8-2-5. IL-1α

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

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.	Classi-fication by MTT assay	Classi-fication 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	
Spescificity	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
Spescificity	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
Spescificity	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 ofsSensitivity, 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

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.

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

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.

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

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 (Appendix 7 and 8).

11. Acknowledgement

This validation study has supported by the Health and Labour Sciences Research Grant, Japan.

12. References

ECVAM : Performance standards for applying human skin models to *in vitro* skin irritation testing (2007)

EPISKIN®-MTTreduction
scientifically validated as replacement to the Draize skin irritation test (2007)

ESAC statement on the validity of in-vitro tests for skin irritation (2007)

ESAC STATEMENT ON THE SCIENTIFIC VALIDITYTY OF IN-VITRO TESTS FOR SKIN IRRITATION TESTING (2008)

ESAC STATEMENT ON THE PERFORMANCE UNDER UN GHS OF THREE IN-VITRO ASSAYS FOR SKIN IRRITATION TESTING ANDTHE ADAPTATION OF THE REFERENCE CHEMICALS AND DEFINED ACCURACY VALUES OF THE ECVAM SKIN IRRITATION PERFORMANCE STANDARDS (2009)

Globally Harmonised System of Classification and Labelling of Chemicals (GHS). Part 3: Health and Environmental Hazards, pp. 107–228.New York, NY, USA, and Geneva, Switzerland: United Nations Organisation(2003).

Green H. (1978): Cyclic AMP in relation to proliferation of the epidermal cell: new view. Cell, 15, 801-811.

Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi A.J., Prieto, P., Sabbioni, E., Scott, L., Worth, A. and Zuang. V. (2004) A Modular Approach to the ECVAM Principles on Test Validity. *ATLA* 32, 467-72.

Masakazu Katoh, Fumiyasu Hamajima, Takahiro Ogasawara, and Ken-ichiro Hata (2009) Assessment of the Human Epidermal Model LabCyte EPI-MODEL for *In Vitro* Skin Irritation Testing According to the ECVAM-Validated Protocol , Journal of Toxicological Science, 34(3) 327-334.

OECD (1999) *OECD series on principles of good laboratory practice and compliance monitoring No 5, Compliance of Laboratory Suppliers with GLP Principles.* Paris, France: Organisation for Economic Cooperation and Development.

OECD (2002) *OECD Guidelines for the Testing of Chemicals No. 404: Acute Skin Irritation/Corrosion.* Paris, France: Organisation for Economic Cooperation and Development.

OECD (2005) Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Environmental Health and Safety Monograph Series on Testing and Assessment No. 34. Available at: http://www.oecd.org/document/30/0,3343,en_2649_34377_1916638_1_1_1_1,00.html. Accessed on 12.02.2008.

REGULATION (EC) No 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006)

Rheinwald J.G. and Green H. (1975): Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell, **6**, 331-344

JSAAE REPORT:

VALIDATION STUDY OF IN VITRO SKIN IRRITATION TEST USING LABCYTE EPI-MODEL 24 (2ND REPORT)

JULY 22, 2009

LABCYTE VALIDATION MANAGEMENT TEAM

MEMBERS OF LABCYTE VALIDATION MANAGEMENT TEAM

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Mr. Isao Yoshimura; Tokyo University of Science

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Mr. Masakazu Kato; Japan Tissue Engineering Co. Ltd.

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Participant laboratories

- Aiken Co., Ltd. (Ms Yoko Ando and Ms Yui Asako)
- KOBAYASHI Pharmaceutical Co., Ltd. (Mr. Yoshihiro Yamaguchi and Ms Maki Nakamura)
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- Drug Safety Testing Center Co., Ltd. (Mr Shinsuke Shinoda and Ms Saori Hagiwara)

Contents

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

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 using additional 6 chemicals in accordance with the revised reference chemicals described by the new ESAC statement 2009.

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 an additional test chemicals shown in ECVAM performance standard (ESAC statement, 2009). 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

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.

3-2. MODEL SUPPLIER

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).

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.

