

Validation Study of the *In Vitro* Skin Irritation Test with the LabCyte EPI-MODEL24

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Summary — A validation study on an *in vitro* skin irritation assay was performed with the reconstructed human epidermis (RhE) LabCyte EPI-MODEL24, developed by Japan Tissue Engineering Co. Ltd (Gamagori, Japan). The protocol that was followed in the current study was an optimised version of the EpiSkin protocol (LabCyte assay). According to the United Nations Globally Harmonised System (UN GHS) of classification for assessing the skin irritation potential of a chemical, 12 irritants and 13 non-irritants were validated by a minimum of six laboratories from the Japanese Society for Alternatives to Animal Experiments (JSAAE) skin irritation assay validation study management team (VMT). The 25 chemicals were listed in the European Centre for the Validation of Alternative Methods (ECVAM) performance standards. The reconstructed tissues were exposed to the chemicals for 15 minutes and incubated for 42 hours in fresh culture medium. Subsequently, the level of interleukin-1 alpha (IL-1 α) present in the conditioned medium was measured, and tissue viability was assessed by using the MTT assay. The results of the MTT assay obtained with the LabCyte EPI-MODEL24 (LabCyte MTT assay) demonstrated high within-laboratory and between-laboratory reproducibility, as well as high accuracy for use as a stand-alone assay to distinguish skin irritants from non-irritants. In addition, the IL-1 α release measurements in the LabCyte assay were clearly unnecessary for the success of this model in the classification of chemicals for skin irritation potential.

Key words: *in vitro*, interleukin-1 alpha (IL-1 α), MTT, reconstructed human epidermis, skin irritation, validation.

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Introduction

Since 1946, the Draize rabbit test for skin irritation has been widely used to evaluate the skin irritation potential of xenobiotics (1, 2). However, the relevance to humans of the data provided by the test is limited by species differences, so a significant number of alternative testing methods have been developed to date, including the use of *in vitro* tissue constructs based on human keratinocytes (3, 4). These constructs closely resemble human epidermis with respect to biochemical profile (e.g. lipid composition), tissue architecture (e.g. cell layering and formation of a stratum corneum), and the presence of a functional skin barrier.

Three commercially available test methods based on reconstructed human epidermis (RhE) have been validated by the European Centre for

the Validation of Alternative Methods (ECVAM; 5–7) as being suitable for determining the potential hazardous (i.e. skin irritant) properties of xenobiotics. These methods are also in compliance with the new United Nations Globally Harmonised System (UN GHS) rules for the classification and labelling of substances, implemented in the EU through regulations on the Classification, Labelling and Packaging of Substances and Mixtures. In December 2008, the EU adopted a new classification system based on the UN GHS system for Classification and Labelling (8), but which continues to use two categories to distinguish non-irritant (No Category) chemicals from irritant (Category 2) chemicals. According to the new UN GHS rules for the classification and labelling of skin irritants, the cut-off *in vivo* score to distinguish between No Category and Category

2 chemicals has changed from a value of 2.0 to 2.3. Consequently, chemicals with an *in vivo* score of between 2.0 and 2.3 had been considered irritants under the existing EU classification system, but are now classified as non-irritants under the new UN GHS system, which does not use the optional GHS Category 3.

The three *in vitro* test methods validated by ECVAM are based on identical tissue engineering technology, and, essentially, the same test protocol was followed through several validation studies (9–17), as is evident from their associated Standard Operating Procedures (SOPs). For these reasons, they are suitable for the development of a general test method procedure, which will include minimal performance criteria for similar and modified methods (6, 7, 18, 19). The EU system for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; 20), the EU Cosmetics Directive (21), and other legislative requirements worldwide, are a clear indication of the need for an internationally-harmonised and consistent test procedure for *in vitro* skin irritation testing based on RhE, similar to test guidelines promoted by the Organisation for Economic Co-operation and Development (OECD; 22).

A RhE model, the LabCyte EPI-MODEL24, was developed by Japan Tissue Engineering Co. Ltd (J-TEC; Gamagori, Aichi, Japan), and this was the skin model used in an *in vitro* skin irritation assay that has undergone protocol optimisation based on the EpiSkin protocol (23). According to this protocol, tissues are exposed to the test chemicals for 15 minutes, and are then incubated for 42 hours in fresh culture medium without the test chemicals. After this period, the amount of interleukin-1 alpha (IL-1 α) released into the conditioned medium is measured, as is tissue viability (via the MTT assay). This system is referred to as the 'LabCyte assay' throughout this paper.

A multi-laboratory assessment of the LabCyte assay was performed under the direction of the

Japanese Society for Alternatives to Animal Experiments (JSAAE) and the Japanese Centre for the Validation of Alternative Methods (JaCVAM), and was based on the ECVAM performance standards for *in vitro* skin irritation test methods based on reconstructed human epidermis (18, 19). The present paper reports the results of a three-phase validation study, which was performed by a minimum of six laboratories. The test substances were chosen in accordance with the ECVAM performance standards, and also from the revised list described in the new ECVAM Scientific Advisory Committee (ESAC) statement from 2009 (19). The objective of this investigation was to conduct a series of validation studies to assess the reliability (within-laboratory and between-laboratory reproducibility) and relevance (predictive capacity) of this assay, by using a challenge set of 25 coded test chemicals (12 irritants and 13 non-irritants) for which high-quality *in vivo* data were available. The validation study was 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* (24), and according to the modular approach to validation described by Hartung *et al.* (25).

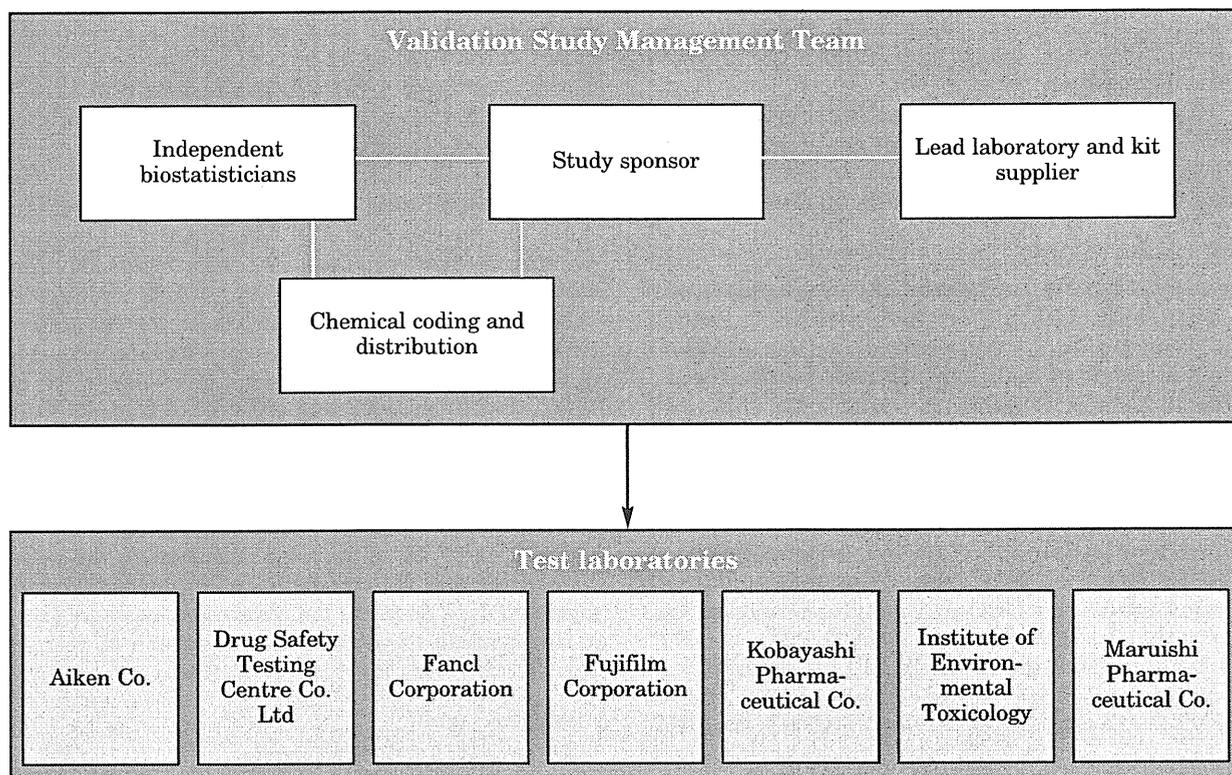
Materials and Methods

Validation study management structure

The LabCyte skin irritation assay validation study management team (VMT) was organised by the JSAAE. The management structure and members of the study are shown in Figure 1 and Table 1. The VMT played a central role in overseeing the conduct of the validation study, and was responsible for: selecting test chemicals, producing goal statements, planning the project (including the

