CERI

Receipt Number	832-16-T-8814
Study Number	K10-0227

FINAL REPORT

In vitro skin irritation test of C6OLF using LabCyte EPI-MODEL24 SIT

March, 2017

Chemicals Evaluation and Research Institute, Japan, Hita

GLP STATEMENT

Chemicals Evaluation and Research Institute, Japan, Hita

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: *In vitro* skin irritation test of C6OLF using LabCyte EPI-MODEL24 SIT

Study Number: K10-0227

The study described in this report was conducted in compliance with the following GLP principle.

OECD Principles of Good Laboratory Practice, November 26, 1997, ENV/MC/CHEM (98)17

I also confirmed that this report accurately reflected the raw data and the test data were valid.

Study Director:

March 10, 2017

Date

TABLE OF CONTENTS

1. TITLE

In vitro skin irritation test of C6OLF using LabCyte EPI-MODEL24 SIT

2. SPONSOR

Name DAIKIN INDUSTRIES, LTD.

Address 1-1, Nishi Hitotsuya, Settsu-shi, Osaka 566-8585, Japan

3. TESTING FACILITY

Name Chemicals Evaluation and Research Institute, Japan, Hita (CERI Hita)

Address 3-822, Ishii-machi, Hita-shi, Oita, 877-0061, Japan

4. OBJECTIVE

The ability of the test substance to induce skin irritation is investigated using LabCyte EPI-MODEL24 SIT.

5. TEST METHOD

"OECD Guidelines for the Testing of Chemicals, No. 439, *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method" (Adopted: July 28, 2015)

6. GLP PRINCIPLE

OECD Principles of Good Laboratory Practice, November 26, 1997, ENV/MC/CHEM (98)17

7. DATES

Study Initiation Date	February 3, 2017
Experiment Starting Date	February 7, 2017
Experiment Completion Date	February 13, 2017
Study Completion Date	March 10, 2017

8. PERSONNEL CONCERNED WITH STUDY

Study Director:

Section 3, CERI Hita

Study Staff:

(Exposure of test substance, rinse of tissue and measurement of optical density (OD))

9. STORAGE AND RETENTION PERIOD OF DATA

The original study plan, the original final report, the raw data, documents concerning the study presented by the sponsor and other reports are stored in the archives of the testing facility. The storage period is 10 years after the study completion date.

Treatment of data after the end of the retention period (continued retention at the testing facility, reject at the testing facility or return to the sponsor) will be carried out with the approval of the sponsor.

10. APPROVAL BY AUTHOR

Study Director:

March 10, 2017

Date

11. SUMMARY

The ability of C6OLF to induce skin irritation was investigated using LabCyte EPI-MODEL24 SIT.

As a result of the skin irritation test, the cell viability treated by C6OLF was 118.0%, it was over 50%.

Consequently, it was concluded that C6OLF was "Non-irritant" (UN GHS Category: not classified (including UN GHS Category 3)) under the present test conditions.

12. MATERIALS

12.1 Test Substance and Control Substances

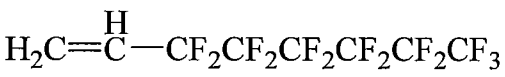
a) Test substance (information provided by the sponsor)

1) Chemical name, etc.

Chemical name	3,3,4,4,5,5,6,6,7,7,8,8,8-Tridecafluorooct-1-ene
Other name	C6OLF
CAS number	25291-17-2

2) Structural formula, etc.

Structural formula



Molecular formula	$C_8H_3F_{13}$
Molecular weight	346.09

3) Purity, etc.

Purity	99.95%
Impurity	Unknown; 0.05%
Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	C2160215

4) Physicochemical properties

Boiling point	106°C (760 mmHg)
Density	1.560 g/cm^3
Appearance at ordinary temperature	Colorless transparent liquid
Stability	Stable in storage condition

5) Storage conditions

The test substance was put into a light-resistant and airtight container and stored at room temperature in the test substance storage room (permissible range: from 10°C to 30°C).

