Testing Method Development and Validation for *in Vitro* Skin Irritation Testing (SIT) by Using Reconstructed Human Epidermis (RhE) Skin Equivalent - EPiTRI®

Yu-Chun Lin[1], Hui-Chun Hsu[1], Chiu-Hsing Lin[1], Cheng-Yi Wu[1], Wannhsin Chen[1], and Huey-Min Lai[2(✉)]

[1] Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute (ITRI), Hsinchu TW 300, Taiwan
[2] Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute (ITRI), Rm 207A Bldg. 13, No. 321, Sect. 2, Kwan-Fu Road, Hsinchu TW 300, Taiwan
HMLai@itri.org.tw

Abstract. Current regulatory requirements focus on assessment of acute irritation potential of chemicals and cosmetics in order to support risk management. A trend has been changed from *in vivo* to *in vitro* testing due to 3R (replacement, reduction, refinement) requirements has resulted from a recent animal testing ban in Taiwan. RhE (Reconstructed human Epidermis) models use normal human keratinocytes to form a multi-layered epidermis including a stratum corneum at the top, which function as a barrier. Some commercialized RhEs passed validation of skin irritation test (SIT) examined by ECVAM, but none of them are originated from Chinese heredity. Therefore, ITRI started a RhE project some years ago based on our long-term developed cell culture experiences, such as isolation of cells from human donors, cell expansion technology, and our own GTP/GMP qualified facilities. So far, a well differentiated and with reproducible barrier function epidermis named EPiTRI® has been reconstructed. We developed a protocol for EPiTRI® SIT in accordance to OECD TG 439. The protocol displays a result of sensitivity of 100%, specificity of 70%, and accuracy of 85% in international validation study. Thus, the human epidermal skin equivalent EPiTRI® can be provided as an *in vitro* model for evaluation of skin irritation and a reliable method has been developed accordingly.

Keywords: Reconstructed human epidermis · Skin irritation test
Validation of protocol

Introduction

Topical exposure to chemicals and cosmetic products can lead to various adverse skin effects. Corrosion and irritation are commonly regarded as two major categories among these adverse effects. Corrosive substances irreversibly damage the skin beyond repair, while irritant substances lead to a reversible local inflammatory reaction caused by innate immune system of the affected tissue. Some chemicals trigger an irritant response after

H. Kojima et al. (Eds.): *Alternatives to Animal Testing*, pp. 8–18, 2019.
https://doi.org/10.1007/978-981-13-2447-5_2

repeated exposure to the same skin area, while other chemicals may cause irritation after a single exposure. Current regulatory requirements focus on assessment of acute irritation potential of chemicals and cosmetics in order to support the risk management. Data on skin irritation effects are required by regulatory regimes for chemicals, pesticides, pharmaceuticals and medical devices as a condition of marketing in many countries.

Internationally accepted test methods for skin irritation testing (SIT) include the traditional *in vivo* animal test as well as *in vitro* test methods. However, there is a trend away from *in vivo* to *in vitro* testing due to the 3R (replacement, reduction, refinement) requirement resulted from recent cosmetic animal testing bans in Taiwan and elsewhere. All accepted *in vitro* test methods are based on the RhE technology (Reconstructed human Epidermis) validated by ECVAM. RhE models use normal human keratinocytes which, during culturing, form a multi-layered epidermis including a stratum corneum at the top and can function as a barrier. There are only few RhEs that have been validated for SIT and approved by ECVAM. For the growing Chinese and Asian cosmetic markets, we aimed to develop a human epidermis skin equivalent that contains normal human epidermal structure and function derived from a Chinese population. ITRI has started some years ago in-house based on our culture experience of cells isolated from human donors, the cell expansion (scale up) technology, and our own GTP/GMP facilities. So far, a multi-layered epidermis composed of well differentiated stratified stratum corneum, granulosum, stratum spinosum and basal layer, and with reproducible barrier function, was developed and used for skin irritation testing in accordance to OECD TG 439 guideline.

