eurofins | BioPharma Product Testing

In vitro Skin Irritation: Human Skin Model Test (EpiDerm™) with DyeMansion Sample

Report

Version: Final

Study Completion Date: 21 AUG 2015

Eurofins Munich Study No.: 152634

Sponsor:
DyeMansion GmbH
Lochhamer Straße 29a
82152 Planegg-München
(ehemalige Trindo GmbH)
Germany

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D-82152 Planegg/Munich
Germany

Tel +49 (0)89 899 650-0
Fax : +49 (0)89 899 650-11

GLP / GMP FDA approved

1. Copy of the GLP Certificate

Bayerisches Landesamt für
Gesundheit und Lebensmittelsicherheit

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

EUROFINS BIOPHARMA PRODUCT TESTING MUNICH GMBH
BEHRINGSTRAßE 6-8
82152 PLANEGG

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

Kategorie 2/ Category 2
Kategorie 3/ Category 3
Kategorie 8/ Category 8
Kategorie 9*/ Category 9*

Sonstige Prüfungen:
biologische und mikrobiologische Sicherheitsprüfungen an Medizinprodukten und Arzneimitteln; Auftragsarchivierung

other tests:
biological an microbiological safety evaluation on medical devices and pharmaceuticals; contract archiving

Datum der Inspektion/Date of Inspection
(Tag.Monat.Jahr/day.month.year)

18. bis 19.03.2015

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Schwabach, 05.06.2015

Dr. Peter Franke
Leiter der GLP-Landesleitstelle Bayern

GLP- Landesleitstelle Bayern
Bayerisches Landesamt für Gesundheit
und Lebensmittelsicherheit
Rathausgasse 4
91126 Schwabach

2. Contents

3. List of Tables

4. Preface

4.1. Abbreviations

Aqua dest.	Aqua destillata *(distilled water)*
Art.	Artikel *(article)*
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
CV	Coefficient of variation
DPBS	Dulbecco's Phosphate Buffered Saline
e.g.	exempli gratia *(for example)*
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EU CLP	European Union Regulation on the Classification, Labelling and Packaging of Substances and Mixtures
Eurofins Munich	Eurofins BioPharma Product Testing Munich GmbH
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
I	irritant
ISO	International Organization for Standardization
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	negative control
NHEK	normal human epidermal keratinocytes
NI	non-irritant
OD	optical density
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate Buffered Saline
PC	positive control
QA	Quality Assurance
QAU	Quality Assurance Unit
RhE	reconstructed human epidermis
SD	Standard Deviation
SDS	sodium dodecyl sulfate
SOPs	Standard Operating Procedures
TM	Test Item
UN GHS	United Nations Globally Harmonized System on the Classification and Labelling of Chemicals
VC	vehicle control

4.2. General

Sponsor:	DyeMansion GmbH Lochhamer Straße 29a 82152 Planegg-München (ehemalige Trindo GmbH) Germany
Study Monitor:	Mr Felix Ewald
Test Facility:	Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany
Eurofins Munich Study No.:	152634
Test Item:	DyeMansion Sample
Title:	*In vitro* Skin Irritation: Human Skin Model Test (EpiDerm™) with DyeMansion Sample

4.3. Project Staff

Study Director:	Dr. Helge Gehrke
Management:	Dr. Angela Lutterbach Dr. Katrin Witschital
Head of GLP Quality Assurance Unit:	Dipl.-Biol. Carolin Schmidt

4.4. Schedule

Arrival of the Test Item:	07 May 2015
Study Initiation Date:	24 June 2015
Experimental Starting Date:	19 July 2015
Experimental Completion Date:	24 July 2015

5. Quality Assurance

5.1. GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998 [3].

The OECD Principles of Good Laboratory Practice are accepted by regulatory authorities throughout the European Community, USA and Japan.

This study was assessed for compliance with the study plan and the Standard Operating Procedures of Eurofins Munich. The study and/or the test facility are inspected periodically by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

5.2. Guidelines

This study followed the procedures indicated by internal Eurofins Munich SOPs and the following internationally accepted guidelines and recommendations:

OECD Guideline for the Testing of Chemicals No. 439: *In Vitro* Skin Irritation: Reconstructed human Epidermis Test Method, 26 July 2013 [4].

Commission Regulation (EC) No. 640/2012, L 193, Part B.46. "*In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method" 06-Jul-2012 [5].

MatTek Corporation Protocol for: *In Vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT) For use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm (EPI-200-SIT); Version 26-Mar-2012 [6].

ECVAM Performance Standards for *in* vitro Skin Irritation Test Methods based on Reconstructed human Epidermis (RhE). Updated Version 24-Aug-2009 [7].

ISO 10993-10, Annex D: 2010, "Biological evaluation of medical devices - Part 10: Tests for irritation and skin sensitization" [8].

ISO 10993-12: 2012 "Sample preparation and reference materials" [9].

5.3. Archiving

For a period of 15 years (or shorter if in compliance with the GLP regulations) Eurofins Munich will store the records, materials and specimens in their scientific archives according to the GLP regulations.

The following records have to be stored according to the GLP regulations:

A copy of the final report, the study plan and documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study. Any document relating to the study will be discarded only with the prior consent of the sponsor.

The following materials and samples have to be stored according to the period of time specified in the GLP regulations:

A retained sample of the test item will be archived according to the GLP regulations, if possible, and will be discarded without the sponsor’s prior consent.

Other materials and specimens have to be stored according to the GLP regulations and disposed of after the respective archiving period with the sponsor’s prior consent.

As requested the remaining test item will be returned to the sponsor.

6. Statement of Compliance

Eurofins Munich Study No.:	152634
Test Item:	DyeMansion Sample
Title:	*In vitro* Skin Irritation: Human Skin Model Test (EpiDerm™) with DyeMansion Sample
Study Director:	Dr. Helge Gehrke

This study performed in the test facility Eurofins Munich was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998 [3].

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dr. Helge Gehrke

.....

Date: 21 Aug 2015

7. Statement of the Quality Assurance Unit

Eurofins Munich
Study No.: 152634

Test Item: DyeMansion Sample

Title: *In vitro* Skin Irritation: Human Skin Model Test (EpiDerm™) with DyeMansion Sample

Study Director: Dr. Helge Gehrke

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates:

Phase of QAU Inspection	**Date of QAU Inspection**	**Date of Reporting to the Study Director and Management**
Audit Final Study Plan:	24 June 2015	24 June 2015
Audit Experimental Phase (process-based):	20 October 2014	20 October 2014
Audit Final Report:	20 AUG 2015	20 AUG 2015

This report reflects the raw data.

Member of the
Quality Assurance Unit:

K. Seidel
.....
Print Name: Katrin Seidel

Date: 25 Aug. 2015

8. Summary

8.1. Summary Results

In the present study the skin irritant potential of DyeMansion Sample was analysed. The EpiDerm™-Standard Model (EPI-200™), a reconstituted three-dimensional human epidermis model, was used as a replacement for the Draize Skin Irritation Test (OECD TG 404) to distinguish between UN GHS “Category 2” skin irritating test substances and not categorized test substances (“No Category”) which may be considered as non-irritant. Hereby, a polar (0.9% NaCl solution) and unpolar extract (sesame seed oil) of the test item was applied topically. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT after an 18 h exposure compared to those of the concurrent negative controls.

8.2. Conclusion

In this study under the given conditions the both extracts of the test item showed no irritant effects. The relative mean tissue viability after 60 min of exposure and 42 h post incubation was > 50%. The test item is therefore classified as “non-irritant” in accordance with UN GHS “No Category”.

9. Introduction

Acute irritation is a local, reversible inflammatory response of normal living skin to direct injury caused by the application of an irritant substance. The potential to induce skin irritation is an important consideration included in procedures for the safe handling, packing and transports of chemicals.

Current guidelines include OECD guideline 404 for acute dermal irritation and corrosion of chemicals. This guideline is based on the method described by Draize [10], and generally involves the rabbit as the experimental animal. In order to replace *in vivo* testing on skin irritation validation studies on alternative *in vitro* methods were conducted under the auspices of ECVAM [11], [12], [13], [14]. It was concluded that the modified *in vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT) showed evidence of being a reliable and relevant stand-alone replacement test for *in vivo* skin irritation testing [15], [16]. Recently studies demonstrated the applicability of the *in vitro* RhE models for medical device extracts [17], when adapted to the special needs of the extraction procedure mentioned in Annex D of ISO 10993-10 and -12 [8], [9].