6) Safety

In order to avoid inhalation and contact with the skin and eyes, chemically resistant gloves, a mask, a head cap, safety glasses and a lab coat were worn.

b) Negative control substance

1) Name

Distilled water

2) Manufacturer, lot number and grade

Manufacturer	Otsuka Pharmaceutical Factory
Lot number	K6G73
Grade	For injection

3) Reason for selection

Distilled water is recommended in the test method.

4) Storage conditions

Distilled water was stored at room temperature in the preparation room No. 2.

c) Positive control substance

1) Name

5 w/v% Sodium dodecyl sulfate (SDS) solution

2) Manufacturer, lot number and grade for SDS

Manufacturer	Wako Pure Chemical Industries
Lot number	LKM9213
Grade	For biochemistry

3) Preparation method

SDS was dissolved in distilled water (Lot number: K6G73, for injection, Otsuka Pharmaceutical Factory) to prepare 5 w/v% SDS solution.

4) Reason for selection

5 w/v% SDS solution is recommended in the test method.

5) Storage conditions

SDS was stored at room temperature in the test substance storage room (permissible range: from 10°C to 30°C). 5 w/v% SDS solution was prepared just before use and stored at room temperature in the cell experimental room No. 1.

12.2 Test Kit

a) Name

LabCyte EPI-MODEL24 SIT

b) Manufacturer

Japan Tissue Engineering Co., Ltd. (J-TEC)

c) Receipt date

February 7, 2017

d) Components

LabCyte tissue (Lot number: LCE24-170206-A, expiration date: February 10, 2017)
Assay medium (medium, Lot number: 1000442310)

e) Reason for selection

LabCyte EPI-MODEL24 SIT is recommended in the test method.

f) Storage conditions

LabCyte tissue was stored at room temperature in the cell experimental room No. 1. The medium was stored in a cold place in the cell experimental room No. 1 (permissible range: from 1°C to 10°C).

g) Quality of three-dimensional skin model

The results of quality verification (MTT assay, barrier function and morphology) performed by the manufacturer were shown in Appendix 1.

12.3 Culture Condition (setting value)

Incubator	CO_2 incubator (MCO-18AIC, SANYO Electric)
Temperature	37°C
Humidity	Under humid condition
CO_2 concentration	5%

12.4 Buffer Solution, Medium Containing MTT Solution and MTT Extraction Solvent

a) Buffer solution

Phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS(−))

b) Medium containing MTT solution

1) Preparation method

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Lot number: JQ009, for research, DOJINDO Laboratories) was dissolved in the medium to prepare medium containing 0.5 mg/mL MTT solution (MTT medium).

2) Timing of preparation and storage conditions

MTT medium was prepared just before use. MTT medium was put into a light-resistant container and stored at room temperature until use.

c) MTT extraction solvent

2-Propanol (Lot number: DSN3412, Special grade, Wako Pure Chemical Industries)

13. TEST PROCEDURE

13.1 Preliminary Test

The preliminary test was conducted in, "*In vitro* Eye Irritation Test of C6OLF using EpiOcular™EIT (OCL-200)" (Study number: K10-0229) at the testing facility.

a) Test for reactivity with MTT

Fifty microliters of the test substance and 1 mL of MTT medium were mixed, the mixture was incubated for 180 minutes. After the incubation, the change in color of the MTT medium was evaluated. As a result, the change in color was not observed. It was judged that the test substance had no reactivity with MTT. Therefore, interference of the test substance with MTT (interference test) was not conducted in the skin irritation test.

13.2 Skin Irritation Test

Triplicate tissues were used for the test substance, negative control substance and positive control substance, respectively. Duplicate tissues were used for the test substance and negative control substance to check the tissue-binding of the test substance (tissue-binding test).

a) Pre-incubation

1) Tissue inserts were placed in the first row of 24-well plate filled with 0.5 mL/well of the medium that was heated at 37°C and incubated for 15-30 hours.

b) Exposure of the test substance

1) One milliliter per well of the medium that was heated at 37°C was added in the third row of 24-well plate.
2) Twenty five microliters of the control substances and the test substance were applied onto each tissue surface at 1 minute interval.
3) Each tissue insert was placed until 15 minutes ± 30 seconds was completed.

c) Rinsing and post-incubation

1) Tissues were rinsed fifteen times or more with PBS(−).
2) Remaining PBS(−) was removed from outside of tissue insert with a sterile cotton swab. The tissue inserts were transferred into the third row of 24-well plate.
3) The plate was incubated for 42 ± 1 hours.

d) MTT reaction and extraction

1) Zero point five milliliters per well of MTT medium that was heated at 37°C was added in fourth row of 24-well plate.
2) The tissue inserts were transferred into the fourth row of 24-well plate and incubated for 180 ± 5 minutes.
3) The epidermis tissue was taken out from the tissue insert and transferred into 1.5 mL-micro tube.
4) Three hundred microliters per tube of 2-propanol was added into each micro tube.
5) The lid of the tube was closed and the tube was put into a light-resistant container and stored in a cold place for 15 hours or more.

e) Measuring of optical density (OD)

1) The extracts were mixed to obtain homogeneous solutions.
2) Two hundred microliters per well of the extracts were transferred into a 96-well plate (n = 1). Two hundred microliters per well of 2-propanol was used as blank (n = 1).
3) OD of each extract was measured spectrophotometrically using Multimode Microplate Reader (FLUOstar OPTIMA, BMG LABTECH) at 570 nm and 650 nm. The OD at 650 nm was substracted from OD at 570 nm. The blank OD was substracted from the value was measurement value.
4) The cell viability of each tissue was calculated by the following formula.

$$\text{Cell viability (\%)} = \frac{\text{[Mean measurement value of each treatment group]}}{\text{[Mean measurement value of the negative control substance group]}} \times 100$$

Standard deviation (SD) of cell viabilities used three tissue inserts were calculated in each treatment group.

f) Tissue-binding test

The tissue-binding test was carried out using the same procedure as described in 13.2 a) to e), except medium without MTT was used instead of MTT medium. After the measuring of OD, the staining ratio was calculated by the following formula.

$$\text{Staining ratio (\%)} = \frac{\text{[Mean measurement value of the test substance group (without MTT)]} - \text{[Mean measurement value of the negative control substance group (without MTT)]}}{\text{[Mean measurement value of the negative control substance group (with MTT)]}} \times 100$$

The mean staining ratio was below 5%. Therefore, correction of measurement value was not conducted.

14. JUDGEMENT CRITERIA OF THE RESULTS

Skin irritation was judged according to the following criteria.

Cell viability (%)	Category
≤ 50	Irritant (UN GHS*1 Category 1 or 2)
> 50	Non-irritant (UN GHS not classified*2)

*1 : Globally Harmonized System of Classification and Labelling of Chemicals

*2 : Including UN GHS Category 3

15. ACCEPTABLE CRITERIA OF THE TEST

a) Mean of measurement value in the negative control substance group is ≥ 0.7 and ≤ 2.5.

b) Cell viability in the positive control substance group is ≤ 40%.

c) SDs of cell viabilities in each treatment group used three tissue inserts are ≤ 18%.

16. FACTORS AFFECTED RELIABILITY OF TEST

There were no factors that might have affected the reliability of the test.

17. TEST RESULTS AND DISCUSSION

The test results are shown in Tables 1 and 2.

As a result of tissue-binding test, the staining ratio was −0.2%. The staining ratio was below 5%. Therefore, correction of measurement value was not conducted.

The OD treated by the negative control substance was 0.810. The cell viability treated by the positive control substance was 2.7%. SDs of cell viabilities in the negative control, positive control and test substance groups were 10.8%, 0.1% and 1.8%, respectively. These results indicated that the present test data were valid.

As a result of skin irritation test, the cell viability treated by C6OLF was 118.0%.

18. CONCLUSION

C6OLF was judged to be "Non-irritant" (UN GHS Category: not classified (including UN GHS Category 3)) under the present test conditions.

Table 1 Results of skin irritation test

Group	Tissue No.	Measurement value[a]		Cell viability[b] (%)		SD[e] (%)	Category
		Correction[c]	Mean		Mean[d]		
Negative control (Distilled water)	1	0.902	0.810	111.4	**100**	10.8	
	2	0.801		98.9			
	3	0.727		89.8			
Positive control (5 w/v% SDS solution)	1	0.022	0.022	2.7	2.7	0.1	
	2	0.022		2.7			
	3	0.021		2.6			
Test substance[c] (C6OLF)	1	0.960	0.956	118.5	118.0	1.8	Non-irritant
	2	0.940		116.0			
	3	0.967		119.4			

a) Measurement value which the mean of blank OD was subtracted from each tissue OD was shown.

b) Cell viability in the negative control substance was regarded as 100%.

c) The staining ratio was below 5%. Therefore, correction of measurement value was not conducted.

d) The mean cell viability was calculated from mean measurement value of each group.

e) The SD was calculated from the cell viability (n=3) of each tissue.

Table 2 Results of tissue-binding test

Group	Tissue No.	Measurement value[a]		Staining ratio[b] (%)
			Mean	
Negative control[c] (Distilled water)	1	0.002	0.005	
	2	0.008		
Test substance[c] (C6OLF)	1	0.002	0.003	-0.2
	2	0.003		

a) Measurement value which the mean of blank OD was subtracted from each tissue OD was shown.

b) Staining ratio (%) = $\dfrac{\text{[Mean measurement value of the test substance group (without MTT)] - [Mean measurement value of the negative control substance group (without MTT)]}}{\text{Mean measurement value of the negative control substance group (with MTT)}} \times 100$

c) Medium without MTT was used instead of MTT medium.

Appendix 1

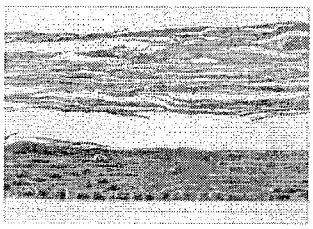
J-TEC

13-Feb-17

Data Sheet

LabCyte EPI-MODEL 24

Product Code : 401124 401150
Lot Number : LCE24-170206-A

Test(検査項目)	Acceptance criteria(判定基準)	Result(結果)
Viability(MTT assay) 生細胞数(MTT試験)	0.8≦OD≦2.5	OD = 1.1
Barrier Function バリア機能試験	0.14%≦IC_{50}≦0.40% 1.4mg/mL≦IC_{50}≦4.0mg/mL	IC50 = 0.26
Morphology 組織評価	Observing multilayered epidermis with a stratum corneum 角質層が形成されていること、 重層化した表皮組織が構築されていること	 conform 適合

QUALITY ASSURANCE STATEMENT

Chemicals Evaluation and Research Institute, Japan, Hita

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: *In vitro* skin irritation test of C6OLF using LabCyte EPI-MODEL24 SIT

Study Number: K10-0227

I assure that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of this study. The inspections of the study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance Unit as follows.

Item of inspection	Date of inspection	Date of report
Study plan	February 7, 2017	February 7, 2017
Cell pre-culture	February 7, 2017	February 7, 2017
Exposure of test substance	February 8, 2017	February 8, 2017
MTT assay	February 10 and 13, 2017	February 13, 2017
Raw data and draft final report	March 8, 2017	March 8, 2017
Final Report	March 10, 2017	March 10, 2017

Date: March 10, 2017

Quality Assurance Manager: