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Final Report

In Vitro Skin Corrosion Test with Wunderrein in the EPISKIN Model

Study Number: **960-431-5173**

Date of Final Report: **March 30, 2020**

(Report including Appendices total pages 33)

Sponsor:

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Statement of the Study Director

This study has been performed in accordance with the study plan, the OECD Guidelines for Testing of Chemicals (No. 431, 18 June 2019), Commission Regulation (EC) No.440/2008, Annex Part B, B.40Bis: "In Vitro Skin Corrosion: Human Skin Model Test", Official Journal of the European Union No. L142, dated May 31st, 2008, *INVITTOX* Protocol No. 118; "EPISKIN™ Skin Corrosivity Test" (updated December 2011 / February 2012) and the Good Laboratory Practice Regulations as specified by national Hungarian Good Laboratory Practice Regulation: 42/2014 (VIII. 19.) EMMI decree of the Minister of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM(98)17.
I declare that this report constitutes a true record of the actions undertaken and the results obtained in this study.

Signature: _____ Date: 30 MARCH 2020

István Buda

Statement of the Management

I, the undersigned managing director hereby declare that the "*In Vitro* Skin Corrosion Test with Wunderrein in the EPISKIN Model" was performed in accordance with the agreed study plan in Toxi-Coop ZRT.

Signature: _____

Dr. Gábor Hirka

Date: March 30, 2020

Quality Assurance Statement

Study Number: **960-431-5173**

Study Title: ***In Vitro* Skin Corrosion Test with Wunderrein in the EPISKIN Model**

Test Item: **Wunderrein**

This study has been inspected and this report audited by the Quality Assurance in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established the methods described and the results incorporated in this report accurately reflect the raw data produced during this study.

All inspections, data reviews and the report audit were reported in writing to the study director and to management. The dates of such inspections and of the report audit are given below:

Date	Inspection/Audit	Date of report to Management	Date of report to Study Director
December 02, 2019	Study Plan	December 02, 2019	December 02, 2019
August 08- 09, 2019	Handling of Test Item; Adequacy of Test System; Identification; Treatment; MTT test, Formazan Extraction; Measurement of Samples (Process Based)	August 09, 2019	August 09, 2019
January 10, 2020	Draft Report	January 10, 2020	January 10, 2020
March 30, 2020	Final Report	March 30, 2020	March 30, 2020

Signature: _____ Date: March 30, 2020

Alexandra Széles
Quality Assurance

General Statements and Responsibilities

Study title:	***In Vitro* Skin Wunderrein in the EPISKIN Model**
Study number:	**960-431-5173**
Sponsor:	**PUROLEX Betriebshygiene & Gastroservice GmbH** Mario Hofer Natschlag 47 4160 Aigen Schlägl Österreich
Study Monitor:	**INVITRO-CONNECT GmbH** Dr. Ute Hassmann Weitenunger Straße 42 77815 Bühl Germany
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Test facility management:	**Dr. Gábor Hirka**
Study director:	**István Buda**
Quality Assurance:	**Ildikó Hermann** **Alexandra Széles**
Technical assistance:	**Krisztina Fejes Pátkainé**

Experimental Schedule

Start of experimental phase:	**December 04, 2019**
End of experimental phase:	**December 05, 2019**
Date of Draft Report:	**January 10, 2020**
Date of Final Report:	**March 30, 2020**

1.0 Summary

EpiSkin™ SM test of the test item Wunderrein has been performed to predict its corrosion potential by measurement of its cytotoxic effect, as reflected in the MTT assay according to the OECD Test Guideline No. 431, 18 June 2019.

Disks of EPISKIN (two units) were treated with the test item and incubated for 4 hours (±10 min) at room temperature. Exposure of the test material was terminated by rinsing with PBS 1x solution. The viability of each disk was assessed by incubating the tissues for 3 hours (±15 min) with MTT solution at 37±1 °C in an incubator with 5±1 % CO_2 in a ≥ 95 % humidified atmosphere and protected from light. The formazan precipitated was then extracted using acidified isopropanol and quantified spectrophotometrically.

NaCl (9 g/L saline) and glacial acetic acid treated epidermis were used as negative and positive controls, respectively.

The test item is a MTT-reducer, therefore additional controls (test item treated killed tissues and negative control treated killed tissues) were used to detect and correct for test substance interference with the viability measurement.

For each treated tissue viability was expressed as a % relative to negative control. The test item is considered to be non-corrosive to skin, if the mean relative viability after 4 hours of exposure is above or equal 35 % of the negative control.

The test item did not show significantly reduced cell viability in comparison to the negative control after 4 hours of exposure. The average test item treated tissue viability was 89 % (corrected values) at 4 hours of exposure. The test item treated tissue viability was above 35 % of the mean negative control value after 4 hours of exposure.

