Receipt Number	832-16-T-8816
Study Number	K10-0229

FINAL REPORT

In vitro eye irritation test of C6OLF using EpiOcular^{TM}EIT (OCL-200)

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* eye irritation test of C6OLF using EpiOcular™EIT (OCL-200)

Study Number: K10-0229

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 eye irritation test of C6OLF using EpiOcular™EIT (OCL-200)

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 eye irritation is investigated using EpiOcular™EIT (OCL-200).

5. TEST METHOD

"OECD Guidelines for the Testing of Chemicals, No. 492, Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage" (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 14, 2017
Experiment Completion Date	February 22, 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))

(Rinse of tissue)

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 eye irritation was investigated using EpiOcular™EIT (OCL-200).

As a result of the eye irritation test, the cell viability treated by C6OLF was 72.4%, it was over 60%.

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

12. MATERIALS

12.1 Test Substance and Control Substances

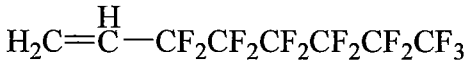
a) Test substance (information provided by the sponsor)

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 sample 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

Methyl acetate

2) Manufacturer and lot number

Manufacturer	MatTek Corporation (kit component)
Lot number	020917ALA

3) Reason for selection

Methyl acetate is recommended in the test method.

4) Storage conditions

Methyl acetate was stored at room temperature in the cell experimental room No. 1.

12.2 Test Kit

a) Name

OCL-200EIT

b) Manufacturer

MatTek Corporation

c) Receipt date

February 20, 2017

d) Components

EpiOcular tissue (Lot number: 20978, manufactured on February 16, 2017)

Assay medium (medium, Lot number: 021317MWKD)

Methyl acetate

e) Storage conditions

EpiOcular tissue and the medium were stored in a cold place in the cell experimental room No. 1 (permissible range: from 1°C to 10°C).

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 PBS(−) to prepare 5 mg/mL MTT solution. This solution was diluted with the medium to

prepare medium containing 1 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

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 eye irritation test.

13.2 Eye Irritation Test

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

a) Pre-incubation

1) Tissue inserts were placed in 6-well plate (Asahi Glass) filled with 1 mL/well of the medium and incubated for 60 ± 5 minutes.

2) The medium was aspirated and 1 mL/well of the fresh medium was added to each well. Then, the tissue inserts were incubated for 16-24 hours.

b) Exposure of the test substance

1) Twenty microliters of PBS(−) was added onto each tissue surface at 1 minute interval. Two tissues were treated at once. Each plate was incubated until 30 ± 2 minutes was completed for the first exposed tissue in each plate.

2) Fifty microliters of the control substances and the test substance were applied onto each tissue surface at 1 minute interval. Two tissues were treated at once. Each plate was incubated until 30 ± 2 minutes was completed for the first exposed tissue in each plate.

c) Rinsing and post-incubation

1) After the incubation, each tissue insert was completely submerged three times about 100 mL of PBS(−). Two tissues were treated at once. Rinsing was repeated twice further. PBS(−) was removed from the tissue surface.

2) The tissue inserts were transferred into a 12-well plate (Corning) filled with 5 mL/well of the fresh medium and soaked 12 ± 2 minutes.

3) The tissue inserts were transferred into 6-well plates filled with 1 mL/well of the fresh

medium and incubated for 120 ± 15 minutes.

d) MTT reaction and extraction

1) All tissue inserts were transferred into a 24-well plate (Corning) filled with 0.3 mL/well of MTT medium and incubated for 180 ± 10 minutes.
2) The outside of tissue inserts was wiped. All tissues were transferred into new 24-well plates and added 2 mL/well of 2-propanol inside tissue inserts.
3) The plate was put into a plastic bag, and extraction was performed at room temperature for 2 hours or more using a plate shaker.
4) The extracts were moved from the inside of tissue inserts to plate and mixed to obtain homogeneous solutions.

e) Measuring of optical density (OD)

1) Two hundred microliters per well of the extracts were transferred into a 96-well plate (Corning) (n = 2). Two hundred microliters of 2-propanol was used as blank (n = 8).
2) OD of each extract was measured spectrophotometrically using Multimode Microplate Reader (FLUOstar OPTIMA, BMG LABTECH) at 570 nm.
3) The mean of blank OD was subtracted from ODs of each tissue and the mean value was calculated in each tissue to obtain OD of each tissue. The cell viability of each tissue was calculated by the following formula.

$$\text{Cell viability (\%)} = \frac{\text{OD of each tissue}}{\text{Mean OD of the negative control substance group}} \times 100$$

The mean cell viability of each treatment group was calculated from the cell viability of each tissue.

