U.S. ARMY PUBLIC HEALTH CENTER

8252 Blackhawk Road, Aberdeen Proving Ground, Maryland 21010-5403

Toxicity Report No. S.0055513-18, February 2018-September 2018
Toxicology Directorate

***In Vitro* Dermal Irritation Proficiency Demonstration, February 2018-September 2018**

Prepared by Emily May Lent

Toxicology Directorate
Toxicity Evaluation Division
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ARIMS designation: 500C Toxicity Test

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In Vitro Dermal Irritation Proficiency Demonstration, February 2018 - September 2018

Data Requirement

OECD Guidelines for the Testing of Chemicals, Section 4, Test No. 439: *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method

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Study Completed On

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Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. Manufacturer reported purity of the neat compounds was not verified analytically. This is not considered to have affected the outcome of the study as certificates of analyses were provided with each compound.

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PROTOCOL NO. 68-IV18-02-01
***IN VITRO* DERMAL IRRITATION PROFICIENCY DEMONSTRATION**
FEBRUARY 2018-SEPTEMBER 2018

1 Summary

1.1 Purpose

The *in vitro* dermal irritation test assesses the potential for chemicals to produce reversible damage to the skin when applied for up to 4 hours. This method uses tissue damage (measured by assessing viability), an initiating event in the skin irritation cascade, in reconstructed human epidermis (RhE) to predict chemical-induced skin irritation. The following study was conducted to demonstrate technical proficiency using one of the four validated test methods, EpiDerm™ Skin Irritation Test (EPI-SIT-200, MatTek, Ashland, Massachusetts).

1.2 Conclusions

The proficiency substances were tested using the EPI-SIT-200 assay as a demonstration of technical proficiency of the laboratory prior to routine use of this test method. All assay acceptance criteria were met and 100% of the proficiency substances were correctly classified, therefore the performing lab is technically proficient and EPI-SIT-200 may be routinely used for *in vitro* prediction of skin irritation.

2 References

See Appendix A for a listing of references.

3 Authority

This study was sponsored by the U.S. Army Medical Command, Office of the Surgeon General and identified as WBS element S.0055513.

4 Background

Chemical-induced skin irritation has historically been assessed in laboratory animals by qualitative evaluation of erythema and edema. The *in vitro* test system utilizes cell and tissue damage, the initial step in the *in vivo* inflammatory cascade that occurs during skin irritation. *In vivo*, damaged cells release inflammatory mediators which act on the endothelial cells of the blood vessels to produce dilation and increased permeability and the observed erythema and edema. The RhE-based test methods lack vascularization, therefore the initial event in the cascade, cell / tissue damage [1, 2], is measured using cell viability. Cell viability in RhE models is measured by dehydrogenase conversion of MTT [(3-4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue], into a blue formazan salt that is quantitatively measured after extraction from tissues. Reduction in the viability of tissues exposed to chemicals in comparison with negative controls is used to predict skin irritation potential. The RhE model tests allow discrimination between irritants of United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) category 2 and non-irritants. The tests do not discriminate between non-mandatory subcategories of the UN GHS (i.e., do not distinguish between GHS category 2 and category 3 irritants).

Prior to routine use of any of the four validated test methods specified in the OECD guideline, technical proficiency must be demonstrated using the ten Proficiency Substances listed in the OECD guideline [3]. The present study was conducted as a technical proficiency demonstration.

5 Materials and Methods

5.1 Materials

5.1.1 Test Substances

The ten proficiency substances listed in the OECD Guideline (Table 1) were purchased from Sigma Aldrich (St. Louis, MO).

Table 1. Proficiency Chemicals.

