Paper towel

Convenient consumable items are shown followings.

Also, it would be convenient to have the following.

- Capillary & piston for positive-displacement-type pipette (10-100µL)

2.3 OTHERS

2.3.1 EQUIPMENT/INSTRUMENTS

- Safety cabinet (or clean bench)
- Water bath (37°C)
- CO_2 incubator (37°C, 5% CO_2, capable of maintaining high humidity)
- Autoclave
- 96-well multi-plate reader (required filters: 450 nm, 650 nm)
- Precision balance (0.1 mg)
- Aspirator
- Stop-watches
- Adjustable micro-pipette (10–200 µL, 200–1000 µL)
- Sharp-edged forceps (sterile)
- Micro spatula (sterile)
- Beaker (1–2 L: sterile)
- Sterilizable poly wash bottle (500–1000 mL: sterile) with wide mouth (mouth > 3-mm dia.)
- Mortar with pestle

Also, it would be convenient to have the following.

- Positive-displacement-type pipette (10–100 µL)

3. TEST METHOD

*Procedures described in Sections 3.1.1 to 3.1.3 and Sections 3.3.1 to 3.3.3 are to be performed aseptically in a safety cabinet or clean bench. Procedures other than those mentions in the previous sentence need not be performed aseptically. Refer to **Section 2.1.3 "INSTRUCTIONS FOR USE OF LabCyte CORNEA-MODEL24"**.

3.1 PREPARATIONS

3.1.1 POSITIVE CONTROL

(1) Use Ethanol as a positive control for liquid test chemicals.

(2) Use Lauric acid as a positive control for solid test chemicals.

3.1.2 NEGATIVE CONTROL

(1) Use PBS as a negative control for liquid test chemicals.

(2) Use non-treatment as a negative control for solid test chemicals.

3.1.3 POLY WASH BOTTLE FOR PBS

(1) Sterilize poly wash bottle using an autoclave.

(2) Fill the sterilized poly wash bottle with sterile PBS.

3.2 TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT

There are two kinds of test chemicals that interfere with the WST-8 assay.

(a) Test chemicals that stain corneal epithelial tissues directly.

(b) Test chemicals that react directly with WST-8.

Test chemical that stain the corneal epithelial tissues could possibility transfer from the corneal epithelial tissues to the WST-8 reaction buffer and affect the OD measurements. Test chemicals that react directly with WST-8 can also affect OD measurements, if the test chemical is present in the corneal epithelial tissues when the WST-8 viability test is performed. The procedure for detecting such test chemicals is described below.

3.2.1 DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE

3.2.1.1 STEP 1 (PRELIMINARY TEST)

(1) Add 50μL (Liquid) or 10mg (Solid) of the test chemical into wells of 24-well assay plate preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as control.

(2) Close the lid of 24-well assay plate and incubate the mixture in CO_2 incubator for 15 minutes.

(3) After incubation, shake the mixture gently and evaluate the staining of the distilled water macroscopically.

(4) When the color of the solution changes significantly, the test chemical is presumed to have the potential to stain the tissue and a functional check on viable tissues (Step2) should be performed. When the color of the solution does not change significantly, the test chemical is determined not to have a potential to stain the tissue.

(5) Record the details of steps 1 to 4 above in **MDS 1-2**.

3.2.1.2 STEP 2 (FUNCTIONAL CHECK ON VIABLE TISSUE)

(1) dd 50 μL (liquid) or 10 mg (solid) of the test chemical, which clearly changed the color of the distilled water in step 1, onto the surface of the epidermis tissues. Distilled water is used as negative control.

(2) Follow all procedures described in the Section 3.3 EXECUTION OF THE TEST. However, incubate the tissue for 3 hours in medium (EBSS) without WST-8 instead of incubating in the diluted WST-8 medium to evaluate the staining of the WST-8 medium (correcting tissue).

(3) The corrected OD is calculated using the following formula.

Corrected OD = A − (B − C),

where

A is the OD of viable tissue exposed to a test chemical,

B is the mean OD of correcting tissue exposed to a test chemical, and

C is the mean OD of correcting tissue exposed to the negative control.

(4) If a corrected OD is below 0, the OD is considered to be 0.

(5) When a cell viability that is calculated according to the procedures described in **Section 3.3.4.4** is ≤ 40%, the test chemical is predicted to be an irritant (GHS Category 1 or 2) and there is no need to calculate a corrected value.

3.2.2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE WST-8

3.2.2.1 STEP 3 (PRELIMINARY TEST)

(1) Dilute the cell counting kit-8 (WST-8 stock solution) with EBSS (Cell Counting Kit-8:EBSS = 1:10), and then prepare the diluted WST-8 medium.

*Prepare immediately prior to use and use as soon as possible.

Fill each well of the 24-well assay plate with 0.3 mL of diluted WST-8 medium.

(2) Add 50 µL of a liquid test chemical or 10 mg of a test chemical to the wells of 24-well assay plate. The diluted WST-8 medium is used as control.

(3) Close the lid of 24-well assay plate and incubate the in a CO_2 incubator for about 4 hours.

(4) After incubation, shake the mixture gently and evaluate the staining of the diluted WST-8 medium macroscopically.

Ethanol | 5% Triton X100 | Glycolic acid | m-Phenylene diamine | Monoethanolamine | Calcium thioglycollate

Photo 1 Example of test chemicals to interfere the WST-8 assay directly (STEP 1).

When test chemicals like m-Phenylene diamine, Monoethanolamine, or Calcium thioglycollate have colored the diluted WST-8 medium, Step 2 must be performed.

(5) Significant coloring of the diluted WST-8 medium by the test chemical can interfere with the WST-8 assay and therefore requires the additional functional check of Step 2.

(6) Record the details of steps 1 to 5 above in **MDS 1-3**.

3.2.2.2 STEP 4 (FUNCTIONAL CHECK ON FREEZE-KILLED TISSUE)

(6) Add 50 µL of a liquid test chemical or 10 mg of solid a test chemical that clearly changed the color of the diluted WST-8 medium (**3.2.2.1. STEP 3**) to the surface of the corneal epithelial tissues.

(7) Follow all procedures described in **Section 3.3 "EXECUTION OF THE TEST"**. Instead of using viable corneal epithelial tissues, however, use corneal epithelial tissues that were freeze-killed at -80°C or lower for 30 min.

Record the details of freeze-killing the tissue in **MDS 1-3**.

(8) The corrected OD is calculated using the following formula.

Corrected OD = A − (B − C),

where

A is the OD of viable tissue exposed to a test chemical,

B is the mean OD of freeze-killed tissue (correcting tissue) exposed to a test chemical, and

C is the mean OD of freeze-killed tissue (correcting tissue) exposed to the negative control.

(9) If a corrected OD is below 0, the OD is considered to be 0.

(10) When a cell viability that is calculated according to the procedures described in **Section 3.3.4.4** is ≤ 40%, the test chemical is predicted to be an irritant (GHS Category 1 or 2) and there is no need to calculate a corrected value.

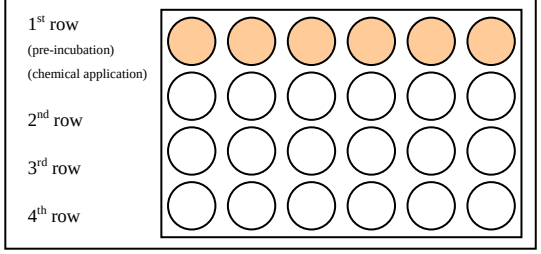
3.3 EXECUTION OF THE TEST

3.3.1 PREPARATION OF LabCyte CORNEA-MODEL24 (DAY -1)

(1) Warm the assay medium to 37°C for 30 minutes in a water bath.

(2) Fill the six wells of the 1st row of each assay plate for LIQUID/SOLID with 0.5 mL/well of the warm assay medium.
See Fig. 1.

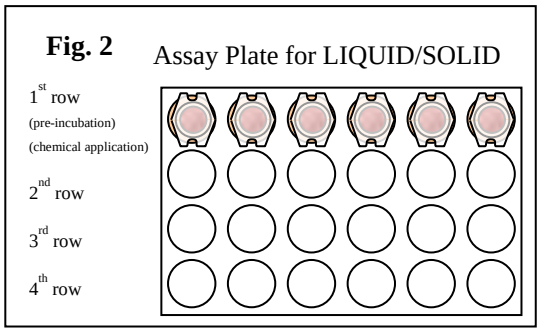
Fig. 1 Assay Plate for LIQUID/SOLID



(3) Open the LabCyte CORNEA-MODEL24 aluminum package.

(4) Open the LabCyte CORNEA-MODEL24 plate lid and pick up the culture inserts using sterile forceps.
*Do not touch the surface of the corneal epithelial tissue in the culture inserts.
*Use forceps to remove any agar medium sticking to the outside of the culture inserts.

Fig. 2 Assay Plate for LIQUID/SOLID



(5) Transfer the culture inserts to the assay medium in the wells of the 1st row using sterile forceps.
See Fig. 2.
*Do not allow air bubbles to form under the tissue inserts.

(6) Close the lid on the plate and place it in a CO_2 incubator.

(7) Incubate overnight (15–30 hours) until ready to perform **Section 3.3.2 "APPLICATION OF LIQUID TEST CHEMICALS, RINSING AND POST-INCUBATION"** or **Section 3.3.3 "APPLICATION OF SOLID TEST CHEMICALS AND RINSING"**.

(8) Record the details of steps 1 to 7 above in **MDS 2**.

3.3.2 APPLICATION OF LIQUID TEST CHEMICALS AND RINSING (DAY 0~1)

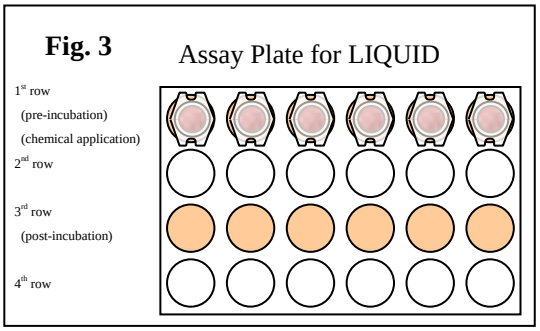
3.3.2.1 PREPARATION OF WELLS FOR POST-INCUBATION (3RD ROW)

(1) Warm the assay medium to 37°C for 30 minutes using a water bath.

(2) Remove the assay plate for LIQUID from the CO_2 incubator.

(3) Open the lid of the assay plate for LIQUID, and use a micropipette to fill the six wells of the 3rd row with 0.5 mL/well of the warm assay medium. **See Fig. 3.**

Fig. 3 Assay Plate for LIQUID



(4) Close the lid of the assay plate and perform **Section 3.3.2.2 "APPLICATION OF LIQUID TEST CHEMICALS AND RINSING"** continuously.

(5) If the test chemicals are not applied immediately, store the Assay Plate for LIQUID in a CO_2 incubator until ready apply but for no more than 12 hours.

(6) Record the details of steps 1 to 5 above in **MDS 3-1**.

3.3.2.2 APPLICATION OF LIQUID TEST CHEMICALS AND RINSING

(1) Remove the assay plate for LIQUID from the CO_2 incubator.

(2) Using a micropipette, apply 50 µL of a liquid test chemical to the surface of the corneal epithelial tissues in the 1st row of the assay plate. Each test chemical is to be tested in three wells (N=3). Carefully apply the test chemical to the central part of each corneal epithelial tissue. After application, close the lid of the assay plate and tap the sides of the plate to spread the liquid test chemicals to spread out over the entire corneal epithelial surface. If necessary, use a micro spatula to spread the liquid test chemical over the entire surface. Take care not to press down on the surface of the corneal epithelial with the spatula.

Photo 2 Pipette tips for viscous liquid test chemicals

*For viscous LIQUID test chemicals, use a wide orifice cell saver tip (**See Photo 2.**) or positive displacement type pipette.

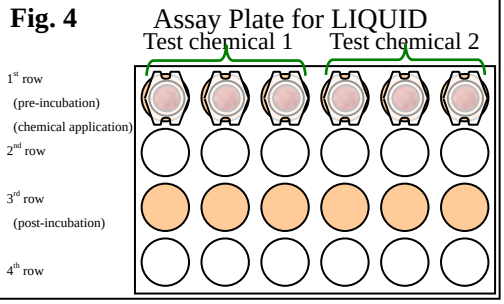
*Use a pipette or other equipment to familiarize yourself beforehand with the characteristics the test chemicals.

*Assay no more than two test chemicals on one 24-well assay plate.

See Fig. 4.

Each chemical is tested in three wells, using three tissues (N = 3).

Fig. 4 Assay Plate for LIQUID



(3) Apply test chemicals to each well at an interval of one to three minutes.

(4) Close the lid and incubate each well for 60 ±10 seconds in the cabinet at room temperature.

*Keep the lid of the assay plate closed at all times except when applying test chemicals. Leaving the lid open could affect the quantity of the test chemical in the well due to air circulation in the cabinet.

(5) Wait for 60 ±10 seconds after applying a test chemical, then open the assay plate for LIQUID and pick up a culture insert with sterile forceps.

(6) Discard the test chemical on the tissue by tilting the insert and tapping it on a beaker. Fill the culture insert to overflowing with PBS from a poly wash bottle.

*Apply a stream of PBS directly to the tissue surface to wash away the test chemical. Maintain a uniformly strong stream of PBS while washing the tissue.

See Photo 3.

*To avoid damaging the tissue with too forceful a stream, use a wide-mouth nozzle on the poly wash bottle.

Photo 3 Rinse 1

(7) Tilt the insert to discard the PBS into the beaker. Remove as much of the PBS inside the culture insert as possible by tapping it on the beaker.

See Photo 4.

Photo 4 Rinse 2

(8) Repeat steps 6 and 7 at least 10 times to remove as much as possible of the residual test chemical on the tissue surface.

*Depending upon the physical properties of the test chemical, bubbles might form in an insert during washing. Continue washing until all bubbles disappear.

(9) Using a sterile cotton bud, gently remove as much as possible of the leftover PBS both inside and outside the culture insert.

See Photo 5.

*Take care not to press down on the surface of the tissue

Photo 5 Rinse 3

with the sterile cotton bud.

(10) Repeat steps 6 to 9 to remove any residual test chemical from the corneal epithelial tissue surface.

(11) Blot the culture insert and then place it in the well of the same column in the 3rd row.

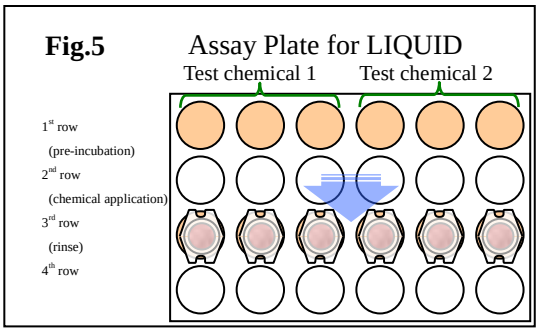
See Fig. 5.

*Take care to prevent air bubbles from forming under the culture inserts.

(12) Repeat steps 1 to 11 for all culture inserts at one- to three-minute intervals.

(13) Record the details of steps 1 to 12 above in **MDS 3-1**.

Fig.5 Assay Plate for LIQUID



3.3.2.3 POST-EXPOSURE INCUBATION

(1) After performing **Section 3.3.2.2 "APPLICATION OF LIQUID TEST CHEMICALS AND RINSING"**, close the lid of the assay plate for LIQUID and place it in a CO_2 incubator as soon as possible.

(2) Incubate for 24 ±1 hours.

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

3.3.3 APPLICATION OF SOLID TEST CHEMICALS AND RINSING (DAY 0–1)

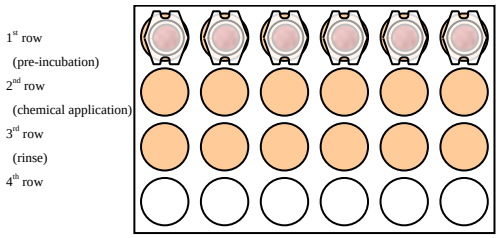
3.3.3.1 PREPARATION OF WELLS FOR SOLID TEST CHEMICAL APPLICATION (2ND ROW) AND FOR POST-INCUBATION (3RD ROW)

(1) Warm the assay medium to 37°C for 30 minutes using a water bath.

(2) Remove the assay plate for SOLID from the CO_2 incubator.

(3) Open the lid of the assay plate for SOLID and use a micropipette to fill the 12 wells in the 2nd and 3rd rows with 0.5 mL/well of warm assay medium . **See Fig. 6.**

Fig.6 Assay Plate for SOLID



(4) Close the lid of the assay plate and perform **Section 3.3.3.2 "APPLICATION OF SOLID TEST CHEMICALS"** continuously.

(5) If the test chemicals are not applied immediately, store the Assay Plate for LIQUID in a CO2 incubator until ready apply but for no more than 12 hours.

(6) Record the details of steps 1 to 5 above in **MDS 3-2**.

3.3.3.2 APPLICATION OF SOLID TEST CHEMICALS

(1) Remove the assay plate for SOLID from the CO_2 incubator.

Using a precision balance, weigh out 10 ±2 mg of the solid test chemicals. If necessary, crush and grind the solid test chemicals in a mortar with pestle. Apply the solid test chemical to the surface of the corneal epithelial tissue. **See Photo 6.**

Photo 6 Application of SOLID

If necessary, use a micro spatula to spread the test chemical gently over the entire surface. Use three wells per test chemical (N = 3).

See Fig. 7.

Each chemical is tested in three wells, using three tissues (N = 3).

(2) Place the exposed culture insert in the well of the same column in the 2nd row (chemical application).

See Fig. 8.

*Take care to prevent air bubbles from forming under the culture inserts.

(3) Close the lid of the assay plate for SOLID and place it in a CO_2 incubator. Incubate for 24 ±1 hours.

(4) Record the details of steps 1 to 4 above in **MDS 3-2**.

Fig.7 Assay Plate for SOLID

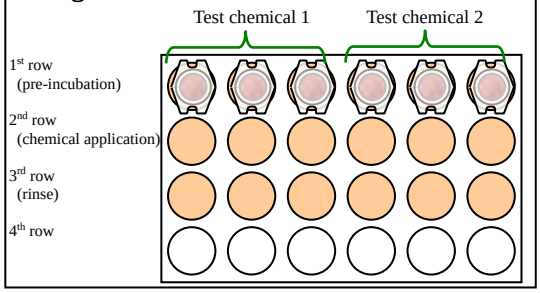
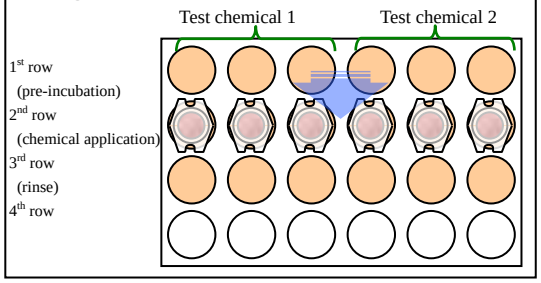


Fig.8 Assay Plate for SOLID



3.3.3.3 RINSING OF SOLID TEST CHEMICALS

(1) After incubation, remove the assay plate for SOLID from the CO_2 incubator.

(2) Open the assay plate for SOLID and pick up a culture insert with sterile forceps.

(3) Discard test chemicals on the tissue by tilting the insert and tapping if on a beaker. Fill the culture insert to overflowing with PBS from a poly wash bottle.

*Apply a stream of PBS directly to the tissue surface to wash away the test chemical. Maintain a uniformly strong stream of PBS while washing the tissue.

See Photo 7.

*To avoid damaging the tissue with too forceful a stream, use a wide-mouth nozzle on the poly wash bottle.

Photo 7 Rinse 1

(4) Tilt the insert to discard the PBS into the beaker.

Remove as much of the PBS inside the culture insert as possible by tapping it on the beaker.

See Photo 8.

(5) Repeat steps 3 and 4 at least 10 times to remove as much as possible of the residual test chemical on the tissue surface.

(6) Using a sterile cotton bud, gently remove as much as possible of the leftover PBS both inside and outside the culture insert.

See Photo 9.

(7) If it proves difficult to remove completely all the residual test chemical from the corneal epithelial tissue surface, remove as much as possible and continue to step 8.

(8) Place the rinsed culture insert in the well of the same column in the 3rd row (rinse).

See Fig. 9.

*Take care to prevent air bubbles from forming under the culture inserts.

(9) Record the details of steps 1 to 8 above in **MDS 3-2**.

After rinsing step, perform **Section 3.3.4 "WST-8 assay"** continuously.

Photo 8 Rinse 2

Photo 9 Rinse 3

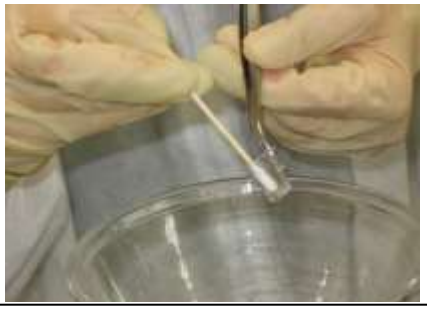
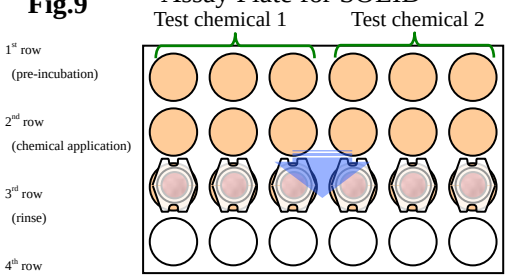


Fig.9 Assay Plate for SOLID



3.3.4 WST-8 ASSAY (DAY 1)

3.3.4.1 PREPARATION OF WELLS FOR WST-8 ASSAY

(1) Warm the EBSS to 37°C for 30 minutes using a water bath.

(2) Dilute the cell counting kit-8 (WST-8 stock solution) with EBSS (Cell Counting Kit-8:EBSS = 1:10), and then warm the diluted WST-8 medium. Blanks for the WST-8 assay are prepared using the diluted WST-8 medium as follows.

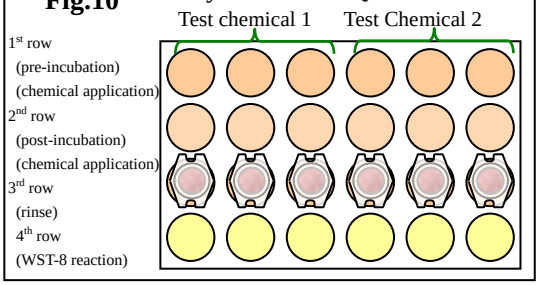
*Prepare immediately prior to performing the WST-8 assay.

(3) Remove the assay plate for LIQUID from the CO_2 incubator or prepare the assay plate for SOLID.

(4) Open the lid of the assay plate and use a micropipette to fill each well of the 4th row with 0.3 mL/well of the warm diluted WST-8 medium.

See Fig. 10.

Fig.10 Assay Plate for LIQUID/SOLID



Close the lid of the assay plate and perform **Section 3.3.4.2 "WST-8 REACTION"** continuously.

(5) Record the details of steps 1 to 4 above in **MDS 4-1**.

3.3.4.2 WST-8 REACTION

(1) Add about 20 mL of PBS each to two dishes: PBS dish 1 and PBS dish 2.

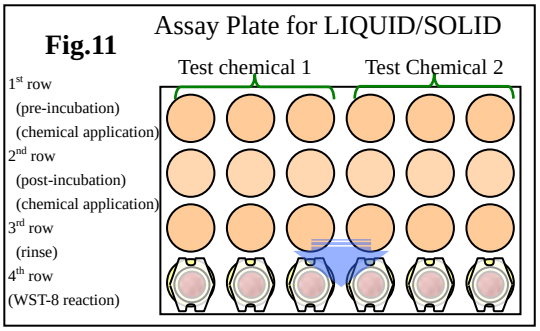
(2) Open an assay plate (for either LIQUID or SOLID) and pick up a culture insert with sterile forceps.