In this study, we reported the progress about development of reconstructed human epidermis (EPiTRI®) and the aim to develop the skin irritation testing protocol by using EPiTRI® for validation. Quality control parameters for EPiTRI®, such as structure morphology of tissue, thickness, TEER (trans-epithelium electrical resistant), and lipid profile were investigated to study the correlation with barrier function. After obtaining satisfactory quality control data, we conducted the validation process. During protocol development, several important parameters were evaluated for obtaining better statistical accuracy of data when compared with data from *in vivo* testing. These important parameters include pre-incubation time, post-incubation volume, chemical exposure time, washing method, etc. Resulting sensitivity of 100%, specificity of 70% and accuracy of 85% were obtained in current Phase I validation status. The study shows that the human epidermal skin equivalent EPiTRI® could possibly provide as an *in vitro* model to evaluate the skin irritation, and a reliable SIT method has been developed accordingly.

Materials and Methods

EPiTRI® Reconstructed Human Epidermis

EPiTRI® RhE tissue was developed by Biomedical Technology and Device Research Laboratories, ITRI (www.itri.org.tw). The tissue was a three-dimensional and fully differentiated human epidermal skin equivalent, grown from normal human keratinocytes in defined growth medium on the air liquid interface. On day 14, tissues were transferred on nutritive agarose plates and enclosed in a temperature-controlled

container for shipment to customers. Each experiment was performed in triplicate on one single tissue production batch, but different batches were used for each repeated experiment. On day 15, EPiTRI® RhE tissues were transferred in 2 mL ITRI culture medium in 6-well plates for an 18–30 h pre-incubation step at 37 ± 1°C, 5 ± 1% CO_2.

Selection of Test Substances

Twenty test substances with diversity of functional chemical groups and physico-chemical properties were selected according to OECD TG 439 (2015) and evaluated by using of EPiTRI®. Details of the 20 test substances are described in Table 2. Phosphate Buffer Saline solution (PBS) treated RhE tissues were used as negative control, Sodium Dodecyl Sulphate (SDS 5% W/V aqueous solution) treated RhE tissues as positive control.

Cell Viability Measurement by MTT Reduction

The MTT (3-[4,5-di-methyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was performed to measure cell viability via converting yellow-colored MTT to blue/purple formazan crystals by dehydrogenases of living cells [3]. Per test conditions, the three EPiTRI® RhE tissues were incubated in 300 μL of MTT solution (1 mg MTT per 1 mL medium) for a 3-h incubation time at 37 ± 1 °C, 5 ± 1% CO_2. Formazan crystal inserts were dissolved in 2 mL isopropanol in 24-well plates for a 3-h extraction time at room temperature.

After extraction, cell viability was analyzed by comparing the optical density of the extracts measured at 570 nm in percentage to the negative PBS treated controls.

In Vitro Skin Irritation Test (SIT)

Step-wised protocol of SIT was shown in Fig. 1. Each test substance was applied topically in triplicate of EPiTRI® RhE tissues. After treatment, tissues were rinsed with PBS and incubated with fresh medium for 42 h at 37 ± 1 °C, 5 ± 1% CO_2. At the end of the incubation, cytotoxicity was determined by MTT conversion test. For each run, the cell viability was calculated and expressed as a percentage relative of the NC. Acceptance criteria for qualified experiment are: (1) 1.0 ≤ OD_{570} of NC ≤2.8; (2) PC ≤20%; (3) SD of test substance <18%.

