	STANDARD OPERATING PROCEDURE	Version: 2.6 Pages: 35

EpiSensA

Epidermal Sensitization Assay

Kao Corporation

Revision history

Revision	Date	Comments
Version 1.1	27th Jun. 2018	Created by H. Mizumachi and M. Miyazawa
Version 2.1	1st Oct. 2018	-Positive control was changed from 6.25 w/v% BADGE to 0.78 w/v% clotrimazole and 0.10 w/v% 4-nitrobenzylbromide. -The time criteria to check whether the suspension is stable or not was added. -The reason why dose-range of main study can differ was clarified. -The note when RNA concentration is less than 100 ng/μL was added. -The acceptance criteria for viability of vehicle control was changed from 80% to 90%. -The acceptance criteria about GAPDH Ct value of test chemicals was added.
Version 2.2	4th Sep. 2019	-The example of 0.78 w/v% clotrimazole preparation was changed. -The storage condition of prepared PCR plate was added. - Example 96-well plate layout for LDH assay at Appendix 1 was corrected. - The criteria about RNA correction ratio was modified. - The prediction model about negative judgment was corrected. - The procedure about lysis of *epidermis* was described in more detail. - REQUIREMENT FOR QUALIFIED TESTING about vehicle control was changed. - The Note of Prediction Model describing about retesting was modified.

Version 2.3	12th May 2020	-The trade name and catalogue number of primers were changed. -Solubilization procedure was slightly revised. -LDH assay protocol was modified in accordance with the kit manual. -Note of topical application was revised. -cDNA synthesis method was slightly revised.
Version 2.4	23rd Sep. 2020	-Note about liquid chemical exposure was added to avoid cross-contamination. - Examples of plate layouts were changed. - Examples of fold induction calculation sheets were slightly revised.
Version 2.5	27th Jul. 2021	-Topical application procedure was modified to avoid cross-contamination. -Note about topical application was revised.
Version 2.6	22nd Aug. 2022	- The purity for clotrimazole was added. - The tissue size of LabCyte EPI-MODEL 24 was added.

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INDEX

Abbreviations and Definitions 4

Abstract 5

Description of Assay 5

Contact person 5

Materials and Preparations 6

Method 10

Data Analysis 23

Prediction Model 25

Abbreviations and Definitions

AOO: acetone: olive oil = 4: 1 v/v

AOP: adverse outcome pathway

ATF3: activating transcription factor 3

Ct: cycle threshold

CXCL8: chemokine (C-X-C motif) ligand 8

DNAJB4: dnaJ (Hsp40) homolog, subfamily B, member 4

DW: distilled water

EpiSensA: Epidermal Sensitization Assay

50% EtOH: 50 v/v% ethanol in DW

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GCLM: glutamate-cysteine ligase, modifier subunit

HSC: highest soluble concentration

IL-8: interleukin-8

I_{max}: maximal fold induction

J-TEC: Japan Tissue Engineering Co., Ltd.

K_{ow}: octanol-water partition coefficient

LDH: lactate dehydrogenase

4NBB: 4-Nitrobenzyl bromide

PCR: polymerase chain reaction

RhE: reconstructed human epidermis

Abstract

The EpiSensA is an *in vitro* method for measuring the expression of four marker genes (*ATF3*, encoding Activating transcription factor 3; *GCLM*, encoding Glutamate-cysteine ligase, modifier subunit; *DNAJB4*, encoding DnaJ (Hsp40) homolog, subfamily B, member4; *IL-8*, encoding Interleukin-8) of the reconstructed human *epidermis* (RhE) following controlled exposure of test chemicals (Saito et al., 2013, 2017). The two main important keratinocyte responses that occur in the skin sensitization adverse outcome pathway are Inflammatory response and Induction of cytoprotective gene pathways (OECD, 2012). This assay is based on the induction of multiple marker genes related to these two keratinocyte responses in the induction of skin sensitization. In addition, EpiSensA is applicable for not only hydrophilic chemicals but also lipophilic ones (e.g. $LogK_{ow}$>3.5) because lipophilic vehicle which is used in animal test can be used (Saito et al., 2017). Moreover, because RhE models exhibit metabolic capability similar to that of human skin (Tokudome et al., 2015), EpiSensA can correctly detect pre/pro-haptens. Therefore, information from the EpiSensA method is considered relevant for the assessment of the skin sensitisation potential of various chemicals.

Description of Assay

Biological Endpoint and Endpoint Measurement:

Modulation of the expression of four marker genes (*ATF3, GCLM, DNAJB4,* and *IL-8*) and one endogenous control gene (*GAPDH*, encoding the housekeeping protein Glyceraldehyde 3-phosphate dehydrogenase): quantified by real-time PCR analysis following 6 hour exposure of test chemicals on the surface of *epidermis*.

Endpoint Value:

Fold induction of four marker genes (*ATF3*, *GCLM*, *DNAJB4* and *IL-8*).

Experimental System:

The test system is RhE and the recommended model is LabCyte EPI-MODEL24 (diameter is 6.4 mm) purchased from Japan Tissue Engineering Co., Ltd. (J-TEC). LabCyte EPI-MODEL is reconstructed from normal human epidermal keratinocyte.

Materials and Preparations

CELL OR TEST SYSTEM

The test system is the reconstructed human *epidermis*, and the recommended model is LabCyte EPI-MODEL24 purchased from Japan Tissue Engineering Co., Ltd. (J-TEC), #401124. LabCyte EPI-MODEL24 is reconstructed from normal human epidermal keratinocytes. The kit #401124 contains 24 *epidermises*, an additional 24-well culture plate and ready-to-use assay medium.

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TEL: +81-533-66-2020 FAX: +81-533-66-2019
http://www.jpte.co.jp/index.html

FIXED EQUIPMENT

- Sterile hood for cell culture work
- CO_2 incubator (37°C, 5% CO_2, 95% humidity)
- Micro-pipettes and tips
- Multi-step pipette and combitips (e.g. Multipette M4, eppendorf)
- Positive displacement pipette and tips for volumes of 10 µL, preferred model is the Microman M10 (Gilson)
- Precision tweezers
- Vortex mixer
- 96-well plate absorbance reader equipped for reading at 490 (or 492) nm and more than or equal to 600 nm
- Microtube centrifuge
- Compact centrifuge (applicable to 1.5 mL tube and 0.2 mL PCR tube)
- Plate centrifuge (applicable to 96-well PCR plate)
- Spectral photometer for measurement of RNA concentration, preferred model is the NanoDrop (Thermo Scientific)
- Thermal cycler for cDNA synthesis
- Real-time PCR system, preferred model is the 7500 Fast Real-Time PCR System (Applied Biosystems)

- Other models that have been used successfully with this protocol:

  CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories)

  ABI PRISM 7900HT machine (Applied Biosystems)

  QuantStudio 3/5 Real-Time PCR System (Applied Biosystems)

FIXED REAGENTS, CHEMICALS, MATERIALS

	Product	Company	Catalog Number
Probe, Primer	TaqMan™ Gene Expression Assay Assay ID: ATF3: Hs00231069_m1 GCLM: Hs00157694_m1 DNAJB4: Hs00199826_m1 CXCL8 (IL-8): Hs00174103_m1 GAPDH: Hs99999905_m1	Applied Biosystems	XS: 4453320 S: 4331182 M: 4351370 L: 4351368
PCR master mix	TaqMan™ Universal PCR Master Mix	Applied Biosystems	4304437

REAGENTS, CHEMICALS, MATERIALS AND OTHER ITEMS

Below are listed the reagents, chemicals, materials and other items used for the routine testing. For most alternative products from other manufactures will work equally.

	Product	Company	Catalog Number
AOO	Acetone : Olive oil = 4 : 1 v/v Acetone, >99.5% Olive oil	 Kanto Chemical Co., Inc. Wako Pure Chemical Industries, Ltd.	 01026-00 150-00276
DW	Distilled water	Otsuka Pharmaceutical Co., Ltd.	
EtOH	Ethanol, >99.5%	Kanto Chemical Co., Inc.	14033-00
D-PBS	Dulbecco's phosphate buffered saline, no calcium, no magnesium	GIBCO	14190-144
LDH assay kit	Cytotoxicity Detection Kit (LDH)	Roche	11644793001
MTT	Thiazolyl Blue Tetrazolium Bromide, 98%	Sigma-Aldrich	M2128
Isopropanol	Isopropanol, >99.5%	Sigma-Aldrich	I9516

1 mol/L HCl	1 mol/L Hydrochloric acid (1 N)	Kanto Chemical Co., Inc.	18591-08
Lysis reagent	TRIzol reagent	Invitrogen	15596026
Chloroform	Chloroform, >99.0%	Tokyo Chemical Industry	C0819
Ultra-pure water	UltraPure™ DNase/RNase-Free Distilled Water	Invitrogen	10977-023
Homogenization kit	QIA shredder	QIAGEN	79654
2-Mercaptoethanol	2-Mercaptoethanol, >99.0%	SERVA Electrophoresis GmbH	28625.01
RNA extraction kit	RNeasy Mini Kit	QIAGEN	74104
cDNA synthesis kit	Superscript III First-Strand Synthesis System	Invitrogen	18080051
24-well plate	Multi-well culture plate, 24-well, flat bottom, non-treated	BD falcon	351147
96-well plate	Multi-well culture plate, 96-well, flat bottom, non-treated	BD falcon	351172
1.5 mL tube	DNA LoBind Tube 1.5 mL	Eppendorf	0030108051
PCR tube	PCR Tube Strips 0.2 mL	Eppendorf	0030124359
PCR plate	MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL	Applied Biosystems	4346907
Plate sealer	MicroAmp™ Optical Adhesive Film	Applied Biosystems	4311971
Positive control	Clotrimazole >98.0% CAS No. 23593-75-1 4-Nitrobenzyl bromide, >98.0% CAS No. 100-11-8	Sigma-Aldrich Tokyo Chemical Industry	C6019 N0181
Killed control	Triton X-100, average mol wt 625 CAS No. 9002-93-1	Sigma-Aldrich	T9284

PREPARATIONS

Assay Medium

Ready-to-use assay medium is included with the LabCyte EPI-MODEL24 kit (#401124). The medium should be stored at 4°C and used within one month. The assay medium should be pre-warmed to 37°C just before use.

Test Compound Solutions (vehicle selection and solubility check)

The solubility of each chemical is evaluated and confirmed visually. An appropriate vehicle will dissolve the test chemical completely, i.e. the solution should not be cloudy or have noticeable precipitate. The solubilization procedure listed below for selection of the appropriate vehicle should be followed.

Solubilization procedure

- First, try to dissolve the chemical in AOO at 50%. For example, 0.1 g of test chemical is measured, and 0.1mL of AOO is added. If the chemical is soluble or form a stable dispersion (i.e. a colloid or suspension in which the test chemical does not settle or separate from the vehicle into different phase in less than 10 minutes after preparation) in AOO at 50%, AOO is used as the vehicle.
- If not, the chemical should be dissolved in DW at 50% as described above. If the chemical is soluble or form a stable dispersion in DW at 50%, DW is used as the vehicle.
- If not, the chemical should be dissolved in 50% EtOH at 50% as described above. If the chemical is soluble or form a stable dispersion in 50% EtOH at 50%, 50% EtOH is used as the vehicle.
- If the chemical is not soluble or do not forms a stable dispersion at 50% in any vehicle, the highest soluble concentration should be determined by diluting the solution from 50% in a common ratio of two (e.g. 25% → 12.5% → 6.25% → continue if needed). The dilution is performed by adding the vehicle until the volume is doubled. The vehicle that dissolves the test chemical or forms a stable dispersion of the chemical at the highest concentration is used as the appropriate vehicle.
- The vehicle priority is AOO → DW → 50% EtOH.
- If the chemical is not soluble or do not forms a stable dispersion at 0.0122% in any vehicle, the chemical is judged as “not applicable” to EpiSensA.
- It should be verified whether the highest concentration determined can be prepared at weight per volume (e.g. 50% → 50 w/v%) in a volumetric flask.

Note:

- If the test chemical is not soluble, try sonicating or warm at 37°C for several minutes.
- Other vehicle may be used if sufficient scientific rationale can be provided.

Positive Control

Clotrimazole dissolved to a final concentration of 0.78 w/v% in AOO and 4-nitrobenzyl bromide (4NBB) dissolved to a final concentration of 0.10 w/v% is used as the positive control. For example, 0.0312 g of clotrimazole is measured in a 1 mL volumetric flask, and AOO is added for a total solution volume of 1 mL, and after that, dilute 4 times with AOO in a glass vial or a microtube. In addition, 0.0100 g of 4NBB is measured in a 1mL volumetric flask, and AOO is added for 1 mL, and after that, dilute 10 times with AOO in a glass vial or a microtube.

Negative Control

There is no negative control chemical tested in each run. The vehicle control is used as the control.

Killed Control

Triton X-100 dissolved to a final concentration of 10 w/v% in DW is used as the killed control. For example, 0.1 g of Triton X-100 is measured in a 1 mL volumetric flask, and DW is added for a total solution volume of 1 mL.

Method

TEST SYSTEM PROCUREMENT

For contact details on purchasing the kit, see the Materials and Preparations section. The quality of each EPI-MODEL24 batch is checked by the manufacturer. Results of the quality control assessments are supplied with the kits.

DAY 0: EXPERIMENTAL SYSTEM PROCUREMENT

- Receipt of LabCyte EPI-MODEL24:

After the EPI-MODEL24 kit 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.

- Preparation and pre-incubation for dose finding study:

This step should be performed under sterile conditions. Start pre-incubating all tissue units soon after opening the package on the day it is received. Do not store the tissue units in the nutritive agar after opening the aluminum package.

- Use a sterile 24-well plate (supplied) for each EPI-MODEL24.
- Pre-warm the supplied assay medium in a water bath at 37°C.
- Fill each well of the 24-well plate with 0.5 mL of the medium.
- Open the EPI-MODEL24 kit and transfer one tissue unit into each of the 24 medium-filled wells using sterile forceps.
- Be careful not to carry over any agar. If necessary, carefully remove the agar with forceps.
- Make sure there are no air bubbles under the tissue units. If necessary, tap the plate or remove the tissue units and place them in the wells again.
- Incubate the plate at 37°C (5% CO_2) overnight.

DAY 1: DOSE FINDING STUDY

- Preparation:

- Prepare the test chemical solution at the highest concentration in the appropriate vehicle using a volumetric flask (e.g. when preparing 1 mL of 12.5 w/v% solution, add the vehicle to 0.125 g of test chemical in a volumetric flask for a final volume of 1 mL). Transfer the solution from the flask to a glass vial or a microtube.
- Prepare working solutions of each test chemical as 4-fold serial dilutions from the highest concentration to concentrations of 0.024 w/v% or below in the appropriate vehicle (e.g. 6.25, 1.56, 0.39, 0.098, 0.024 w/v%).
- If the test chemical is a liquid, perform the serial dilution from 25 w/v% (100 (neat), 25, 6.25, 1.56, 0.39, 0.098, 0.024 w/v%).
- Prepare a 10 w/v% Triton X-100 solution in DW. For example, measure 0.1 g of Triton X-100 in a 1 mL volumetric flask, and add DW for a final solution volume of 1 mL.

- Topical applications (6-hour treatment):

This step should be performed under sterile conditions.

- Pre-warm the supplied assay medium in a water bath at 37°C.
- Make a plate layout as "Examples of plate layout". If a test chemical is liquid at 100% (neat) at room temperature, separate the tissue units which are used for the liquid test

chemical from other test chemicals and controls (e.g. positive controls and vehicle controls) into individual 24-well plates.

- Fill each well of a new 24-well plate with 0.5 mL of the medium, and transfer one pre-cultured tissue unit into each of the 24 medium-filled wells using sterile forceps.
- Apply an aliquot (5 μL) of working solution to the center of each *epidermis* surface (one tissue unit per each concentration) using a positive displacement pipette and tips.
- Prepare one tissue unit for the non-treated control and two tissue units for the killed control (treated with 10 μL of 10 w/v% Triton X-100) for the cell viability measurement.
- Incubate the treated *epidermis* for 6 hours at 37°C under 5% CO_2.

Caution;

- If liquid test chemicals (potentially volatile) or their solutions are applied on epidermises, the vapor of the chemicals may cross-contaminate other epidermises which are used for controls or other test chemicals. If epidermises for vehicle control are cross-contaminated, marker gene expressions may increase at vehicle control, and gene expressions of test chemicals and positive controls may be under-evaluated. On the other hand, if epidermises for test chemicals are cross-contaminated, gene expressions of the test chemicals may be over-evaluated.
- Cross-contamination of volatile chemicals can be avoided by separating the tissue units which are used for the volatile chemicals from others into individual 24-well plates.

- LDH assay:

In this protocol, an LDH assay method using Cytotoxicity Detection Kit (LDH) (Roche) is explained as an example.

- Collect 50 μL of the medium in a 96-well plate and perform a lactate dehydrogenase (LDH) assay using an LDH cytotoxicity detection kit according to the manufacturer's instructions. Briefly, prepare the following reaction mix (Solution A + Solution B) for the requisite amount, and add an equal volume (50 μL) of the reaction mix to the collected medium in a 96-well plate using a multi-step pipette and combitips. Incubate the plate for 30 minutes at room temperature with protecting from light, and stop the reaction by adding 25 μL/well of 1 mol/L HCl using a multi-step pipette and combitips.

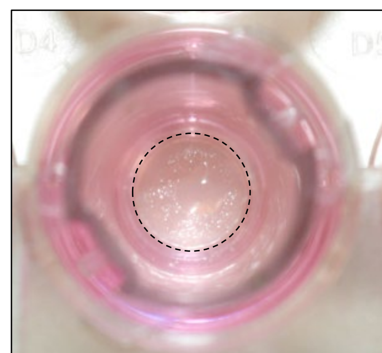
Component of reaction mix	For 1 sample	For 10 samples
Solution A (Catalyst)	1.25 µL	12.5 µL
Solution B (Dye solution)	56.25 µL	562.5 μL

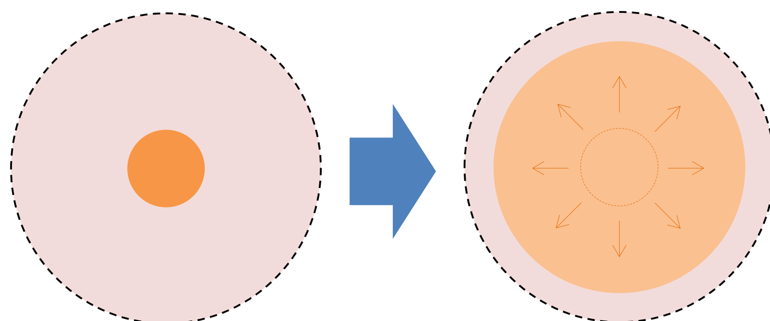
- Measure the absorbance at 490 or 492 nm and the reference wavelength (should be equal to or more than 600 nm) using a 96-well plate absorbance reader. The measurement should be performed immediately (at most within one hour) after adding HCl.
- Calculate Δabs. by subtracting the absorbance at reference wavelength from the absorbance at 490 or 492 nm.
- Calculate cell viability using the following equation.
- The lowest concentration showing less than 80% cell viability is used for the subsequent main study.

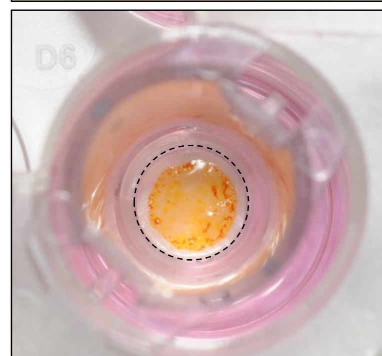
$$Cell\ viability\ (\%)$$
$$= 100 - \frac{\Delta abs.of\ test\ chemical\ treatment - \Delta abs.of\ non\text{-}treated\ control}{mean\ \Delta abs.of\ killed\ control - \Delta abs.of\ non\text{-}treated\ control} \times 100$$

Note:

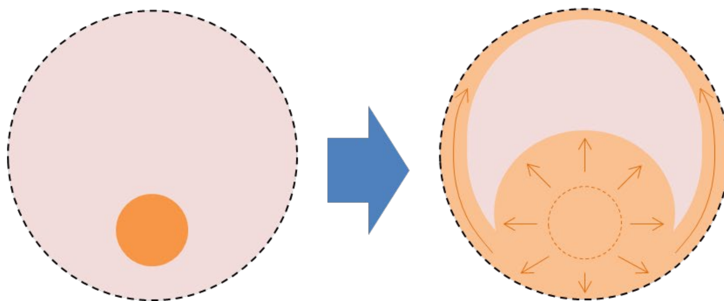
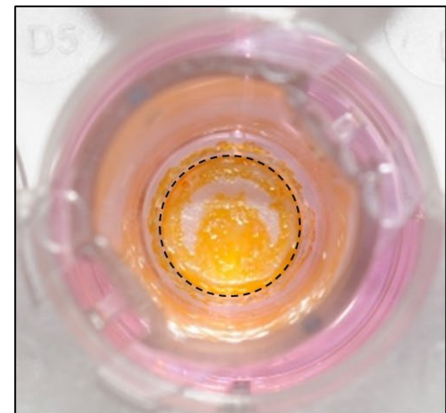
- Working solution should be put on the center of the *epidermis* surface. The solution spreads along the surface but does not usually reach the outer edge of the *epidermis* (AOO and 0.4% fluorescein isothiocyanate in AOO are applied at below photographs). In addition, even if the solution reaches the outer edge after spreading, this *epidermis* could be used as long as the solution is put on the center of the *epidermis* surface.



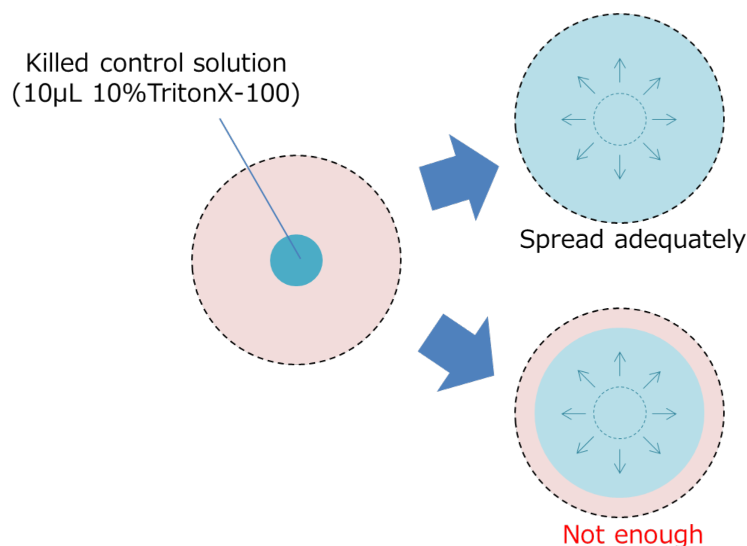




- In a rare case, when a liquid test chemical is applied at 100% (neat) or at a high concentration, the test chemical can immediately spread along the surface due to high affinity to *epidermis* surface and low viscosity. Nevertheless, this *epidermis* could be used as long as the solution is put on the center of the *epidermis* surface.
- If a working solution is not put on the center of the *epidermis* and immediately reaches the outer edge, the solution will run along the outer edge (0.4% fluorescein isothiocyanate in AOO is exposed at below photograph). In this case, the *epidermis* should be discarded and the solution should be applied again on fresh *epidermis*.

- Since acetone is volatile chemical, the concentration would immediately decrease after application of AOO solution. When the solution is put on the center of the *epidermis*, it takes several seconds to spread the surface. Therefore, the concentration of acetone after applying and spreading would decrease compared with fresh AOO (80v/v%). On the other hand, if AOO solution is not put on the center and immediately reaches the outer edge of *epidermis*, the concentration would be near to fresh AOO. In this case, the acetone could induce cell toxicity and non-specific gene expression because acetone can easily penetrate the *epidermis*.
- As an exception, the solution should reach the outer edge of the *epidermis* when applying the killed control (treated with 10 µL of 10 w/v% Triton X-100) because twice the volume of solution is used. If needed, it is possible to spread the solution by tips.



- When more than two EPI-MODEL24 plates are used simultaneously (i.e. same batch), it is only necessary to put the non-treated control and killed control in the 1st plate.
- Each test chemical solution and vehicle cannot be re-used and should be prepared fresh for each experiment.
- If the *epidermis* applied with a test chemical shows high cell viability (e.g. >90%) but is likely damaged compared with non-treated control, the test chemical may lead to the false estimate of viability by inhibiting the reaction of LDH.
- In order to identify a potential inhibition of LDH reaction, the RNA extraction may be used by following the collection and lysis of *epidermis* after the chemical exposure. When the mean cell viability at the tested concentration is high (e.g. >90%) but the mean total RNA concentration is less than 50% of vehicle control, the test chemical may lead to the false estimate of viability. See **DAY 8~: MAIN STUDY**.
- Instead of LDH assay, other cytotoxicity assay such as MTT assay may be used. See Appendix 1.

- Examples of plate layout

Example 24-well EPI-MODEL plate layout for chemical exposure:

Plate #1

1. Non-treated	2. Killed Ctrl.	3. Killed Ctrl.	13. Sample B 0.012 w/v%	14. Sample B 0.049 w/v%	15. Sample B 0.20 w/v%
4. AOO	5. DW	6. 50% EtOH	16. Sample B 0.78 w/v%	17. Sample B 3.13 w/v%	
7. Sample A 0.024 w/v%	8. Sample A 0.098 w/v%	9. Sample A 0.39 w/v%			
10. Sample A 1.56 w/v%	11. Sample A 6.25 w/v%	12. Sample A 25 w/v%			

Plate #2

18. Sample C 0.024 w/v%	19. Sample C 0.098 w/v%	20. Sample C 0.39 w/v%			
21. Sample C 1.56 w/v%	22. Sample C 6.25 w/v%	23. Sample C 25 w/v%			
24. Sample C 100% (Liquid)					

Plate #3

25. Sample D 0.024 w/v%	26. Sample D 0.098 w/v%	27. Sample D 0.39 w/v%			
28. Sample D 1.56 w/v%	29. Sample D 6.25 w/v%	30. Sample D 25 w/v%			
31. Sample D 100% (Liquid)					

Samples C and D should be separated from other test chemicals and controls because they are liquid chemicals.

Example 96-well plate layout for LDH assay:

1	2	3	13	14	15	25	26	27			
4	5	6	16	17	18	28	29	30			
7	8	9	19	20	21	31					
10	11	12	22	23	24						

Each number corresponds to the number shown in the upper figure (24-well plate format)

Note:

- It is possible to start the experiments for the main study by skipping the dose finding study. However, the results of tested concentrations that show a mean cell viability of less than 80% should not be considered. The results of at least one tested concentration that shows equal to or greater than 80% mean cell viability should be used to judge test chemicals as positive or negative.
- If the cytotoxicity of the test chemical is unclear, it is recommended that at least 9 working solutions at 2-fold serial dilutions from the highest soluble concentration are tested in one main study. If all of the mean cell viability at the tested concentrations are less than 80%, an additional study needs to be performed at 2-fold serial dilutions from the lowest concentration in the first main study.

DAY 7: EXPERIMENTAL SYSTEM PROCUREMENT

- Receipt of LabCyte EPI-MODEL24:

After the EPI-MODEL24 kit 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.

- Preparation and pre-incubation for main study:

This step should be performed under sterile conditions.

- Use a sterile 24-well plate for each EPI-MODEL24 plate received.
- Pre-warm the supplied assay medium in a water bath at 37°C.
- Fill each well of the 24-well plate with 0.5 mL of the medium.
- Open the EPI-MODEL24 kit and transfer one tissue unit into each of the 24 medium-filled wells using sterile forceps.
- Be careful not to carry over any agar. If necessary, carefully remove the agar with forceps.
- Make sure there are no air bubbles under the tissue units. If necessary, tap the plate or remove the tissue units and place them in the wells again.
- Incubate the plate at 37°C (5% CO_2) overnight.

DAY 8~: MAIN STUDY

- Preparation:

- Prepare the test chemical solution at the lowest concentration showing less than 80% cell viability in the dose finding study using a volumetric flask. Transfer the solution from the flask to a glass vial or a microtube.
- Prepare working solutions of each test chemical as 2-fold serial dilutions from the above concentration. It is recommended to dilute the working solution to the highest concentration showing greater than 90% cell viability in the dose finding study depending upon the slope of cell viability (basically from 3 (in steep slope) to 5 (in shallow slope) working solutions at 2-fold serial dilutions).
- When the cell viability is not less than 80% in the dose finding study, test the chemical in the main study by preparing at least 3 working solutions at 2-fold serial dilutions from the highest soluble concentration or 100% (neat).
- Prepare a 10 w/v% Triton X-100 solution in DW. For example, measure 0.1 g of Triton X-100 in a 1 mL volumetric flask, and add DW for a final solution volume of 1 mL.
- Prepare a 0.78 w/v% clotrimazole solution and 0.10 w/v% 4NBB solution in AOO. For example, measure 0.0312 g of clotrimazole in a 1 mL volumetric flask and add AOO for a total solution volume of 1 mL, and after that, dilute 4 times with AOO in a glass vial or a microtube. In addition, measure 0.0100 g of 4NBB in a 1mL volumetric flask, and add AOO for 1 mL, and after that, dilute 10 times with AOO in a glass vial or a microtube.

\- Topical applications (6-hour treatment):

This step should be performed under sterile conditions.

- Pre-warm the supplied assay medium in a water bath at 37°C.
- Make a plate layout as "Examples of plate layout". If a test chemical is liquid at 100% (neat) at room temperature, separate the tissue units which are used for the liquid test chemical from other test chemicals and controls (e.g. positive controls and vehicle controls) into individual 24-well plates.
- Fill each well of a new 24-well plate with 0.5 mL of the medium, and transfer one pre-cultured tissue unit into each of the 24 medium-filled wells using sterile forceps.
- Apply an aliquot (5 μL) of working solution to the center of each *epidermis* surface using a positive displacement pipette and tips. Three tissue units per each concentration should be used at each tested concentration.
- Prepare one tissue unit for the non-treated control and two tissue units for the killed control (treated with 10 μL of 10 w/v% Triton X-100) for the cell viability measurement.

- Prepare three tissue units for 5 μL of 0.78 w/v% clotrimazole, three tissue units for 5 μL of 0.10 w/v% 4NBB and three tissue units for the vehicle control (treated with 5 μL of AOO, DW and/or 50% EtOH if used).
- Incubate the treated *epidermis* for 6 hours at 37°C under 5% CO_2.

Caution;

- If liquid test chemicals (potentially volatile) or their solutions are applied on epidermises, the vapor of the chemicals may cross-contaminate other epidermises which are used for controls or other test chemicals. If epidermises for vehicle control are cross-contaminated, marker gene expressions may increase at vehicle control, and gene expressions of test chemicals and positive controls may be under-evaluated. On the other hand, if epidermises for test chemicals are cross-contaminated, gene expressions of the test chemicals may be over-evaluated.

Cross-contamination of volatile chemicals can be avoided by separating the tissue units which are used for the volatile chemicals from others into individual 24-well plates.

- LDH assay:

- After exposure, collect 50 μL of the medium in a 96-well plate and perform the LDH assay described on page 11. Calculate the cell viability.

Note:

- The solution should preferably avoid reaching the outer edge of the *epidermis* because the test chemical or vehicle can easily penetrate the *epidermis* here, which can lead to overestimation of cell toxicity or gene expression. Therefore, when the solution reaches the outer edge of the *epidermis*, it is recommended that the result from the *epidermis* is not considered.
- When more than two EPI-MODEL24 plates are used simultaneously (i.e. same batch), it is only necessary to put the non-treated control, killed control, positive control, and vehicle control in the 1st plate.
- Each test chemical solution and vehicle cannot be re-used and should be prepared fresh for each experiment.
- To avoid cross-contamination by volatile compounds, the tissue units which are used for liquid test chemicals should be separated from other test chemicals and controls into individual 24-well plates.

- Examples of plate layout

Example 24-well EPI-MODEL plate layout for chemical exposure:

Plate #1

1. Non-treated	2. Killed Ctrl.	3. Killed Ctrl.	13. DW	14. DW	15. DW
4. AOO	5. AOO	6. AOO	16. 50% EtOH	17. 50% EtOH	18. 50% EtOH
7. Clotrimazole 0.78 w/v%	8. Clotrimazole 0.78 w/v%	9. Clotrimazole 0.78 w/v%	19. Sample A 0.78 w/v% (DW)	20. Sample A 0.78 w/v% (DW)	21. Sample A 0.78 w/v% (DW)
10. 4NBB 0.10 w/v%	11. 4NBB 0.10 w/v%	12. 4NBB 0.10 w/v%	22. Sample A 1.56 w/v% (DW)	23. Sample A 1.56 w/v% (DW)	24. Sample A 1.56 w/v% (DW)

Plate #2

25. Sample A 3.13 w/v% (DW)	26. Sample A 3.13 w/v% (DW)	27. Sample A 3.13 w/v% (DW)	37. Sample B 3.13 w/v% (AOO)	38. Sample B 3.13 w/v% (AOO)	39. Sample B 3.13 w/v% (AOO)
28. Sample A 6.25 w/v% (DW)	29. Sample A 6.25 w/v% (DW)	30. Sample A 6.25 w/v% (DW)			
31. Sample B 0.78 w/v% (AOO)	32. Sample B 0.78 w/v% (AOO)	33. Sample B 0.78 w/v% (AOO)			
34. Sample B 1.56 w/v% (AOO)	35. Sample B 1.56 w/v% (AOO)	36. Sample B 1.56 w/v% (AOO)			

Plate #3

40. Sample C 25 w/v% (50%EtOH)	41. Sample C 25 w/v% (50%EtOH)	42. Sample C 25 w/v% (50%EtOH)			
43. Sample C 50 w/v% (50%EtOH)	44. Sample C 50 w/v% (50%EtOH)	45. Sample C 50 w/v% (50%EtOH)			
46. Sample C 100% (neat)	47. Sample C 100% (neat)	48. Sample C 100% (neat)			

Sample C should be separated from other test chemicals and controls because it is liquid chemical.

Example 96-well plate layout for LDH assay:

1	2	3	13	14	15	25	26	27	37	38	39
4	5	6	16	17	18	28	29	30	40	41	42
7	8	9	19	20	21	31	32	33	43	44	45
10	11	12	22	23	24	34	35	36	46	47	48

Each number corresponds to the number shown in the upper figure (24-well plate format)

- Collection and lysis of *epidermis*:

In this protocol, two *epidermis* lysis methods are explained as examples.

(i) Using TRIzol reagent (Invitrogen) and vortex mixer

- Add 500 μL of TRIzol reagents to 1.5-mL microtubes and store on ice.
- After the test chemical exposure, transfer the treated tissue units to another 24-well plate containing 0.5 mL/well D-PBS using tweezers. Discard the two tissue units for the killed control.
- Fill a multi-step pipette and combitips (adjusted for a 0.5 mL distribution) with D-PBS. Add 0.5 mL of D-PBS to the tissue units and remove the residual test chemical and D-PBS by turning the tissue unit upside down. Repeat this washing three times.
- Pick up the *epidermis* gently from the insert cup using tweezers. Wipe off any remaining D-PBS gently with absorbent paper and put the *epidermis* into the 1.5-mL microtube containing TRIzol reagents.
- Homogenize by vortex mixing for at least 90 seconds (preferably crush to pieces from visual appearance).
- These homogenized samples can be stored at -80°C.
- Add chloroform (100 μL) to the homogenized samples, and shake the samples vigorously and centrifuge at 12,000 × g for 15 minutes at 4°C.
- Transfer the supernatant (about 250 μL) to another 1.5-mL microtube, and add the same volume of 70% ethanol. Mix well. Do not centrifuge because precipitation of RNA may be formed by adding ethanol and centrifuging may cause low RNA yield.

(ii) Using shredder column (QIAGEN) and centrifuge

- Add 350 μL of RLT lysis buffer (supplied with the RNeasy Mini Kit) with 1% 2-mercaptoethanol and store on ice.

- After the test chemical exposure, transfer the treated tissue units to another 24-well plate containing 0.5 mL/well D-PBS using tweezers. Discard the two tissue units for the killed control.
- Fill a multi-step pipette and combitips (adjusted for a 0.5 mL distribution) with D-PBS. Add 0.5 mL of D-PBS to the tissue units and remove the residual test chemical and D-PBS by turning the tissue unit upside down. Repeat this washing three times.
- Pick up the *epidermis* gently from the insert cup using tweezers. Wipe off any remaining D-PBS gently with absorbent paper and put the *epidermis* into a 1.5-mL microtube containing RLT lysis buffer with 1% 2-mercaptoethanol. Perform vortex mixing for at least 2 minutes.
- These lysis samples can be stored at -80°C.
- Add the lysis sample to a shredder column and centrifuge at 12,000 × g for 5 minutes at room temperature.
- Transfer the supernatant (about 300 μL) to another 1.5-mL microtube, and add the same volume of 70% ethanol. Mix well. <u>Do not centrifuge because precipitation of RNA may be formed by adding ethanol and centrifuging may cause low RNA yield.</u>

\- <u>RNA extraction:</u>

In this protocol, an RNA extraction method using an RNeasy Mini Kit (QIAGEN) is explained as an example. Perform all centrifugation steps at 20–25°C and ensure that the centrifuge does not cool below 20°C because RNA collection efficiency may be decreased at cool temperature.

- Transfer the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 mL collection tube (supplied). Centrifuge for 1 minute at ≥8000 x g. Discard the flow-through.
- Add 700 µL Buffer RW1 (supplied) to the RNeasy spin column. Centrifuge for 1 minute at ≥8000 x g to wash the spin column membrane. Discard the flow-through.
- Add 500 µL Buffer RPE (supplied) to the RNeasy spin column. Centrifuge for 1 minute at ≥8000 x g to wash the spin column membrane. Discard the flow-through.
- Add 500 µL Buffer RPE to the RNeasy spin column and centrifuge for 2 minutes at ≥8000 x g to wash the spin column membrane.
- Place the RNeasy spin column in a new 2 mL collection tube, and discard the old collection tube with the flow-through. Centrifuge at ≥8000 x g for 1 minute to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column.

- Place the RNeasy spin column in a new 1.5 mL collection tube (supplied). Add 30 µL RNase-free water (supplied) directly to the center of the spin column membrane. Centrifuge for 1 minute at ≥8000 x g to elute the RNA.
- Measure the total RNA concentration using a spectral photometer (e.g. NanoDrop).
- The eluted RNA samples can be stored at -80°C.

Note:

- When the mean cell viability at the tested concentration is high (e.g. >90%) but the mean total RNA concentration is less than 50% of vehicle control, the test chemical may lead to the false estimate of viability by inhibiting the reaction of LDH.
- Instead of LDH assay, other cytotoxicity assay such as MTT assay may be used. See **Appendix 1**.

- cDNA synthesis:

In this protocol, a cDNA synthesis method using the Superscript III First-Strand Synthesis System (Invitrogen) is explained as an example. Thaw all components on ice and briefly centrifuge before use.

- Adjust the total RNA concentration to 100 ng/µL with DEPC-treated water (supplied) in a 0.2 mL PCR tube.
- Transfer 5 µL of 100 ng/µL RNA samples to another 0.2 mL PCR tube.
- Prepare the following primer mix for the requisite amount in a 1.5 mL tube.

Component of primer mix	For 1 sample	For 10 samples
10 mM dNTP	1 µL	10 µL
50 µM oligo$(dT)_{20}$	0.4 µL	4 µL
DEPC-treated water	3.6 µL	36 µL

- Add 5 µL of primer mix to each RNA sample, mix gently, and collect by brief centrifugation.
- Incubate the tube at 65°C for 5 minutes, and then keep on ice for at least 1 minute.
- Prepare the following cDNA synthesis mix for the requisite amount in a 1.5 mL tube, by adding each component in the indicated order.

Component of cDNA synthesis mix	For 1 sample	For 10 samples
10X RT buffer	2 µL	20 µL
25 mM $MgCl_2$	4 µL	40 µL
0.1 M DTT	2 µL	20 µL
RNaseOUT (40U/µL)	1 µL	10 µL
SuperScript III RT (200 U/µL)	1 µL	10 µL

- Add 10 μL of cDNA synthesis mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation.
- Incubate the tube at 50°C for 50 minutes to synthesize cDNA. Terminate the reactions at 85°C for 5 minutes, and then keep on ice.
- Collect the reactions by brief centrifugation. Add 1 μL of RNase H to each tube and incubate the tubes for 20 minutes at 37°C, and then keep on ice.
- These cDNA samples can be stored at -20°C.

Note:

- If RNA concentration is less than 100 ng/μL, transfer the RNA solution with 500 ng RNA into 0.2 mL PCR tube, and add 1 μL of dNTP and 0.4 μL of oligo $(dT)_{20}$, and after that, add DEPC-treated water up to 10 μL.

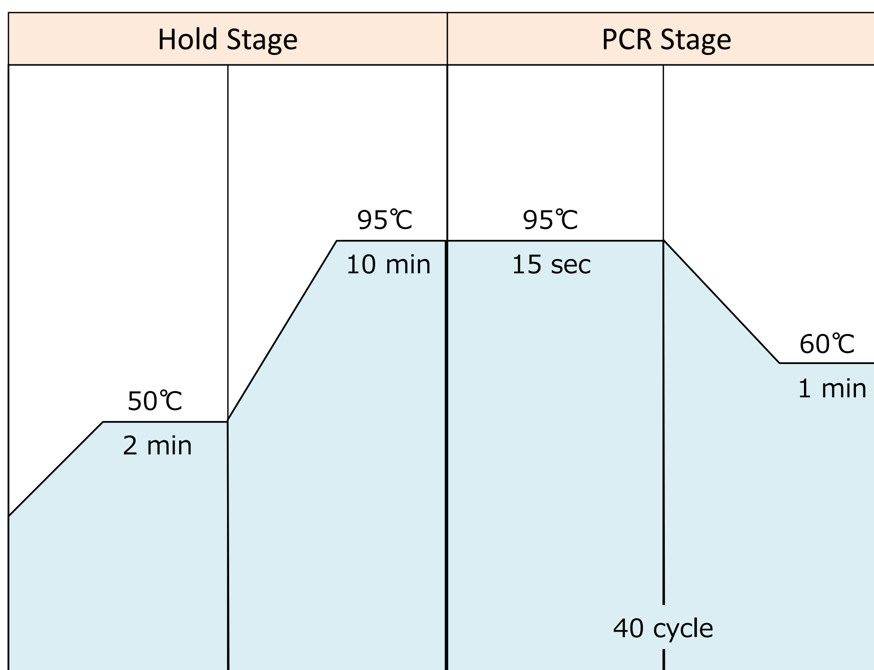
- Real-time PCR:

Quantitative RT-PCR is performed using the TaqMan Gene Expression Assay and TaqMan Universal PCR Master Mix.

