

## Assessment of the *in vitro* skin irritation of chemicals using the Vitrolife-Skin™ human skin model

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### Abstract

Currently, the European Centre for the Validation of Alternative Methods (ECVAM) has supported formal validation studies on *in vitro* tests for predicting skin irritancy and corrosivity, which included three of the *in vitro* tests employed human skin models, EPISKIN™ and EpiDerm™. When skin models are used to evaluate skin irritancy and corrosivity, it is important that suitable chemical application procedures are utilized. We used 44 chemicals and evaluated their skin irritancy using post-incubation method (10-minute treatment and 18-hour post-treatment incubation) that we have originally developed to predict skin irritancy. This method is quite similar to a refined protocol for the EPISKIN™ had been proposed in the ECVAM validation study. The sensitivity, specificity, and accuracy with the MTT reduction assay-based prediction model were 77.8%, 76.9%, and 77.3%, respectively. On the other hand, the sensitivity, specificity, and accuracy with the interleukin-1 $\alpha$  secretion assay-based prediction model were 61.1%, 92.3%, and 79.5%, respectively. Combining these endpoints, this resulted in a clear increase in sensitivity and accuracy to 94.4% and 81.8%, respectively. Vitrolife-Skin™ showed basic utility for irritancy testing by this method and it is possible to confidently predict skin irritancy, provided that the appropriate chemical application procedures are used.

**Keywords:** human skin model, skin irritancy, Vitrolife-Skin™, EPISKIN™, ECVAM

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### Introduction

Currently, the European Centre for the Validation of Alternative Methods (ECVAM) has supported formal validation studies on *in vitro* tests for predicting skin irritancy, which included two of the *in vitro* tests employed human skin models, EPISKIN™ (Cotovio *et al.*, 2005) and EpiDerm™ (Kandarova *et al.*, 2005). When skin models are used to evaluate skin irritancy, it is important that suitable chemical application procedures are utilized. We used 44 chemicals and evaluated their skin irritancy using post-incubation method (10-minute treatment and 18-hour post-treatment incubation) that we have originally developed to predict skin irritancy. This method is quite similar to a refined protocol for the EPISKIN™ had been proposed in the ECVAM validation study (Cotovio *et al.*, 2005).

### Materials and methods

#### Materials

A total of 44 test chemicals were selected from the chemicals tested in the ECVAM skin irritancy

validation study (Cotovio *et al.*, 2005). All test chemicals were purchased from Aldrich (Milwaukee, USA) and Acros Organics (New Jersey, USA), and used as received. The Vitrolife-Skin™ human skin model consisting of dermis and epidermis with cornified layers was prepared as previously described (Morikawa *et al.*, 2002, Morota *et al.*, 1998, Morota *et al.*, 1999).

#### Chemical application procedure by the post-incubation (PI) method

The experimental steps were performed as follows, using a method that is referred to as the post-incubation (PI) method with slightly modifications, as described previously (Morikawa *et al.*, 2002, Morikawa *et al.*, 2005, Morota *et al.*, 1998, Morota *et al.*, 1999). The Vitrolife-Skin™ models were placed in 250  $\mu$ L of Dulbecco's modified Eagle's medium (DMEM) + 5% fetal bovine serum (FBS) in 24-well plates and equilibrated in a 1-hour incubation (37 °C, 5% CO<sub>2</sub>, 90% humidity). Test chemicals were applied directly to the stratum corneum (8 mm diameter) of

six replicate models per chemical. Liquids (100  $\mu$ L) were applied using a positive displacement pipette. Solids were crushed to a powder, if necessary, and 50 mg was applied using a spatula, with addition of 50  $\mu$ L of distilled water to ensure good contact with the surface. Six replicate models were applied with 100  $\mu$ L distilled water as a negative control. After exposure for 10 minutes at room temperature, six replicate models per chemical were washed thoroughly with Dulbecco's phosphate-buffered saline (PBS) to remove the test chemical from the tissue surface. Six replicate models were then immersed in 1.5 mL of DMEM + 5% FBS in 24-well plates and triplicate models were cultured for an additional 18 hours or 42 hours at 37°C in a 5% CO<sub>2</sub>, 90% humidity environment. These chemical application procedures, which were 10-minute chemical exposure and additional 18-hour or 42-hour post-incubation, are quite similar to the refined or optimized EPISKIN™ skin irritation protocol in the ECVAM validation study, respectively (Cotovio *et al.*, 2005).

### Calculation of the cell viability

The effects of the test chemicals on cell viability were determined using 3-(4,5-dimethylthiazol-2-yl)-

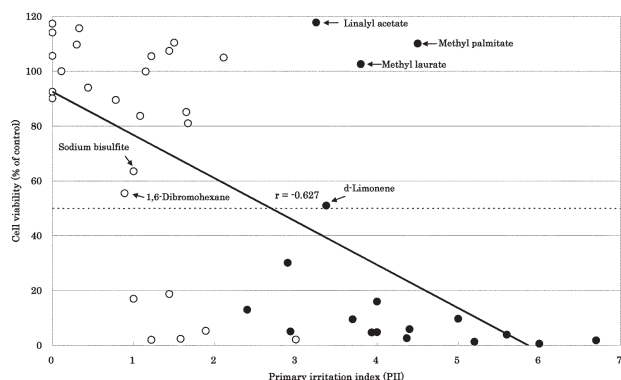


Fig. 1. Comparison of cell viability after exposure to the test chemical for 10 minutes and 18-hour post-incubation and the PII. Closed circles; *in vivo* irritant (I), open circles; *in vivo* non irritant (NI) chemicals.

