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Assessment of the Effects of Pre-Incubation Time on Irritation Potential Using the EpiSkin® *in vitro* Irritation Test

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Introduction

The EpiSkin® reconstructed human epidermis model (commercialised by SkinEthic) has been validated by ECVAM (European Centre for the Validation of Alternative Methods) as an *in vitro* alternative for the assessment of skin irritation potential. Charles River performs EpiSkin® *in vitro* irritation studies in accordance with the ECVAM validation SOP. The skin units have a limited and defined period of viability from the day of production and the SOP states that they should be pre-incubated for at least 24 h prior to dosing. Delays in delivery of the Episkin® units can, therefore, result in the 24 h pre-incubation being impractical. This study was designed to assess the effect of a shortened, 2 h, pre-incubation on the performance of the assay.

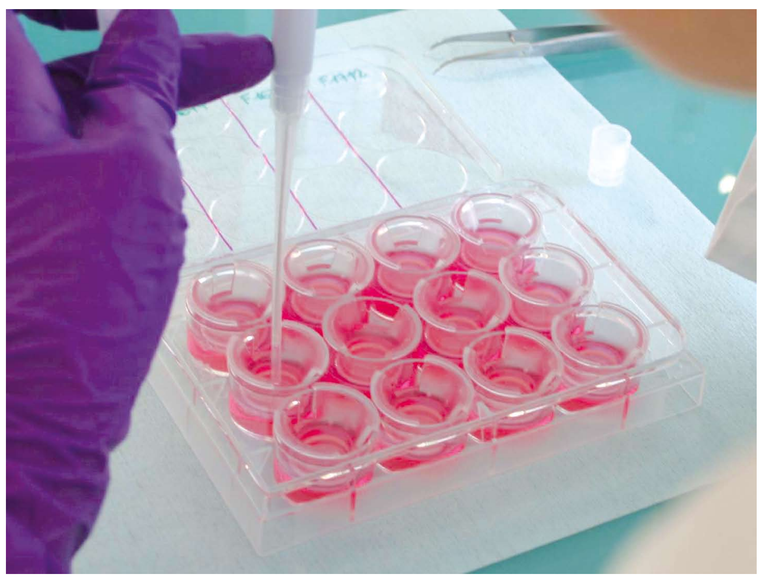


Figure 1. EpiSkin® in transwell plate

Methods

The 20 reference chemicals from the OECD competency list were investigated using both a 2 h and a 24 h pre-incubation period. These chemicals are shown in Table 1, below.

Charles River Reference Number	Chemical	CAS No.	Physical State	*In vivo* score	VRM *in vitro* Cat.	GHS *in vivo* Cat.
1	1-bromo-4-chlorobutane	6940-78-9	Liquid	0	Cat. 2	No Cat.
2	diethyl pthalate	84-66-2	Liquid	0	No Cat.	No Cat.
3	napthalene acetic acid	86-87-3	Solid	0	No Cat	No Cat.
4	allyl phenoxy-acetate	7493-74-5	Liquid	0.3	No Cat	No Cat.
5	isopropanol	67-63-0	Liquid	0.3	No Cat	No Cat.
6	4-methyl-thiobenzaldehyde	3446-89-7	Liquid	1	Cat. 2	No Cat.
7	methyl stearate	112-61-8	Solid	1	No Cat	No Cat.
8	heptyl butyrate	5870-93-9	Liquid	1.7	No Cat	No Cat.*
9	hexyl salicylate	6259-76-3	Liquid	2	No Cat	No Cat.*
10	cinnamaldehyde	104-55-2	Liquid	2	Cat. 2	No Cat.*
11	1-decanol	112-30-1	Liquid	2.3	Cat. 2	Cat. 2
12	cyclamen aldehyde	103-95-7	Liquid	2.3	Cat. 2	Cat. 2
13	1-bromo-hexane	111-25-1	Liquid	2.7	Cat. 2	Cat. 2
14	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	Solid	2.7	Cat. 2	Cat. 2
15	di-n-propyl disulphide	629-19-6	Liquid	3	No Cat	Cat. 2
16	potassium hydroxide (5%, w/v)	1310-58-3	Liquid	3	Cat. 2	Cat. 2
17	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	7340-90-1	Liquid	3.3	Cat. 2	Cat. 2
18	1-methyl-3-phenyl-1-piperazine	5271-27-2	Solid	3.3	Cat. 2	Cat. 2
19	heptanal	111-71-7	Liquid	4	Cat. 2	Cat. 2
20	1,1,1-trichloroethane	71-55-6	Liquid	4	Cat. 2	Cat. 2

* Optional Category 3

Table 1. Reference chemicals from OECD competency list

A preliminary test was conducted with each of the test substances to assess their inherent ability to chemically reduce MTT to formazan. Where a reaction was observed, additional killed-skin controls were employed to quantify the effect. The absorbance value associated with this specific chemical reduction was subtracted prior to the calculation of cell viability for the exposed skin units.

EpiSkin® units were sent to Charles River on transport agar in sterile plates. Upon arrival, half of the EpiSkin® units were transferred to 12 well plates containing maintenance medium and pre-incubated for *ca* 24 h in a humidified atmosphere (*ca* 37°C, *ca* 5% CO_2). The remaining EpiSkin® units were stored overnight in the transport packaging to simulate late delivery. On the day of testing, these tissues were pre-incubated for *ca* 2 h as described above.

An initial irritation test was conducted with 19 of the 20 reference chemicals. 1,1,1-trichloroethane could not be obtained in time for inclusion in this assay. For each chemical, at each pre-incubation period, three EpiSkin® units (0.38 cm^2) were dosed with *ca* 10 μL (liquids) or *ca* 10 mg (solids) for *ca* 15 min. The exposure period was terminated by washing each unit with Dulbecco's phosphate buffered saline (PBS). Tissues were then incubated in fresh maintenance medium for *ca* 42 h to allow for either cell recovery or the manifestation of clear cytotoxic effects. After this period, the skin units were transferred to assay medium containing MTT (0.3 mg/mL) and returned to the incubator for *ca* 3 h. Biopsies of the EpiSkin® membranes were then removed and added to acidified isopropanol. The formazan produced was assessed by measuring the optical density of the extract at 550 nm. The viability of each individual tissue was then calculated as a percentage of the mean negative control viability.

A second test was conducted to further investigate any chemicals that gave the incorrect classification in the initial test or that gave different classifications at the two pre-incubation times. 1,1,1-trichloroethane was included in this assay.

Results

A graphical representation of the results of the initial and second experiment can be found in Figures 2 to 6.

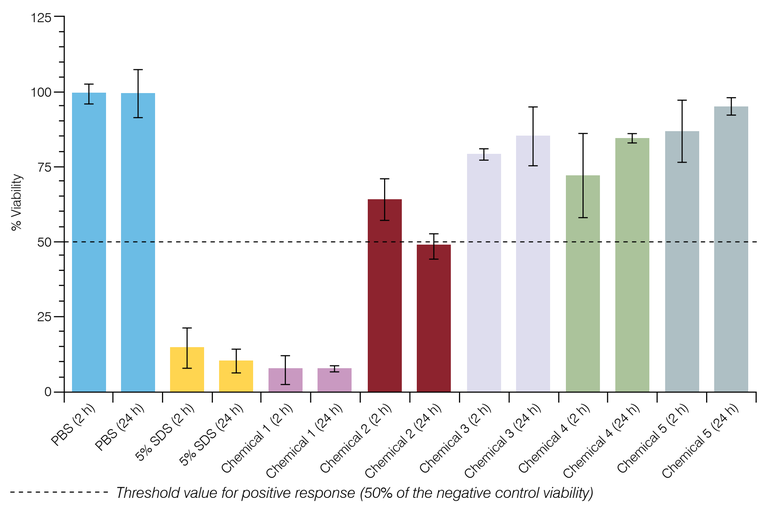
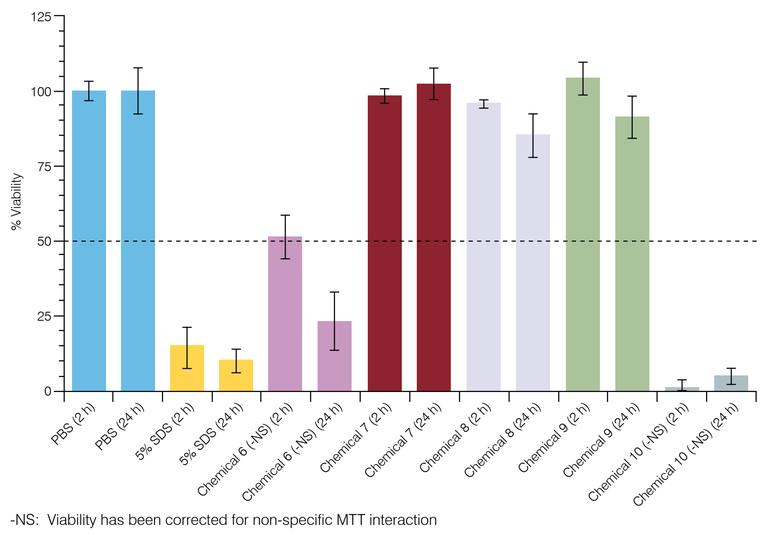
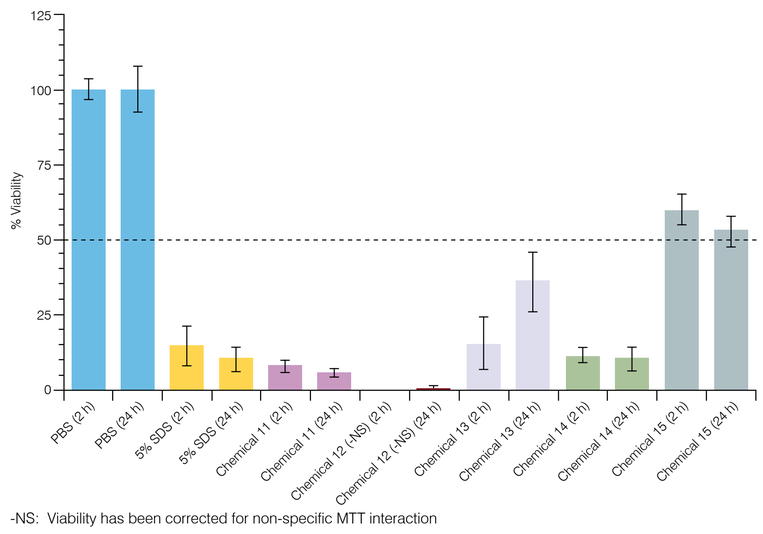


Figure 2. Percentage viability of treated EpiSkin® cultures after 2 h versus 24 h pre-incubation (mean ± SD, n = 6) - initial test



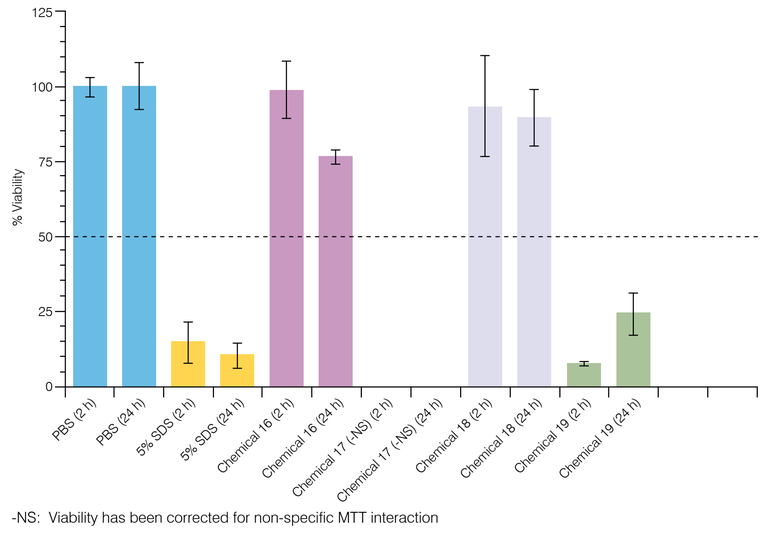
-NS: Viability has been corrected for non-specific MTT interaction

Figure 3. Percentage viability of treated EpiSkin® cultures after 2 h versus 24 h pre-incubation (mean ± SD, n = 6) - initial test



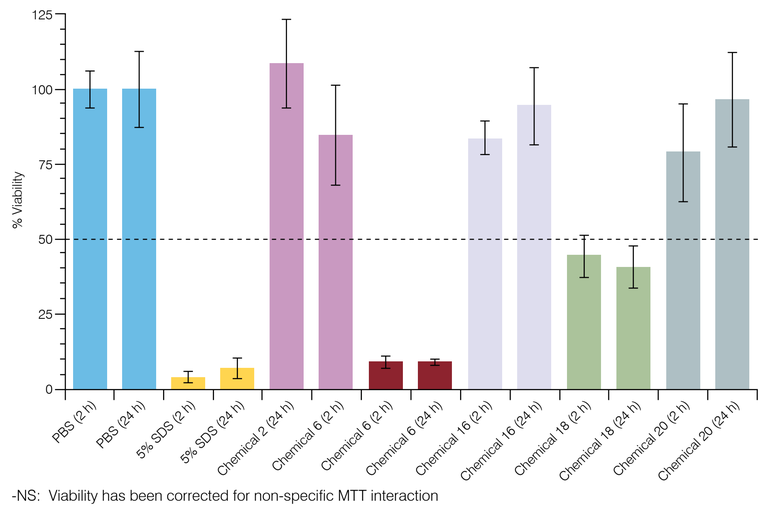
-NS: Viability has been corrected for non-specific MTT interaction

Figure 4. Percentage viability of treated EpiSkin® cultures after 2 h versus 24 h pre-incubation (mean ± SD, n = 6) - initial test



-NS: Viability has been corrected for non-specific MTT interaction

Figure 5. Percentage viability of treated EpiSkin® cultures after 2 h versus 24 h pre-incubation (mean ± SD, n = 6) - initial test



-NS: Viability has been corrected for non-specific MTT interaction

Figure 6. Percentage viability of treated EpiSkin® cultures after 2 h versus 24 h pre-incubation (mean ± SD, n = 6) - repeat test

Discussion

Some minor variations in viabilities were observed between the 2 h and 24 h data. Where the viability values were close to the threshold value for a positive response (i.e. 50% of the negative control viability) this sometimes led to the misclassification of a chemical at one of the pre-incubation times. These differences in classification are expected to be the result of normal variability associated with the assay and are not considered to be attributable to the pre-incubation time. There was no correlation between the pre-incubation time and the viability results. Of the 26 individual experimental "runs" conducted over the initial and repeat tests (19 chemicals and 1 positive control in the initial test, 5 chemicals and 1 positive control in the second test), the viability increased with shortened pre-incubation for 13 of the runs and decreased with shortened pre-incubation for 13 of the runs.

Conclusion

In conclusion, reducing the pre-incubation time of the EpiSkin® tissues from 24 h to 2 h had no effect on the performance of the *in vitro* skin irritation assay.

References

1 ECVAM Statement on the validity of *in vitro* tests for skin irritation (2007).

2 ECVAM skin irritation validation study. Validation of the EpiSkin® skin irritation test – 42 hours assay for the prediction of acute skin irritation of chemicals, L'Oreal Recherche, January 2005.

3 ECVAM skin irritation validation study (SIVS). Performance standards for applying human skin models to *in vitro* skin irritation testing (2007).

4 Draft OECD Guideline. *In vitro* skin irritation: reconstructed human epidermis (RhE) test method. 20 March 2009 (Version 6) 2nd Circulation.

Ackowledgements

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