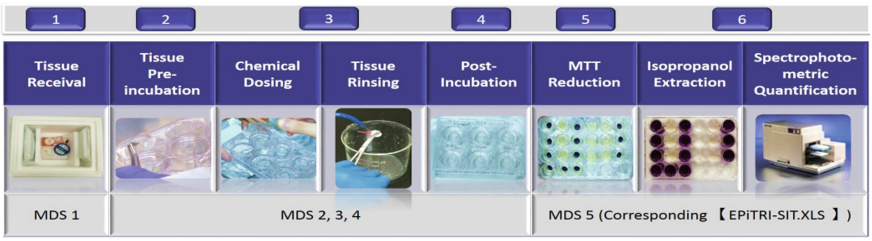

Fig. 1. Protocol of skin irritation test (SIT) developed by ITRI for EPiTRI®

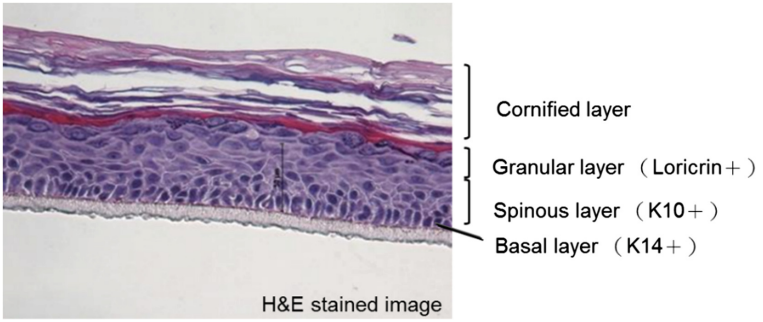

Fig. 2. Structure morphology of EPiTRI® by H&E and immunohistochemical stain

Prediction Model

The SIT protocol allows for prediction of skin irritation potential of test substances according to the United Nations Globally Harmonized System of Classification and Labeling (UN-GHS). Tissue viability that was equal or below 50% of the negative control was used to classify the substance as "Category 2" (Irritant). Tissue viability that was above 50% resulted in classifying the substance as "No category" (Non-irritant).

Statistical Analysis

Mean optical density (OD) and standard deviation (SD) were determined. SD values should be dealt carefully since ECVAM performance standards considers a test as valid only if SD obtained from the three concurrently tested tissues is $\leq 18\%$. The test was evaluated using contingency tables. Sensitivity (percent of Irritant (Category 2) substances correctly predicted *in vitro*), specificity (percent of No category substances correctly predicted *in vitro*) and the concordance (percent of substances correctly classified *in vitro*) were calculated.

Results and Discussions

Change of TER and Structural Morphology during the Reconstruction of EPiTRI®

We have established an efficient protocol in our laboratory for the expansion, differentiation and reconstruction of human skin epidermis equivalent EPiTRI® from primary human keratinocyte. Using our proprietary induction protocol, a multi-layered epidermis composed of stratified stratum corneum, granulosum, stratum spinosum and basal layer were formed after air-lift culture and resembled *in vivo* skin epidermis structure. Moreover, immunohistochemically staining showed that loricrin, cytokeratin 10 and cytokeratin 14 were expressed in the granulosum, spinosum and basal layer respectively, indicating that the reconstructed human epidermis skin equivalent was

closely mimic the histology and morphology of human epidermis (Fig. 2). During formation of EPiTRI®, transcutaneous electrical resistance (TER) measurements were performed on EPiTRI® from Day 1 to Day 15. As shown in Fig. 3(A), the TER value was very low at the first three days of differentiation. However, as the epidermis gradually developed, the TER value increased dramatically from Day 3 to Day 9. Process of EPiTRI® differentiation was shown in Fig. 3(B), which displayed a time-coursed maturation of EPiTRI®. As time went by, a multi-layered epidermis was formed. We found that Transcutaneous Electrical Resistance (TER) correlated to the differentiation process from Day 1 to Day 9 or 10. After epidermis matured, the TER maintained at a higher value.

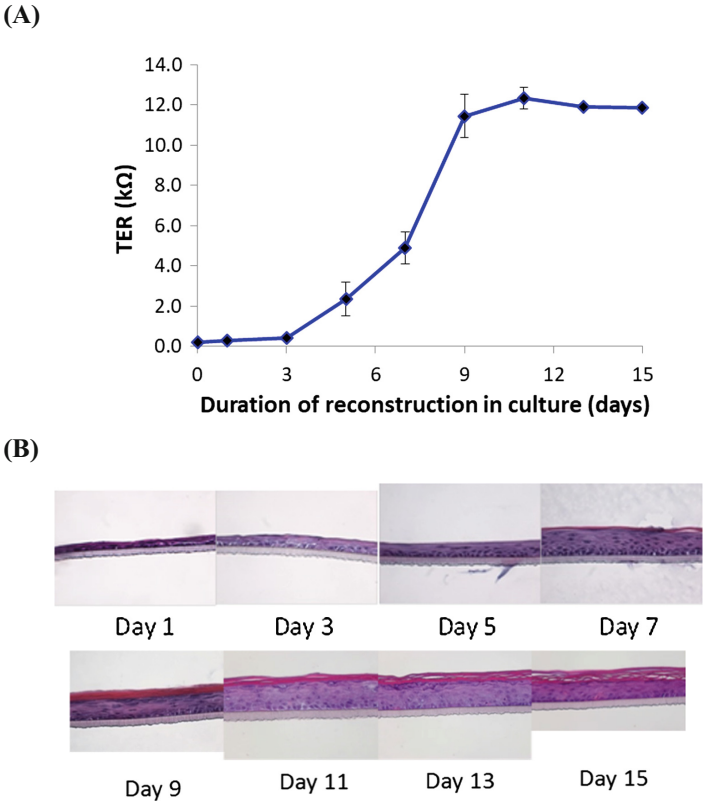

Fig. 3. TER and structural morphology during the reconstruction of EPiTRI®

(A) Transcutaneous electrical resistance (TER) measurements were performed on EPiTRI® from Day1 to Day15. Data represented the average TER of three different tissues at each day point. (B) EPiTRI® skin equivalent shows a time-dependent cell maturation, generating a multi-layer epidermis composed of stratified stratum corneum, granulosum, stratum spinosum and basal layer.

Quality Control of EPiTRI®

In order to meet the requirement of OECD TG 439, the quality control of EPiTRI® is important. Quality factors that we measured include: the thickness of EPiTRI®, the barrier property, the cell viability of negative control and positive control, and transcutaneous electrical resistance. As shown in Fig. 4, the variation on these parameters was small for EPiTRI®. Histology was used to measure the thickness of EPiTRI®. The thickness of EPiTRI® was in the range from 67.06 μm to 85.20 μm (76.32 ± 4.85 μm, CV = 6.36%) (Fig. 4(A)). Negative control OD values of EPITRI® was in the range of 1.33−1.58 (1.44 ± 0.07, CV = 5.2%) (Fig. 4(B)). Measured barrier function of EPiTRI® was in the range of 62.48%−75.01% (70.92 ± 2.53, CV = 4.97%) (Fig. 4(C)). Measurements of TER: 11.3 kΩ−11.9 kΩ (11.6 ± 0.2 kΩ, CV = 1.4%) (Fig. 4(D)).

(A) Thickness of EPiTRI®: 67.06 μm−85.20 μm (76.32 ± 4.85 μm, CV = 6.36%); **(B)** OD values of Negative control: 1.33−1.58 (1.44 ± 0.07, CV = 5.2%); **(C)** Barrier function: of 62.48%−75.01% (70.92 ± 2.53, CV = 4.97%); **(D)** Measurements of TER: 11.3 kΩ−11.9 kΩ (11.6 ± 0.2 kΩ, CV = 1.4%).

Protocol Refinements for the Skin Irritation Test of EPiTRI®

Modification of Washing Method for SIT

Washing method is important in terms of being able to effectively wash away chemicals without damaging the epidermis tissue when considering the development of SIT protocol. We have tried many washing method. Finally, we found an efficient procedure (the last group as shown in Fig. 5(A)) which resulted in the least standard deviation of testing chemicals. Thus we finalized the washing method for SIT in our protocol: Washing off the chemical with continuous stream of PBS at a distance of 3 cm. Squeeze the bottle to maintain a continuous stream of PBS. Then flush the stream against the wall of the insert near the six-o'clock direction for 3 s (4 ± 1 mL). Make sure tissue surface will be fully covered by PBS stream during washing. After washing, discard the PBS into a beaker by taping the insert for three times. Then, gently place the insert on a sterile gauze sponge, tilt the insert on the gauze and tap for three times. Repeat the flushing for another 2 times. Wash off the residual chemical from the insert with PBS for 35 times. To avoid damaging tissues, it is strongly recommended to maintain stable stream of PBS against the wall of the insert.

