Table 7. Classification using three independent viabilities

			Lab.						
Chem.	Score	Exp.	a	b	c	d	f	g	
A	2	1	P	P	P	P	P	P	
		2	P	P	P	P	P	P	
		3	P	P	P	P	P	P	
B	2.7	1	P	P	P	P	P	P	
		2	P	P	P	P	P	P	
		3	P	P	P	P	P	P	
C	3.0	1	P	P	P	P	P	P	
		2	P	P	P	P	P	P	
		3	P	P	P	P	P	P	
D	3.3	1	P	P	P	P	P	P	
		2	P	P	P	P	P	P	
		3	P	P	P	P	P	P	
E	3.3	1	P	P	P	P	P	P	
		2	P	P	P	P	P	P	
		3	P	P	P	P	P	P	
F	4.0	1	P	P	P	P	P	P	
		2	P	P	P	P	P	P	
		3	P	P	P	P	P	P	

「P」:Positive、「N」: Negative

Table 8 Sensitivity, specificity and accuracy on MTT assay vs GHS-EU

Index	Lab.						
	a	b	c	d	f	g	
Sensitivity	5/5	5/5	5/5	5/5	5/5	5/5	
Spescificity	0/1	0/1	0/1	0/1	0/1	0/1	
Accuracy	5/6	5/6	5/6	5/6	5/6	5/6	

9. Discussion

9-1. Reliability

All data of negative control and positive control each laboratory in 3rd phase study was sufficient with the acceptance criteria as shown in Tables 3 and 4. Compared with 2nd phase study, there were high repeat abilities within laboratory in this model.

All data of 6 chemicals was judged all positive each laboratory as shown in Tables 5, 6 and 7, Fig 2. Therefore, there were high repeatabilities without laboratories in this model.

9-2. Predictivity

In December 2008, the EU adopted the UN Globally Harmonised System for Classification and Labelling and will implement this by means of the so-called CLP regulation (Regulation EC 1272/2008). The new EU classification system based on UN GHS (abbreviated here as "GHS-EU")

continues to use two categories to distinguish non-irritant (no-category) from irritant (category 2) substances. However, according to the new rules for skin irritation classification and labelling, the cut-off score to distinguish between no-category and category 2 substances was shifted to 2.3 from a value of 2.0 (EU classification system). Consequently substances with an in vivo score between 2.0 and 2.3 that are considered irritant under the existing EU classification system will be considered non-irritants under the future GHS-EU classification system, which does not use the optional UN GHS category 3.

According to this rules, the prediction values of the skin irritation test with LabCyte EPI-MODEL24 when it was only evaluated MTT as an indicator and GHS-EU classification in this phase validation study, is shown in Table 8. Sensitivity, specificity and accuracy of this prediction model are 5/5, 0/1, and 5/6 respectively.

9-3. Proposal

Based on the 1st and 2nd report, the reliability and predicitivity should be recalculated.

9-4. Conclusion

Based on the GHS-EU classification, 5 irritants and 1 non-irritant in the new ECVAM Performance Standards were tested by the 6 labs using **LabCyte** EPI-MODEL24. The assay demonstrated high reliability with and without laboratories on the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

10. Acknowledgement

This validation study has supported by the Health and Labour Sciences Research Grant, Japan.

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JSAAE DRAFT PROJECT PLAN ON:

VALIDATION OF THE HCE TESTS : LABCYTE EPI-MODEL 24 ACUTE SKIN IRRITATION PREDICTION

1. Goal statement

- The aim of this study is to validate *in vitro* skin irritation tests in a formal inter-laboratory study, The ultimate goal of the test strategy will be to replace the regulatory Draize skin irritation test according OECD TG 404 (OECD, 2002).
- The primary goal of this validation study is an evaluation of the ability of the *in vitro* tests to reliably discriminate skin irritant (I) from non-irritant (NI) chemicals, as defined according to the OECD and United Nations proposal for a Globally Harmonised System (GHS) for the classification and labelling of ocular irritation (category 1/category 2; no category; Anon., 2003) .