Positive and negative controls showed the expected cell viability values within acceptable limits.

All assay acceptance criteria were met, the experiment was considered to be valid.

The results obtained from this *in vitro* skin corrosion test, using the EPISKIN model (OECD 431), indicate that the test item reveals no skin corrosion potential under the utilised testing conditions. In conclusion, the test item Wunderrein can be classified as Non-corrosive to skin.

2.0 Study purpose and introduction

The corrosivity potential of a chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS: 298-93-1] assay, on the EPISKIN™ reconstituted human epidermis. This method is approved by international regulatory agencies as a replacement for the identification of corrosives in the *in vivo* Rabbit skin assay (OECD 404) and is specifically approved as a replacement for the *in vivo* skin corrosivity test within OECD 431.

The present test is based on the experience that corrosive chemicals show cytotoxic effects following short-term exposure of the stratum corneum of the epidermis. The purpose of this study is to predict the skin corrosivity potential of a chemical by assessment of its effect on a reconstituted human epidermis.

EPISKIN Standard Model™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use for skin corrosivity testing involves the topical application of test materials to the surface of the skin, and the subsequent assessment of their effects on cell viability. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT (Fentem *et al*., 1998).

3.0 Regulatory guidelines and test methods

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD Guidelines for Testing of Chemicals, Section 4, No. 431, "In Vitro Skin Corrosion: reconstructed human epidermis (RHE) test method" adopted 18 June 2019.
- Commission Regulation (EC) No 440/2008, Annex Part B, B.40Bis: "In Vitro Skin Corrosion: Human Skin Model Test", Official Journal of the European Union No. L142, dated May 31st, 2008.
- *INVITTOX* Protocol No. 118; "EPISKIN™ Skin Corrosivity Test" updated December 2011 / February 2012 (ECVAM Database Service on Alternative Methods to Animal Experimentation).

4.0 Archiving

The study documents and samples as listed below will be archived according to the OECD GLP and to the Toxi-Coop Zrt. SOPs in the archives of Toxi-Coop Zrt. (H-8230 Balatonfüred, Galamb u. 12/A., Hungary):

- Study plan and any amendment(s) (15 years)
- All raw data (15 years)
- Retained sample of the test item and reference item (5 years)
- Correspondence (15 years)
- Study report and any amendments (15 years)

After the retention time all the archived materials listed above will be returned to the Sponsor or retained for a further period if agreed by a contract or destroyed on their behalf.
None of the above cited documents or material will be discarded without the explicit written consent of the Sponsor.
At the end of the study, any remaining test item will be discarded, unless otherwise instructed by the Sponsor.

5.0 Materials and methods

5.1 Test Item

5.1.1 Name and Data of Test Item

Test Item name	Wunderrein
Batch No.	Production from 10.10.2019
Appearance	white creamy substance (cold condition) liquid clean (heated condition)
Expiry date	29 October 2021 (24 months after opening)
Storage	room temperature

5.1.2 Identification, Receipt

The test item of a suitable chemical purity was supplied by the Sponsor. All precautions required in the handling and disposal of the test item were outlined by the Sponsor and archived with the raw data.

Identification of the test item was performed in the laboratory of Toxi-Coop ZRT. on the basis of the information provided by the Sponsor.

5.1.3 Formulation

The test item was applied in its original form, no formulation was required. However, the test item was preheated by an incubator before the needed amount of test item was measured and applied the following way:

Used preheating procedure for Check-method for possible direct MTT reduction with the test item and Check-method to detect the colouring potential of test item:

The incubator was heated to 37 °C before the plastic tube with test item was put in it. The plastic tube with the test item was in the incubator for approximately one hour and after that it was removed and approximately 50µL and 10 µL test item, respectively were measured form the preheated container to be used in the check tests.

Used preheating procedure for the treatment:

The incubator was heated to 37 °C before the plastic tube with the test item was put in it. The plastic tube with the test item was in the incubator for approximately one hour and after that it was removed and approximately 50µL test item was measured from the preheated container to each test item treated tissues.

5.2 Controls

Positive and negative controls were included parallel in the experiment.

5.2.1 Negative Control

NaCl (9 g/L saline):

Sodium chloride:
Supplier: lach:ner
Batch No.: PP/2018/05962
Retest date: 31 January 2021
Storage condition: Room temperature

Diluted with ultra-pure water (prepared by MILLIPORE Synergy UV HF ASTM 1: F8JA80461C water purification system) in Toxi-Coop ZRT.

5.2.2 Positive Control

Glacial acetic acid:

Supplier: MERCK
Batch No.: STBG9991
Retest date: April 2022
Storage condition: Room temperature

5.3 Additional materials

5.3.1 MTT stock solution

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1] was dissolved to a final concentration of 3 mg/mL in saline buffer (1x PBS). The obtained stock solution can be stored in refrigerator (2-8 °C), protected from light up to 15 days.

5.3.2 MTT ready to use solution

The MTT stock solution was diluted with pre-warmed “assay medium” to a final concentration of 0.3 mg/mL. The obtained solution was used within two hours; it was protected from light before used.

5.3.3 Acidified isopropanol

Isopropanol was diluted with HCl acid to a final concentration of 0.04N HCl. The obtained solution can be stored in refrigerator (2-8 °C), protected from light for one month.

5.3.4 Chemicals used in the experiment

The chemicals used in the experiment, are summarised in the following table:

Table 1: Chemicals used in the experiment

Chemical	Supplier/Manufacturer	Lot/Batch Number	Retest/Expiry date
MTT	SIGMA-ALDRICH	MKCG3023	September 2023
Isopropanol	SIGMA-ALDRICH	BCBZ5307	08 July 2020
HCl	CARLO ERBA	V3N459023N	December 2019
10x PBS*	SIGMA-ALDRICH	SLBX1610	March 2020

* 1x PBS is prepared by appropriate diluting with ultra-pure water (prepared by MILLIPORE Synergy UV HF ASTM 1: F8JA80461C water purification system) in Toxi-Coop ZRT.

5.4 Test System

5.4.1 Human Skin

EpiSkin™ Small Model (EpiSkin™SM), EPISKIN SNC Lyon, France, is a three-dimensional human epidermis model. Adult human-derived epidermal keratinocytes are seeded on a dermal substitute consisting of a collagen type I matrix coated with type IV collagen. A highly differentiated and stratified epidermis model is obtained after 13-day culture period comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum (Tinois *et al*., 1994). Its use for skin corrosion testing involves topical application of test materials to the surface of the epidermis, and the subsequent assessment of their effects on cell viability.

Supplier: EPISKIN Laboratories
4, rue Alexander Fleming, 69366 Lyon Cedex 07 - France
Batch No.: 19-EKIN-049
Expiry date: 09 December 2019

5.4.2 Justification for selection of the test system

The EPISKIN model has been validated for corrosivity testing in an international trial; it is considered to be suitable for this study (STATEMENT ON THE SCIENTIFIC VALIDITY OF THE EPISKIN™ TEST (AN *IN VITRO* TEST FOR SKIN CORROSIVITY); ECVAM JRC Environment Institute, European Commission; Ispra; 03 April 1998).

5.4.3 Demonstration of proficiency

The routine use of the method Toxi-Coop ZRT. demonstrated the technical proficiency in a separate study (study no.: 392-431-4224) using the twelve Proficiency Chemicals according to OECD Test Guideline No. 431.

5.4.4 Quality Control

EPISKIN-SM kits are manufactured according to defined quality assurance procedures (certified ISO 9001). All biological components of the epidermis and the kit culture medium have been tested for the presence of viruses, bacteria and mycoplasma. The quality of the final product is assessed by undertaking an MTT cell viability test and a cytotoxicity test with sodium dodecyl sulphate (SDS).

5.4.5 EpiSkin™SM KIT Contents

Units:	EpiSkin™SM plate containing up to 12 reconstructed epidermis units (area: 0.38 cm^2). Each reconstructed epidermis is attached to the base of a tissue culture vessel with an O-ring set and maintained on nutritive agar for transport.
Plate:	12-well assay plate
Punch:	EpiSkin™SM biopsy punch for easy sampling of epidermis
Medium:	A flask of sterile “Maintenance Medium” for incubations (Batch No.: 19-MAIN3-057; Exp. Date: 11 December 2019).
	A flask of sterile “Assay Medium” for test item application and for use in MTT assays (Batch No.: 19-ESSC-051; Exp. Date: 11 December 2019).

5.4.6 Number of replicate wells

In this assay 2 replicates per test item and 2 replicates of negative control, 2 replicates of the positive control were used. Furthermore, 2 killed test item treated tissues and 2 killed negative control treated tissues are used for the MTT evaluation.

5.4.7 EpiSkin™SM KIT Reception Procedure

The colour of the agar medium used for transport was checked for its pH:

- orange colour = good
- yellow or violet colour = not acceptable

The colour of the temperature indicator was inspected to verify that the kit has not been exposed to a temperature above 40 °C:

- the indicator changes from white to grey at 40 °C

The kit was found to be in good order at reception.

5.4.8 EpiSkin™SM KIT Storage

The EpiSkin™SM units were kept in their packaging at room temperature until the pre-incubation was started. The maintenance and assay medium were stored at 2-8 °C.

5.5 Indicator for potential false viability

Optical properties of the test item or its chemical action on MTT may interfere with the assay leading to a false estimate of viability. This may occur when the test item is not completely removed from the tissue by rinsing or when it penetrates the *epidermis*. If the test material acts directly on MTT (MTT-reducer), is naturally coloured, or becomes coloured during tissue treatment, additional controls should be used to detect and correct for test item interference with the viability measurement.

