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VALIDATION REPORT FOR THE SKIN IRRITATION TEST METHOD USING LABCYTE EPI-MODEL24

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or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80 E-mail: ehscont@oecd.org

E-mail: ehscont@oecd.org

FOREWORD

This document presents the validation report for the "*Skin Irritation Test Using the LabCyte EPI-MODEL24*". The project for developing a Test Guideline for an *in vitro* epidermal model to assess skin irritation using the LabCyte EPI-MODEL24, led by Japan, was included in the work plan of the Test Guidelines Programme in 2009. The Working of National Coordinators of the Test Guidelines Programme endorsed this validation at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) agreed to its declassification on 5 August 2011.

A validation peer review report, accompanied by a report on additional validation work, is also expected to be published in the Series on Testing and Assessment.

This document is published under the responsibility of the Joint Meeting.

MEMBERS OF LABCYTE VALIDATION MANAGEMENT TEAM

Mr. Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods
Mr. Takashi Omori; Kyoto Univ.
Mr. Kenji Idehara; Daicel Chemical Co.
Mr. Isao Yoshimura; Tokyo University of Science
Mr.Etsuyoshi Miyaoka; Tokyo University of Science
Mr. Masakazu Kato; Japan Tissue Engineering Co. Ltd.
Mr. Toshiro Yokouchi; Japan Tissue Engineering Co. Ltd.

Participant laboratories

- Aiken Co., Ltd. (Ms Yoko Ando and Ms Yui Asako)
- KOBAYASHI Pharmaceutical Co., Ltd. (Mr. Yoshihiro Yamaguchi and Ms Maki Nakamura)
- The Institute of Environmental Toxicology (Mr Tadashi Kosaka and Mr Koichi hayashi)
- Fancl Corp. (Ms. Tamie Suzuki and Ms Runa Izumi)
- FUJIFILM Corporation (Ms. Atsuko Yuasa, and Mr Shinichi Akimoto)
- Maruishi Pharmaceutical Co., Ltd. (Mr Yukihiko Watanabe and Mr Osamu Mitani)
- Drug Safety Testing Center Co., Ltd. (Mr Shinsuke Shinoda and Ms Saori Hagiwara)

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1. Goal statement

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

2. Objective

1. The *in vitro* test system, employing reconstructed human epidermis model (RhE: LabCyte EPI-MODEL24), has progressed through protocol optimisation as in vitro skin irritation test. The multi-laboratory assessment of this system performed based on the a few ECVAM performance standards (ESAC statement, 2007, 2008, 2009). This report shows the results of 3rd phase validation study in accordance with the revised reference chemicals described by the new ESAC statement 2009.

2. The present objective was to conduct a validation study to assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of this test system with a challenging set of coded 25 test chemicals 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* (No. 34, OECD, 2005) and according to the Modular Approach to validation (Hartung *et al.* 2004).

3. Test Method

3-1. Reconstructed human cultured epidermal model

3. LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by Japan Tissue Engineering Co. Ltd. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, they were cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978). Reconstruction of human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface 0.3 cm^2) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. It constructs a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18°C (Kato, 2009).

3-2. MODEL SUPPLIER

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

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

6. The audits focused on the procedures established to guarantee a defined quality of the tissue models.

4. Validation Management structure

7. This validation study was performed by the Japanese Society for the Alternative to Animal Experiments (JSAAE).

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

4-1. Validation Management Group

8. The Validation Management Team (VMT), which plays a central role overseeing the conduct of the validation study, includes:

1) Goal statement
2) Project plan including objective
3) Study protocol / amendments
4) Outcome of QC audits
5) Test chemicals
6) Data management procedures
7) Timeline/ study progression
8) Study interpretation and conclusions
9) Reports and publication

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

Members:

A chair (Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods)
The sponsor representative: representatives of JSAAE (Takashi Omori; Kyoto Univ., Kenji Idehara; Daicel Chemical Co. and Isao Yoshimura; Tokyo University of Science)
The sponsor representative, LabCyte EPI-MODEL24suppliers and lead lab (Masakazu Kato : Japan Tissue Engineering Co., Ltd, J-TEC)

4-2. Chemical selection, acquisition, coding and distribution

1) Definition of selection criteria
2) Chemical selection
3) Liaise with suppliers
4) Final check of chemicals provided
5) Acquisition
6) Coding
7) Distribution

Member

Hajime Kojima, JaCVAM

4-3 . Independent biostatisticians

1) Approve spreadsheets
2) Collect data
3) Analyse data

Members:
Takashi Omori: Kyoto Univ., Etsuyoshi MIyaoka and Kenya Ishiyama: Tokyo University of Science

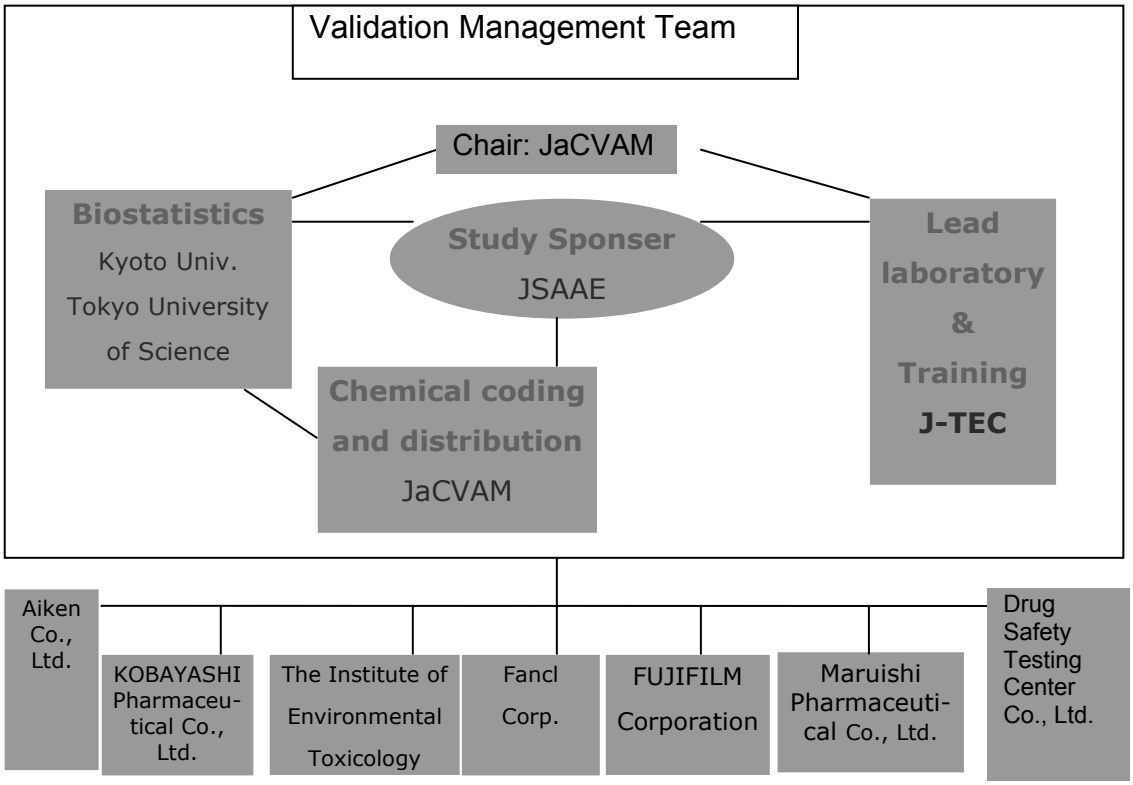


Figure 1. Management structure of the JSAAE skin irritation validation study

4-4. Participating laboratories

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

The following 6 laboratories participated in the validation study for the evaluation of the LabCyte EPI-MODEL24 assays:

- Laboratory 1 – Aiken Co., Ltd. (Yoko Ando and Yui Asako)
- Laboratory 2 – KOBAYASHI Pharmaceutical Co., Ltd. (Yoshihiro Yamaguchi and Maki Nakamura)
- Laboratory 3 – The Institute of Environmental Toxicology (Tadashi Kosaka and Koichi hayashi)
- Laboratory 4 – Fancl Corp. (Tamie Suzuki and Runa Izumi)
- Laboratory 5 – FUJIFILM Corporation (Atsuko Yuasa, and Shinichi Akimoto)

  This laboratory was not participated at the 3rd Phase study.
- Laboratory 6 – Maruishi Pharmaceutical Co., Ltd. (Yukihiko Watanabe and Osamu Mitani)
- Laboratory 7 – Drug Safety Testing Center Co., Ltd. (Shinsuke Shinoda and Saori Hagiwara)

A lead laboratory is also identified as J-TEC (Mr. Masakazu Kato and Mr Toshihiro Yokouchi). This laboratory was not participated in the validation study.

Each laboratory also was responsible for complying with GLP-like principles and specifying QA aspects.

4-5. Sponsorship

The study was managed and finance by JSAAE and J-TEC .

1) JSAAE finance:

- the management of the study (VMT meetings)
- the independent statistical support (biostatistician)
- the responsible for the chemicals purchase, coding and distribution to the laboratories
- the independent QC audit of the data
- the publication of the study

2) *J-TEC finance:*

- the lead laboratories for the test method
- training for the participating laboratories
- the independent QC audit on the LabCyte EPI-MODEL24
- the financial assistance for the participated laboratories

5. Study design

10. Before this validation study, the training course using LabCyte EPI-MODEL24 was performed by J-TEC on April, 2008. All technicians from each laboratory participated at this training course.

11. Three phases of validation studies were performed. In the 1st phase, we confirmed the transferability of the test protocol and assessed its reproducibility, by testing three coded chemicals (ethanol, glycerol and napthalen acetic acid) and a positive control (5% sodium lauryl sulfate solution) in seven laboratories between June and July of 2008.

12. In the 2nd phase study, we confirmed the intra- and inter-laboratory reproducibility robustness, and the correlation of test using 19 new chemicals tested in reference to the original EPISKIN performance standards (ECVAM, 2007) . These tests were conducted by 7 laboratories between September 2008 and January of 2009.

13. In the 3rd phase study, we confirmed the intra- and inter-laboratory reproducibility robustness, and the correlation of test using 6 chemicals tested in reference to the new EPISKIN performance standards (ESAC statement, 2009). This study was conducted by 6 laboratories, which attend the 1st and 2nd phase validation study between April to May, 2009.

6. Test Chemical

6-1. Chemicals Selection and list

14. In 1st phase study, JaCVAM selected three coded chemicals (ethanol, glycerol and napthalen acetic acid) to test.

15. According to the original ESAC Performance Standard (ESAC statement,2007) in 2nd Phase, the VMT selected 19 new chemicals to test in Table 1. One chemical, tri-isobuthyl phosphate (No. 13) on the chemical list reference for the original ECVAM Performance Standard cannot be purchased on the Japanese market. The VMT is responsible for the final approval of the chemicals proposed by JaCVAM. To avoid any potential bias in the final selection, the laboratory representatives on the VMT were not party to these discussions, nor were they informed of the final list of test chemicals for either phase of the validation study.

16. According to the new ECVAM performance standard (ESAC statement, 2009) in 3rd phase, the VMT selected 6 new chemicals tested in Table 2. The final approval of the chemicals proposed by JaCVAM is the responsibility of the VMT. To avoid any potential for bias in the final selection, the laboratory representatives on the VMT did not be party to these discussions, nor were they made aware of the chemicals finally approved for testing in either phase of the validation study.

Table 1. Reference test chemicals and codes

No.	Chemical	CAS number	GHS label	In vivo score (PII)	Laboratory						
					a	b	c	d	e	f	g
01	1-bromo-4-chlorobutane	6940-78-9	no	0	A-01	B-099	C-077	D-115	E-133	F-031	G-049
02	diethyl phthalate	84-66-2	no	0	A-02	B-100	C-078	D-116	E-134	F-032	G-050
03	di-propylene glycol	25265-71-8	no	0	A-03	B-081	C-079	D-117	E-135	F-033	G-051
04	naphtalen acetic acid	86-87-3	no	0	A-04	B-082	C-080	D-118	E-136	F-034	G-052
05	allyl phenoxy-acetate	7493-74-5	no	0.3	A-05	B-083	C-061	D-119	E-137	F-035	G-053
06	isopropanol	67-63-0	no	0.3	A-06	B-084	C-062	D-120	E-138	F-036	G-054
07	4-methyl-thio-benzaldehyde	3446-89-7	no	1	A-07	B-085	C-063	D-101	E-139	F-037	G-055
08	methyl stearate	112-61-8	no	1	A-08	B-086	C-064	D-102	E-140	F-038	G-056
09	allyl heptanoate	142-19-8	no	1.7	A-09	B-087	C-065	D-103	E-121	F-039	G-057
10	heptyl butyrate	5870-93-9	no	1.7	A-10	B-088	C-066	D-104	E-122	F-040	G-058
11	hexyl salicylate	6259-76-3	no	2	A-11	B-089	C-067	D-105	E-123	F-021	G-059
12	terpinyl acetate	80-26-2	no	2	A-12	B-090	C-068	D-106	E-124	F-022	G-060
13	5(W/V %) SLS				A-13	B-091	C-069	D-107	E-125	F-023	G-041
14	1-decanol	112-30-1	Category 2	2.3	A-14	B-092	C-070	D-108	E-126	F-024	G-042
15	cyclamen aldehyde	103-95-7	Category 2	2.3	A-15	B-093	C-071	D-109	E-127	F-025	G-043
16	1-bromohexane	111-25-1	Category 2	2.7	A-16	B-094	C-072	D-110	E-128	F-026	G-044
17	α-terpineol	98-55-5	Category 2	2.7	A-17	B-095	C-073	D-111	E-129	F-027	G-045
18	di-n-propyl disulphide	629-19-6	Category 2	3	A-18	B-096	C-074	D-112	E-130	F-028	G-046
19	butyl methacrylate	97-88-1	Category 2	3	A-19	B-097	C-075	D-113	E-131	F-029	G-047
20	heptanal	111-71-7	Category 2	4	A-20	B-098	C-076	D-114	E-132	F-030	G-048

1) CAS No.: Chemical abstracts service registry number.
2) PII: Primary irritation index.

Table 2. Test chemicals and code.

No.	Chemical	CAS number	GHS label	In vivo Score	Laboratory					
					a	b	c	d	f	g
A	Cinnamaldehyde	104-55-2	no	2	A-151	B-176	C-196	D-216	F-236	G-256
B	2-Chroromethyl-3,5-dimethyl-4-methoxypyridine HCl	322-76821	Category 2	2.7	A-154	B-173	C-192	D-211	F-233	G-253
C	Potassium hydroxide (5%aq)	168-21815	Category 2	3	A-156	B-175	C-194	D-213	F-232	G-251
D	Benzenethiol, 5-(1,1-dimethyethyl)-2-methyl	7340-90-1	Category 2	3.3	A-153	B-172	C-191	D-214	F-234	G-254
E	1-Methyl-3-phenyl-1-piperazine	5271-27-2	Category 2	3.3	A-152	B-171	C-195	D-215	F-235	G-255
F	1,1,1-Torichloroethane	200-02463	Category 2	4	A-155	B-174	C-193	D-212	F-231	G-252

1)CAS No.: Chemical abstracts service registry number.

6-2. Deficit chemical

17. In Table1, tri-isobuthyl phosphate (No. 13) could not be used in the examination because it was not available in Japan. Therefore, a 5% SLS solution was used instead of tri-isobuthyl phosphate. The data obtained with the 5% SLS solution were not used for calculating the predictivity of the test.

6-3. Chemical Coding and distribution

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

7. Protocol

7-1. Protocol of the skin irritation test with LabCyte EPI-MODEL

19. In 2nd phase study, we used the SOP (ver. 5.0) and we used the SOP (ver. 6.1) in 3rd phase study. The revised points, which make the deletion measurement of IL-1α, revise calculating formula of viability, classification used median of 3trails and how to treat of volatile substances were shown in change tracking of the SOP (ver. 6.1). The VMT made judgments that these revise points were minor and difference with the SOP (ver.5.0) used by 2nd phase study and this version was little in the VMT meeting on July 17, 2009.

20. LabCyte EPI-MODEL tissues were shipped from the supplier on Mondays and delivered to recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 mL), and incubated overnight (37°C, 5% CO_2 humidified atmosphere). On the following day, the tissues were topically exposed to the test chemicals. Liquids (25 µL) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25 µL sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25 µL distilled water, and three tissues serving as

positive controls were exposed to 5% SLS. After a 15-minute exposure, each tissue was carefully washed with PBS (Invitrogen, CA, USA) 10 times using a washing bottle to remove any remaining test chemical from the surface. The blotted tissues were then transferred to new 24-well plates containing 1 mL of fresh assay medium.

21. The treated and control tissues were incubated for 42 hours (37°C, 5% CO_2 humidified atmosphere). When the 42-hour post-incubation period was complete, blotted tissues were transferred to new 24-well plates containing 0.5 mL of freshly prepared MTT medium (1 mg/mL; Dojindo Co., Kumamoto, Japan) for the MTT assay and conditioned medium was collected to determine the interleukin-1 alpha (IL-1α) levels. Tissues were incubated for three hours (37°C, 5% CO_2 humidified atmosphere) and then transferred to microtubes containing 0.3 mL isopropanol, which completely immersed the tissue. Formazan extraction was performed at room temperature, and the tissues were allowed to stand overnight. Subsequently, 200-µL extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and 650 nm as a reference absorbance, with isopropanol as a blank.

22. The tissue viability was calculated as a percentage relative to the viability of the negative controls. The median of three values from identically treated tissues was used to classify a chemical according to the prediction model.

23. The amount of IL-1α released in the conditioned medium after 42 hours was determined using an IL-1α ELISA kit (Invitrogen, CA, USA), following the manufacturer's detailed instructions.