2. Objective

The *in vitro* test systems, employing human reconstructed tissue models will be evaluated. This model (LabCyte^{TM}) comprises normal, human-derived epidermal keratinocytes. This assay has progressed through protocol optimisation and multi-laboratory assessment. The present objective is to conduct a validation study to assess the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of this test system with a challenging set of coded test chemicals for which high quality *in vivo* data are available. The validation study will be undertaken in accordance with the principles and criteria documented in the OECD *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (No. 34, OECD, 2005) and according to the Modular Approach to validation (Hartung *et al.* 2004).

3. Validation Management structure

The management structure of the study is shown in Figure 1.

3-1. Validation Management Group

The Validation Management Group (VMG), which plays a central role overseeing the conduct of the validation study, includes:

1) Goal statement

2) Project plan

3) Study protocol / amendents

4) Outcome of QC audits

5) Test chemicals

6) Data management procedures

7) Timeline/ study progression

8) Study interpretation and conclusions

9) Reports and publication

The final decision on which laboratories participate in the validation study is the responsibility of the VMG.

Members:

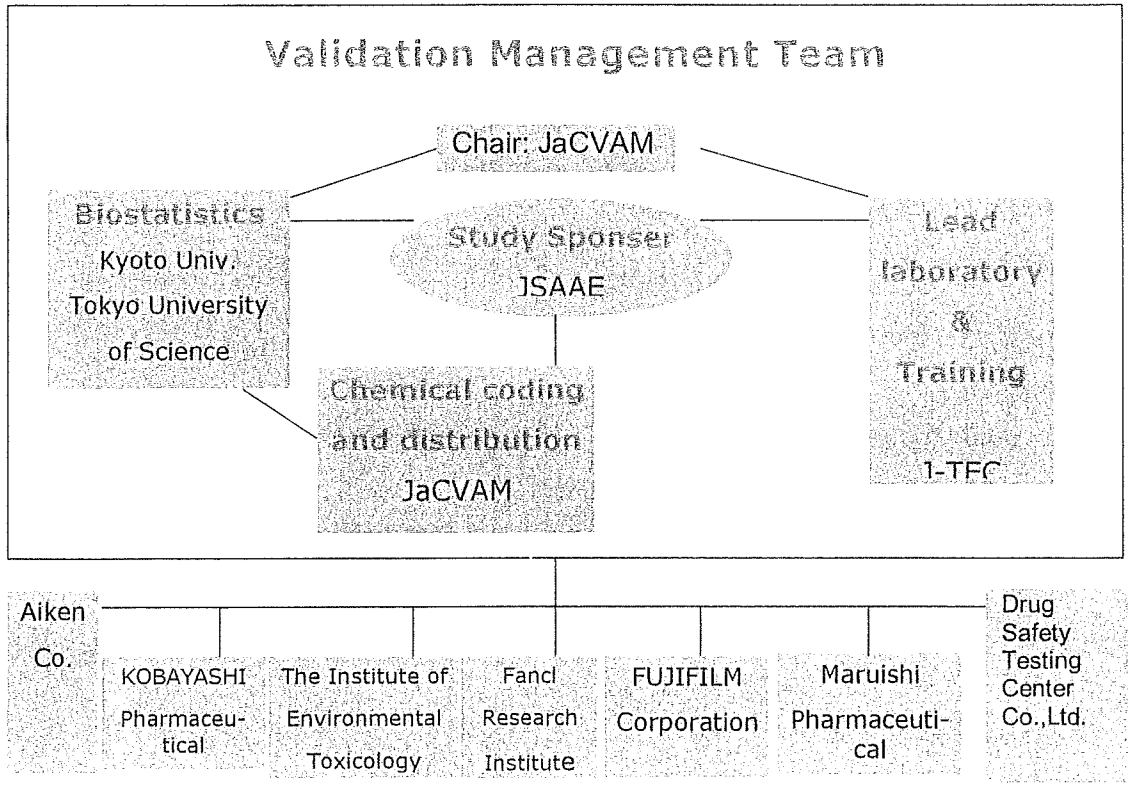
A chair (Hajime Kojima, JaCVAM)

The sponsor representative: representatives of JSAAE (Takashi Omori; Kyoto Univ., Kenji Idehara; Daicel Chemical Co. and Isao Yoshimura; Tokyo University of Science)
The sponsor representative, LabCyte™ suppliers and lead lab (Masaichi Kato : Japan Tissue Engineering Co., Ltd (J-TEC)

3-2 Chemical selection, acquisition, coding and distribution
1) Definition of selection criteria
2) Chemical selection
3)Liaise with suppliers
4) Final check of chemicals provided
5) Acquistion
6) Coding
7) Distribution
Member
Hajime Kojima, JaCVAM

3-3 Independent biostatisticians
1) Support chemical selection
2) Approve spreadsheets
3) Collect data
4) Analyse data
Members:
Takashi Omori; Kyoto Univ.
Miyaoka, E.,and Ishiyama, K.; Tokyo University of Science

Figure 1: Management Structure of the JSAAE Skin Irritation Validation Study



4. Sponsorship

The study will be managed by JSAAE and financed by J-TEC .