5.5.1 Check-method for possible direct MTT reduction with test item

The test item was preheated (see section 5.1.3) and after approximately 50 µl test item was added to 2 mL MTT 0.3 mg/mL solution and mixed. The mixture was incubated for three hours at 37±1 °C protected from light and then any colour change observed:

- Test items which do not interact with MTT: yellow
- Test items interacting with MTT: blue or purple

If the MTT solution colour becomes blue or purple, the test substance interacts with the MTT. It is then necessary to evaluate the part of optical density (OD) due to the non-specific reduction of the MTT (i.e. by using killed epidermis).

The test item showed direct interaction with MTT. Using of additional controls was necessary.

Additional controls for direct MTT interacting chemicals (MTT reducers):

In addition to the normal procedure, 2 killed test item treated tissues and 2 killed negative control treated tissues were used for the MTT evaluation in one run (untreated killed tissues may exhibit little residual NADH and dehydrogenase associated activity). The batch of killed tissues was different than the batches of the living tissues (batch no. of killed epidermis: 19-EKIN-040). The same treatment steps are followed for these tissues as for the living tissues.

Water-killed epidermis for MTT-interaction items:

- Place the living epidermis in a 12 well plate with 2 mL of distilled water (replacing the culture medium).
- Incubate at 37 °C, 5 % CO_2, ≥95 % humidified atmosphere for 48 hrs +/- 1 hour. At the end of the incubation, discard the water.
- Keep dead epidermis frozen (dry) in freezer at -18 °C to -20 °C (killed epidermis can be stored and used up to 6 months).
- Before use, the killed tissues are de-frozen at room temperature (app. 1 hour in 2 mL of maintenance medium).
- Further use of killed tissues is similar to living tissues.

Results of this check-test are detailed in section 10.2.

5.5.2 Check-method to detect the colouring potential of test item

Prior to treatment, the test item was evaluated for its intrinsic colour or ability to become coloured in contact with water (simulating a tissue humid environment).
The test item was preheated (see section 5.1.3) and after approximately 10 µL test item was added to 90 µL of water (prepared in Toxi-Coop ZRT. by MILLIPORE Synergy UV HF ASTM 1: F8JA80461C water purification system) and mixed. The mixture was shaken for 15 minutes at room temperature and then colour checked (unaided eye assessment).

Results of this check-test are detailed in section 10.2.

6.0 Description of the test procedure

6.1 Pre-incubation

The “maintenance medium” was pre-warmed to 37 °C. The appropriate number of assay plate wells were filled with the pre-warmed medium (2 mL per well). The epidermis units were placed with the media below them, in contact with the epidermis into each prepared well and then incubated overnight at 37±1 °C in an incubator with 5±1 % CO_2 in a ≥ 95 % humidified atmosphere.

6.2 Application

Before application the skin units were placed into pre-warmed “assay medium” (37 °C; 2 mL per well).

Two replicates were used for the test item and control(s) respectively.

Test Item

The test item was preheated (see section 5.1.3) before the treatment and after approximately 50 µL test item was applied evenly to the epidermal surface of the two test skin units respectively. The test item may be spread gently with the pipette tip in order to cover evenly all the epidermal surface if necessary.

Positive and negative control

A volume of 50 µL positive control (glacial acetic acid) or negative control (NaCl 9 g/L) was applied on the skin surface by using a suitable pipette. Chemicals were spread gently with the pipette tip in order to cover evenly the complete epidermal surface if necessary.

Additional controls for MTT direct interacting chemicals

In addition to the normal procedure, 2 killed test item treated tissues and 2 killed negative control treated tissues were used for the MTT evaluation in one run.

6.3 Exposure

The plates with the treated epidermis units were incubated for the exposure time of 4 hours (±10 min) at room temperature (23.2-24.4 °C).

6.4 Rinsing

After the incubation time the EpiSkin^{TM}SM units were rinsed thoroughly with approximately 25 mL 1x PBS solution to remove the test item from the epidermal surface. The rest of the PBS was removed from the epidermal surface with suitable pipette tip linked to a vacuum source (care was taken to avoid the damage of epidermis).

6.5 MTT test

To terminate the exposure with the test item, the EpiSkin^{TM}SM units were rinsed with PBS. Thereafter, the EpiSkin^{TM}SM units were transferred into the MTT solution filled wells (2 mL of 0.3 mg/mL MTT per well) and then incubated for 3 hours (± 15 min) at 37±1 °C in an incubator with 5±1 % CO_2 protected from light, ≥95 % humidified atmosphere.