Substance	CAS No.	Lot No.	Purity	Physical state	UN GHS Category
NON-CLASSIFIED SUBSTANCES (UN GHS No Category)					
naphthalene acetic acid	86-87-3	SLBT1922	99	Solid	No Cat.
isopropanol	67-63-0	SHBJ4945	100.0	Liquid	No Cat.
methyl stearate	112-61-8	BCBV9185	99	Solid	No Cat.
heptyl butyrate	5870-93-9	MKBP3552V	99.8	Liquid	No Cat. (Opt Cat. 3)
hexyl salicylate	6259-76-3	BCBN6342V	≥99.0	Liquid	No Cat. (Opt Cat. 3)
CLASSIFIED SUBSTANCES (UN GHS Category 2)					
cyclamen aldehyde	103-95-7	MKCB4641	97.6	Liquid	Cat. 2
1-bromohexane	111-25-1			Liquid	Cat. 2
potassium hydroxide (45%)	1310-58-3	MKCC7148	45.6	Liquid	Cat. 2
1-methyl-3-phenyl-1-piperazine	5271-27-2	MKBV3605V	99.0	Solid	Cat. 2
heptanol	111-71-7	S7448918741		Liquid	Cat. 2

5.1.2 Test System, Controls, and Reagents

The reconstructed human epidermal model EpiDerm™ was acquired from MatTek (EPI-200, MatTek, Ashland). The EpiDerm™ tissues are shipped as kits, containing 24 tissues on shipping agarose together with the necessary amount of culture media – Dulbeco's Modified Eagle's Medium (DMEM) based, Dulbeco's Phosphate Buffered Saline (DPBS), positive control (5% sodium dodecyl sulphate (SDS) solution), 6-well plates, and 24-well plates. In addition the MTT kit (containing MTT concentrate, diluent, and extractant) was also purchased from MatTek. Additional DPBS without calcium, magnesium, or phenol red was purchased from Gibco, Inc. (a subsidiary of ThermoFisher, Waltham, MA). All test systems, reagents and chemicals were stored according to the manufacturer's instructions.

5.2 Quality Assurance

5.2.1 Quality Control of Test System

The EpiDerm™ System is manufactured according to defined quality assurance procedures. All biological components of the epidermis and the culture medium are tested by the manufacturer for viral, bacterial, fungal, and mycoplasma contamination. MatTek determines the effective time for 50% viability (ET-50 value) following exposure to Triton X-100 (1%) for each EpiDerm™ lot. The ET-50 must fall within the range of the EpiDerm historical database of 4.77 – 8.72 hours. If tissue lots fail quality control (QC) or sterility testing, the manufacturer notifies the customer. All of the tissue lots used in this proficiency demonstration passed QC and sterility testing.

5.2.2 Assay Acceptance Criteria

The absolute optical density (OD) of the negative control (NC) tissues (treated with sterile DPBS) in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures and under specific conditions of use. The assay meets the acceptance criterion if the mean OD at 570 nanometers (OD_{570}) of the NC tissues is ≥ 1.0 and ≤ 2.8.

A 5% SDS (in H_2O) solution is used as a positive control (PC) and tested concurrently with the test chemicals. Viability of the positive control should be within 95±1 % confidence interval of the historical data. The assay meets the acceptance criterion if the mean viability of PC tissues expressed as % of the negative control tissues is ≤ 20%.

Since in each test, skin irritancy potential is predicted from the mean viability determined on 3 single tissues, the variability of tissue replicates should be acceptably low. The assay meets the acceptance criterion if the SD calculated from individual % tissue viabilities of the 3 identically treated replicates is < 18%. Chemicals that provide tissue viabilities in a range of 30 – 70 % may provide high SD. If the high SD (above acceptance limits) is typical for the chemical and the classification of the chemical is consistent in all independent runs, it is recommended that this result be accepted [4].

5.2.3 Quality Compliance

The APHC Quality Systems and Regulatory Compliance Office audited critical study phases. Appendix B provides the dates of these audits, the phases audited, and the dates the results were reported to the Study Director (SD) and Management.

5.3 Study Personnel

Appendix C lists the names of individuals contributing to the study performance.

5.4 Methods

All tests were conducted according to the manufacturer's instructions.

5.4.1 Test for Interference with MTT Endpoint

Because test substances may interfere with the MTT endpoint if they are colored and/or able to directly reduce MTT, tests for interference of chemicals with the MTT endpoint were conducted.

Some non-colored test materials may change into colored materials in wet or aqueous conditions and thus stain tissues during the 60 ± 5 min exposure. Therefore, a functional check for this possibility should be performed. However, if the colored test material or the MTT reducing chemical is classified as irritant by the skin irritation test (SIT) (tissue viability <50 %), the correction procedures are not necessary.

