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FINAL REPORT

SKIN CORROSIVITY
IN VITRO WITH
Ethylene Dibromide Industrial (EDB)
IN THE EPISKIN MODEL

Date of Final Report: 25 October 2010

STUDY CODE: 10/112-039B

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, 13 April 2004), Commission Regulation (EC) No.440/2008, Annex Part B, B.40Bis and the Principles of Good Laboratory Practice Regulations as specified by national Hungarian GLP Regulations (9/2001.(III.30.) EüM-FVM joint decree of the Minister of Health and the Minister of Agriculture and Regional Development) which corresponds to the OECD GLP, ENV/MC/CHEM(98)17.

I the undersigned declare that this report constitutes a true record of the actions undertaken and the results obtained in the course of this study. By virtue of my dated signature I accept the responsibility for the validity of the data and the following conclusion drawn from them:

In this in vitro EPISKIN model test with Ethylene Dibromide Industrial (EDB), the results indicate that the test item is not corrosive to the skin.

Signature:_____ Date: 25 October 2010

István Ágh, MSc.
Study Director

STATEMENT OF THE MANAGEMENT

According to the conditions of the research and development agreement between CHEMTURA CORPORATION (as Sponsor) and LAB Research Ltd. (as Testing Facility) Acute Skin Corrosivity In Vitro with Ethylene Dibromide Industrial (EDB) in the EPISKIN Model" has been performed in compliance with the study plan and the Principles of Good Laboratory Practice.

Signature: _____ Date: 25 Oct. 2010

Christopher Banks, DABT.
Managing Director

QUALITY ASSURANCE STATEMENT

Study Code: 10/112-039B

Study Title: Skin Corrosivity In Vitro with Ethylene Dibromide Industrial (EDB) in the EPISKIN Model

Test Item: Ethylene Dibromide Industrial (EDB)

This study has been inspected, and this report audited by the Quality Assurance Unit 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 written form to the study director and to management. The dates of such inspections and of the report audit are given below:

Date of Inspection	Phase(s) Inspected/Audited	Date of report to	
		Management	Study Director
31 May 2010	Study Plan	31 May 2010	31 May 2010
08 September 2010	Treatment	08 September 2010	08 September 2010
23 September 2010	Draft Report	23 September 2010	23 September 2010
25 October 2010	Final Report	25 October 2010	25 October 2010

Signature: Fábián Makovi Éva Date: 25 October 2010

Éva Makovi-Fábián B.Sc.
On behalf of QAU

STUDY TITLE	:	Skin Corrosivity In Vitro with Ethylene Dibromide Industrial (EDB) in the EPISKIN Model
TEST ITEM	:	Ethylene Dibromide Industrial (EDB)
SPONSOR	:	CHEMTURA CORPORATION Address: 199, Benson Road, Middlebury, Connecticut 06749 USA
TEST FACILITY	:	LAB Research Ltd. Address: H-8200 Veszprém, Szabadságpuszta Hungary Phone: 36 88 545-300 Fax: 36 88 545-301
STUDY DIRECTOR	:	István Ágh, M.Sc.
QUALITY ASSURANCE	:	Éva Makovi-Fábián B.Sc.
TECHNICAL ASSISTANT	:	Krisztina Pátkai-Fejes
START OF EXPERIMENT	:	08 September 2010
END OF EXPERIMENT	:	08 September 2010
BASIS OF STUDY	:	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. OECD Guidelines for Testing of Chemicals, Section 4, No. 431, "In Vitro Skin Corrosion: Human Skin Model" adopted 13 April 2004.

CONTENTS

1. SUMMARY

Disks of EPISKIN were treated with positive control, negative control or test item and incubated for 4 hours. After rinsing, viability of each disk was assessed by use of an MTT method. Viability below 35% of the negative control is considered to indicate a corrosive effect.

The positive control result showed zero viability. The optical density (OD) of the extract dye from the negative control tissues were more than 20 fold greater than the OD of the extraction solvent alone and in the acceptable range of 0.115 – 0.4 based on historical data [6.].

The test item did not show significantly reduced cell viability in comparison to the negative control. All test item results were far above 35% of the mean negative control value.

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

In conclusion, in this in vitro EPISKIN model test with Ethylene Dibromide Industrial (EDB), the results indicate that the test item is not corrosive to the skin.

2. 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 number 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. MATERIALS AND METHODS

3.1. TEST ITEM

3.1.1. Name and Data of the Test Item

Name:	Ethylene Dibromide Industrial (EDB)
Chemical name:	1,2-Dibromoethane
Batch No.:	510100003
Active component:	>99.94 % 1,2-Dibromoethane (CAS 106-93-4)
Description:	clear to amber liquid
Manufacture date:	February 2010
Expiry date:	February 2011
Storage:	room temperature; 15-25°C (humidity 50 % ± 20); protect from light
Safety Precautions:	see Safety Data Sheet
Manufacturer:	Chemtura Manufacturing UK Limited
Address:	Tenax Road, Trafford Park Manchester United Kingdom M17 1WT

3.1.2. 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. These documents are part of the raw data. Identification of the test item was performed in the Central Dispensary of LAB Research Ltd. on the basis of the information provided by the Sponsor (name, batch number, appearance and colour).

3.1.3. Preparation of the Test Item

The test item was applied in its original form, no formulation was required.

3.2. CONTROLS

Positive and negative controls were included in the experiment.

3.2.1. Negative Control

NaCI (9 g/l saline):

Supplier:	HUNGAROPHARMA ZRT.
Batch No.:	3390210
Expiry date:	February 2013

3.2.2. Positive Control

Glacial acetic acid:

Supplier:	MERCK
Batch No.:	K37658156
Expiry date:	31 July 2012

3.3. TEST SYSTEM

3.3.1. Human Skin

The EPISKIN Standard Model™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum.

Each reconstructed epidermis unit consists of a plastic well and a human collagen (Types III and I) matrix, representing the dermis, covered with a film of Type IV human collagen, upon which stratified differentiated epidermis derived from human keratinocytes has been laid.

Supplier : Skinethic, Nice, France.
Batch No. : 10-EKIN-030
Expiry date : 13 September 2010

3.3.2. 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 dodecylsulphate (SDS).

The release form certifying the conformity of the batch was sent by the supplier (see Appendix 2).

3.3.3. 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.

3.3.4. 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 “Assay Basic Medium” for use in assays was provided as part of the kit.

3.3.5. Number of Replicate Wells

In this assay 3 replicates per test item and 3 negative controls + 3 positive controls were used.

3.3.6. Kit Reception

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 the start of the experiment.

3.3.7. Storage

The EPISKIN-SM kit was stored at room temperature and the assay medium supplied with the kit was stored at 2-8°C until the initiation of the test.

3.4. ADDITIONAL MATERIALS

3.4.1. MTT solution

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1] was dissolved in saline buffer (PBS) to a final concentration of 3 mg/mL. The obtained stock solution was diluted with pre-warmed (37°C) assay medium to a final concentration of 0.3 mg/mL and used within one hour.

3.4.2. Acidified isopropanol

Isopropanol was diluted with HCl acid to a final concentration of 0.04N HCl.

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

Chemical	Supplier	Batch Number	Expiry date
MTT	SIGMA-ALDRICH	MKBB9557	October 2014
Isopropanol	REANAL	KBM55858	November 2010
HCl acid	REANAL	KBM59614	August 2012
PBS	SIGMA-ALDRICH	BCBB8247	July 2014

3.4.3. Equipments

Normal laboratory equipment and the following were necessary for determination of the parameters of the test:

- Wash bottle
- 1 mL tubes with caps
- 200 µL, 1 mL and 5 mL micropipette
- Vacuum source and Pasteur pipettes
- Small forceps
- Timers
- Microplate reader with filter of 540 nm and 96-well microplates
- Vortex mixer
- Orbital shaker
- Laboratory centrifuge

3.5. PERFORMANCE OF THE STUDY

3.5.1. Application

The Assay medium was pre-warmed to 37°C. The appropriate number of an assay plate wells were filled with the pre-warmed medium (2.2 mL per well). The epidermis units were placed with the media below them, in contact with the epidermis into each prepared well. Three skin units were used for each test or control material.

- 50 µl of test item was added to each of the three test skin units
- 50 µl NaCI (9 g/l saline) was added to each of the three negative control skin units
- 50 µl glacial acetic acid was added to each of the three positive control skin units

The plates with the treated epidermis units were incubated for the exposure time of 4 hours at room temperature (18-28°C) covered with the plate lids.

3.5.2. Rinsing

After the incubation time (4 hours), each skin unit was removed and rinsed thoroughly with PBS 1x solution (0.9 %) to remove all of the test material from the epidermal surface. The rest of the PBS was removed from the epidermal surface using a Pasteur pipette linked to a vacuum source (without touching the epidermis) and the culture medium was removed from the assay plate wells.

MTT solution (2.2 mL of 0.3 mg/mL MTT) was added to each well below the skin units. The lid was replaced and the plate incubated at room temperature (20-28°C) for 3 hours, protected from light.

3.5.3. Indicator for potential false viability

Approximately 10 µl of test item was added to 2.2 mL MTT and any colour change was observed: the information from this test can help to avoid a false estimation of viability.

3.5.4. 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 0.85 mL acidified isopropanol (One tube corresponding to one well of the tissue culture plate).
The capped tubes were thoroughly mixed by using a vortex mixer and stored at room temperature overnight, protected from light. It was ensured that the acidified isopropanol was in good contact with all of the material.

3.5.5. Cell viability measurements

Following the formazan extraction each tube was centrifuged for 10 minutes at 3000 rpm (so that any cell fragments do not interfere with the absorbance readings). After the solution settled by centrifugation, 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 at a wavelength of 540 nm using acidified isopropanol solution as the blank.

Note: The validity of the microplate reader was verified with a standard verification plate daily before use. The standard plate was calibrated yearly by the manufacturer.

3.6. CALCULATIONS OF VIABILITY PERCENTAGES

Viability (%) = 100 x (OD test material/mean OD negative control at 4 hours)

The mean OD of the 3 negative control values was calculated: this corresponds to 100% viability.

The mean OD of the 3 positive control values was calculated: the % viability of the positive control is calculated relative to the mean negative control.

The % viability of each individual test well was calculated following exposure to the test material as the OD expressed as a percentage of the mean negative control value.

Note: the measured OD values were corrected with the mean OD of the plate wells and the mean OD of the blank solution.

3.7. VALIDITY OF THE TEST

The optical density of the extract dye from the negative control tissue should be at least 20 fold greater than the OD of the extraction solvent alone.
The acceptable mean percentage viability range for positive controls is 0-20% (as per the manufactures specification, validated by ECVAM (Fentem, 1998)).

3.8. INTERPRETATION OF TEST RESULTS

The prediction model below corresponds to the methods agreed by EU regulatory agencies in line with OECD 431 (OECD, 2004).
The interpretation model is for 2 disks; in this assay 3 disks are used so the wells with the two closest results are used in this model. The same calculation applies to each incubation period.
The cut-off value of 35% and classification method was validated in an international validation of this kit (Fentem, 1998).

For 2 disks:

If both disks have viability of >35% = Non Corrosive
If both disks have viability of <35% = Corrosive (at the corresponding incubation period)

Otherwise:
If the mean value is >35% and the variability is less than 50% = Non Corrosive
If the mean value is <35% and the variability is less than 50% = Corrosive

Otherwise:
If the classification is not made with these criteria, retest with 2 more disks. Take the mean of the 4 disks to classify as above or below 35%. Outlier values may be excluded where there are scientific grounds, such as where application or rinsing is difficult and that the Study Director considers that a result is not representative.

Variability for 2 disks is calculated by :-
(Disk1-Disk2)/((Disk1+Disk2)/2) x 100%

Classification	**Packing group**	**Criteria for *In Vitro* interpretation**
UN	Corrosive class I	If corrosive after 3 min exposure
	Corrosive class II	If not corrosive after 3 min exposure and corrosive after 1 hour exposure
	Corrosive class III	If not corrosive after 1 hour exposure and corrosive after 4 hours exposure
	Non corrosive	If not corrosive after 4 hours exposure
EU	Corrosive class R35	If corrosive after 3 min exposure
	Corrosive class R34	If not corrosive after 3 min exposure and corrosive after 4 hours exposure
	Non corrosive	If not corrosive after 4 hours exposure

3.9 ARCHIVES

The study documents and samples:

- study plan and amendment,
- all raw data,
- retained sample of the test item,
- correspondence,
- study report and any amendments

are stored in the archives of LAB Research Ltd., H-8200 Veszprém, Szabadságpuszta, Hungary according to the Hungarian GLP regulation and to test facility SOPs.
After the retention time has elapsed all the archived materials listed above would be offered to the Sponsor or retained for a further period if agreed by a contract.
No raw data or material relating to the study will be discarded without the sponsor's prior consent.

3.10. DEVIATION FROM THE STUDY PLAN

Concerning:	Storage of Test Item
According to the Study Plan:	room temperature
Deviation:	room temperature; 15-25°C (humidity 50 % ± 20); protect from light
Reason for this change:	Typing error
Presumed Effect on the Study:	None
Concerning:	Technical assistants
According to the Study Plan:	Irén Lisztes-Fekete, Tamás Buda
Deviation:	Krisztina Pátkai-Fejes
Reason for this change:	Organization changes
Presumed Effect on the Study:	None

Concerning:	MTT stock solution
According to the Study Plan:	MTT will be diluted in saline buffer (PBS) by a mechanical dispersion of 15 minutes using Vortex mixer.
Deviation:	MTT was dispersed until all the amount was dissolved.
Reason for this change:	technical
Presumed Effect on the Study:	None

3.11. DISTRIBUTION OF THE FINAL REPORT

Sponsor: 1x copy, bound
1x copy, unbound
1x PDF file

Archive: 1x original, bound

3.12. REFERENCES

1. OECD Guidelines for Testing of Chemicals Section 4: Health Effects Acute Dermal Toxicity (No.: 431; 13 April 2004) Organisation for Economic Co-Operation and Development, Paris.
2. OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.
3. Hungarian Good Laboratory Practice Regulations: 9/2001 (III. 30) EÜM-FVM joint decree of the Minister of Health and the Minister of the Agriculture and Regional Development which corresponds to the OECD GLP 1997.
4. Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).
5. Anon. (1991) Code of Federal Regulation. Method of Testing Corrosion to the Skin. Transportation Title 49, Part 173.136, Appendix A., Office of the Federal Register, National Archives and Records Administration, Washington D.C., USA.
6. Fentem J.H., Archer G.E.B., Balls M., Botham P.A., Curren R.D., Earl L.K., EsdaileD.J., Holzhütter H.G. and Liebsch M. (1998). The ECVAM international validation study on in vitro tests for skin corrosivity. II. Results and evaluation by the Management Team. Toxicology in Vitro 12, 483-524 Pergamon Press/ Elsevier, Oxford, UK
7. 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.

4. RESULTS

4.1. VALIDITY OF THE TEST

The positive control result showed zero viability. The optical density (OD) of the extract dye from the negative control tissues were more than 20 fold greater than the OD of the extraction solvent alone and in the acceptable range of 0.115 – 0.4 based on historical data [6.]. All validity criteria were within acceptable limits and therefore the study can be considered as valid.

4.2. CELL VIABILITY

The results of the optical density (OD) measured at 540 nm of each extract and the calculated % viability of the cells is presented below:

Substance	**Optical Density (OD)**		**Viability (%)**
Test Item: Ethylene dibromide Industrial (EDB)	**1**	0.121	61
	2	0.164	83
	3	0.157	79
	mean	**0.148**	**74**
Positive Control: Glacial acetic acid	**1**	0.001	
	2	0.003	
	3	0.007	**2**
	mean	**0.004**	
Negative Control: NaCl (9 g/L saline)	**1**	0.178	
	2	0.151	
	3	0.265	**100**
	mean	**0.198**	

Mean Negative Control OD = 0.198 units
Mean Positive Control OD = 0.004 units
Mean Blank OD = 0.001 units

All Test Item results were above 35% of the mean negative control value.

The test to verify if the test item was capable of inducing the colour reaction gave negative result; no colour change was detected and therefore a false estimation of viability can be precluded.

5. DISCUSSION AND CONCLUSION

Disks of EPISKIN were treated with positive control, negative control or test item and incubated for 4 hours. After rinsing, viability of each disk was assessed by use of an MTT method. Viability below 35% of the negative control is considered to indicate a corrosive effect.

The test item did not show significantly reduced cell viability in comparison to the negative control. All test item results were far above 35% of the mean negative control value.

The positive and negative control results were found to meet the acceptability criteria.

In conclusion, in this in vitro EPISKIN model test with Ethylene Dibromide Industrial (EDB), the results indicate that the test item is not corrosive to the skin.

A P P E N D I C E S

APPENDIX 1

COPY OF THE GLP-CERTIFICATE

ORSZÁGOS GYÓGYSZERÉSZETI INTÉZET
National Institute of Pharmacy

H-1051 Budapest, Zrinyi u. 3.
Mail: 1372 P.O. Box 450.
Phone: +36 1 8869-300
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E-mail: ogyi@ogyi.hu

Budapest, 20th December 2008
No: 38625/48/2007
Our ref.: Szilvia Karsai
Subject: GLP Certificate

GOOD LABORATORY PRACTICE (GLP) CERTIFICATE

Based on the Inspection report and the discussion of follow up activities it is hereby certified that the test facility

LAB Research Ltd.
H-8201 Veszprém, Szabadságpuszta, Hungary

is able to carry out **Physical-chemical testing, Toxicity studies, Mutagenicity studies, Environmental toxicity studies on aquatic and terrestrial organisms, Studies on behaviour in water, soil and air; bioaccumulation, Bioanalytical, Analytical and clinical chemistry testing** compliance with the Principles of GLP (Good Laboratory Practice).

Date of the inspection: **13-22 October 2008.**

This GLP Certificate is valid for 2 years.

Zsuzsanna Szepezdi, Ph. D.
Director-General

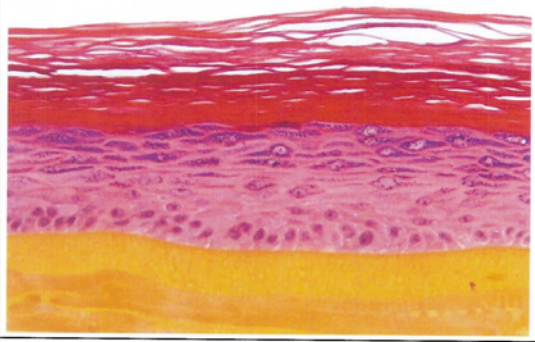
APPENDIX 2

COPY OF THE TEST KIT QUALITY CONTROL

SkinEthic laboratories

TECHNICAL DATA, SAFETY SHEET AND CERTIFICATE OF ANALYSIS
RECONSTRUCTED HUMAN EPIDERMIS

CCE-037/11

Description:	**Episkin Small Model** 0.38 cm² reconstructed epidermis of normal human keratinocytes. Cells are grown on a collagen matrix, for 13 days.
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% CO_2 with saturated humidity.
Passage:	Second (Strains n° : 01-KERA-008, 01-KERA-011, 08-KERA-003, 08-KERA-004)
Batch N°:	**10-EKIN-030**
Origin:	Adult donors.
Histology:	 **Control n° E10444**
Quality Controls:	**. Histology scoring** (HES stained vertical paraffin sections, n = 6) : specification ≥ 19.5, **result = 21.3 ± 0.5 , CV = 2.5 %** Well-differentiated epidermis consisting of a basal layer, several spinous and granular layers and a thick stratum corneum **. IC 50 determination** (SDS concentration, MTT test, n = 14) : specification ≥ 1,5 mg/ml, **result = 2.3 mg/ml** **. Statistical Analysis :** → Histology : probability 0.95 that 95 % of the batch > 20 → IC 50 : probability 0.95 that IC 50 ≥ 1.8 mg/ml (threshold value)
Biological safety:	On blood of the same donors, we have verified: . the absence of HIV1 and 2 antibodies (Abbott + Centaur Bayer) . the absence of hepatitis C antibodies (Centaur Bayer) . the absence of hepatitis B antigen HBs (Centaur Bayer) On epidermal cells of the same donors, we have verified: . the absence of bacteria, fungus and mycoplasma
Expiration date	September 13, 2010.

Lyon, September 7, 2010.
Certified and released by
Carole Amsellem, PhD, Quality Control Manager

Manufactured in accordance to the ISO9001 quality system of Episkin.

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