6.6 Formazan extraction

At the end of incubation with MTT a formazan extraction was undertaken:

A disk of epidermis was cut from the unit (this involves the maximum area of the disk) using a biopsy punch (supplied as part of the kit). The epidermis was separated with the aid of forceps and both parts (epidermis and collagen matrix) were placed into a tube of 500 µL acidified isopropanol (one tube corresponding to one well of the tissue culture plate).
The capped tubes were thoroughly mixed by using a vortex mixer to achieve a good contact of all of the material with the acidified isopropanol then incubated overnight at room temperature protected from light for formazan extraction. At the end of the incubation period, each tube was additionally mixed using a vortex mixer to help extraction.

6.7 Cell viability measurements

Following the formazan extraction, 2×200 µL sample from each tube was placed into the wells of a 96-well plate (labelled appropriately) and read the OD (Absorbance / Optical Density) of the samples in a 96-well plate spectrophotometer (Thermo Scientific; Multiscan FC) at 570 nm (±10 nm; Read out range: 0-3.5 Abs, Linearity range: 0.2908 – 2.6589) using acidified isopropanol solution as the blank (6×200 µL).

7.0 Evaluation of Experimental Data

7.1 Calculations of viability percentages

The calculations were performed using Microsoft Excel software.

Blank:

– The mean of the 6 blank OD values was calculated.

Negative control:

– Individual negative control OD values were corrected with the mean blank OD:

OD Negative Control (OD_{NC1}) = OD_{NCraw1} – $OD_{blank\ mean}$

OD Negative Control (OD_{NC2}) = OD_{NCraw2} – $OD_{blank\ mean}$

Mean OD Negative Control (mean OD_{NC}) = [(OD_{NC1}) + (OD_{NC2})] / 2

– The corrected mean OD of the 2 negative control values was calculated: this corresponds to 100 % viability.

Positive control:

– Individual positive control OD values were corrected with the mean blank OD:

OD Positive Control (OD_{PC}) = OD_{PCraw} – $OD_{blank\ mean}$

– The corrected mean OD of the 2 positive control values was calculated.
– The % viability for each positive control replicate was calculated relative to the mean negative control:

% Positive Control 1 = (OD_{PC1} / mean OD_{NC}) ×100
% Positive Control 2 = (OD_{PC2} / mean OD_{NC}) ×100

– The mean value of the 2 individual viability % for positive control was calculated:

Mean PC % = (% PC1 + % PC2) / 2

Test item:

– Individual test item OD values were corrected with the mean blank OD:

OD Treated Tissue (OD_{TT}) = OD_{TTraw} – $OD_{blank\ mean}$

– The corrected mean OD of the 2 test item values was calculated
– The % viability for each test item replicate was calculated relative to the mean negative control:

% Treated Tissue 1 = (OD_{TT1} / mean OD_{NC}) ×100
% Treated Tissue 2 = (OD_{TT2} / mean OD_{NC}) ×100

– The mean value of the 2 individual viability % for test item was calculated:

Mean TT % = (% TT1 + % TT2) / 2

7.2 Data Calculation for MTT-interacting Items

Test items that interfere with MTT can produce non-specific reduction of the MTT. It is necessary to evaluate the OD due to non-specific reduction and to subtract it before calculations of viability %.

– Non-specific MTT reduction calculation (NSMTT):

Tissue 1 $NSMTT_1$ % = [(OD_{KT1} – mean OD_{KNC}) / mean OD_{NC}] × 100

Tissue 2 $NSMTT_2$ % = [(OD_{KT2} – mean OD_{KNC}) / mean OD_{NC}] × 100

Mean NSMTT% = ($NSMTT_1$ % + $NSMTT_2$ %) / 2

OD_{KNC}: negative control treated killed tissues OD (similar calculations as for mean OD_{NC})
OD_{KT}: test item treated killed tissues OD (mean)
OD_{NC}: negative control OD

If *NSMTT %* is > 50 % relative to the negative control: additional steps must be undertaken if possible, or the test item must be considered as incompatible with the test.

– True MTT metabolic conversion (TOD_{TT}) is undertaken if NSMTT is ≤ 50 %:

TOD_{TT} = [OD_{TT} – (mean OD_{KT} – mean OD_{KNC})]

OD_{TT}: test item treated viable tissues

– The % relative viability (% RV) for each test item replicate is calculated relative to the mean negative control:

% RV 1 = [TOD_{TT1} / mean OD_{NC}] × 100
% RV 2= [TOD_{TT2} / mean OD_{NC}] × 100

– The mean value of the 2 individual relative viability % for test item is calculated:

Mean Relative Viability % = (% RV 1 + % RV 2) / 2

7.3 Assay Acceptance Criteria

- The mean OD value of the two negative control tissues should be between 0.6 and 1.5.
- The acceptable percentage viability for positive control (each of two tissues) is 0 – 20 % (as per the manufactures specification, validated by ECVAM (Fentem *et al*, 1998)).
- In the range 20-100 % viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not exceed 30 %.

7.4 Interpretation of test results

The prediction model below corresponds to the methods agreed by EU regulatory agencies in line with INVITTOX Protocol No. 118; "EPISKINTM Skin Corrosivity Test" updated December 2011 / February 2012.

The cut-off value of 35 % and classification method was validated in an international validation of this kit (Fentem *et al*, 1998).

The interpretation of test results is summarized in the following table:

Table 2: The EPISKIN prediction model

Classification	Packing group / classification categories	Criteria for *In Vitro* interpretation
UN	Corrosive: Optional Sub- category 1A	Mean tissue viability is < 35 % after 3 min exposure
	Corrosive: Optional Sub- category 1B and 1C	Mean tissue viability is ≥ 35 % after 3 min exposure and < 35 % after 1 hour exposure **OR** Mean tissue viability is ≥ 35 % after 1 hour exposure and < 35 % after 4 hours exposure
	Non corrosive	Mean tissue viability is ≥ 35 % after 4 hours exposure

8.0 Deviations from the Study Plan

No. 1:

Concerning:	Date of Final Report
According to the Study Plan:	not later than 4 weeks after Draft Report revision and correction
Deviation:	The Final Report is available later than 4 weeks after Draft Report revision and correction
Reason:	Unplanned delay
Presumed effect on the study:	None

9.0 Amendment to the Study Plan

There was no amendment to the Study Plan.

10.0 Results

10.1 Validity of the Test

- The mean OD value of the two negative control tissues was 0.987
- The positive control result showed 5 % viability.
- The difference of viability between the two tissue replicates:
  - Negative control: 10.1 %
  - Positive control: 5.1 %
  - Test item: 3.8 %

All validity criteria were within acceptable limits and therefore the study can be considered as valid.

10.2 Indicator for potential false viability

Possible direct MTT reduction with test item:

During the check-method for possible direct MTT reduction, colour change was observed after three hours of incubation. The test item interacted with the MTT, therefore additional controls and data calculations were necessary.

The non-specific MTT reduction (NSMTT) was determined to be 4.741 %. As the NSMTT was below 50 % the true MTT metabolic conversion in all occasions and the correction of viability percentages were undertaken.

Colouring potential of test item:

The test item showed no ability to become coloured in contact with water. The intrinsic colour of test item is white and therefore considered not to be able to significantly stain the tissues and lead to a false estimate of viability. Furthermore, the test item was completely removed from the epidermal surface at rinsing period. Additional controls and data calculations were not necessary. A false estimation of viability can be precluded.

10.3 Cell viability

The results of the optical density (OD) measured at 570 nm of each replicate and the calculated % viability of the cells is presented below:

Table 3: OD values and cell viability percentages of the positive and negative control:

Controls	Optical Density (OD)		Viability (%)	Δ%
Negative Control: NaCl (9 g/L saline)	1 2	1.037 0.937	105 95	10.1
	Mean	**0.987**	**100**	
Positive Control: Glacial acetic acid	1 2	0.078 0.028	8 3	5.1
	Mean	**0.053**	**5**	

Table 4: OD values and viability percentages of the test item (including corrected values):

Test Item	Optical Density (OD)		TOD_{TT}	Viability (%)	Relative Viability (%)	Δ%
Wunderrein	1 2	0.941 0.903	0.894 0.857	95 92	91 87	3.8
	Mean	**0.922**	**0.875**	**93**	**89**	
	standard deviation (SD)			2.66	2.66	

Table 5: OD values of additional controls for MTT-interacting test item:

Additional controls	Optical Density (OD)	
Negative control treated killed tissues: NaCl (9 g/L saline)	1 2	0.057 0.055
	mean	**0.056**
Test item treated killed tissues: Wunderrein	1 2	0.097 0.108
	mean	**0.103**

Remark: **Δ %**: The difference of viability between the two relating tissues
***TOD*$_{TT}$: true MTT metabolic conversion**
Mean blank value was 0.0402

11.0 Discussion and Conclusion

Disks of EPISKIN (two units) were treated with the test item and incubated for 4 hours (±10 min) at room temperature. Exposure of the test material was terminated by rinsing with PBS 1x solution. The viability of each disk was assessed by incubating the tissues for 3 hours (±15 min) with MTT solution at 37±1 °C in an incubator with 5±1 % CO_2 in a ≥ 95 % humidified atmosphere and protected from light. The formazan precipitated was then extracted using acidified isopropanol and quantified spectrophotometrically.

NaCl (9 g/L saline) and glacial acetic acid treated epidermis were used as negative and positive controls, respectively.

The test item is a MTT-reducer, therefore additional controls (test item treated killed tissues and negative control treated killed tissues) were used to detect and correct for test substance interference with the viability measurement.