7-2. Prediction model of skin irritation

24. In this study, the prediction model of skin irritation potential with LabCyte EPI-MODEL was set to refer to the conditions for EPISKIN described in the ECVAM Performance Standards. This prediction model is described in Table 3. In the event that the three independent results within an individual batch were not consistent, the result that occurred twice was used.

Acceptance criteria

1) OD_{NC} of the negative control is greater than 0.7.
2) The viability of the positive control is less than 40%.

Table 3. Positive Criteria.

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

7-3. Difference between LabCyte EPI-MODEL 24 protocol and EPISKIN protocol

25. The differences between the LabCyte EPI-MODEL 24 protocol and EPISKIN protocol are summarized in Table 3. Although the amount of medium (Table 4(A)), amount of test chemicals (Table 4(B)), and threshold of IL-1α content (Table 4(C)) for the LabCyte EPI-MODEL 24 protocol are different from the EPISKIN protocol, their conditions meet the descriptions of the Performance Standards.

Table 4. Differences between the LabCyte EPI-MODEL 24 protocol and EPISKIN protocol.
(A) Amount of medium.

	LabCyte EPI-MODEL 24 SOP	EPISKIN SOP	Reason
Pre-incubation	0.5 mL	2 mL	LabCyte EPI-MODEL 24 cultures are performed in 24-well culture plates. A medium volume of 0.5 mL to 1 mL is appropriate to add to the 24-well culture plate. A medium volume of 1 mL is necessary for a 42-hour culture.
Post-incubation	1 mL	2 mL	
MTT assay	0.5 mL	2 mL	

These conditions meet the descriptions of the Performance Standards.

(B) Amount of test chemicals.

Test chemical	LabCyte EPI-MODEL 24 SOP	EPISKIN SOP	Reason
Liquid	25 μL (75 μL/cm^2)	10 μL (25 μL/cm^2)	The lowest amount of the test chemical that spread uniformly was applied to the test model.
Solid	25 mg+25 μL DW (75 μL/cm^2)	10 mg+10 μL DW (25 μL/cm^2)	

These conditions meet the descriptions of the Performance Standards.

(C) Amount of test chemicals.

LabCyte EPI-MODEL 24 SOP	EPISKIN SOP	Performance Standards (EPISKIN)
IL-1α content ≥ 120 pg/tissue (IL-1α content ≥ 120 pg/mL)	IL-1α content ≥ 100 pg/tissue (IL-1αcontent ≥ 50 pg/mL)	IL-1α content ≥ 120 pg/tissue (IL-1α ≥ 60 pg/mL)

The threshold of IL-1α released in LabCyte EPI-MODEL was set based on the conditions for EPISKIN described in the Performance Standards.

7-4. Data collection, handling, and analysis

26. The independent biostatisticians for the study collected and organised the data using specific data collection software (Datasheet4.0:20080910.xls in 2nd phase study and Datasheet5.0:20090430.xls in 3rd phase study). They will work in close collaboration with the biostatisticians, (Takashi Omori, Etsuyoshi Miyaoka, and Kenya Ishiyama). After decoding the data, they will perform statistical analyses. The data management procedures and statistical tools applied will be approved by the VMT.

7-5. Quality assurance, GLP

LABORATORIES

27. All participating laboratories worked in the spirit of OECD GLP-like principles.

QA aspects

28. Takashi Omori, Kenya Ishiyama and Hajime Kojima assured the quality of all the data and records.

8. Results

8-1 1st Phase

8-1-1 Negative control

29. In 1st phase data, Table 5 shows the absorbance values for the negative control. All data for the negative control met the acceptance criteria.

Table 5. Absorbance of negative control by 1st phase study.

	Exp.				
	1	2	3		
Lab.	Value	Value	Value	Mean	SD
a	1.073	0.928	1.007	1.003	0.073
b	0.93	1.245	1.042	1.072	0.16
c	0.96	0.869	0.761	0.863	0.1
d	0.987	0.928	0.939	0.951	0.031
e	0.84	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

8-1-2 Positive control and test chemicals

30. Table 6 shows the testing chemicals did not show any great score when the scores on tests were repeated in each laboratory. Furthermore, there was no significant inter-laboratory variation. These experiments suggested the feasibility of the LabCyte EPI-MODEL24 through the experiment. All laboratories were judged to participate at the Phase II by the validation management team.

Table 6. Viability of the positive control and three coded chemicals by 1st phase study

		1	2	3		
Chem.	**Lab.**	**Viability**	**Viability**	**Viability**	**Mean**	**SD**
PC	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
	d	11.74	7.22	14.08	11.02	3.49
	e	31.6	9.76	38.61	26.66	15.05
	f	3.1	2.89	2.93	2.97	0.11
	g	4.46	7.17	2.62	4.75	2.29
P01	a	62.67	39.12	46.61	49.46	12.03
Ethanol	b	41.08	50.86	86.58	59.51	23.95
	c	68.13	34.13	67.31	56.53	19.4
	d	68.57	40.52	33.03	47.37	18.73
	e	54.19	72.08	60.55	62.27	9.07
	f	.	64.16	47.98	56.07	11.44
	g	4.68	5.23	6.67	5.53	1.03
P02	a	103.63	104.17	98.48	102.09	3.14
Glycerol	b	85.5	100.58	67.97	84.68	16.32
	c	101.24	99.41	104.84	101.83	2.76
	d	103.3	101.35	89.73	98.13	7.34
	e	101.75	98.06	99.04	99.62	1.91
	f	.	97.23	96	96.62	0.87
	g	94	98.16	103.6	98.59	4.82
P03	a	109.13	90.73	97.78	99.22	9.28
naphtalen acetic acid	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	.	101.34	102.07	101.7	0.52
	g	92.2	101.04	105.52	99.59	6.78

8-2. 2nd phase & 3rd phase

8-2-1. Comments at the Datasheet

31. All tests were sufficient with acceptance criteria. There were a few comments from each laboratory in Tables 7 -9. By an application of Potassium hydroxide (5%aq) (B175, D213 and F232), the model's layers were desquamated. By an application of cinnamicaldehyde (D216 and G256), the cups were discoloured and crystallized.

Table 7. Comments on the datasheets (Viability) by 2nd phase

Lab ID	Exp.No.	Lot	Date	Comments
a	Main-2	LCE24-081013-B	2008/10/20	This test was recorded as the Main-1.
a	Main-3	LEC24-081117-B	2008/11/1	This test was recorded as the Main-2.
a	Main-4	LCE24-081117-B	2008/11/22	This test was recorded as the Main-3.
b	Main-1	LCE24-081013-B	2008/10/20	
b	Main-2	LCE24-081027-B	2008.11.04	
b	Main-3	LCE24-081117-B	2008/11/25	
c	1	LCE24-080929-B	2008.10.6	
c	2	LCE24-081020-B	2008/10/27	
c	3	LCE24-081027-B	2008.11.3	
d	81021	LCE24-081020-B	2008/10/27	
d	81028	LCE24-081027-B	2008/11/4	
d	81118	LCE24-081117-B	2008/11/25	
e	Main-1	LCE24-081006-B	2008/10/14	
e	Main-2	LCE24-081013-B	2008/10/20	
e	Main-3	LCE24-081020-B	2008/10/27	
f	LAB-08VAL	LCE24-080929-B	2008/10/6	
f	Maruishi	LCE24-081013-B	2008/10/20	
f	LAB-08VAL	LCE24-081103-B	2008/11/10	
g	Main-1	LCE24-080929-B	2008.10.06	By an application of G49,G53,G55, the model's cap was discolored.
g	Main-2	LCE24-081013-B	2008.10.20	By an application of G49,G53,G55, the model's cap was discolored.
g	Main-3	LCE24-081027-B	2008.11.03	By an application of G49,G53,G55, the model's cap was discolored.