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

4. Validation Management structure

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

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

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. and Etsuyoshi Miyaoka; Tokyo University of Science

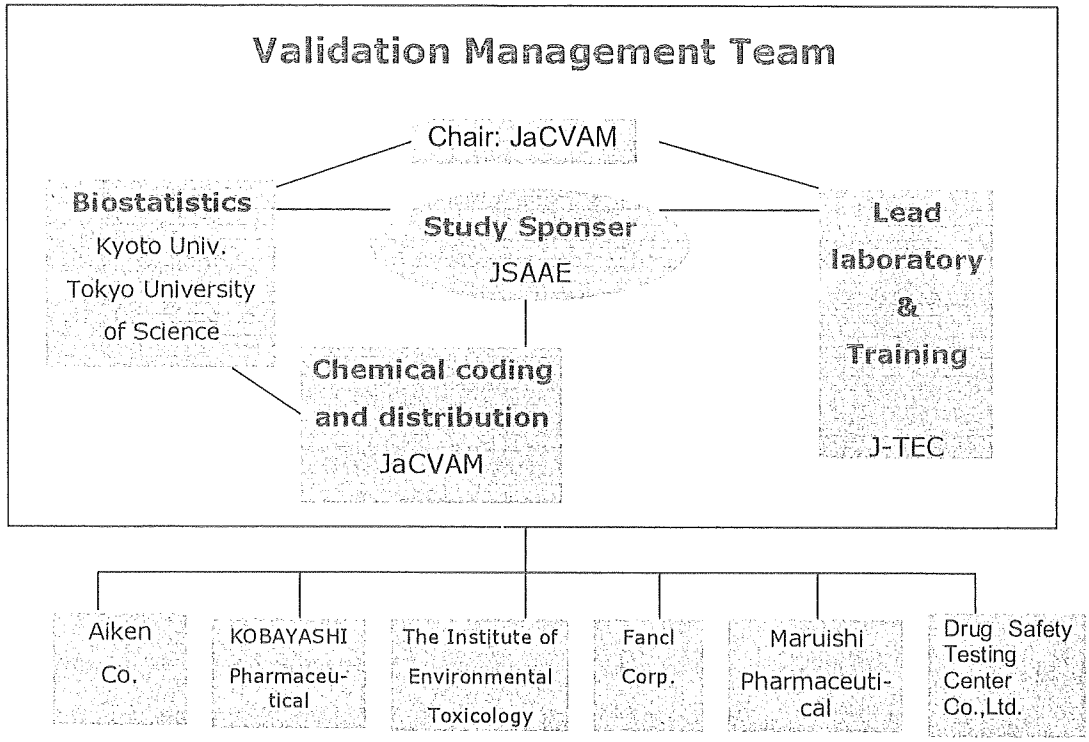

Fig. 1: Management Structure of the 3rd phase Skin Irritation Validation Study

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 – Maruishi Pharmaceutical Co., Ltd. (Yukihiko Watanabe and Osamu Mitani)
- Laboratory 6 – Drug Safety Testing Center Co., Ltd. (Shinsuke Shinoda and Saori Hagiwara)

A lead laboratory is also identified as J-TEC (Masakazu Kato and 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 .

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

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

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.

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. 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 ECVAM performance standards (2007). These tests were conducted by 7 laboratories between September 2008 and January of 2009.

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 ECVAM 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

According to the new ECVAM performance standard (ESAC statement, 2009), we selected 6 new chemicals tested. 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.

6-2. Chemical list

Reference chemicals added in "ECVAM performance standard (ESAC,2009)" are shown in Table 1.

Table 1. Test chemicals and code.

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

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

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6-3. Chemical Coding and distribution

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. Revised protocol of skin irritation test with LabCyte EPI-MODEL24

In this phase study, we used the SOP (ver. 6.1). 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.

A rough outline of LabCyte EPI-MODEL24 SOP base on the EPSKIN SOP (EPISKIN®-MTTreduction,2007) was presented below. LabCyte EPI-MODEL24 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 next 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 by 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 15 minutes of exposure, each tissue was carefully rinsed with PBS (Invitrogen, CA, USA) ten times by using a washing bottle to remove any remaining test chemical from the surface. The blotted tissues were then transferred to new wells of 24-well plates containing 1 mL of fresh assay medium.