For each treated tissue viability was expressed as a % relative to negative control. The test item is considered to be non-corrosive to skin, if the mean relative viability after 4 hours of exposure is above or equal 35 % of the negative control.

The test item did not show significantly reduced cell viability in comparison to the negative control after 4 hours of exposure. The average test item treated tissue viability was 89 % (corrected values) at 4 hours of exposure. The test item treated tissue viability was above 35 % of the mean negative control value after 4 hours of exposure.

Positive and negative controls showed the expected cell viability values within acceptable limits.

All assay acceptance criteria were met, the experiment was considered to be valid.

The results obtained from this *in vitro* skin corrosion test, using the EPISKIN model (OECD 431), indicated that the test item reveals no skin corrosion potential under the utilised testing conditions. In conclusion, the test item Wunderrein can be classified as Non-corrosive to skin.

12.0 References

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6. *INVITTOX* Protocol No. 118; "EPISKIN™ Skin Corrosivity Test" updated December 2011 / February 2012 (ECVAM Database Service on Alternative Methods to Animal Experimentation).
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9. OECD (2015). OECD Guideline for Testing of Chemicals. No. 404: "Acute Dermal Irritation/Corrosion" adopted 28 July 2015.
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12. United nations (UN) (2017). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Seventh revised edition, UN New York and Geneva, 2017.

APPENDICES

APPENDIX I

COPY OF THE GLP CERTIFICATE OF Toxi-Coop ZRT.

OGYÉI
Országos Gyógyszerészeti
és Élelmezés-egészségügyi Intézet

1051 Budapest, Zrínyi u. 3.
Levélcím: 1372 Postafiók 450.
Tel.: (1) 8869-300, Fax: (1) 8869-460
E-mail: ogyei@ogyei.gov.hu, Web: www.ogyei.gov.hu

Ref. no: OGYÉI/8623-5/2019
Admin.: dr. Szaller Zoltán
Date: 22 May, 2019

GOOD LABORATORY PRACTICE (GLP)
CERTIFICATE

It is hereby certified that the test facility

TOXI-COOP Toxicolgical Research Center Zrt.

H-1103 Budapest, Cserkesz u. 90.,
H-1045 Budapest, Berlini u. 47-49.,
H-8230 Balatonfüred, Arácsi u. 97-99.,
H-8230 Balatonfüred, Vasút u. 3.,
H-8230 Balatonfüred, Galamb u. 12/A ,
H-8230 Balatonfüred, Ady E. u. 12,
8354 Karmacs, hrsz 4150/2

is able to carry out

physico-chemical testing, toxicity studies, mutagenicity studies, environmental toxicity studies on aquatic and terrestrial organisms, studies on behaviour in water, soil and air; bio-accumulation studies, analytical and clinical chemistry, safety pharmacology testing, metabolism and toxico/pharmacokinetics testing, testing of toxicological properties of operative procedures and equipment, reproduction toxicological studies, tolerance studies, inhalation toxicology and in vitro studies

in compliance with the Principles of GLP (Good Laboratory Practice) and also complies with the corresponding OECD/European Community requirements.

Date of the inspection: **18-26. February 2019.**

dr. Mittner András
Digitálisan aláírta: dr. Mittner András
Dátum: 2019.05.23 09:01:24 +02'00'

Dr. András Mittner
Head of Inspectorate

APPENDIX II

COPY OF THE TEST SYSTEM QUALITY CONTROL

EPISKIN

TECHNICAL DATA, SAFETY SHEET AND CERTIFICATE OF ANALYSIS

CCE-091-SM D13-5/04

NAME

EpiSkin™ Small / Human Epidermis (SM/13)

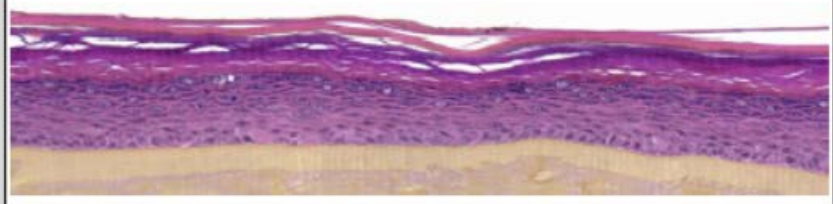
DESCRIPTION

0.38 cm² reconstructed epidermis of normal human keratinocytes. Cells are grown on a collagen matrix, for 13 days

BATCH : **19-EKIN-049**
ORIGIN : Adult donors
USAGE : FOR SCIENTIFIC USE ONLY - PRODUCT OF HUMAN ORIGIN
STORAGE : This product was prepared and packaged using aseptic techniques. Store in an incubator at 37°C, 5% CO2 with saturated humidity