Chemical Exposure Time

Some parameters in the protocol are critical to develop a successful protocol, for example, chemical exposure time. We found in our experiment for some specific chemicals such as no. 26 and 29, change of exposure time may result in a totally different classification as an irritant or a non-irritant. With 10 min of exposure, they could exhibit non-irritant. However, with 30 min of exposure they apparently are irritant (Fig. 5B).

Pre-incubation Time and Post-incubation Volume

The incubation volume and time are also important to develop a good protocol. Because skin irritation is a reversible reaction, for some chemicals such as No. 19,

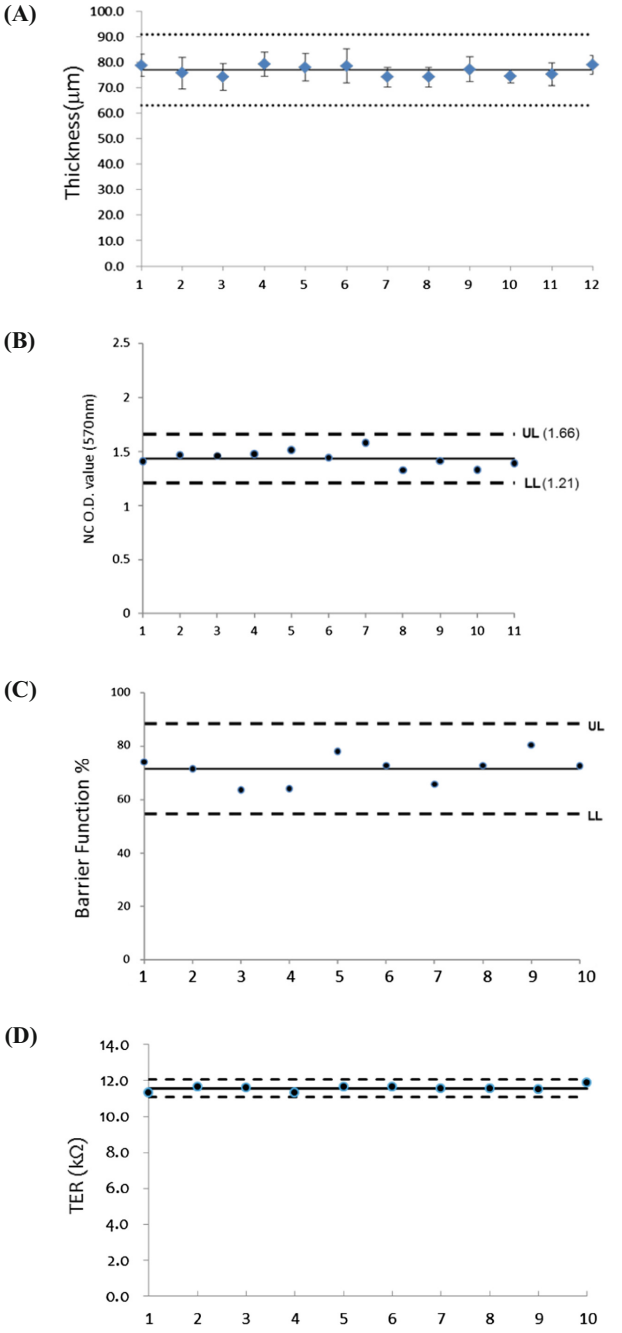

Fig. 4. Quality control of EPiTRI® on **(A)** thickness, **(B)** OD of NC, **(C)** barrier function, and **(D)** TER.

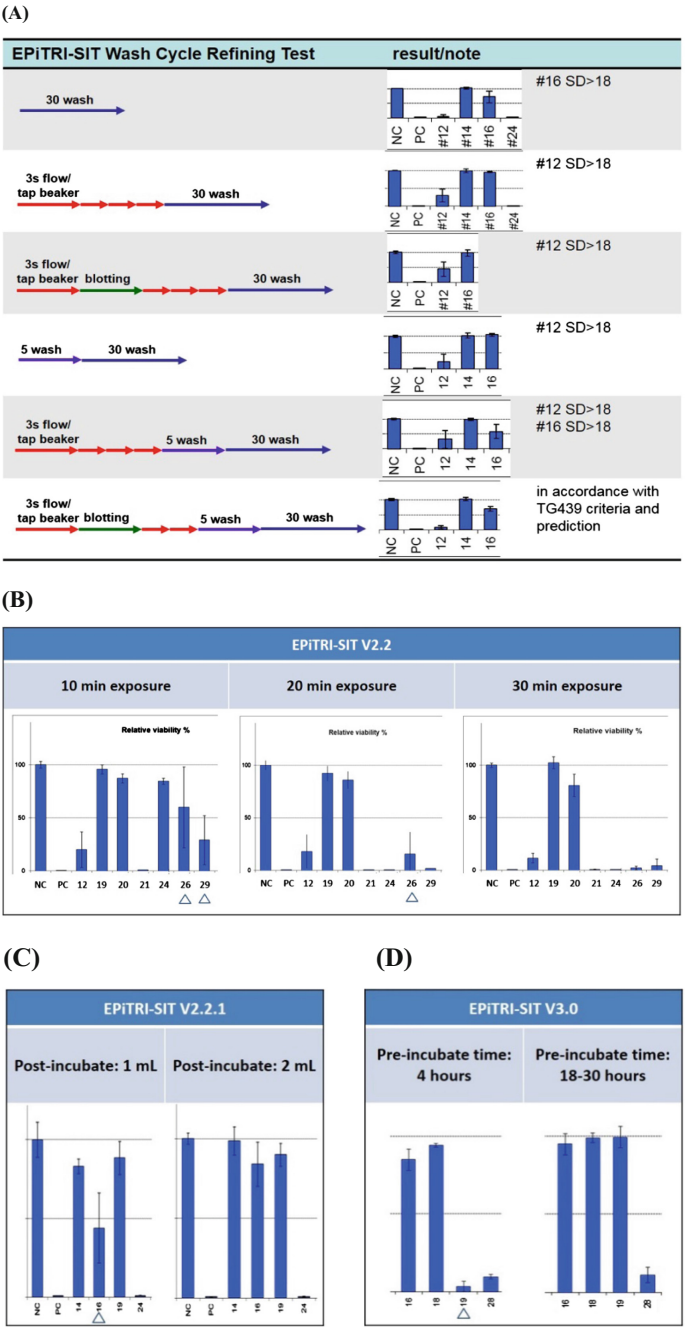

Fig. 5. Protocol refinements for the skin irritation test of EPiTRI®

the increase of pre-incubation time resulted in its classification from irritant to non-irritant. For some chemicals such as No. 16 in our experiment, a post-incubation volume could improve the variation of results (Fig. 5**C, D**). The important parameters of SIT using EPiTRI® when compared with other ECVAM proved products are listed in Table 1.