JSAAE finance:

- the management of the study (VMG meetings)

- the independent statistical support (biostatistician)

- an independent laboratory responsible for the chemicals purchase, coding and distribution to the laboratories

- the IL-1 α kits purchase and distribution to the laboratories

- the independent QC audit of the data

- the publication of the study

J-TEC will finance:

- the lead laboratories for the test method

- training for the participating laboratories

- the independent QC audit on the LabCyte models

-the financial assistance for the participated laboratories

5. Chemicals Selection

According to the EPISKIN performance standards, we have selected 19 new chemicals tested. One chemical can not be purchased at the Japanese market in the chemical list reference to EPISKIN performance standard. The final approval of the chemicals proposed by JaCVAM is the responsibility of the VMG. To avoid any potential for bias in the final selection, the laboratory representatives on the VMG will not be party to these discussions nor will they be made aware of the chemicals finally approved for testing in either phase of the validation study.

6. Chemical Coding and distribution

Independent coding and distribution of chemicals will be contracted out by JaCVAM to an independent company. The (company's name) is certified according to ISO 9001, EN 4500 and GLP, and has proven experience of reliable services. The codes will be provided by JaCVAM.

7. Participating laboratories

The laboratories participating in the study are to be defined as shown in **Figure 1**.

The 7 laboratories which will participate in the validation study for the evaluation of the LabCyte assays are:

- Laboratory 1– ***Aiken Co., Ltd.*** (***Ando, Yoko***)
- Laboratory 2 – ***KOBAYASHI Pharmaceutical Co., Ltd.*** (***Yamagichi, Y.***)
- Laboratory 3 – ***The Institute of Environmental Toxicology*** (***Kosaka, T.***)
- Laboratory 4 – ***Fancl Research Institute*** (***Suzuki, T.***)
- Laboratory 5 – ***FUJIFILM Corporation*** (***Yuasa, A.***)
- Laboratory 6 – ***Maruishi Pharmaceutical Co., Ltd.*** (***Watanabe Y.,***)
- Laboratory 7- ***Drug Safety Testing Center Co.,Ltd.*** (***Shinoda, S.)***

A reserve laboratory is also identified as J-TEC (Masaichi Kato).

All responsibilities of the laboratories (in particular, work programme and data submission deadlines to be met) will be specified in contracts between JaCVAM and the laboratories. Each laboratory will also be responsible for assuring compliance with GLP-like principles, and for specifying QA aspects.

8. Study design

Before this validation study, the training course using LabCyte was performed by J-TEC on April, 2008. All technicians from each laboratory participated at this training course.

Two phases of validation studies is planned. In Phase I, we will confirme the transferability of the test protocol and assessed its reproducibility using suitable statistical analyses, by testing three coded chemicals (ethanol, glycerol and napthalen acetic acid) and a positive control (5% sodium lauryl sulfate solution) in seven laboratories between June and July of 2008. In Phase II, we are planning to confirm the intra- and inter-laboratory reproducibility robustness, and the correlation of test using 19 new chemicals tested in reference to the EPISKIN performance standards[2]. These tests will be conducted by 7 laboratories between September and December of 2008.

9. Data Collection, handling, and analysis

The independent biostatistician of the study will collect the data and organise it in specific data collection software. They will work in close collaboration with the biostatisticians, (T.Omori, Miyaoka, E.,and Ishiyama, K.). After decoding they will analyse the data statistically. The data management procedures and statistical tools applied are to be approved by the VMG.

10. Quality assurance, GLP

LABORATORIES

All participating laboratories will work in the spirit of OECD GLP principles.

