Pusat Penyelidikan Teknologi Alam Sekitar
Environmental Technology Research Centre
Building 15, SIRIM Berhad, Shah Alam, Selangor Darul Ehsan
Tel: 60-3-5544 6550 / 6598 Fax: 60-3-5544 6590 / 6579

RESULTS SUMMARY

Company Name : Changkat Rembian Mineral Sdn. Bhd.
(Attn to: Mr Shah Salik Al-Mujaddidi)

Address : PT2992, KM10 Jalan Teluk Intan
35500 Bidor
Perak

Your Ref No.	QG20000059
SIRIM Ref. No.	ETRC 257/16/1535
Job No.	J026/20
Report No.	R120/20
Date of issue	2 June 2020
No. of pages	2 pages

Request : Analysis of Clay Samples for Microbiological, Heavy Metal, Skin and Eye Irritation.

SAMPLE DESCRIPTION
One (1) samples were received and coded as below.

No.	Client Code	Physical Appearance	Laboratory Code	Date of received
1	A8	Raw Powder Form	J026-1/20	20 Jan 2019

TEST METHOD:
Please refer page 2.

RESULT:
Please refer Table 1, 2 and 3

INFERENCE:
Not applicable
(The inferences expressed herein are outside the scope of accreditation.)

Name : **Rafindde Ramli**
Designation : **Juruperunding Kanan**
Tel. No : **03-5519 4411**
Fax. No : **03-55107292**

TEST METHOD:

1. ** Compendium of Methods for the Microbiological Examination of Foods, 5th Edition 2015
   1. Chapter 8, Mesophilic Aerobic Plate Count
   2. Chapter 21, Yeasts and Molds Count
2. * Inductively Couple Plasma Mass Spectrometry (ICP-MS) Analysis using QES Optima DV2000.
3. ** OECD (2015), Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage –OECD Guidelines for Testing of Chemicals No. 492
4. ** OECD (2013), In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method – OECD Guidelines for Testing of Chemicals No. 439

Note: ** Non-accredited method*
*** Subcontracted and non-accredited*

RESULT:

Table 1: Mesophilic Aerobic Plate Count and Yeasts and Molds Count

No.	Sample	Mesophilic Aerobic Plate Count (CFU/g)	Yeasts and Molds Count (CFU/g)	Date of Analysis
1.	Clay A8	< 10	60	29 January 2020

Notes: CFU – Colony Forming Unit, Microbial Contamination ≤1000 CFU/g according to European Pharmacopoeia (EP 4th, 2002)

Table 2: Heavy Metal Analysis

Heavy Metal Elements	Parts per million (ppm)	Date of Analysis
Arsenic	13.6	30 January 2020
Cadmium	2.2	
Chromium	35.3	
Copper	12.6	
Nickel	22.6	
Tin	36.6	
Zinc	48.4	

Notes: Heavy Metal ≤50 ppm for kaolinite according to European Pharmacopoeia (EP 4th, 2002)

Table 3: Eye and Skin Irritation Test

Test Method	**Results**	**Date of Analysis**
OECD Guidelines for Testing of Chemicals No. 492 - Eye Irritation Test	Non- irritant	21 May 2020
OECD Guidelines for Testing of Chemicals No. 439 - Skin Irritation Test	Non- irritant	

INDUSTRIAL BIOTECHNOLOGY RESEARCH CENTRE
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TEST REPORT

EVALUATION OF SKIN IRRITATION ON CLAY A8 USING *IN VITRO* RECONSTRUCTED HUMAN EPIDERMAL MODEL EPIDERM™ SKIN IRRITATION TEST

Job No. J187/20

Report No. R187/20/B19/43

Sponsor:
Environmental Technology Research Centre (ETRC),
Building 15, SIRIM Berhad
(Rafindde Ramli)

Test Facility:
Industrial Biotechnology Research Centre (IBRC),
Building 19, SIRIM Berhad

Study Initiation Date:
17 February 2020

Experimental Start Date:
17 March 2020

Experimental End Date:
27 March 2020

Study Completion Date:
03 April 2020

TEST REPORT

REPORT NO:**R187/20/B19/43**	PAGE: 2 of 18
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APPROVAL SIGNATURES

We, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected throughout the study.

(SUZAINI BADRUDIN)
Reviewer
Industrial Biotechnology Research Centre

03 APR 2020
Date

(NURHAYATI ARIFFIN)
Analyst
Industrial Biotechnology Research Centre

03 APR 2020
Date

TEST REPORT

REPORT NO:**R187/20/B19/43**	PAGE: 3 of 18

SUMMARY

EVALUATION OF SKIN IRRITATION ON CLAY A8 USING *IN VITRO* EPIDERM™ SKIN IRRITATION TEST (SIT)

In vitro skin irritation test on Clay A8 was performed according to the requirement of *In vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method –OECD Guidelines for Testing of Chemicals No. 439. This *in vitro* standard method was validated by European Centre of the Validation of Alternative Methods (ECVAM) as *in vitro* test method based on reconstructed human epidermis (RhE) technology. The test was conducted in line with Standard Operating Procedure (SOP) developed at MatTek Corporation. This test assesses irritability of both cosmetic ingredients and finished products.

The test was conducted to determine whether the test item cause irritation to the *in vitro* skin model EpiDerm™.

In vitro dermal irritation test consists of topical exposure of the Clay A8 to reconstructed human epidermal model EpiDerm™ tissues, followed by a cell viability test. After 60 minutes of exposure, tissues were thoroughly rinsed, blotted to remove the test extract, and transferred to fresh medium. After a 24 hours incubation period, the medium was changed and tissues were incubated for another 18 hours. MTT [(3-4, 5 dimethyl triazole 2-yl) 2, 5-diphenyltetrazoliumbromide] assay was then performed by transferring the tissues to 6-well plates containing MTT medium (1 mg/mL). After 3 hours of incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2.0 mL isopropanol / tissue. The optical density of the extracted formazan was determined using a spectrophotometer at 570 nm. Relative cell viability was calculated for each tissue as percentage (%) of the mean of the negative control tissues. The skin irritation potential was classified according to the remaining cell viability obtained after test item treatment.

The Clay A8 did not reduce viability of the EpiDerm™ tissue to below 50 % of the negative control. Under the condition of this test, Clay A8 is considered as **Non-Irritant** to *in vitro* skin model EpiDerm™.

BACKGROUND

Skin irritation refers to reversible damage to the skin following the application of a test chemical for up to 4 hours as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

For chemical, the reconstructed human epidermal model was developed and designed to predict skin irritation potential of neat test substances in the context of identification and classification of skin irritation hazard according to the European Union (EU) classification system (R 38 or no label). Since the EU and GHS systems were harmonized in 2008, the procedure described in the SOP also allows for hazard identification of irritant substances in accordance to UN GHS.

In vitro skin irritation: Reconstructed Human Epidermis Test allows for assessment of irritation. A sufficient amount of extract was applied on the surface of the three dimensional reconstructed human epidermis (RhE). The RhE model is comprised of non-transformed human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human *epidermis*. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers representing main lipid classes analogous to those found *in vivo*. After a certain incubation period, cell viability is assessed by MTT [(3-4, 5 dimethyl triazole 2-yl) 2, 5-diphenyltetrazoliumbromide] colorimetric test. The reduction of the viability of tissues exposed to chemicals in comparison to negative controls (treated with water) is used to predict the skin irritation potential.

Irritant chemicals are identified by their ability to decrease cell viability below defined threshold levels (*i.e.* ≤ 50 %, for UN GHS Category 2). Depending on the regulatory framework and applicability of the Test Guideline, chemicals that produce cell viability above the defined threshold level may be considered non-irritants (*i.e.* > 50 %, No Category).

BIOTECHNOLOGY RESEARCH CENTRE * INDUSTRIAL SIRIM BERHAD

TEST REPORT

CONTENTS

INDUSTRIAL BIOTECHNOLOGY RESEARCH CENTRE * SIRIM BERHAD

1.0 OBJECTIVE

The objective of this study is to predict skin irritation potential of test item using *in vitro* skin model EpiDerm™.

2.0 STUDY TIMETABLE

2.1 **Receipt of reconstructed human epidermal model EpiDerm™ tissues:**
17 March 2020

2.2 **Tissue Conditioning**
17 March 2020

2.3 **Pre-Incubation**
17 March 2020

2.4 **Treatment**
18 March 2020

2.5 **Change Medium**
19 March 2020

2.6 **MTT Viability Test**
20 March 2020

2.7 **Optical Density Reading**
20 March 2020

2.8 **Data Analysis**
20 March 2020 – 27 March 2020

3.0 MATERIALS

3.1 **Test Item**

3.1.1 Test item: Clay A8

3.1.2 Sample marking : Changkat Rembian

3.1.3 Date received: 17 February 2020

3.1.4 Physical appearance: Powder

3.1.5 Colour: Brownish

3.1.6 Physical Chemical Properties Data: Not provided

3.1.7 Quantity received : 1 bottle

3.1.8 pH: Not provided

3.1.9 Storage condition: Ambient

3.1.10 Solubility: Not provided

3.1.11 Stability: Not provided

3.1.12 Expiration date: Not provided

3.2 **Test System**

3.2.1 Test System: Reconstructed human epidermal model EpiDerm™

The reconstructed human epidermal model EpiDerm™ (EPI-200, MatTek, Ashland, USA) consists of normal human-derived epidermal keratinocytes, which have been cultured to form a multilayered highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo.*

The EpiDerm™ tissues are cultured on specially prepared cell culture inserts, containing 24 tissues using serum free medium. Ultrastructurally, the EpiDerm Skin Model closely parallels human skin, thus providing a useful *in vitro* means to assess dermal irritancy and toxicology.

3.2.1.1 Lot No: 32193

3.2.1.2 Production Date: 12 March 2020

3.2.1.3 Date of Shipping: 13 March 2020

3.2.1.4 Receipt of EpiDerm™:17 March 2020, Tuesday

3.2.1.5 Visual quality control of the skin: All tissues in good condition

3.2.1.6 The EpiDerm™ System is manufactured according to defined quality assurance procedures. All biological components of the epidermis and the culture medium are tested by manufacturer for viral, bacterial, fungal and mycoplasma contamination. MatTek determines the ET-50 value following exposure to Triton X-100 (1%) for each EpiDerm™ lot. The ET-50 must fall within a range established based on a historical database of results or acceptability ranges for quality control based on OECD Test Guidelines. The quality control value is presented in 10.3.

3.3 **Reagent**

3.3.1 Assay Medium: EPI-100-NMM-SIT / Assay Medium
3.3.1.1 Lot No. : 031120TVKD
3.3.1.2 Sterility : Sterile
3.3.1.3 Expiration Date : 08/04/2020
3.3.1.4 Storage : Refrigerator (5 ± 3 °C)
3.3.1.5 Manufacturer : Mattek Corporation

3.3.2 Phosphate Buffered Saline without Calcium and Magnesium
Lot No.: 111219RAHA
Expiration Date : 12/11/2020
Manufacturer : Mattek Corporation

3.3.3 Phosphate Buffered Saline without Calcium and Magnesium, contain the following:
3.3.3.1 Sodium Chloride 8 g/L
Lot No.: K50722004912
Manufacturer : MERCK

3.3.3.2 Potassium chloride 0.2 g/L
Lot No.: 1409BI4J35501
Manufacturer : Bio Basic Canada Inc

3.3.3.3 Anhydrous potassium dihydrogen orthophosphate 0.2 g/L
Lot No.: 1211ACN1K12062801
Manufacturer : Bio Basic Canada Inc

3.3.3.4 Anhydrous disodium hydrogen orthophosphate 1.15 g/L
Lot No.: 1306ACK2NA12020101
Manufacturer : Bio Basic Canada Inc

3.3.4 MTT – 2mL (5mg/mL)
Lot No. : MKBW0025V
CAS No. : 298-93-1
Manufacturer : SIGMA

3.3.5 MTT Diluent – 8mL
As above 3.3.1

3.3.6 Extractant Solution – Isopropanol
Lot No. : 632261
Storage : Room Temperature
CAS No. : 67-63-0
Manufacturer : Fisher Scientific

3.3.7 Positive Control: 5 % SDS Solution
Part No. : TC-SDS-5 %
Lot No. : 121219BBA
Expiration Date : 12/12/2020
Storage : Room Temperature
Manufacturer : Mattek Corporation

3.3.8 Negative Control: As above 3.3.2

3.4 **Material**

3.4.1 Nylon MesH (EPI-MESH)
3.4.2 Lot no. : 0551428-00
3.4.3 Expiry Date : 31/12/2029
3.4.4 Manufacturer : MatTek Corporation

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4.0 METHOD

Upon receipt of the reconstructed human epidermal model EpiDerm™, the tissue kits and the solutions were stored according to the manufacturer's directions for unpacking and storage.