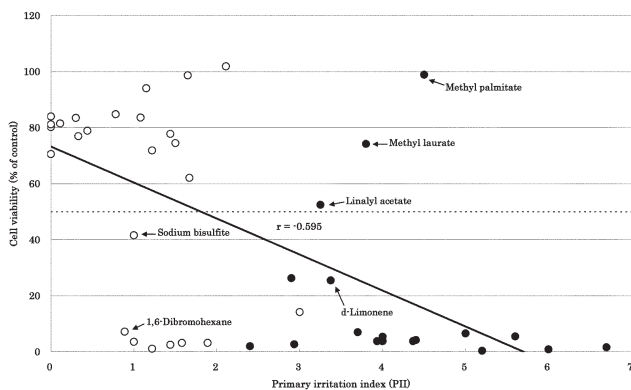


Fig. 2. Comparison of cell viability after exposure to the test chemical for 10 minutes and 42-hour post-incubation and the PII. Closed circles; *in vivo* irritant (I), open circles; *in vivo* non irritant (NI) chemicals.

2,5-diphenyltetrazolium bromide (MTT) reduction assay. After blotting, the models were incubated (5% CO<sub>2</sub>, 90% humidity) in 1.0 mL of DMEM + 5% FBS containing 0.5 mg of MTT for an additional 3 hours at 37 °C. After the models were washed with PBS, biopsies of the models were taken using a biopsy punch (6 mm diameter). The biopsies were separated from the models with the aid of forceps, and placed into isopropanol (1.0 mL), after removing excess water by placing the units on absorbent paper. The precipitated formazan was extracted overnight at room temperature, with protection from light. The absorbance of the extracts was measured at 570 nm using a UV-VIS spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). The cell viability determined by the MTT reduction assay method was expressed as a percentage of a negative control.

### Quantification of cytokines

The release of the pro-inflammatory cytokines was determined, as described previously (Morota *et al.*, 1999). At the end of the post-incubation period, the culture media from each treated or negative control model were collected in polypropylene vials and stored at -30 °C until cytokine assay. The collected media were diluted appropriately with fresh culture media (DMEM + 5% FBS). The cytokines, human interleukin (IL)-1 $\alpha$  and IL-8, concentrations were determined with use of commercially available ELISA kits (Pierce Endogen, Rockford, IL, USA).

### Prediction models

Predictions of *in vitro* irritant/non irritant were made according to the refined or optimized prediction model used in the EPISKIN™ skin irritancy test (Cotovio *et al.*, 2005). Hence, chemicals that reduced the cell viability to less than 50% upon exposure to the Vitrolife-Skin™ model are predicted to be *in vivo* irritant.

## Results

### Skin irritancy test using MTT reduction assay

Results of cell viability and classification predictions (50% threshold) are shown in Table 1. Comparisons of cell viability after exposure to the test chemical for 10-minute treatment period and 18-hour post treatment period (10 min/18 hr) or 10-minute treatment period and 42-hour post-incubation (10 min/42 hr), and the primary irritation index (PII) are shown in Figs. 1 and 2, respectively. Based on the MTT results of 10 min/18 hr protocol, 14 of the 18 *in vivo* irritant chemicals were correctly classified *in vitro*. Among these, *d*-limonene appeared to have a borderline result close to the 50% defined threshold (with viability value of 51.0%). On the other hand, linalyl acetate, methyl palmitate, and methyl laurate showed very high viabilities, close to 100%.

Table 1. Results for cell viability (MTT assay) and classification predictions.

No.	Chemical	10 min/18 hr <sup>1)</sup>			10 min/42 hr <sup>2)</sup>		
		Viability (%)		<i>In vitro</i> class <sup>3)</sup>	Viability (%)		<i>In vitro</i> class <sup>3)</sup>
1	Sodium lauryl sulfate (20%, aq.)	1.8 ± 0.1		I	1.6 ± 0.2		I
2	Sodium lauryl sulfate (50%, aq.)	0.6 ± 0.1		I	0.9 ± 0.2		I
3	Tetrachloroethylene	3.9 ± 1.5		I	5.5 ± 3.3		I
4	Pottasium hydroxide (5%, aq.)	1.3 ± 0.0		I	0.4 ± 0.2		I
5	Heptanal	9.7 ± 2.8		I	6.6 ± 0.9		I
6	Lilestralis/lilial	4.8 ± 0.8		I	5.4 ± 0.4		I
7	Methyl palmitate	110.1 ± 11.4		NI	98.9 ± 17.7		NI
8	1-Bromopentane	5.9 ± 0.7		I	4.2 ± 0.9		I
9	α-Terpineol	2.6 ± 0.0		I	3.8 ± 0.2		I
10	β-Citronellol	4.7 ± 0.3		I	3.8 ± 0.6		I
11	1-Bromohexane	16.0 ± 15.9		I	3.8 ± 0.8		I
12	Methyl laurate	102.6 ± 4.3		NI	74.2 ± 4.7		NI
13	Cinnamaldehyde	9.5 ± 1.1		I	7.0 ± 1.3		I
14	Linalyl acetate	117.8 ± 6.6		NI	52.5 ± 30.2		NI
15	<i>d</i> -Limonene	51.0 ± 20.7		NI	25.5 ± 12.4		I
16	Eugenol	30.1 ± 5.6		I	26.3 ± 4.6		I
17	10-Undecenoic acid	13.0 ± 13.0		I	2.0 ± 0.2		I
18	Linalool	5.1 ± 0.2		I	2.7 ± 0.3		I
19	Dimethyl disulfide	2.1 ± 0.1		I	14.2 ± 14.9		I
20	Methyl stearate	105.0 ± 1.3		NI	101.9 ± 37.0		NI
21	Benzyl alcohol	85.1 ± 31.3		NI	98.7 ± 27.4		NI
22	<i>cis</i> -Cyclooctene	5.3 ± 0.3		I	3.2 ± 0.5		I
23	2-Ethoxyethyl methacrylate	81.0 ± 8.1		NI	62.1 ± 8.9		NI
24	Benzyl benzoate	110.4 ± 7.9		NI	74.5 ± 5.1		NI
25	2-Methyl-4-phenyl-2-butanol	2.4 ± 0.6		I	3.2 ± 0.5		I
26	Benzyl acetate	99.9 ± 4.7		NI	94.1 ± 13.6		NI
27	Isopropyl palmitate	107.4 ± 10.1		NI	77.8 ± 8.3		NI
28	2,4-Xylidine	18.7 ± 11.4		I	4.5 ± 0.4		I
29	Sodium metasilicate (10%, aq.)	2.0 ± 0.3		I	1.1 ± 0.2		I
30	Isopropyl myristate	105.5 ± 6.1		NI	71.9 ± 9.0		NI
31	Hydroxycitronellal	17.0 ± 5.0		I	3.6 ± 0.4		I
32	<i>n</i> -Butyl propionate	83.7 ± 23.3		NI	83.6 ± 12.9		NI
33	Sodium bisulfite	63.5 ± 20.9		NI	41.6 ± 11.8		I
34	1,6-Dibromohexane	55.5 ± 11.2		NI	7.2 ± 0.8		I
35	2-Propanol	89.5 ± 6.0		NI	84.8 ± 10.8		NI
36	Benzyl salicylate	115.7 ± 4.6		NI	77.0 ± 7.2		NI
37	Lauric acid	94.0 ± 5.5		NI	78.9 ± 6.0		NI
38	Dipropylene glycol	109.7 ± 1.2		NI	83.5 ± 8.8		NI
39	Sodium bicarbonate	100.0 ± 5.1		NI	81.5 ± 4.2		NI
40	3,3'-Dithiopropionic acid	114.1 ± 26.1		NI	80.2 ± 6.5		NI
41	4,4'-Methylenebis(2,6-di- <i>tert</i> -butylphenol)	117.4 ± 29.3		NI	84.0 ± 3.8		NI
42	4-Amino-1,2,4-triazole	92.5 ± 9.3		NI	81.2 ± 23.5		NI
43	1-Chloro-3-nitrobenzene	90.1 ± 2.9		NI	127.5 ± 23.7		NI
44	Erucamide	105.6 ± 23.9		NI	70.6 ± 8.9		NI

1) 10 min/18 hr: 10-minute treatment period and 18-hour post-incubation period.

2) 10 min/42 hr: 10-minute treatment period and 42-hour post-incubation period.

3) I = irritant (viability &lt; 50 %), NI = non irritant (viability &gt; 50%).

Comparatively, the 10 min/42 hr protocol correctly classified as irritant, 15 of the 18 chemicals classified as irritant *in vivo*. *d*-Limonene detected as borderline in the 10 min/18 hr protocol was moved to the correct class (irritant) with viability clearly under the 50% threshold (25.5%). Also, linalyl acetate detected as very high viability in the 10 min/18 hr protocol was moved to a borderline result close to the 50% defined threshold (with viability value of 52.5%). On the other hand, of the other 26 chemicals, which were non irritant *in vivo*, 20 were correctly classified with the 10 min/18 hr protocol. Two chemicals, sodium bisulfite and 1,6-dibromohexane, correctly classified as non irritant with the 10 min/18 hr protocol, were moved to over classification as irritant with the 10 min/42 hr protocol.

#### Skin irritancy test using IL-1 $\alpha$ release assay

Results of IL-1 $\alpha$  release are expressed as fold

increase release to negative control. Comparison of IL-1 $\alpha$  release after exposure to the test chemical for 10 minutes and 18-hour or 42-hour post-incubation, and the PII are shown in Figs. 3 and 4, respectively. As shown in Figs. 3 and 4, most of *in vivo* non irritant chemicals had IL-1 $\alpha$  release value of less than 3.5-fold. In this study, therefore, IL-1 $\alpha$  release value of less than 3.5-fold was considered to be indicative of a non irritant chemical. Results of IL-1 $\alpha$  release and classification predictions are shown in Table 2. Methyl laurate, linalyl acetate, and *d*-limonene, detected wrongly as non irritant with MTT assay, were correctly classified *in vitro* with IL-1 $\alpha$  release assay in the 10 min/18 hr protocol. Also, linalyl acetate, detected wrongly as non irritant with MTT assay, was correctly classified *in vitro* with IL-1 $\alpha$  release assay in the 10 min/42 hr protocol. On the other hand, *n*-butyl propionate, detected correctly as non irritant with MTT assay, were wrongly classified

Table 2. Results for interleukin-1 $\alpha$  (IL-1 $\alpha$ ) release and classification predictions.

No.	Chemical	10 min/18 hr <sup>1)</sup>		10 min/42 hr <sup>2)</sup>	
		IL-1 $\alpha$ release <sup>3)</sup>	<i>In vitro</i> class <sup>4)</sup>	IL-1 $\alpha$ release <sup>3)</sup>	<i>In vitro</i> class <sup>4)</sup>
1	Sodium lauryl sulfate (20%, aq.)	6.06 $\pm$ 1.75	I	4.10 $\pm$ 2.00	I
2	Sodium lauryl sulfate (50%, aq.)	1.83 $\pm$ 0.74	NI	0.86 $\pm$ 0.17	NI
3	Tetrachloroethylene	25.68 $\pm$ 1.44	I	7.99 $\pm$ 1.00	I
4	Pottasium hydroxide (5%, aq.)	1.16 $\pm$ 0.08	NI	0.76 $\pm$ 0.00	NI
5	Heptanal	3.27 $\pm$ 1.47	NI	5.25 $\pm$ 0.69	I
6	Lilestralis/lilial	3.28 $\pm$ 0.57	NI	3.13 $\pm$ 0.53	NI
7	Methyl palmitate	2.01 $\pm$ 0.09	NI	1.03 $\pm$ 0.12	NI
8	1-Bromopentane	5.21 $\pm$ 0.92	I	3.33 $\pm$ 1.03	NI
9	$\alpha$ -Terpineol	2.84 $\pm$ 0.27	NI	7.03 $\pm$ 0.71	I
10	$\beta$ -Citronellol	6.04 $\pm$ 1.09	I	4.02 $\pm$ 1.92	I
11	1-Bromohexane	6.25 $\pm$ 0.27	I	3.76 $\pm$ 0.99	I
12	Methyl laurate	6.64 $\pm$ 0.37	I	0.95 $\pm$ 0.27	NI
13	Cinnamaldehyde	1.29 $\pm$ 0.06	NI	0.86 $\pm$ 0.04	NI
14	Linalyl acetate	3.57 $\pm$ 1.07	I	6.10 $\pm$ 0.91	I
15	<i>d</i> -Limonene	5.53 $\pm$ 1.55	I	5.46 $\pm$ 2.48	I
16	Eugenol	16.62 $\pm$ 4.11	I	2.56 $\pm$ 0.43	NI
17	10-Undecenoic acid	7.19 $\pm$ 0.95	I	6.90 $\pm$ 3.21	I
18	Linalool	4.07 $\pm$ 0.11	I	7.18 $\pm$ 0.84	I
19	Dimethyl disulfide	1.68 $\pm$ 0.49	NI	2.79 $\pm$ 1.17	NI
20	Methyl stearate	1.00 $\pm$ 0.11	NI	0.66 $\pm$ 0.26	NI
21	Benzyl alcohol	1.21 $\pm$ 0.23	NI	1.93 $\pm$ 0.25	NI
22	<i>cis</i> -Cyclooctene	3.15 $\pm$ 0.13	NI	3.77 $\pm$ 0.78	I
23	2-Ethoxyethyl methacrylate	1.10 $\pm$ 0.44	NI	2.72 $\pm$ 0.44	NI
24	Benzyl benzoate	1.17 $\pm$ 0.36	NI	0.17 $\pm$ 0.24	NI
25	2-Methyl-4-phenyl-2-butanol	2.91 $\pm$ 0.90	NI	2.77 $\pm$ 0.77	NI
26	Benzyl acetate	1.64 $\pm$ 0.38	NI	1.95 $\pm$ 0.59	NI
27	Isopropyl palmitate	0.96 $\pm$ 0.14	NI	0.17 $\pm$ 0.19	NI
28	2,4-Xylidine	8.69 $\pm$ 3.33	I	2.39 $\pm$ 0.32	NI
29	Sodium metasilicate (10%, aq.)	0.25 $\pm$ 0.04	NI	1.16 $\pm$ 0.02	NI
30	Isopropyl myristate	1.09 $\pm$ 0.09	NI	0.28 $\pm$ 0.07	NI
31	Hydroxycitronellal	1.55 $\pm$ 0.30	NI	1.04 $\pm$ 0.18	NI
32	<i>n</i> -Butyl propionate	7.72 $\pm$ 0.99	I	7.70 $\pm$ 2.59	I
33	Sodium bisulfite	3.29 $\pm$ 1.00	NI	2.99 $\pm$ 0.04	NI
34	1,6-Dibromohexane	2.49 $\pm$ 0.38	NI	4.00 $\pm$ 0.91	I
35	2-Propanol	2.56 $\pm$ 0.12	NI	2.77 $\pm$ 0.53	NI
36	Benzyl salicylate	2.55 $\pm$ 0.29	NI	2.97 $\pm$ 0.82	NI
37	Lauric acid	1.25 $\pm$ 0.12	NI	1.10 $\pm$ 0.09	NI
38	Dipropylene glycol	2.47 $\pm$ 0.06	NI	3.47 $\pm$ 1.09	NI
39	Sodium bicarbonate	1.63 $\pm$ 0.35	NI	1.06 $\pm$ 0.25	NI
40	3,3'-Dithiopropanoic acid	1.34 $\pm$ 0.24	NI	1.42 $\pm$ 0.46	NI
41	4,4'-Methylenebis(2,6-di- <i>tert</i> -butylphenol)	1.13 $\pm$ 0.20	NI	1.28 $\pm$ 0.26	NI
42	4-Amino-1,2,4-triazole	2.20 $\pm$ 0.23	NI	1.48 $\pm$ 0.56	NI
43	1-Chloro-3-nitrobenzene	0.88 $\pm$ 0.16	NI	1.00 $\pm$ 0.11	NI
44	Erucamide	0.75 $\pm$ 0.16	NI	0.63 $\pm$ 0.27	NI

1) 10 min/18 hr; 10-minute treatment period and 18-hour post-incubation period.

2) 10 min/42 hr; 10-minute treatment period and 42-hour post-incubation period.

3) IL-1 $\alpha$  release; results are expressed as fold increase release to control; control mean  $\pm$  SD release = 110.9  $\pm$  44.8 pg/18 hr and 87.3  $\pm$  23.8 pg/42 hr.

4) I = irritant (IL-1 $\alpha$  release > 3.5-fold), NI = non irritant (IL-1 $\alpha$  release < 3.5-fold).

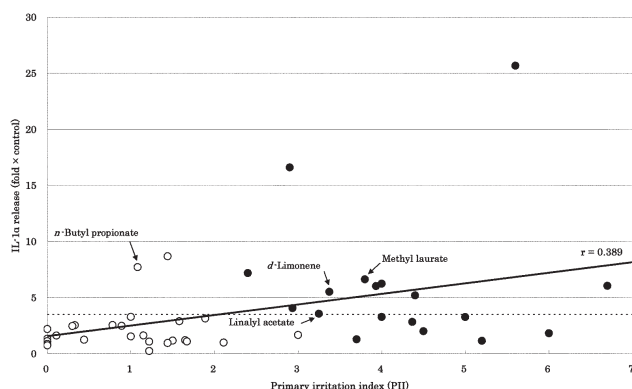


Fig. 3. Comparison of IL-1 $\alpha$  release after exposure to the test chemical for 10 minutes and 18-hour post-incubation and the PII. Closed circles; *in vivo* irritant (I), open circles; *in vivo* non irritant (NI) chemicals.

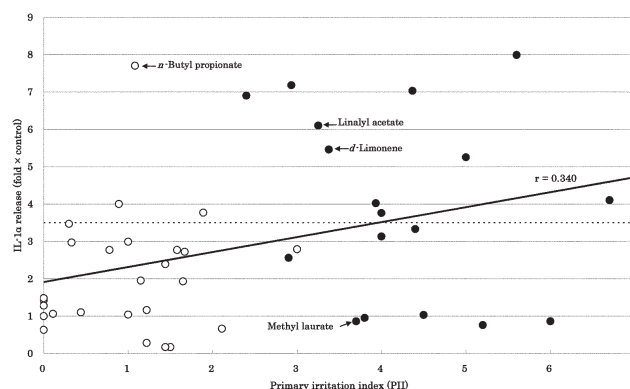


Fig. 4. Comparison of IL-1 $\alpha$  release after exposure to the test chemical for 10 minutes and 42-hour post-incubation and the PII. Closed circles; *in vivo* irritant (I), open circles; *in vivo* non irritant (NI) chemicals.

Table 3. Results for interleukin-8 (IL-8) release and classification predictions.