MODEL SUPPLIER

According to OECD GLP Consensus Document No.5 "*Compliance of Laboratory Suppliers with GLP Principles*" the responsibility for the quality and fitness for use of equipment and materials rests entirely with the management of the test facility (OECD, 1999).

The acceptability of equipment and materials in laboratories complying to GLP-like principles should therefore be guaranteed to any regulatory authority to whom studies are submitted. In some countries where GLP has been implemented, suppliers belong to national regulatory or voluntary accreditation schemes (for example, for laboratory animals) which can provide users with additional documentary evidence that they are using a test system of a defined quality.

The audits will focus on the procedures established to guarantee a defined quality of the tissue models.

11. Acknowledgement

This validation study has supported by the Health and Labour Sciences Research Grant, Japan.

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-JSAAE Skin Irritation Validation Study-

VALIDATION OF THE SKIN IRRITATION TEST ASSAY USING THE RECONSTRUCTED HUMAN MODEL "LABCYTE EPI-MODEL24".

LabCyte EPI-MODEL24 SKIN IRRITATION TEST-42 HOURS

S.O.P

TABLE OF CONTENTS

1. PURPOSE OF THE PROTOCOL

The LabCyte EPI-MODEL 24 skin irritation test protocol is shown in order to conduct a validation study of skin irritation test method using the human epidermis model "LabCyte EPI-MODEL 24".

2. MATERIALS

2.1 LabCyte EPI-MODEL24

2.1.1 LabCyte EPI-MODEL24 KIT COMPONENTS

Table 1 shows the components of a LabCyte EPI-MODEL 24 kit

Table 1 - LabCyte EPI-MODEL24 Kit Components

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

Three kits of LabCyte EPI-MODEL 24 are required to assay 20 test substances in accordance with this protocol. Other optional materials below are required and supplied with LabCyte EPI-MODEL 24 kits.

- Assay Medium, 100mL (Optional: 402250) , 1 bottle
- MTT, 25mg (Optional: 403026), 1 bottle

2.1.2 DELIVERY 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 Icompo is delivered, examine the contents of the Icompo and make sure that all kit components (LabCyte EPI-MODEL24 plate, assay medium, and 24-well assay plate) are packaged. Also confirm lot numbers and expiration dates. Record details in the Methods Documentation Sheet (MDS) 1.

NIPPON EXPRESS will pick up Icompo at a later date (generally, the day after the delivered date), and return it with a receipt as well as insulating materials.

2.1.3 INSTRUCTION FOR USE OF LabCyte EPI-MODEL24

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

The human epidermis cells used in LabCyte EPI-MODEL24 originate from a normal donor that was HIV-, HBV-, HCV-, and HPV-negative. However, handle them with enough care and in accordance with the laboratory biosafety guidelines since it contains raw materials of human origin

2.2 IL-1α ELISA KIT

2.2.1 IL-1α ELISA KIT USED IN THE STUDY

Product Name: Interleukin-1 Alpha (IL-1α) ELISA kit (96 Test kit)

Product Number: 01-031-078 (Product Code: KAC1191)

Distributor: ASAHI TECHNO GLASS CO., LTD. **Manufacturer:** Invitrogen, Biosource™

Sensitivity: 1 pg/mL **Detection Range:** 3.9 ~ 250 pg/mL

2.2.2 IL-1α ELISA KIT COMPONENTS

Table 2 shows the components of IL-1α ELISA kit used in the study.

Table 2 - IL-1α ELISA kit components

Name	Qty	Description
HuIL-1α Standard	2 vials	Recombinant human IL-1α for drawing standard curve
Standard Diluent Buffer	1 bottle	Recombinant human IL-1α diluting solution
HuIL-1α Antibody-coated wells	1 plate	Anti-IL-1α antibody coated plate (96-well plate)
HuIL-1α Biotin conjugate	1 bottle	Biotin-labeled anti-IL-1α antibody (secondary antibody)
Incubation buffer	1 bottle	
Streptavidin-Peroxidase (HRP)	1 vial	Luminescent enzyme (HRP) labeled Streptavidin
HRP Diluent	1 bottle	HRP diluting solution
Wash Buffer Concentrate(x25)	1 bottle	Concentrated solution to rinse (x25)
Stabilized Chromogen	1 bottle	Solution to catalyze chromogenic reaction
Stop Solution	1 bottle	Solution to stop the reaction
Plate Covers	3	To cover a plate for reactions

If all the 20 test substances are assayed in accordance with the protocol, 79 wells (79 tests) are required.