4.1 Tests for Interference of Test Item

Tissue-Binding of Coloured or Staining Materials

25 µL of sterile pure water and 25 mg of test item (25 µL sterile pure water + 25 mg of test item) was applied onto three single EpiDerm™ tissues. In parallel, a tissue was exposed to negative control. After 60 minutes of exposure, tissues were thoroughly rinsed, blotted to remove the test extract, and transferred to fresh medium.

After a 24 hours incubation period, the medium was changed and tissues were incubated for a further 18 hours. The medium was then changed again and incubated for 3 hours. After incubation was completed, the tissues were rinsed and extracted using 2.0 mL of isopropanol per tissue. The optical density of the extracted tissue was determined using spectrophotometer at 570 nm.

4.1.2 Test for Interference of Test Item with MTT

25 mg of the test item was added to 1 mL of the 1 mg/mL MTT and incubated at (37 ± 1)° C, (5 ± 1)% CO_2, 95 % relative humidity for 60 minutes. Untreated MTT medium was used as control. If the MTT solution turns blue/purple, the test item reduces MTT and additional functional check must be performed.

4.2 Tissue Conditioning – Day 0

Under sterile conditions, a sealed 24-well plate containing the EpiDerm™, was opened. Visual inspection of each insert containing the epidermal tissue was done prior to tissue conditioning.

0.9 mL of assay medium was dispensed into each well of six-well plate, the EpiDerm™ tissue cultures were transferred into the wells and the plates was incubated for (60 ± 5) minutes at (37 ± 1)° C, (5 ± 1)% CO_2, 95 % relative humidity. At the end of the first 60 minutes pre-incubation, the assay medium was renewed for further pre-incubation. Each insert was aseptically transferred into well containing 0.9 mL assay medium and pre-incubation was done at (37 ± 1)° C, (5 ± 1)% CO_2, 95 % relative humidity for (18 ± 3) hours.

4.3 **Dosing protocol – Day 1**

25 µL of sterile pure water and 25 mg of test item (25 µL sterile pure water + 25 mg of test item) was applied onto three single EpiDerm™ tissues.Negative and positive controls were conducted in parallel using identical method to the dosed cultures. The cultures were incubated at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % relative humidity for (35 ± 1) minute.

After incubation, each dosed EpiDerm™ tissue was removed from the incubator and placed at room temperature in the biological safety cabinet for 25 minutes. At the end of the exposure period, the EpiDerm™ tissue cultures were removed from the assay plates and gently rinsed with phosphate buffered saline to eliminate any residual test material. The EpiDerm™ tissue cultures were then transferred into wells containing 0.9 mL assay medium and the plates incubated at (37 ± 1) °C,(5 ± 1) % CO_2, 95 % relative humidity for (24 ± 2) hours.

4.4 **Change Medium – Day 2**

After 24 hour's incubation, the medium was changed and EpiDerm™ tissues were incubated for a further 18 hours.

4.5 **MTT Viability Test**

4.5.1 Day 3

The MTT assay was performed by transferring each EpiDerm™ tissues to 6-well plates containing 300 µL MTT medium (1 mg/mL) in each well. After 3 hours of MTT incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2.0 mL isopropanol / tissue (extractant solution). The extraction plates were sealed with parafilm and agitated for at least 2 hours at room temperature. As alternative, overnight extraction at room temperature in the dark also possible.

4.5.2 At the end of the extraction period, EpiDerm™ tissue was pierced with an injection needle and the extract was decanted into the well from which insert was taken. The insert was then discarded. The extraction solution was pipetted up and down to ensure complete mixing. Finally, 200 µL was transferred into a 96 well microtiter plate for absorbance measurement (OD=optical density) at 570 nm without using a reference filter. 200 µL of isopropanol was used as blank.

Relative cell viability for each tissue was calculated as percentage (%) relative to mean of the negative control tissues viability.

5.0 DATA ANALYSIS

5.1 Tests for Interference of Test Item

5.1.1 Assessment of Coloured or Staining Materials

The percentage difference of the optical density between a coloured test item (CTI) and the negative control (NC) was calculated according to the following formula:

$$[(\text{Mean OD}_{CTI} - \text{Mean OD}_{NC}) / \text{Mean of OD}_{NC}] \times 100$$

OD reading of treated tissue by coloured test item	Action
Below 5 % (<5 %) of the negative control ; Tissue viability determined in MTT Assay not close to classification cut-off (50%)	Correction of the results is not necessary
Between 5 % and 30 % of the negative control	Further test on more tissue
Above 30 % (>30 %) of the negative control	Additional step and expert judgment ; Incompatible with the test system

The real MTT OD (unaffected by interference with the color or staining materials) was calculated using the following formula:

OD = OD Coloured tissue (MTT assay) – OD Coloured tissue (no MTT assay)

5.1.2 Test for Interference of Test Item with MTT

The test item is presumed to have reduced the MTT if the MTT solution colour turns blue/ purple.

5.2 Workbook EpiDerm™-SIT

A blank, password protected MS EXCEL workbook EpiDerm™-SIT-SPREAD.XLS was provided by MatTek Corporation. The workbook consists of two single spreadsheets named: Import and Spread.

5.3 Raw Data of Optical Densities (ODs)

Raw data of optical densities generated by the microplate reader (without blank subtraction) were copied from the reader software and then pasted into the Import spreadsheet of the Excel workbook. The blank corrections, calculation of results and statistical parameters are automatically calculated in the Spread spreadsheet of the workbook.

5.4 **Calculation**

5.4.1 After data entry, the spreadsheet performs the following calculations:

5.4.2 Blank correction

5.4.3 For each individual tissue treated with a test item (TI), the positive control (PC) and the negative control (NC) the individual relative tissue viability was calculated according to the following formulas;

Relative viability TI (%) = $[OD_{TI} / \text{Mean of } OD_{NC}] \times 100$

Relative viability NC (%) = $[OD_{NC} / \text{mean of } OD_{NC}] \times 100$

Relative viability PC (%) = $[OD_{PC} / \text{mean of } OD_{NC}] \times 100$

5.4.4 For each test item, negative control and positive control, the mean relative viability of the three individual tissues was calculated and used for classification according to the Prediction Model (Refer to 8.0).

6.0 ACCEPTABILITY RANGES FOR QUALITY CONTROL

	Lower acceptance limit	Upper acceptance limit
EpiDerm™ SIT (EPI-200) (1% Triton X-100)	ET_{50} = 4.0 hour	ET_{50} = 8.7 hour

7.0 ACCEPTABILITY RANGES FOR NEGATIVE CONTROL OD VALUES OF THE TEST METHODS

	Lower acceptance limit	Upper acceptance limit
EpiDerm™ SIT (EPI -200)	≥ 1.0	≤ 2.5

8.0 ACCEPTANCE CRITERIA FOR POSITIVE CONTROL

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as percent of the negative control tissues is ≤ 20%. The standard deviation shall be below 18 for all substances and controls.