(3) Clean the bottom of the culture insert so it is free of residual culture medium by washing it first in PBS dish 1 and the in PBS dish 2. After washing, wipe the bottom with a paper towel.

(4) After washing and wiping, place the culture insert in the well of the same column in the 4th row.

See Fig. 11.

Fig.11 Assay Plate for LIQUID/SOLID



*Take care to prevent air bubbles from forming under the culture inserts.

(4) Close the lid of the assay plate and place it in the CO_2 incubator.

Incubate for 4 hours ± 20 minutes.

Record the details of steps 1 to 5 above in **MDS 4-1.**

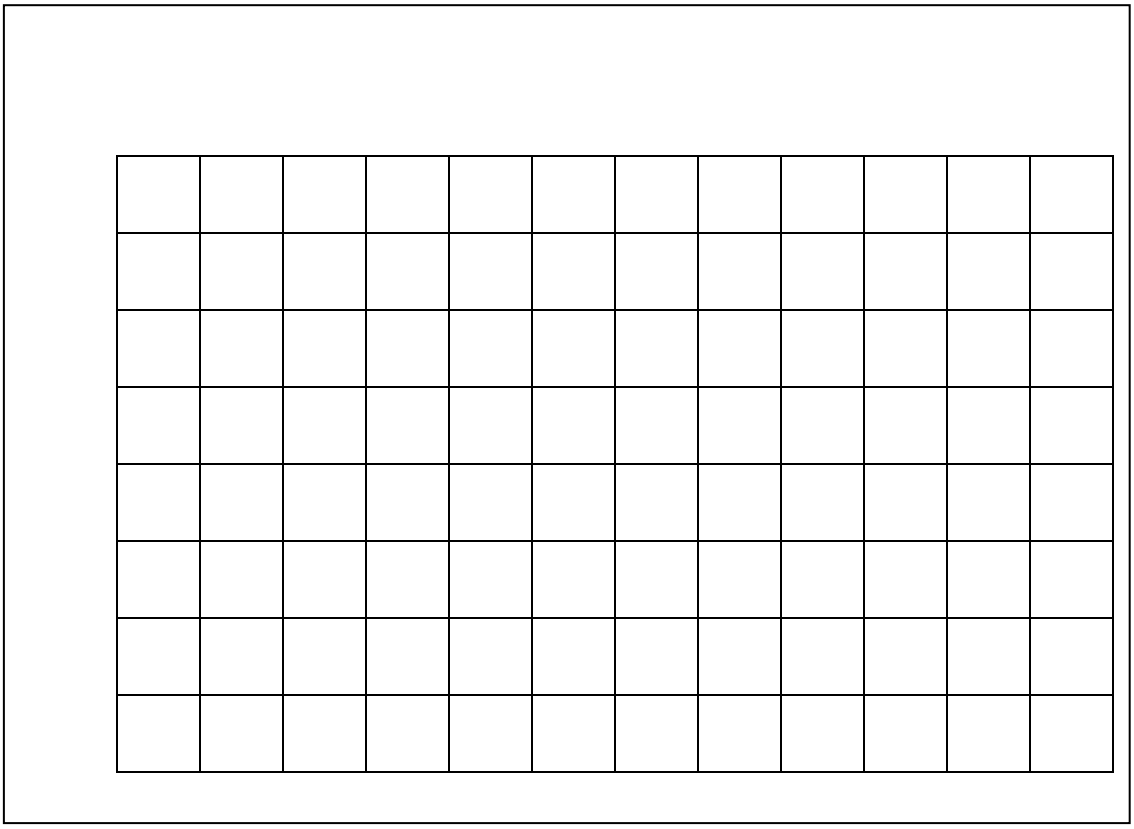
3.3.4.3 SAMPLING THE REACTED WST-8 MEDIUM

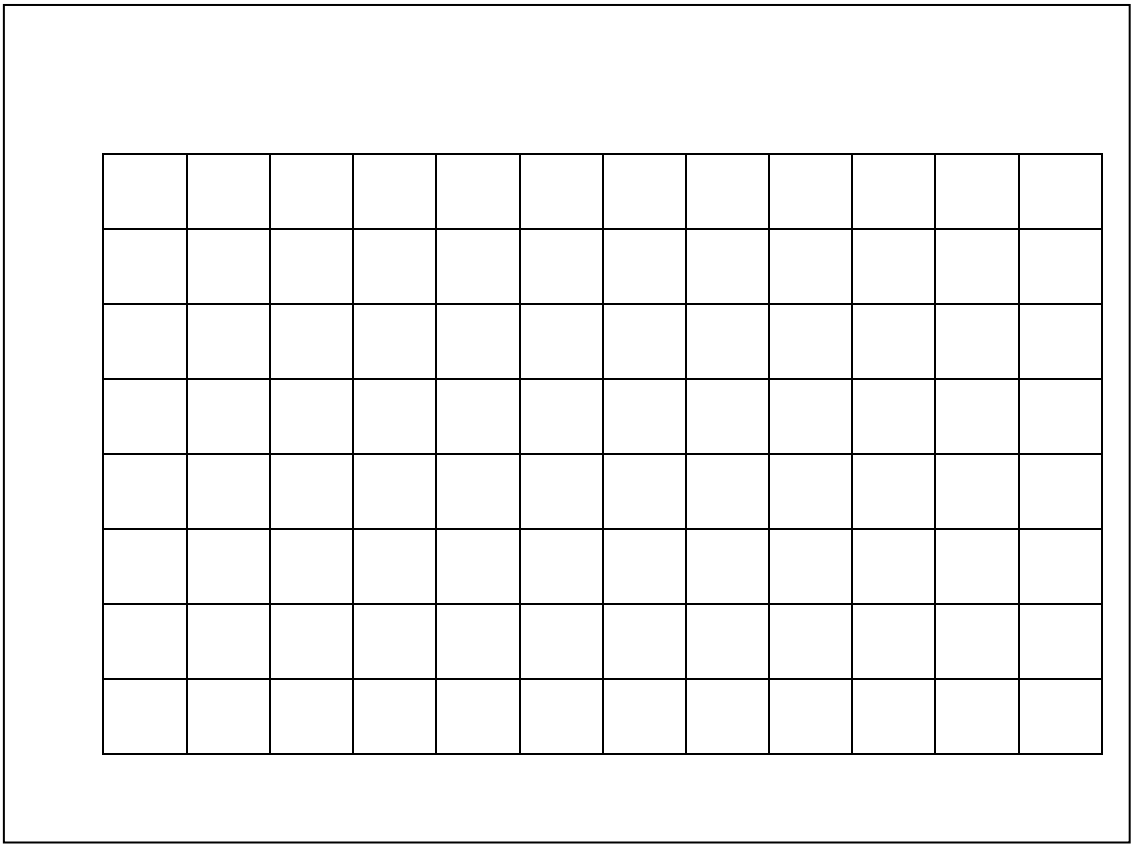
(1) After incubation, remove the assay plate from the CO_2 incubator.

(2) Open the lid of the assay plate and remove the culture inserts from the 4th row with forceps.

Transfer 200 µL of the reacted WST-8 dilution medium into the wells of a 96-well plate.

*Figs. 12A and 12B show typical allocations on a 96-well plate for both living and correcting tissue.





(3) Record the details of steps 1 to 2 above in **MDS 4-2**.

3.3.4.4 OPTICAL DENSITY MEASUREMENTS OF THE REACTION MEDIUM

(1) Using a 96-well plate reader, measure OD at 450 nm and 650 nm and then used the following equation to determine a composite OD for each well.

Composite OD = (OD_{TC} at 450 nm – OD_{blank} at 450 nm) – (OD_{TC} at 650 nm – OD_{blank} at 650 nm)

*If the plate reader can be programmed to perform this calculation automatically, then only the composite OD value need be recorded.

(2) Calculate the Mean OD for the negative control, a cell viability for each individual tissue, and a mean cell viability (including SD) for each test chemical using the following equations.

$$\text{Mean } OD_{NC} = \frac{\text{Sum of the } OD_{NC} \text{ for three replicate tissues}}{3}$$

554

$$\text{Tissue cell viability (\%)} = \frac{\text{Each tissue } OD_{TC}}{\text{Mean } OD_{NC}} \times 100$$

555

$$\text{Mean cell viability (\%)} = \frac{\text{Sum total of cell viability (\%) for three replicate tissues}}{3}$$

556

557 (3) Record the details of steps 1 and 2 above in **MDS 4-2**.

558

559

4. ASSESSMENT

4.1 CONDITIONS FOR A VALID TEST

An eye irritation test is considered valid if all three of the following criteria have been met.

- Tissue viability: 0.5 ≤ mean OD (A450/650) measured value for negative control ≤ 1.3
- Positive control: mean tissue viability for positive control ≤ 40%
- SD: SD (negative control, positive control and each test chemicals) of tissue viability of 3 identically treated replicates ≤ 18 %

4.2 ASSAY CRITERIA

The criteria for in vitro prediction are shown below.

After exposure to a chemical, if cell viability is 40% or less, the chemical is predicted to be an irritant (GHS Category 1 or 2), otherwise it is predicted to be a non-irritant (GHS No Category).

See Table 3.

Table 3 Prediction model of LabCyte24 EIT

Tissue Viability	Prediction
≤ 40%	Category 1 or 2
> 40%	No Category

5. References

Green H. (1978) Cyclic AMP in relation to proliferation of the epidermal cell: New view. Cell, 15, 801-811.

Rheinwald J.G. and Green H. (1975) Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. Cell, 6, 331-343.

MDS 1-1:

RECEIPT OF LabCyte CORNEA-MODEL24 (2.1.2)

Laboratory name:_____ Test name: _____ Test no. : _____

1. LabCyte CORNEA-MODEL24

Date received: _____

Lot no.: _____

Expiration date: _____

Accessories: Assay medium, 30mL □ (Lot no.:_____ Expiration date:_____)

24 well assay plate □

Note

2. Assay medium

Date received: _____

Lot no.: _____

Expiration date: _____

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

MDS 1-2:

TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT

STEP-1 (3.2.1.1)

Laboratory name:_ Test name: _____ Test no. : _____

1. Add distilled water (0.5 mL) to the wells of the 24-well assay plate.

To add distilled water (0.5 mL) □ Execution date/time:_____

2. Apply test chemicals to the wells of the 24-well assay plate.

3. Culture the 24-well assay plate in CO_2 incubator for 4 hours.

Time of WST-8 reaction started: _____

Time of WST-8 reaction completed: _____

4. Check the color of water.

5. Test chemical information and check list of coloring potential.

Test chemical	Physical state	Amount	Coloring	Test chemical.	Physical state	Amount	Coloring
PBS (NC)	LIQUID			Non treatment (NC)			
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

MDS 1-3:

TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT

STEP-3 (3.2.2.1)

Laboratory name:_ Test name: _____ Test no. : _____

1. Preparation of WST-8 dilution medium

Warm EBSS for 30 minutes. □ Time/date:_____

CCK-8: (Lot no.:_____ Expiration date:_____)
EBSS: (Lot no.:_____ Expiration date:_____)
Volume _____mL Time/date completed:_____

2. Add WST-8 dilution medium (0.3mL) to the wells of the 24-well assay plate.

To add WST-8 dilution medium (0.3mL) □ Time/date executed:_____

3. Apply test chemicals to the wells of the 24-well assay plate.

4. Culture the 24-well assay plate in CO_2 incubator for 4 hours.

Time of WST-8 reaction started: _____

Time of WST-8 reaction completed: _____

5. Check the color of WST-8 medium.

6. Test chemical information and checked list of WST-8 assay interfere.

Test chemical	Physical state	Amount	WST-8 assay interfere	Test chemical.	Physical state	Amount	WST-8 assay interfere
PBS (NC)	LIQUID			Non treatment (NC)			
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

MDS 1-4:

PREPARATION OF FREEZE KILLED TISSUE (3.2.2)

Laboratory name:_____ Test name: _____ Test no. : _____

1. Transfer LabCyte CORNEA-MODEL24 tissues to 50 mL tube or appropriate sterile container.

2. Freeze tissues in the -80℃ deep-freezer for 30 minutes (1st freezing).

   Store for 30 minutes. □ Time/date:_____

3. Thaw tissues in the 37℃ incubator for 15 minutes.

   Store for 15 minutes. □ Time/date:_____

4. Freeze tissues in the -80℃ deep-freezer for more than 30 minutes (2nd freezing).

   Store for more 30minutes. □ Time/date:_____

5. Just before using, thaw tissues in the 37℃ incubator for 15 minutes.

   Store for 15 minutes. □ Time/date:_____

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

MDS 2:

PREPARATION OF LabCyte CORNEA-MODEL24 (3.3.1)

Laboratory name: _____ Test name: _____ Test no. : _____

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

Assay medium: (Lot no.:_____ Expiration date:_____)

Warm for 30 minutes. □ Time/date:_____

Add 0.5mL of assay medium to each well □ Time/date:_____ Number of plate:_____

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

LabCyte CORNEA-MODEL24(Lot no.:_____ Expiration date:_____)

Time/date executed:_____

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

3. LabCyte CORNEA-MODEL24 is cultured in CO_2 incubator overnight.

.

Time/date of culture started: _____

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

MDS 3-1(LIQUID):

APPLICATION OF LIQUID TEST CHEMICALS, RINSING AND POST-INCUBATION (3.3.2)

Laboratory name: _________ Test name: ___________ Test no. : _________________________

1. Warm up the assay medium and add 0.5mL of the assay medium to the wells of the 3rd row on the 24-well assay plate for LIQUID.

Assay medium: (Lot no.:_________________________ Expiration date:_____________________)

Warm for 30 minutes. □ Time/date:_______________

Add 0.5mL of assay medium to each well □ Time/date:_______________ Number of plate:__________

2. Apply test chemicals to the LabCyte CORNEA-MODEL24.

Time/date execution started: _____________________

3. LIQUID test chemical information

Test chemical.	Lot no.	Physical state	Test chemical vol.	Time of application	Time of rinsing	Exposure period (1 minute)
PBS (Negative control)		LIQUID	50 µL	1 : 2 : 3 :	: : :	□ □ □
Ethanol (Positive control)		LIQUID	50 µL	1 : 2 : 3 :	: : :	□ □ □
		LIQUID, viscous	50 µL	1 : 2 : 3 :	: : :	□ □ □
		LIQUID, viscous	50 µL	1 : 2 : 3 :	: : :	□ □ □
		LIQUID, viscous	50 µL	1 : 2 : 3 :	: : :	□ □ □
		LIQUID, viscous	50 µL	1 : 2 : 3 :	: : :	□ □ □
		LIQUID, viscous	50 µL	1 : 2 : 3 :	: : :	□ □ □
		LIQUID, viscous	50 µL	1 : 2 : 3 :	: : :	□ □ □
		LIQUID, viscous	50 µL	1 2 3		□ □ □
		LIQUID, viscous	50 µL	1 2 3		□ □ □
		LIQUID, viscous	50 µL	1 2 3		□ □ □
		LIQUID, viscous	50 µL	1 2 3		□ □ □

4. After exposure to test chemical for 1 minute, wash out the LabCyte CORNEA-MODEL24 and transfer the culture inserts to the 3rd row on the 24-well assay plate.

PBS: (Lot no.:_________________________ Expiration date: _____________________)

Hit PBS stream on the tissue surface directly. □

Confirm that there are no bubbles under the cell culture insert. □ Time/date completed:___________

5. Culture LabCyte CORNEA-MODEL24 in CO_2 incubator for 24 hours.

Time/date post-incubation started: ________________________

Time/date post-incubation completed: ________________________

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

MDS 3-2(SOLID):

APPLICATION OF SOLID TEST CHEMICALS AND RINSING (3.3.3)

Laboratory name: _____ Test name: _____ Test no. _____

1. Warm up the assay medium and add 0.5mL of the assay medium to the wells of 2nd and 3rd row on the 24-well assay plate for SOLID.

Assay medium: (Lot no.:_____ Expiration date:_____)

Warm for 30 minutes. □ Time/date:_____

Add 0.5mL of assay medium to each well □ Time/date:_____ Number of plate:_____

2. Apply test chemicals to the LabCyte CORNEA-MODEL24 and transfer the culture inserts to the 2nd row on the 24-well assay plate.

Time/date started:_____ Time/date completed:_____

3. SOLID test chemical information

Test chemical	Lot no.	Physical state	Crush and grind	Test chemical amount.	Time of application	Exposure period (24hours)
Non treatment (Negative control)					:	□
Lauric acid (Positive control)		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□
		SOLID	□	(mg, mg, mg)	:	□

4. Culture LabCyte CORNEA-MODEL24 in CO_2 incubator for 24 hours.

Time/date exposure started: _____

Time/date exposure completed: _____

5. After exposure to test chemical, wash out the LabCyte CORNEA-MODEL24 and transfer the culture inserts to the 3rd row on the 24-well assay plate.

PBS: (Lot no.:_____ Expiration date: _____)

Hit PBS stream on the tissue surface directly. □

Time/date started:_____ Time/date completed:_____

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

MDS 4-1:

WST-8 ASSAY (3.3.4.1, 3.3.4.2)

Laboratory name:_____ Test name: _____ Test no. : _____

1. Preparation of WST-8 dilution medium

Warm EBSS for 30 minutes. □ Time/date:_____

CCK-8: (Lot no.:_____ Expiration date:_____)
EBSS: (Lot no.:_____ Expiration date:_____)
Volume _____mL Time/date completed:_____

2. Add WST-8 dilution medium (0.3mL) to the wells in the 4th row on the 24-well assay plate.

To add WST-8 dilution medium (0.3mL) □ Time/date executed:_____

3. After the operation, the blotted tissue transfer to wells of 4th row of 24-well assay plate.

Time/date started:_____ Time/date completed:_____

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

4. Culture LabCyte CORNEA-MODEL24 in CO_2 incubator for 4 hours.

Time of WST-8 reaction started: _____

Time of WST-8 reaction completed: _____

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

MDS 4-2:
SAMPLING OF REACTING WST-8 SOLUTION (3.3.4.3) AND MEASUREMENT (3.3.4.4)

Laboratory name:_____ Test name: _____ Test no. : _____

1. Reacting WST-8 solution (200 µL) is transferred to each well on the 96-well plate.

Transfer to the 96-well plate. □

Time/date executed:_____

Sample location on 96-well plate.

	LIQUID						SOLID					
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B	PBS-1	PBS-2	PBS-3	Ethanol-1	Ethanol-2	Ethanol-3	Non-treatment-1	Non-treatment-2	Non-treatment-3	Lauric acid-1	Lauric acid -2	Lauric acid -3
C												
D												
E												
F												
G												
H												

2. Analyze extract OD at 450nm and 650nm, and calculate the OD(450nm-650nm).

Analyze OD at 450nm and 650nm. □
Calculate the OD (450nm-650nm). □
Calculate cell viability and SD. □
Cell viability and SD are recorded on a separate data sheet. □
The data sheet is attached to the back of this sheet. □
Check for input errors. □
Time/date executed:_____

Note

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

REVISION HISTORY

Rev.	CONTENT	Date Revised
Ver.1.1	1) First version	Aug., 2012
Ver.1.2	1) Revised clerical error.	Aug., 2012
Ver.1.3	1) Revised clerical error.	Sep., 2012
Ver.1.3PS	Added the following supplementary explanation. 1) Added more detail explanation about the conditions of WST-8 reaction. 2) Added the formula of SD.	Sep., 2012
Ver.2.1	1) Revised clerical error. 2) In the section 1.2 “BACKGROUND”, changed statement about animal testing (Draize test). 3) In the section 3.3. “TEST METHOD”, explained the washing protocol of the LIQUID and SOLID test chemical more briefly. 4) In the section 3.3. “TEST METHOD”, changed the WST-8 dilution rate with PBS from 1:10 to 1:5. 5) In the section 3.3. “TEST METHOD”, changed the reaction period of WST-8 from 5 hours to 4 hours. 6) In the section 3.3. “TEST METHOD”, changed the condition of WST-8 reaction from shaking to standing. 7) In the section 3.3. “TEST METHOD”, changed the application-amount of SOLID chemicals from 50mg to 10mg. 8) As assay acceptance criteria, added that SD (test chemicals) of tissue viability of 3 identically treated replicates ≤ 20 %. 9) In the prediction model of this EIT, changed the cut-off value of the mean viability from 50% to 40%.	May, 2013

Ver.2.2	1) Revised clerical error. 2) In the section 3.3. “TEST METHOD”, additionally explained that the solid test chemical is crush and grind in a mortar with pestle if necessary. 3) In the section 3.3.2 “APPLICATION OF LIQUID TEST CHEMICALS AND RINSING (DAY 0~1)”, explained temperature condition (room temperature) at chemical application.	Sep., 2013
Ver.2.3	1) Revised clerical error. 2) In the section 3.3. “TEST METHOD”, explained about a blank preparation at WST-8 assay. 3) In the section 3.3. “TEST METHOD”, changed the condition of WST-8 reaction from standing to shaking. 5) In the section 3.3. “TEST METHOD”, explained about a blank preparation at WST-8 assay.	Feb., 2014
Ver.2.3.1	1) Revised clerical error. 2) About the prediction result of eye irritation, changed the classification from irritation/no irritation to GHS classification.	Mar., 2014
Ver.2.3.2MTT	1) At the analysis of cell viability, changed the assay method from WST-8 assay to MTT assay.	Jul., 2014
Ver.2.4.1	1) At the analysis of cell viability, changed the assay method from MTT assay to WST-8 assay. 2) In the section 3.3. “TEST METHOD”, changed the condition of WST-8 reaction from shaking to standing. 3) In the section 3.3. “TEST METHOD”, changed the dilution solution of WST-8 reaction from PBS to EBSS. 4) In the section 3.3. “TEST METHOD”, changed the reaction period of WST-8 from 5 hours to 4 hours. 5) The standard of the additional testing was mentioned about a	Jan., 2015

	borderline result. 9) In the prediction model of this EIT, changed the cut-off value of the mean viability from 40% to 50%.	
Ver.2.4.2	1) Changed the assay acceptance criterion of negative control from 0.5 < and <2.0 to 0.6 < and <1.5.	Mar., 2015
Ver.2.4.2m	1) The judgement of the chemical which is a result of the borderline was added.	Jul., 2015
Ver.2.5.1	1) Revised clerical error. 2) In the prediction model of this EIT, changed the cut-off value of the mean viability from 50% to 40%. 3) Changed the judgement of equivocal results. 4) Changed the assay acceptance criterion of negative control from 0.6 < and <1.5 to 0.5 < and <1.3. 5) In the section 3.2. “TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT”, revised description has clearly. 6) Added MDS1-2 and MDS 1-3.	Aug., 2015
Ver.2.5.1m	1) Revised clerical error. 2) Changed allocation for a 96-well plate of pattern which consists of freeze killed tissue (Fig.12B).	Sep., 2015
Ver. 2.5.1mr	1) Revised Fig.2B	Sep.,2015
Ver. 2.5.2	1) In the section 3.3. “TEST METHOD”, explained the washing protocol of the LIQUID and SOLID test chemical more briefly. 2) In the MDS 3-1 and the MDS 3-2, added the check box about the washing procedure. 3) In the MDS 3-2, added the check box about crush and grind of	Sep.,2015

	test chemicals.	
Ver. 2.5.4	1) In the section 3.3. “TEST METHOD”, added attention point of the washing procedure. 2) In the section 4.3 “ASSESSMENT FLOWCHART”, Change numbers of test run from three independent run to single run.	June, 2016
Ver. 2.5.5	1) Revised clerical error. 2) As assay acceptance criteria, changed that SD (negative control, positive control and test chemicals) of tissue viability of 3 identically treated replicates from ≤ 20 % to ≤ 18 %.	October, 2016
Ver.2.5.6	1) Revised clerical error. 2) Detection protocol of coloring interference is changed from using WST-8 medium to distilled water and correction of coloring interference is changed from using freeze-killed tissue to using living tissue without WST-8 reaction.	February, 2017

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