- Dilute cDNA samples 4.5 times with ultra-pure water in a 0.2 mL PCR tube (e.g. 15 μL of cDNA + 52.5 μL of ultrapure water).
- Prepare the following real-time PCR mix for the requisite amount in a 1.5 mL tube for the respective 5 genes.

Component of real-time PCR mix	For 1 sample	For 10 samples
TaqMan Universal PCR Master Mix	10 μL	100 μL
TaqMan Gene Expression Assay	1 μL	10 μL

- Transfer 9 μL of diluted cDNA to individual wells of a 96-well PCR reaction plate. Transfer 9 μL of ultrapure water to another well for non-template control.
- Add 11 μL of real-time PCR mix to each diluted cDNA in the PCR reaction plate. Seal the plate and collect by brief centrifugation (e.g. 250 × g for 1 minute).
- Measure the cycle threshold (Ct) values of four skin sensitization marker genes (*ATF3*, *GCLM*, *DNAJB4*, and *IL-8*) and one endogenous control gene (*GAPDH*) using the 7500 Fast Real-Time PCR System. The PCR program is shown below. The calculation of baseline and threshold should be performed by automatic baseline and threshold determination software.



Note:

- Do not separate the cDNA samples for measurement of one gene into different real-time PCR plates. All cDNA samples should be measured on the same plate for each gene.
- All the PCR plates of one experiment should be prepared at the same time in order to reduce the error deriving from micropipette dispensing.
- Prepared PCR plate should be measured within 24 hours if stored on ice with protecting from light.

<u>- Examples of 96-well real-time PCR plate layout</u>

Plate #1

1 *ATF3*	4 *ATF3*	5 *ATF3*	6 *ATF3*	7 *ATF3*	8 *ATF3*	9 *ATF3*	10 *ATF3*	11 *ATF3*	12 *ATF3*	13 *ATF3*	14 *ATF3*
15 *ATF3*	16 *ATF3*	17 *ATF3*	18 *ATF3*	19 *ATF3*	20 *ATF3*	21 *ATF3*	22 *ATF3*	23 *ATF3*	24 *ATF3*	25 *ATF3*	26 *ATF3*
27 *ATF3*	28 *ATF3*	29 *ATF3*	30 *ATF3*	31 *ATF3*	32 *ATF3*	33 *ATF3*	34 *ATF3*	35 *ATF3*	36 *ATF3*	37 *ATF3*	38 *ATF3*
39 *ATF3*	40 *ATF3*	41 *ATF3*	42 *ATF3*	43 *ATF3*	44 *ATF3*	45 *ATF3*	46 *ATF3*	47 *ATF3*	48 *ATF3*	NTC *ATF3*	
1 *GCLM*	4 *GCLM*	5 *GCLM*	6 *GCLM*	7 *GCLM*	8 *GCLM*	9 *GCLM*	10 *GCLM*	11 *GCLM*	12 *GCLM*	13 *GCLM*	14 *GCLM*
15 *GCLM*	16 *GCLM*	17 *GCLM*	18 *GCLM*	19 *GCLM*	20 *GCLM*	21 *GCLM*	22 *GCLM*	23 *GCLM*	24 *GCLM*	25 *GCLM*	26 *GCLM*
27 *GCLM*	28 *GCLM*	29 *GCLM*	30 *GCLM*	31 *GCLM*	32 *GCLM*	33 *GCLM*	34 *GCLM*	35 *GCLM*	36 *GCLM*	37 *GCLM*	38 *GCLM*
39 *GCLM*	40 *GCLM*	41 *GCLM*	42 *GCLM*	43 *GCLM*	44 *GCLM*	45 *GCLM*	46 *GCLM*	47 *GCLM*	48 *GCLM*	NTC *GCLM*	

Plate #2

1 *DNAJB4*	4 *DNAJB4*	5 *DNAJB4*	6 *DNAJB4*	7 *DNAJB4*	8 *DNAJB4*	9 *DNAJB4*	10 *DNAJB4*	11 *DNAJB4*	12 *DNAJB4*	13 *DNAJB4*	14 *DNAJB4*
15 *DNAJB4*	16 *DNAJB4*	17 *DNAJB4*	18 *DNAJB4*	19 *DNAJB4*	20 *DNAJB4*	21 *DNAJB4*	22 *DNAJB4*	23 *DNAJB4*	24 *DNAJB4*	25 *DNAJB4*	26 *DNAJB4*
27 *DNAJB4*	28 *DNAJB4*	29 *DNAJB4*	30 *DNAJB4*	31 *DNAJB4*	32 *DNAJB4*	33 *DNAJB4*	34 *DNAJB4*	35 *DNAJB4*	36 *DNAJB4*	37 *DNAJB4*	38 *DNAJB4*
39 *DNAJB4*	40 *DNAJB4*	41 *DNAJB4*	42 *DNAJB4*	43 *DNAJB4*	44 *DNAJB4*	45 *DNAJB4*	46 *DNAJB4*	47 *DNAJB4*	48 *DNAJB4*	NTC *DNAJB4*	
1 *IL-8*	4 *IL-8*	5 *IL-8*	6 *IL-8*	7 *IL-8*	8 *IL-8*	9 *IL-8*	10 *IL-8*	11 *IL-8*	12 *IL-8*	13 *IL-8*	14 *IL-8*
15 *IL-8*	16 *IL-8*	17 *IL-8*	18 *IL-8*	19 *IL-8*	20 *IL-8*	21 *IL-8*	22 *IL-8*	23 *IL-8*	24 *IL-8*	25 *IL-8*	26 *IL-8*
27 *IL-8*	28 *IL-8*	29 *IL-8*	30 *IL-8*	31 *IL-8*	32 *IL-8*	33 *IL-8*	34 *IL-8*	35 *IL-8*	36 *IL-8*	37 *IL-8*	38 *IL-8*
39 *IL-8*	40 *IL-8*	41 *IL-8*	42 *IL-8*	43 *IL-8*	44 *IL-8*	45 *IL-8*	46 *IL-8*	47 *IL-8*	48 *IL-8*	NTC *IL-8*	

Plate #3

1 *GAPDH*	4 *GAPDH*	5 *GAPDH*	6 *GAPDH*	7 *GAPDH*	8 *GAPDH*	9 *GAPDH*	10 *GAPDH*	11 *GAPDH*	12 *GAPDH*	13 *GAPDH*	14 *GAPDH*
15 *GAPDH*	16 *GAPDH*	17 *GAPDH*	18 *GAPDH*	19 *GAPDH*	20 *GAPDH*	21 *GAPDH*	22 *GAPDH*	23 *GAPDH*	24 *GAPDH*	25 *GAPDH*	26 *GAPDH*
27 *GAPDH*	28 *GAPDH*	29 *GAPDH*	30 *GAPDH*	31 *GAPDH*	32 *GAPDH*	33 *GAPDH*	34 *GAPDH*	35 *GAPDH*	36 *GAPDH*	37 *GAPDH*	38 *GAPDH*
39 *GAPDH*	40 *GAPDH*	41 *GAPDH*	42 *GAPDH*	43 *GAPDH*	44 *GAPDH*	45 *GAPDH*	46 *GAPDH*	47 *GAPDH*	48 *GAPDH*	NTC *GAPDH*	

Each number corresponds to the number shown in the example 24-well EPI-MODEL plate layout of the main study

Data Analysis

CALCULATION OF FOLD INDUCTION

Relative gene expression levels versus control (fold induction) are calculated using the $2^{-\Delta\Delta Ct}$ method described below.

- Calculate the ΔCt value by subtracting the Ct value of *GAPDH* from the Ct value of the respective marker genes.
- Calculate the mean ΔCt value of the vehicle control.

- Calculate the ΔΔCt value by subtracting the mean ΔCt value of the vehicle control from the ΔCt value of the test chemical or positive control.
- Calculate the fold induction by $2^{-\Delta\Delta Ct}$.

- Examples of the fold induction calculation focusing on one marker gene (*ATF3*)

Sample	Conc. (w/v%)	Ct value		ΔCt value	ΔΔCt value	Fold induction
		ATF3	*GAPDH*	*ATF3*	*ATF3*	*ATF3*
AOO **(vevicle of clotrimazole)**		31.24	18.87	12.37		
		30.96	18.92	12.03		
		31.33	19.18	12.15		
		Mean ΔCt value of vehicle control ⇒		**12.18**		
Clotrimazole	0.78	25.59	19.59	6.00	-6.19	**72.8**
		24.50	19.22	5.27	-6.91	**120.1**
		25.21	19.21	5.99	-6.19	**73.0**

- Examples of a fold induction calculation sheet (all marker genes)

Sample	Conc. (w/v%)	Ct value					ΔCt value				ΔΔCt value				Fold induction			
		ATF3	*GCLM*	*DNAJB4*	*IL8*	*GAPDH*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*
AOO		31.24	25.79	26.71	27.58	18.87	12.37	6.92	7.83	8.70								
		30.96	25.97	26.69	27.73	18.92	12.03	7.04	7.77	8.81								
		31.33	26.23	26.97	28.12	19.18	12.15	7.05	7.80	8.95								
		Mean ΔCt value of vehicle control ⇒					**12.18**	**7.00**	**7.80**	**8.82**								
Clotrimazole	0.78	25.59	26.12	26.14	21.83	19.59	6.00	6.53	6.54	2.24	-6.19	-0.47	-1.26	-6.58	**72.8**	1.4	**2.4**	**95.8**
		24.50	26.29	24.31	22.15	19.22	5.27	7.07	5.08	2.92	-6.91	0.06	-2.72	-5.90	**120.1**	1.0	**6.6**	**59.6**
		25.21	26.23	25.50	23.54	19.21	5.99	7.02	6.29	4.33	-6.19	0.01	-1.51	-4.49	**73.0**	1.0	**2.9**	**22.5**
4NBB	0.10	24.90	23.80	24.32	24.82	18.93	5.97	4.87	5.39	5.89	-6.22	-2.14	-2.41	-2.93	**74.4**	**4.4**	**5.3**	**7.6**
		23.72	24.78	24.93	24.14	19.24	4.48	5.54	5.69	4.90	-7.70	-1.46	-2.11	-3.92	**208.2**	**2.8**	**4.3**	**15.1**
		25.60	24.42	25.36	24.45	19.35	6.25	5.06	6.01	5.09	-5.94	-1.94	-1.79	-3.73	**61.2**	**3.8**	**3.5**	**13.2**
Vehicle of sample A		31.92	27.11	27.02	27.22	18.05	13.88	9.07	8.98	9.17								
		32.06	26.86	27.22	27.43	17.96	14.10	8.90	9.26	9.47								
		32.43	27.26	27.36	27.51	18.08	14.34	9.17	9.28	9.42								
		Mean ΔCt value of vehicle control ⇒					**14.11**	**9.05**	**9.17**	**9.36**								
Sample A	0.78	32.61	27.30	27.55	28.19	18.59	14.02	8.71	8.96	9.59	-0.09	-0.33	-0.21	0.24	1.1	1.3	1.2	0.8
		31.49	27.05	27.22	27.16	18.05	13.43	9.00	9.17	9.10	-0.67	-0.05	0.00	-0.25	1.6	1.0	1.0	1.2
		31.01	26.91	27.07	26.37	17.97	13.04	8.93	9.10	8.40	-1.07	-0.11	-0.07	-0.96	2.1	1.1	1.0	1.9
	1.56	31.75	26.83	27.14	27.28	18.14	13.61	8.69	9.00	9.15	-0.50	-0.36	-0.17	-0.21	1.4	1.3	1.1	1.2
		32.24	26.83	27.12	27.10	18.18	14.06	8.64	8.93	8.91	-0.05	-0.40	-0.24	-0.44	1.0	1.3	1.2	1.4
		31.67	26.40	26.88	26.77	17.96	13.71	8.44	8.92	8.81	-0.40	-0.60	-0.25	-0.54	1.3	1.5	1.2	1.5
	3.13	27.27	27.40	26.96	26.07	18.23	9.04	9.16	8.73	7.83	-5.06	0.12	-0.44	-1.52	**33.4**	0.9	1.4	2.9
		28.04	27.42	27.01	26.41	18.15	9.89	9.27	8.86	8.26	-4.21	0.22	-0.31	-1.10	**18.5**	0.9	1.2	2.1
		28.74	27.57	27.02	26.87	18.48	10.26	9.09	8.54	8.39	-3.84	0.04	-0.63	-0.97	14.3	1.0	1.5	2.0
	6.25	25.94	24.88	23.38	23.16	18.49	7.45	6.39	4.89	4.67	-6.66	-2.66	-4.28	-4.69	**100.9**	**6.3**	**19.5**	**25.8**
		25.53	24.91	23.04	22.03	18.51	7.02	6.40	4.53	3.52	-7.08	-2.64	-4.64	-5.84	**135.6**	**6.2**	**25.0**	**57.2**
		25.33	24.98	22.78	22.28	18.70	6.64	6.28	4.08	3.58	-7.47	-2.76	-5.09	-5.77	**177.3**	**6.8**	**34.0**	**54.8**
Sample	Conc. (w/v%)	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*GAPDH*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*	*ATF3*	*GCLM*	*DNAJB4*	*IL8*

of *epidermis* corresponds to the number shown in the example 24-well *epidermis* plate layout of the main study.

Red highlight indicates the fold induction that exceeds the respective cut-off value for the marker genes.

REQUIREMENT FOR QUALIFIED TESTING

- Each cell viability of at least two *epidermises* of vehicle control should be equal to or greater than 95%. (If the viability of only one *epidermis* is less than 95%, Ct values from the remaining two *epidermises* should be used.)
- Mean cell viability of both positive controls should be equal to or greater than 80%.
- In the positive control of 0.78 w/v% clotrimazole, the mean values of fold induction for *ATF3* and *IL-8* should exceed the cut-off value.

- In the positive control of 0.10 w/v% 4NBB, the mean values of fold induction for *GCLM* and *DNAJB4* should exceed the cut-off value.

If these requirements are not met, the run should be discarded.

REQUIREMENT FOR DATA ACCEPTANCE

- The results of tested concentrations that show a mean cell viability of less than 80% should not be considered.
- The results of at least one tested concentration that shows equal to or greater than 80% mean cell viability should be used to judge test chemicals as positive or negative.
- When mean GAPDH Ct value of each tested concentration (n=3) is within mean GAPDH Ct value of corresponding vehicle control (n=3) +/- 1, the result at the concentration can be acceptable.

Prediction Model

- Each chemical is tested in one run, and three tissue units per tested concentration should be used.
- The mean value of maximum fold induction (I_{max}) is obtained using the data from the concentrations that show equal to or greater than 80% mean cell viability.
- When the mean I_{max} of at least one out of the four marker genes exceeds the respective cut-off value (*ATF3*, 15-fold; *GCLM*, 2-fold; *DNAJB4*, 2-fold; and *IL-8*, 4-fold), the chemical is judged as positive in EpiSensA.
- When the mean I_{max} of any marker genes does not exceed the respective cut-off value, and if at least one mean cell viability at the tested concentrations is less than 80%, the chemical is judged as negative in EpiSensA.
- When the mean I_{max} of any marker genes does not exceed the respective cut-off value, and if all mean cell viability at the tested concentrations are greater than 80%, the chemical should not be judged as negative. An additional study should be performed at 2-fold serial dilutions from the higher concentration than the highest concentration in the first main study. However, if the test chemical does not indicate less than 80% mean cell viability at the highest soluble concentration (solid) or 100% (liquid), the chemical is judged as negative.

Note:

- In rare cases, there are some test chemicals that show steeply or greatly fluctuating dose response curves for cell viability and/or fold induction, and the fold induction exceeds the cut-off value just at the lowest concentration with less than 80% mean cell viability. Such test chemicals should be retested with a narrower dose-response analysis using a lower dilution factor (e.g. $\sqrt{2}$ (=1.41) fold dilution), to determine whether induction has occurred at cytotoxic levels (80 to 95% mean cell viability).
- If the GAPDH Ct value does not meet the acceptance criteria (see Page 25) at the highest concentration with equal to or greater than 80% mean cell viability and the fold induction does not exceed respective cut-off values at the lower concentrations, the test chemical should be retested.

Appendix 1: MTT assay

DAY 1: DOSE FINDING STUDY (MTT assay)

- Preparation:

- Prepare the test chemical solution at the highest concentration in the appropriate vehicle using a volumetric flask (e.g. when preparing 1 mL of 12.5 w/v% solution, add the vehicle to 0.125 g of test chemical in a volumetric flask for a final volume of 1 mL). Transfer the solution from the flask to a glass vial or a microtube.
- Prepare working solutions of each test chemical as 4-fold serial dilutions from the highest concentration to concentrations of 0.024 w/v% or below in the appropriate vehicle (e.g. 6.25, 1.56, 0.39, 0.098, 0.024 w/v%).
- If the test chemical is a liquid, perform the serial dilution from 25 w/v% (100 (neat), 25, 6.25, 1.56, 0.39, 0.098, 0.024 w/v%).

- Topical applications (6-hour treatment):

This step should be performed under sterile conditions.

- Pre-warm the supplied assay medium in a water bath at 37°C.
- Make a plate layout. If a test chemical is liquid at 100% (neat) at room temperature, separate the tissue units which are used for the liquid test chemical from other test chemicals and controls (e.g. positive controls and vehicle controls) into individual 24-well plates.
- Fill each well of a new 24-well plate with 0.5 mL of the medium, and transfer one pre-cultured tissue unit into each of the 24 medium-filled wells using sterile forceps.
- Apply an aliquot (5 μL) of working solution to the center of each *epidermis* surface (one tissue unit per each concentration) using a positive displacement pipette and tips.
- Prepare three tissue units for the non-treated control of the cell viability measurement.
- Incubate the treated *epidermis* for 6 hours at 37°C under 5% CO_2.

- MTT assay:

- After the test chemical exposure, fill a multi-step pipette and combitips (adjusted for a 0.5 mL distribution) with D-PBS. Add 0.5 mL of D-PBS to the tissue units and remove the residual test chemical and D-PBS by turning the tissue unit upside down. Repeat this washing three times.

- Prepare 0.5 mg/mL-medium MTT solution. Fill each well of a new 24-well plate with 0.5 mL of the MTT medium, and transfer an washed tissue unit into each of the 24 MTT medium-filled wells using forceps. Incubate for 3 hours at 37°C under 5% CO_2.
- Pick up the *epidermis* gently from the insert cup using tweezers. Wipe off any remaining D-PBS gently with absorbent paper and put the *epidermis* into a 1.5-mL microtube containing 250 μL isopropanol.
- Incubate for at least 2 hours (within 24 hours) at room temperature with protecting from light, and extract MTT formazan dye.
- Collect 200 μL of the MTT formazan dye solution in a 96-well plate. Prepare blank well by adding 200 μL of isopropanol. Measure the absorbance at between 540 to 590 nm (usually 570 nm) using a 96-well plate absorbance reader.
- Calculate cell viability using the following equation.
- The lowest concentration showing less than 80% cell viability is used for the subsequent main study.

$$Cell\ viability\ (\%) = \frac{abs.of\ test\ chemical\ treatment - abs.of\ blank}{mean\ abs.of\ non\text{-}treated\ control - abs.of\ blank} \times 100$$

<u>- Examples of plate layout</u>

Example 24-well EPI-MODEL plate layout for chemical exposure:

Plate #1

1. Non-treated	2. Non-treated	3. Non-treated	13. Sample B 0.012 w/v%	14. Sample B 0.049 w/v%	15. Sample B 0.20 w/v%
4. AOO	5. DW	6. 50% EtOH	16. Sample B 0.78 w/v%	17. Sample B 3.13 w/v%	18. Sample C 0.012 w/v%
7. Sample A 0.024 w/v%	8. Sample A 0.098 w/v%	9. Sample A 0.39 w/v%	19. Sample C 0.049 w/v%	20. Sample C 0.20 w/v%	21. Sample C 0.78 w/v%
10. Sample A 1.56 w/v%	11. Sample A 6.25 w/v%	12. Sample A 25 w/v%	22. Sample C 3.13 w/v%	23. Sample C 12.5 w/v%	24. Sample C 50 w/v%

Example 96-well plate layout for MTT assay measurement:

1	2	3	13	14	15						
4	5	6	16	17	18						
7	8	9	19	20	21						
10	11	12	22	23	24						

Each number corresponds to the number shown in the upper figure (24-well plate format)

DAY 8: MAIN STUDY (MTT assay)

- Preparation:

- Prepare the test chemical solution at the lowest concentration showing less than 80% cell viability in the dose finding study using a volumetric flask. Transfer the solution from the flask to a glass vial or a microtube.
- Prepare working solutions of each test chemical as 2-fold serial dilutions from the above concentration to the highest concentration showing greater than 90% cell viability depending upon the slope of cell viability (basically from 3 (in steep slope) to 5 (in shallow slope) working solutions at 2-fold serial dilutions).
- When the cell viability is not less than 80% in the dose finding study, test the chemical in the main study by preparing at least 3 working solutions at 2-fold serial dilutions from the highest soluble concentration or 100% (neat).
- Prepare a 0.78 w/v% clotrimazole solution and 0.10 w/v% 4NBB solution in AOO. For example, measure 0.0312 g of clotrimazole in a 1 mL volumetric flask, and add AOO for a total solution volume of 1 mL, and after that, dilute 4 times with AOO in a glass vial or a microtube. In addition, measure 0.0100 g of 4NBB in a 1mL volumetric flask, and add AOO for 1 mL, and after that, dilute 10 times with AOO in a glass vial or a microtube.

- Topical applications (6-hour treatment):

This step should be performed under sterile conditions.

- Pre-warm the supplied assay medium in a water bath at 37°C.

- Make a plate layout. If a test chemical is liquid at 100% (neat) at room temperature, separate the tissue units which are used for the liquid test chemical from other test chemicals and controls (e.g. positive controls and vehicle controls) into individual 24-well plates.
- Fill each well of a new 24-well plate with 0.5 mL of the medium, and transfer one pre-cultured tissue unit into each of the 24 medium-filled wells using sterile forceps.
- Apply an aliquot (5 μL) of working solution to the center of each *epidermis* surface using a positive displacement pipette and tips. Six tissue units per each concentration (three is for gene expression assay and other three are for MTT assay) should be used at each tested concentration.
- Prepare one tissue unit for the non-treated control and two tissue units for the killed control (treated with 10 μL of 10 w/v% Triton X-100) for the LDH assay.
- Prepare three tissue units for the non-treated control for the MTT assay.
- Prepare three tissue units for 5 μL of 0.78 w/v% clotrimazole, three tissue units for 5 μL of 0.10 w/v% 4NBB and three tissue units for the vehicle control (treated with 5 μL of AOO, DW and/or 50% EtOH if used).
- Incubate the treated *epidermis* for 6 hours at 37°C under 5% CO_2.

- LDH assay:

- After exposure, collect 50 μL of the medium in a 96-well plate. Perform the LDH assay described on page 11 for positive control and vehicle control. Calculate the cell viability.

- MTT assay:

- After exposure, wash the epidermis which is used for MTT assay three times. Perform MTT assay described above. Calculate the cell viability.

- Examples of plate layout

Example 24-well EPI-MODEL plate layout for chemical exposure:

Plate #1

1. Non-treated (LDH assay)	2. Killed Ctrl.	3. Killed Ctrl.	13. DW	14. DW	15. DW
4. AOO	5. AOO	6. AOO	16. Sample A 1.56 w/v% (DW)	17. Sample A 1.56 w/v% (DW)	18. Sample A 1.56 w/v% (DW)
7. Clotrimazole 0.78 w/v%	8. Clotrimazole 0.78 w/v%	9. Clotrimazole 0.78 w/v%	19. Sample A 3.13 w/v% (DW)	20. Sample A 3.13 w/v% (DW)	21. Sample A 3.13 w/v% (DW)
10. 4NBB 0.10 w/v%	11. 4NBB 0.10 w/v%	12. 4NBB 0.10 w/v%	22. Sample A 6.25 w/v% (DW)	23. Sample A 6.25 w/v% (DW)	24. Sample A 6.25 w/v% (DW)

Plate #2

25. Non-treated (MTT assay)	26. Nom-treated (MTT assay)	27. Non-treated (MTT assay)			
28. Sample A 1.56 w/v% (DW)	29. Sample A 1.56 w/v% (DW)	30. Sample A 1.56 w/v% (DW)			
31. Sample A 3.13 w/v% (DW)	32. Sample A 3.13 w/v% (DW)	33. Sample A 3.13 w/v% (DW)			
34. Sample A 6.25 w/v% (DW)	35. Sample A 6.25 w/v% (DW)	36. Sample A 6.25 w/v% (DW)			

Example 96-well plate layout for LDH assay:

1	2	3	13	14	15						
4	5	6									
7	8	9									
10	11	12									

Each number corresponds to the number shown in the upper figure (24-well plate format)

Example 96-well plate layout for MTT assay measurement:

25	26	27									
28	29	30									
31	32	33									
34	35	36									

Each number corresponds to the number shown in the upper figure (24-well plate format)