This test may be used for the hazard identification of irritant chemicals/extracts in accordance with UN GHS “Category 2”. It does not allow the classification of chemicals/extracts to the optional UN GHS “Category 3” (mild irritants). Therefore all remaining substances will not be classified, i.e. UN GHS “No Category” [18], [19], [20]. The endpoint is evaluated by MTT reduction [16].

9.1. Aim of the Study

This *in vitro* method is designed to predict and classify the skin irritation potential of extracts from medical devices by assessment of its effect on a RhE model (EpiDerm™, EPI-200, MatTek) a reconstituted three-dimensional human epidermis model.

9.2. Justification for the Selection of the Test System

This test uses the EpiDerm™ reconstructed human epidermis model (MatTek) which consists of human keratinocytes (NHEK) and therefore represents *in vitro* the target organ of the species of interest and closely mimics the biochemical and physiological properties of the upper parts of the human, i.e. the epidermis.

9.3. Justification for the Selection of the Test Method

This test method is able to detect chemicals that cause skin irritation, i.e. produce reversible damage to the skin and allows for hazard identification in accordance with UN GHS “Category 2”.

10. Materials and Methods

10.1. Characterisation of the Test Item

The identity of the test item was inspected upon delivery at the test facility (e.g. test item name, batch no. and additional data were compared with the label) based on the following specifications provided by the sponsor.

Name:	DyeMansion Sample
Batch No.:	not specified by sponsor
Aggregate State at RT:	solid
Colour:	black
Type of Material:	synthetic polymer (Polyamid 12)
Storage Conditions:	room temperature
Surface according to sponsor:	418 cm²
Surface (calculated):	70.4 cm² per test item
Sterilisation Method:	not sterile
Expiry Date:	not applicable ***/*** not provided by the sponsor
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

10.2. Extraction of the Test Item

The extraction was done according to ISO 10993-12. A polar and non-polar extract was generated by using 0.9% saline solution and sesame seed oil (pharmaceutical grade), respectively. The test items were extracted under agitation for 72 ± 2 h at 37 ± 1°C. The final surface/volume ratio in the assay was 3 cm²/mL, which corresponds to 100% extract concentration. Extracts were sterile filtered.

10.3. Preparation of the Test Items

The respective extracts were applied undiluted. 100 µL of the test item extracts were dispensed directly atop the EpiDerm™ tissue.

10.4. Controls

Controls were set up in parallel to the test item in order to confirm the validity of the test.

Negative Control

Dulbecco's phosphate buffered saline (DPBS; Gibco, Cat. No. 14040-091, Lot No.: 1660068).

Positive Control

As positive control 1% sodium dodecyl sulphate (SDS) in the respective vehicle (0.9 mL saline solution, Braun, Lot No.: 141418002 and sesame seed oil, Fluka, Lot No.: BCBN4680V) was used. 500 µL of 20% SDS (Fluka, Cat No.: 05030, Lot No.: BCBP8067V) solution was mixed with 9.5 mL of particular vehicle and thoroughly vortex. Extracts were sterile filtered.

Vehicle Control

Vehicle controls (VCs), consisting of extraction vehicle alone (0.9% saline solution Braun, Lot No.: 141418002 and sesame seed oil (pharmaceutical grade), Fluka, Lot No.: BCBN4680V) and treated in the same way as the treatment groups were included. Extracts were sterile filtered.

10.5. Dose Groups

1.	Negative Control	100 µL DPBS
2.	Positive Control	100 µL 1% SDS solution in vehicle (0.9% saline solution and sesame seed oil)
3.	Vehicle Control	100 µL (undiluted)
4.	Test Item	100 µL (undiluted)

The test was performed on a total of 3 tissues per dose group.

10.6. Test System

The test was carried out with the reconstituted three-dimensional human skin model EpiDerm™ (MatTek). This skin model consists of normal (non-cancerous), human-derived epidermal keratinocytes (NHEK) which have been cultured to form a multilayered, highly differentiated model of the human epidermis. The NHEK are cultured on chemically modified, collagen-coated cell culture inserts (Millicell®). The EpiDerm™ skin model exhibits *in vivo*-like morphological and growth characteristics which are uniform and highly reproducible. It consists of organised basal, spinous, granular and cornified layers analogous to those found *in vivo*

10.7. Provided Materials

The EpiDerm™ tissues were provided as kits (EPI-200-SIT, MatTek), consisting of the following components relevant for this study:

1x sealed 24-well plate containing 24 inserts with tissues on Agarose (Lot No.: 21681 Kit C + E)

2x 24-well plates

8x 6-well plates

1x bottle of assay medium (DMEM-based medium, Lot No.: 071615ZSC)

1x bottle of DPBS Rinse Solution (Lot No.: 031715MHA / 043015ZSJ)

25 pieces Nylon Mesh circles (8 mm diameter, 200 µm pore)

For MTT reduction assay the MTT assay kit (MTT-100; MatTek) was provided, consisting of:

1x vial MTT concentrate (Lot:063015TMA)

1x vial MTT diluent (supplemented DMEM; Lot:1644369)

1x bottle extractant solution (Isopropanol; Lot:041415MHA)

10.8. Further Reagents

Sodium Dodecyl Sulphate (20 %); Cat No.: 05030, Lot No.: BCBP8067V

10.9. Pre-Experiments

To check the MTT-reducing capability of the test item 30 µL of the respective test item extract was mixed per 1 mL MTT medium and incubated for 1 h in an incubator at 37 ± 1 °C. If the mixture turns blue/purple, the test item is presumed to have reduced MTT.

To check the colouring potential of the test item 30 µL of the respective test item extract was mixed per 300 µL aqua dest. in a transparent recipient and incubated at 37 ± 1°C for 60 min.

10.10. Experimental Procedure

Upon receipt of the EpiDerm™, the tissues were transferred into 6-well plates containing 0.9 mL pre-warmed assay medium (room temperature) per well and were incubated for 1 h ± 5 min at 37 ± 1 °C, 5.0% CO_2. Subsequently the tissues were transferred in new wells containing 0.9 mL pre-warmed assay medium per well and were incubated overnight in a humidified incubator at 37 ± 1 °C, 5.0% CO_2.

After this pre-incubation the surface of the tissues was evaluated and moisture was removed using a sterile cotton tip. Before test substance exposure tissues were transferred in new wells containing 0.9 mL pre-warmed assay medium per well. Tissues were treated with each dose group in triplicate, starting with the negative control. Start time was recorded with dosing of the first tissue staggered in one-minute intervals. After dosing of all tissues, all plates were transferred to the incubator for 18 h ± 30 min. After exposure, all plates were removed from the incubator and tissues were washed intensively with DPBS, staggered in one-minute intervals. Excess DPBS was removed by blotting the bottom with blotting paper. The inserts were placed in a prepared 24-well plate containing 0.3 mL pre-warmed fresh assay medium until all tissues are rinsed. After all inserts were rinsed tissue surface was carefully dried using a sterile cotton bud. The assay medium from the incubation was collected for further IL-1α determination. Before collecting the media, plates were shaked using a plate shaker for 10 min at 500 rpm prior to media collection. Samples were pipetted into suitable tubes and stored at - 20°C until determination of IL-1α.

After this incubation period the inserts were transferred in a prepared 24-well plate containing 300 µL pre-warmed MTT medium and further incubated for 3 h ± 5 min at 37 ± 1 °C, 5.0% CO_2, humidified to 95%.

After the 3 h ± 5 min MTT incubation period the tissues were rinsed three times with DPBS and afterwards placed on blotting paper to dry. The tissues were immersed in 2 mL isopropanol, sealed to inhibit evaporation and incubated at room temperature for at least two hours.

At the end of the formazan extraction period the plate was mixed by shaking until the solution colour became homogeneous.

Per each tissue 2 x 200 µL aliquots of the extracts were transferred into a 96-well plate and OD was measured at 570 nm without reference wavelength in a plate spectrophotometer.

10.11. Data Analysis

Irritant potential of the test item was predicted from the relative mean tissue viabilities compared to the negative control tissues concurrently treated with DPBS. The test item is considered to be irritant to skin in accordance with regulation EC 1272/2008 (UN GHS “Category 2”) [19], [20], if the tissue viability after 15 min of exposure and 42 h of post-incubation is less or equal to 50%. The test substance may be considered as non-irritant to skin in accordance with UN GHS “No Category” if the tissue viability after exposure and post-treatment incubation is higher than 50%.