5.4.1.1 Test for Colored Materials

To determine if non-colored test materials change into colored test materials, 30 µl (liquid) or 25 mg (solid - using a sharp spoon) of the test substance was added to 0.3 ml of deionized water in a glass vial and placed in the incubator (37±1°C, 5±1 % CO2, 95% RH) for 60 ± 5 min. At the end of the exposure time, the mixture was evaluated for the presence and intensity of staining. If the solution changed color, a functional check on viable tissues was performed.

5.4.1.2 Test for Tissue Binding

To check the tissue-binding of a colored test substance (or a chemical that changes into a colored substance), one viable tissue was exposed to 50 µl of liquid test substance or 25 mg of solid test substance. In parallel, one tissue was exposed to water (negative control). Tissues were treated as described in section 5.4.2 except tissues were incubated for 3 hours ± 5 min in culture media without MTT (37±1°C, 5±1% CO_2, 95% RH) instead of media containing MTT.

5.4.1.3 Data Correction Procedure

The actual MTT OD (unaffected by interference with the colored test materials) is calculated using following formula:

$$OD = OD_{\text{colored tissue (MTT assay)}} – OD_{\text{colored tissue (no MTT assay)}}$$

5.4.1.4 Test for Direct MTT Reduction

To test if a material directly reduces MTT, 30 µl (liquid) or 25 mg (solid - using sharp spoon) of the test substance was added to 1 ml of the MTT medium and incubated in the incubator (37±1°C, 5±1 CO2, 95% RH) for 60 ± 5 min. Untreated MTT medium was used as control.

If the MTT solution turned blue/purple, the test substance directly reduces MTT. The additional functional check was performed unless the extract from tissues treated by test substance had an OD <5% of the negative control tissue and the tissue viability (determined in MTT assay) was not close to the classification cut-off (50%).

5.4.1.5 Functional Test of Interference from Test Chemical

Each MTT reducing chemical was applied to two freeze-killed tissues. In addition, two freeze-killed tissues were left untreated. The entire assay protocol was performed on the frozen tissues.

5.4.1.6 Data Correction Procedure

$$\text{True viability} = \text{Viability of treated tissue} - \text{Interference from test chemical}$$
$$= OD_{tvt} - OD_{kt}$$

Where:
OD_{kt} = (mean OD_{tkt} – mean OD_{ukt})
tvt = treated viable tissue
kt = killed tissues
tkt = treated killed tissue
ukt = untreated killed tissue (NC treated tissue)

5.4.2 EPI-200-SIT Test

5.4.2.1 Day 0

Upon receipt of assay kit, all components were stored according to the manufacturer's instructions. The assay medium was brought to room temperature (20-25 °C) and 0.9 ml assay medium was pipetted into each well of 8, 6-well plates. The 24-well plates containing epidermal tissues were opened under sterile conditions in a biological safety cabinet (BSC) and quickly inspected for excess surface moisture or defects. The tissue surfaces were dried with a sterile cotton swab and tissue inserts transferred to the upper row of pre-filled 6-well plates. Plates were placed in an incubator at (37±1°C, 5±1% CO_2, 95% RH) for 60 ± 5 minutes. At the end of the 60 minute pre-incubation, the inserts were transferred from the upper well to the lower wells of the 6-well plates. Plates were returned to the incubator for 18 ± 3 hours.

5.4.2.2 Day 1

After 18 ± 3 hours, plates were transferred from the incubator to the BSC and the tissue surfaces evaluated. Very wet and physically defective tissues were excluded. Moisture was removed from tissue surfaces with a sterile cotton swab. Tissues were dosed by applying 30 µl or 25 mg of undiluted test compound, negative control (NC), or positive control (PC) to 3 tissue replicates at 1 minute intervals. Liquid compounds were applied directly using a pipette. For solids, the surface was pre-wetted with 25 µl of sterile DPBS and then a level spoonful of compound was applied using a sharp application spoon (bone curette #1). For both materials a glass rod was then used to gently distribute the material across the surface of the skin. For liquids that would not disperse evenly, a nylon mesh insert was placed on the surface as a spreading tool. After dosing was complete, all plates were transferred to the incubator (37±1°C, 5±1% CO2, 95% RH) for 35 ± 1 minutes. After 35 minutes, all plates were removed from the incubator and placed in the sterile hood until a period of 60 minutes had elapsed from application of the first tissue dose. After 60 ± 1 minutes test compound exposure, tissues were rinsed with DPBS by filling and emptying the tissue inserts 15X using a constant stream applied from a wash bottle at a distance of approximately 1.5 cm. The inserts were then submerged 3X in 150ml DPBS (separate clean beaker of DPBS per test chemical), shaken to remove test material then rinsed inside and out and blotted on sterile blotting paper. Tissues were transferred to new 6-well plates pre-filled with 0.9 ml assay medium and tissue surfaces dried with sterile cotton swabs. Plates were placed in the incubator for 24 ± 2 hours.

5.4.2.3 Day 2

At the end of the 24 hour incubation period, inserts were transferred from the upper row of the 6-well plate to the pre-filled lower row and returned to incubator for 18 ± 2 hours.

5.4.2.4 Day 3

The MTT concentrate was thawed and diluted with the MTT diluent then covered with foil and stored at 4 ± 2°C for same day use. After the 18 ± 2 hours incubation (18 hr, 39 min ± 15 min) was complete, plates were transferred from the incubator to the BSC. Inserts were removed from 6-well plates, blotted on sterile paper, and transferred to 24-well plate pre-filled with 300 µl diluted MTT solution. Plates were placed in the incubator for 3 hours ± 5 minutes. After 3 hour ± 5 minutes incubation, the MTT medium was gently aspirated from all wells. All wells were rinsed twice by refilling with DPBS and aspirating. Tissue surfaces were dried with sterile cotton swabs and inserts blotted on sterile paper and transferred to a new 24-well plate. Isopropanol/MTT extractant solution (2ml) was pipetted into each insert. Plates were sealed with adhesive plate covers and/or parafilm and placed on a plate shaker (120 rpm) for at least 2 hours at room temperature. Inserts were pierced with forceps to release solution into the well and the solution mixed by pipetting up and down. Two aliquots (200 µl) were transferred to a 96-well flat bottom plate according to the plate layout. The OD was read at 570 nm (540-595nm acceptable) without a reference filter, using isopropanol/MTT extractant as a blank.

5.5 Data Calculations, Analyses, and Interpretation

Experimental data generated during the course of this study were recorded by hand and tabulated, summarized, and/or analyzed using Microsoft® Excel.

The mean OD of the blank (MTT extractant solution) aliquots was subtracted from the OD reading for each well (blank corrected OD). Mean ODs were calculated by aliquot then by tissue replicate for each test substance. For each individual tissues treated with a test substance (TS), the positive control (PC), and the negative control (NC), the individual relative tissue viability was calculated according to the following formulas:

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

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

Results were compared to the assay acceptance criteria (see Section 5.2.2) and the prediction model for skin irritation (Table 2).

Table 2. Prediction Model for Skin Irritation.

In vitro result	In vivo prediction
Mean tissue viability ≤50%	Irritant (I), GHS category 2
Mean tissue viability >50%	Non-irritant (NI)

6 Results and Discussion

6.1 Test for Interference with MTT Endpoint

6.1.1 Test for Colored Materials

None of the test materials were colored or changed color significantly, therefore the functional check on viable tissues indicated in the manufacturer's instructions was not necessary.

6.1.2 Test for Direct MTT Reduction

Direct MTT reduction (i.e., MTT solution turned blue/purple) was observed for cyclamen aldehyde, potassium hydroxide, and 1-methyl-3-phenyl-1-piperazine.

6.1.3 Functional Test of Interference from Test Chemical

Because potassium hydroxide and 1-methyl-3-phenyl-1-piperazine were classified as irritants by the SIT, the functional check was not performed with these test chemicals. Because percent viability values were borderline for cyclamen aldehyde, a functional check of interference from the test chemical in the assay was performed for cyclamen aldehyde.

6.1.4 Data Correction Procedure

True viability = Viability of treated tissue – Interference from test chemical
= $OD_{tvt} - OD_{kt}$

Where:
OD_{kt} = (mean OD_{tkt} – mean OD_{ukt})
tvt = treated viable tissue
kt = killed tissues
tkt = treated killed tissue
ukt = untreated killed tissue (NC treated tissue)

True viability =1.43 – (0.060-0.068)
=1.43-(-0.008)
=1.438

The OD_{ukt} and OD_{kt} values were very similar, with the untreated cells demonstrating greater MTT reduction, therefore direct reduction of MTT by cyclamen aldehyde did not interfere with the MTT assay.

6.2 EPI SIT-200 Test

As recommended by the manufacturer, proficiency test substances were tested in groups of 4 and tested concurrently with the NC and PC. One chemical, 1-bromohexane, was unavailable at the time of this testing; however, it was tested at a later date, by another investigator, using these procedures and was correctly classified. Solid test substances were ground with a mortar and pestle to a fine powder, as necessary, and tested in neat form. Liquid test substances were tested undiluted with the exception of potassium hydroxide (5%) which was purchased as a 45% solution and diluted (1:9) with distilled water.

Three EPI-SIT-200 assays were conducted with the proficiency chemicals. The mean OD and percent viability for each test substance, NC, and PC were within expected limits (Table 3). Data for each run, from individual replicate tissues can be found in Appendix D.

Table 3. EPI-SIT-200 assay results. Mean optical density and relative viability (%) for proficiency chemicals, negative control (NC), and positive control (PC).

Test Substance	Date	Mean of OD	SD of OD	Mean Relative Viability %	SD of viability	CV %
NC	2/16/2018	1.99	0.08	100.0	4.0	4.0
PC	2/16/2018	0.10	0.02	4.9	1.2	23.6
hexyl salicylate	2/16/2018	1.98	0.16	99.4	8.1	8.1
isopropanol	2/16/2018	2.00	0.36	100.3	18.1	18.1
heptanol	2/16/2018	0.11	0.10	5.7	5.0	87.0
methyl stearate	2/16/2018	2.04	0.10	102.1	5.0	4.9
NC	3/3/2018	2.50	0.03	100.0	1.3	1.3
PC	3/3/2018	0.04	0.00	1.6	0.1	5.8
heptyl butyrate	3/3/2018	1.98	0.34	79.4	13.4	16.9
cyclamen aldehyde	3/3/2018	1.04	0.44	41.7	17.5	42.1
potassium hydroxide	3/3/2018	0.07	0.03	2.8	1.2	44.6
1-methyl-3-phenyl-1-piperazine	3/3/2018	0.04	0.00	1.7	0.1	7.5
heptyl butyrate	3/3/2018	2.32	0.13	93.0	5.1	5.4
1-methyl-3-phenyl-1-piperazine	3/3/2018	0.04	0.01	1.7	0.5	27.8
NC	3/9/2018	2.55	0.16	100.0	6.4	6.4
PC	3/9/2018	0.03	0.00	1.3	0.0	1.7
cyclamen aldehyde	3/9/2018	1.31	0.10	51.3	4.0	7.8
isopropanol	3/9/2018	2.32	0.02	90.9	0.8	0.9
heptanol	3/9/2018	0.03	0.00	1.2	0.1	11.4

napthaleneacetic acid	3/9/2018	2.46	0.10	96.4	3.8	3.9
SD: standard deviation; OD: optical density; CV: coefficient of variation; NC: negative control; PC: positive control						

6.3 Criteria for Valid Assay

6.3.1 Test Acceptance Criterion for the NC

Tissue viability of the DPBS-treated tissues was determined in the MTT assay. In the 3 assays conducted, the mean OD_{570} of the NC tissues was 1.99, 2.50, and 2.55. This meets the acceptance criterion of mean $OD_{570} \geq 1.0$ and ≤ 2.8.

6.3.2 Test Acceptance Criterion for the PC

In the 3 assays, 3 replicate tissues were treated with a 5% SDS solution as a positive control. The relative tissue viability was 4.9, 1.6, and 1.3%. The assay meets the acceptance criterion of mean viability of PC being ≤ 20% of the negative control tissues.

6.3.3 Variability of the Tests

To determine if the 3 replicate tissues reacted similarly, the tissue viability standard deviations for each test substance were examined. Only one test substance, isopropanol, exceeded the 18% acceptance cut-off. Testing was repeated for this chemical and the SD of the re-test (0.8) met the acceptance criteria.

6.4 Classification of Proficiency Chemicals

The *in vitro* classification of the test substances was determined by comparing the mean relative viability to the prediction model for skin irritation. According to the EU and GHS classification, an irritant is predicted if the mean relative tissue viability of three individual tissues exposed to the test substance is reduced below 50% of the mean viability of the negative controls. The *in vitro* classification was then compared to the GHS *in vivo* classification to determine whether the test substances were correctly classified in the assay (Table 4). Only one test substance, cyclamen aldehyde, was misclassified. However, cyclamen aldehyde was tested twice and was correctly classified as an irritant (I) in the first assay and was repeated due to high, though acceptable, SD. On repeat testing it was misclassified as non-irritant (NI) though the viability (51.3%) was only slightly greater than the cut-off value (50%). Cyclamen aldehyde is a borderline chemical with an *in vivo* score of 2.3 which may explain the differing results for this chemical.

Therefore, the sensitivity (prediction of irritants), specificity (prediction of non-irritants), and accuracy (overall concordance) were all 100%.

Table 4. Classification of Proficiency Chemicals. In vitro classification using EPI-SIT-200 compared with GHS in vivo classification.

Test Substance	Date	GHS *in vivo*	*in vitro*	Concordance
hexyl salicylate	2/16/2018	No Cat.	NI	yes
2-propanol	2/16/2018	No Cat.	NI	yes
heptanol	2/16/2018	Cat. 2	I	yes
methyl stearate	2/16/2018	No Cat.	NI	yes
heptyl butyrate	3/2/2018	No Cat.	NI	yes
cyclamen aldehyde	3/2/2018	Cat. 2	I	yes
potassium hydroxide (5% aq)	3/2/2018	Cat. 2	I	yes
1-methyl-3-phenyl-1-piperazine	3/2/2018	Cat. 2	I	yes
heptyl butyrate	3/2/2018	No Cat.	NI	yes
1-methyl-3-phenyl-1-piperazine	3/2/2018	Cat. 2	I	yes
cyclamen aldehyde	3/9/2018	Cat. 2	NI	no
2-propanol	3/9/2018	No Cat.	NI	yes
heptanol	3/9/2018	Cat. 2	I	yes
1-napthaleneacetic acid	3/9/2018	No Cat.	NI	yes
No Cat.: no category/not classified as irritant; Cat. 2: category 2 irritant; NI: non-irritant; I: irritant				

7 Conclusions

The proficiency substances were tested using the EPI-SIT-200 assay as a demonstration of technical proficiency of the laboratory prior to routine use of this test method. All assay acceptance criteria were met and 100% of the proficiency substances were correctly classified, therefore the performing lab is technically proficient and EPI-SIT-200 may be routinely used for *in vitro* prediction of skin irritation.

8 Point of Contact

Questions pertaining to this report should be referred to Emily May Lent at DSN 584-3980, commercial 410-436-3980, or by e-mail: usarmy.apg.medcom-aphc.mbx.tox-info@mail.mil.

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Toxicity Report No. S.0055513-18, February 2018-September 2018

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Date

Appendix A

References

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2. Spielmann, H., et al., *The ECVAM international validation study on in vitro tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test.* Altern Lab Anim, 2007. **35**(6): p. 559-601.

3. OECD, *Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method*. 2015, OECD: Paris.

4. MatTek, *In Vitro EpiDerm^{TM} Skin Irritation Test (EPI-200-SIT)*. 2014: Ashland, MA.

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Appendix B

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. S.0055513-18, Protocol No. 68-iv18-02-01 entitled *In vitro* dermal irritation proficiency demonstration, February 2018-March 2018", the following Good Laboratory Practice Standard Inspections were conducted:

Study Specific Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Type Protocol Good Laboratory Practice Standard Review	2/09/2018	02/09/2018
Skin Irritation Verification/Qualification Study - Day 0 - Day Prior to Dosing Procedures	2/27/2018	3/13/2018
Skin Irritation Verification/Qualification Study - Maintenance and Calibration of Equipment	2/27/2018	3/13/2018
Skin Irritation Verification/Qualification Study - Test System Receipt, Control and Characterization	2/27/2018	3/13/2018
Skin Irritation Verification/Qualification Study-Test Article-Facilities, ID, Control, and Handling	2/28/2018	3/13/2018
Skin Irritation Verification/Qualification Study-Reagents, Negative and Positive Controls handling	2/28/2018	3/13/2018
Skin Irritation Verification/Qualification Study - Day 1 Chemical Exposure Procedures	2/28/2018	3/13/2018
Skin Irritation Verification/Qualification Study - Day 2 Change Medium Procedures	3/01/2018	3/13/2018
Skin Irritation Verification/Qualification Study - Day 3 - MTT Viability Test and Plate Reading	3/02/2018	3/13/2018
Study Raw Data Good Laboratory Practice Standard Review	10/18/2018	10/19/2018
Final Study Good Laboratory Practice Standard Report Review	10/18/2018	10/19/2018

Note 1: *All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.*

Note 2: *This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and of the procedures conducted.*

Note 3: *In addition to the study specific critical phase inspections listed here, general facility and process based inspection not specifically related to this study are done monthly or annually in accordance with QA Standard Operating Procedure.*

Michael P. Kefauver
Good Laboratory Practice Standards
Quality Assurance Specialist, QSARC

08 FEB 2019
Date

Appendix C

Archives and Study Personnel

C-1 Archives

All raw data, documentation, records, protocol, and a copy of the final report generated as a result of this study will be archived in room 1026, building E-2100, APHC, for a minimum of ten (10) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

Some ancillary records pertaining to this study, such as instrument maintenance logs will not be archived until those logbooks have been completed. Once complete they will be archived in room 1026, building E-2100, APHC.

C-2 Personnel

Management: Dr. Mark S. Johnson, Director, Toxicology; MAJ Jarod Hanson, Executive Officer, Toxicology; Mr. Arthur J. O'Neill, Chief, Toxicity Evaluation Division (TEV); Dr. Michael J. Quinn, Chief, Health Effects Research Division (HEF).

Study Director: Dr. Emily May Lent, Toxicologist, TEV.

Quality Assurance: Michael P. Kefauver, Quality Assurance Specialist, Quality Systems and Regulatory Compliance Office.

Archivist: Lee C.B. Crouse, Biologist, TEV.

Appendix D

EPI-SIT-200 Data

Exp. No.:	1
Tissue Lot No.:	27943
Date:	16-Feb-18
Operator:	Emily Lent

							mean
Blanks:	0.041	0.040	0.041	0.040	0.040	0.040	0.04

Code	Tissue	Raw Data		Blank Corrected		Mean of Aliquots	% Viability
		Aliquot 1	Aliquot 2	Aliquot 1	Aliquot 2		
NC	1	1.965	1.962	1.92	1.92	1.92	96.45
	2	2.129	2.114	2.09	2.07	2.08	104.37
	3	2.002	2.034	1.96	1.99	1.98	99.18
PC	1	0.133	0.134	0.09	0.09	0.09	4.67
	2	0.118	0.118	0.08	0.08	0.08	3.90
	3	0.164	0.163	0.12	0.12	0.12	6.18
C1-hexyl salicylate	1	2.199	2.153	2.16	2.11	2.14	107.10
	2	2.074	1.996	2.03	1.96	1.99	100.03
	3	1.875	1.833	1.83	1.79	1.81	90.96
C2-isopropanol	1	1.682	1.673	1.64	1.63	1.64	82.10
	2	1.926	2.158	1.89	2.12	2.00	100.38
	3	2.500	2.302	2.46	2.26	2.36	118.39
C3-heptanol	1	0.264	0.273	0.22	0.23	0.23	11.44
	2	0.082	0.083	0.04	0.04	0.04	2.11
	3	0.114	0.113	0.07	0.07	0.07	3.67
C4-methyl stearate	1	2.153	2.195	2.11	2.15	2.13	107.00
	2	2.074	2.082	2.03	2.04	2.04	102.19
	3	1.974	1.977	1.93	1.94	1.94	97.05

Toxicity Report No. S.0055513-18, February 2018-September 2018

Exp. No.:	2
Tissue Lot No.:	27961
Date:	3-Mar-18
Operator:	Emily Lent

							mean
Blanks:	0.040	0.040	0.041	0.040	0.041	0.042	0.04

Code	Tissue	Raw Data		Blank Corrected		Mean of Aliquots	% Viability
		Aliquot 1	Aliquot 2	Aliquot 1	Aliquot 2		
NC	1	2.494	2.538	2.45	2.50	2.48	99.07
	2	2.564	2.561	2.52	2.52	2.52	100.93
	3						
PC	1	0.078	0.077	0.04	0.04	0.04	1.47
	2	0.080	0.08	0.04	0.04	0.04	1.57
	3	0.083	0.081	0.04	0.04	0.04	1.65
C1-heptyl butyrate	1	1.805	1.795	1.76	1.75	1.76	70.41
	2	1.867	1.861	1.83	1.82	1.82	72.97
	3	2.407	2.414	2.37	2.37	2.37	94.85
C2-cyclamen aldehyde	1	0.667	0.669	0.63	0.63	0.63	25.11
	2	1.028	1.046	0.99	1.01	1.00	39.88
	3	1.537	1.546	1.50	1.51	1.50	60.07
C3-potassium hydroxide	1	0.077	0.077	0.04	0.04	0.04	1.45
	2	0.116	0.115	0.08	0.07	0.07	3.00
	3	0.140	0.137	0.10	0.10	0.10	3.92
C4-1-methyl-3-phenyl-1-piperazine	1						
	2	0.081	0.081	0.04	0.04	0.04	1.61
	3	0.085	0.086	0.04	0.05	0.04	1.79
C1repeat	1	2.508	2.5	2.47	2.46	2.46	98.59
	2	2.323	2.334	2.28	2.29	2.29	91.57
	3	2.268	2.25	2.23	2.21	2.22	88.78
C4repeat	1	0.079	0.079	0.04	0.04	0.04	1.53
	2	0.097	0.097	0.06	0.06	0.06	2.25
	3	0.075	0.074	0.03	0.03	0.03	1.35

Toxicity Report No. S.0055513-18, February 2018-September 2018

Exp. No.:	3
Tissue Lot No.:	27973
Date:	9-Mar-18
Operator:	Emily Lent

							mean
Blanks:	0.041	0.040	0.041	0.040	0.040	0.040	0.04

Code	Tissue	Raw Data		Blank Corrected		Mean of Aliquots	% Viability
		Aliquot 1	Aliquot 2	Aliquot 1	Aliquot 2		
NC	1	2.740	2.718	2.70	2.68	2.69	105.27
	2	2.662	2.624	2.62	2.58	2.60	101.90
	3	2.413	2.41	2.37	2.37	2.37	92.84
PC	1	0.074	0.074	0.03	0.03	0.03	1.32
	2	0.073	0.073	0.03	0.03	0.03	1.28
	3	0.073	0.073	0.03	0.03	0.03	1.28
C1-cyclamen aldehyde	1	1.471	1.461	1.43	1.42	1.43	55.82
	2	1.279	1.259	1.24	1.22	1.23	48.10
	3	1.321	1.316	1.28	1.28	1.28	50.04
C2-isopropanol	1	2.364	2.349	2.32	2.31	2.32	90.68
	2	2.388	2.384	2.35	2.34	2.35	91.84
	3	2.336	2.359	2.30	2.32	2.31	90.33
C3-heptanol	1	0.064	0.075	0.02	0.03	0.03	1.14
	2	0.063	0.074	0.02	0.03	0.03	1.10
	3	0.076	0.074	0.04	0.03	0.03	1.36
C4-napthaleneacetic acid	1	2.524	2.516	2.48	2.48	2.48	97.08
	2	2.384	2.411	2.34	2.37	2.36	92.29
	3	2.584	2.589	2.54	2.55	2.55	99.69