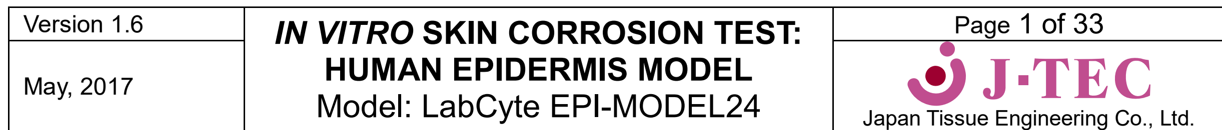

SKIN CORROSION TEST USING THE RECONSTRUCTED HUMAN MODEL “LABCYTE EPI-MODEL24”

Ver. 1.6

LabCyte EPI-MODEL24 SKIN CORROSION TEST

S.O.P.

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1. RATIONALE AND BACKGROUND

1.1 LabCyte EPI-MODEL24 SKIN CORROSION TEST (LabCyte EPI-MODEL24 SCT)

The LabCyte EPI-MODEL24 skin corrosion test (LabCyte EPI-MODEL24 SCT) is designed for the prediction of acute skin corrosion of chemicals by measurement of their cytotoxic effect, as reflected in the MTT assay, on the Reconstructed Human Epidermis (RhE) model. LabCyte EPI-MODEL24 tissues are commercially available with a minimum of 24 tissues (one 24-well plate) per order.

1.2 BACKGROUND OF THE LabCyte EPI-MODEL24 SCT

Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test chemical, as defined by the Globally Harmonized System (GHS) for Classification and Labeling of Chemical Substances and Mixtures[1).

The potential for chemical induced skin corrosion is an important consideration in establishing procedures for the safe handling, packaging and transport of chemicals. Various systems for classification of corrosive potential are included in international regulatory requirements.

The present test is based on the fact that corrosive chemicals are cytotoxic after a short term exposure to the stratum corneum of the epidermis, if cytotoxicity is immediately determined after chemical exposure. It is designed to predict and classify the skin corrosive potential of a chemical by using a three dimensional human epidermis model.

In 1998, EPISKIN and TER *in vitro* corrosive tests were successfully validated and met the acceptance criteria previously defined by the Management Team of the ECVAM International Validation Study[2)]. Because EPISKIN was not available after the study, a catch up validation study was performed with the RhE model, EpiDerm[3)].

In 2002, national coordinators of the OECD Test Guideline Programme (WNT) endorsed New Draft Test Guidelines (TG) 430 (TER) and 431 (Human Skin Model; adopted in 13th April 2004) for In Vitro Skin Corrosion Testing. In the OECD TG431, general functional and performance criteria were defined in case other (or new) skin or epidermis models were to be used in the context of this guideline[4)]. The current SOP describes a generally applicable method for Skin Corrosion Testing, here applied to the LabCyte EPI-MODEL24 SCT.

1.3 BASIS OF THE METHOD

Most international regulatory classification schemes define chemically induced dermal corrosion as full thickness destruction (necrosis) of the skin tissue, while some extend the definition of corrosion to include any irreversible alterations caused to the skin. The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packaging and transport of chemicals.

The test is designed to predict and classify the skin corrosive potential of a test substance by assessment of its effect on a reconstructed human epidermis. The relative viability of the treated tissues is measured at the end of the treatment exposure (3 minutes and 60 minutes), by the MTT [(3-4,5-dimethyl thiazole 2-yl) 2,5-diphenyltetrazoliumbromide] assay. The cell viability of the negative control is then considered and used to classify test substances. 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 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 maintain their phenotype during expansion, human keratinocytes are cultured on a feeder layer of 3T3-J2 cells (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. The model reconstructs 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.

1.3.1.1 QUALITY CONTROL OF THE TEST SYSTEM

The LabCyte EPI-MODEL24 is manufactured according to defined quality assurance procedures. Each product is provided with directions concerning storage conditions, instructions for use, lot number and origin. The quality of each batch production is assessed by histological examination (demonstration of human epidermis-like structure with multilayered stratum corneum), MTT assay to determine cell viability, and by IC50 with SLS (sodium lauryl sulfate) to analyze barrier function integrity ($0.14 \leq IC50 \leq 0.4$).

1.3.1.2 PRECAUTIONS

The epidermal cells are taken from a 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 of the model and kit components.
b) After use, the epidermis, as well as the material and all media that were in contact with it, should be decontaminated prior 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 tissues (NC, treated with sterile distilled water) in the MTT assay is an indicator of cell 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

8N (8 mol/L) potassium hydroxide (KOH) solution is used as positive control (PC) and tested concurrently with the test chemicals. Here, concurrent means that the PC has to be tested in each assay, but not more than one PC is required per testing day.

Mean cell viability (60 minutes) < 15%

1.3.2.3 ASSAY ACCEPTANCE CRITERION 3: DIFFERENCE OF VIABILITY

Since skin corrosion potential is predicted in each test from the mean viability determined on 2 single tissues, the variability of tissue replicates should be acceptably low.

