-JSAAE Skin Irritation Test Validation Study-

SKIN IRRITATION TEST USING THE RECONSTRUCTED HUMAN MODEL "LABCYTE EPI-MODEL 24"

Ver. 8.3

LabCyte EPI-MODEL24 SKIN IRRITATION TEST-42 HOURS

S.O.P.

TABLE OF CONTENTS

1. RATIONAL AND BACKGROUND

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

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

1.2 BACKGROUND OF SIT using LabCyte EPI-MODEL24

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

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

1.3 BASIS OF THE METHOD

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

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

1.3.1 TEST SYSTEM DESCRIPTION

LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, they were cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978).

Reconstruction of human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface 0.3 cm_2) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. It constructs a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18°C.

1.3.1.1 Quality control of the test system

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

1.3.1.2 Precautions

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

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

1.3.2 ASSAY QUALITY CONTROL

1.3.2.1 Assay Acceptance Criterion 1: Negative Control

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

$0.7 \leq$ Mean OD (A570/650) measured value ≤ 2.5

1.3.2.2 Assay Acceptance Criterion 2: Positive Control

A 5% SDS (in H_2O) solution (see 7.6.3) is used as positive control (PC) and tested concurrently with the test chemicals. Concurrent means here the PC has to be tested in each assay, but not more than one PC is required per testing day. Viability of positive control should be within 95±1 % confidence interval of the historical data.

Mean tissue viability ≤ 40%

1.3.2.3 Assay Acceptance Criterion 3: Standard Deviation (SD)

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

Standard Deviation (SD) of tissue viability of 3 identically treated replicates for negative control and positive control ≤ 18 %

1.4 LIMITATION OF THE METHOD

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

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

1.5 BRIEF BASIC PROCEDURE

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

1.6 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

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

In vitro results	In vivo prediction
Tissue viability is ≤ 50%	Irritant
Tissue viability is > 50%	Non Irritant

2. MATERIALS

2.1 LabCyte EPI-MODEL 24

2.1.1 LabCyte EPI-MODEL 24 KIT COMPONENTS

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

Table 1 - LabCyte EPI-MODEL24 Kit Components

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

2.1.2 SHIPMENT OF LabCyte EPI-MODEL24

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

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

2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL 24

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

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

2.2 TEST CHEMICALS

Coded test chemicals are delivered to each laboratory.

2.3 CONSUMABLES

The following consumables are required.

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

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

2.4 OTHERS

2.4.1 EQUIPMENT / INSTRUMENTS

- Safety cabinet (or clean bench)
- Water bath (37 °C)
- CO_2 incubator (37 °C, 5%CO_2, capable of maintaining high humidity)
- Autoclave
- 96-well multi-plate reader (required filters: 450nm, 570nm, 650nm)
- Precision balance (0.1mg)
- Aspirator
- Stop-watches
- Adjustable micro-pipette (10-200µL, 200-1000µL)
- Sharp-edged forceps (sterile)
- Micro spatula (sterile)
- Beaker (1~2L: sterile)
- Sterilizable poly wash bottle (500~1000mL: sterile)

2.4.2 CONSUMMABLE ITEMS

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

3. TEST METHOD

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

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

3.1 PREPARATIONS

3.1.1 MTT SOLUTION

(1) Dissolve MTT in the assay medium to prepare the MTT medium (final concentration: 0.5mg/mL)
Use ultrasonic cleaning equipment or a vortex mixer as necessary in order to completely dissolve the MTT.
*Store in a dark, cold place and use it within 24 hours.

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

3.1.2 POSITIVE CONTROL SUBSTANCE

(1) Weigh 500mg of SLS precisely.

(2) To prepare a positive control solution, put the SLS into a graduated cylinder or measuring flask and dilute to 10mL with distilled water (final concentration: 5% w/v)]
* Store in a dark, cold place and use it within 24 hours.

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

3.1.3 NEGATIVE CONTROL SUBSTANCE

(1) Use distilled water.

3.1.4 POLY WASH BOTTLE FOR PBS

(1) Sterilize poly wash bottle using an autoclave.

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

3.2 TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH MTT ENDPOINT

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

(a) Chemical that stains epidermis tissues.

(b) Chemical that is able to directly reduce MTT.

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

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

3.2.1 DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE

3.2.1.1 STEP1 (PRELIMINARY TEST)

(1) Add 25µL (Liquid) or 25mg (Solid) of the test chemical into wells of 24-well assay plate preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as control.

(2) Close the lid of 24-well assay plate and incubate the mixture in CO_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.

3.2.1.2 STEP2 (FUNCTIONAL CHECK ON VIABLE TISSUE)

(1) Add 25µL (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the distilled water (Step1), onto the surface of the epidermis tissues. Distilled water is used as negative control.