Table 8. Comments on the datasheets (ELISA) by 2nd phase

Lab_ID	Exp.No.	Lot	Date	Comments
a	Main-2	LCE24-081013-B	2008/10/20	This test was recorded as the Main-1.
a	Main-3	LEC24-081117-B	2008/11/1	This test was recorded as the Main-2.
a	Main-4	LCE24-081117-B	2008/11/22	This test was recorded as the Main-3.
b	Main-1	LCE24-081013-B	2008/12/12	
b	Main-2	LCE24-081027-B	2008/12/12	
b	Main-3	LCE24-081117-B	2008.12.26	
c	1	LCE24-080929-B	2008/10/7	
c	2	LCE24-081020-B	2008/10/30	
c	3	LCE24-081027-B	2008.11.3	
d	81021	LCE24-081020-B	2008/11/11	
d	81028	LCE24-081027-B	2008/11/26	
d	81118	LCE24-081117-B	2009/1/7	
e	Main-1	LCE24-081006-B	2008/12/2	
e	Main-2	LCE24-081013-B	2008/12/2	
e	Main-3	LCE24-081020-B	2008/12/19	
f	Maruishi	LCE24-081013-B	2008/11/25	
f	Maruishi	LCE24-081013-B	2008/11/27	
f	LAB-08VAL	LCE24-081103-B	2008/12/25	
g	Main-1	LCE24-080929-B	2008.10.09	
g	Main-2	LCE24-081013-B	2008.10.22	
g	Main-3	LCE24-081027-B	2008.11.05	

Table 9. Comments on the datasheets (Viability) by 3rd phase study

Lab ID	Exp.No.	Lot	Date	Comments
a	No.1	LCE24-090420-A	2009/4/27	
a	No.2	LEC24-090511-A	2009/5/18	
a	No.3	LEC24-090518-A	2009/5/25	
b	20090421-1	LCE24-090420-A	2009/4/27	By an application of B175, the model's layers were desquamated.
b	20090421-2	LEC24-090511-A	2009/5/20	By an application of B175, the model's layers were desquamated.
b	20090421-3	LEC24-090518-A	2009/5/25	By an application of B175, the model's layers were desquamated.
c	1	LCE24-090420-A	2009/4/27	
c	2	LEC24-090511-A	2009/5/18	
c	3	LEC24-090518-A	2009/5/25	
d	90512	LEC24-090511-A	2009/5/18	By an application of D213, the model's layers were desquamated. By an application of D216, white crystallizations in the cup were detected.
d	90519	LEC24-090518-A	2009/5/25	By an application of D213, the model's layers were desquamated. By an application of D216, white crystallizations in the cup were detected.
d	90526	LEC24-090525-A	2009/6/1	By an application of D213, the model's layers were desquamated. By an application of D216, white crystallizations in the cup were detected.
f	LAB-09VAL	LCE24-090420-A	2009/4/27	By an application of F232, the model's layers were desquamated.
f	LAB-09VAL	LEC24-090511-A	2009/5/18	
f	LAB-09VAL	LEC24-090518-A	2009/5/25	By an application of F232, the model's layers were desquamated.
g	①	LCE24-090420-A	2009/4/27	By an application of G256, the model's caps were discolored.
g	②	LCE24-090427-A	2009/5/4	By an application of G256, the model's caps were discolored.
g	③	LEC24-090511-A	2009/5/18	By an application of G256, the model's caps were discolored.

8-2-2. Negative control

32. In Table 10 and Fig.2, absorbances of negative control are shown. All data of negative control were sufficient with acceptance criteria excluding Lab a, test1. The mean OD of lab a, test 1 is 0.59 (0.61, 0.58, 0.57). We were not accepted at this result, and accepted the results of test 2-4 re-tested at Lab a.

Table 10 Absorbance of negative control

		Lab.					
Study	Run	a	b	c	d	f	g
2	1	0.75 (0.02)	0.93 (0.01)	0.91 (0.01)	0.82 (0.02)	0.84 (0.01)	1.13 (0.01)
	2	0.86 (0.02)	0.85 (0.04)	1.01 (0.02)	0.90 (0.04)	0.79 (0.02)	1.18 (0.02)
	3	0.82 (0.04)	0.84 (0.03)	0.93 (0.02)	0.96 (0.03)	0.83 (0.00)	1.05 (0.05)
3	1	0.90 (0.02)	0.96 (0.02)	1.04 (0.02)	1.11 (0.05)	0.90 (0.02)	0.91 (0.04)
	2	0.72 (0.02)	1.01 (0.02)	1.06 (0.01)	1.11 (0.04)	0.94 (0.02)	1.08 (0.01)
	3	0.80 (0.02)	0.97 (0.04)	1.01 (0.02)	1.13 (0.03)	0.92 (0.03)	0.88 (0.03)
Mean		0.81	0.93	0.99	1.01	0.87	1.04
Median		0.81	0.94	1.01	1.03	0.87	1.06
Min		0.72	0.84	0.91	0.82	0.79	0.88
Max		0.9	1.01	1.06	1.13	0.94	1.18
SD		0.07	0.07	0.06	0.13	0.06	0.12
Range		0.17	0.17	0.15	0.31	0.15	0.3

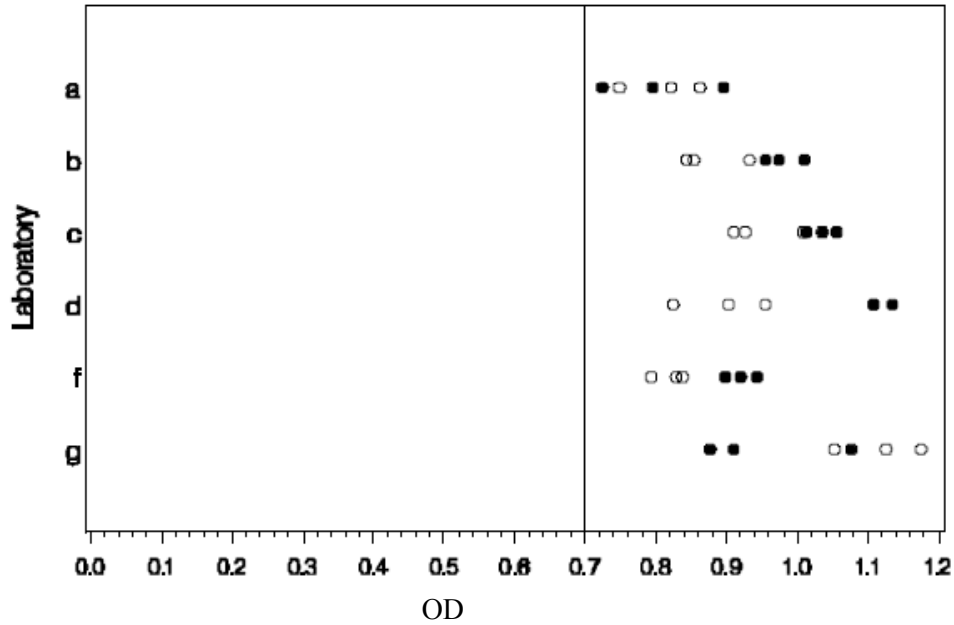


Fig.2 Distribution of Absorbance on negative control

8-2-3. Positive control

33. Table 11 and Fig.3 show three independent viabilities and statistical analysis of positive control at each laboratory. All data were sufficient with acceptance criteria of positive control.

Table 11. Viability of positive control

		Lab.					
Study	Run	a	b	c	d	f	g
2	1	5.9 (1.3)	5.2 (2.3)	4.1 (0.5)	5.7 (2.3)	3.5 (0.4)	3.1 (0.2)
	2	8.8 (4.8)	12.3 (6.9)	5.4 (3.0)	2.6 (0.3)	2.9 (0.3)	10.7 (5.3)
	3	2.5 (0.4)	7.8 (2.4)	3.8 (0.0)	3.3 (0.3)	3.2 (0.3)	4.2 (1.3)
3	1	6.4 (1.8)	9.3 (6.8)	8.2 (3.4)	3.5 (0.9)	8.5 (1.9)	11.7 (2.5)
	2	2.2 (0.4)	2.2 (0.1)	7.3 (2.2)	2.5 (0.3)	4.1 (1.3)	2.5 (0.1)
	3	1.8 (0.2)	1.6 (0.3)	2.4 (0.2)	2.1 (0.4)	2.7 (0.0)	3.3 (0.3)
Mean		4.6	6.4	5.2	3.3	4.1	5.9
Median		4.2	6.5	4.7	2.9	3.3	3.7
Min		1.8	1.6	2.4	2.1	2.7	2.5
Max		8.8	12.3	8.2	5.7	8.5	11.7
SD		2.9	4.2	2.2	1.3	2.2	4.1
Range		7	10.7	5.7	3.6	5.8	9.2

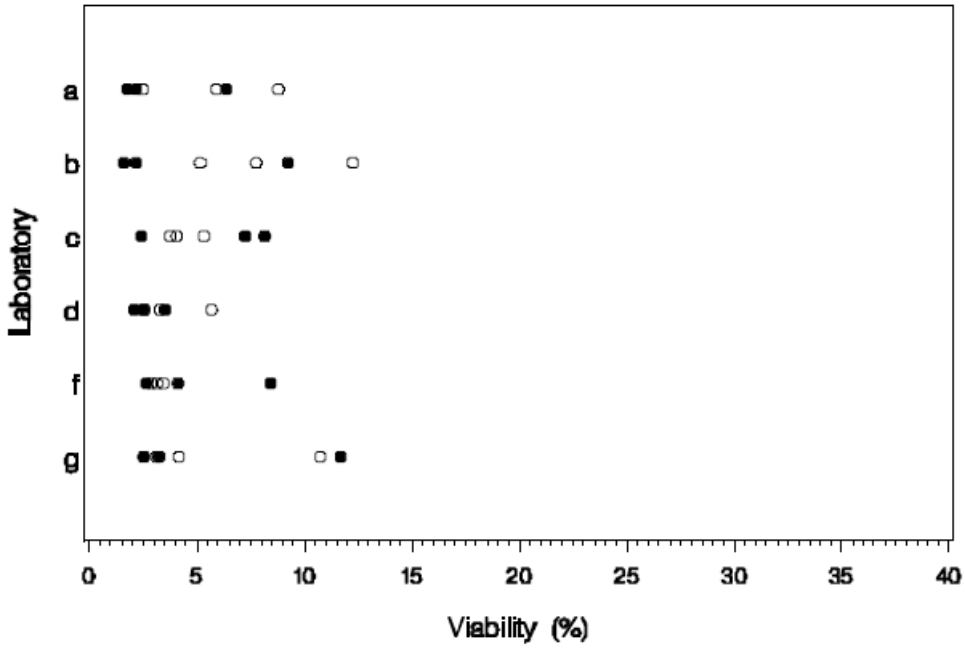


Fig.3 Distribution of viability on positive control

8-2-4. Skin irritation test by cell viability

34. The results of the skin irritation test with LabCyte EPI-MODEL 24 when it was only evaluated cell viabilities as indicator are shown in Table 12 in 2nd phase study and Table 14 in 3rd phase study. Summary statistical analysis of viability each chemical are shown in Table 13 and Fig.4 in 2nd phase study and Table 15 and Fig.5 in 3rd phase study.

35. Invalid data obtained only Lab a, run 1. This lab performed at retesting. Therefore, the data of lab a were accepted among run 2-4.

Table 12. Viability of chemicals at each laboratory by 2nd phase study.

				Lab.						
Chem.	Vivo	Score	Exp.	a	b	c	d	e	f	g
01	no	0	1	31.0	47.1	10.6	14.3	38.1	14.3	10.6
			2	11.2	10.4	20.3	9.1	25.2	11.2	10.6
			3	11.6	16.1	12.4	9.6	32.3	10.4	14.0
02	no	0	1	79.8	66.9	88.1	102.3	101.8	75.3	96.0
			2	76.5	61.7	89.7	89.8	76.4	67.2	94.8
			3	65.2	88.7	85.8	67.6	85.8	75.7	103.3
03	no	0	1	109.1	93.3	94.6	105.1	129.6	94.2	100.5
			2	103.9	99.8	93.1	112.8	106.6	97.9	93.4
			3	100.9	102.3	95.7	101.4	103.9	92.5	111.1
04	no	0	1	106.3	94.4	97.1	106.1	127.1	100.1	104.8
			2	95.2	100.2	99.9	100.9	113.6	92.8	103.3
			3	96.5	98.6	97.8	98.4	105.2	92.7	109.8
05	no	0.3	1	78.5	61.7	91.4	79.4	103.0	71.9	96.8
			2	78.5	71.9	95.2	70.5	90.3	39.3	89.9
			3	74.1	84.5	89.2	66.1	89.6	55.1	88.4
06	no	0.3	1	92.5	77.9	81.0	91.3	97.0	87.8	87.2
			2	79.4	83.5	79.1	102.4	81.5	94.4	81.2
			3	82.4	80.5	83.6	82.7	90.7	81.1	54.1
07	no	1	1	24.1	10.8	20.8	21.7	17.5	15.8	31.5
			2	12.6	12.6	16.2	13.8	22.2	31.1	22.5
			3	17.8	13.2	15.2	19.8	21.3	15.6	19.9
08	no	1	1	111.9	86.7	75.3	109.4	114.9	89.7	101.1
			2	90.2	100.6	82.3	107.5	100.9	97.8	100.9
			3	95.3	104.8	77.2	103.0	100.9	96.5	109.0
09	no	1.7	1	112.8	96.7	106.6	105.0	115.8	98.8	102.3
			2	97.1	110.1	96.8	103.4	108.6	86.5	103.4
			3	101.1	109.5	93.5	98.1	103.9	97.7	112.1
10	no	1.7	1	115.9	115.4	107.5	114.3	132.0	104.0	107.9
			2	104.1	110.1	103.6	108.2	117.0	101.2	108.4
			3	86.5	111.3	103.7	105.5	107.5	101.2	113.1

Table 12. continued

				Lab.						
Chem.	Vivo	Score	Exp.	a	b	c	d	e	f	g
11	no	2	1	113.7	105.0	101.0	102.4	123.1	103.1	102.8
			2	98.1	106.6	94.6	105.8	110.4	98.0	100.5
			3	112.6	103.7	94.1	102.7	105.5	94.6	109.0
12	no	2	1	28.2	24.6	24.9	54.3	55.6	27.2	87.7
			2	18.4	24.6	44.8	76.2	57.8	65.2	98.0
			3	15.3	15.9	28.1	27.4	57.2	66.0	112.6
14	Category 2	2.3	1	11.1	12.1	14.7	10.7	14.2	13.1	13.5
			2	6.6	8.3	9.5	11.7	12.0	16.7	12.0
			3	6.8	8.8	9.1	10.2	10.4	17.0	10.6
15	Category 2	2.3	1	11.1	9.3	13.1	8.0	11.0	8.6	9.2
			2	7.1	10.2	19.3	8.6	11.3	5.9	24.7
			3	8.2	9.9	8.1	9.2	8.7	7.1	9.2
16	Category 2	2.7	1	67.9	92.0	51.5	18.1	98.2	59.6	64.9
			2	32.2	54.1	86.3	79.2	90.6	50.4	79.6
			3	59.8	98.3	81.7	37.7	78.7	67.5	86.5
17	Category 2	2.7	1	6.1	4.5	5.3	6.6	8.9	6.9	6.2
			2	4.8	4.7	6.0	5.3	6.3	5.5	5.3
			3	5.6	5.7	5.9	3.9	5.4	4.5	5.3
18	Category 2	3	1	82.1	46.5	91.2	83.7	98.9	69.2	92.4
			2	78.3	50.6	87.3	69.9	87.2	80.6	85.9
			3	25.3	100.0	87.5	59.0	69.1	71.9	94.4
19	Category 2	3	1	15.0	74.6	10.0	30.4	83.1	40.1	35.8
			2	19.9	10.9	22.4	28.3	26.1	87.0	44.7
			3	51.1	32.0	35.0	18.2	69.4	71.8	38.7
20	Category 2	4	1	31.1	24.8	10.4	9.6	10.7	8.1	8.8
			2	9.3	8.0	7.6	16.9	8.2	7.8	6.7
			3	29.5	9.3	7.6	30.9	6.2	8.2	8.6

Table 13. Summary of the statistical analysis of the viability for each chemical by 2nd phase study.

		Lab.						
Chem.	Stat.	a	b	c	d	e	f	g
01	Mean	17.9	24.5	14.4	11.0	31.9	12.0	11.7
	Median	11.6	16.1	12.4	9.6	32.3	11.2	10.6
	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
02	Mean	73.8	72.4	87.8	86.6	88.0	72.7	98.0
	Median	76.5	66.9	88.1	89.8	85.8	75.3	96.0
	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
03	Mean	104.7	98.5	94.5	106.4	113.3	94.8	101.7
	Median	103.9	99.8	94.6	105.1	106.6	94.2	100.5
	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
04	Mean	99.3	97.8	98.2	101.8	115.3	95.2	105.9
	Median	96.5	98.6	97.8	100.9	113.6	92.8	104.8
	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
05	Mean	77.0	72.7	91.9	72.0	94.3	55.4	91.7
	Median	78.5	71.9	91.4	70.5	90.3	55.1	89.9
	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
06	Mean	84.8	80.7	81.2	92.1	89.7	87.8	74.2
	Median	82.4	80.5	81.0	91.3	90.7	87.8	81.2
	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
07	Mean	18.2	12.2	17.4	18.4	20.3	20.8	24.6
	Median	17.8	12.6	16.2	19.8	21.3	15.8	22.5
	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
08	Mean	99.1	97.4	78.3	106.6	105.6	94.7	103.7
	Median	95.3	100.6	77.2	107.5	100.9	96.5	101.1
	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
09	Mean	103.7	105.4	98.9	102.2	109.4	94.3	105.9
	Median	101.1	109.5	96.8	103.4	108.6	97.7	103.4
	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
	Median	104.1	111.3	103.7	108.2	117.0	101.2	108.4
	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

Table 13. continued.

		Lab.						
Chem.	Stat.	a	b	c	d	e	f	g
11	Mean	108.1	105.1	96.6	103.6	113.0	98.6	104.1
	Median	112.6	105.0	94.6	102.7	110.4	98.0	102.8
	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
	Median	18.4	24.6	28.1	54.3	57.2	65.2	98.0
	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
14	Mean	8.2	9.7	11.1	10.9	12.2	15.6	12.0
	Median	6.8	8.8	9.5	10.7	12.0	16.7	12.0
	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
	Median	8.2	9.9	13.1	8.6	11.0	7.1	9.2
	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
	Median	59.8	92.0	81.7	37.7	90.6	59.6	79.6
	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
	Median	5.6	4.7	5.9	5.3	6.3	5.5	5.3
	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
	Median	78.3	50.6	87.5	69.9	87.2	71.9	92.4
	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
	Median	19.9	32.0	22.4	28.3	69.4	71.8	44.7
	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
	Median	29.5	9.3	7.6	16.9	8.2	8.1	8.6
	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

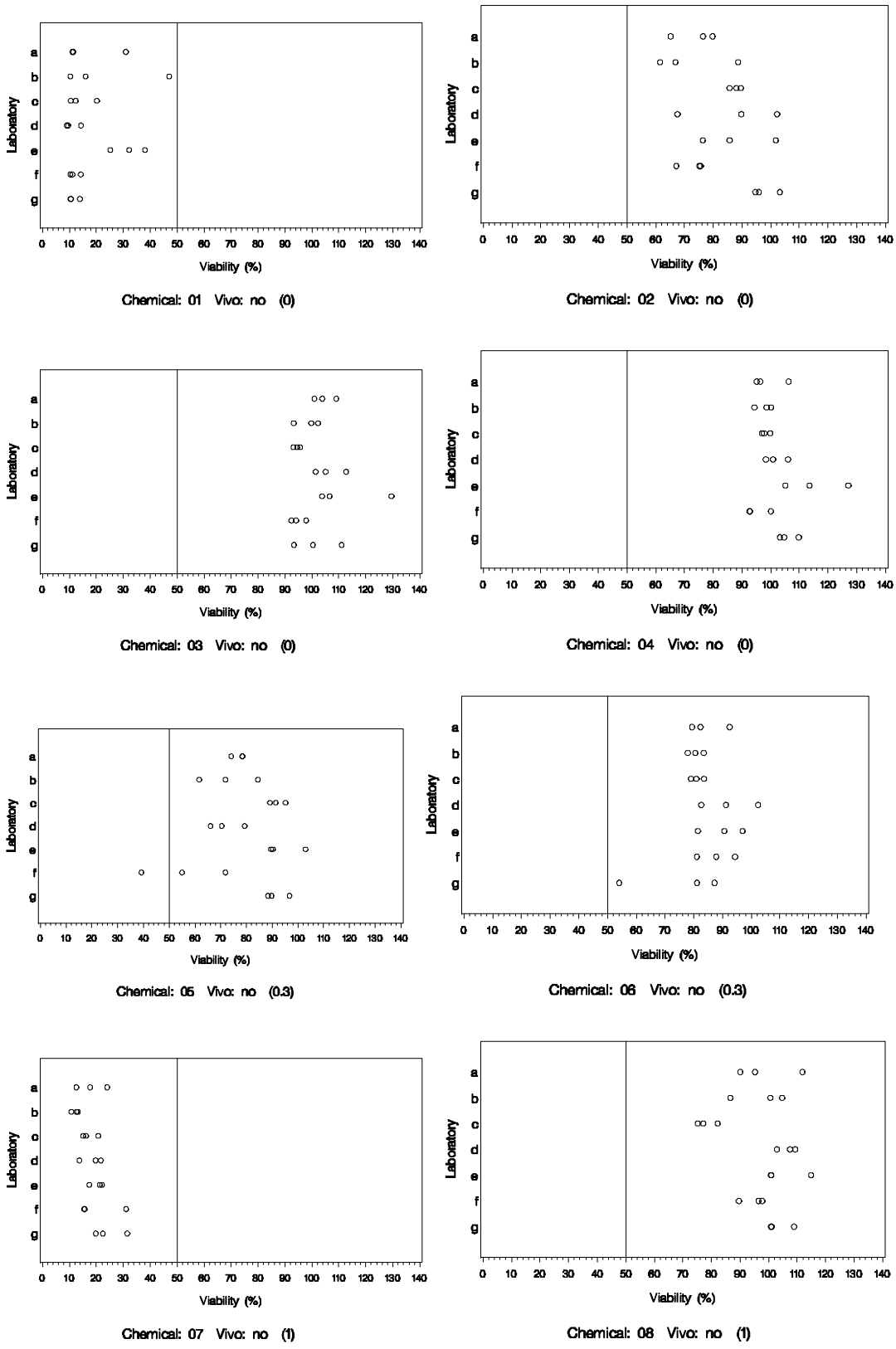


Fig. 4. Distribution of the viability for each chemical.

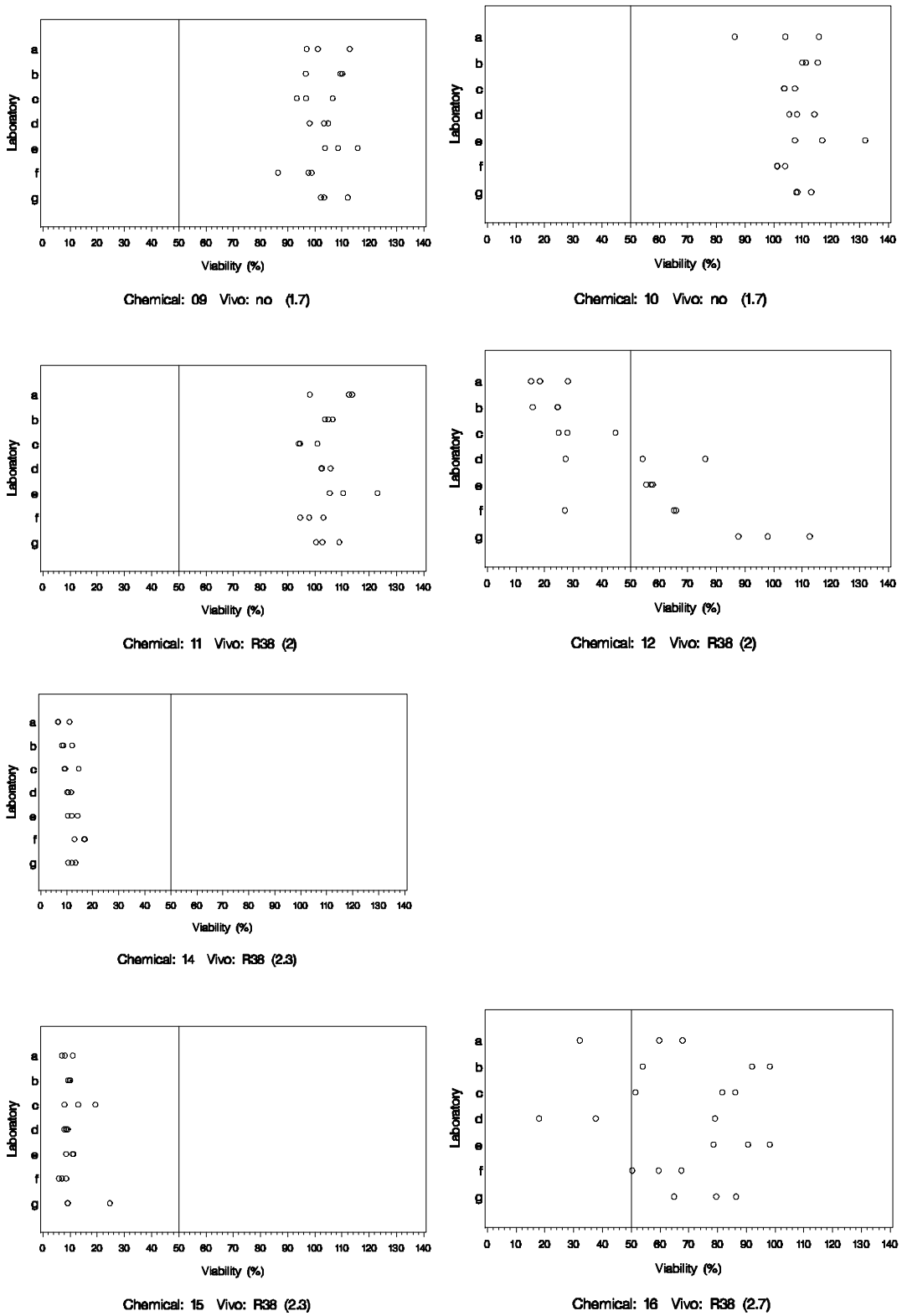


Fig. 4. continued

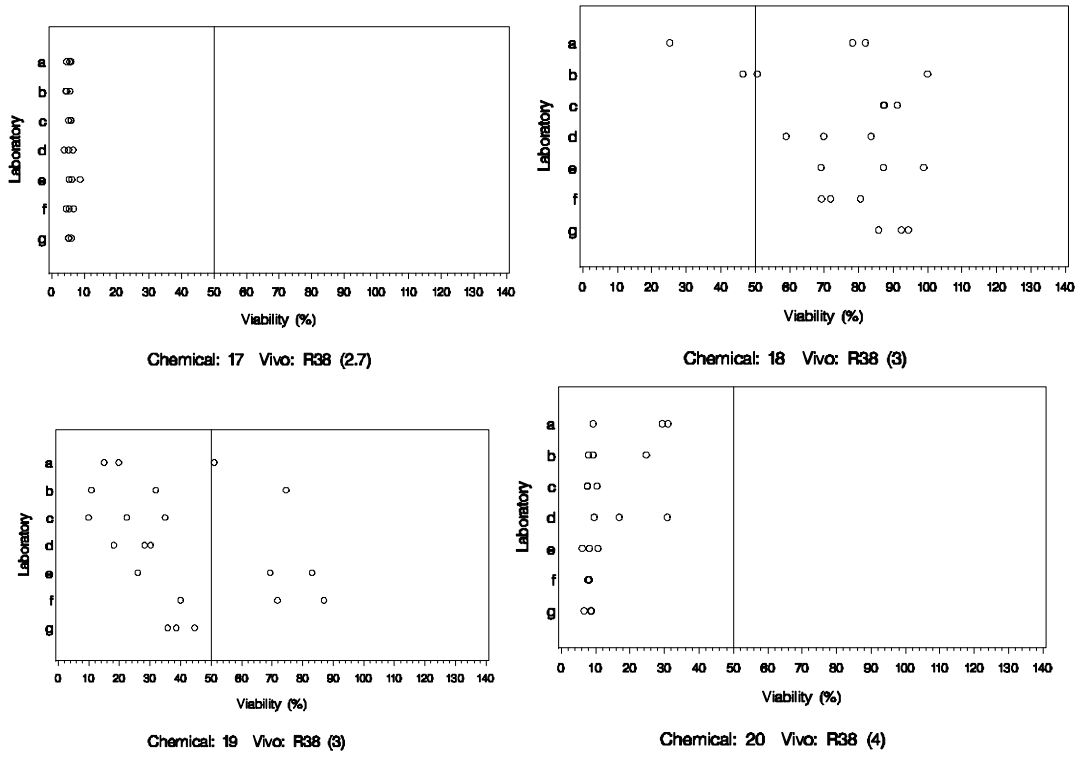


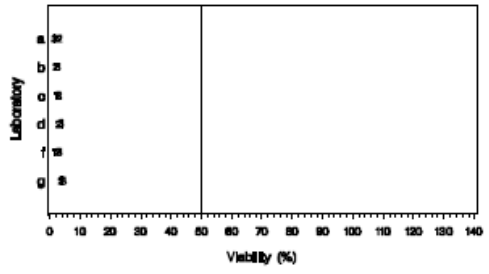
Fig. 4. continued.

Table 14. Viability of chemicals each laboratory by 3rd phase study

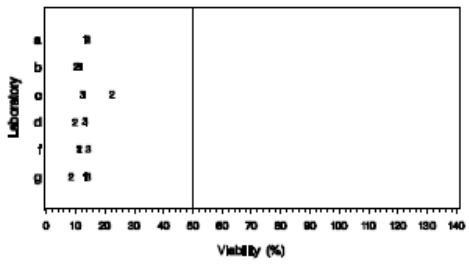
				Lab.					
Chem.	Vivo	Score	Exp.	a	b	c	d	f	g
A	no	2	1	13.3	11.8	13.2	13.8	11.4	13.7
			2	14.2	10.2	22.5	9.9	11.3	8.7
			3	14.0	11.1	12.3	13.2	14.3	14.3
B	Category 2	2.7	1	1.5	2.2	2.5	4.0	1.7	3.9
			2	3.1	2.2	2.9	3.0	2.6	3.7
			3	1.5	2.5	3.0	3.9	3.2	4.7
C	Category 2	3	1	0.7	0.7	0.7	6.9	0.8	1.0
			2	1.3	1.1	1.4	2.0	4.8	0.4
			3	0.5	0.8	1.0	0.8	1.0	0.3
D	Category 2	3.3	1	14.5	24.0	12.7	10.3	13.8	19.3
			2	13.6	16.0	12.5	18.3	8.8	15.2
			3	18.6	15.5	12.6	23.0	19.2	14.1
E	Category 2	3.3	1	3.9	3.4	3.4	8.2	3.2	4.1
			2	4.5	2.7	3.3	3.9	4.2	3.1
			3	1.8	3.5	3.5	3.7	5.0	5.1
F	Category 2	4	1	5.6	7.2	6.5	6.4	5.2	7.2
			2	5.7	6.1	6.8	5.4	7.4	6.8
			3	5.4	4.2	6.5	5.4	5.0	7.6

Table 15 Summary statistical analysis of viability each chemical by 3rd phase study

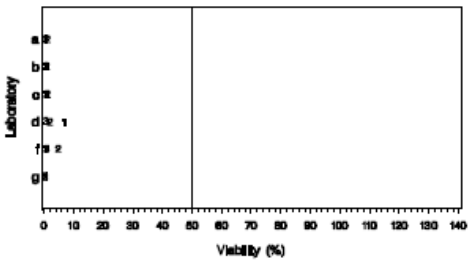
		Lab.					
Chem.	Stat.	a	b	c	d	f	g
A	Mean	13.8	11.0	16.0	12.3	12.3	12.2
	Median	14.0	11.1	13.2	13.2	11.4	13.7
	Min	13.3	10.2	12.3	9.9	11.3	8.7
	Max	14.2	11.8	22.5	13.8	14.3	14.3
B	Mean	2.0	2.3	2.8	3.6	2.5	4.1
	Median	1.5	2.2	2.9	3.9	2.6	3.9
	Min	1.5	2.2	2.5	3.0	1.7	3.7
	Max	3.1	2.5	3.0	4.0	3.2	4.7
C	Mean	0.8	0.8	1.0	3.2	2.2	0.6
	Median	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
D	Mean	15.6	18.5	12.6	17.2	13.9	16.2
	Median	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
E	Mean	3.4	3.2	3.4	5.3	4.2	4.1
	Median	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
F	Mean	5.5	5.8	6.6	5.7	5.9	7.2
	Median	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



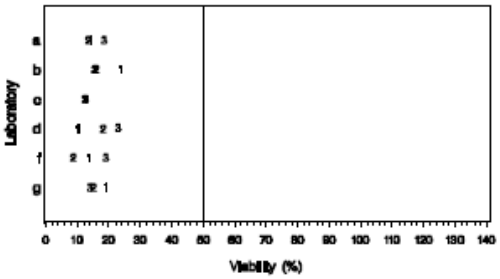
2−chloromethyl−3,5−dimethyl−4−methoxypyridine HCl



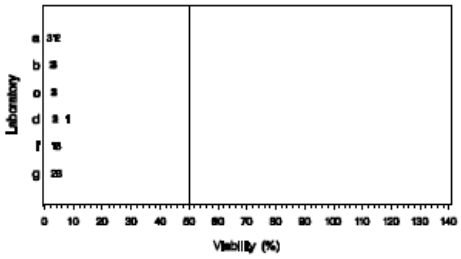
cinnamaldehyde



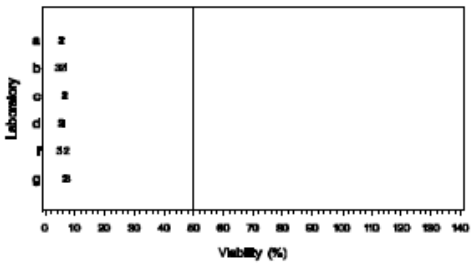
potassium hydroxide (5% aq.)



benzenethiol, 5−(1,1−dimethylethyl)−2−methyl



1−methyl−3−phenyl−1−piperazine



1,1,1−trichloroethane

Fig.5 Distribution of viability each chemical

8-2-5. IL-1α

36. The results of the LabCyte EPI-MODEL 24 skin irritation test when IL-1α was evaluated as an indicator are summarized in Table 16.

Table 16. IL-1α levels from each laboratory.

				Lab.						
Chem.	GHS	Score	Exp.	a	b	c	d	e	f	g
01	no	0	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.
02	no	0	1	132.8	52.9	59.3	41.2	60.7	61.3	9.4
			2	68.1	56.5	37	89.1	68.4	99.3	9.6
			3	97.6	41.1	76	72.4	46	70.1	12.6
03	no	0	1	12	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
04	no	0	1	10	6	8	11.7	9.5	2.5	6.3
			2	5.3	8	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
05	no	0.3	1	122	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	191.6	33.2
06	no	0.3	1	59	85.7	114	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
07	no	1	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.
08	no	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	4.1	33.1	5.2	6.7	2.1	7.2
09	no	1.7	1	10.9	17.1	11.2	42.6	29.5	33	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	1.7	1	27.9	7.4	31.3	41.2	46.5	39.3	9.8
			2	17.1	12.7	15	50.4	26.7	26.7	14.5
			3	66.2	12.2	30	42.1	26.3	24.2	13.2

Table 16. continued.

				Lab.						
Chem.	GHS	Score	Exp.	a	b	c	d	e	f	g
11	no	2	1	5	31.1	18	15.3	10.4	16.2	6.4
			2	3.3	11.9	15.8	19	9.7	8.1	7.5
			3	18.2	5	8.9	8.7	8.6	12.6	11.9
12	no	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
17	Category 2	2.7	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.
18	Category 2	3	1	61.5	.	60.6	90.3	86.9	114.5	18
			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	59.5	.
20	Category 2	4	1	.	.	.	.	.	.	.
			2	.	.	.	.	.	.	.
			3	.	.	.	.	.	.	.

Cells highlighted in yellow indicate that the classification changed based on the IL-1α data.