2.3 TEST SUBSTANCES

Coded test substances are delivered to each laboratory.

2.4 MATERIALS PROVIDED BY J-TEC

J-TEC purchases the following materials in bulk quantity and supplies them to each laboratory.

* The described quantities are distributed per each laboratory, so that 20 samples can be assayed three times.

- Wide orifice cell saver tips for micro-pipette (sterile) 96 tips — 1 box
- 24-well assay plate (Becton,Dickinson and Company: 353047) — 27 plates
- 96-well plate (Becton,Dickinson and Company: 353072) — 3 plates
- Phosphate buffered saline (PBS) 500mL (Invitrogen: 14190-144) — 12 bottles
- Isopropanol 500mL (Wako Pure Chemical Industries: 164-08335) — 1 bottle
- SLS 25g (SIGMA:L4390) — 1 bottle
- Sterile distilled water 20mL (Otsuka Pharmaceutical: 36A1X00001) — 5 bottles
- Sterile cotton buds (JAPAN COTTON BUDS: 10A754D) — 1 box

2.5 MATERIALS NOT PROVIDED WITH THE J-TEC KITS

The following materials should be prepared at each laboratory.

2.5.1 EQUIPMENT/INSTRUMENT

- Safety cabinet (or clean bench)
- Water bath (37 °C)
- CO_2 incubator (37 °C, 5%CO_2, capable to maintain 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)

2.5.2 EXPENDABLE SUPPLIES

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

3. METHODS

*Perform operations in Section 3.1.1~3.1.4 and Section 3.2.1~3.2.3 aseptically in a safety cabinet (or clean bench).

*Operations other than above do not need to be performed with aseptic technique, and refer to **Section 2.1.3 INSTRUCTION FOR USE OF LabCyte EPI-MODEL24**

3.1 PREPARATIONS

3.1.1 MTT SOLUTION PREPARATION

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

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

3.1.2 POSITIVE CONTROL SUBSTANCE

(1) Weigh 500mg of SLS precisely.

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

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

3.1.3 NEGATIVE CONTROL SUBSTANCE

(1) Use distilled water.

3.1.4 POLY WASH BOTTLE FOR PBS

(1) Sterilize poly wash bottle using autoclave preliminarily.

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

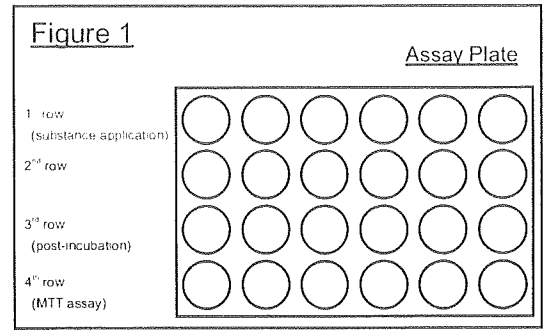
3.2 STUDY METHODS

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

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

(2) Fill 6 wells of the 1st row of each 24-well assay plate with the pre-warmed assay medium (0.5mL/well).
→ Figure 1

Figure 1

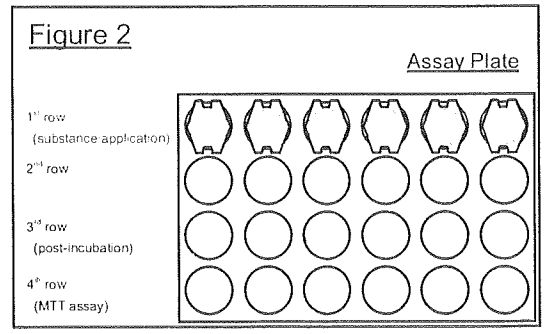


(3) Open the LabCyte EPI-MODEL24 aluminum package.

(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.
*Remove the agar medium stick to outside of the culture inserts using forceps.

(5) Transfer the culture inserts into assay medium filled wells of the 1st row using sterile forceps.
→ Figure 2
*Avoid air bubbles 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 SUBSTANCES AND RINSING."

(8) Record details in the MDS 2 regarding steps (1) ~ (7).