(2) Follow all procedures described in this SOP Section 3.3 EXECUTION OF THE TEST. However, incubate the tissue for 3 hours in culture media without MTT instead of incubating in media containing MTT to evaluate the staining of the epidermis tissues.

(3) Calculate ratio of staining by test chemical from the following formula.

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

(4) When the ratio of staining by test chemical is <5%, correction of the results is not necessary.

When the ratio is between 5% and 30%, the corrected MTT OD is calculated using the following formula.test chemical

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

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

3.2.2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE MTT

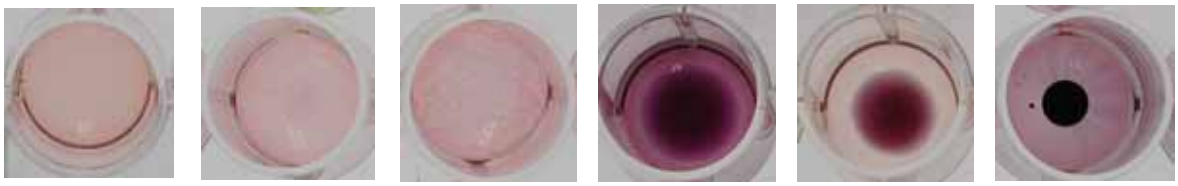
3.2.2.1 STEP3 (PRELIMINAY TEST)

(1) Add 25µL (Liquid) or 25mg (Solid) of the test chemical into wells of 24-well assay plate preliminarily filled with 0.5mL of MTT medium. Untreated MTT medium is used as control.

(2) Close the lid of 24-well assay plate and incubate the mixture in CO_2 incubator for 1 hour.

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

(4) When the MTT medium turns blue/purple significantly, the test chemical can reduce MTT and additional functional check (Step4) must be performed.



5% SLS | 1-bromo hexane | 1,1,1-tetrachloroethane | 5-(1,1-dimethylethyl)-2-methyl | cinnamaldehyde | eugenol

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

3.2.2.2 STEP4 (FUNCTIONAL CHECK ON VIABLE TISSUE)

(1) Add 25µL (Liquid) or 25mg (Solid) of the test chemical, which clearly changed the color of the MTT medium into blue/purple (Step3), onto the surface of the epidermis tissues.

Distilled water is used as negative control.

(2) Follow all procedures described in this SOP Section 3.3 EXECUTION OF THE TEST. However, use the epidermis tissues that has been freeze-killed at -20 °C or lower for more than 24 hours instead of viable epidermis tissues.

(3) Calculate ratio of staining by test chemical from the following formula.

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

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

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

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

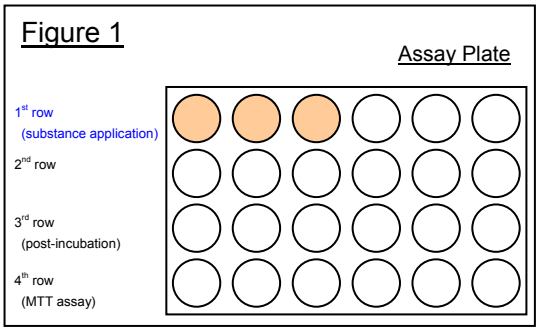
3.3 EXECUTION OF THE TEST

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

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

(2) Fill 3 wells of the 1st row of each 24-well assay plate with the pre-warmed assay medium (0.5mL/well).
→ Figure 1

Figure 1

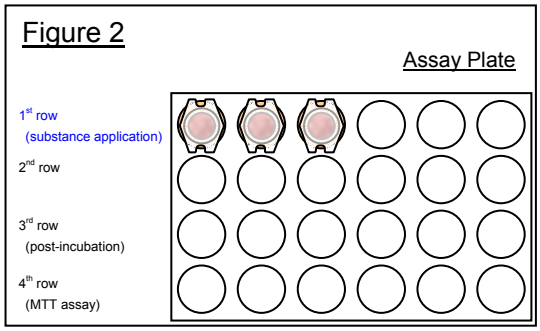


(3) Open the LabCyte EPI-MODEL 24 aluminum package.

(4) Open the LabCyte EPI-MODEL 24 plate lid and pick up the culture inserts using sterile forceps.
*Do not touch the epidermis surface of culture inserts.
*Use forceps to remove agar medium sticking to the outside of the culture inserts.

(5) Transfer the culture inserts into assay medium filled wells of the 1st row using sterile forceps.
→ Figure 2
*Avoid air bubble formation under the tissue inserts.

Figure 2



(6) Place the plate (lid on) in a CO_2 incubator.

(7) Incubate overnight (15~30 hours) until Section 3.2.2 "APPLICATION OF TEST CHEMICALS AND RINSING."