Table 1: Prediction I/NI

Mean tissue viability (% negative control)	**Prediction I / NI**
≤ 50 %	Irritant (I): UN GHS – “Category 2”
> 50 %	Non-Irritant (NI): UN GHS “No Category”

10.12. Test Acceptance Critria

The test meets acceptance criteria if:

- mean $OD_{570\ nm}$ of the three negative control tissues is ≥ 0.8 and ≤ 2.8
- mean relative tissue viability of the three positive control tissues is ≤ 20%
- standard deviation (SD) of relative tissue viability obtained from each three concurrently tested tissues is < 18%.

11. Deviations from the Study Plan

There were the following deviations from the study plan:

- **Concerning:**

  7.1 Characterisation of the Test Item, study plan, p. 10

  Study Plan:

  Calculated surface: 418 cm²

  Report:

  Surface according to sponsor: 418 cm²

  Surface (calculated): 70.4 cm² per test item

  Reason:

  Typing error

- **Concerning:**

  7.6 Provided Materials, study plan, p. 11

  Study Plan:

  -

  Report:

  For MTT reduction assay the MTT assay kit (MTT-100; MatTek) was provided, consisting of:

  1 vial MTT concentrate (Lot:063015TMA)

  1 vial MTT diluent (supplemented DMEM; Lot:1644369)

  1 bottle extractant solution (Isopropanol; Lot:041415MHA)

  Reason:

  MTT kit from the tissue supplier was used.

- **Concerning:**

  7.7 Further reagents, study plan, p. 12

  Study Plan:

  MTT solution

  - MTT stock solution: 5 mg/mL MTT in PBS

    MTT medium: MTT stock solution will be diluted 1 + 4 with assay medium (final concentration 1 mg/mL)

  Isopropanol

  Report:

  -

  Reason:

  MTT kit from the tissue supplier was used.

- **Concerning:**

  7.12 Test Acceptance criteria, study plan, p. 14

  Study Plan:

  The test meets acceptance criteria if:

  - mean OD570 nm of the three negative control tissues is ≥ 1.0 and ≤ 2.8
  - mean relative tissue viability of the three positive control tissues is < 50%
  - standard deviation (SD) of relative tissue viability obtained from each three concurrently tested tissues is < 20%.

  Report:

  The test meets acceptance criteria if:

  - mean OD570 nm of the three negative control tissues is ≥ 0.8 and ≤ 2.8
  - mean relative tissue viability of the three positive control tissues is < 20%
  - standard deviation (SD) of relative tissue viability obtained from each three concurrently tested tissues is < 18%.

  Reason:

  Adjusting the acceptance criteria according to OECD guideline 439.

These deviations did not influence the quality or integrity of the present study.

12. Results and Discussion

12.1. Results

12.1.1. Pre-Experiments

The mixture of 30 µL test item extract per 1 mL MTT medium showed no reduction of MTT compared to the solvent. The mixture did not turn blue/purple.

The mixture of 30 µL of the test item extract per 300 µl aqua dest. showed no colouring detectable by unaided eye-assessment.

12.1.2. Experiment

Table 2: Result of the Test Item DyeMansion Sample extracted with 0.9% saline solution

Name	**NC**			**PC**			**TM**			**VC**		
Tissue	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
absolute OD550	1.836	2.036	1.820	0.070	0.089	0.074	1.749	1.682	1.687	1.783	1.696	1.459
	1.857	2.033	1.848	0.070	0.089	0.078	1.830	1.705	1.713	1.823	1.691	1.514
OD_{570} (blank-corrected)	1.794	1.994	1.779	0.029	0.048	0.032	1.708	1.640	1.646	1.742	1.655	1.417
	1.816	1.991	1.807	0.029	0.048	0.037	1.789	1.664	1.672	1.782	1.650	1.473
mean OD_{570} of the duplicates (blank-corrected)	1.805	1.993	1.793	0.029	0.048	0.034	1.748	1.652	1.659	1.762	1.652	1.445
total mean OD_{570} of 3 replicate tissues (blank-corrected)	1.864*			0.037			1.687			1.620		
SD OD_{570}	0.112			0.010			0.054			0.161		
relative tissue viability [%]	**96.9**	**106.9**	**96.2**	**1.5**	**2.6**	**1.8**	**93.8**	**88.7**	**89.0**	**94.6**	**88.7**	**77.5**
mean relative tissue viability [%]	**100.0**			**2.0****			**90.5**			**86.9**		
SD tissue viability [%]***	**6.0**			**0.5**			**2.9**			**8.6**		