3.2.2 APPLICATION OF TEST SUBSTANCES AND RINSING (DAY 0)

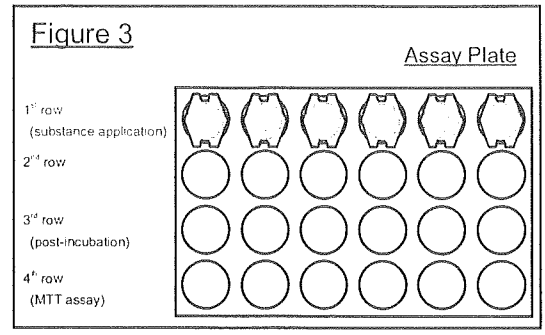
3.2.2.1 PREPARATION OF WELLS FOR POST-INCUBATION (3RD ROW)

(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 6 wells of the 3rd row with the pre-warmed assay medium (1.0mL/well) using a micropipette.
→ Figure 3
*Be precise on the amount of added medium since it affects the IL-1α measurement.

Figure 3



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

(5) Incubate until applying test substances (0~12 hours).

(6) Record details in the MDS 3 regarding steps (1) ~ (5).

3.2.2.2 APPLICATION OF TEST SUBSTANCES

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

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

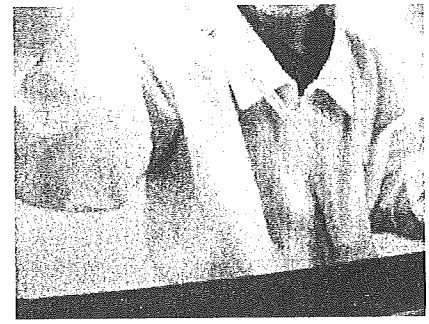
FOR LIQUIDs: Carefully apply 25µL of test substances onto the central part of each epidermis using a micropipette. After applied, close the lid of the assay plate and tap the side of the plate outside a safety cabinet (or clean bench) in order for a liquid to be spread out all over the epidermis surface. 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 1

Know the nature of test substances with a pipette etc. in advance.

Photo 1 - Pipette tips for viscous liquids



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

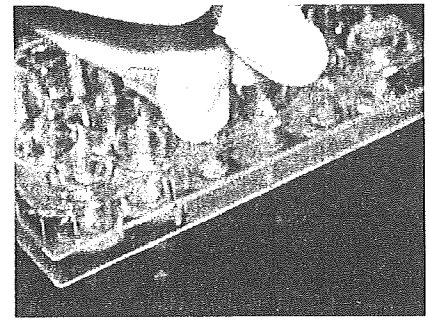
→ Photo 2

*One 24-well assay plate should be used to assay 2 kinds of test substances.

→ Figure 4

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

Photo 2 – Applying a solid substance

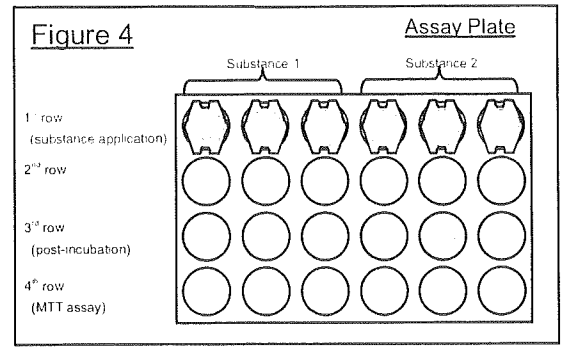


(3) Apply test substances onto each well at 1~3-minute(s) interval.

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

*Close the lid of assay plate at times other than applying samples. It might affect the amount of test samples if the lid is kept open due to air circulation in a safety cabinet (or clean bench).

Figure 4



(5) Record details in the MDS 3 regarding steps (1) ~ (4).

3.2.2.3 REMOVAL OF THE TEST SUBSTANCES

(1) After 15 minutes (±30 seconds) of applying a chemical, open the assay plate and pick up a culture insert with sterile forceps.

(2) Directly fill the culture insert with PBS using a PBS filled poly wash bottle.

→ Photo 3

*Be careful not to damage the epidermis surface.

(3) Discard the filled PBS into a beaker by tilting the insert. Remove the PBS inside the culture insert as much as possible by tapping it right above the beaker.

→ Photo 4

(4) Repeat steps (2) and (3) more than 10 times, and remove all residual test substance from the culture insert.