No.	Chemical	10 min/18 hr <sup>1)</sup>		10 min/42 hr <sup>2)</sup>	
		IL-8 release <sup>3)</sup>	<i>In vitro</i> class <sup>4)</sup>	IL-8 release <sup>3)</sup>	<i>In vitro</i> class <sup>4)</sup>
1	Sodium lauryl sulfate (20%, aq.)	0.33 ± 0.22	I	0.11 ± 0.05	I
2	Sodium lauryl sulfate (50%, aq.)	0.17 ± 0.02	I	0.13 ± 0.02	I
3	Tetrachloroethylene	0.12 ± 0.08	I	0.15 ± 0.05	I
4	Pottasium hydroxide (5%, aq.)	1.16 ± 0.04	I	0.16 ± 0.03	I
5	Heptanal	0.15 ± 0.04	I	0.13 ± 0.01	I
6	Lilestralis/lilial	0.31 ± 0.07	I	0.10 ± 0.01	I
7	Methyl palmitate	1.26 ± 0.23	NI	0.94 ± 0.67	NI
8	1-Bromopentane	0.25 ± 0.07	I	0.12 ± 0.03	I
9	α-Terpineol	0.08 ± 0.01	I	0.13 ± 0.00	I
10	β-Citronellol	0.37 ± 0.15	I	0.13 ± 0.04	I
11	1-Bromohexane	0.45 ± 0.16	I	0.14 ± 0.02	I
12	Methyl laurate	1.52 ± 0.02	NI	5.75 ± 1.14	NI
13	Cinnamaldehyde	0.03 ± 0.00	I	0.11 ± 0.01	I
14	Linalyl acetate	1.36 ± 0.37	NI	8.48 ± 3.68	NI
15	d-Limonene	1.21 ± 0.82	NI	0.25 ± 0.11	I
16	Eugenol	0.09 ± 0.01	I	0.12 ± 0.01	I
17	10-Undecenoic acid	0.19 ± 0.05	I	0.24 ± 0.01	I
18	Linalool	0.26 ± 0.02	I	0.14 ± 0.01	I
19	Dimethyl disulfide	0.10 ± 0.03	I	0.32 ± 0.38	I
20	Methyl stearate	0.79 ± 0.32	NI	5.39 ± 1.30	NI
21	Benzyl alcohol	1.08 ± 0.66	NI	3.03 ± 0.37	NI
22	cis-Cyclooctene	0.32 ± 0.30	I	0.12 ± 0.00	I
23	2-Ethoxyethyl methacrylate	1.07 ± 0.04	NI	2.56 ± 0.89	NI
24	Benzyl benzoate	1.63 ± 0.09	NI	3.46 ± 1.36	NI
25	2-Methyl-4-phenyl-2-butanol	0.18 ± 0.00	I	0.13 ± 0.01	I
26	Benzyl acetate	1.43 ± 0.31	NI	3.38 ± 0.70	NI
27	Isopropyl palmitate	1.32 ± 0.51	NI	1.45 ± 0.56	NI
28	2,4-Xylidine	0.26 ± 0.09	I	0.13 ± 0.04	I
29	Sodium metasilicate (10%, aq.)	0.04 ± 0.01	I	0.52 ± 0.16	I
30	Isopropyl myristate	1.64 ± 0.08	NI	2.87 ± 0.95	NI
31	Hydroxycitronellal	0.04 ± 0.01	I	0.07 ± 0.00	I
32	n-Butyl propionate	1.36 ± 0.12	NI	1.58 ± 0.38	NI
33	Sodium bisulfite	0.85 ± 0.42	NI	1.09 ± 0.33	NI
34	1,6-Dibromohexane	0.35 ± 0.09	I	0.10 ± 0.01	I
35	2-Propanol	1.65 ± 0.06	NI	3.84 ± 1.59	NI
36	Benzyl salicylate	1.68 ± 0.11	NI	7.04 ± 1.62	NI
37	Lauric acid	2.13 ± 0.46	NI	1.79 ± 0.70	NI
38	Dipropylene glycol	1.40 ± 0.31	NI	2.62 ± 0.46	NI
39	Sodium bicarbonate	1.35 ± 0.34	NI	2.97 ± 1.73	NI
40	3,3'-Dithiopropionic acid	1.29 ± 0.11	NI	4.51 ± 1.53	NI
41	4,4'-Methylenebis(2,6-di-tert-butylphenol)	1.20 ± 0.22	NI	4.13 ± 2.30	NI
42	4-Amino-1,2,4-triazole	1.26 ± 0.28	NI	2.99 ± 0.90	NI
43	1-Chloro-3-nitrobenzene	1.30 ± 0.10	NI	1.94 ± 1.11	NI
44	Erucamide	1.30 ± 0.45	NI	3.25 ± 1.91	NI

1) 10 min/18 hr: 10-minute treatment period and 18-hour post-incubation period.

2) 10 min/42 hr: 10-minute treatment period and 42-hour post-incubation period.

3) IL-8 release; results are expressed as fold increase release to control; control mean ± SD release = 773.7 ± 529.8 ng/18 hr and 1084.1 ± 802.6 ng/42 hr.

4) I = irritant (IL-8 release < 0.7-fold), NI = non irritant (IL-8 release > 0.7-fold).

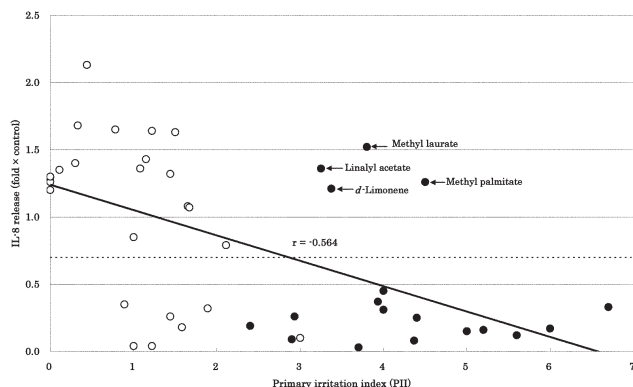


Fig. 5. Comparison of IL-8 release after exposure to the test chemical for 10 minutes and 18-hour post-incubation and the PII. Closed circles; *in vivo* irritant (I), open circles; *in vivo* non irritant (NI) chemicals.

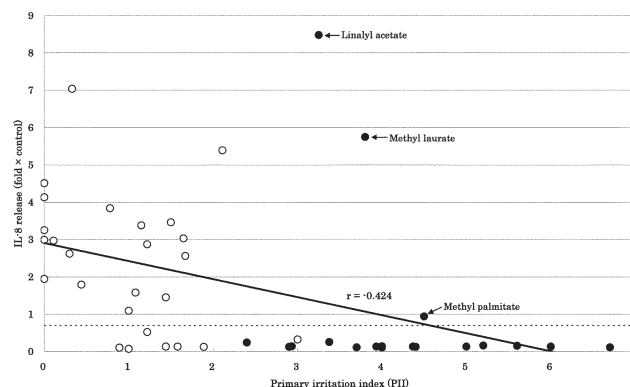


Fig. 6. Comparison of IL-8 release after exposure to the test chemical for 10 minutes and 42-hour post-incubation and the PII. Closed circles; *in vivo* irritant (I), open circles; *in vivo* non irritant (NI) chemicals.

Table 4. Comparison of statistical parameters for key performance indicators using individual parameters MTT, IL-1 $\alpha$ , and IL-8, and combined parameters (MTT + IL-1 $\alpha$ ).

Parameter	10 min/18 hr <sup>1)</sup>				10 min/42 hr <sup>2)</sup>			
	MTT <sup>3)</sup>	IL-1 $\alpha$ <sup>4)</sup>	MTT + IL-1 $\alpha$ <sup>5)</sup>	IL-8 <sup>6)</sup>	MTT <sup>3)</sup>	IL-1 $\alpha$ <sup>4)</sup>	MTT + IL-1 $\alpha$ <sup>5)</sup>	IL-8 <sup>6)</sup>
Sensitivity	14/18 77.8%	11/18 61.1%	17/18 94.4%	14/18 77.8%	15/18 83.3%	10/18 55.6%	16/18 88.9%	15/18 83.3%
Specificity	20/26 76.9%	24/26 92.3%	19/26 73.1%	19/26 73.1%	18/26 69.2%	23/26 88.5%	17/26 65.4%	19/26 73.1%
Accuracy	34/44 77.3%	35/44 79.5%	36/44 81.8%	33/44 75.0%	33/44 75.0%	33/44 75.0%	33/44 75.0%	34/44 77.3%
Positive predictivity	14/20 70.0%	11/13 84.6%	17/24 70.8%	14/21 66.7%	15/23 65.2%	10/13 76.9%	16/25 64.0%	15/22 68.2%
Negativepredictivity	20/24 83.3%	24/31 77.4%	19/20 95.0%	19/23 82.6%	18/21 85.7%	23/31 74.2%	17/19 89.5%	19/22 86.4%
False negatives	4/24 16.7%	7/31 22.6%	1/20 5.0%	4/23 17.4%	3/21 14.3%	8/31 25.8%	2/19 10.5%	3/22 13.6%
False positives	6/20 30.0%	2/13 15.4%	7/24 29.2%	7/21 33.3%	8/23 34.8%	3/13 23.1%	9/25 36.0%	7/22 31.8%

1) 10 min/18 hr; 10-minute treatment period and 18-hour post-incubation period.

2) 10 min/42 hr; 10-minute treatment period and 42-hour post-incubation period.

3) MTT; 50 % viability cut-off (Table 1, Figures 1 & 2).

4) IL-1 $\alpha$ ; 3.5-fold release cut-off (Table 2, Figures 3 & 4).

5) MTT + IL-1 $\alpha$ ; Two-step selection according to the EPISKIN<sup>TM</sup> prediction model in the ECVAM validation study (Cotovio *et al.*, 2005).

6) IL-8; 0.7-fold release cut-off (Table 3, Figures 5 & 6).

Table 5. Matrix of correlation coefficients.

	10 min/18 hr <sup>1)</sup>			10 min/42 hr <sup>2)</sup>		
	MTT	IL-1 $\alpha$	IL-8	MTT	IL-1 $\alpha$	IL-8
MTT	1			1		
IL-1 $\alpha$	-0.326	1		-0.414	1	
IL-8	0.916	-0.311	1	0.698	-0.233	1

1) 10 min/18 hr; 10-minute treatment period and 18-hour post-incubation period.

2) 10 min/42 hr; 10-minute treatment period and 42-hour post-incubation period.

*in vitro* with IL-1 $\alpha$  release assay in the both 10 min/18 hr protocol and 10 min/42 hr protocol.

### Skin irritancy test using IL-8 release assay

Results of IL-8 release are expressed as fold increase release to negative control. Comparison of IL-8 release after exposure to the test chemical for 10 minutes and 18-hour or 42-hour post-incubation, and the PII are shown in Figs. 5 and 6, respectively. As shown in Figs. 5 and 6, most of *in vivo* irritant chemicals had IL-8 release value of less than 0.7-fold. In this study, therefore, IL-8 release value of less than 0.7-fold was considered to be indicative of an irritant chemical. Results of IL-8 release and classification predictions are shown in Table 3.

### Discussion

Table 4 shows the key performance parameters for the two protocols. Comparatively, the sensitivity of the MTT assay results with 10 min/42 hr protocol rose to 83.3% from 77.8%, thus showing an improvement. On the other hand, the specificity was decreased from 76.9% (10 min/18 hr protocol) to 69.2% (10 min/42 hr protocol). The accuracy was also slightly decreased from 77.3% (10 min/18 hr protocol) to 75.0% (10 min/42 hr protocol). In the EPISKIN<sup>TM</sup> validation

study, the sensitivity, specificity, and accuracy were improved from 75.0%, 75.0%, and 75.0% (15 min/18 hr protocol) to 85.0%, 78.6%, and 81.3% (15 min/42 hr protocol), respectively (Cotovio *et al.*, 2005). These results were considered to be due to barrier function between EPISKIN<sup>TM</sup> and Vitrolife-Skin<sup>TM</sup> or detail of chemical application procedures (chemical volume and washing method).

Table 4 also shows classification predictions of cell viability and IL-1 $\alpha$  release combinations. Using this two-step selection according to the EPISKIN<sup>TM</sup> prediction model in the ECVAM validation study (Cotovio *et al.*, 2005), this resulted in a clear increase in sensitivity and accuracy to 94.4% and 81.8% with the 10 min/18 hr protocol, respectively. These results were quite similar to results of the EPISKIN<sup>TM</sup> validation study (Cotovio *et al.*, 2005). On the other hand, combining these endpoints, this resulted in a slightly increase in sensitivity and accuracy to 88.9% and 75.0% with the 10 min/42 hr protocol, respectively.

As shown in Table 4, classifications by IL-8 release were quite similar to classifications by cell viability except for 1,6-dibromohexane with the 10 min/18 hr protocol and sodium bisulfite with the 10 min/42 hr protocol. As a result, key statistical parameters were also quite similar between IL-8 release and cell viability. These results were quite different from results of the EPISKIN<sup>TM</sup> validation study (Cotovio *et al.*, 2005).

Correlations between the three endpoints measured are shown in Table 5. IL-8 release was significantly correlated with the cell viability ( $r = 0.916$  for 10 min/18 hr protocol and  $r = 0.698$  for 10 min/42 hr protocol). On the other hand, IL-1 $\alpha$  release was

not so highly correlated with the cell viability ( $r = -0.326$  for 10 min/18 hr protocol and  $r = -0.414$  for 10 min/42 hr protocol). IL-1 $\alpha$  which is stored in cells as pro-IL-1 $\alpha$  and released only damaged leaky cells, so that IL-1 $\alpha$  release from the non damaged Vitrolife-Skin™ was quite low level (data not shown). On the other hand, there is no intracellular protein storing of IL-8, so that its expression and sequent release need time to process. IL-8 release from the non damaged Vitrolife-Skin™ was increased gradually, in contrast, IL-8 release from the Vitrolife-Dermis™ which was composed only dermis (fibroblasts) was constant level (data not shown). IL-1 $\alpha$  release from Vitrolife-Skin™ was the same level as that from EPISKIN™ (Cotovio *et al.*, 2005). On the other hand, IL-8 release from Vitrolife-Skin™ and Vitrolife-Dermis™ was quite higher level than that from EPISKIN™ (Cotovio *et al.*, 2005). It was considered that the difference of IL-8 production profile was caused to the difference of IL-8 assay results between Vitrolife-Skin™, human skin model and EPISKIN™, human epidermal model.

In conclusion, Vitrolife-Skin™ showed basic utility for irritancy testing by this method and it is possible to confidently predict skin irritancy, provided that the appropriate chemical application procedures and combination of suitable endpoints, such as MTT assay and IL-1 $\alpha$  release assay, are used.

## References

- Cotovio, J., Grandidier, M.H., Portes, P., Roguet, R., and Rubinstenn, G. (2005) The *in vitro* skin irritation of chemicals: optimisation of the EPISKIN prediction model within the framework of the ECVAM validation process, *Altern Lab Anim*, 33, 329-349.
- Kandarova, H., Liebsch, M., Gerner, I., Schmidt, E., Genschow, E., Traue, D., and Spielmann, H. (2005) The EpiDerm test protocol for the upcoming ECVAM validation study on *in vitro* skin irritation tests--an assessment of the performance of the optimised test, *Altern Lab Anim*, 33, 351-367.
- Morikawa, N., Morota, K., Morita, S., Kojima, H., Nakata, S., and Konishi, H. (2002) Prediction of human skin irritancy using a cultured human skin model: comparison of chemical application procedures and development of a novel chemical application procedure using the Vitrolife-Skin™ model, *AATEX*, 9, 1-10.
- Morikawa, N., Morota, K., Suzuki, M., Kojima, H., Nakata, S., and Konishi, H. (2005) Experimental study on a novel chemical application procedure for *in vitro* skin corrosivity testing using the Vitrolife-Skin™ human skin mode, *AATEX*, 11, 68-78.
- Morota, K., Morikawa, N., Morita, S., Kojima, H., and Konishi, H. (1998) Development and evaluation of the cultured skin model, *Tiss Cult Res Commun*, 17, 87-93.
- Morota, K., Morikawa, N., Morita, S., Kojima, H., and Konishi, H. (1999) Alternative to primary Draize skin irritation test using cultured human skin model: comparison of six end points, *AATEX*, 6, 41-51.