* Corrected mean $OD_{570\ nm}$ of the negative control corresponds to 100% absolute tissue viability.

** Mean relative tissue viability of the three positive control tissues is ≤ 20%.

*** Standard deviation (SD) obtained from the three concurrently tested tissues is < 18%

Table 3: Result of the Test Item DyeMansion Sample extracted with sesame seed oil

Name	NC			PC			TM			VC		
Tissue	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
absolute OD_{570}	1.863	2.036	1.820	0.095	0.101	0.098	1.763	1.747	1.811	1.096	1.068	1.905
	1.857	2.033	1.848	0.096	0.103	0.099	1.848	1.709	1.829	1.161	1.122	1.902
OD_{570} (blank-corrected)	1.794	1.994	1.779	0.053	0.060	0.057	1.722	1.706	1.770	1.055	1.027	1.864
	1.816	1.991	1.807	0.054	0.061	0.058	1.807	1.667	1.787	1.120	1.081	1.861
mean OD_{570} of the duplicates (blank-corrected)	1.805	1.993	1.793	0.054	0.061	0.057	1.764	1.687	1.779	1.087	1.054	1.862
total mean OD_{550} of 3 replicate tissues (blank-corrected)	1.864*			0.057			1.743			1.334		
SD OD_{550}	0.112			0.003			0.049			0.458		
relative tissue viability [%]	**96.9**	**106.9**	**96.2**	**2.9**	**3.3**	**3.1**	**94.7**	**90.5**	**95.4**	**58.3**	**56.5**	**99.9**
mean relative tissue viability [%]	**100.0**			**3.1****			**93.5**			**71.6**		
SD tissue viability [%]***	**6.0**			**0.2**			**2.7**			**24.6**		

* Corrected mean $OD_{570\ nm}$ of the negative control corresponds to 100% absolute tissue viability.

** Mean relative tissue viability of the three positive control tissues is ≤ 20%.

*** Standard deviation (SD) obtained from the three concurrently tested tissues is < 18%. The VC is excluded from this statement.

12.1.3. Quality Criteria

Table 4: Quality Criteria for Results with 0.9% saline solution

	Value	Cut off	pass/fail
Mean absolute $OD_{570\ nm}$ NC	1.864	0.8 < NC < 2.8	pass
Relative Viability [%] PC	2.0	≤ 20%	pass
SD Viability [%]	0.5 – 8.6	< 18%	pass

Table 5: Quality Criteria for Results with sesame seed oil

	Value	Cut off	pass/fail
Mean absolute $OD_{570\ nm}$ NC	1.864	0.8 < NC < 2.8	pass
Relative Viability [%] PC	3.1	≤ 20%	pass
SD Viability [%]*	0.2 – 24.6	< 18%	fail

* SD tissue viability of the VC treated tissues is slightly above the threshold value. Since the mean relative tissue viability is clearly above 50% (56.5 – 99.9%) for all three replicates, the VC can be clearly considered as non-irritant and the high standard deviation is regarded as not biologically relevant.

12.1.4. Historical Data

Table 6: Historical Data

	Mean $OD_{550\text{-}570}$ NC	Mean Relative Viability [%] PC	SD Viability [%]
Mean	1.916	3.7	4.4
SD	0.452	1.516	4.985
n	12	12	37

12.2. Discussion

The potential of the test item to induce skin irritation was analysed by using the three-dimensional human skin model EpiDerm™ (MatTek) comprising a reconstructed epidermis with a functional stratum corneum.

In the present study a polar (0.9% NaCl solution) and unpolar extract (sesame seed oil) of DyeMansion Sample was generated and applied topically to the EpiDerm™ tissue for 18 h, followed by an immediate determination of cytotoxic effects via MTT reduction assay.

Irritant potential of the test item was predicted from the relative mean tissue viabilities obtained compared to the corresponding negative control tissues concurrently treated with DPBS.

The polar extract (0.9% NaCl solution) of the test item showed no irritant effects. The mean relative tissue viability (% negative control) was > 50% (90.5%, extracted with NaCl) after 18 h treatment.

The non-polar extract (sesame seed oil) of the test item showed no irritant effects. The mean relative tissue viability (% negative control) was > 50% (93.5% extracted with sesame seed oil) after 18 h treatment. The standard deviation of tissue viability of the replicate tissues treated with solvent control was slightly above the threshold value. Since the mean relative tissue viability was clearly above 50% (56.5 – 99.9%) for all three replicates and the solvent control has no direct influence on the test item extract, the non-polar test item extract can be clearly classified as non-irritant irrespective of the high standard deviation which is regarded as not biologically relevant.

The controls confirmed the validity of the study. The mean absolute OD_{570} of the three negative control tissues was ≥ 0.8 and ≤ 2.8. The mean relative tissue viability (% negative control) of the positive controls was $\leq 20\%$ (2.0% for 0.9% NaCl solution extract; 3.1% for sesame seed oil extract). The maximum standard deviation of viability of replicate tissues of all dose groups with exception of the VC of the sesame seed oil extract was < 18% (0.2% - 8.6%).

13. Conclusion

In this study under the given conditions polar and non-polar extracts of the test item showed no irritant effects. The test item is therefore classified as “non-irritant” in accordance with UN GHS “No Category”.

14. Distribution of the Report

1 original (paper):	Sponsor
1 copy (paper):	Eurofins Munich
1 copy (electronic):	Sponsor

15. References

15.1. Guidelines

[1] Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498)

[2] Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998

[3] OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998

[4] OECD (2013). OECD Guideline for the Testing of Chemicals No. 439: *In Vitro* Skin Irritation: Reconstructed human Epidermis Test Method. 26 July 2013

[5] Commission Regulation (EC) No. 640/2012 of 06 July 2012 amending, for the purpose of its adaption to technical progress, Regulation (EC) No. 440/2008 laying down test methods pursuant to Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), L 193, Part B.46. "*In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method"

[6] MatTek Corporation Protocol for In Vitro EpiDerm™ Skin Irritation Test (EPI-200-SIT). For use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm (EPI-200-SIT); Version 26 March 2012

[7] ECVAM Performance Standards for Skin Irritation Testing: *In vitro* Skin Irritation Test Methods based on Reconstructed human Epidermis (RhE). Updated Version 24 August 2009

[8] ISO 10993-10, 2010(E), "Biological evaluation of medical devices - Part 10: Tests for irritation and skin sensitization". Annex D: *In vitro* tests for skin irritation

[9] ISO 10993-12: 2012 "Sample preparation and reference materials"

15.2. Literature

[10] Draize J.H., Woodand G.& Calvery H.O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of Pharmacology and Experimental Therapeutics.* 82, pp 337-390

[11] Fentem J.H., Briggs D., Chesné C, Elliot G.R., Harbell J.W., Heylings J.R., Portes P., Roguet R., van de Sandt J.J.M. and Botham P. (2001). A prevalidation study on *in vitro* tests for acute skin irritation. Results and evaluation by the Management Team. *Toxicol. in Vitro* 15, 57-93

[12] Faller C., Bracher M., Dami N. and Roguet R. (2002) Predictive ability of reconstructed human epidermis equivalents for assessment of skin irritation of cosmetics. *Toxicol. in vitro* 16, 557-572

[13] Cotovio J., Grandidier M.-H., Portes P., Roguet R. and Rubinstenn G. (2005). The *in vitro* acute skin irritation of chemicals: optimization of the EpiSkin prediction model within the framework of the ECVAM validation process. *ATLA* **33**, 329-349

[14] Zuang V., Balls M., Botham P.A., Coquettte A., Corsini E., Curren R.D., Elliot G.R., Fentem J.H., Heylings J.R., Liebsch M., Medina J., Roguet R., van de Sandt J.J.M., Wiemann C., Worth A.P. (2002) Follow-up to the ECVAM prevalidation study on *in vitro* tests for acute skin irritation. ECVAM Skin irritation Task Force Report 2. *ATLA* 30, 109-1029