The treated and control tissues were post-incubated for 42 hours (37°C, 5%, CO_2, humidified atmosphere). When the 42-hour post-incubation period was completed, blotted tissues were transferred to new wells of 24-well plates containing 0.5 ml of freshly prepared MTT medium (1 mg/mL; Dojindo Co., Kumamoto, Japan) for MTT assay. Tissues were incubated for three hours (37°C, 5% CO_2, humidified atmosphere) and were then transferred to microtubes containing 0.3 ml isopropanol, completely immersing the tissue. Formazan extraction was performed at room temperature and the tissues were allowed to stand overnight. Subsequently, 200µl extracts were trans-

ferred to a 96-well plate. The optical density was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.

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

7-2. Prediction Model of Skin Irritation

In this study, the prediction model of skin irritation potential with LabCyte EPI-MODEL24 was set refer to the conditions for EpiSkin described in the original ECVAM performance standards (ECVAM, 2007). This prediction model is described in Table 2. In the event that the three independent results within an individual batch were not in agreement, the result that occurred twice was used.

Acceptance criteria

1) OD_{NC} of negative control is less than 0.7.

2) The viability of positive control is up to 40%

Positive criteria

The test substance is considered to be irritant to skin in accordance with regulation GHS category 2 if the tissue viability after exposure and post-treatment incubation is equal or lower (≤)that 50%.

The test substance may be considered as no-category if the tissue viability after exposure and post-treatment incubation is higher (>) than 50%.

7-3. Data Collection, handling, and analysis

The independent biostatisticians for the study collected and organised the data using specific data collection software (Datasheet5.0:20090430.xls). They worked in close collaboration with the biostatisticians, (Takashi Omori, and Etsuyoshi Miyaoka). After decoding the data, they performed statistical analyses. The data management procedures and statistical tools applied was approved by the VMT.

7-4. Quality assurance, GLP

Laboratories

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

QA aspects

Takashi Omori and Hajime Kojima assured the quality of all the data and records.

8. Results

8-1. Comments at the Datasheet

All tests were sufficient with acceptance criteria. There were a few comments from each laboratory. 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 2. 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. Negative control

In Table 3, absorbances of negative control are shown. All data of negative control were sufficient with acceptance criteria.

Table 3 Absorbance of negative control

	Lab.					
Exp.	a	b	c	d	f	g
1	0.9	0.96	1.04	1.11	0.90	0.91
2	0.72	1.01	1.06	1.11	0.94	1.08
3	0.8	0.97	1.1	1.03	0.92	0.88
Mean	0.81	0.98	1.07	1.08	0.92	0.96
Sd	0.09	0.03	0.03	0.05	0.02	0.11

8-3. Positive control

Table 4 shows three independent viabilities and statistical analysis of positive control at each laboratory. All data were sufficient with acceptance criteria of positive control.

Table 4. Viability of positive control

	Lab.					
Exp.	a	b	c	d	f	g
1	6.4	9.3	8.2	3.5	8.5	11.7
2	2.2	2.2	7.3	2.5	4.1	2.5
3	1.8	1.6	2.4	2.1	2.7	3.3
Mean	3.5	4.4	6.0	2.7	5.1	5.8
Sd	2.5	4.3	3.1	0.7	3.0	5.1

8-4. Skin irritation test by cell viability

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 5. Summary statistical analysis of viability each chemical are shown in Table 6 and Fig.2

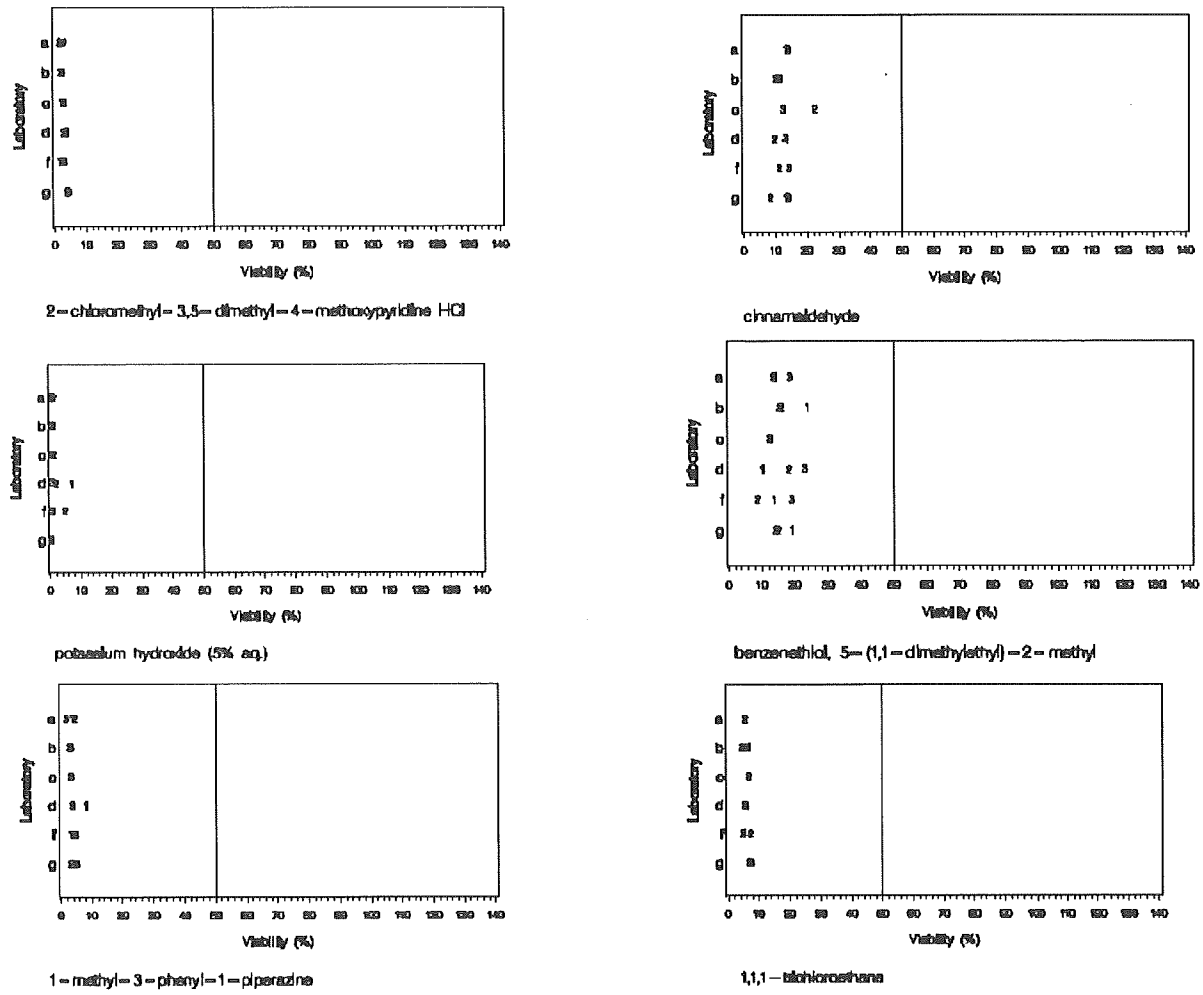
Table 5. Viability of chemicals each laboratory

				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	Cat.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	Cat.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	Cat.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	Cat.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	Cat.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 6 Summary statistical analysis of viability each chemical

		Lab.					
Chem.	Stat.	a	b	c	d	f	g
A	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
B	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
C	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
D	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
E	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
F	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

Fig.2 Distribution of viability each chemical



8-5. Classification of three independent viabilities at each labortatory

The classifications of three independent viabilities only evaluated MTT assay were shown in Table 7. All laboratories evaluated positives from the results obtained by 6 chemicals.