Table 1. Comparison of EPiTRI®-SIT with validated test tissues in OECD TG 439

Model					
Procedure	EpiSkin™	EpiDerm™ SIT (EPI-200)	SkinEthic™ RHE	LabCyte EPI-MODEL24 SIT	EPiTRI Ver 3.2
Model surface	0.38 cm^2	0.63 cm^2	0.5 cm^2	0.3 cm^2	0.47 cm^2
a) Pre-incubation					
Incubation time	18−24 h	18−24 h	<2 h	15−30 h	18−30 h
Medium volume	2 mL	0.9 mL	0.3 or 1 mL	0.5 mL	2 mL
b) Test chemical application					
For liquids	10 μL (26 μL/cm^2)	30 μL (47 μL/cm^2)	16 μL (32 μL/cm^2)	25 μL (83 μL/cm^2)	35 μL (75 μL/cm^2)
For solids	10 mg (26 mg/cm^2) +DW (5 μL)	25 mg (39 mg/cm^2) +DPBS (25 μL)	16 mg (32 mg/cm^2) +DW (10 μL)	25 mg (83 mg/cm^2) +DW (25 μL)	25 mg (53mg/cm^2) + 20 μL DW
Use of Nylon mesh	not used	if necessary	Applied	not used	S: Applied L: if necessary
Total application time	15 min	60 min	42 min	15 min	30 min
Application temperature	RT	a. RT, 25 min b. 37 °C, 35 min	RT	RT	RT
c) Post-incubation volume					
Medium volume	2 mL	0.9 mL×2	2 mL	1 mL	2 mL
d) Maximum acceptable variability					
SD between tissue replicates	SD ≦ 18	SD ≦ 18	SD ≦ 18	SD ≦ 18	SD ≦ 18

(A) Wash cycle refining test: the last group was selected due to the success of SD < 18 for #12, #14, and #16 chemicals; (**B**) Optimal chemical exposure time was 30 min by using 7 non-coded chemicals. Δ represented SD $\geq$ 18; **(C)** Because the test result of #16 chemical was different from UN GHS Cat. Data exhibited SD $\geq$ 18%, thus 2 mL of medium volume in post-incubation was selected rather than 1 mL; **(D)** Because the test result of #19 chemical was different from UN GHS Cat., thus 18−30 h pre-incubation time was better than 4 h.

In the Phase 1 study of EPiTRI® for SIT, the lead lab ITRI tested the protocol by 20 chemicals (Table 2). Distribution of the testing results of 20 chemicals, with triplicates on each, was shown in Fig. 6 expressed as % cell viability of negative control. 50% viability cut-off was used for the prediction of irritants and non-irritants. The predictive capacity in terms of sensitivity, specificity and accuracy, we obtained scores of 100%, 70%, and 85%, respectively (Table 3). Meet the requirement of OECD TG 439.

Three independent experiments performed for each reference chemical. Distribution of the 20 chemical test results, the average of the three experiments for each data point, shown as % of tissue viability of negative control. 50% viability cut-off was used for the prediction of irritants from non-irritants.

Table 2. Twenty selected test substances and predictive capacity of EPiTRI® in comparison with GHS Category or OECD VRM

Test substances	CAS No.	Storage	Physical state	VRM Cat. based on in vitro	UN GHS Cat. based on in vivo results	In vivo score
l-bromo-4-chlorobutane	6940-78-9	RT	Liquid	Cat. 2	No Cat.	0
Diethyl phthalate	84-66-2	RT	Liquid	No Cat.	No Cat.	0
Naphthalene acetic acid	86-87-3	RT	Solid	No Cat.	No Cat.	0
Allyl phenoxy-acetate	7493-74-5	RT	Liquid	No Cat.	No Cat.	0.3
Isopropanol	67-63-0	RT	Liquid	No Cat.	No Cat.	0.3
4-{methylthio)-benzaldehyde	3446-89-7	RT	Liquid	Cat. 2	No Cat.	1
Methyl stearate	112-61-8	-20 °C	Solid	No Cat.	No Cat.	1
Heptyl butyrate	5870-93-9	RT	Liquid	No Cat.	NoCat.(Optional Cat. 3)	1.7
Hexyl salicylate	6259-76-3	RT	Liquid	No Cat.	NoCat.(Optional Cat. 3)	2
Cinnamaldehyde	104-55-2	4 °C	Liquid	Cat. 2	No Cat.(Optional Cat. 3)	2
1-decanol	112-30-1	RT	Liquid	Cat. 2	Cat. 2	2.3
Cyclamen aldehyde	103-95-7	RT	Liquid	Cat. 2	Cat. 2	2.3
1-bromohexane	111-25-1	RT	Liquid	Cat. 2	Cat. 2	2.7
2-chloromethyl-3,S-dimethyl-4- methoxypyridine HCI	86604-75-3	RT	Solid	Cat. 2	Cat. 2	2.7
Di-n-propyl disulphide	629-19-6	RT	Liquid	No Cat.	Cat. 2	3
Potassium hydroxide (5% aq.)	1310-58-3	RT	Liquid	Cat. 2	Cat. 2	3
Benzenethiol, 5 (1,1-dimethylethyl)-2-methyl	7340-90-1	RT	Liquid	Cat. 2	Cat. 2	3.3
l-methyl-3-phenyl-l-piperazine	5271-27-2	RT	Solid	Cat. 2	Cat. 2	3.3
Heptanal	111-71-7	RT	Liquid	Cat. 2	Cat. 2	3.4
Tetrachloroethylene	127-18-4	RT	Liquid	Cat. 2	Cat. 2	4