(5) Gently remove the left moisture of PBS inside and outside the culture insert with a sterile cotton bud.

→ Photo 5

(6) If test material is remained on the epidermis surface, gently remove it using a sterile cotton bud etc and repeat steps (2) ~ (5) again.

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

→ Figure 5

*Avoid air bubble formation under the culture inserts.

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

(9) Record details in the MDS 3 regarding steps (1) ~ (8).

Photo 3 - Rinse 1

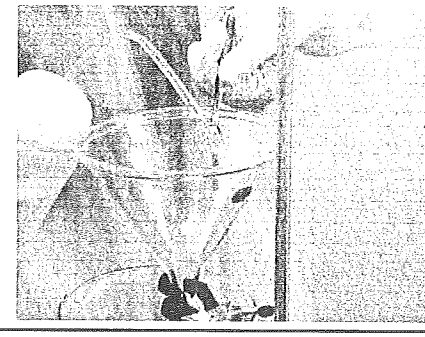


Photo 4 - Rinse 2

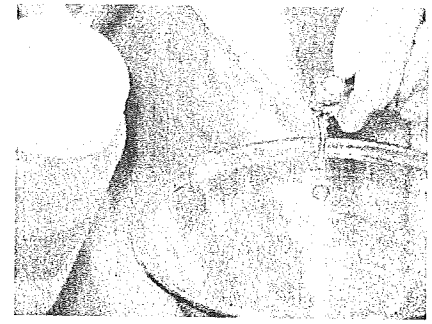


Photo 5 - Rinse 3

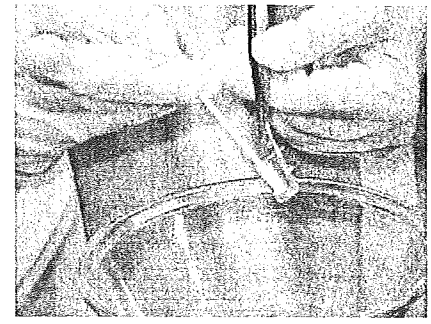
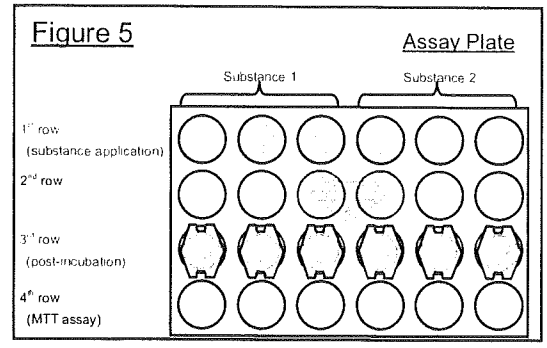


Figure 5



3.2.3 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.

3.2.4 MTT ASSAY AND MEDIA SAMPLING FOR MEDIATORS AND ENZYME RELEASE MEASUREMENT (DAY 2)

3.2.4.1 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.

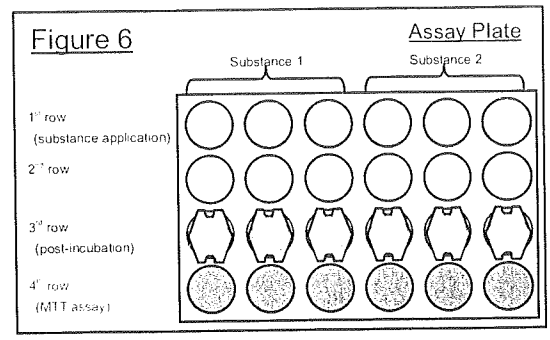
(3) Open the lid of the assay plate, and fill 6 wells of the 4th row with the pre-warmed MTT medium (0.5mL/well) using a micropipette.
→ Figure 6

(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 in the MDS 4 regarding steps (1) ~ (5)

Figure 6



3.2.4.2 MTT ASSAY

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

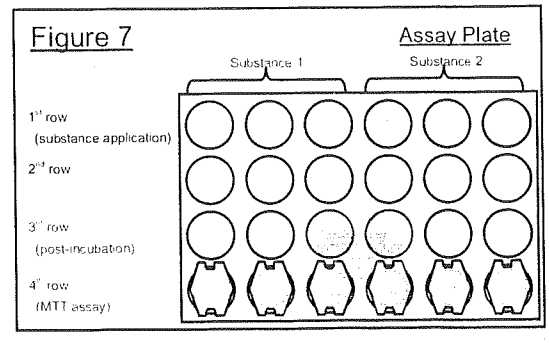
(2) Transfer each culture insert from the 3rd row to the 4th row of the corresponding column.
→ Figure 7
*Avoid the dripping from the base end surface of the culture insert into other wells.
*Avoid air bubbles formation under the culture inserts.

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

(4) Incubate for 3 hours.

(5) Record details in the MDS 4 regarding steps (1) ~(4).

Figure 7



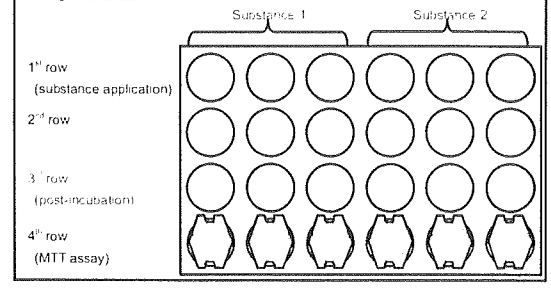
3.2.4.3 MEDIA SAMPLING

(1) After performing steps in **Section 3.4.3.2 MTT ASSAY**, transfer the entire medium of each well in the 3rd row into a 1.5mL micro tube.
→ Figure 8
*Shake the plates to help to homogenize the medium before transferring.

(2) Store micro tubes under -20°C until the measurement of IL-1α release.

(3) Record details in the MDS 4 regarding steps (1) and (2).

Figure 8 Assay Plate



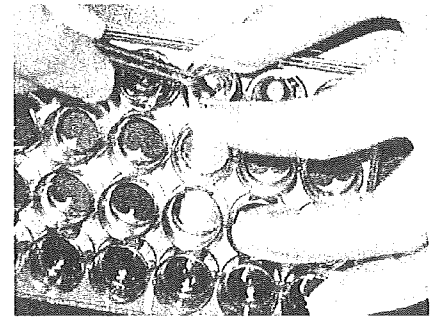
3.2.5 FORMAZAN EXTRACTION AND MEASUREMENT (DAY 2~3)

3.2.5.1 FORMAZAN EXTRACTION

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

(2) Open the lid of the assay plate and pinch the cultured epidermis from each culture insert of the 4th row using forceps.
→ Photo 6
*Use a micro spatula to scratch up the epidermis or a scalpel to cut the membrane filter on the base of a culture insert if cultured epidermis cannot be pinched due to the damage by a test substance.

Photo 6 - Detachment of epidermis



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

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

(5) Incubate them in the dark cold place (or refrigerator) for overnight (more than 15 hours) to extract pigments completely.
*Tighten a seal of the micro tube.
*Shaking micro tubes would help extranction efficiently.

(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 solution in each micro tube into each well of a 96-well plate.
*200µL of isopropanol should be set as blank.
*Figure 9 shows an example of allocation in a 96-well plate.

Figure 9 – Allocation of 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 in the MDS 5 regarding steps (1) ~ (7).

3.2.5.2 OPTICAL DENSITY MEASUREMENTS OF EXTRACTS

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

$$\text{Measured OD} = [570\text{nm OD}_{\text{sample}} - 570\text{nm OD}_{\text{blank}}] - [650\text{nm OD}_{\text{sample}} - 650\text{nm OD}_{\text{blank}}]$$

*Set the plate reader-calculated value as a measured OD if a 96-well plate reader performs automated calculations.

(2) Calculate the cell viability of a sample using the equation below.

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

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

3.2.6 MEASUREMENT OF IL-1α RELEASE

Measure IL-1α production in the distilled water (negative control) and supernatant liquids using the IL-1α ELISA kit.

3.2.6.1 IL-1α ELISA REAGENTS

3.2.6.1.1 IL-1α STANDARD SOLUTION (DILUTION SERIES)

(1) Reconstitute IL-1α standard to 10,000 pg/mL by diluting HuIL-1α Standard with Standard Diluent Buffer in a 1.5mL micro tube labeled as 10,000pg/mL. Follow the direction on the label of HuIL-1α Standard for the dilution ratio. After mixed thoroughly, incubate for 10 minutes to help dissolve.