In the viability range of 20-100%, and for ODs≥ 0.3, difference of viability between the two tissue replicates should not exceed 30%.

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 substances are a problem only if at the time of the MTT test (i.e. 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 transferred to fresh assay medium and conditioned by incubation to release transport-stress related compounds and debris overnight. After overnight pre-incubation, LabCyte EPI-MODEL24 tissues are topically exposed to the test chemicals for 3 minutes and 60 minutes. Two tissues each are used per treatment, negative control and positive control. After exposure, tissues are rinsed and blotted, and assay medium is replaced by MTT-assay medium. After 3 hours incubation, the blue formazan salt is extracted with isopropanol. The optical density of the formazan extract is determined spectrophotometrically at 570 nm and 650 nm as reference, and cell viability is calculated for each tissue as % of the mean of the negative control tissues. Skin corrosive potential of the test materials is classified according to the remaining cell viability obtained after 3 minutes or 60 minutes exposure with the test chemical.

1.6 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

Corrosive potential of the test materials is predicted from the relative mean tissue viabilities obtained after 3 minutes as well as 60 minutes treatment compared to the negative control tissues

concurrently treated with H_2O. The prediction models for the LabCyte EPI-MODEL24 SCT methods, associated with the UN GHS classification system, are shown in Table 1.

Table 1. LabCyte EPI-MODEL24 SCT prediction model

	Step 1			Step 2	
	3 minutes	60 minutes	Prediction	3 minutes	Prediction
Viability	< 50%		Corrosive	< 15%	Sub-category 1A
	≥ 50%	< 15%		≥ 15%	Sub-category 1B and 1C
		≥ 15%	Non-corrosive		

2. MATERIALS

2.1 LabCyte EPI-MODEL24

2.1.1 LabCyte EPI-MODEL24 KIT COMPONENTS

LabCyte EPI-MODEL24 kit components are shown in Table 2.

Table 2. LabCyte EPI-MODEL24 Kit Components

Component	Qty	Description
LabCyte EPI-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). 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-MODEL24 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. Verify lot number and expiration date, and 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 the insulating materials.

2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL24

Start incubating all culture inserts soon after opening the package. Do not store the culture inserts in the nutritive agar after opening the aluminum package.

The human epidermis cells used in LabCyte EPI-MODEL24 originate from a healthy 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 2 samples can be assayed once.

- Assay Medium, 100 mL (J-TEC: 402100) 1 bottle
- MTT (e.g. J-TEC: 403026) 1 bottle
- 24-well assay plate (Corning: 353047) 3 plates
- 96-well plate (e.g. Corning: 353072) 1 plates
- Phosphate buffered saline (PBS) 500 mL (e.g. Invitrogen: 14190-144) 3 bottles
- Isopropanol 500 mL (e.g. Wako Pure Chemical Industries: 164-08335) 1 bottle
- 8 mol/l KOH 500 mL (e.g. Wako Pure Chemical Industries: 169-20365) 1 bottle
- Sterile distilled water 20 mL (e.g. Otsuka Pharmaceutical: 36A1X00001) 1 bottles
- Sterile cotton buds (e.g. 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: 570 nm, 650 nm)
- Precision balance (0.1 mg)
- Aspirator
- Stop-watches
- Adjustable micro-pipette (10-200 μL, 200-1000 μL)
- Positive-displacement pipette (50 μL)
- Sharp-edged forceps (sterile)
- Micro spatula (sterile)
- Beaker (1-2 L: sterile)
- Sterilizable poly wash bottle (500-1000 mL: sterile)

2.4.2 CONSUMMABLE ITEMS

- Micro-pipette tips (sterile: 10-200 μL, 200-1000 μL)
- Capillary Pistons for positive-displacement pipette (50 μL)
- Microtubes (1.5 mL)
- 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-MODEL24**

3.1 PREPARATIONS

3.1.1 NEGATIVE CONTROL SUBSTANCE

Distilled water is used as negative control.

3.1.2 POSITIVE CONTROL SUBSTANCE

8 mol/L KOH is used as positive control.

3.1.3 POLY WASH BOTTLE FOR PBS

(1) Sterilize poly wash bottle using an autoclave.

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

3.1.4 MTT SOLUTION

(1) Dissolve MTT in the assay medium to prepare the MTT medium (final concentration: 0.5 mg/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.

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) Chemicals that stain epidermal tissues.

(b) Chemicals able to directly reduce MTT.

Test chemicals that stain the epidermal tissues have the possibility to transfer from the tissues to the extraction solution, and to affect the optical density (OD) measurements.

Test chemicals able to directly reduce MTT can affect the optical density (OD) measurements, if the test chemical is present in the epidermal 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 STEP 1 (PRELIMINARY TEST)

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

(2) Close the lid of 24-well assay plate and incubate the mixture in a CO_2 incubator for 60 minutes.

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

(4) If 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 (step 2) should be performed. If the color of the solution does not change significantly, the test chemical is determined not to have the potential to stain the tissue.

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

3.2.1.2 STEP 2 (MEASUREMENT OF TISSUE STAINING FOR OD CORRECTION)

(1) Add 50 μL (liquid) or 50 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. Set a negative control using distilled water.

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

$$\text{Corrected OD} = A - (B - C),$$

where:

A is the OD of tissue exposed to a test chemical using MTT medium;

B is the mean OD of tissue exposed to the test chemical using assay medium instead of MTT medium; and

C is the mean OD of tissue exposed to the negative control using assay medium instead of MTT medium.

If the OD of (B-C) is below 0, the OD of (B-C) is considered to be 0.

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

3.2.2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE MTT

3.2.2.1 STEP 3 (PRELIMINAY TEST)

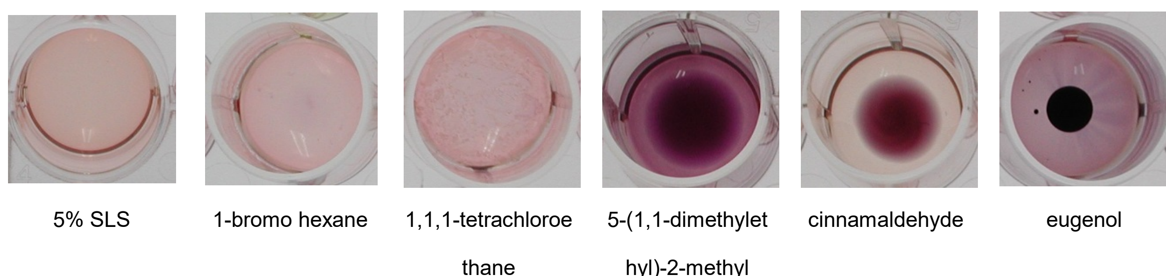
(1) Add 50 μL (liquid) or 50 mg (solid) of the test chemical into wells of 24-well assay plate pre-filled with 0.5 mL of MTT medium. Untreated MTT medium is used as control.

(2) Close the lid of 24-well assay plate and incubate the mixture in CO_2 incubator for 60 minutes.

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

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

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



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 have directly reduced MTT. In these cases, step 4 must be performed.

3.2.2.2 STEP 4 (MEASUREMENT OF DIRECT REDUCE MTT FOR OD CORRECTION)

(1) Add 50 μL (liquid) or 50 mg (solid) of the test chemical, which clearly changed the color of the MTT medium into blue/purple (step 3), onto the surface of the epidermal tissues. Set a negative control using distilled water.

(2) Follow all procedures described in the SOP Section 3.3 EXECUTION OF THE TEST.

Instead of using viable epidermal tissues, however, use freeze-killing epidermal tissues. Freeze-killing tissues were prepared that tissues were frozen at -80°C or lower for 1 hour and warm-up at 37°C for 30 minutes, and repeat their freeze-thawing step twice. Record the preparation details of freeze-killing the tissue in the MDS 1-4.

(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 freeze-killed tissue exposed to a test chemical; and

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

If the OD of (B-C) is below 0, the OD of (B-C) is considered to be 0.

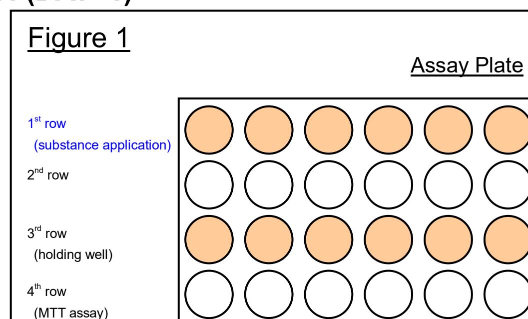
If a corrected OD is below 0, the OD is considered to be 0.

3.3 EXECUTION OF THE TEST

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

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

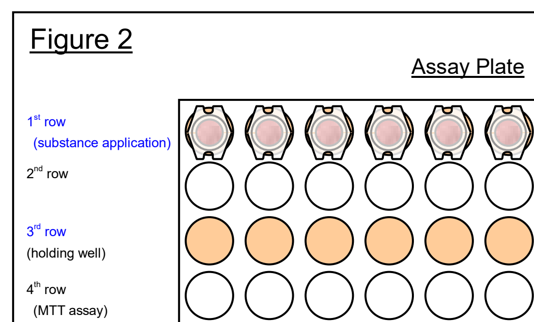
(2) Fill 12 wells of the 1st row and the 3rd row of each 24-well assay plate with the pre-warmed assay medium (0.5 mL/well).
→ Figure 1



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

(4) Open the LabCyte EPI-MODEL24 plate lid and pick up the culture inserts using sterile forceps.
*Do not touch the epidermis model surface.
*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 of the plate using sterile forceps.
→ Figure 2
*Avoid trapping air bubbles under the tissue inserts.



(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.