8-2-6. Classification of three independent viabilities at each laboratory

37. The classifications from mean of three independent viabilities only evaluated MTT assay were shown in Table 17 in 2nd phase study and Table 19 in 3rd phase study. Refer to Table 18, the IL-1α results changed the classification for only 3 data points. The classification of **Allyl phenoxy-acetate** by Lab f was changed the misunderstood classification. The other two chemicals were changed the correct classification. Regarding the ILα only a few chemicals showed different results but the overall call was that ILα did not significantly contribute to the performance of the assay.

Table 17.Classification using three independent viabilities by 2nd phase study
「P」:Positive、「N」: Negative

			Lab.						
Chem.	**GHS**	**Score**	**a**	**b**	**c**	**d**	**e**	**f**	**g**
01	no	0	P	P	P	P	P	P	P
02	no	0	N	N	N	N	N	N	N
03	no	0	N	N	N	N	N	N	N
04	no	0	N	N	N	N	N	N	N
05	no	0.3	N	N	N	N	N	N	N
06	no	0.3	N	N	N	N	N	N	N
07	no	1	P	P	P	P	P	P	P
08	no	1	N	N	N	N	N	N	N
09	no	1.7	N	N	N	N	N	N	N
10	no	1.7	N	N	N	N	N	N	N
11	no	2	N	N	N	N	N	N	N
12	no	2	P	P	P	N	N	N	N
14	Category 2	2.3	P	P	P	P	P	P	P
15	Category 2	2.3	P	P	P	P	P	P	P
16	Category 2	2.7	**N**	**N**	**N**	P	**N**	**N**	**N**
17	Category 2	2.7	P	P	P	P	P	P	P
18	Category 2	3	**N**	**N**	**N**	**N**	**N**	**N**	**N**
19	Category 2	3	P	P	P	P	**N**	**N**	P
20	Category 2	4	P	P	P	P	P	P	P

Table.18. Classification of chemicals by MTT assay demolished by additional IL-1α measurement

No.	**Chemical**	**CAS number**	**GHS label**	**In vivo score (PII)**	**Lab.**	**Classification by MTT assay**	**Classification by** MTT+IL-1α
05	**Allyl phenoxy-acetate**	**7493-74-5**	**no**	**0.3**	f	N	P
16	**1-bromohexane**	**111-25-1**	**Category 2**	**2.7**	**a**	**N**	**P**
18	**di-n-propyl disulphide**	**629-19-6**	**Category 2**	**3**	**d**	**N**	**P**

Table 19 Classification using three independent viabilities by 3rd phase study
「P」:Positive、「N」: Negative

			Lab.					
Chem.	**in vivo**	**Score**	**a**	**b**	**c**	**d**	**f**	**g**
A	no	2	P	P	P	P	P	P
B	Category 2	2.7	P	P	P	P	P	P
C	Category 2	2.7	P	P	P	P	P	P
D	Category 2	3.3	P	P	P	P	P	P
E	Category 2	3.3	P	P	P	P	P	P
F	Category 2	4	P	P	P	P	P	P

Table 20. Sensitivity, specificity and accuracy on MTT assay vs GHS-EU classification in the 2nd + 3rd Phase validation study (25 substances)

Index	Lab.						
	a	b	c	d	f	g	
Sensitivity	10/12	10/12	10/12	11/12	9/12	10/12	
	83.3	83.3	83.3	91.6	75	83.3	
Specificity	9/13	9/13	9/13	10/13	10/13	10/13	
	69.2	69.2	69.2	76.9	76.9	76.9	
Accuracy	19/25	19/25	19/25	21/25	19/25	20/25	
	76	76	76	84	76	80	

Table 21. Sensitivity, specificity and accuracy on MTT assay vs GHS-EU classification in 2nd phase study (19 substances).

Index	Lab.						
	a	b	c	d	e	f	g
Sensitivity	5/7	5/7	5/7	6/7	4/7	4/7	5/7
	71.4	71.4	71.4	85.7	57.1	57.1	71.4
Specificity	9/12	9/12	9/12	10/12	10/12	10/12	10/12
	75	75	75	83.3	83.3	83.3	83.3
Accuracy	14/19	14/19	14/19	16/19	14/19	14/19	15/19
	73.7	73.7	73.7	84.2	73.7	73.7	78.9

Table 22. Sensitivity, specificity and accuracy of the MTT assay and IL-1α vs. the GHS-EU classification in 2nd phase study (19 substances).

Index	Lab.						
	a	b	c	d	e	f	g
Sensitivity	6/7	5/7	5/7	7/7	4/7	4/7	5/7
	85.7	71.4	71.4	100	57.1	57.1	71.4
Specificity	9/12	9/12	9/12	10/12	10/12	9/12	10/12
	75	75	75	83.3	83.3	75	83.3
Accuracy	15/19	14/19	14/19	17/19	14/19	13/19	15/19
	78.9	73.7	73.7	89.5	73.7	68.4	78.9

Table 23(A). Mean and range of Sensitivity, specificity and accuracy on the MTT assay using LabCyte EPI-MODEL vs. GHS-EU classification in the 2nd + 3rd Phase validation study (25 substances)

	N	Mean	Min.	Max.	ECVAM criteria
Sensivitity (%)	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

Table 23(B). Mean and range of sensitivity, specificity and accuracy of the MTT assay vs. the GHS-EU classification in 2nd phase study (19 substances).

	N	Mean	Min.	Max.	ECVAM criteria
Sensivitity (%)	7	69.4	57.1	85.7	80.0
Specificity (%)	7	79.7	75.0	83.3	70.0
Accuracy (%)	7	75.9	73.7	84.2	75.0

Table 23(C). Mean and range of sensitivity, specificity and accuracy of the MTT assay and IL-1α vs. the GHS-EU classification in 2nd phase study (19 substances).

	N	Mean	Min.	Max.	ECVAM criteria
Sensivitity (%)	7	73.4	57.1	100.0	80.0
Specificity (%)	7	78.6	69.2	76.9	70.0
Accuracy (%)	7	76.7	68.4	89.5	75.0

9. Discussion

9-1. Reliability

38. All data of negative control and positive control each laboratory in 2nd and 3rd phase study was sufficient with the acceptance criteria as shown in Tables 10 and 11. There were high respectabilities within and between laboratories in this model.

39. In all data, Invalid data obtained only one data (Lab a, run 1). This lab performed at retesting and we accepted data of run 2-4. Therefore, the rate of invalid at this assay is 0.2% (total 1/508, 400 data: 3runs X 7 labs X 19 chemicals+1 run in 2nd phase study & 108 data; 3 runs X 6 labs X 6 chemicals in 3rd phase study). Based on a comparison of the results from the seven laboratories, the classification of 3 chemicals (No. 12, 16 and 19) should be potentially changed. However, the classifications of the remaining chemicals were not changed. The variations of these chemicals and No.18 are larger than those of others. The IL-1α data changed the classification for No. 5, 16 and 18 at Lab. f (No. 5), Lab. a (No. 16), and Lab. d (No. 18). The effect of IL-1α on the reliability of these results is small.

9-2. Predictivity

40. In December 2008, the EU adopted the UN Globally Harmonised System for Classification and Labelling and will implement this by means of the so-called CLP regulation (Regulation EC 1272/2008). The new EU classification system based on UN GHS (abbreviated here as "GHS-EU") continues to use two categories to distinguish non-irritant (no-category) from irritant (category 2) substances. However, according to the new rules for skin irritation classification and labelling, the cut-off score to distinguish between no-category and category 2 substances was shifted to 2.3 from a value of 2.0 (EU classification system). Consequently substances with an in vivo score between 2.0 and 2.3 that are considered irritant under the existing EU classification system will be considered non-irritants under the future GHS-EU classification system, which does not use the optional UN GHS category 3.

41. The prediction values of the LabCyte EPI-MODEL 24 skin irritation test when it was evaluated by cell viabilities (MTT) as an indicator, and the GHS-EU classifications are shown in Table 20. The sensitivity, specificity and accuracy of this prediction model at each laboratory were 75-91.6 %, 69.2-76.9 %, and 76-84 %, respectively. These predictivities were similar with each laboratory. The mean and range of prediction values of the skin irritation test with LabCyte EPI-MODEL 24 when it was only evaluated by MTT as an indicator and the GHS-EU classification are shown in Table 23(A). The mean sensitivity, specificity and accuracy of this prediction model are 83.3%, 73.1%, and 78.0%, respectively. Some deviations from the ESAC Performance standard (sensitivity of 80%, specificity of 70% and an accuracy of 75%) that were specific adaptations for the Japanese model.The effect of IL-1α on the predictivity was small compared with results in Tables 21,22, 23 (B) and 23(c).

10. Conclusions

42. Based on the GHS-EU classification, 12 irritants and 13 non-irritants in the ECVAM Performance Standards(2007,2009) were tested by the 7 labs using **LabCyte** EPI-MODEL. The assay demonstrated high reliability within and between laboratories, and acceptable reliability of the positive control (100%) and accuracy (77.5% overall accuracy, 82.3% overall sensitivity, 72.6% overall specificity) on the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants.

This report summarized at JSAAE 1st report and 2nd report on this validation study.

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