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.

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

The mean staining ratio was below 60%. Therefore, the cell viability was corrected by the following formula.

Corrected cell viability (%) =

Mean cell viability of the test substance group (%) – Mean staining ratio (%)

14. JUDGEMENT CRITERIA OF THE RESULTS

Eye irritation was judged according to the following criteria.

Cell viability (%)	Category
≤ 60	Irritant (UN GHS*1 Category 1 or 2)
> 60	Non-irritant (UN GHS Category: not classified)

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

15. ACCEPTABLE CRITERIA OF THE TEST

a) OD in the negative control substance group is > 0.8 and < 2.5.
b) Cell viability in the positive control substance group is < 50%.
c) Differences of two tissue cell viabilities in each treatment group are < 20%.

16. FACTORS AFFECTED RELIABILITY OF TEST

In the first eye irritation test, the OD in the negative control substance group was "0.532", which was less than the range of acceptable criteria of the test (> 0.8 and < 2.5). Because it was suspected that the defect of the test kit was caused, the test result was rejected and a retest was carried out using a new test kit. Therefore, it was considered that there was no effect on the reliability of the test.

17. TEST RESULTS AND DISCUSSION

The test results are shown in Tables 1 and 2.

As a result of the tissue-binding test, the staining ratio was 0.8% that was below 60%. Therefore, the cell viability was corrected.

OD in the negative control substance group was 1.056. The cell viability in the positive control substance group was 36.0%. Differences of two tissue cell viabilities in the negative control substance, the positive control substance and the test substance groups were 5.5%, 4.2% and 2.4%, respectively. These results indicated that the present test data were valid.

As a result of the eye irritation test, the cell viability treated by C6OLF was 72.4%.

18. CONCLUSION

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

Table 1 Results of eye irritation test

Group	Tissue No.	OD[a]		Cell viability[b] (%)		Difference[c] (%)	Category
			Mean		Mean / Corrected value[d]		
Negative control substance (Distilled water)	1	1.117, 1.085, 1.052	1.056	102.7	**100**	5.5	
	2	1.005, 1.026, 1.047		97.2			
Positive control substance (Methyl acetate)	1	0.394, 0.402, 0.410	0.380	38.1	36.0	4.2	
	2	0.351, 0.358, 0.364		33.9			
Test substance (C6OLF)	1	0.775, 0.786, 0.797	0.773	74.4	73.2 / 72.4	2.4	Non-irritant
	2	0.745, 0.760, 0.774		72.0			

a) OD which the mean of blank OD was subtracted from was shown.

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

c) The difference of cell viability between the two tissues was shown.

d) The cell viability was corrected by the stainning ratio.

Corrected cell viability (%) = Mean cell viability of the test substance group (%) - Mean staining ratio (%)

Table 2 Results of tissue-binding test

Group	Tissue No.	OD[a]		Staining ratio[b] (%)	
			Mean		Mean
Tissue-binding test[c]	1	0.005, 0.006, 0.006	0.008	0.6	**0.8**
	2	0.008, 0.010, 0.011		0.9	

a) OD which the mean of blank OD was subtracted from was shown.

b) Staining ratio (%) = $\frac{\text{OD of the test substance group (without MTT)}}{\text{Mean OD of the negative control substance group (with MTT)}} \times 100$

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

QUALITY ASSURANCE STATEMENT

Chemicals Evaluation and Research Institute, Japan, Hita

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: *In vitro* eye irritation test of C6OLF using EpiOcular™EIT(OCL-200)

Study Number: K10-0229

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 14, 2017	February 14, 2017
Exposure of test substance	February 15, 2017	February 15, 2017
MTT assay	February 15, 2017	February 15, 2017
Record of accident or deviation from study plan	March 8, 2017	March 8, 2017
Raw data and draft final report	March 9, 2017	March 9, 2017
Re-inspection of raw data and draft final report	March 9, 2017	March 9, 2017
Final Report	March 10, 2017	March 10, 2017

Date: March 10, 2017

Quality Assurance Manager: