SOP of LabCyte EPI-MODEL24 Skin Irritation Test method (LabCyte EPI-MODEL24 SIT) for medical device

Ver. 2.2

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1. RATIONALE AND BACKGROUND

1.1 LabCyte EPI-MODEL24 SKIN IRRITATION TEST (LabCyte EPI-MODEL24 SIT) FOR MEDICAL DEVICE EXTRACTS

The LabCyte EPI-MODEL24 SIT for medical device extracts is designed for the prediction of acute skin irritation of medical device extracts by measurement of their cytotoxic effect, as reflected in the MTT assay, on the Reconstructed Human Epidermis (RhE) model, LabCyte EPI-MODEL24.

1.2 LabCyte EPI-MODEL24

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. The model constructs a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum.

1.2.1 Quality control of the test system

The LabCyte EPI-MODEL24 is manufactured according to defined quality assurance procedures. Each batch production is 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, and barrier function integrity ($0.14 \leq IC50 \leq 0.4$).

1.2.2 Precautions

The epidermal cells were derived from a healthy donor negative to HIV, HBV and HCV. Nevertheless, handling procedures for biological materials should be followed:

a) It is recommended to wear gloves during handling of the skin 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 BASIS OF THE METHOD

1.3.1 Endpoints

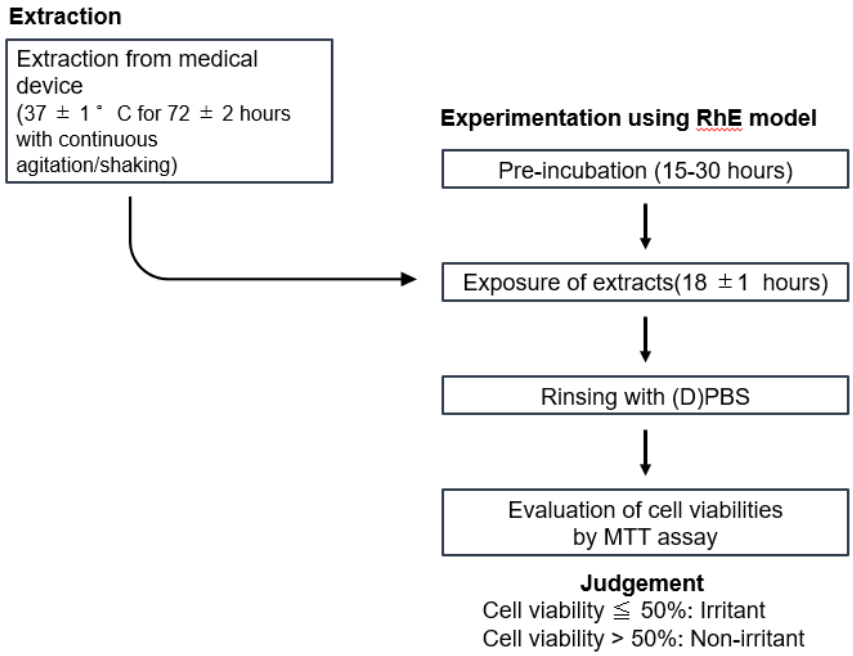
Cell viability determination is based on cellular reduction of MTT, and subsequent conversion to a purple formazan salt that is quantitatively measured after extraction from tissues (Faller et al., 2002; Mosmann, 1983). The reduction of cell viability in treated tissues is compared to negative controls and expressed as a percentage. The percent reduction in viability is used to predict the irritation potential of the tested substances.

1.3.2 Brief Procedure

Before starting experiments, LabCyte EPI-MODEL24 tissues are conditioned by incubation to release transport stress related compounds and debris overnight. After pre-incubation, tissues are topically exposed to medical device extracts for 18 hours. Preferably, three tissues are used per

medical device extracts and for the positive, negative and vehicle controls. Tissues are then thoroughly rinsed, blotted to remove the test substances, and transferred to fresh medium. After exposure of medical device extracts and rinsing, the MTT assay is performed by transferring the tissues to wells containing MTT medium (1.0 mg/mL). After 3 hours of MTT incubation, the purple formazan salt formed by cellular mitochondria is extracted with 500 µL/tissue of isopropanol and the optical density at 570 nm and 650 nm of the extracted formazan is determined using a spectrophotometer. 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% (v/v) solution of SDS in saline solution are used as positive controls and DPBS-treated epidermis is used as the negative control.



1.3.3 Rinsing Procedure

A. Rinsing by wash bottle

Time	No.						
	1	2	3	・・・・・	22	23	24
0min	Apply						
1min		Apply					
2min			Apply				
・ ・ ・							
21min					Apply		
22min						Apply	
23min							Apply
24min							
	Exposure for 18 hours						
18h 0min	Rinsing						
18h 1min		Rinsing					
18h 2min			Rinsing				
・ ・ ・							
18h 21min					Rinsing		
18h 22min						Rinsing	
18h 23min							Rinsing

- Above indicates the rinsing schedule for one plate. Perform rinsing of other plates as well as first plate.
- The time interval is set as 1-3 minutes.

B. Rinsing by pipetting

No.			
1 - 6	7 - 12	13 - 18	19 - 24
Apply			
Exposure for 18 hours	Apply		
		Apply	
			Apply
Rinsing			
	Rinsing		
		Rinsing	
			Rinsing

- Perform rinsing procedure per 6-well unit.
- Perform rinsing of other plates as well as first plate.

2. MATERIALS

2.1 Skin Irritation Test kit

2.1.1 COMPONENTS

Components of Skin Irritation Test kit are shown in Table 1.

Table 1 - 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: 0.3cm^2).
Assay Medium	2 bottles	Basic medium for incubation (30mL). Store at refrigeration temperature.
24-well plate	4 plates	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 transportation box and delivered. After delivered, please check that all kit components (LabCyte EPI-MODEL24 plate, assay medium, and 24-well assay plates) are included in the transportation box. Verify lot numbers and expiration dates. Record details in the Methods Documentation Sheet (MDS) 3.

2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL24

Start the incubation of the culture inserts soon after opening the package. Do not store the culture inserts again after opening the aluminum package.

The human epidermis cells used in LabCyte EPI-MODEL24 originate from a healthy donor and are HIV-, HBV-, and HCV-negative. However, handle them with enough care and in accordance with the laboratory biosafety guidelines since they contain raw materials of human origin.

2.3 CONSUMABLES

The following consumables are required.

* The described quantities are necessary so that 1 to 5 samples extracted by 2 kinds of vehicle (sesame oil and saline) can be assayed once.

- Assay Medium, 100 mL (J-TEC: 402100) 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(s) (Corning: 353047) 3 plates
- 96-well plate 1 plate
- Dulbecco's Phosphate buffered saline (DPBS) 500 mL 2 bottles
- Isopropanol 500 mL 1 bottle
- Sodium dodecyl sulfate (SDS), 20% solution in water 1mL
- Sesame oil
  (Sigma: #85067 or reagent listed in Japanese Pharma Pharmacopoeia) 30 mL

- Saline (Otsuka Pharmaceutical: #10095-3) 30 mL
- Sterile cotton bud 1BOX

2.4 OTHERS

2.4.1 EQUIPMENT / INSTRUMENTS

- Safety cabinet (or clean bench)
- Water bath (37 ± 1°C)
- CO_2 incubator (37 ± 1 °C, 5% CO_2, capable of maintaining high humidity)
- Autoclave
- Timer
- 96-well multi-plate reader (required filters: 570 nm, 650 nm)
- Precision balance (1 mg)
- Adjustable micro-pipette (10-200 µL, 200-1000 µL)
- Adjustable positive displacement Pipette (10-200 µL, 200-1000 µL)
- Electronic pipette controller
- Sharp-edged forceps (sterile)
- Micro spatula (sterile)
- Paper towel
- Shaker for micro-tube
- Beaker (1~2L)
- Sterilizable poly wash bottle (500~1000mL: sterile)

2.4.2 CONSUMMABLE ITEMS

- Micro-pipette tips for micro pipette and positive displacemnet pipette (sterile: 10-200 µL, 200-1000 µL)
- Serological pipettes (sterile: 10 mL, 25 mL)
- Micro-tubes (1.5mL)
- Scalpel (KEISEI MEDICAL INDUSTRIAL: Keisei Scalpel 11A)

3. TEST METHOD

*Perform operations in Section 3.1.1~3.1.5 and Section 3.2.1~3.2.2 aseptically in a safety cabinet (or clean bench).

*Operations other than above do not need to be performed with aseptic techniques. For these operations, refer to **Section 2.1.3 INSTRUCTIONS FOR USE OF LabCyte EPI-MODEL24**

3.1 PREPARATIONS

3.1.1 POSITIVE CONTROL SUBSTANCE

(1) Mix 500 µL of 20% SDS solution with 9.5 mL of the particular extraction vehicle (saline) and vortex thoroughly.
* Store in a dark, cold place and use it within 24 hours.

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

3.1.2 MTT MEDIUM

(1) Dissolve MTT in the assay medium to prepare the MTT medium (final concentration: 1.0 mg/mL)
Use an ultrasonic cleaner equipment or a vortex mixer, as necessary, in order to completely dissolve the MTT powder. Keep the solution in a dark, cold place and use it within 24 hours in the case of ready-to use.
*Stocking condition: Under -20 °C for 6 months.

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

3.1.3 NEGATIVE CONTROL SUBSTANCE

(1) The negative control is (D)PBS.

3.1.4 VEHICLES SUBSTANCE

(1) The vehicle controls (saline and Sesame oil) should be kept in amber glass vial extraction vessels and subjected to the same extraction procedure (72 ± 2hours at 37 ± 1°C) adopted for the medical device extraction.

(2) Refer ISO 10993-12 extraction procedure.

3.1.5 PREPARATION OF MEDICAL DEVICE EXTRACTS

(1) Document the information on the test substances/medical devices in the table of **Annex 1**: Characterization of test substances.

(2) The preparation of device extracts is done according to ISO 10993-12 guidelines and is based on surface area to volume ratios described in this standard.

(3) Polar extracts are prepared in saline.

(4) Non-polar extracts are prepared in sesame oil.

(5) Extraction is conducted at (37 ± 1) °C for (72 ± 2) hours.

(6) Store extracted solution at room temperature and use within 24 hours after the extraction.

3.2 EXECUTION OF THE TEST

3.2.1 PREPARATION OF LabCyte EPI-MODEL24

(1) Pre-warm the assay medium to 37 ± 1°C for 30 minutes using a water bath.
(2) Dispense 500 µL of pre-warmed assay medium into each well of 24-well assay plate(s).
(3) Take out the LabCyte EPI-MODEL24 from aluminum package.
(4) Take the plate lid of LabCyte EPI-MODEL24 out 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) Using sterile forceps, transfer the culture inserts to the 24-well assay plate(s) pre-filled with assay medium.
*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) before proceeding to Section 3.2.2 "APPLICATION OF EXTRACTS AND RINSING."
(8) Record details of steps (1) – (7) above in the MDS 5-1.

3.2.2 APPLICATION OF EXTRACTS AND RINSING

3.2.2.1 PREPARATION FOR EXTRACTS

(1) Pre-warm the assay medium to 37 ± 1°C for 30 minutes using a water bath.
(2) Dispense 500 µL of pre-warmed assay medium into each well of 24-well assay plate(s).
(3) Put the lid on the 24-well assay plate(s) and proceed to Section **3.2.2.2 "APPLICATION OF TEST** EXTRACTS**"**.
(4) Record details of steps (1) – (3) above in the MDS 5-2.

3.2.2.2 APPLICATION OF EXTRACTS

(1) Take the pre-incubated plate(s) from the CO_2 incubator.
(2) Apply 100 µL of the undiluted medical device extracts, negative control, positive control and vehicle control on the surface of tissues (inside of culture inserts). Use 3 tissues per extract (N=3).Negative and positive controls should be set in each plate.
*Check the extracts spread over entire of tissues.
*Tilt the plate to spread extracts throughout entire tissues if necessary.
(3) Transfer the culture inserts applied medical device extracts to each wells of the 24-well assay plate(s) prepared in section 3.2.2.1.
*Avoid trapping air bubbles under the culture inserts.
(4) Put on the lid of the 24-well assay plate(s) and place it in a CO_2 incubator.
(5) Incubate for 18 ±1 hours.
(6) Record details of steps (1) - (5) above in the MDS 5-2.

3.2.2.3 PREPARATION FOR AFTER-RINSE CONDITIONING

(1) Pre-warm the assay medium for 30 minutes to 37 ± 1°C using a water bath.
(2) Dispense 500 µL of pre-warmed assay medium into each well of 24-well assay plate(s) for transferring rinsed insert cups.
(3) Put on the lid of 24 well assay plate(s) and proceed to Section **3.2.2.4 "REMOVAL OF THE EXTRACTS"**.
(4) Record details of steps (1) – (3) above in the MDS 5-3.

3.2.2.4 REMOVAL OF EXTRACTS

A. Rinsing by using wash bottle

(1) After exposure of extracts for 18 ± 1 hours, pick up a culture insert with sterile forceps. Discard extracts onto the tissue by tilting and then tapping the insert on the beaker.
(2) Fill the culture insert with (D)PBS using a PBS filled poly wash bottle. Flow (D)PBS stream from the washing bottle against the side-wall of the culture insert and wash on the tissue surface by the PBS current (Photo 1).

Attention: Avoid flowing the (D)PBS stream on the tissue surface directly. Be careful not to damage the tissue surface.

Photo 1 - Rinsing by poly wash bottle

(3) Remove the PBS from inside of the culture inserts by tapping on the beaker (Photo 2).
(4) Repeat steps (2) and (3) at least 10 times or more as much as possible and remove all residual extracts on the tissue surface almost completely.
(5) Gently wipe the leftover PBS inside and outside of the culture inserts by using a sterile cotton bud (Photo 3).
*Wipe the inside of cell culture insert to remove PBS almost completely.
(6) Transfer the rinsed culture inserts to the corresponding wells in the 24-well assay plate(s) prepared in section 3.2.2.3 (2).
*Avoid trapping air bubbles under the culture inserts.
(7) Repeat steps (1) ~ (7) for all the culture inserts at 1~3-minute intervals.
(8) Record details of steps (1) – (4) above in the MDS 5-4.
(9) After the rinsing step, proceed to Section 3.2.3.2 "MTT ASSAY".

Photo 2 - Discarding the PBS

Photo 3 - Wipe the cell culture insert

B. Rinsing by pipetting

(1) After the substance application period (18 ± 1 hours), carefully remove the test substances from the culture inserts using micro pipette.

(2) Apply 0.5 mL/well of (D)PBS to the tissue surface.

(3) Remove (D)PBS from inside of cell culture insert by using micro pipette.

(4) Repeat steps (2) ~ (3) at least 10 times for all the culture inserts to remove extracts almost completely from tissues.

(5) Transfer the rinsed culture inserts to the corresponding wells in the 24-well assay plate(s)prepared in section 3.2.2.3 (2).

*Avoid trapping air bubbles under the culture inserts.

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

(7) After the rinsing step, proceed to Section 3.2.3.2 “MTT ASSAY”.

3.2.2.5 INFLAMATORY CYTOKINES MEASUREMENT (OPTIONAL)

For inflammatory cytokines measurement, collect medium after exposure of 18 ± 1 hours. Medium can be stored in a freezer at ≤ -20 °C.

3.2.3 MTT ASSAY

3.2.3.1 PREPARATION FOR MTT ASSAY

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

(2) Dispense 300 µL of pre-warmed MTT medium into each well of 24-well assay plate(s).

(3) Put the lid on the assay plate and place it in the CO_2 incubator until the start of the MTT assay.

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

3.2.3.2 MTT ASSAY

(1) After rinsing procedure completed, transfer cell culture inserts to 24-well assay plate(s) dispensed with MTT medium. Before transfer cell culture insert, absorb the assay medium on the outside of cell culture inset into paper rag to remove remaining assay medium

*Avoid cross contamination of assay medium dropping from outside of cell culture inset when transferred cell culture inserts to 24-well assay plate(s).

*Avoid trapping air bubbles under the culture inserts after cell culture transferred.

(2) Put the lid on cell culture transferred 24-well assay plate(s) and place the plate(s) in the CO_2 incubator.

(3) Incubate for 3 hours ± 5min.

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

3.2.4 FORMAZAN EXTRACTION AND MEASUREMENT

3.2.4.1 FORMAZAN EXTRACTION

(1) After MTT reaction for 3 hours ± 5min, take out the 24-well assay plate(s) containing the culture inserts in MTT medium from the CO_2 incubator.

(2) Take off the lid of the 24-well assay plate(s) and pinch the cultured epidermis from each culture insert with forceps.

→ Photo 4

*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 substance.

Photo 4 - Detachment of epidermis

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

(4) Add 500 µL of isopropanol to the micro-tubes and immerse the entire epidermis tissue in the isopropanol.

(5) To extract formazan, shaking the micro-tube for at least 2 hours at room temperature or place in dark overnight in order to extract pigments completely.

*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 sediment or gently centrifuge them (if a centrifuge is available).

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

(7) Fill two wells of a 96-well plate with 200 µL, each, of the solution from each micro-tube.

*Eight wells of 200 µL of isopropanol should be set as a blank.

*Figure 1 shows an example of sample allocation in a 96-well plate.

Record details of step (7) above in the MDS 5-7.

Figure 1 – Allocation for a 96-well plate

For saline Extracts

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank-1	Blank-2	Blank-3	Blank-4	Blank-5	Blank-6	Blank-7	Blank-8				
B	NC (DPBS)1–1	Vehicle control (Saline)1-1	Extract No. 1 1-1	Extract No. 2 1-1	Extract No. 3 1-1	Extract No. 4 1-1	Extract No. 5 1-1	PC (1%SDS)1-1				
C	NC (DPBS)1–2	Vehicle control (Saline)1-2	Extract No. 1 1-2	Extract No. 2 1-2	Extract No. 3 1-2	Extract No. 4 1-2	Extract No. 5 1-2	PC (1%SDS)1-2				
D	NC (DPBS)2-1	Vehicle control (Saline)2-1	Extract No. 1 2-1	Extract No. 2 2-1	Extract No. 3 2-1	Extract No. 4 2-1	Extract No. 5 2-1	PC (1%SDS)2-1				
E	NC (DPBS)2-2	Vehicle control (Saline)2-2	Extract No. 1 2-2	Extract No. 2 2-2	Extract No. 3 2-2	Extract No. 4 2-2	Extract No. 5 2-2	PC (1%SDS)2-2				
F	NC (DPBS)3-1	Vehicle control (Saline)3-1	Extract No. 1 3-1	Extract No. 2 3-1	Extract No. 3 3-1	Extract No. 4 3-1	Extract No. 5 3-1	PC (1%SDS)3-1				
G	NC (DPBS)3-2	Vehicle control (Saline)3-2	Extract No. 1 3-2	Extract No. 2 3-2	Extract No. 3 3-2	Extract No. 4 3-2	Extract No. 5 3-2	PC (1%SDS)3-2				
H												

For sesame oil Extracts

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank-1	Blank-2	Blank-3	Blank-4	Blank-5	Blank-6	Blank-7	Blank-8				
B	NC (DPBS)1–1	Vehicle control (Sesame oil)1-1	Extract No. 1 1-1	Extract No. 2 1-1	Extract No. 3 1-1	Extract No. 4 1-1	Extract No. 5 1-1	PC (1%SDS)1-1				
C	NC (DPBS)1–2	Vehicle control (Sesame oil)1-2	Extract No. 1 1-2	Extract No. 2 1-2	Extract No. 3 1-2	Extract No. 4 1-2	Extract No. 5 1-2	PC (1%SDS)1-2				
D	NC (DPBS)2-1	Vehicle control (Sesame oil)2-1	Extract No. 1 2-1	Extract No. 2 2-1	Extract No. 3 2-1	Extract No. 4 2-1	Extract No. 5 2-1	PC (1%SDS)2-1				
E	NC (DPBS)2-2	Vehicle control (Sesame oil)2-2	Extract No. 1 2-2	Extract No. 2 2-2	Extract No. 3 2-2	Extract No. 4 2-2	Extract No. 5 2-2	PC (1%SDS)2-2				
F	NC (DPBS)3-1	Vehicle control (Sesame oil)3-1	Extract No. 1 3-1	Extract No. 2 3-1	Extract No. 3 3-1	Extract No. 4 3-1	Extract No. 5 3-1	PC (1%SDS)3-1				
G	NC (DPBS)3-2	Vehicle control (Sesame oil)3-2	Extract No. 1 3-2	Extract No. 2 3-2	Extract No. 3 3-2	Extract No. 4 3-2	Extract No. 5 3-2	PC (1%SDS)3-2				
H												

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

3.2.4.2 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 650 nm OD from the 570 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 variability (SD) of tissue replicates.

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

$$\text{Cell Viability (\%)} = \frac{\text{Mean of measured OD}_{\text{sample}}}{\text{Mean of measured OD}_{\text{NC}}} \times 100$$

4. ASSESSMENT

4.1 CONDITIONS FOR A SUCCESSFUL STUDY

The skin irritation test should be considered successful if all 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 1% SDS (positive control) ≤ 40%.
- SD: SD (negative control, positive control and test substance) of tissue viability of 3 identically treated replicates ≤ 20%
- Vehicle control: 80% of tissue viability of negative control < mean tissue viability of vehicle control < 120% of tissue viability of negative control

4.2 ASSAY CRITERIA

The criteria for *in vitro* interpretation are shown below.

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

The Methods Documentation Sheet

Test schedule (For 2 plates)

Procedure	Date (yy/mm/dd)	Plate 1 (Saline)		Plate 2 (Sesame oil)	
		Starting time	Ending time	Starting time	Ending time
Pre-incubation (15-30 hours)					
Exposure (18 ± 1 hours)					
Rinsing					
MTT assay (3 hours ± 5 min)					
Extraction (At least 2 hours)					
Measurement					

MDS 1. Equipment Check

Operator: _____

Date: _____

Manufacturer name of CO_2 incubator : _____ Model: _____

☐ The concentration of CO_2 is 5 ± 1%

☐ The temperature of incubator is 37 ± 1℃

☐ The torey in incubator is filled with water

Manufacturer name of refrigerator : _____ Model: _____

☐ The temperature of refrigerator is 5 ± 2℃

Check of micropipettes

1. Transfer each volume of distilled water to each tubes using micropipettes
2. Confirm the consistency of the volume transferred by micropipette and weight measured by precision balance

*Repeat 3 times above procedures and confirm the accuracy of the micropipettes

*Operator must confirm the accuracy of micropipettes before every experiments.

For micro pipette

	2 mL	900 µL	300 µL	200 µL	100 µL
1					
2					
3					
Average					
SD					
Tolerance	5%	5%	5%	5%	5%

Filled the name of micropipettes in gray columns

For positive displacement pipette

	2 mL	900 µL	300 µL	200 µL	100 µL
1					
2					
3					
Average					
SD					
Tolerance	5%	5%	5%	5%	5%

Filled the name of micropipettes in gray columns

MSD 2. The information of test samples

Operator: _____

Date: _____

- Saline

Manufacturer name of saline : _____

Lot. No. : _____

Expiration date: _____

- Sesame oil

Manufacturer name of saline : _____

Lot. No. : _____

Expiration date: _____

☐Extraction by shaker （Condition rpm）

Manufacturer name of shaker: _____ Model: _____

☐Extraction by hand mixing （Number of mixing ）

Manufacturer name of incubator: _____ Model: _____

Preparation of extracts

Name of samples	Vehicle	Volume of vehicle(ml)	Name of used incubator	Starting date _____ Starting time	Ending date _____ Ending time	Gross appearance after extraction
Vehicle control Saline	Saline					
	Saline					
	Saline					
	Saline					
	Saline					
Vehicle control Sesame oil	Sesame oil					
	Sesame oil					
	Sesame oil					
	Sesame oil					
	Sesame oil					

MSD 3. Receipt of EPI-MODEL24

Operator:

Date of receipt:

Lot No.

Expiration date:

Assay media: (Lot No. Expired date:)

Defective appearance: ☐ No ☐ Yes (Outline of defective:)

MSD 4-1. Preparation of 1% SDS as positive control

Operator:

Preparation date:

- 1% SDS solution

20% SDS solution μL

Saline mL

MSD 4-2 .Preparation of MTT solution

Operator:

Preparation date:

Dissolve MTT in assay media to prepare 1.0mg/mL of MTT solution

(Recommend: Dissolve the 16 mg of MTT with 16 mL of assay media)

Supplier:

Lot No.:

Weight of MTT mg

Assay media mL

MSD 5-1. Pre-incubation (15-30 hr)

Operator:

☐ Pre-incubated the assay media at 37 ± 1 ℃ before use

Manufacturer name of incubator: Model:

☐ Added 500 µL/well of assay media

Starting date (time): (:)

Ending date (time): (:)

MSD 5-2. Exposure of test sample (18 ± 1hr)

Operator:

☐Pre-incubated the assay media at 37 ± 1℃ before use

☐Added 500 µL/well of assay media to the 24-well assay plate prepared for exposure

☐Added 100 µL of test sample to the surface of tissues

☐ Starting incubation time of PLATE 1:

☐ Ending incubation time of PLATE 1:

☐ Starting incubation time of PLATE 2:

☐ Ending incubation time of PLATE 2:

☐Abnormal appearance is not occurred in the tissues after sample exposure for 18 ± 1 hr.

Fill in the remarks column if there is any abnormal appearance

Remarks.

MSD 5-3. Preparation before rinse

Operator:

☐Pre-incubated the assay media at 37 ± 1℃ before use

☐Added 500 μL/well of assay media to the assay plate prepared for rinsing

MSD 5-4. Rinsing

Operator:

Supplier of PBS: Lot No:

☐ Rinsing by micropipette ☐ Rinsing by poly wash bottle

☐ Rinsing more than 10 times

Starting time of PLATE 1 (time): (: ～)

Ending time of PLATE 1 (time): (～ :)

Starting time of PLATE 2 (time): (: ～)

Ending time of PLATE 2 (time): (～ :)

Fill in the remarks column if there are any issues. For example, the tissues are detached from culture inserts.

Remarks

5-5 MTT test（3 hours ± 5min）

Operator:

☐ Preincubated MTT solution at 37 ± 1℃ before use

☐ Added 300 μL/well of MTT solution to the 24-well assay plate

Starting time of PLATE 1 (time): (: ～)

Ending time of PLATE 1 (time): (～ :)

Starting time of PLATE 2 (time): (: ～)

Ending time of PLATE 2 (time): (～ :)

5-6 Extraction (at least 2 hours)

Operator:

☐ Soaked each tissues in 500 μL of iso-propanol

☐ Shaked during extraction (Not needed this item if extraction is preceded overnight)

Manufacturer name of shaker: Model:

☐Shaded during extraction

Starting date (time): (: ～)

Ending date (time): (～ :)

5-7 Measurement

Operator:

☐ OD values were measured at 570 nm and 650 nm

Manufacture name of microplate reader: Model:

Starting date (time): (: ～)

Ending date (time): (～ :)