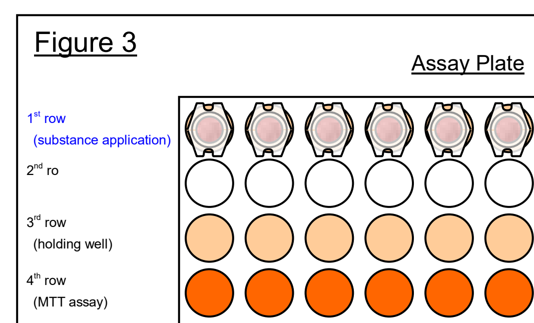
3.3.2 APPLICATION OF TEST CHEMICALS AND RINSING (DAY 0)

3.3.2.1 PREPARATION OF WELLS FOR MTT ASSAY (4TH ROW)

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

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

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



(4) Record details of steps (1) – (3) 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 2 wells per test chemical (N=2).

FOR LIQUIDs: Carefully apply 50 μL of the test chemical onto the center of each epidermis using a micropipette. After application, close the lid and tap the side of the plate so the liquid spread over the entire epidermis surface. If necessary, use a micro spatula to coat the unapplied tissue surface with the chemical. Do not apply pressure to the epidermis surface.

*Use a positive displacement pipette and tip for viscous liquids. Use the pipette to familiarize yourself with the nature of the test chemicals in advance.

Photo 2 – Applying a solid substance

FOR SOLIDs: Weigh out 50 mg (±2 mg) of the solid chemical with a precision balance in advance. If necessary, crush and grind the solid test chemicals in a mortar with pestle. Apply first 50 μL 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 2

*One 24-well assay plate should be used to assay three test chemicals.

→ Figure 4

(3 samples x 2(n) = 6(culture inserts))

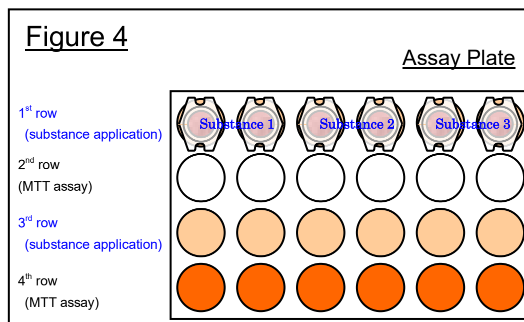
(3) Apply test chemical onto each well at 1-minute intervals.

(4) Incubate each well as described below;

3 minutes exposure: Incubate each well for 3 minutes in the safety cabinet (lid on between the intervals).

*Close the lid of the assay plate at all times except when applying samples. Leaving the lid opened might affect the amount of test sample due to air circulation in the safety cabinet (or clean bench).

Figure 4



60 minutes exposure: Place the 24-well plates into the incubator (37°C, 5% CO_2) for the rest of the exposure time until 60 minutes is reached for first tissue dosed.

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

3.3.2.3 REMOVAL OF THE TEST CHEMICALS

(1) After chemical exposure, open the assay plate and pick up a culture insert with sterile

forceps.

(2) Discard the test chemical on the tissue by tilting the insert and tapping it on a beaker. Fill the culture insert by overflowing it with PBS applied 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.

→ Photo 3

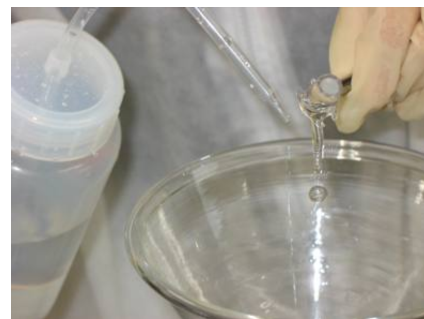
*To avoid damaging the tissue with too forceful a stream, use a wide-mouth nozzle on the poly wash bottle.

Photo 3 - Rinse 1

(3) 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.

→ Photo 4

Photo 4 - Rinse 2



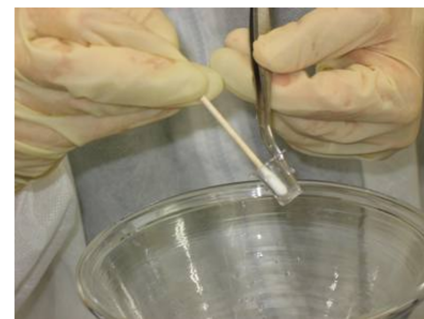
(4) Repeat steps (2) and (3) 10 times or more to remove any residual test chemical as much as possible from the tissue surface.

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

→ Photo 5

*Take care to not apply pressure on the surface of the tissue with the sterile cotton bud.

Photo 5 - Rinse 3



(6) If test material remains on the epidermis surface, gently remove it using a sterile cotton bud and repeat steps (2)-(5).

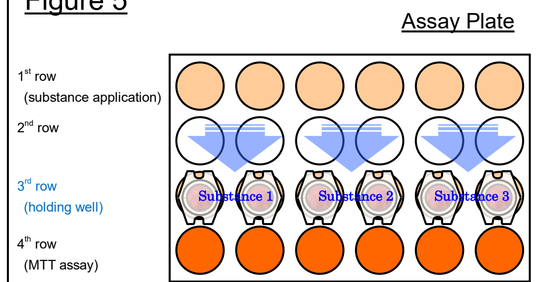
*If it proves difficult to remove completely all the residual test chemical from the epidermal tissue surface, remove as much as possible and continue to step 7.

(7) Transfer the blotted culture insert to a well in the 3rd row of the corresponding column (holding well).

→ Figure 5

*Avoid trapping air bubbles under the culture

Figure 5



inserts.

(8) Repeat steps (1)-(7) for all the culture inserts at 1-3-minute intervals in order to keep exposure periods of each test chemical.

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

3.3.3 MTT ASSAY (DAY 0)

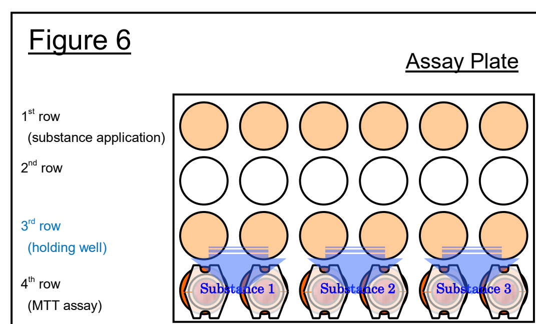
(1) Open the lid of the assay plate, transfer the blotted culture insert to a well in the 4th row of the corresponding column (for MTT assay). → Figure 6

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

(3) Incubate for 3 hours.

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

Figure 6



3.3.4 FORMAZAN EXTRACTION AND MEASUREMENT (DAY 0-1)

3.3.4.1 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 pick-up the tissue from each culture insert by the pinching it with forceps (Photo 6) or by cutting both tissue and membrane filter off from the culture insert with a scalpel.

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

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

Photo 6 – scratch up the epidermis

(5) Incubate the micro tubes in a dark cold place 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 epidermis tissue fragments are suspended, wait until they sink or gently centrifuge them (if a centrifuge is available).

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

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

*Figs. 7A and 7B show typical sample allocation on a 96-well plate for both for non-corrected tissues and for corrected tissues.

Figure 7A – Sample allocation for a 96-well plate

(same pattern for 3 or 60 minutes application)

	1	2	3	4	5	6	7	8	9	10	11	12
A		Blank		DW-1	DW-2			KOH-1 [Only 60 min]	KOH-2 [Only 60 min]			
B		S1-1	S1-2	S2-1	S2-2	S3-1	S3-2	S4-1	S4-2	S5-1	S5-2	
C		S6-1	S6-2	S7-1	S7-2	S8-1	S8-2	S9-1	S9-2	S10-1	S10-2	
D		S11-1	S11-2	S12-1	S12-2	S13-1	S13-2	S14-1	S14-2	S15-1	S15-2	
E		S16-1	S16-2	S17-1	S17-2	S18-1	S18-2	S19-1	S19-2	S20-1	S20-2	
F		S21-1	S21-2	S22-1	S22-2	S23-1	S23-2	S24-1	S24-2	S25-1	S25-2	
G		S26-1	S26-2	S27-1	S27-2	S28-1	S28-2	S29-1	S29-2	S30-1	S30-2	
H												

Figure 7B – Sample allocation for a 96-well plate for OD-correcting tissue

(same pattern for 3 or 60 minutes application)

	1	2	3	4	5	6	7	8	9	10	11	12
A		Blank		DW-1	DW-2							
B		S1-1	S1-2	S2-1	S2-2	S3-1	S3-2	S4-1	S4-2	S5-1	S5-2	
C		S6-1	S6-2	S7-1	S7-2	S8-1	S8-2	S9-1	S9-2	S10-1	S10-2	
D		S11-1	S11-2	S12-1	S12-2	S13-1	S13-2	S14-1	S14-2	S15-1	S15-2	
E		S16-1	S16-2	S17-1	S17-2	S18-1	S18-2	S19-1	S19-2	S20-1	S20-2	
F		S21-1	S21-2	S22-1	S22-2	S23-1	S23-2	S24-1	S24-2	S25-1	S25-2	
G		S26-1	S26-2	S27-1	S27-2	S28-1	S28-2	S29-1	S29-2	S30-1	S30-2	
H												

(8) Record details of steps (1)-(6) above in the MDS 5.

3.3.4.2 OPTICAL DENSITY MEASUREMENTS OF THE EXTRACTS

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

The equation is shown below:

Measured OD = [570 nm OD_{sample} – 570 nm OD_{blank}] – [650 nm OD_{sample} – 650 nm 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 difference of viability.

(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 corrosion test should be considered successful if the following criteria have been met.

- Cell viability: 0.7 ≤ mean OD (A570/650) measured value for negative control ≤ 2.5.
- Positive control (60 minutes exposure): mean cell viability for 8N KOH < 15%.
- In the viability range of 20-100%, and ODs ≥0.3, the difference of viability between the two tissue replicates should not exceed 30%.

4.2 ASSAY CRITERIA

Corrosive potential of the test materials is predicted from the relative mean tissue viabilities obtained after 3 minutes, as well as 60 minutes treatment, compared to the negative control tissues concurrently treated with H_2O. The prediction models for the LabCyte EPI-MODEL24 SCT methods, associated with the UN GHS classification system, are shown in Table 3.

Table 3. LabCyte EPI-MODEL24 SCT prediction model

<table>
<tr><th rowspan="2"></th><th colspan="3">Step 1</th><th colspan="2">Step 2</th></tr>
<tr><th>3 minutes</th><th>60 minutes</th><th>Prediction</th><th>3 minutes</th><th>Prediction</th></tr>
<tr><td rowspan="3">Viability</td><td>< 50%</td><td></td><td rowspan="2">Corrosive</td><td>< 15%</td><td>Sub-category 1A</td></tr>
<tr><td rowspan="2">≥ 50%</td><td>< 15%</td><td>≥ 15%</td><td>Sub-category 1B and 1C</td></tr>
<tr><td>≥ 15%</td><td>Non-corrosive</td><td colspan="2"></td></tr>
</table>

5. REFFERENCE

(1) OECD (2001) Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures. OECD Series on Testing and Assessment Number 33. ENV/JM/MONO(2001)6, Paris. http://www.olis.oecd.org/olis/2001doc.nsf/LinkTo/env-jm-mono(2001)6

(2) Fentem, J.H., et al. (1998). The ECVAM international validation study on in vitro tests for skin corrosivity. 2. Results and evaluation by the Management Team. *Toxicol. in Vitro* 12, 483-524.

(3) Liebsch, M., et al. (2000). The ECVAM prevalidation study on the use of EpiDerm for skin corrosivity testing. *ATLA* 28, 371-401.

(4) OECD (2016) OECD Guideline for the testing of chemicals, No.431: In vitro skin corrosion: reconstructed human epidermis (RHE) test method. Paris, France: Organization for economic cooperation and development.

MDS 1-1:
RECEIPT OF LABCYTE EPI-MODEL24

Laboratory name: ________________ Test name: ________________ Test No. : ____________

1. LabCyte EPI-MODEL24

Date received : ________________________________

Lot No. : ________________________________

Expiration date : ________________________________
(MM/DD/YYYY)

Accessories : Assay medium, 30mL ☐ （Lot No. : _________ Expiration date : _____________)
(MM/DD/YYYY)

24 well assay plate ☐

Note

2. Assay medium

Date received : ________________________________

Lot No. : ________________________________

Expiration date : ________________________________
(MM/DD/YYYY)

Note

Date:____________________ Operator:____________________ Check date:____________ Study director:_______________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:________________________ Name:____
(MM/DD/YYYY)

MDS 1-2:
MTT REACTION INTERFERENCE TEST (DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE)
STEP-1 (Section 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. Incubate the 24-well assay plate in a CO_2 incubator for 60 minutes.

Incubation starting time: ______________________________________

Incubation ending time: ______________________________________

4. Check the color of water.

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

Test chemical	Physical state	Amount	Coloring potential
H_2O (NC)	Liquid	50μL	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	

Note

Date:________________ Operator:_________________ Check date:___________ Study director:_______________

Secretariat Check date:________________________ Name:____

MDS 1-3:
MTT REACTION INTERFERENCE TEST (DETECTION OF CHEMICALS THAT DIRECTLY REDUCE MTT)
STEP-3 (Section 3.2.2.1)

Laboratory name:_________________ Test name: _____________________Test no. : _______

1. Preparation of MTT medium

Preparation vol. ______mL Lot No. _________ Execution date/time:_________________
(MM/DD/YYYY HH/MM)

2. Add MTT medium (0.5 mL) to the wells of the 24-well assay plate.

To add MTT medium (0.5 mL) □ Execution date/time:________________________

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 60 minutes.

MTT reaction starting time: ___________________

MTT reaction ending time: ___________________

5. Check the color of MTT medium.

6. Test chemical information and checked list of MTT reducing potential.

Test chemical	Physical state	Amount	Coloring potential
H_2O (NC)	Liquid	50μL	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	
	Liquid, viscous, solid	50μL, (mg)	

Note

Date:________________ Operator:_________________ Check date:___________ Study director:_______________

Secretariat Check date:_________________________ Name:____

MDS 1-4:

PREPARATION OF FREEZE-KILLED TISSUE (Section 3.2.2.2)

Laboratory name:_ Test name: ______ Test no. : ________

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

2. Freeze tissues in a -80℃ deep-freezer for 1 hour (1st freezing).

   Store for 1 hour. □ Date/time:_______________

3. Thaw tissues in a 37℃ incubator for 30 minutes.

   Store for 30 minutes. □ Date/time:_______________

4. Freeze tissues in a -80℃ deep-freezer for more than 1 hour (2nd freezing).

   Store for 1 hour. □ Date/time:_______________

5. Thaw tissues in a 37℃ incubator for 30 minutes.

   Store for 30 minutes. □ Date/time:_______________

Note

Date:__________________ Operator:_________________ Check date:___________ Study director:_______________

Secretariat Check date:_________________________ Name:____

MDS 2:
PRE-INCUBATION OF LABCYTE EPI-MODEL24 (Section 3.3.1)

Laboratory name: ________________ Test name: ________________ Test No. : ____________

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

Assay medium : (Lot No. : ______________________ Expiration date : __________________)
(MM/DD/YYYY)

Warm for 30 minutes. ☐

Add 0.5 mL of assay medium to each well. ☐

Number of plates : __________________________

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

LabCyte EPI-MODEL24 : (Lot No. : __________________ Expiration date : __________________)
(MM/DD/YYYY)

Execution date/time : ________________________
(MM/DD/YYYY HH:MM)

Release any air bubbles trapped under the cell culture insert. ☐

3. LabCyte EPI-MODEL24 is cultured in a CO_2 incubator overnight.

Culture starting date/time : ____
(MM/DD/YYYY HH:MM)

Planned date/time of chemical exposure : ____
(MM/DD/YYYY HH:MM)

Note

Date:________________ Operator:________________ Check date:__________ Study director:______________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:______________________ Name:____________________________________
(MM/DD/YYYY)

MDS 3-1:
APPLICATION OF TEST CHEMICALS AND RINSING: *3 MINUTES EXPOSURE*
(Section 3.3.2)

Laboratory name: ______________ Test name: ______________ Test No.: ___________

1. Preparation of MTT medium

Preparation vol. _____mL Lot No. _________ Execution date/time : ________________
(MM/DD/YYYY HH/MM)

2. Warm up the MTT medium and add 0.5mL to the wells in the 4th row on the 24-well assay plate.

MTT medium. : (Lot No. : ______________________ Expiration date : ____________)
(MM/DD/YYYY)

Warm for 30 minutes ☐ Add 0.5 mL of the MTT medium ☐ Execution date/time : __________
(MM/DD/YYYY HH/MM)

3. Apply test chemicals to the LabCyte EPI-MODEL24.

Starting date/time : ______________________ Ending date/time : ___________________________
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

4. Test chemical information

Test chemical code No.	Lot No.	Physical state	Test chemical vol.(weight) (measured weight)	Time of application	Exposure time (3 minutes.)
Distilled Water (Negative control)		Liquid	50μL	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐

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

PBS : (Lot No. : ______________________ Expiration date : ___________________)
(MM/DD/YYYY)

Starting date/time : ______________________ Ending date/time : ___________________________
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

Release any air bubbles trapped under the cell culture insert. ☐

Note

Date:____________________ Operator:___________________ Check date:_____________ Study director:________________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:___________________________ Name:____
(MM/DD/YYYY)

MDS 3-2:
APPLICATION OF TEST CHEMICALS AND RINSING: *60 MINUTES EXPOSURE*
(Section 3.3.2)

Laboratory name: ________________ Test name: ________________ Test No.: ____________

1. Preparation of MTT medium
   Preparation vol. ______mL Lot No. _________ Execution date/time : ________________
   (MM/DD/YYYY HH/MM)

2. Warm up the MTT medium and add 0.5mL to the wells in the 4th row on the 24-well assay plate.
   MTT medium. : (Lot No. : ________________________ Expiration date : ____________)
   (MM/DD/YYYY)
   Warm for 30 minutes ☐ Add 0.5 mL of the MTT medium ☐ Execution date/time : ___________
   (MM/DD/YYYY HH/MM)

3. Apply test chemicals to the LabCyte EPI-MODEL24.
   Starting date/time : ________________________ Ending date/time : ______________________________
   (MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

4. Culture LabCyte EPI-MODEL 24 in a CO_2 incubator for 60 minutes.
   Culture starting date/time : ____
   (MM/DD/YYYY HH:MM)
   Culture ending date/time : ____
   (MM/DD/YYYY HH:MM)

5. Test chemical information

Test chemical code No.	Lot No.	Physical state	Test chemical vol.(weight) (measured weight)	Time of application	Exposure time (60 minutes)
Distilled Water (Negative control)		Liquid	50μL	:	☐
8N KOH (Positive control)		Liquid	50μL	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐
		Liquid, viscous, solid	50μL, (mg, mg)	:	☐

6. After exposure to test chemical for 60 minutes, wash out the LabCyte EPI-MODEL24 and transfer the culture inserts to the 3rd row on the 24-well assay plate.
   PBS : (Lot No. : ________________________ Expiration date : ____________________)
   (MM/DD/YYYY)
   Starting date/time : ________________ Ending date/time : ______________________________
   (MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

   Released any air bubbles trapped under the cell culture insert. ☐

Note

Date:____________________ Operator:___________________ Check date:_____________ Study director:_________________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:___________________________ Name:____
(MM/DD/YYYY)

MDS 4:
MTT ASSAY (Section 3.3.3)

Laboratory name: ________________ Test name: ________________ Test No. : ____________

1. After washing, transfer the LabCyte EPI-MODEL24 to wells of the 4th row of 24-well assay plate.

3 minutes exposure:
Starting date/time : ________________________ Ending date/time : ____________________________
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

60 minutes exposure:
Starting date/time : ________________________ Ending date/time : ____________________________
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

Release any air bubbles trapped under the cell culture insert. ☐

2. Incubate LabCyte EPI-MODEL24 in a CO_2 incubator for 3 hours.

3 minutes exposure:
MTT reaction starting date/time : _____________ MTT reaction ending date/time : _________________
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

60 minutes exposure:
MTT reaction starting date/time : _____________ MTT reaction ending date/time : _________________
(MM/DD/YYYY HH/MM) (MM/DD/YYYY HH/MM)

Note

Date:___________________ Operator:__________________ Check date:___________ Study director:_______________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:________________________ Name:____
(MM/DD/YYYY)

MDS 5:
FORMAZAN EXTRACTION AND MEASUREMENT (Section 3.3.4)

Laboratory name: ________________ Test name: ________________ Test No. : ____________

1. After MTT reaction, use forceps to pick up the cultured epidermis from the cell culture insert and put it in a 1.5 mL microtube.

Did you use a scalpel to cut out the cultured epidermis? ☐
Execution date : __________________________
(MM/DD/YYYY)

2. Add isopropanol (300μL) to a microtube and completely immerse the tissue in isopropanol.

Isopropanol Lot No.__________________________ Added isopropanol (300μL) ☐
Immersion of the cultured epidermis in isopropanol. ☐
Execution date : __________________________
(MM/DD/YYYY)

3. For MTT formazan extraction, leave the micro tubes in a cold and dark space for more than 15 hours.

Place micro tube in a cold and dark space. ☐

4. Transfer the extracted solution (200μL) to a well of a 96-well plate.

Transfer to the 96-well plate. ☐
Execution date/time : ______________________
(MM/DD/YYYY HH/MM)

Sample location on 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A		Blank		DW-1	DW-2			KOH-1 [Only 60 min]	KOH-2 [Only 60 min]			
B		S1-1	S1-2	S2-1	S2-2	S3-1	S3-2	S4-1	S4-2	S5-1	S5-2	
C		S6-1	S6-2	S7-1	S7-2	S8-1	S8-2	S9-1	S9-2	S10-1	S10-2	
D		S11-1	S11-2	S12-1	S12-2	S13-1	S13-2	S14-1	S14-2	S15-1	S15-2	
E		S16-1	S16-2	S17-1	S17-2	S18-1	S18-2	S19-1	S19-2	S20-1	S20-2	
F		S21-1	S21-2	S22-1	S22-2	S23-1	S23-2	S24-1	S24-2	S25-1	S25-2	
G		S26-1	S26-2	S27-1	S27-2	S28-1	S28-2	S29-1	S29-2	S30-1	S30-2	
H												

5. Analyze extract OD at 570 nm and 650 nm, and calculate the OD (570 nm-650 nm).

Analyze OD at 570 nm and 650 nm. ☐
Calculate the OD (570 nm-650 nm). ☐
Calculate cell viability and difference of viability. ☐
Cell viability and difference of viability are recorded on a separate data sheet. ☐
The data sheet is attached to the back of this sheet. ☐
Check for input errors. ☐
Execution date/time : ______________________
(MM/DD/YYYY HH/MM)

Note

Date:____________________ Operator:___________________ Check date:____________ Study director:________________
(MM/DD/YYYY) (MM/DD/YYYY)

Secretariat Check date:___________________________ Name:____
(MM/DD/YYYY)

REVISION HISTORY

Rev.	Content	Date Revised
Ver. 1.1	First version	01/05/2015
Ver. 1.2	Revised clerical error.	02/03/2017
Ver. 1.3	Replaced the term “tissue viability” by “cell viability” throughout entire document. In the section 3.3. “EXECUTION OF THE TEST”, additionally explained that the solid test chemical is crush and grind in a mortar with pestle if necessary.	02/06/2017
Ver. 1.4	Revised clerical error. In the section 3.2. “TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH MTT ENDPOINT”, additionally explained the preparation of freeze-killing tissues.	03/03/2017
Ver. 1.5	In the section 3.2. “TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH MTT ENDPOINT”, supplementary explained calculating formula for correcting OD.	03/23/2017
Ver. 1.6	Revised clerical error. In the section 3.2. “TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH MTT ENDPOINT”, added directions concerning the negative control in STEP 2 and STEP 4. In the section 3.3.3. “REMOVAL OF THE TEST CHEMICALS”, added detailed explanation of the washing procedure. In the section 3.3.4. “FORMAZAN EXTRACTION AND MEASUREMENT”, added description of two different methods to remove tissues from the culture inserts.	05/12/2017