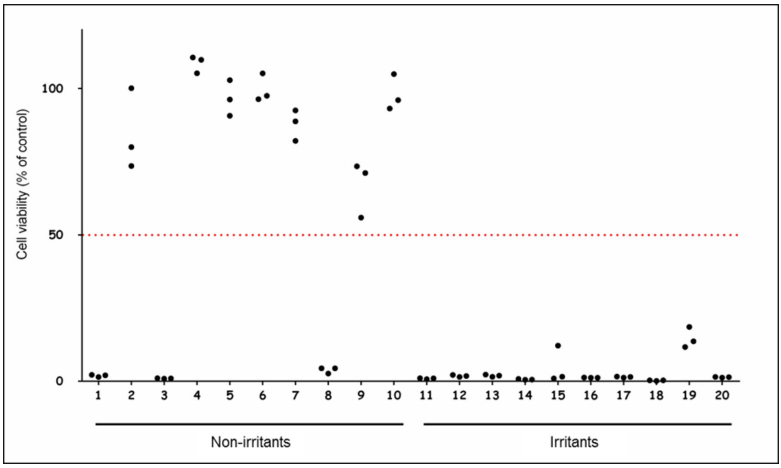

Fig. 6. Distribution of the relative tissue viability obtained with the treatment of 20 selected chemical substances on EPiTRI®.

Table 3. The predictive capacity recommended by OECD TG 439 and tested result on EPiTRI®

Predictive capacity	OECD TG439 recommendation (%)	EPiTRI-SIT
Sensitivity	80%	100%(10/10)
Specificity	70%	70% (7/10)
Accuracy	75%	85% (17/20)

Conclusion

In this study, the good quality of RhE (EPiTRI®) model was presented in terms of its stable thickness, TER, structural morphology and barrier function. Furthermore, we demonstrated the development of a reliable SIT protocol using of EPiTRI®, producing an overall sensitivity of 100%, specificity of 70% and accuracy of 85%. These results meet the OECD guidelines. Based on its high predictive capacity, our *in vitro* SIT with EPiTRI® could be a promising alternative to *in vivo* SIT.

References

1. Protocol of *In Vitro* Skin Irritation: Human Epidermis Model: EPiTRI®-SIT V3.2
2. OECD Guidelines for the Testing of Chemicals (2015), *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method
3. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
4. Frank N (2005) The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. Eur J Pharm Biopharm 60:167–178
5. Carine T (2006) *In vitro* skin irritation testing on reconstituted human epidermis: reproducibility for 50 chemicals tested with two protocols. Toxicol in Vitro 20:401–416
6. Martin M (2009) A tiered approach to the use of alternatives to animal testing for the safety assessment of cosmetics: skin irritation. Regul Toxicol Pharmacol 54:188–196
7. Carine T (2010) Assessment of the optimized SkinEthic™ Reconstructed Human Epidermis (RHE) 42 bis skin irritation protocol over 39 test substances. Toxicol in Vitro 24:245–256
8. Masakazu K (2011) Refinement of LabCyte EPI-MODEL24 skin Irritation test method for adaptation to the requirements of OECD test guideline TG 439. Altern Anim Test Exp 16:111–122
9. Hajime K (2013) A catch-up validation study of an *in vitro* skin irritation test method using reconstructed human epidermis LabCyte EPI-MODEL24. J Appl Toxicol 34:766–774