QUALITY CONTROLS

Control # E191892

	Process	Specification	Result
HISTOLOGY	HES stained paraffin section	Multi-layered, highly differentiated epidermis consisting of organized basal, spinous and granular layers, and a multilayered *stratum corneum*	Satisfactory
		Number of cell layers ≥ 4	9 cell layers
			
IC50 DETERMINATION	SDS concentration, MTT test.	1.5 mg/mL ≤ IC50 ≤ 3.0 mg/mL	2.9 mg/mL

BIOLOGICAL SAFETY:

On blood of the donors, we have verified the absence of HIV1 and 2 antibodies, hepatitis C antibodies and hepatitis B antigen HBs.
On cells from the donors, we have verified the absence of bacteria, fungus and mycoplasma.

SUGGESTED EXPIRATION DATE:

December 9, 2019

Lyon, December 3, 2019
Certified and released by **Anaïs JENSEN**, Quality Control Manager

Manufactured in accordance to the ISO9001 quality system of Episkin.

The use of this human tissue is strictly limited to *in vitro* testing. All other manipulations of this tissue such as: extraction and maintenance of single cells in culture, use of the tissue for diagnostic or therapeutic purposes and in human subjects, are strictly prohibited.

ISO 9001 Certified
4, rue Alexander Fleming - 69366 Lyon Cedex 07 - France - Tél : +33 (0)4 37 28 72 00 - Fax : +33 (0)4 37 28 72 28
S.A. au capital de 13 608 807 € - 412 127 565 R.C.S. Lyon - NAF : 7211 Z - N° TVA Intracommunautaire FR 46 412 127 565
www.episkin.com

EPISKIN

TECHNICAL DATA, SAFETY SHEET AND CERTIFICATE OF ANALYSIS

CCE-091-SM D13-S/04

NAME

EpiSkin™ Small / Human Epidermis (SM/13)

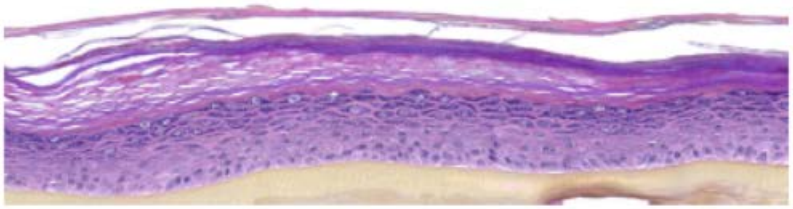
DESCRIPTION

0.38 cm² reconstructed epidermis of normal human keratinocytes. Cells are grown on a collagen matrix, for 13 days

BATCH : **19-EKIN-040**
ORIGIN : Adult donors
USAGE : FOR SCIENTIFIC USE ONLY - PRODUCT OF HUMAN ORIGIN
STORAGE : This product was prepared and packaged using aseptic techniques. Store in an incubator at 37°C, 5% CO2 with saturated humidity

QUALITY CONTROLS

Control # E191524

	Process	Specification	Result
HISTOLOGY	HES stained paraffin section	Multi-layered, highly differentiated epidermis consisting of organized basal, spinous and granular layers, and a multilayered *stratum corneum*	**Satisfactory**
		Number of cell layers ≥ 4	**8 cell layers**
			
IC50 DETERMINATION	SDS concentration, MTT test.	1.5 mg/mL ≤ IC50 ≤ 3.0 mg/mL	**2.3 mg/mL**

BIOLOGICAL SAFETY:

On blood of the donors, we have verified the absence of HIV1 and 2 antibodies, hepatitis C antibodies and hepatitis B antigen HBs.
On cells from the donors, we have verified the absence of bacteria, fungus and mycoplasma.

SUGGESTED EXPIRATION DATE:

October 7, 2019

Lyon, October 1, 2019
Certified and released by Michel BATAILLON, Quality Control Manager

Manufactured in accordance to the ISO9001 quality system of Episkin.

The use of this human tissue is strictly limited to *in vitro* testing. All other manipulations of this tissue such as: extraction and maintenance of single cells in culture, use of the tissue for diagnostic or therapeutic purposes and in human subjects, are strictly prohibited.

ISO 9001 Certified

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S.A. au capital de 13 608 807 € - 412 127 565 R.C.S. Lyon - NAF : 7211 Z - N° TVA Intracommunautaire FR 46 412 127 565
www.episkin.com

APPENDIX III

HISTORICAL CONTROL DATA

Table 6: Historical Control Data

Historical Control Data (Period of 2013-2019 December)			
	Negative Control data NaCl (9g/L saline)	**Positive Control data** Glacial acetic acid	
	Optical Density (OD)	Optical Density (OD)	% viability
Mean	0.999	0.024	3
Minimum	0.571	0.002	0
Maximum	1.614	0.084	10