[15] EC-ECVAM (2007), Statement on the validity of *in vitro* tests for skin irritation, issued by the ECVAM Scientific Advisory Committee (ESAC26), 27 April 2007

[16] EC-ECVAM (2008), Statement on the scientific validity of *in vitro* tests for skin irritation testing, issued by the ECVAM Scientific Advisory Committee (ESAC29), 5 November 2008

[17] Casas J.W., Lewerenz G.M., Rankin E.A., Willoughby J.A. Sr., Blakeman L.C. McKim J.M.Jr.,and Coleman K.P. (2013). In Vitro Human Skin irritation Test for Evaluation of Medical Device Extracts. *Toxicol. In Vitro* 27, 2175-2183.

[18] UN (2013), United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva. Available at: http://www.unece.org/trans/danger/publi/ghs/ghs_rev05/05files_e.html

[19] EC-ECVAM (2009), Statement on the "Performance under UN GHS of three *in vitro* assays for skin irritation testing and the adaption of the Reference Chemicals and Defined Accuracy Values of the ECVAM skin irritation Performance Standards", issued by the ECVAM Scinetific Advisory Committee (ESAC30), 9 April 2009

[20] EC (2008), Regulation (EC) No. 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No. 1907/2006. Official Journal of the European Union L353, 1-1355

16. Appendix 1: EpiDerm™ Skin Tissue: Certificate of Analysis

Certificate of Analysis

MatTek | IN VITRO LIFE SCIENCE LABORATORIES

Product: EpiDerm™ Reconstructed Human Epidermis

Lot Number: 21681

Part#: EPI-200, EPI-212, EPI-218
Description: Reconstructed human epidermis tissue containing normal human keratinocytes. This product is for research use only. Not for use in animals, humans or diagnostic purposes.

I. Cell source

All cells used to produce EpiDerm™ are purchased or derived from tissue obtained by MatTek Corporation from accredited institutions. In all cases, consent was obtained by these institutions from the donor or the donor's legal next of kin, for use of the tissues or derivatives of the tissue for research purposes.

Keratinocyte Strain: 00267

II. Analysis for potential biological contaminants

The cells used to produce EpiDerm™ tissue are screened for potential biological contaminants. Tests for each potential biological contaminant listed below were performed according to the test method given. Results of "Not detected" indicate that testing for the potential biological contaminant was not observed as determined by the stated test method.

HIV-1 virus – Oligonucleotide-directed amplification	Not detected
Hepatitis B virus – Oligonucleotide- directed amplification	Not detected
Hepatitis C virus – Oligonucleotide- directed amplification	Not detected
Bacteria, yeast, and other fungi – long term antibiotic, antimycotic free culture	Not detected

III. Analysis for tissue functionality and quality

Test	Specification	Acceptance criteria	Result and QA Statement	
Tissue viability	MTT QC assay, 4 hours, n=3	OD (540-570 nm) [1.0-3.0]	1.983±0.102	Pass
Barrier function	ET-50 assay, 100 µL 1% Triton X-100, 4 time-points, n=3, MTT assay	ET-50 [4.77-8.72 hrs]	6.13 hrs	Pass
Sterility	Long term antibiotic and antimycotic free culture	No contamination	Sterile	Pass

Tissue viability and the barrier function test are within the acceptable ranges and indicate appropriate formation of the epidermal barrier, the presence of a functional stratum corneum, a viable basal cell layer, and intermediate spinous and granular layers. Results obtained with this lot conform to the requirements of the **OECD TG 431 and 439.**

Initials: PK

Date: 22.07.2015

Paul Kearney
Paul Kearney
Quality Assurance Manager

July 22, 2015
Date

CAUTION: Whereas all information herein is believed to be correct, no absolute guarantee that human derived material is non-infectious can be made or is implied by this certificate of analysis. All tissues should be treated as potential pathogens. The use of protective clothing and eyeware and appropriate disposal procedures are strongly recommended.

MatTek In Vitro Life Science Laboratories
Mlynské Nivy 73, Bratislava - Slovakia
+421-2-3260-7401

www.mattek.com
information@mattek.com