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H. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

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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-1α) 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-1α 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-1α), 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-1α) 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

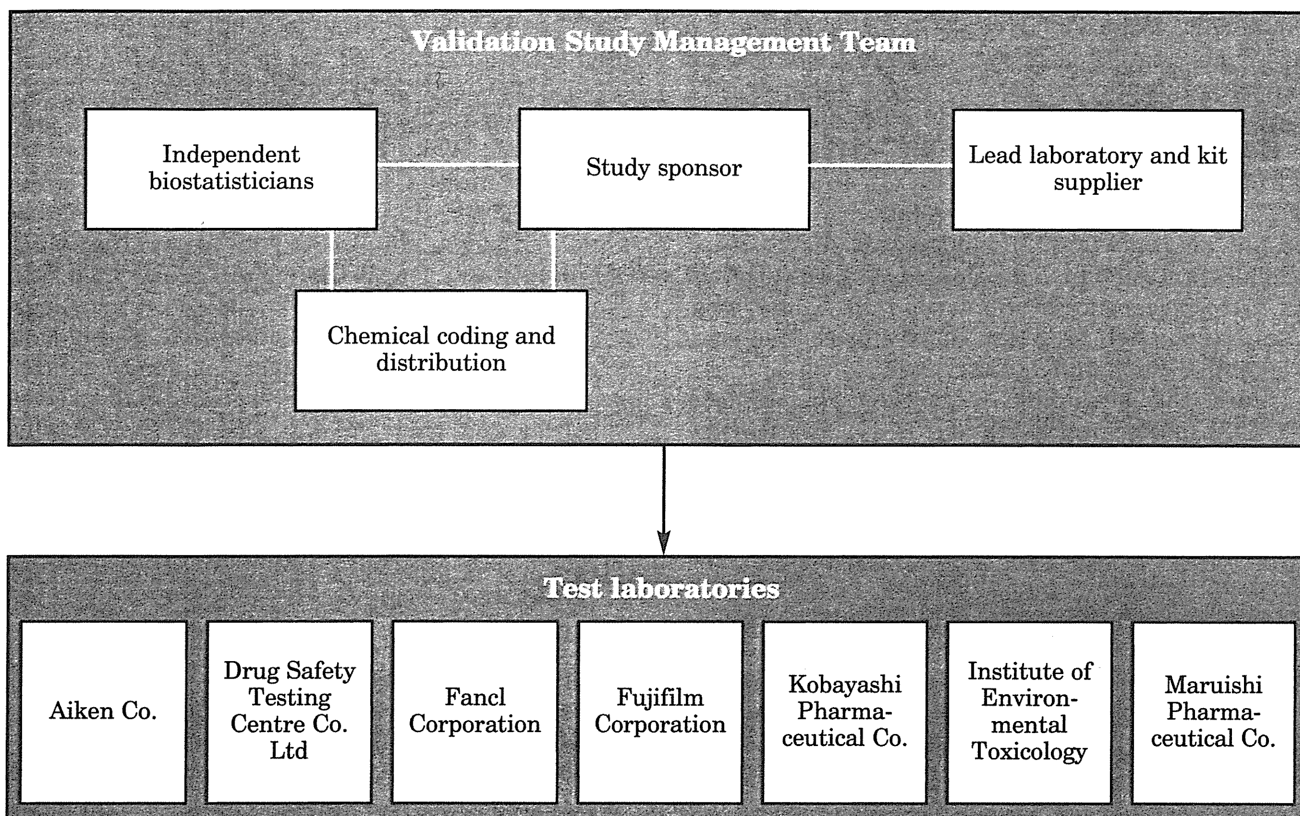
Validation study management structure

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 $0.3cm^2$ — 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 multilayer 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-*n*-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-dimethyethyl)-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

[a]*Replacement 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-1α 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-1α 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-1α 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 CO_2, humidified atmosphere). On the following day, the test chemicals were applied topically to the tissues. Liquids (25μl) were applied with a micropipette, and solids (25mg) were premoistened with 25μl 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, 25μl of distilled water were added to three tissues, which served as the negative control, while three tissues were exposed to 25μl 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% CO_2, humidified atmosphere), then the conditioned medium was collected for determining the levels of IL-1α, 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-diphenyltetrazolium bromide (MTT; Dojindo Co., Kamimashiki, Kumamoto, Japan), for the MTT assay. The tissues were incubated for 3 hours (37°C, 5% CO_2, humidified atmosphere), then transferred to microtubes containing 0.3ml isopropanol, which completely immersed the tissues. Formazan extraction was performed at room temperature, and the tissues were allowed to stand overnight in the extraction solution. Subsequently, 200μl 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-1α released into the conditioned medium after the 42-hour incubation was determined by using an IL-1α 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-1α 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 ≥ 50%

[Equation1]

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

where measured OD = $[570\text{nm OD}_{\text{sample}} - 570\text{nm OD}_{\text{blank}}] - [650\text{nm OD}_{\text{sample}} - 650\text{nm OD}_{\text{blank}})$.

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

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

IL-1α levels were not assessed, and IL-1α 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 laboratory. 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	A	6.35	27.55	15.67	16.52	10.63
(5% w/v sodium lauryl sulphate)	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

[a]*Chemical 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-1α release were measured in those cases where the mean tissue viability was > 50% (Table 10). As shown in Tables 10 and 11, IL-1α 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-1α 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-1α 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

[a]*The 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	*In vivo* 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-1α 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-1α release ≥ 120pg/tissue; — = not tested.*
The values in bold refer to cases where IL-1α release data changed the classification assigned by using the MTT assay data.

Table 10: continued

No.	GHS label	*In vivo* 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-1α release ≥ 120pg/tissue; — = not tested.*
The values in bold refer to cases where IL-1α 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 values 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-1α release data

No.	Name	CAS number	GHS label	*In vivo* score	Laboratory	Classification (MTT assay)	Classification (MTT and IL-1α 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-*n*-Propyl disulphide	629-19-6	Category 2	3.0	D	–ve	+ve

+ve = positive; –ve = negative.