Table 1: Details of the JSAAE skin irritation assay validation study management team

No.	Role	Name	Affiliation
1	Chair, chemical coding and distribution	Hajime Kojima	JaCVAM and NIHS
2	Protocol check	Kenji Idehara	Daicel Corporation
3	Protocol check	Isao Yoshimura	Tokyo University of Science
4	Lead laboratory and kit supplier	Masakazu Kato	J-TEC
5	Independent biostatistician and study sponsor	Takashi Omori	Kyoto University (present post: Doshisya University, Japan)
6	Independent biostatistician	Etsuyoshi Miyaoka	Tokyo University of Science
7	Independent biostatistician	Kenya Ishiyama	Tokyo University of Science

Figure 1: Structure of the JSAAE skin irritation assay validation study management team

study protocol and amendments), the outcome of quality control (QC) audits, data management procedures, timeline and study progression, study interpretation, conclusions, and the publication of reports. In addition, the VMT was responsible for making the final decision on which laboratories were to participate in the validation study. The chemical selection group, JaCVAM, was in charge of defining the test chemical selection criteria and selecting the test chemicals, liaising with suppliers, performing final checks on the chemicals provided, coding the test substances, and distributing them to the different laboratories. The independent biostatistics group was responsible for the collection, screening and analysis of the data, and for preparing spreadsheets.

The following seven laboratories (and study directors) participated in the first and second phases of the validation study for the evaluation of the LabCyte assay:

- Laboratory A: Aiken Co. Ltd (Yoko Ando and Yui Asako)
- Laboratory B: Kobayashi Pharmaceutical Co. Ltd (Yoshihiro Yamaguchi and Maki Nakamura)
- Laboratory C: The Institute of Environmental

Toxicology (Tadashi Kosaka and Koichi Hayashi)

- Laboratory D: Fancl Corporation (Tamie Suzuki and Runa Izumi)
- Laboratory E: Fujifilm Corporation (Atsuko Yuasa and Shinichi Akimoto)
- Laboratory F: Maruishi Pharmaceutical Co. Ltd (Yukihiko Watanabe and Osamu Mitani)
- Laboratory G: Drug Safety Testing Center Co. Ltd (Shinsuke Shinoda and Saori Hagiwara)

Six of the laboratories also participated in the third phase. Only Laboratory E did not participate in all three phases of the study. J-TEC, the lead laboratory in the VMT, did not participate in the practical application of the protocol in the validation study.

Study design

Before this validation study on the operation of the LabCyte EPI-MODEL24 was carried out, a one-day training course was held by J-TEC, in April 2008. All of the technicians from each laboratory participated in this training course.

The validation study was conducted in three stages, as follows. In the first phase of the study, the proposed test protocol was confirmed and its transferability was assessed by testing three coded chemicals (ethanol, glycerol and naphthalene acetic acid) and a positive control (5% w/v sodium lauryl sulphate [SLS]) in seven laboratories, between June and July 2008.

During the second phase, the VMT confirmed within-laboratory and between-laboratory reproducibility, as well as the correlation between the results obtained and the identities of the 20 coded chemicals, 19 of which are described in the original ECVAM performance standards (18). These tests were conducted by seven laboratories, between September 2008 and January 2009.

Since the statement regarding the revised ECVAM performance standards (7, 19) became available after the second phase of the study had finished, the VMT decided to conduct a third phase. During this last phase, six additional chemicals were tested for within-laboratory and between-laboratory reproducibility, and the correlations between the results obtained and the available *in vivo* data were assessed. Phase three of the study was conducted by six laboratories, all of which had participated in both the first and second phases of the study, between April and May 2009.

Test chemicals

Throughout all phases of the study, the negative control consisted of distilled water and the positive control was 5% w/v SLS (Wako Pure Chemical Industries Ltd, Osaka, Japan).

In the first phase, the VMT selected and JaCVAM distributed the three coded chemicals (ethanol, glycerol and naphthalene acetic acid; Wako Pure Chemical Industries Ltd) to each of the laboratories taking part in the study (see Table 2).

In the second phase, the VMT selected 19 of 20 chemicals for testing, according to the reference list provided in the original ECVAM performance standards (18). Their chemical names, suppliers, CAS numbers, GHS labels and *in vivo* scores are listed in Table 2. One of the chemicals in the original ECVAM performance standards reference list, tri-isobutyl phosphate, was not available for purchase in Japan. Ultimately, the VMT approved the use of a 5% w/v SLS solution — the same chemical that JaCVAM had proposed for use as the positive control — to replace tri-isobutyl phosphate as chemical No. 13. To avoid any potential bias in the final selection, the VMT did not inform the laboratory representatives about these discussions.

In the third phase of the study, the VMT selected six new chemicals to be tested, according to the reference list in the revised ECVAM performance

standards (19). Their chemical names, suppliers, CAS numbers, GHS labels and *in vivo* scores are listed in Table 2. JaCVAM suggested the final list of chemicals, which was then approved by the VMT. As before, in order to avoid any bias in the final selection, the VMT did not inform the laboratory representatives of these discussions.

As shown in Table 2, the chemicals were purchased from Wako Pure Chemical Industries Ltd, Kanto Chemical Co. Inc. (Tokyo, Japan), Sigma-Aldrich Corporation (St Louis, MO, USA), Sigma-Fluka (St Louis, MO, USA), Alfa Aesar (Haverhill, MA, USA) and Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). JaCVAM contracted an independent laboratory to code and distribute the chemicals, and a chemical manager (or safety officer) carried out these tasks. The Material Safety Data Sheet for each chemical was also distributed with the chemicals.

Reconstructed human cultured epidermal model

LabCyte EPI-MODEL24 consists of normal human epidermal keratinocytes, whose biological origin is neonatal foreskin. In order to expand the number of human keratinocytes while maintaining their phenotype, the cells were cultured with 3T3-J2 cells as a feeder layer (26, 27). LabCyte EPI-MODEL24 involves growing keratinocytes on an inert substrate — a tissue culture filter with a surface of 0.3cm² — at the air-liquid interface for 13 days in an optimised medium. The composition of the optimised medium for the culture of LabCyte EPI-MODEL24 is based on Dulbecco's modified Eagle's medium and Ham's F12 medium (in a 3:1 ratio), with epidermal growth factor, insulin, hydrocortisone, other proprietary stimulators of epidermal differentiation, antibiotics, and 5% v/v fetal bovine serum (FBS). Although the FBS was purchased from several different suppliers, after reviewing safety data and performance, FBS from Japan Bio Serum (Fukuyama, Japan) was used.

Ultimately, this tissue model results in a multi-layer structure consisting of a fully-differentiated epithelium that has features of the normal human epidermis, including a stratum corneum. For dispatch, the LabCyte EPI-MODEL24 samples are embedded in an agarose gel containing appropriate nutrients, and shipped in 24-well plates at around 18°C (13).

J-TEC audited the batch release criteria for LabCyte EPI-MODEL24, in order to ensure compliance with the principles of Good Laboratory Practice (GLP), and to guarantee that only certified tissues were used for the prediction and classification of irritants (28). For this purpose, the viability of the tissue was assessed, via the MTT

Table 2: Reference test chemicals used in the validation study

No.	Chemical	Supplier	CAS number	GHS label	In vivo score
a) In the first phase					
I	Ethanol	WPCI	64-17-5	No cat.	0
II	Glycerol	Sigma-Aldrich	56-81-5	No cat.	0
III	Naphthalene acetic acid	WPCI	86-87-0	No cat.	0
IV	Sodium lauryl sulphate (SLS) 5% w/v (positive control)	WPCI	151-21-3	—	—
b) In the second phase					
1	1-Bromo-4-chlorobutane	WPCI	6940-78-9	No cat.	0.0
2	Diethyl phthalate	WPCI	84-66-2	No cat.	0.0
3	di-Propylene glycol	WPCI	25265-71-8	No cat.	0.0
4	Naphthalene acetic acid	WPCI	86-87-3	No cat.	0.0
5	Allyl phenoxyacetate	WPCI	7493-74-5	No cat.	0.3
6	Isopropanol	WPCI	67-63-0	No cat.	0.3
7	4-Methylthio-benzaldehyde	WPCI	3446-89-7	No cat.	1.0
8	Methyl stearate	KCC	112-61-8	No cat.	1.0
9	Allyl heptanoate	WPCI	142-19-8	No cat.	1.7
10	Heptyl butyrate	Sigma-Aldrich	5870-93-9	No cat.	1.7
11	Hexyl salicylate	Sigma-Fluka	6259-76-3	No cat.	2.0
12	Terpinyl acetate	Alfa Aesar	80-26-2	No cat.	2.0
13	Sodium lauryl sulphate (SLS) 5% w/v ^a	WPCI	151-21-3	—	—
14	1-Decanol	WPCI	112-30-1	Category 2	2.3
15	Cyclamen aldehyde	WPCI	103-95-7	Category 2	2.3
16	1-Bromohexane	WPCI	111-25-1	Category 2	2.7
17	α -Terpineol	KCC	98-55-5	Category 2	2.7
18	di- <i>n</i> -Propyl disulphide	WPCI	629-19-6	Category 2	3.0
19	Butyl methacrylate	WPCI	97-88-1	Category 2	3.0
20	Heptanal	KCC	111-71-7	Category 2	4.0
c) In the third phase					
21	Cinnamaldehyde	Sigma-Aldrich	104-55-2	No cat.	2.0
22	2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	WPCI	322-76821	Category 2	2.7
23	Potassium hydroxide (5% w/v)	WPCI	168-21815	Category 2	3.0
24	Benzenethiol 5-(1,1-dimethylethyl)-2-methyl	TCI	7340-90-1	Category 2	3.3
25	1-Methyl-3-phenyl-1-piperazine	TCI	5271-27-2	Category 2	3.3
26	1,1,1-Trichloroethane	WPCI	200-02463	Category 2	4.0

^aReplacement for tri-isobutyl phosphate, and also used as the positive control.

KCC = Kanto Chemical Co. Inc.; TCI = Tokyo Chemical Industry Co. Ltd; WPCI = Wako Pure Chemical Industries Ltd; No cat. = no category.

assay, after exposure to distilled water (i.e. the negative control) or to various concentrations of an SLS solution for 18 hours.

Study protocol

The protocol for the LabCyte assay was developed by J-TEC, and was based on the EpiSkin protocol (29). In the first phase of the study, the SOP (version 4.1) did not include the measurement of IL-1 α release. At the second VMT meeting, in August 2008, the VMT discussed with the participating

laboratories the results of the first phase and the content of the SOP, including the acceptance criteria for this validation study. Subsequent SOPs reflected the results of this discussion.

The second and third phases used different versions of the SOP, versions 5.0 and 6.1, respectively. A major difference between these versions was the elimination of the measurement of IL-1 α release from version 6.1, in which the protocol specified that judgements on the classification had to be based on the LabCyte MTT assay alone. Other revisions were minor, and included changes in the description of the formula for calculating viability,

in the description of the use of a median of three runs for classification, and to the procedure for handling volatile substances. At a VMT meeting in July 2009, it was concluded that these revisions were minor, as long as the judgement for the classification was based on the MTT assay only. It was further decided that there was little difference between versions 5.0 and 6.1 of the SOP, apart from the elimination of the IL-1 α release measurement.

LabCyte EPI-MODEL24 tissues were shipped from the supplier on Mondays and delivered to the recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA), with 0.5ml per well of assay medium (which had the same composition as the culture medium), and incubated overnight (37°C, 5% v/v CO₂, humidified atmosphere). On the following day, the test chemicals were applied topically to the tissues. Liquids (25 μ l) were applied with a micropipette, and solids (25mg) were pre-moistened with 25 μ l sterile water before application. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied by using a micropipette with a cell saver-type tip. Each test chemical was applied to three tissues. In addition, 25 μ l of distilled water were added to three tissues, which served as the negative control, while three tissues were exposed to 25 μ l 5% w/v SLS as the positive control. After a 15-minute exposure, each tissue was carefully washed ten times with PBS (Invitrogen, Frederick, MD, USA) by using a wash bottle, to ensure the removal of any remaining test chemical from the surface. The washed tissues were then transferred to new 24-well plates, containing 1ml of fresh assay medium per well.

The test and control tissues were incubated for 42 hours (37°C, 5% CO₂, humidified atmosphere), then the conditioned medium was collected for determining the levels of IL-1 α , and the washed tissues were transferred to new 24-well plates with 0.5ml of freshly prepared medium containing 1mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Dojindo Co., Kamimashiki, Kumamoto, Japan), for the MTT assay. The tissues were incubated for 3 hours (37°C, 5% CO₂, humidified atmosphere), then transferred to microtubes containing 0.3ml isopropanol, which completely immersed the tissues. Formazan extraction was performed at room tem-

perature, and the tissues were allowed to stand overnight in the extraction solution. Subsequently, 200 μ l of the formazan-containing solutions were transferred to each well of a 96-well plate. The optical density of the samples was measured at 570nm with a reference wavelength of 650nm, and with isopropanol as a blank.

Tissue viability was calculated as a percentage, relative to the viability of the negative control, by using Equation 1 (see bottom of page).

The amount of IL-1 α released into the conditioned medium after the 42-hour incubation was determined by using an IL-1 α ELISA kit (Invitrogen), according to the manufacturer's instructions.

Prediction model of skin irritation

The acceptance criteria for the negative and positive controls, and the model for the prediction of skin irritation potential by using the LabCyte assay, were established to correspond to the equivalent conditions for EpiSkin, as described in the revised ECVAM performance standards (19). The acceptance criteria were:

1. a negative control OD greater than 0.7; and
2. a positive control viability lower than 40%.

The positive criteria are described in Table 3. If the mean viability was equal to or less than 50%, no measurable amount of IL-1 α was released.

The agreement between three independent viability measurements was used to indicate within-laboratory reproducibility, and the majority classification for each chemical was used to evaluate between-laboratory reproducibility.

The median value of three independent viability measurements was used to classify each chemical for the accuracy of the results. When only the tissue viability measurement was used for the classification, the classification was based entirely on the median of the three measurements. In the event that the three independent results from within an individual run were not in agreement, the mean of the two most similar results was used for the classification (i.e. minority classification), according to the positive criteria.

In the third phase of the study, the criteria for classification were revised as follows:

- a substance is irritant if the median tissue viability is < 50%
- a substance is non-irritant if the median tissue viability is \geq 50%

[Equation1]

$$\text{Tissue viability (\%)} = \frac{[\text{Mean measured OD}_{\text{sample}}]}{[\text{Mean measured OD}_{\text{negative control}}]} \times 100$$

where measured OD = [570nm OD_{sample} - 570nm OD_{blank}] - [650nm OD_{sample} - 650nm OD_{blank}].

Table 3: Positive criteria used for classification in the second phase of the study

Tissue viability (primary)	IL-1 α ELISA (secondary)	Classification
Mean tissue viability \leq 50%	—	Irritant
Mean tissue viability > 50%	Mean IL-1 α release \geq 120 pg/tissue	Irritant
Mean tissue viability > 50%	Mean IL-1 α release < 120 pg/tissue	Non-irritant

IL-1 α levels were not assessed, and IL-1 α release was not used in the classification.

The independent biostatisticians had developed a standard Microsoft Excel[®] datasheet for use in the collection of data. The biostatisticians assembled all the submitted datasheets, decoded the chemical identifiers, and created the datasets that formed the basis of all the analyses. The data management procedures and statistical tools applied were approved by the VMT.

Quality assurance

All the participating laboratories worked according to the OECD GLP principles. The independent biostatisticians confirmed the authenticity of the data entered on the datasheet by comparing outputs from the spectrophotometers. Furthermore, JaCVAM assured the quality of all the documents and datasheets.

Results

Quality control (QC) of the tissue models

The QC data for the tissue models employed during this validation study, which are not shown, demonstrated that the OD after the MTT assay, and therefore the viability of the tissue when exposed to distilled water and after treatment for 18 hours with various concentrations of an SLS solution, was stable among the different batches provided to each lab-

oratory. From these data, the VMT was able to confirm the completeness of the epithelial tissue layers used in this validation study. The participants did not need to perform any histological analysis after receiving LabCyte EPI-MODEL24, because J-TEC, the supplier of the tissues, had assessed the structure of all the batches prior to shipment. All the batches used for the validation study had passed the manufacturer’s model supply criteria of LabCyte EPI-MODEL24.

First phase

In the first phase of the study, all the data for the negative and positive controls met the acceptance criteria, as shown in Tables 4 and 5, respectively. With the sole exception of ethanol, none of the chemicals tested in this phase of the study showed a significant variation in the triplicate tests performed at each laboratory (for Laboratory F, data for one run is missing; Table 5). The VMT judged cell viabilities from ethanol exposure at most laboratories to be around 50%, and although the data obtained from the different laboratories were similar, the within-laboratory variation was considerable. The experiments performed in this first phase indicated the transferability of the LabCyte MTT assay throughout the laboratories participating in the study. Based on the results obtained, the VMT decided that the transferability of this assay was high, and that all seven laboratories would participate in the subsequent phase of the study.

Table 4: Absorbance data obtained for the negative control used in the first phase of the study

Laboratory	Experiment 1 Absorbance	Experiment 2 Absorbance	Experiment 3 Absorbance	Mean	SD
A	1.073	0.928	1.007	1.003	0.073
B	0.930	1.245	1.042	1.072	0.160
C	0.960	0.869	0.761	0.863	0.100
D	0.987	0.928	0.939	0.951	0.031
E	0.840	0.884	0.973	0.899	0.068
F	1.049	0.934	0.968	0.984	0.059
G	1.147	1.159	1.074	1.127	0.046

Table 5: Tissue viability data obtained for the positive control and three chemicals used in the first phase of the study

Chemical	Laboratory	Exp. 1 Viability (%)	Exp. 2 Viability (%)	Exp. 3 Viability (%)	Mean	SD
Positive control (5% w/v sodium lauryl sulphate)	A	6.35	27.55	15.67	16.52	10.63
	B	3.94	3.51	3.97	3.81	0.26
	C	5.45	4.81	3.49	4.58	1.00
	D	11.74	7.22	14.08	11.02	3.49
	E	31.60	9.76	38.61	26.66	15.05
	F	3.10	2.89	2.93	2.97	0.11
	G	4.46	7.17	2.62	4.75	2.29
Ethanol	A	62.67	39.12	46.61	49.46	12.03
	B	41.08	50.86	86.58	59.51	23.95
	C	68.13	34.13	67.31	56.53	19.40
	D	68.57	40.52	33.03	47.37	18.73
	E	54.19	72.08	60.55	62.27	9.07
	F	ND	64.16	47.98	56.07	11.44
	G	4.68	5.23	6.67	5.53	1.03
Glycerol	A	103.63	104.17	98.48	102.09	3.14
	B	85.50	100.58	67.97	84.68	16.32
	C	101.24	99.41	104.84	101.83	2.76
	D	103.30	101.35	89.73	98.13	7.34
	E	101.75	98.06	99.04	99.62	1.91
	F	ND	97.23	96.00	96.62	0.87
	G	94.00	98.16	103.6	98.59	4.82
Naphthalene acetic acid	A	109.13	90.73	97.78	99.22	9.28
	B	93.96	103.91	103.96	100.61	5.76
	C	103.66	102.11	117.3	107.69	8.36
	D	102.28	98.15	94.56	98.33	3.86
	E	107.11	104.39	97.36	102.95	5.03
	F	ND	101.34	102.07	101.7	0.52
	G	92.20	101.04	105.52	99.59	6.78

Exp. = experiment, *ND* = No data.

Second and third phases

One of the limitations of this study was that the experiments could not be performed under full GLP compliance, because some of the participating laboratories were not in GLP-compliant facilities. However, the VMT conducted the experiments according to GLP principles, and all the submitted datasheets and documentation sheets were verified.

There were a few comments from each laboratory in the document sheets; for instance: ‘the application of coded chemical No. 21 (cinnamaldehyde) caused the cups to become discoloured and crystallised’; and ‘the application of coded chemical No. 23 (potassium hydroxide 5% w/v) caused the model’s layers to become desquamated’.

With regard to the absorbance (i.e. mean OD) of the negative control for each run, in Laboratory A, the mean OD for run 1 was 0.59 (0.61, 0.58 and 0.57) in the second phase of the study. The VMT did not accept this result under the acceptance criteria and, instead, accepted the results of tests

2–4 from Laboratory A (runs 1, 2 and 3, respectively). The reason for this was unclear (Data not shown).

Excluding these data, all the negative control data from each laboratory involved in the study satisfied the pre-specified acceptance criteria, as shown in Table 4 for phase 1 (data for the second and third phases are not shown). Therefore, for this assay, the rate of invalid tests was 1/508 or 0.2% — i.e. 400 data points (3 runs × 7 laboratories × 19 chemicals + 1 run) in the second phase of the study, plus 108 data points (3 runs × 6 labs × 6 chemicals) in the third phase of the study.

Given the results of three independent cell viability readings, and the summary statistics for the positive control at each laboratory, the data were sufficient for the positive control to meet the acceptance criteria. The distribution of the data for cell viability after treatment with chemical No. 13 (5% w/v SLS, i.e. the same chemical as used as the positive control) showed high repeatability and reproducibility in the second and third phases of the study (Tables 6 and 7, respectively). The sum-

Table 6: Tissue viability data obtained for the positive control and chemical No. 13 in the second phase of the study

Chemical	Laboratory	Experiment 1 Viability (%)	Experiment 2 Viability (%)	Experiment 3 Viability (%)	Mean	SD
No. 13 ^a	A	12.2	3.6	2.2	6.0	4.4
	B	5.2	3.2	12.5	7.0	4.0
	C	9.9	5.0	3.3	6.1	2.8
	D	3.8	3.6	2.5	3.3	0.6
	E	12.9	6.7	4.7	8.1	3.5
	F	12.0	3.1	7.4	7.5	3.6
	G	10.7	8.0	3.3	7.3	3.1
Positive control	A	5.9	8.8	2.5	5.7	2.6
	B	5.2	12.3	7.8	8.4	2.9
	C	4.1	5.4	3.8	4.4	0.7
	D	5.7	2.6	3.3	3.9	1.3
	E	4.1	12.6	5.6	7.4	3.7
	F	3.5	2.9	3.2	3.2	0.2
	G	3.1	10.8	4.2	6.0	3.4

^aChemical No. 13 is 5% w/v sodium lauryl sulphate, which replaced the tri-isobutyl phosphate in the second phase of the study and which was also used as the positive control.

mary statistics for tissue viability data for each chemical in the second and third phases are shown in Table 8. As mentioned above, all the data obtained were valid.

The classifications based on the median of three independent cell viability results (i.e. taking into consideration data from the MTT assay only) are shown in Table 9. In the second phase of the study, the levels of IL-1 α release were measured in those cases where the mean tissue viability was > 50% (Table 10). As shown in Tables 10 and 11, IL-1 α results altered the classification of three chemicals. The classification of chemical No. 5 (allyl phenoxyacetate) by Laboratory F was changed, leading to its misclassification as a false positive result. In contrast, the classification of the other two chemicals had been underestimated, and they subsequently were placed in the correct category. Thus, while the use of IL-1 α release data was responsible for changing the classification of a few

chemicals, in general, it did not have a major effect. That is, the determination of IL-1 α release did not significantly contribute to the performance of the assay.

The VMT, therefore, decided that the following analysis should be performed only on tissue viability data (by using the MTT assay). With regard to the within-laboratory reproducibility of the LabCyte MTT assay, some disagreements occurred between measurements: discrepancies were detected between three triplicate viability measurements in Laboratories A and F, two in Laboratories B and D, and one in Laboratory E (none occurred in Laboratories C or G; data not shown). Of the total of 170 tests performed (on 25 chemicals in six laboratories, plus 20 chemicals in one laboratory), there were 11 (6.5%) discrepancies, resulting in a within-laboratory reproducibility ratio of 93.5%. Therefore, the VMT concluded that this assay showed high within-laboratory

Table 7: Tissue viability data obtained for the positive control in the third phase of the study

	Laboratory	Experiment 1 Viability (%)	Experiment 2 Viability (%)	Experiment 3 Viability (%)	Mean	SD
Positive control ^a	A	6.4	2.2	1.8	3.5	2.6
	B	9.4	2.3	1.7	4.4	4.3
	C	8.2	7.3	2.4	6.0	3.1
	D	3.5	2.5	2.1	2.7	0.7
	F	8.5	4.1	2.7	5.1	3.0
	G	11.7	2.5	3.3	5.8	5.1

^aThe positive control was 5% w/v sodium lauryl sulphate.

Table 8: A summary of the tissue viability data obtained for each chemical in the second and third phases of the study

No.	Statistics	Laboratory						
		A	B	C	D	E	F	G
a) In the second phase								
1	Mean	17.9	24.5	14.4	11.0	31.9	12.0	11.7
	SD	11.3	19.8	5.2	2.9	6.4	2.0	2.0
	Min	11.2	10.4	10.6	9.1	25.2	10.4	10.6
	Max	31.0	47.1	20.3	14.3	38.1	14.3	14.0
2	Mean	73.8	72.4	87.8	86.6	88.0	72.7	98.0
	SD	7.7	14.3	1.9	17.6	12.8	4.8	4.6
	Min	65.2	61.7	85.8	67.6	76.4	67.2	94.8
	Max	79.8	88.7	89.7	102.3	101.8	75.7	103.3
3	Mean	104.7	98.5	94.5	106.4	113.3	94.8	101.7
	SD	4.1	4.6	1.3	5.8	14.1	2.7	8.9
	Min	100.9	93.3	93.1	101.4	103.9	92.5	93.4
	Max	109.1	102.3	95.7	112.8	129.6	97.9	111.1
4	Mean	99.3	97.8	98.2	101.8	115.3	95.2	105.9
	SD	6.1	3.0	1.5	3.9	11.0	4.2	3.4
	Min	95.2	94.4	97.1	98.4	105.2	92.7	103.3
	Max	106.3	100.2	99.9	106.1	127.1	100.1	109.8
5	Mean	77.0	72.7	91.9	72.0	94.3	55.4	91.7
	SD	2.5	11.4	3.1	6.8	7.6	16.3	4.5
	Min	74.1	61.7	89.2	66.1	89.6	39.3	88.4
	Max	78.5	84.5	95.2	79.4	103.0	71.9	96.8
6	Mean	84.8	80.7	81.2	92.1	89.7	87.8	74.2
	SD	6.9	2.8	2.3	9.9	7.8	6.7	17.7
	Min	79.4	77.9	79.1	82.7	81.5	81.1	54.1
	Max	92.5	83.5	83.6	102.4	97.0	94.4	87.2
7	Mean	18.2	12.2	17.4	18.4	20.3	20.8	24.6
	SD	5.7	1.3	3.0	4.2	2.5	8.9	6.1
	Min	12.6	10.8	15.2	13.8	17.5	15.6	19.9
	Max	24.1	13.2	20.8	21.7	22.2	31.1	31.5
8	Mean	99.1	97.4	78.3	106.6	105.6	94.7	103.7
	SD	11.4	9.5	3.6	3.3	8.1	4.4	4.6
	Min	90.2	86.7	75.3	103.0	100.9	89.7	100.9
	Max	111.9	104.8	82.3	109.4	114.9	97.8	109.0
9	Mean	103.7	105.4	98.9	102.2	109.4	94.3	105.9
	SD	8.2	7.6	6.8	3.6	6.0	6.8	5.4
	Min	97.1	96.7	93.5	98.1	103.9	86.5	102.3
	Max	112.8	110.1	106.6	105.0	115.8	98.8	112.1
10	Mean	102.1	112.2	104.9	109.3	118.8	102.1	109.8
	SD	14.8	2.8	2.2	4.5	12.3	1.6	2.9
	Min	86.5	110.1	103.6	105.5	107.5	101.2	107.9
	Max	115.9	115.4	107.5	114.3	132.0	104.0	113.1
11	Mean	108.1	105.1	96.6	103.6	113.0	98.6	104.1
	SD	8.7	1.4	3.8	1.9	9.0	4.3	4.4
	Min	98.1	103.7	94.1	102.4	105.5	94.6	100.5
	Max	113.7	106.6	101.0	105.8	123.1	103.1	109.0
12	Mean	20.7	21.7	32.6	52.6	56.9	52.8	99.5
	SD	6.7	5.0	10.7	24.4	1.1	22.2	12.5
	Min	15.3	15.9	24.9	27.4	55.6	27.2	87.7
	Max	28.2	24.6	44.8	76.2	57.8	66.0	112.6

Table 8: continued

No.	Statistics	Laboratory						
		A	B	C	D	E	F	G
a) In the second phase								
13	Mean	6.0	7.0	6.1	3.3	8.1	7.5	7.3
	SD	5.4	4.9	3.4	0.7	4.3	4.5	3.7
	Min	2.2	3.2	3.3	2.5	4.7	3.1	3.3
	Max	12.2	12.5	9.9	3.8	12.9	12.0	10.7
14	Mean	8.2	9.7	11.1	10.9	12.2	15.6	12.0
	SD	2.6	2.1	3.1	0.8	1.9	2.2	1.4
	Min	6.6	8.3	9.1	10.2	10.4	13.1	10.6
	Max	11.1	12.1	14.7	11.7	14.2	17.0	13.5
15	Mean	8.8	9.8	13.5	8.6	10.3	7.2	14.4
	SD	2.1	0.4	5.6	0.6	1.4	1.4	9.0
	Min	7.1	9.3	8.1	8.0	8.7	5.9	9.2
	Max	11.1	10.2	19.3	9.2	11.3	8.6	24.7
16	Mean	53.3	81.4	73.1	45.0	89.1	59.1	77.0
	SD	18.7	23.9	18.9	31.2	9.8	8.6	11.0
	Min	32.2	54.1	51.5	18.1	78.7	50.4	64.9
	Max	67.9	98.3	86.3	79.2	98.2	67.5	86.5
17	Mean	5.5	4.9	5.8	5.3	6.9	5.6	5.6
	SD	0.7	0.6	0.4	1.4	1.8	1.2	0.5
	Min	4.8	4.5	5.3	3.9	5.4	4.5	5.3
	Max	6.1	5.7	6.0	6.6	8.9	6.9	6.2
18	Mean	61.9	65.7	88.7	70.9	85.1	73.9	90.9
	SD	31.7	29.8	2.2	12.4	15.0	6.0	4.5
	Min	25.3	46.5	87.3	59.0	69.1	69.2	85.9
	Max	82.1	100.0	91.2	83.7	98.9	80.6	94.4
19	Mean	28.7	39.2	22.5	25.6	59.5	66.3	39.8
	SD	19.6	32.4	12.5	6.5	29.7	23.9	4.5
	Min	15.0	10.9	10.0	18.2	26.1	40.1	35.8
	Max	51.1	74.6	35.0	30.4	83.1	87.0	44.7
20	Mean	23.3	14.0	8.6	19.2	8.4	8.0	8.1
	SD	12.1	9.3	1.6	10.8	2.3	0.2	1.2
	Min	9.3	8.0	7.6	9.6	6.2	7.8	6.7
	Max	31.1	24.8	10.4	30.9	10.7	8.2	8.8
b) In the third phase								
21	Mean	13.8	11.0	16.0	12.3	—	12.3	12.2
	SD	14.0	11.1	13.2	13.2	—	11.4	13.7
	Min	13.3	10.2	12.3	9.9	—	11.3	8.7
	Max	14.2	11.8	22.5	13.8	—	14.3	14.3
22	Mean	2.0	2.3	2.8	3.6	—	2.5	4.1
	SD	1.5	2.2	2.9	3.9	—	2.6	3.9
	Min	1.5	2.2	2.5	3.0	—	1.7	3.7
	Max	3.1	2.5	3.0	4.0	—	3.2	4.7
23	Mean	0.8	0.8	1.0	3.2	—	2.2	0.6
	SD	0.7	0.8	1.0	2.0	—	1.0	0.4
	Min	0.5	0.7	0.7	0.8	—	0.8	0.3
	Max	1.3	1.1	1.4	6.9	—	4.8	1.0
24	Mean	15.6	18.5	12.6	17.2	—	13.9	16.2
	SD	14.5	16.0	12.6	18.3	—	13.8	15.2
	Min	13.6	15.5	12.5	10.3	—	8.8	14.1
	Max	18.6	24.0	12.7	23.0	—	19.2	19.3

Table 8: continued

No.	Statistics	Laboratory						
		A	B	C	D	E	F	G
b) In the third phase								
25	Mean	3.4	3.2	3.4	5.3	—	4.2	4.1
	SD	3.9	3.4	3.4	3.9	—	4.2	4.1
	Min	1.8	2.7	3.3	3.7	—	3.2	3.4
	Max	4.5	3.5	3.5	8.2	—	5.0	5.1
26	Mean	5.5	5.8	6.6	5.7	—	5.9	7.2
	SD	5.6	6.1	6.5	5.4	—	5.2	7.2
	Min	5.4	4.2	6.5	5.4	—	5.0	6.8
	Max	5.7	7.2	6.8	6.4	—	7.4	7.6

‘—’ indicates that the data are not available from this laboratory.

Table 9: The classification obtained by using three independent tissue viability results from the second and third phases of the study

No.	GHS label	<i>In vivo</i> score	Laboratory						
			A	B	C	D	E	F	G
1	No category	0.0	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2	No category	0.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
3	No category	0.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
4	No category	0.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
5	No category	0.3	–ve	–ve	–ve	–ve	–ve	–ve	–ve
6	No category	0.3	–ve	–ve	–ve	–ve	–ve	–ve	–ve
7	No category	1.0	+ve	+ve	+ve	+ve	+ve	+ve	+ve
8	No category	1.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
9	No category	1.7	–ve	–ve	–ve	–ve	–ve	–ve	–ve
10	No category	1.7	–ve	–ve	–ve	–ve	–ve	–ve	–ve
11	No category	2.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
12	No category	2.0	+ve	+ve	+ve	–ve	–ve	–ve	–ve
21	No category	2.0	+ve	+ve	+ve	+ve	+ve	+ve	+ve
14	Category 2	2.3	+ve	+ve	+ve	+ve	—	+ve	+ve
15	Category 2	2.3	+ve	+ve	+ve	+ve	+ve	+ve	+ve
16	Category 2	2.7	–ve	–ve	–ve	+ve	–ve	–ve	–ve
17	Category 2	2.7	+ve	+ve	+ve	+ve	+ve	+ve	+ve
22	Category 2	2.7	+ve	+ve	+ve	+ve	—	+ve	+ve
23	Category 2	2.7	+ve	+ve	+ve	+ve	—	+ve	+ve
18	Category 2	3.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
19	Category 2	3.0	+ve	+ve	+ve	+ve	–ve	–ve	+ve
24	Category 2	3.3	+ve	+ve	+ve	+ve	—	+ve	+ve
25	Category 2	3.3	+ve	+ve	+ve	+ve	—	+ve	+ve
26	Category 2	4.0	+ve	+ve	+ve	+ve	—	+ve	+ve
20	Category 2	4.0	+ve	+ve	+ve	+ve	+ve	+ve	+ve

— = Not tested. Bold type indicates minority classification. +ve = positive (irritant); –ve = negative (non-irritant).

Table 10: An overview of the levels of IL-1 α release measured in each laboratory

No.	GHS label	In vivo score	Experiment	Laboratory						
				A	B	C	D	E	F	G
1	No category	0	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
2	No category	0	1	132.8*	52.9	59.3	41.2	60.7	61.3	9.4
			2	68.1	56.5	37.0	89.1	68.4	99.3	9.6
			3	97.6	41.1	76.0	72.4	46.0	70.1	12.6
3	No category	0	1	12.0	9.5	15.5	8.6	23.2	12.7	8.1
			2	7.1	8.6	11.7	19.9	10.5	9.2	11.9
			3	10.7	10.3	12.9	9.4	11.3	6.7	15.7
4	No category	0	1	10.0	6.0	8.0	11.7	9.5	2.5	6.3
			2	5.3	8.0	5.5	13.2	15.1	2.6	8.6
			3	6.3	4.7	7.2	7.9	9.7	3.4	6.8
5	No category	0.3	1	122.0*	97.6	24.3	81.2	57.7	183.5*	15.4
			2	35.7	63.5	35.1	115.3	36.6	—	28.5
			3	44.4	26	31.2	49.4	33.0	191.6*	33.2
6	No category	0.3	1	59.0	85.7	114.0	85.6	94.4	60.8	112.5
			2	62.9	93.6	104.9	139.5*	81.4	48.1	62.1
			3	68.8	85.1	82.9	64.5	52.9	54.8	147.1
7	No category	1	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
8	No category	1	1	8.2	9.4	84.1	4.1	6.9	21.4	5.3
			2	3.6	6.4	31.6	10.4	8.5	4.9	5.8
			3	6.0	4.1	33.1	5.2	6.7	2.1	7.2
9	No category	1.7	1	10.9	17.1	11.2	42.6	29.5	33.0	7.4
			2	19.8	8.8	8.8	32.2	6.5	25.3	9.7
			3	31.3	6.8	20.1	21.3	11.2	24.7	10.6
10	No category	1.7	1	27.9	7.4	31.3	41.2	46.5	39.3	9.8
			2	17.1	12.7	15.0	50.4	26.7	26.7	14.5
			3	66.2	12.2	30.0	42.1	26.3	24.2	13.2
11	No category	2	1	5.0	31.1	18.0	15.3	10.4	16.2	6.4
			2	3.3	11.9	15.8	19.0	9.7	8.1	7.5
			3	18.2	5.0	8.9	8.7	8.6	12.6	11.9
12	No category	2	1	—	—	—	157.2	120.4	—	34.5
			2	—	—	—	113	118.6	90.2	27.3
			3	—	—	—	—	58.3	66.2	13.6
14	Category 2	2.3	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
15	Category 2	2.3	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
16	Category 2	2.7	1	86.9	68.1	129.4	—	126.8	116.5	90.8
			2	—	100.2	74.4	169.7	76.1	107.5	70.9
			3	121.2*	42.5	83.6	—	73.1	87.3	79.2

*The mean IL-1 α release \geq 120pg/tissue; — = not tested.
 The values in bold refer to cases where IL-1 α release data changed the classification assigned by using the MTT assay data.

Table 10: continued

No.	GHS label	<i>In vivo</i> score	Experiment	Laboratory						
				A	B	C	D	E	F	G
17	Category 2	2.7	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
18	Category 2	3	1	61.5	—	60.6	90.3	86.9	114.5	18.0
			2	57.7	104.9	45.8	221.3*	98.7	76.4	45.1
			3	—	17.2	51.4	138.1*	63.9	102.2	22.1
19	Category 2	3	1	—	57.3	—	—	109.2	—	—
			2	—	—	—	—	—	69.2	—
			3	102.3	—	—	—	68.0	59.5	—
20	Category 2	4	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—

*The mean IL-1 α release \geq 120pg/tissue; — = not tested.

The values in bold refer to cases where IL-1 α release data changed the classification assigned by using the MTT assay data.

reproducibility. For the between-laboratory reproducibility of the LabCyte MTT assay, the minority classifications were for six results (chemical No. 12: three results; chemical No. 16: one result; and chemical No. 19: two results) among all the 170 classifications, as shown in Table 9. This corresponded to only 3.5% of the total, so the between-laboratory reproducibility was 96.5%. Based on these results, the VMT decided this assay showed high between-laboratory reproducibility.

For the evaluation of accuracy, the data for chemical No. 13 were excluded, because this chemical was not included in the lists within the original ECVAM performance standards (18) or in the ESAC statements (5, 6). The sensitivity, specificity and accuracy of the MTT assay (in relation to the UN GHS category) for the 25 chemicals tested in the second and third phases of the study are shown in Table 12. Collectively, the sensitivity, specificity and accuracy of this prediction model at the different laboratories varied between 75–91.6%, 69.2–76.9% and 76–84%, respectively. Similar val-

ues were obtained within each laboratory. The mean prediction values of the LabCyte MTT assay were: sensitivity 83.3%, specificity 73.1%, and accuracy 78.0% (Table 13). The ESAC statement proposed that the performance of a skin irritation model should be as follows: sensitivity 80%, specificity 70% and accuracy 75% (18, 19), so this requirement was fully satisfied.

Discussion

This validation study refers to the original, or the revised, ECVAM performance standards (18, 19). While the study was being conducted, the draft OECD performance standards, which were based on the ECVAM performance standards, were the subject of discussion by OECD international skin irritation experts. Therefore, we were unable to follow current OECD test guidelines for the calculation of within-laboratory and between-laboratory reproducibility, or during the data analysis (22).

Table 11: The chemicals that had their classification changed as a result of IL-1 α release data

No.	Name	CAS number	GHS label	<i>In vivo</i> score	Laboratory	Classification (MTT assay)	Classification (MTT and IL-1 α release)
5	Allyl phenoxyacetate	7493-74-5	No category	0.3	F	–ve	+ve
16	1-Bromohexane	111-25-1	Category 2	2.7	A	–ve	+ve
18	di- <i>n</i> -Propyl disulphide	629-19-6	Category 2	3.0	D	–ve	+ve

+ve = positive; –ve = negative.

Table 12: A comparison of the classification based on the LabCyte MTT assay data with the actual GHS classifications, in the second and third phases of the study

Index	Laboratory					
	A	B	C	D	F	G
Sensitivity	10/12 83.3%	10/12 83.3%	10/12 83.3%	11/12 91.6%	9/12 75%	10/12 83.3%
Specificity	9/13 69.2%	9/13 69.2%	9/13 69.2%	10/13 76.9%	10/13 76.9%	10/13 76.9%
Accuracy	19/25 76%	19/25 76%	19/25 76%	21/25 84%	19/25 76%	20/25 80%

A total of 25 substances were tested.

Reliability

All the negative control data from the LabCyte MTT assay showed high repeatability, as well as within-laboratory and between-laboratory reproducibility (data for the first phase is shown in Table 4). The same was true for the positive control data — high repeatability, within-laboratory and between-laboratory reproducibility were reported throughout the study (Tables 5–7).

When we conducted this study, we referred to the original ECVAM performance standards (18). In this document, one of the defined acceptance criteria was that the data range should have a standard deviation $\leq 18\%$. After the first phase of the study, the VMT discussed the topic in relation to the LabCyte assay. Ultimately, the VMT decided that this particular criterion (i.e. standard deviation $\leq 18\%$) was not appropriate, because, at the time, the amount of data was not enough to set this kind of range. Instead of this criterion, the VMT set another indicator of within-laboratory reproducibility — the agreement between three independent viability measurements. In order to evaluate between-laboratory reproducibility, majority classification for each chemical was used. As a result, the ratio of within-laboratory repro-

ducibility was 93.5%, and for between-laboratory reproducibility it was 96.5%. Based on these values, the VMT decided that the LabCyte MTT assay showed high within-laboratory and between-laboratory reproducibility. As shown in the next section, the reliability of the model was high. Therefore, the criteria with regard to the range may not be required for this *in vitro* tissue model, even though the variation should be assessed.

For three of the test chemicals, the classifications based on the LabCyte MTT assay were not consistent among all seven laboratories. These chemicals were No. 12 (terpinyl acetate), No. 16 (1-bromohexane) and No. 19 (butyl methacrylate), as shown in Table 9. We consider this a peculiarity of viability measurements close to the threshold, since when chemical treatment results in a tissue viability of about 50%, laboratory-dependent discrepancies might occur. Chemicals that elicit this sort of response might not be suitable for this type of validation study. It should be noted that chemicals No. 12 and No. 19 have been eliminated from the list of chemicals in the revised ECVAM performance standards (19).

On the other hand, the other 22 chemicals exhibited consistent classifications between the laboratories (Table 9), while chemical No. 18 (di-*n*-propyl

Table 13: The mean and range of sensitivity, specificity and accuracy of the LabCyte MTT assay classifications versus UN GHS classifications, in the second and third phases of the study

	<i>n</i>	Mean	Minimum	Maximum	ECVAM criterion
Sensitivity (%)	6	83.3	75.0	91.6	80.0
Specificity (%)	6	73.1	69.2	76.9	70.0
Accuracy (%)	6	78.0	76.0	84.0	75.0

A total of 25 substances were tested.

disulphide) exhibited large variation. These results indicate that the model is highly reliable.

Predictivity

The mean sensitivity, specificity and accuracy of this prediction model are 83.3%, 73.1% and 78.0%, respectively (Table 13). The ESAC statement proposed that the performance of a skin irritation model should be as follows: sensitivity 80%, specificity 70% and accuracy 75% (18, 19). Therefore, our results satisfied these values.

The VMT detected four false positives: chemicals No. 1 (1-bromo-4-chlorobutane), No. 7 (4-methylthio-benzaldehyde), No. 12 (terpinyl acetate) and No. 21 (cinnamaldehyde) were among the 13 chemicals classified as non-irritant. Furthermore, there were three false negatives: chemicals No. 16 (1-bromohexane), No. 18 (di-*n*-propyl disulphide) and No. 19 (butyl methacrylate), which were among the 12 chemicals classified as irritant. In order for this model to be of use in regulatory assessment, it is important to determine the causes of false-negative or false-positive results. Although chemical No. 18 is an irritant in rabbits, it is a non-irritant chemical with human tissue. We propose that chemical No. 18, like chemical No. 16, was scored as a false negative because its viability was around 50%, which caused different results to be obtained in different laboratories for these two coded chemicals.

The necessity of IL-1 α release measurement

IL-1 α is a cytokine produced by keratinocytes, and is a well-known irritation marker. It is a key player when the mode of action of skin irritation is considered (30). Previously, Coquette *et al.* reported that the up-regulation of IL-1 α mRNA was observed after topical application of sensitizers and irritants, but only the latter significantly increased extracellular IL-1 α (31). The determination of IL-1 α release levels, in association with MTT conversion to formazan, is necessary to discriminate and classify between irritant and sensitising agents in a single assay, and thus represents a potential *in vitro* alternative to two classical *in vivo* assays. Spielmann *et al.* (16) reported the necessity of these endpoints with EpiSkin. For the MTT assay only, the sensitivity was 75% and the specificity was 81%. When the MTT and IL-1 α release assays were combined, the sensitivity increased to 91% and the specificity was 79% (16). The ESAC has also made recommendations on the same combination of assays, MTT and IL-1 α release, when using EpiSkin (6).

In contrast, our data show that IL-1 α release determination changed the classification only for

chemicals No. 5 (allyl phenoxyacetate) in Laboratory F, No. 16 (1-bromohexane) in Laboratory A and No. 18 (di-*n*-propyl disulphide) in Laboratory D, as shown in Tables 10 and 11. The effect of IL-1 α release data on the reliability of these results was small. Therefore, the VMT considered the IL-1 α release measurement in the LabCyte assay to be unnecessary for this protocol, although additional validation studies involving IL-1 α release will be required to confirm this decision. Spielmann *et al.* have also reported the sensitivity of the EpiDerm assay (MTT data only) to be 57% and the specificity 85%, while the predictive capacity of the EpiDerm assay did not improve by the measurement of IL-1 α release (16). In addition, OECD Test Guideline No. 439 has not approved the use of IL-1 α release determination in the *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method (22).

Conclusion

The LabCyte MTT assay demonstrated high reliability, both within-laboratory and between-laboratory, and good reliability for the positive control (100%). In addition, the data showed acceptable relevance (77.5% overall accuracy, 82.3% overall sensitivity and 72.6% overall specificity) by using solely the MTT assay. Therefore, we found the assay to be suitable for use as a stand-alone method to distinguish between skin irritants and non-irritants. However, these results were based on, at most, 25 chemicals. Since this model demonstrated high reliability, we plan further investigations with additional chemicals, which will be conducted by the lead laboratory.

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動物実験代替法における国際協調

小島 肇夫

要約：動物実験代替法（以下、代替法と記す）の開発を促すために各国に設立された代替法バリデーションセンターの協調を図るため、2009年に日米欧カナダの参加で、代替試験法協力国際会議（ICATM: International Cooperation on Alternative Test Methods）の覚書が交わされ、代替法の開発に国際協調がより重要視されることになった。さらに、2011年には韓国の追加参加が決まり、新たな覚書が交わされた。このICATMの取り決めに準じた日本動物実験代替法評価センター（JaCVAM: Japanese Center for the Validation of Alternative Methods）の活動状況および今後の国際対応についてまとめた。本来なら、ICATMの傘下においてJaCVAMは欧米間での調整役を果たすべきであるが、体制が未熟で層が薄いJaCVAMにはその役割が望まれていない。国際的な位置付けを高めるためにも、日本の技術力を利用して、より多くの画期的な試験法をテストガイドラインに提案していきたい。そのためには日本人専門家の協力が不可欠であると考えている。

はじめに

欧米の影響を受け、日本でも動物実験の3R（削減、苦痛の軽減、置き換え）(1)のニーズが社会的にも、経済的にも増している(2)。しかし、動物実験の3Rの促進には総論として賛成される方が多いが、各論となると種々の問題点が挙げられ、その必要性に苦言を呈する方が多い。たとえば、3Rの最終目的である動物実験代替法（以下、代替法と記す）の開発には否定論が多い。これは、現在の代替法の適用が有害性の同定に限られており、動物実験で得られるようリスク評価ができない。その試験法の多くが遺伝毒性や局所毒性の検出に限定されているにもかかわらず、1つの試験法のみでは代替が不可能であること等の理由による。代替法で複雑な生体を模倣することは極めて難しく、反復投与が必要な毒性は検出できず、これまで以上に経費が掛かってしまう状況である。人類の進歩を考慮した場合、質が悪く、高コストのものにどうして変えていく必要があるのか。このような科学的・経済的な必要性への疑問もあり、日本では代替法を促進する社

会的な必要性が少ない。欧州のように動物福祉ありきで成立された法律により、その方向性が明示されている社会と比較して、その必要性が認知されていない日本では、その対策も国際社会に歩調を合わせるに留まっている。

このような状況の中、本書では代替法の公定化に関する国際協調の状況をまとめた後、日本の活動現状をまとめた。これらを把握した上で、今後の日本の国際対応を考えてみたい。

1. 国際協調の動向

代替法の開発に関して、化学物質等の安全性試験の公定化には厳密な国際ルールが作られている。これが2005年に発行されたOECDガイダンス文書（GD: Guidance Document）No.34である(3)。この文書の中には、今後、新規試験法が公定化される場合のバリデーションや専門家による第三者評価（以後、第三者評価と記す）に関する手順、手法が記載されている。すなわち、新規試験法が公定化されるにはバリデーションや第三者評価、行政的な受入れのための評価を経なければいけない。ところが、バリデーションや第三者評価を実施すると言っても、バリデーションの実施や組織の構築にはノウハウが多く、正確性や再現性の確認に大変な労力を要する。第三者評価においても種々の専門家への要請、公的な認証までの手順をも考慮する必要がある。そこで、このガイダンスに先立ち、世界各地に代替法バリデーションセンターが設立された。1990年代に米国ではNICEATM（The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods）/ICCVAM（Interagency Coordinating Committee on the Validation of Alternative Methods）(4)、欧州にはECVAM（European Center for the Validation of Alternative Methods）が設立された(5)。これらのセンターの役割・規模は表1に示すように、それぞれ法律で規定されている。特に、ICCVAMやECVAMはその役割を果たすために必要な規模を有しており、代替法への関与を精力的に進めている。さらに、遅れて

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2005年に日本に日本動物実験代替法評価センター (JaCVAM: Japanese Center for the Validation of Alternative Methods) が設立された(6)。これらセンターの国際協調を進めるためもあり、2009年4月には日米欧カナダの参加で、代替試験法協力国際会議 (ICATM: International Cooperation on Alternative Test Methods) の覚書が交わされ(7)、代替法の開発に国際協調が重要視されることになった。この組織は化粧品規制協力国際会議 (ICCR: International Cooperation on Cosmetic Regulation) によって設立されたが(8)、化粧品の安全性評価だけでなく、医薬品を含む化学物質全般の代替法開発を進めている。さらに、2009年11月には韓国にバリデーションセンター (KoCVAM: Korean Center for the Validation of Alternative Methods) が設立され(9)、2011年3月にはKoCVAMのICATMへの追加参加が決まった。5ヵ国による新たな覚書が交わされて、拡張が始まっている。

以下に、このICATMの目的を示す。もっとも重要な設立目的は、④に示す代替法開発における効率と効果の向上である。

- ①代替法の有用性と限界を明らかにし、国内外における行政的な受入れを促すために必要なバリデーション研究の適切な計画と実施
- ②透明性と利害関係者が意見を寄せる機会を組み込んだ代替法の科学的な第三者評価
- ③バリデートされた代替法を、有用性と限界を明らかにした上で、行政的な試験法として推奨
- ④代替法への取り組みと活用のため限られた資源の重複を避け、効率と効果の向上
- ⑤代替法の国際的な承認をタイムリーに支援
- ⑥適切な代替法の開発と利用を促進

このようなICATMの取り決めに基づき、それぞれのバリデーションセンターは国際協力を前提に、バリデーションや第三者評価をより積極的に進めることになった。これらの過程を経て、最終的には経済協力開発機構 (OECD: Organisation for Economic Co-operation

and Development) (10)、日米EU医薬品規制調和国際会議 (ICH: The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) (11)等のテストガイドラインの成立を促すことが最終的なゴールである。実際、2009年以降、OECDテストガイドラインになった試験法は、ICATMの合意に基づく国際的協力を経て採択された。具体的な試験法の進捗を表2に示したが、OECDテストガイドラインとなった代替法としては、2009年に、日本から提案していた内分泌かく乱物質の検出 (スクリーニング) を指標としたガイドライン The Stably Transfected Human Estrogen Receptor-alpha Transcriptional Activation Assay for Detection of Estrogenic Agonist (STTA) No.455が採択された。また、眼刺激性試験代替法 BCOP (bovine corneal opacity/permeability assay: 牛摘出角膜試験) No.437 および ICE (isolated chicken eye assay: 鶏摘出眼球試験) No.438 も採択された。いずれも腐食性・強い眼刺激性を検出できる方法としてICCVAMから提案された(4)。これら代替法は異例の早さとなる申請後たった1年でOECDテストガイドラインとなった。2010年には、再構築ヒト表皮モデルを用いた皮膚刺激性試験がOECDテストガイドラインNo.439として採択されるとともに、2002年に採択されていた皮膚感作性試験である局所リンパ節試験 (LLNA: local lymph node assay) テストガイドラインNo.429が改訂された。さらに日本で開発・バリデートされた放射性物質を用いない皮膚感作性試験 LLNA: DAがNo.442Aとして、LLNA: BrdU-ELISAがNo.442Bとして採択された。また、やや時間を要したが、in vitro 哺乳類細胞小核試験がテストガイドラインNo.487として採択された。

さらに、次のテストガイドラインを目指す試験法がICATMのワークプランに挙がっている。これらの試験法を表2に示す。現在、OECDテストガイドライン案として検討対象となっている試験法も多い(12)。こ

表1 VAM (Validation of Alternative Methods) 組織の比較

機関名	正式名	母体組織	基盤法	成立年	規模 (正規職員数)	備考
ECVAM	European Center for the Validation of Alternative Methods	Joint Research Center	Directive 86/609/EEC	1991.1	20名	ポストドク30名以上がサポート
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods	NICATM (The NTP Interagency Center for the Evaluation of Alternative Toxicological Methods)	NIH Revitalization Act of 1993, ICCVAM Authorization Act of 2000	1994	NICATMとして3名	ILSという企業のポストドク10名程がサポート
JaCVAM	Japanese Center for the Validation of Alternative Methods	国立衛研	なし	2005.11	2名	
KoCVAM	Korean Center for the Validation of Alternative Methods	Korean Food and Drug Association	不明	2009.11	3名	
ICATM	International Cooperation on Alternative Test Methods	日米欧カナダ韓国 VAM	なし	2009.4		各国VAMの緩やかな協力体制、カナダにはVAMはないが、Health Canadaが参加