	Mean of Viability (%)	Standard Deviation of Viability (SD %)
EpiDerm™ SIT (EPI-200)	≤ 20	<18

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9.0 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

An irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls.

***In vitro* result**	***In vivo* prediction**
mean tissue viability ≤ 50%	Irritant (I), (R38 or GHS category 2)
mean tissue viability > 50%	non-irritant (NI)

10.0 RESULT AND DISCUSSION

10.1 Tests for Interference of Test Item

10.1.1 Assessment of Coloured or Staining Materials

Tissue-Binding of a Coloured Test Item

The relative OD reading of treated tissue by coloured test item is below 5% of the negative control. Correction of the results is not necessary.

10.1.2 Test for Interference of Test Item with MTT

There was no change in MTT colour therefore the test item did interfere with MTT.

10.2 Viability Measurement

10.2.1 Raw data of optical densities (ODs) of Blank

Refer To Table 1

10.2.2 Blank Correction

Refer to Table 2

10.3 The Quality Control value meets the acceptance range criteria

EpiDerm™ SIT (EPI-200)	**Hour**
ET_{50}	5.02

10.4 The negative control OD value meets the acceptance range criteria

EpiDerm™ SIT (EPI-200)	**Lower OD**	**Upper OD**
Negative Control	1.944	2.127

10.5 **The positive control OD value meets the acceptance range criteria**

EpiDerm™ SIT (EPI-200)	**Mean of Viability (%)**	**Standard Deviation of Viability (SD %)**
Positive Control	6.2	0.13

10.6 **Classification**

Test item, negative control and positive control are qualified according to prediction model. The mean relative viability of the three individual tissues was calculated and used for classification

Refer to Table 3

10.7 **Graph**

10.7.1 The spreadsheet shows a graph of the results (% of relative viability ± standard deviation)

Refer to Figure 1

10.8 **Prediction Model**

	Mean of Viability (%)	**Standard Deviation of Viability (SD %)**	***In vitro* result**	***In vivo* prediction**
Clay A8	93.2	0.65	mean tissue viability > 50 %	**Non-Irritant (NI)**
Negative Control	100.0	4.80	mean tissue viability > 50 %	**Non-Irritant (NI)**
Positive Control	6.2	0.13	mean tissue viability ≤ 50 %	**Irritant (I), (R38 or GHS category 2)**

11.0 CONCLUSION

Under the condition of this test, Clay A8 is considered as **Non-Irritant** to *in vitro* skin model EpiDerm™.

12.0 RETENTION OF RECORDS AND TEST ITEM

One report will be forwarded to the Sponsor. The other report, together with all generated raw data is maintained at the Industrial Biotechnology Research Centre Archives.

13.0 REFERENCES

13.1 OECD (2013), In *Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method – OECD Guidelines for Testing of Chemicals No. 439

13.2 Protocol for: In *Vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT) – Reconstructed Human Epidermal Model EpiDerm (EPI-200-SIT). For use with MatTek Corporation

13.3 (LWI-238-43) : In *Vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT)

INDUSTRIAL BIOTECHNOLOGY RESEARCH CENTRE ★ SIRIM BERHAD

TEST REPORT

Table 1 Optical Densities (ODs) of Blank

Optical Densities (ODs) of Blank	Mean Optical Densities (ODs) of Blank
0.0419	0.0428
0.0416	
0.0414	
0.0441	
0.0455	
0.0425	

Table 2 Blank Corrected Data

	Tissue	Raw data		Blank corrected data		Mean	% of Viability
		1	2	1	2		
Clay A8	**1**	1.8428	1.8362	1.800	1.793	1.797	92.5
	2	1.8706	1.8584	1.828	1.816	1.822	93.8
	3	1.8683	1.8425	1.825	1.800	1.813	93.3
Negative Control	**1**	2.1665	2.1732	2.124	2.130	2.127	103.4
	2	2.0936	2.1936	2.051	2.151	2.101	102.1
	3	1.9494	2.0245	1.907	1.982	1.944	94.5
Positive Control	**1**	0.1669	0.1616	0.124	0.119	0.121	6.3
	2	0.1602	0.1687	0.117	0.126	0.122	6.3
	3	0.1614	0.1585	0.119	0.116	0.117	6.0

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Table 3 Classification of Test Item

	Mean of OD	SD of OD	Mean of Viability [%]	SD of Viability	CV [%]	*In vitro* result	Classification
Clay A8	1.810	0.013	93.2	0.65	0.69	Non-Irritant	Qualified
Negative Control	2.060	0.099	100.0	4.80	4.80	Non-Irritant	Qualified
Positive Control	0.120	0.003	6.2	0.13	2.12	Irritant	Qualified

OD- Optical Density, SD- Standard Deviation, CV- Coefficient of Variation

INDUSTRIAL BIOTECHNOLOGY RESEARCH CENTRE * SIRIM BERHAD

TEST REPORT

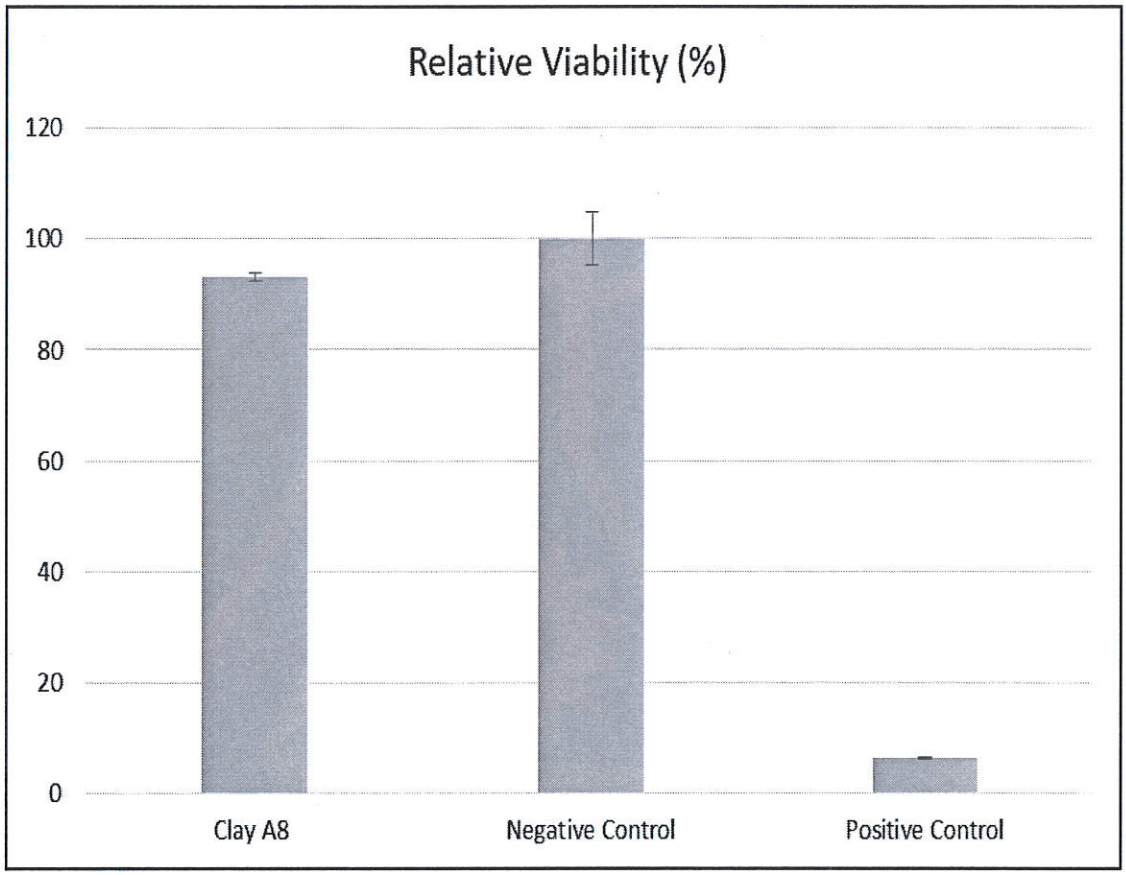

Figure 1 Relative Viability

INDUSTRIAL BIOTECHNOLOGY RESEARCH CENTRE ★ SIRIM BERHAD

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Website: www.sirim.my

TEST REPORT

EVALUATION OF EYE IRRITATION ON CLAY A8 USING *IN VITRO* RECONSTRUCTED HUMAN CORNEA-LIKE EPITHELIUM TISSUE MODEL EPIOCULAR™ EYE IRRITATION TEST

Job No. J188/20

Report No. R188/20/B19/44

Sponsor:
Environmental Technology Research Centre (ETRC),
Building 15, SIRIM Berhad
(Rafindde Ramli)

Test Facility:
Industrial Biotechnology Research Centre (IBRC),
Building 19, SIRIM Berhad

Study Initiation Date:
17 February 2020

Experimental Start Date:
31 March 2020

Experimental End Date:
07 April 2020

Study Completion Date:
14 April 2020

TEST REPORT

REPORT NO: **R188/20/B19/44**	PAGE: 2 of 17
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APPROVAL SIGNATURES

We, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected throughout the study.

14 APR 2020

(SUZAINI BADRUDIN)
Reviewer
Industrial Biotechnology Research Centre

Date

14 APR 2020

(NURHAYATI ARIFFIN)
Analyst
Industrial Biotechnology Research Centre

Date

TEST REPORT

REPORT NO: **R188/20/B19/44**	PAGE: 3 of 17

SUMMARY

EVALUATION OF EYE IRRITATION ON CLAY A8 USING *IN VITRO* EPIOCULAR™ EYE IRRITATION TEST

In vitro eye irritation test on Clay A8 using *in vitro* EpiOcular™ Eye Irritation Test (EIT) was performed according to Standard Operating Procedure developed at MatTek Corporation. This *in vitro* standard method was validated by European Centre of the Validation of Alternative Methods (ECVAM) as *in vitro* test method based on reconstructed human cornea-like epithelium (RhCE) technology. The test was conducted in line with the requirement of OECD Guidelines for Testing of Chemicals No 492. This test assesses irritability of both cosmetic ingredients and finished products.

The test was conducted to determine whether the test item cause irritation to the *in vitro* eye model EpiOcular™.

In vitro EpiOcular™ eye irritation test assessed irritancy level of the Clay A8 via topical exposure of the sample on three-dimensional reconstructed human cornea-like epithelial (RhCE) model EpiOcular™, followed by cell viability test.This concentration was used to be applied on EpiOcular™ tissue as dose treatment. The test was conducted in duplicate; two RhCE tissues. After 6 hours ± 15 minutes of exposure, tissues were thoroughly rinsed, blotted to remove the test item and followed by (25 ± 2) minutes post-treatment immersion (Post-Soak) at room temperature. Then, the medium was changed and tissues were incubated for another (18 hours ± 0.25 hours). Next, MTT assay was performed by transferring the tissues into 24-well plates containing MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) medium (1 mg/mL). After (180 ± 10) minutes of incubation, blue formazan salt formed via chemical conversion of MTT in cellular mitochondria was extracted with 2.0 mL isopropanol / tissue. Optical density of the extracted formazan was determined at 570 nm using spectrophotometer. Relative cell viability was calculated for each tissue as % of the mean of the negative control tissues. The eye irritation potential was classified according to the remaining cell viability obtained after test item treatment.

The data indicates that **Clay A8** did not reduce viability of the EpiOcular™ tissue to below 60 % of the negative control. Under the condition of this test, Clay A8 is considered as **Non-Irritant** to *in vitro* eye model EpiOcular™.

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BACKGROUND

According to United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS), eye irritation refers to the production of changes in the eye following application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application.

The EpiOcular™ OCL-200 Eye Irritation Test (OCL-200-EIT) RhCE tissue construct is similar to the *in vivo* corneal epithelium three-dimensional structure and is produced using cells from the species of interest. The EpiOcular™ tissue construct is a nonkeratinized epithelium prepared from normal human keratinocytes. It offers features appropriate for a model of ocular irritation.

Based on the depth of injury model, the EpiOcular™ Eye Irritation Test is intended to differentiate those materials that are non-irritants (would not require a warning label in the European chemical classification systems) from those that would require labeling as either Globally Harmonized System (GHS) 1 or 2. Liquids and solids are treated with different exposure and post-exposure incubations. Minimum of two construct tissues are used for each treatment and control group. Relative tissue viability is determined against the negative control-treated constructs by the NAD(P)H-dependent microsomal enzyme reduction of MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide (and to a lesser extent, by the succinate dehydrogenase reduction of MTT) in control and test article-treated cultures (Berridge, et al., 1996). Thus, the toxicity of the test item or the ocular irritation potential is evaluated by the relative viability of the treated tissues relative to the negative control-treated tissues.

From validation study and its independent peer review, it was concluded that the EpiOcular™ EIT is able to correctly identify chemicals (both substances and mixtures), not requiring classification and labelling for eye irritation or serious eye damage according to UN GHS, and the test method was recommended as scientifically valid for that purpose. The EpiOcular™ EIT is thus referred to as the Validated Reference Method (VRM) in the present Test Guideline.

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TEST REPORT

CONTENTS

1.0 OBJECTIVE

The objective of this study was to predict eye irritation potential of Clay A8 using an *in vitro* eye irritation test using EpiOcular™ model.

2.0 STUDY TIMETABLE

2.1 **Receipt of Reconstructed Human Cornea-like Epithelium (RhCE) model EpiOcular™ tissues:**
31 March 2020

2.2 **Tissue Conditioning**
31 March 2020

2.3 **Pre-Treatment**
31 March 2020

2.4 **Treatment**
01 April 2020

2.5 **Rinsing**
01 April 2020

2.6 **Post-Soak**
01 April 2020

2.7 **Post Incubation**
01 April 2020

2.8 **MTT Viability Test**
02 April 2020

2.9 **Optical Density Reading**
02 April 2020

2.10 **Data Analysis**
02 April 2020 – 07 April 2020

3.0 MATERIALS

3.1 **Test Item**

3.1.1 Test item: Clay A8

3.1.2 Sample marking : Changkat Rembian

3.1.3 Date received: 17 February 2020

3.1.4 Physical appearance: Solid

3.1.5 Colour: Brownish

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3.1.6 Physical Chemical Properties Data: Not provided

3.1.7 Quantity received : 1 bottle

3.1.8 pH: Not provided

3.1.9 Storage condition: Ambient

3.1.10 Solubility: Not provided

3.1.11 Stability: Not provided

3.1.12 Expiration date: Not provided

3.2 **Test System**

3.2.1 Test System: Reconstructed Human Cornea-like Epithelium (RhCE) model EpiOcular™

The Reconstructed Human Cornea-like Epithelium (RhCE) model EpiOcular™ (OCL-200, MatTek, Ashland, USA) consists of normal, human-derived epidermal keratinocytes which have been cultured to form a stratified, squamous epithelium similar to that found in the cornea.
Cultured on specially prepared cell culture inserts using serum-free culture medium, the cells differentiate to form a multi-layered structure which closely parallels the corneal epithelium.

3.2.2 Product Number: OCL-200 version 2.0

3.2.3 Lot No: 28050

3.2.4 Production date: 26 March 2020

3.2.5 Date of Shipping: 27 March 2020

3.2.6 Receipt of EpiOcular™: 31 March 2020

3.2.7 Visual quality control of the Eye: All tissues in good condition

3.2.8 The EpiOcular™ System is manufactured according to defined quality assurance procedures. All biological components of the EpiOcular™ and the culture medium are tested by manufacturer for viral, bacterial, fungal and mycoplasma contamination. MatTek determines the ET-50 value following exposure to Triton X-100 (0.3%) for each EpiOcular™ lot. The ET-50 must fall within a range established based on a historical database of results.

3.3 **Reagent**

3.3.1 Assay Medium: EPI-100-NMM-SIT / Assay Medium
3.3.1.1 Lot No. : 032320ALB
3.3.1.2 Sterility : Sterile
3.3.1.3 Expiration Date : 19/04/2020
3.3.1.4 Storage : Refrigerator (5 ± 3 °C)
3.3.1.5 Manufacturer : Mattek Corporation

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3.3.2 Phosphate Buffered Saline without Calcium and Magnesium
Lot No.: 111219RAHA
Expiration Date : 12/11/2020
Manufacturer : Mattek Corporation

3.3.3 Phosphate Buffered Saline without Calcium and Magnesium, contain the following:

3.3.3.1 Sodium Chloride 8 g/L
Lot No.: K50722004912
Manufacturer : MERCK

3.3.3.2 Potassium chloride 0.2 g/L
Lot No.: 1409BI4J35501
Manufacturer : Bio Basic Canada Inc

3.3.3.3 Anhydrous potassium dihydrogen orthophosphate 0.2 g/L
Lot No.: 1211ACN1K12062801
Manufacturer : Bio Basic Canada Inc

3.3.3.4 Anhydrous disodium hydrogen orthophosphate 1.15 g/L
Lot No.: 1306ACK2NA12020101
Manufacturer : Bio Basic Canada Inc

3.3.4 MTT – 2mL (5mg/mL)
Lot No. : MKBW0025V
CAS No. : 298-93-1
Manufacturer : SIGMA

3.3.5 MTT Diluent – 8mL
As above 3.3.1

3.3.6 Extractant Solution – Isopropanol
Lot No. : 632261
Storage : Room Temperature
CAS No. : 67-63-0
Manufacturer : Fisher Scientific

3.3.7 Positive Control: Methyl Acetate
Part No. : TC-MA
Lot No. : 112619ALA
Expiration Date : 26/02/2020
Storage : Room Temperature
Manufacturer : MatTek Corporation

3.3.8 Negative Control: Sterile pure water

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4.0 METHOD

Upon receipt of the reconstructed human cornea-like epithelial model EpiOcular™, the tissue kits and the solutions were stored according to the manufacturer's directions for unpacking and storage.

4.1 Assessment of direct test item reduction by MTT

50 mg of the test item was added to 1 mL of the 1 mg/mL MTT and incubated at (37 ± 1)° C, (5 ± 1) CO_2, 95 % RH for 60 minutes. Untreated MTT medium was used as control.

4.2 Assessment of Coloured material

4.2.1 Isopropanol

50 mg of the test item was added to 2 mL of isopropanol and agitated for 3 hours at room temperature. Isopropanol was run in parallel as negative control

4.3 Tissue Conditioning – Day 0

Under sterile conditions, the sealed 24-well plates containing the EpiOcular™ tissues, was opened. Visual inspection of each insert containing the EpiOcular™ tissue was done prior to tissue conditioning.

1.0 mL of assay medium was dispensed into each well of six-well plates, the EpiOcular™ tissue cultures were transferred into the wells and the plates were incubated for one hour at (37 ± 1) ° C, (5 ± 1) % CO_2, 95 % RH. At the end of the first hour of pre-incubation, the assay medium was renewed for further pre-incubation. Each insert was aseptically transferred into well containing 1.0 mL assay medium and pre-incubation was conducted at (37 ± 1) ° C, (5 ± 1) % CO_2, 95 % RH for overnight (16 – 24 hours).

4.4 Dosing protocol – Day 1

4.4.1 Pre-Treatment

After overnight incubation, each tissues were pre-wetted with 20 µL of Phosphate Buffered Saline without Calcium and Magnesium (PBS without Ca^{2+} and Ma^{2+}). The tissues were incubated at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH for (30 ± 2) minutes.

4.4.2 Treatment

50 mg of test item was applied directly on the tissue. Negative and positive controls were run in parallel in an identical way to the dosed cultures. The cultures were incubated at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH for 6 hours ± 15 minutes.

4.4.3 Rinsing

At the end of the exposure period, the EpiOcular™ tissue cultures were removed from the incubator and extensively rinsed with phosphate buffered saline to eliminate any residual test item.

4.4.4 Post-Soak

After rinsing, immediately immerse each EpiOcular™ tissues in 5 ml of Assay Medium previously warmed to room temperature in a pre-labeled 12-well plate. The cultures were incubated at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH for (25 ± 2) minutes.

4.4.5 Post-Incubation
After (25 ± 2) minutes incubation, the medium was changed and EpiOcular™ tissues were incubated in 1 mL Assay medium for a further (18 hours ± 15 minutes) at (37 ± 1) °C, (5 ± 1) % CO_2, 95 % RH.

4.5 MTT Viability Test

4.5.1 The MTT assay was performed by transferring the each EpiOcular™ tissues to 24-well plates containing 300 mg MTT medium (1 mg/mL) in each well. After (180 ± 10) minutes of MTT incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2.0 mL isopropanol / tissue (extractant solution MTT-100-EXT). The extraction plates were sealed with parafilm and agitated for at least 2 hours at room temperature.

4.5.2 At the end of the extraction period, EpiOcular™ tissue was pierced with an injection needle and the extract allowed to run into the well from which insert was taken. The insert was then discarded. The extraction solution was pipetted up and down to ensure complete mixing. Finally, 200 µL were transferred into a 96 well microtiter plate for absorbance measurement (OD=optical density) at 570 nm without using a reference filter. 200 µL of isopropanol was used as blank.

Relative cell viability was calculated for each tissue as % of the mean of the negative control tissues.

5.0 DATA ANALYSIS

5.1 Assessment of direct test item reduction by MTT

5.1.1 The test item is presumed to have reduced the MTT if the MTT solution colour turns blue/ purple.

5.2 Assessment of Coloured material

5.2.1 Isopropanol
The test item has to be considered as possibly interacting with the MTT measurement if after subtraction of the OD for isopropanol, the OD of the test item solution is more than 0.08 (approximately 5% of the mean viability of the negative control). An additional test on colorant controls has to be performed.

5.3 Workbook EpiOcular-EIT

A blank, password protected MS EXCEL workbook EpiOcular-EIT-SPREAD.XLS was provided by MatTek Corporation. The workbook consists of two spreadsheets named: Import and Spread.

5.4 Raw Data of Optical Densities (ODs)

Raw data of optical densities (ODs) generated by the microplate reader (without blank subtraction) were copied from the reader software and then pasted into the Import spreadsheet of the Excel workbook. The blank corrections, calculation of results and statistical parameters are done automatically in the Spread spreadsheet of the workbook.

5.5 **Calculation**

5.5.1 After data entry, the spreadsheet performs the following calculations:

5.5.2 Blank correction

5.5.3 For each individual tissue treated with a test substance (TS), the positive control (PC) and the negative control (NC) the individual relative tissue viability was calculated according to the following formulas

Relative Viability TS (%) = [Corrected OD_{TS} / Corrected Mean of OD_{NC}] x 100
Relative Viability NC (%) = [Corrected OD_{NC} / Corrected mean of OD_{NC}] x 100
Relative Viability PC (%) = [Corrected OD_{PC} / Corrected mean of OD_{NC}] x 100

5.5.4 For each test item, negative control and positive control, the mean relative viability of the two individual tissues was calculated and used for classification according to the Prediction Model (Refer to 8.0).

6.0 ACCEPTABILITY RANGES FOR QUALITY CONTROL

	Lower Acceptance Limit	Upper Acceptance Limit
EpiOcular™ EIT (OCL-200)	ET_{50} = 12.2 min	ET_{50} = 37.5 min

7.0 ACCEPTABILITY RANGES FOR NEGATIVE CONTROL OPTICAL DENSITY (OD) VALUES OF THE TEST METHODS

	Lower Acceptance Limit	Upper Acceptance Limit
EpiOcular™ EIT (OCL-200)	≥ 0.8*	≤ 2.5

*This acceptance limit considers the possibility of extended shipping / storage time (e.g., > 4 days) which has been shown not to impact on the performance of the test method.

8.0 ACCEPTANCE CRITERIA FOR POSITIVE CONTROL

The assay meets the acceptance criterion if the mean viability of Positive Control tissues expressed as percent of the negative control tissues is ≤ 60%. The standard deviation shall be below 20 for all substances and controls.

	Mean of Viability (%)	Standard Deviation of Viability (SD %)
EpiOcular™ EIT(OCL-200)	≤ 60%	<20

9.0 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)

An irritant is predicted if the mean relative tissue viability of two individual tissues exposed to the test substance is reduced below 60% of the mean viability of the negative controls.

***In vitro* result**	***In vivo* prediction**
mean tissue viability ≤ 60%	Irritant (I) (GHS Categories 1 or 2) Further testing in other test method is required
mean tissue viability > 60%	Non-Irritant (NI) (GHS No Category)

10.0 RESULT AND DISCUSSION

10.1 **Assessment of Direct Test Item Reduction by MTT**

There was no change in MTT colour therefore the test item did not interact with MTT.

10.2 **Assessment of Coloured material**

10.2.1 Isopropanol

The relative OD reading of treated tissue by coloured test item is below 5% of the negative control. No further testing on colorant controls has to be performed.

10.3 **Viability Measurement**

10.3.1 Raw data of optical densities (ODs) of Blank

Refer To Table 1

10.3.2 Blank Correction

Refer to Table 2

10.4 **The Quality Control value meets the acceptance range criteria**

EpiOcular™ EIT (OCL-200)	**Minute**
ET_{50}	18.88

10.5 **The negative control OD value meets the acceptance range criteria**

EpiOcular™ EIT (OCL-200)	**Lower OD**	**Upper OD**
Negative Control	1.156	1.162

10.6 **The positive control OD value meets the acceptance range criteria**

EpiOcular™ EIT (OCL-200)	**Mean of Viability (%)**	**Standard Deviation of Viability (SD %)**
Positive Control	34.5	5.77

10.7 **Classification**

Test substance, negative control and positive control are qualified according to prediction model. The mean relative viability of the two individual tissues was calculated and used for classification

Refer to Table 3

10.8 **Graph**

10.8.1 The spreadsheet shows a graph of the results (% of relative viability ± standard deviation)

Refer to Figure 1

10.9 **Prediction Model**

	Mean of Viability (%)	**Standard Deviation of Viability (SD %)**	***In vitro* result**	***In vivo* prediction**
Clay A8	99.9	0.92	mean tissue viability > 60 %	Non-Irritant (NI)
Negative Control	100.0	0.35	mean tissue viability > 60 %	Non-Irritant (NI)
Positive Control	34.5	5.77	mean tissue viability ≤ 60 %	Irritant (I)

11.0 CONCLUSION

Under the condition of this test, **Clay A8** is considered as **Non-Irritant** to *in vitro* eye model EpiOcular™.

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12.0 RETENTION OF RECORDS AND TEST ITEM

One report will be forwarded to the Sponsor. The other report, together with all generated raw data is maintained at the Industrial Biotechnology Research Centre Archives.

13.0 REFERENCES

13.1 OECD (2013), Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage –OECD Guidelines for Testing of Chemicals No. 492

13.2 Protocol for: In *Vitro* EpiOcular™ Eye Irritation Test (OCL-200-EIT) – Reconstructed Human Cornea-like Epithelium (RhCE) Model EpiOcular (OCL-200-EIT). For use with MatTek Corporation

13.3 (LWI-238-44) - In Vitro EpiOcular ™ Eye Irritation Test (OCL-200-EIT)

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TEST REPORT

Table 1 Optical Densities (ODs) of Blank

Optical Densities (ODs) of Blank	Mean Optical Densities (ODs) of Blank
0.0406	0.0401
0.0400	
0.0403	
0.0411	
0.0391	
0.0390	
0.0409	
0.0400	

Table 2 Blank Corrected Data

	Tissue	Raw data		Blank corrected data		Mean	% of Viability
		1	2	1	2		
Clay A8	1	1.2110	1.2009	1.171	1.161	1.166	100.6
	2	1.1969	1.1847	1.157	1.145	1.151	99.3
Negative Control	1	1.2125	1.1798	1.172	1.140	1.156	99.8
	2	1.2163	1.1875	1.176	1.147	1.162	100.2
Positive Control	1	0.3993	0.3871	0.359	0.347	0.353	30.5
	2	0.4594	0.5161	0.419	0.476	0.448	38.6

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Table 3 Classification of Test Item

	Mean of OD	SD of OD	Mean of Viability [%]	SD of Viability	CV [%]	*In vitro* result	Classification
Clay A8	1.158	0.015	99.9	0.92	0.92	**Non irritant**	Qualified
Negative Control	1.159	0.006	100.0	0.35	0.35	**Non irritant**	Qualified
Positive Control	0.400	0.095	34.5	5.77	16.70	**Irritant**	Qualified

OD- Optical Density, SD- Standard Deviation, CV- Coefficient of Variation

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TEST REPORT

REPORT NO: **R188/20/B19/44**	PAGE: 17 of 17

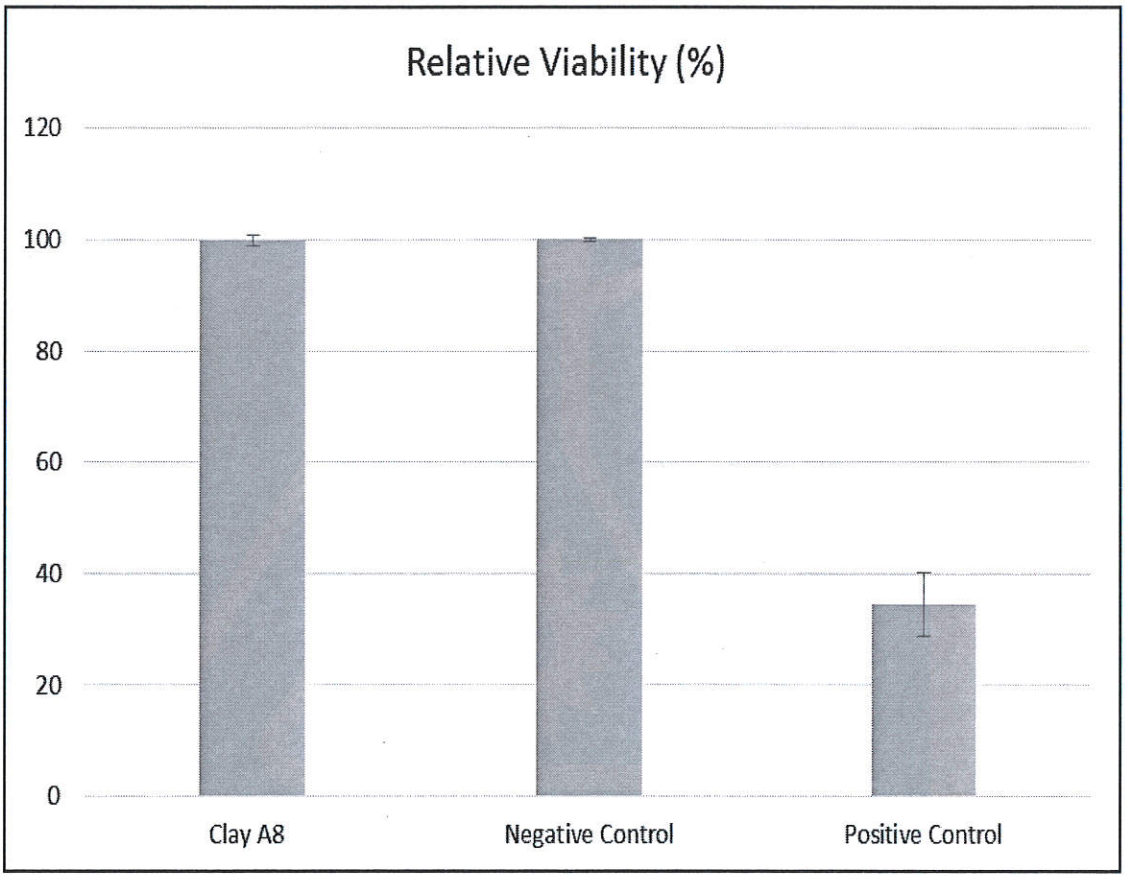

Figure 1 Relative Viability

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