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

3.3.2 APPLICATION OF TEST CHEMICALS AND RINSING (DAY 0)

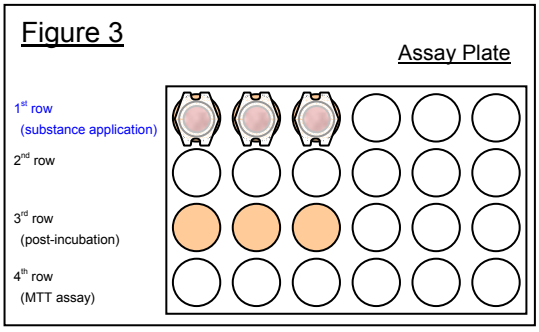
3.3.2.1 PREPARATION OF WELLS FOR POST-INCUBATION (3RD ROW)

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

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

(3) Open the lid of the assay plate, and fill 3 wells of the 3rd row with the pre-warmed assay medium (1.0mL/well) using a micropipette.
→ Figure 3

Figure 3



(4) Place the plate (lid on) in a CO_2 incubator.

(5) Incubate until application of test chemicals (0~12 hours).

(6) Record details of steps (1) – (5) above in the MDS 3.

3.3.2.2 APPLICATION OF TEST CHEMICALS

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

(2) Apply test chemicals onto the surface of epidermis tissues in the 1st row of the assay plate. Use 3 wells per test chemical (N=3).

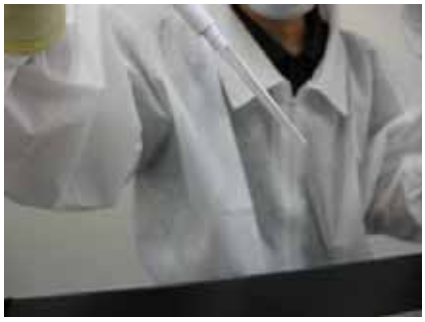
FOR LIQUIDs: Carefully apply 25µL of the test chemical onto the central part of each epidermis using a micropipette. After applied, close the lid of the assay plate and tap the side of the plate outside the safe cabinet (or clean bench) in order for the liquid to spread out over the entire epidermis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

*Use wide orifice cell saver tips for viscous liquids.

→ Photo 2

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

Photo 2 - Pipette tips for viscous liquids



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

→ Photo 3

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

→ Figure 4

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

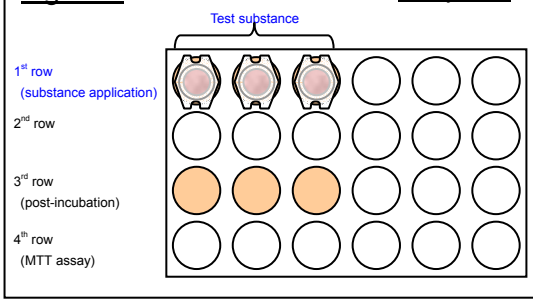
Photo 3 – Applying a solid substance

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

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

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

Figure 4 Assay Plate



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

3.3.2.3 REMOVAL OF THE TEST CHEMICALS

(1) 15 minutes (±30 seconds) after applying a chemical, open the assay plate and pick up a culture insert with sterile forceps. Discard test chemicals on the tissue by tilting and then tapping the insert on the beaker.

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

→ Photo 4

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

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

→ Photo 5

(4) Repeat steps (2) and (3) at least 15 times or more as much as possible, and remove all residual test chemical on the tissue surface almost completely. Must not do discarding by tapping at only the last washing operation.

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

→ Photo 6

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

(6) If test material remains on the epidermis surface, repeat steps (2) ~ (5) again.

(7) Transfer the blotted culture insert to a well in the 3rd row of the same column (for post-incubation).

→ Figure 5

Photo 4 - Rinse 1

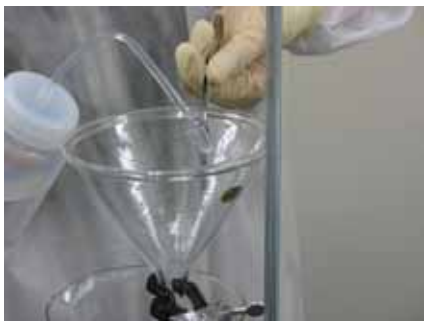
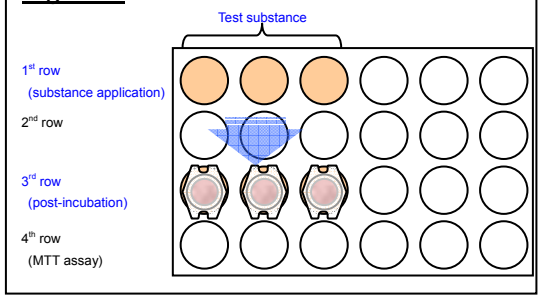


Photo 5 - Rinse 2

Photo 6 - Rinse 3

Figure 5 Assay Plate



*Avoid air bubble formation under the culture inserts.

(8) Repeat steps (1) ~ (7) for all the culture inserts at 1~3-minute intervals.

(9) Record details of steps (1) – (8) above in the MDS 3.

3.3.3 POST TREATMENT INCUBATION (DAY 0~2)

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

(2) Incubate for 42 hours.

3.3.4 MTT ASSAY (DAY 2)

3.3.4.1 PREPARATION OF WELLS FOR MTT ASSAY

(1) Pre-warm MTT medium for 30 minutes to 37°C using a water bath.

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

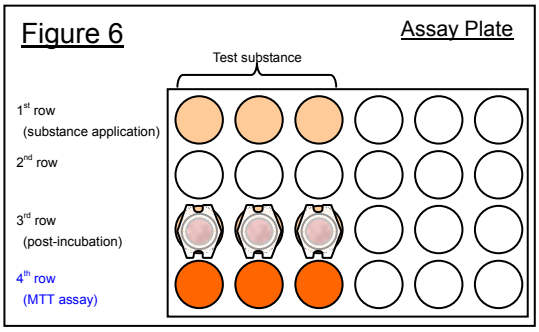
(3) Open the lid of the assay plate, and fill each well of the 4th row with the pre-warmed MTT medium (0.5mL/well) using a micropipette.
→ Figure 6

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

(5) Incubate until starting MTT assay (about 0 ~ 12 hours).

(6) Record details of steps (1) – (5) above in the MDS 4.

Figure 6



3.3.4.2 MTT ASSAY

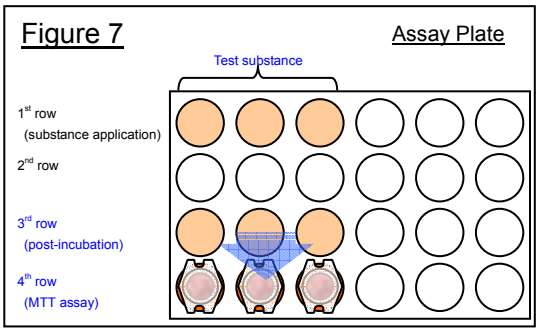
(1) Remove the assay plate from the CO_2 incubator after 42 hours (±1 hour) of post-incubation.

(2) Transfer each culture insert from the 3rd row to the 4th row of the corresponding column.
→ Figure 7
*Avoid dripping from the base end surface of the culture insert into other wells.
*Avoid air bubble formation under the culture inserts.

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

(4) Incubate for 3 hours.

Figure 7



(5) Record details of steps (1) – (4) above in the MDS 4.

3.3.5 FORMAZAN EXTRACTION AND MEASUREMENT (DAY 2~3)

3.3.5.1 FORMAZAN EXTRACTION

(1) Remove the assay plate(s) from the CO_2 incubator 3 hours (±5 minutes) after the MTT assay.

(2) Open the lid of the assay plate and pinch the cultured epidermis from each culture insert of the 4th row with forceps.

→ Photo 7

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

Photo 7 - Detachment of epidermis

(3) Transfer the epidermis tissue into a 1.5mL micro tube.

(4) Add 300µL of isopropanol to the micro tubes and soak the entire epidermis tissue in the isopropanol.

(5) Incubate the micro tubes in a dark cold place (or refrigerator) overnight (more than 15 hours) in order to completely extract pigments.

*Tighten the micro tube seal.

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

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

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

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

*One well of 200µL of isopropanol should be set as a blank.

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

Figure 8 – Allocation for a 96-well plate

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

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

3.3.5.2 OPTICAL DENSITY MEASUREMENTS OF THE EXTRACTS

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

Measured OD = [570nm OD_{sample} – 570nm OD_{blank}] – [650nm OD_{sample} – 650nm OD_{blank}]
*Set the plate reader-calculated value as the measured OD if the 96-well plate reader performs automatic calculations.

(2) Calculate the cell viability of a sample using the equation below. Furthermore, calculate the variability (SD) of tissue replicates.

(3) Record details of steps (1) and (2) above in the MDS 5.

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

4. ASSESSMENT

4.1 CONDITIONS FOR A SUCCESSFUL STUDY

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

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

4.2 ASSAY CRITERIA

The criteria for in vitro interpretation are shown below.

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

Tissue Viability (primary)	Classification
Tissue viability is ≤ 50%	Irritant
Tissue viability is > 50%	Non Irritant

[FLOWCHART] ASSESSMENT FLOWCHART

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

0.7 ≤ Mean OD measured value ≤ 2.5

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

Mean tissue viability ≤ 40%

SD”

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

↓

(All criteria are met)

↓

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

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

↓

(No)

↓

Classified as non irritant

MDS 1:
RECEIPT OF LABCYTE EPI-MODEL 24

施設名: _____ **試験名**: _____ **試験番号**: _____
Laboratory name Test name Test No.

1. LabCyte EPI-MODEL 24

受領日： _____
Date received

ロット番号： _____
Lot No..

使用期限： _____
Expiration date (MM/DD/YYYY)

付属品： **アッセイ培地** 30mL □ **（ロット番号：** _____ **使用期限：** _____**）**
Accessories Assay medium, 30mL Lot No. Expiration date (MM/DD/YYYY)
24 **ウェルアッセイプレート** □
24 well assay plate

特記事項 Note

2. **アッセイ培地**
Assay medium

受領日： _____
Date received

ロット番号： _____
Lot No.

使用期限： _____
Expiration date (MM/DD/YYYY)

特記事項 Note

記録日:_____ **試験担当者**:_____ **確認日**:_____ **試験責任者**:_____
Date (MM/DD/YYYY) Operator Check date (MM/DD/YYYY) Study director

事務局確認 **確認日**:_____ **氏名**:_____
Secretariat Check date (MM/DD/YYYY) Name

MDS 2:
PRE-INCUBATION OF LABCYTE EPI-MODEL 24 (Section 3.3.1)

施設名: _____ **試験名**: _____ **試験番号**: _____
Laboratory name Test Name Test No.

1. **アッセイ培地を温め、24 ウェルアッセイプレート第 1 行に 0.5mL ずつ添加する。**
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.

アッセイ培地： **（ロット番号：** _____ **使用期限：** _____**）**
Assay medium Lot No. Expiration date (MM/DD/YYYY)

約 30 分間加温 Warm for 30 min. □

0.5mL **ずつ添加** Add 0.5mL of assay medium to each well □

プレート数： _____
Number of plates

2. **培養表皮モデルを取り出し、24 ウェルアッセイプレート第 1 行に移す。**
Transfer culture inserts to wells in the 1st row on the 24-well assay plate.

LabCyte EPI-MODEL 24：**（ロット番号：** _____ **使用期限：** _____**）**
Lot No. Expiration date (MM/DD/YYYY)

作業日時： _____
Time/date executed (MM/DD/YYYY HH:MM)

培養カップ底面に気泡が無いことを確認 □
Confirm that there are no bubbles under the cell culture insert.

3. CO_2 **インキュベーターに入れ、被験物質暴露まで一晩静置する。**
LabCyte EPI-MODEL 24 is cultured in CO_2 incubator overnight.

培養開始日時： _____
Time/date of culture start (MM/DD/YYYY HH:MM)

被験物質暴露予定日時： _____
Planned time/date of exposure to test chemical (MM/DD/YYYY HH:MM)

特記事項 Note

記録日: _____ **試験担当者**: _____ **確認日**: _____ **試験責任者**: _____
Date (MM/DD/YYYY) Operator Check date (MM/DD/YYYY) Study director

事務局確認 **確認日**: _____ **氏名**: _____
Secretariat Check date (MM/DD/YYYY) Name

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

施設名: ____________ **試験名**: ____________ **試験番号**: ____________
Laboratory name　Test name　Test No.

1. **陽性対照物質の調製** Preparation of positive control.
   SLS の秤量 ____________mg　調製量____________mL　作業日時：____________
   Weight of SLS　Preparation vol.　Operation date (MM/DD/YYYY HH/MM)

2. **アッセイ培地を温め、24 ウェルアッセイプレート第 3 行に 1.0mL ずつ添加する。**
   Warm up the assay medium and add 1.0mL of the assay medium to the wells of the 3rd row on the 24-well assay plate.
   アッセイ培地：　(ロット番号：____________　使用期限：____________)
   Assay medium　Lot No.　Expiration date (MM/DD/YYYY)
   約 30 分間加温 □　1.0mL ずつ添加 □　作業日時：____________
   Warm for 30 min.　Add 1.0mL of assay medium.　Time/date (MM/DD/YYYY HH/MM) executed

3. **被験物質を適用する。**
   Apply test chemicals to the LabCyte EPI-MODEL 24.
   作業開始日時：____________　作業終了日時：____________
   Time/date execution started (MM/DD/YYYY HH/MM)　Time/date completed (MM/DD/YYYY HH/MM)

4. **暴露 15 分後、培養表皮モデルを洗浄し、24 ウェルアッセイプレート第 3 行に移す。**
   After exposure to test chemical for 15 min., wash out the LabCyte EPI-MODEL 24 and transfer the culture inserts to the 3rd row on the 24-well assay plate.
   PBS：　(ロット番号：____________　使用期限：____________)
   Lot No.　Expiration date (MM/DD/YYYY)
   作業開始日時：____________　作業終了日時：____________
   Time/date execution started (MM/DD/YYYY HH/MM)　Time/date completed (MM/DD/YYYY HH/MM)
   培養カップ底面に気泡が無いことを確認 Confirm that there are no bubbles under the cell culture insert. □

5. **被験物質情報** Test chemical information

被験物質コード番号 Test chemical code No.	ロット番号 Lot No.	物理的性状 Physical state	適用量 Test chemical vol.(weight) (秤量記録 measured weight)	適用時刻 Time of application	暴露時間 Exposure time (15分 15min.)
注射用水（陰性対照） Distilled Water (Negative control)		液体 Liquid	25□L	:	□
5%SLS（陽性対照） (Positive control)		液体 Liquid	25□L	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□
		液体、粘性液体、固体 Liquid, viscous, solid	25μL, (mg, mg, mg)	:	□

記録日:____________ **試験担当者**:____________ **確認日**:____________ **試験責任者**:____________
Date (MM/DD/YYYY)　Operator　Check date (MM/DD/YYYY)　Study director

事務局確認　**確認日**:____________ **氏名**:____________
Secretariat　Check date (MM/DD/YYYY)　Name

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

施設名: ____________________ Laboratory name　　**試験名**: ____________________ Test name　　**試験番号**: ____________________ Test No.

5. **被験物質情報** Test chemical information

被験物質コード番号 Test chemical code No.	**物理的性状** Physical state	**適用量** Test chemical vol.(weight) (**秤量記録** measured weight)	**適用時刻** Time of application	**暴露時間** Exposure time (15分 15min.)
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□
	液体、粘性液体、固体 Liquid, viscous, solid	25μL, (　mg,　mg,　mg)	:	□

6. CO_2**インキュベーターに入れ、**42**時間後培養する。**
Culture LabCyte EPI-MODEL 24 in CO_2 incubator for 42 hrs.

後培養開始日時：____
Time/date post-incubation started (MM/DD/YYYY　HH:MM)

後培養終了予定日時：____________________________________
Time/date post-incubation completed (MM/DD/YYYY　HH:MM)

特記事項 Note

記録日:______________ Date (MM/DD/YYYY)　**試験担当者**:______________ Operator　**確認日**:______________ Check date (MM/DD/YYYY)　**試験責任者**:______________ Study director

事務局確認 Secretariat　**確認日**:______________________ Check date (MM/DD/YYYY)　**氏名**:______________________________ Name

MDS 4:
MTT ASSAY (Section 3.3.4)

施設名: _____ **試験名**: _____ **試験番号**: _____
Laboratory name Test name Test No.

1. MTT **培地の調製** Preparation of MTT medium
調製量 _____ mL **ロット番号**_____ **調製日時：**_____
Preparation vol. Lot No. Time/date (MM/DD/YYYY HH/MM) executed

2. MTT **培地を温め、24 ウェルアッセイプレート第 4 行に** 0.5mL **ずつ添加する。**
Warm up the MTT medium and add 0.5mL of the MTT medium to the wells in the 4th row on the 24-well assay plate.
MTT **培地：(ロット番号：**_____ **使用期限：**_____)
MTT medium. Lot No. Expiration date (MM/DD/YYYY)
約 30 **分間加温** □ 0.5mL **ずつ添加** □ **作業日時：**_____
Warm for 30 min. Add 0.5mL of the MTT medium. Time/date (MM/DD/YYYY HH/MM) executed

3. **後培養終了後、培養表皮モデルを** 24 **ウェルアッセイプレート第** 4 **行に移す。**
After post-incubation, the LabCyte EPI-MODEL24 transfer to wells of 4th row of 24-well assay plate.
作業開始日時：_____ **作業終了日時：**_____
Time/date started (MM/DD/YYYY HH/MM) Time/date completed (MM/DD/YYYY HH/MM)
培養カップ底面に気泡が無いことを確認 Confirm that there are no bubbles under the cell culture insert. □

4. CO_2 **インキュベーターに入れ、**3 **時間** MTT **反応を行う。**
Store LabCyte EPI-MODEL 24 culture overnight in CO_2 incubator for 42 hrs.
MTT **反応時間情報** Information on MTT reaction time

被験物質コード番号 Test chemical code No.	**ロット番号** Lot No.	MTT **反応開始時刻** MTT reaction start time	MTT **反応終了時刻** Time when MTT reaction ends	**被験物質コード番号** Test chemical code No..	**ロット番号** Lot No.	MTT **反応開始時刻** MTT reaction start time	MTT **反応終了時刻** Time when MTT reaction ends
注射用水（陰性対照） Distilled Water (Negative control)		:	:			:	:
5%SLS（**陽性対照**） (Positive control)		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:
		:	:			:	:

特記事項 Note

記録日:_____ **試験担当者**:_____ **確認日**:_____ **試験責任者**:_____
Date (MM/DD/YYYY) Operator Check date (MM/DD/YYYY) Study director

事務局確認 **確認日**:_____ **氏名**:_____
Secretariat Check date (MM/DD/YYYY) Name

MDS 5:
FORMAZAN EXTRACTION AND MEASUREMENT (Section 3.3.5)

施設名: _____ **試験名**: _____ **試験番号**: _____
Laboratory name Test name Test No.

1. MTT **反応終了後、培養皮膚をピンセットでつまんで取り出し、**1.5mL **チューブに移す。**
   After MTT reaction, use forceps to pick up the cultured epidermis from the cell culture insert and put it in a 1.5mL microtube.
   スカルペルの使用 Did you use a scalpel to cut out the cultured epidermis? ☐
   作業日時： _____
   Time/date of execution (MM/DD/YYYY)

2. **イソプロパノール** 300µL **を入れて培養表皮モデルを完全に浸漬する。**
   Add isopropanol (300µL) to microtube so that the cultured epidermis is completely immersed in isopropanol.
   イソプロパノール ロット番号 _____ 300µL **添加** ☐
   Lot No.. To add isopropanol (300µL).
   培養表皮モデルを完全に浸漬 Immersion of the cultured epidermis in isopropanol. ☐
   作業日時： _____
   Time/date executed (MM/DD/YYYY HH/MM)

3. **冷暗所で一晩以上静置し、色素を抽出する。** For MTT formazan extraction, allow micro tube to stand in a cold and dark space.
   冷暗所に静置 Place micro tube in a cold and dark space. ☐

4. **抽出液（**200µL**）を** 96 **ウェルプレートの各ウェルに入れる。**
   Extract solution (200mL) is transferred to each well on the 96-well plate.
   抽出液 200µL **を** 96 **ウェルプレートに移す** Transfer to the 96-well plate. ☐
   作業日時： _____
   Time/date executed (MM/DD/YYYY HH/MM)
   96 **ウェル割り付け記録** Sample location on 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ブランク blank											
B	注射用水-1 Distilled Water-1											
C	注射用水-2 Distilled Water-2											
D	注射用水-3 Distilled Water-3											
E	5% SLS-1											
F	5% SLS-2											
G	5% SLS-3											
H												

5. 570nm**、及び** 650nm **の吸光度を測定し、**570nm **の吸光度から** 650nm **の吸光度を差し引いた値を測定値とする。** Analyze extract OD at 570nm and 650nm, and calculate the OD(570nm-650nm).
   570nm**、及び** 650nm **の吸光度を測定** Analyze OD at 570nm and 650nm. ☐
   測定値（570nm **吸光度-**650nm **の吸光度）を計算** Calculate the OD(570nm-650nm). ☐
   測定値から生細胞率、及び SD **を計算** Calculate cell viability and SD. ☐
   生細胞率、及び SD **を別途配布するファイルに転記** Cell viability and SD are recorded on a separate data sheet. ☐
   データシートをプリントアウトして裏面に貼付 The data sheet is attached to the back of this sheet. ☐
   転記ミスがないことを確認 Check for input errors. ☐
   作業日時： _____
   Time/date executed (MM/DD/YYYY HH/MM)

特記事項 Note

記録日: _____ **試験担当者**: _____ **確認日**: _____ **試験責任者**: _____
Date (MM/DD/YYYY) Operator Check date (MM/DD/YYYY) Study director

事務局確認 **確認日**: _____ **氏名**: _____
Secretariat Check date (MM/DD/YYYY) Name

REVISION HISTORY

Rev.	Content	Date Revised
Ver.1	1) First version	27/02/2008
Ver.2	1) Revised clerical error.	28/02/2008
Ver.3	1) Revised the post-incubation time and assessment criteria in compliance with the EpiSkin method described in “Performance Standards for Applying Human Skin Models to in vitro Skin Irritation Testing” 2) Added photos and figures for instruction.	17/03/2008
Ver.4	1) Added MDS 1~6. 2) Added instruction and operational steps regarding the IL-1α ELISA kit. 3) Added subsections “Delivery of LabCyte EPI-MODEL24” and “Instruction For Use of LabCyte EPI-MODEL24” to Section 2. 4) Added the description regarding test chemicals to Section 2. 5) To Section 2, added the description of materials provided by J-TEC separately from other materials. 6) Stated the specific calculation procedures in Section 3.2.5.2 “OPTICAL DENSITY MEASUREMENTS OF EXTRACTS”.	15/05/2008
Ver.4.1	1) Moved scalpel from Section 2.4 “MATERIALS PROVIDED BY J-TEC” to Section 2.5 “MATERIALS NOT PROVIDED WITH THE J-TEC KITS”. 2) Removed the description regarding how to execute procedures alone. 3) Moved IL-1α ELISA reagents from Section 3.1 “PREPARATIONS” to Section 3.2 “TEST METHOD”. 4) Added a flowchart for the IL-1α ELISA procedures. 5) Changed from “in a cold dark place” to “in a cold dark place (or refrigerator)” regarding formazan extraction. 6) Added the description ~~of~~ “ultrasonic cleaning equipment or vortex mixer” as an example of an MTT dissolution method. 7) Changed the exposure time column from entering actual time to checkboxes on the MDS 3.	21/05/2008
Ver.5.0	1) Corrected typing errors in the section number for IL-1α ELISA reagents.	27/08/2008

	2) Removed the space for SLS lot numbers on the MDS 3. 3) Removed the space for PBS lot numbers on the MDS 3. 4) Added the space for isopropanol lot numbers on the MDS 5. 5) Added a checkbox about using a scalpel when removing tissues in the MDS 5. 6) Added the space for IL-1α ELISA kit lot numbers on the MDS 6. 7) Changed the applicable parts of product codes and kit components in Section 2.2, with the change of IL-1α ELISA kit types to a 96 well test only. 8) Decreased the volume by half to 10mL and changed the storage condition from within 1 month to within 24 hours in Section 3.1.2 “POSITIVE CONTROL SUBSTANCE”. 9) Added the manufacturers and product codes of the 24-well plate and 96-well plate in Section 2.4 “MATERIALS PROVIDED BY J-TEC”. 10) Added specific time frames for incubation or culturing. 11) Added the conditions for a successful study in Section 4 “ASSESSMENT” 12) Changed the specific method of applying liquids in Section 3.2.2.2 “APPLICATION OF TEST CHEMICALS”. 13) Added descriptions in English on the MDS Sheets. 14) Changed the application time interval from 1 minute to 1~3minute(s). 15) Numbered figures and flowcharts. 16) Increased the size of spaces for lot numbers on the MDS Sheets. 17) Changed spaces for dates from MM/DD to MM/DD/YYYY. 18) Added director check date, study director, secretariat check date and name at the end of each MDS. 19) Changed the size of matrixes for sample allocation to a 96-well plate in the MDS 5 & 6. 20) Changed the test chemical name to test chemical code in the MDS 3 & 4. 21) Divided the MDS 3 into MDS 3-1 and 3-2, and added spaces for date, operator, check date, study director at the end of the MDS 3-1, and spaces for laboratory name, test name and test no. at the beginning of the MDS 3-2.	
Ver. 6.0	1) Removed the descriptions regarding the measurement of IL-1α	27/02/2009

	production, since the validation committee decided to use cell viabilities only as an index for the skin irritancy test at the meeting in 2009. 2) Revised the expression “the materials provided by J-TEC” for the validation study to that for a standard skin irritancy test preparation. 3) Clearly stated the cell viability equation to use the mean of measured values. 4) Clearly stated to use the median of cell viabilities from the three-time repeated tests as assay criteria.	
Ver. 6.01	1) In order to avoid the possible influence of volatile test chemicals on the results of other test chemicals, the types of test chemicals per plate was changed from 2 chemicals to just 1 chemical.	23/03/2009
Ver. 7.01	1) Test for detecting chemicals that interfere with MTT endpoint was added to Section 3.2.	03/07/2009
Ver. 7.2	1) Revised clerical error.	30/09/2009
Ver. 8.1	1) Added the description about rational and background as following chapters in Section 1. LabCyte EPI-MODEL 24 SKIN IRRITATION TEST (SIT using LabCyte EPI-MODEL24) BACKGROUND OF SIT using LabCyte EPI-MODEL24 BASIS OF THE METOD LIMITATION OF THE METHOD BRIEF BASIC PROCEDURE DATA INTERPRETATION PROCEDURE (PREDICTION MODEL) 2) Added photo about chemicals that directly reduce MTT in Section 3. 3) Added the washing protocol more detail in Section 3. 4) Added assessment about SD.	30/06/2010
Ver.8.2	1) Changed description about the washing protocol in Section 3. 2) Changed unit of consumable reagents and vessels from per a validation study to per a test.	17/08/2010
Ver.8.3	1) Changed description more briefly about the washing protocol in Section 3.	14/06/2011