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Isocycloseram/Emamectin Benzoate

Isocycloseram/Emamectin Benzoate SC (A23220A) – *In Vitro* Skin Irritation Test in the EpiDerm™ Model (EPI-200-SIT)

Final Report Amendment 1

TEST GUIDELINE(S): OECD No. 439 (2019)
EC No 640/2012, B.46. (2012)

AUTHOR(S): Balázs Orovecz, B.Sc.

COMPLETION DATE: 07 October 2020

REPORT AMENDMENT 1 DATE: 19 November 2020

PERFORMING LABORATORY: Charles River Laboratories Hungary Kft.
H-8200 Veszprém, Szabadságpuszta, hrsz. 028/1.,
Hungary

LABORATORY PROJECT ID: Report Number: 20/080-043B
Study Number: 20/080-043B
Task Number: TK0539400

SPONSOR(S): Syngenta Ltd.
Jealott's Hill International Research Centre
Bracknell, Berkshire, RG42 6EY, United Kingdom

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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Company: Syngenta Crop Protection, LLC
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419-8300 USA

Submitter: _____ Date: _____

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study has been performed in accordance with the study plan and the Principles of Good Laboratory Practice (Hungarian GLP Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities 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.

Signature: _____ Date: 19 November 2020

Balázs Orovecz, B.Sc.
Study Director

Performing Laboratory: Charles River Laboratories Hungary Kft.
H-8200 Veszprém, Szabadságpuszta, hrsz. 028/1.,
Hungary

To be completed for USA EPA submission only:
Representative of Submitter/Sponsor:

_____ _____
Date

Submitter/Sponsor: Syngenta Crop Protection, LLC
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419-8300 USA

FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Number: 20/080-043B

Study Title: Isocycloseram/Emamectin Benzoate SC (A23220A) – *In Vitro* Skin Irritation Test in the EpiDerm™ Model (EPI -200-SIT)

Test Item: Isocycloseram/Emamectin Benzoate SC (A23220A)

This study has been inspected, and The Final Report as well as Final Report Amendment 1 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 Final Report as well as Final Report Amendment 1 audits 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
14 May 2020	Study Plan	14 May 2020	14 May 2020
20 May 2020	Treatment	20 May 2020	20 May 2020
29 June 2020	Draft Report	29 June 2020	29 June 2020
07 October 2020	Final Report	07 October 2020	07 October 2020
19 November 2020	Final Report Amendment 1	19 November 2020	19 November 2020

Signature: Szőcs Csilla Date: 19 November 2020

Csilla Szőcs, B.Sc.
On Behalf of QA

MANAGEMENT STATEMENT

According to the conditions of the research and development agreement between Syngenta Ltd. (as Sponsor) and Charles River Laboratories Hungary Kft. (as Test Facility), the study titled "Isocycloseram/Emamectin Benzoate SC (A23220A) – *In Vitro* Skin Irritation Test in the EpiDerm™ Model (EPI-200-SIT)" was performed, in compliance with the Principles of Good Laboratory Practice.

Signature: _____ Date: 20 November 2020

David J. Esdaile, M.Sc.
Director of Science and Regulatory Affairs

GENERAL INFORMATION

Contributors
The following contributed to this report in the capacities indicated:

Name	Function
Balázs Orovecz, B.Sc.	Study Director
Balázs Tóth, Ph.D.	General Manager
David J. Esdaile, M.Sc.	Director of Science and Regulatory Affairs
Kata Tóth-Gönczöl, B.Sc.	Assistant Scientist
Adrienn Kucska, M.Sc.	Quality Assurance
Csilla Szőcs, B.Sc.	Quality Assurance
Carolina Vaccari	Syngenta Study Manager

Study dates

Study Plan:	15 May 2020
Experimental Starting Date:	20 May 2020
Experimental Completion Date:	22 May 2020
Date of Draft Report:	03 July 2020
Date of Final Report:	07 October 2020
Date of Final Report Amendment 1:	19 November 2020

Deviations from the Study Plan
There were no deviations from the Study Plan.

Performing laboratory test substance reference number
200164

Other
The study documents and samples:
- study plan,
- all raw data,
- sample of the test item,
- original study report and any amendments,
- correspondence

will be archived according to the Hungarian GLP regulations and to applicable SOP's in the Archives of Charles River Laboratories Hungary Kft. (H-8200 Veszprém, Szabadságpuszta, hrsz. 028/1., Hungary). This is for a period of 15 years.

After the retention time of 15 years has elapsed all the archived materials listed above will be returned to the Sponsor or retained for a further period if agreed by a contract. Otherwise the materials will be discarded.

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

An *in vitro* skin irritation test was conducted on isocycloseram/emamectin benzoate SC (A23220A) in a reconstructed human epidermis model. The EpiDerm™ Model is designed to predict and classify the irritation potential of chemicals by measuring 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. The irritation potential of the test item was evaluated according to the OECD guideline No. 439 [1].

Disks of EpiDerm™ Model (three units) were treated with the test item and incubated for 35 minutes 37°C, 5 %CO_2 , >95 RH% humidified atmosphere and 25 minutes at room temperature. Exposure of the test item was terminated by rinsing with Dulbecco's Phosphate Buffered Saline (DPBS). The epidermis units were then incubated at 37°C for 24 hours in an incubator with 5% CO_2, in a >95% humidified atmosphere and 18 hours at 37°C in an incubator with 5% CO_2, in a >95% humidified atmosphere. The viability of each disk was assessed by incubating the tissues for 3 hours with MTT solution at 37°C in an incubator with 5% CO_2 protected from light, in a >95% humidified atmosphere. The precipitated formazan crystals were then extracted using extracting solution (Isopropanol) for 2 hours with shaking at room temperature and quantified spectrophotometrically at 570 nm.

The negative control epidermis units were treated with DPBS, whilst the positive control epidermis units were treated with 5% (w/v) Sodium Dodecyl Sulphate (SDS) (three units / control). Two additional disks were used to provide an estimate of colour contribution (NSC_{living}) from the test item. For each treated tissue, the viability was expressed as a percentage relative to the negative control. If the mean relative viability is less or equal (≤) to 50% of the negative control, the test item is considered to be irritant to skin.

1.2 Results

Following exposure to isocycloseram/emamectin benzoate SC (A23220A), the mean cell viability was 16.2% compared to the negative control. This is below the threshold of 50%, therefore under the condition of this assay the test item was considered to be irritant to skin. The experiment met the validity criteria, therefore the study was considered to be valid.

1.3 Conclusion

In conclusion, under the conditions of this *in vitro* EpiDerm ™ Model irritation assay, the results indicate that isocycloseram/emamectin benzoate SC (A23220A) is irritant to skin.

2.0 INTRODUCTION

Based on the Sponsor's request, the Final Report (issued on 07 October 2020) was reissued on 19 November 2020 in a form of the Final Report Amendment No. 1, "Interpretation of Test Results" section (section 3.11) was updated.

2.1 Purpose

The skin irritation potential of a test item may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the EpiSkin™ reconstituted human epidermis [1-3]. This method is approved by international regulatory agencies as a replacement for the identification of irritants in the *in vivo* rabbit skin assay (OECD No. 404) [4].

The test is designed to predict and classify the skin irritant potential of chemicals/ formulations/products/mixtures according to chemical safety regulations, using the reconstructed human epidermis model EpiDerm™ Model and parameters related to skin irritation.

EpiDerm™ Model is a three-dimensional human skin model comprised of a reconstructed epidermis with a functional stratum corneum. Its use for skin irritation testing involves topical application of test materials to the surface of the epidermis, and the subsequent assessment of their effects on cell viability. Cell viability is determined based on cellular mitochondrial dehydrogenase activity, measured by MTT reduction and conversion into a blue formazan salt that is quantitatively measured after extraction from tissues (Faller C. et al., 2002, Mosmann T., 1983) [5, 10]. The reduction in cell viability in treated tissues is compared to negative controls and expressed as a percentage. The percent reduction in cell viability is used to predict the irritation potential of the test item.

The OECD No. 439 is specifically a test for dermal irritation; it is not a test for corrosivity. A negative or positive result in this assay does not necessarily mean that the test item is non-corrosive.

2.2 Guidelines

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

- OECD Guidelines for Testing of Chemicals, Section 4, No. 439, "*In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method", 18 June 2019
- Commission Regulation (EC) No 640/2012 of 6th July 2012 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008.

2.3 Test Facility

This study was performed in an AAALAC-accredited laboratory. The Institutional Animal Care and Use Committee (IACUC) of Charles River Laboratories Hungary Kft. monitored the conduct of the study.

3.0 MATERIALS AND METHODS

3.1 Test Substance

Information supplied by the Sponsor:

Name:	Isocycloseram/Emamectin Benzoate SC (A23220A)	
Batch number:	TSC002-041-001	
Design code:	A23220A	
Active ingredient content*:	Isocycloseram:	17.5 % w/w corresponding to 201 g/L
	emamectin benzoate:	4.18 % w/w corresponding to 48.1 g/L
Appearance:	Brown liquid	
Recertification date:	31 January 2023	
Storage conditions:	Room temperature (<30°C)	
Safety precautions:	Enhanced safety precautions (half mask at least with P3 filter cartridge, nitrile gloves, lab coat) for unknown materials were applied to assure personnel health and safety.	

* No adjustment for active ingredient content was applied as agreed by the Sponsor.

A Certificate of Analysis supplied by the Sponsor is given in Appendix 1.

The integrity of supplied data relating to the identity, purity and stability of the test material was the responsibility of the Sponsor.

3.2 Identification and Receipt

The test item of a suitable active ingredient content together with all precautions required in the handling and disposal of the test item were supplied by the Sponsor. The identification of the test item was made on the basis of the information provided by Sponsor in the Pharmacy of Charles River Laboratories Hungary Kft.

3.3 Test Item Preparation

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

3.4 Subsidiary Materials

Positive and negative controls were included in the experiment. Furthermore, as the test item was coloured, two additional control tissue samples were used in the experiment for determination of the non-specific colour (as detailed in 3.7.3.).

3.4.1 Negative control

Dulbecco's Phosphate Buffered Saline:

The negative control solution (abbreviated as DPBS in the raw data and report) was prepared freshly in the testing laboratory.

The following chemicals were used for the preparation of the negative control solution:

Abbreviation:	DPBS
Supplier:	Sigma-Aldrich
Batch number:	RNBH7035
Expiry date:	June 2021
Storage conditions:	Room temperature

Distilled water (Aqua Purificata):

Supplier:	Magilab Kft.
Batch number:	202002021
Expiry date:	17 August 2020
Storage conditions:	Room temperature

3.4.2 Positive control

5% (w/v) Sodium Dodecyl Sulphate solution (SDS):

Supplier:	MatTek In Vitro Life Science Laboratories
Batch number:	110519MSA
Expiry date:	05 November 2020
Storage conditions:	Room temperature

3.5 Test System

3.5.1 Human skin

EpiDerm™ Model (EPI-200-SIT) (Source: MatTek, Bratislava, Slovakia, Lot No.:30867, Expiry Date: 22 May 2020) units consist of normal, human-derived epidermal keratinocytes (NHEK) which have been cultured to form a multilayered, highly differentiated model of the human epidermis (0.6 cm^2). It's 3D structure consisting of organized and proliferative basal cells, spinous and granular layers, and cornified

epidermal layers are mitotically and metabolically active. Its use for skin irritation testing involves topical application of test materials to the surface of the epidermis, and the subsequent assessment of their effects on cell viability.

3.5.2 Quality control

EpiDerm™ Model (EPI-200-SIT) kits are manufactured according to defined quality assurance procedures. All biological components of the epidermis and the kit culture medium have been tested for the presence of viruses, bacteria and mycoplasma.

The RhE tissue construct was only used if the developer/supplier demonstrated that each batch of the RhE tissue construct met the defined production release criteria, among which those for viability and barrier function were the most relevant. An acceptability range (upper and lower limits) for the barrier functions as measured by the ET_{50} was established by the RhE tissue construct developer/supplier. The ET_{50} acceptability range used as QC (Quality Control) batch release criterion by the developer/supplier of the RhE tissue constructs was documented in OECD No. 439.

Quality Control batch release criteria

Test Method	**Lower acceptance limit**	**Upper acceptance limit**
EpiDerm™ (EPI-200-SIT) (100 µL of 1% (v/v) Triton X-100)	ET_{50} = 4.77 hours	ET_{50} = 8.72 hours
MTT, QC assay (4 hour, n=3)	OD=1.0	OD=3.0

Note: ET_{50} value refers the barrier function of the tissues, while the MTT assay result represents the tissue viability.

Tissue viability and the barrier function test are within the acceptable ranges and indicate appropriate formation of the epidermal barrier, the presence of a functional stratum corneum, a viable basal cell layer and intermediate spinous and granular layers. Results obtained with this lot conform to the requirements of the OECD TG 439 are documented in Appendix 2.

3.5.3 Justification for selection of the test system

The EpiDerm™ has been validated for irritation testing in an international validation study [3] and its use is recommended by the relevant OECD guideline for irritation testing (OECD No. 439); therefore, it was considered to be suitable for this study.

3.5.4 Kit contents

EPI-200-SIT Kit Components:

Amount	Item	Contains/Used for:
1	Sealed 24-well plate (EPI-200-SIT)	Contains 24 tissues on agarose
2	24-well plates (sterile)	For MTT viability assay
8	6-well plates (sterile)	For irritation assay

1 bottle, 100 ml	Assay Medium (EPI-100-NMM)	DMEM based medium
1 vial, 1 mL	5% SDS Solution (TC-SDS-5%)	Skin irritant reference chemical – Positive Control
1 bottle, 100 ml	DPBS Rinse Solution (TC-PBS)	Used for rinsing the inserts
25 pieces	Nylon Mesh circles 8 mm diameter, 200 μm pore (EPI-MESH)	For spreading test chemicals
1	MK-24-007-023	Complete EpiDerm™ Skin Irritation Test (SIT) protocol is sent electronically

MTT-100 Assay Kit Components:

1 vial, 2 ml	MTT concentrate (MTT-100-CON)	Frozen MTT concentrate
1 vial, 8 ml	MTT diluent (MTT-100-DIL)	For diluting MTT concentrate prior to use in the MTT assay
1 bottle, 60 ml	Extractant Solution (MTT-100-EXT)	For extraction of formazan

Identification data of the components are shown in the table below.

Identification data for the components of the kits:

Name	Batch numbers	Expiry date
Assay Medium	051420LHC	28 May 2020
MTT-100-CON	032720SLA	27 May 2020
MTT-100-DIL	2156402	31 January 2021
MTT-100-EXT	071119ALD	11 July 2020

3.5.5 Number of replicate wells

In this assay, three replicates were used for the test item. Three negative controls and three positive controls were also run in the assay. As the test item was coloured, two additional test item-treated living tissues were used for the non-specific optical density (OD) evaluation.

3.5.6 Kit reception

The date of dispatch written on the package was checked before opening the EpiDerm™ kit. The contents of the package were checked according to the map and instructions provided by the supplier.

3.5.7 Storage

The Assay Medium and MTT diluent (MTT-100-DIL) supplied with the kits were stored at 2-8°C. The MTT concentrate (MTT-100-CON) was stored at freezer (-20 ± 5°C). Additional kit components were stored at room temperature (15-25^{o}C).

3.6 Additional Materials

3.6.1 MTT solution

MTT concentrate (MTT-100-CON) was thawed and diluted with the MTT diluent (MTT-100-DIL). Remaining MTT solution was stored in the dark at 2-8°C for later use on the same day (final concentration: 1 mg MTT / ml medium).

3.6.2 Chemicals used in the experiment

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

Chemical	Supplier	Batch Number	Expiry date
Dulbecco's Phosphate buffered Saline (DPBS)	Sigma-Aldrich	RNBH7035*	June 2021
Distilled water (Aqua Purificata)	Magilab Kft.	202002021	17 August 2020

*The obtained stock solution (prepared on 20 May 2020) was stored in the refrigerator (2-8°C). It was diluted with distilled water (Supplier: Magilab Kft.; Batch number: 202002021, Exp. date: 17 August 2020) to a final concentration of 10x diluted. This control extract was transferred to a sterile dry container, filtered sterile by using a 0.22 µm syringe filter (Supplier: Millipore, Lot No.: MP193704G2, Expiry date: September 2022).

3.7 Indicator for Potential False Viability

Optical properties of the test material or its chemical action on MTT may interfere with the assay leading to a false estimate of cell viability. This may occur when the test item is not completely removed from the tissue by rinsing or when it penetrates the epidermis during the exposure period. If the test material directly acts 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 cell viability measurement. Methods indicating how to correct direct MTT reduction and interferences by colouring agents are detailed in the following paragraphs.

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

30 µL of test item was added to 1 mL MTT working solution and mixed. The mixture was incubated at 37°C for 1 hour in an incubator with 5 % CO2, in a >95 RH % humidified atmosphere protected from light, and then any colour change was recorded:

-Test items which do not react with MTT: other colours
-Test items which directly react with MTT: blue or purple

After the test item, isocycloseram/emamectin benzoate SC (A23220A), had been incubated with MTT for one hour, brown colour of the mixture was detected in the test tube. Thus, the

test item did not react with MTT and therefore, additional controls were not used in the experiment.

3.7.2 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). As the test item had an intrinsic colour, the use of additional control tissues (determination of the non-specific colour percentage) were necessary to evaluate the ability of the test item to stain the epidermis.

3.7.3 Additional control tissues used in respect of colour interference potential (NSC_{living})

In addition to the normal procedure (3.9.1), two additional test item-treated living tissues were used for the non-specific OD evaluation. These tissues followed the same test item application and all steps as the other tissues, except for the MTT step: MTT incubation was replaced by incubation with fresh Assay Medium to mimic the amount of colour from the test item that may be present in the test disks. OD reading was conducted following the same conditions as for the other tissues.

3.8 Performance of the Study

Procedures described in sections 3.8.1., 3.8.2. and 3.8.3. were performed under aseptic conditions (in sterile hood using sterile equipment).

3.8.1 Pre-incubation (Day [-1])

The appropriate number of wells in an assay plate was filled with the EpiDerm Assay medium (0.9 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 1 hour at 37°C in an incubator with 5 % CO_2, in a >95% humidified atmosphere. Afterwards the medium was replaced and continue with overnight pre-incubation.

3.8.2 Application (Day 0)

Test Item

30 µL of test item was applied evenly to each of three test units and each additional control skin units. 30 µL of test item was sufficient to cover the epidermal surface.

Negative and positive controls

30 µL of negative control (DPBS) or positive control (5% (w/v) SDS solution) were added to each skin unit by using a suitable pipette. Chemicals were spread gently with the pipette tip

in order to cover evenly all the epidermal surface if necessary (without damaging the epidermis).

Note: The negative and positive controls were also part of concurrent studies (Charles River Laboratories Hungary study codes: 20/024-043B, 20/025-043B and 20/083-043B) performed in the same experimental period using the same batch of chemicals and same batch of skin units.

Additional control

Two additional test item treated viable tissues for colour control.

Incubation with Test Item

The plates with the test item, negative and positive control treated epidermis units were incubated for 35 minutes (± 1 minute) to the humidified incubator at 37°C with 5 % CO_2, in a >95% humidified atmosphere and for the exposure time of 25 minutes (± 0.5 minute) at room temperature (25.7-26.5°C).

3.8.3 Rinsing (Day 0)

After the incubation time, the EpiDerm ^{TM} units were removed and rinsed into clean beakers containing 100 mL of DPBS each (20 times). Any remaining liquid were removed onto an absorbent paper.

3.8.4 Incubation (Day 0)

After rinsing, the units were placed into the plate wells with fresh Assay Medium (0.9 mL/well) below them and then incubated for 24 hours (± 2hours) at 37°C in an incubator with 5 % CO_2, in a >95 RH % humidified atmosphere.

3.8.5 Incubation (Day 1)

At the end of the 24 hours incubation period the units were placed into the plate wells with fresh Assay Medium (0.9 mL/well) below them and then incubated for 18 hours (± 2hours) at 37°C in an incubator with 5 % CO_2, in a >95 RH % humidified atmosphere.

3.8.6 MTT test after 42 hours incubation (Day 2)

After the 42 hours incubation, 300 µL MTT medium (1 mg MTT / ml medium) was added to each well of plate and the skin units were transferred to the MTT medium (except colour control units which were incubated in Assay Medium). The lid was replaced and the plate incubated at 37°C in an incubator with 5 % CO_2, in a >95% humidified atmosphere for 3 hours (±5 minutes).

3.8.7 Formazan extraction (Day 2)

At the end of incubation with MTT a formazan extraction was undertaken. After the 3-hour MTT incubation period is complete, MTT was gently aspired medium from all the wells, then wells were refilled with DPBS and aspirated again. The rinsing was repeated twice and made sure that tissues are dry after the last aspiration. After than transferred inserts to new 24-well plates. Inserts were immersed by gently pipetting 2 mL extracting solution (MTT-100-EXT) into each insert. The level was rise above the upper edge of the insert, thus completely covering the tissue from both sides. The 24 well plate was sealed to inhibit the extracting solution evaporation. Extraction was performed 2 hours with shaking (~120 rpm) at room temperature.

After the extraction period is complete, the inserts were pierced with an injection needle and allow the extract to run into the well from which the insert was taken. Afterwards the insert was discarded. The 24-well plates were placed on a shaker for 15 minutes until solution is homogeneous in colour.

A blank sample containing 2 mL of extracting solution (MTT-100-EXT) was processed in parallel.

3.8.8 Cell viability measurements (Day 2)

Following the formazan extraction, 2×200 µL sample from each tube were placed into the wells of a 96-well plate (labelled appropriately). The OD (optical density or absorbance) of the samples was measured using a plate reader at 570 nm. The mean of 5 wells of isopropanol solution (MTT-100-EXT) (200 µL/well) was used as blank.

The proper status of the instrument was verified by measuring a Verification plate (Manufacturer: Thermo Fisher Scientific, Catalogue Number: 240 72800, Serial Number: 0920-14, Date of calibration: 13 August 2018, calibration is valid until August 2020) at the required wavelength on each day before use.

3.9 Calculations of Viability Percentages

3.9.1 Data calculation for normal test items

Blank:
– The mean of the five blank OD values was calculated

Negative control:
– Individual negative control OD values ($_{NCraw}$) were corrected with the mean blank OD:
OD Negative Control (OD_{NC}) = $OD_{NCraw} - OD_{blank\ mean}$
– The mean corrected OD of the 3 negative control samples was calculated: this value corresponds to 100% viability

Positive control:

– Individual positive control OD values ($_{PCraw}$) were corrected with the mean blank OD:

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

– The mean corrected OD of the 3 positive control samples 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

Positive Control$_3$ % = (OD_{PC3} / mean OD_{NC}) ×100

– The mean value of the 3 individual relative viability % for positive control was calculated:

Mean PC % = (PC_1 % + PC_2 % + PC_3 %) / 3

Test item:

– Individual test item OD values ($_{TTraw}$) were corrected with the mean blank OD:

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

– The mean corrected OD of the 3 test item samples 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

Treated Tissue$_3$ % = (OD_{TT3} / mean OD_{NC}) ×100

– The mean value of the 3 individual relative viability % for test item was calculated:

Mean TT % = (TT_1 % + TT_2 % + TT_3 %) / 3

3.9.2 Data calculation for test items having MTT-interacting potential

Test items that interfere with MTT can produce non-specific reduction of the MTT. In this case, additional control samples are used to determine the OD value derived from non-specific reduction of the MTT. The measured OD value is corrected by the result of the additional controls before calculation of viability% as detailed below:

– Non-specific MTT reduction calculation (NSMTT%):

NSMTT (%) = [(OD_{KT}- OD_{KNC}) / OD_{NC}] × 100

OD_{KNC}: negative control killed tissues OD

OD_{KT}: test item treated killed tissues OD

OD_{NC}: negative control OD

If NSMTT% is ≤ 30%, then true MTT metabolic conversion (TOD_{TT}) is undertaken as follows:

TOD_{TT} = [OD_{TT} – (OD_{KT} – 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

$RV_2\ \% = [TOD_{TT2} / mean\ OD_{NC}] \times 100$
$RV_3\ \% = [TOD_{TT3} / mean\ OD_{NC}] \times 100$

– The mean value of the 3 individual relative viability % for test item is calculated:

$Mean\ Relative\ Viability\ \% = (RV_1\ \% + RV_2\ \% + RV_3\ \%) / 3$

If NSMTT% is > 30% relative to the negative control, then additional steps must be undertaken if possible, or the test item must be considered as incompatible with the test.

3.9.3 Data calculation for test items having colouring potential

For test items detected as able to stain the tissues the non-specific OD due to the residual chemical colour (unrelated to mitochondrial activity) is evaluated and subtracted before calculation of the "true" viability % as detailed below:

– Non-Specific Colour % with viable tissues (NSC_{living} %):

$NSC_{living}\ \% = (mean\ OD_{CTV} / mean\ OD_{NC}) \times 100$

OD_{CTV}: test substance treated viable tissues (not incubated with MTT)
OD_{NC}: negative control OD (incubated with MTT)

If NSC_{living} % is ≤ 5 % then the normal calculation mode is used (see 3.9.1).

If NSC_{living} % is > 5% and ≤ 30%, then true MTT metabolic conversion (TOD_{TT}) is undertaken as follows.

$TOD_{TT} = [OD_{TT} - mean\ OD_{CTV}]$

OD_{TT}: test substance treated viable tissue (incubated with MTT)
OD_{CTV}: test substance treated viable tissue (not incubated with MTT)

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

$RV_1\ \% = [TOD_{TT1} / mean\ OD_{NC}] \times 100$
$RV_2\ \% = [TOD_{TT2} / mean\ OD_{NC}] \times 100$
$RV_3\ \% = [TOD_{TT3} / mean\ OD_{NC}] \times 100$

– The mean value of the 3 individual relative viability % for test item is calculated:

$Mean\ Relative\ Viability\ \% = (RV_1\ \% + RV_2\ \% + RV_3\ \%) / 3$

If NSC_{living} % is > 30 % relative to the negative control, additional steps must be undertaken if possible, or the test substance must be considered as incompatible with the test.

3.9.4 Data calculation for substance having both MTT interacting potential and colouring potential

For test substances detected as able to stain the tissues (3.9.3) and interfere with MTT (3.9.2), a third set of controls is also required before calculation of the "true" viability %.

– Non-Specific Colour % with killed tissues (NSC_{killed} %):

$NSC_{killed}\ \% = (mean\ OD_{CTK} / mean\ OD_{NC}) \times 100$

OD_{CTK}: test substance treated killed tissues (not incubated with MTT)

OD_{NC}: negative control OD (incubated with MTT)

In that case the true MTT metabolic conversion (TOD_{TT}) is undertaken as follows:

$TOD_{TT} = [OD_{TT} – (OD_{KT} – OD_{KNC}) – mean\ OD_{CTV} + mean\ OD_{CTK}]$
OD_{TT}: test substance treated viable tissues (incubated with MTT)
OD_{KT}: test substance treated killed tissues OD
OD_{KNC}: negative control killed tissues OD
OD_{CTV}: test substance treated viable tissues (not incubated with MTT)
OD_{CTK}: test substance treated killed tissues (not incubated with MTT)

The % relative viability (% RV) for each test substance replicate is calculated relative to the mean negative control:
$RV_1\% = [TOD_{TT1} / mean\ OD_{NC}] \times 100$
$RV_2\% = [TOD_{TT2} / mean\ OD_{NC}] \times 100$
$RV_3\% = [TOD_{TT3} / mean\ OD_{NC}] \times 100$
The mean value of the 3 individual relative viability % for test substance is calculated:
$Mean\ Relative\ Viability\ \% = (RV_1\% + RV_2\% + RV_3\%) / 3$

3.10 Validity of the Test

The mean OD value of the three negative control tissues should be between 0.8 and 2.8 and the standard deviation value (SD) of the % viability should be ≤ 18.
The acceptable mean percentage viability range for positive controls is 0-20% and the standard deviation value (SD) of the % viability should be ≤ 18.
The SD calculated from individual % tissue viabilities of the three identically treated replicates should be ≤18.
The mean OD value of the blank samples (acidified isopropanol) should be <0.1.

3.11 Interpretation of Test Results

The irritation potential of test substances can be classified according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals [7], and a similar system is used in CLP [3]. In the present study, the irritancy potential of test substances is predicted by the mean tissue viability of tissues exposed to the test item. The test chemical is identified as requiring classification and labelling according to UN GHS (Category 2 or Category 1) if the mean relative viability of three individual tissues after 1 hour exposure to the test item and 42 hours post incubation is less or equal (≤) to 50% of the mean viability of the negative controls. In case the test chemical is found to be non-corrosive and shows tissue viability after exposure and post-treatment incubation is less than or equal (≤) to 50%, the test chemical is considered to be irritant to skin in accordance with UN GHS Category 2. The test item may be considered to be non-irritant to skin in accordance with UN GHS (No Category), if the mean relative viability of three individual tissues after 1 hour exposure to the test item and 42 hours post incubation is more than (>) 50% of the mean viability of the negative controls.

The prediction model (PM) is described below:

Criteria for *In Vitro* interpretation	Classification
	UN GHS
Mean tissue viability % is ≤ 50 %	Category 1 or Category 2
Mean tissue viability % is > 50 %	Non-Irritant*

*Note: If there is clear evidence that the test item is not corrosive, then it can be determined as No Category according to the UN GHS. It is plausible that some weaker corrosives could be classified as non-irritant in this *in vitro* assay.

4.0 RESULTS AND DISCUSSION

4.1 Additional Controls

Brown colour was observed after three hours of incubation of the test item in MTT working solution, thus the test material did not interact with MTT. Therefore, additional controls and data calculations were not necessary. The false estimation of viability can be excluded.

As the test item was coloured (brown), two additional test item-treated living tissues were used for the non-specific OD evaluation. The mean optical density (measured at 570 nm) of tissues was 0.004, non-specific colour percent was calculated as 0.2% (see Table 1). This value was below 5%, therefore additional data calculation was not necessary.

4.2 Viability Results

The results of the optical density (OD) measured at 570 nm for the test item, positive and negative controls and the calculated relative viability percentage values are presented in Table 2. The OD values were used to calculate the mean relative viability of the test item treated skin samples, which was shown to be 16.2% compared to the negative control.

4.3 Validity of the Test

Based on the observed colours, the epidermis units were in a suitable condition for use in the assay.

The mean OD value of the three negative control tissues was in the recommended range (1.759). Standard deviation of the viability results for negative control samples was 4.3%.

The positive control treated tissues showed 2.1% viability demonstrating the proper performance of the assay. The standard deviation of the viability results for positive control samples was 0.3%.

The standard deviation of viability values of the three test item-treated tissue samples in the MTT assay was 4.1%.

The mean OD value of the blank samples (isopropanol) was 0.048.

All these parameters met the acceptability criteria, therefore the study was considered to be valid.

Historical control data are presented in Appendix 3.

4.4 Discussion

Following exposure to isocycloseram/emamectin benzoate SC (A23220A), the mean cell viability was 16.2% compared to the negative control. This is below the threshold of 50%, therefore under the condition of this assay the test item was considered to be irritant to skin. The experiment met the validity criteria, therefore the study was considered to be valid.

5.0 CONCLUSIONS

In conclusion, under the conditions of this *in vitro* EpiDerm™ irritation assay conducted on isocycloseram/emamectin benzoate SC (A23220A), the results indicate that the test item is irritant to skin.

6.0 REFERENCES

1. OECD Guidelines for Testing of Chemicals, Section 4, No. 439, "*In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method", 18 June 2019
2. Commission Regulation (EC) No 761/2009 of 23 July 2009 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), ANNEX III, B.46. IN VITRO SKIN IRRITATION: RECONSTRUCTED HUMAN EPIDERMIS MODEL TEST and amended by Commission Regulation (EU) No 640/2012 of 6 July 2012
3. *In Vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT), For use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm™ (EPI-200-SIT), SOP, October 02th 2019
4. OECD (2015), *Acute Dermal Irritation/Corrosion*, OECD Guideline for the Testing of Chemicals No. 404, OECD, Paris
5. Faller C., Bracher M., Dami N. and Roguet R. (2002) Predictive activity of reconstructed human epidermis equivalents for assessment of skin irritation of cosmetics

6. Hungarian Good Laboratory Practice Regulations: 42/2014. (VIII. 19.) EMMI decree of the Ministry of Human Capacities which corresponds to the OECD GLP, ENV/MC/CHEM(98)17

7. UN (2019), United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Eighth revised edition, UN New York and Geneva.

8. EC-ECVAM (2009), Statement on the "Performance under UN GHS of three in vitro assays for skin irritation testing and the adaptation of the Reference Chemicals and Defined Accuracy Values of the ECVAM skin irritation Performance Standards", issued by the ECVAM Scientific Advisory Committee (ESAC30), 9 April 2009

9. Guidance on the Application of the CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 5.0, November 2017

10. Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application toproliferation and cytotoxicity assays. J. Immunological Methods 65, 55-62

7.0 DISTRIBUTION OF THE FINAL REPORT AMENDMENT 1

Sponsor: 1x PDF file, one Word file will be uploaded to collaborative website

Archive: 1x original, bound

TABLES SECTION

TABLE 1 Optical Density (OD) and the Calculated Non Specific Colour % (NSC_{living}%) of the Additional Control Tissues

Additional control		Optical Density (OD)		NSC% (living)
		Measured	Blank corrected	
Treated with	1	0.054	0.006	**0.2**
Isocycloseram/Emamectin Benzoate SC	2	0.049	0.001	
(A23220A)	mean	--	**0.004**	

Notes:

1. Mean blank value was 0.048.
2. Optical density means the mean value of the duplicate wells for each sample (rounded to three decimal places).

TABLE 2 Optical Density (OD) and the Calculated Relative Viability % of the Samples

Substance		Optical Density (OD)		Viability	Standard Deviation
		Measured	Blank corrected	(% RV)	(SD)
Negative Control:	1	1.894	1.846	104.9	--
Dulbecco's Phosphate Buffered Saline	2	1.755	1.707	97.0	--
	3	1.773	1.725	98.0	--
	mean	--	**1.759**	**100.0**	**4.3**
Positive Control:	1	0.085	0.037	2.1	--
5% (w/v) SDS solution	2	0.079	0.031	1.8	--
	3	0.089	0.041	2.3	--
	mean	--	**0.036**	**2.1**	**0.3**
Test Item:	1	0.328	0.280	15.9	--
Isocycloseram/Emamectin Benzoate SC	2	0.408	0.360	20.5	--
(A23220A)	3	0.265	0.217	12.4	--
	mean	--	**0.286**	**16.2**	**4.1**

Notes:

1. Mean blank value was 0.048.
2. Optical density means the mean value of the duplicate wells for each sample (rounded to three decimal places).

APPENDICES SECTION

APPENDIX 1 Certificate of Analysis

syngenta

Syngenta Crop Protection AG
GLP Testing Facility WMU
Analytical Development & Product Chemistr
Breitenloh 5
4333 Münchwilen, Switzerland

Certificate of Analysis

A23220A

isocycloseram/emamectin benzoate SC (200/050)

TSC002-041-001

Batch Identification	TSC002-041-001
Other Batch ID	1122866
Product Code	**A23220A**
Other Product Code(s)	isocycloseram/emamectin benzoate SC (200/050)
Chemical Analysis (Active Ingredient content)	
- **Identity of the Active Ingredient(s)***	**confirmed**
- **Content of isocycloseram***	**17.5 % w/w corresponding to 201 g/l**
- **Content of emamectin benzoate***	**4.18 % w/w corresponding to 48.1 g/l**
	The Active Ingredient(s) content is within the FAO limits.
Methodology used for Characterization / Recertification	LC, chiral LC, oscillating density meter
Physical Analysis	
- **Appearance**	**brown liquid**
- **Density***	**1150 kg/m³**
Stability:	
- **Storage Temperature**	**< 30°C**
- **Recertification Date**	**End of January 2023**

If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.
This Certificate of Analysis summarizes data which originates either from a single study or from several individual studies. Tests marked with an asterisk (*) have been conducted in compliance with GLP.
Raw data, documentation, study plans, any amendments to study plans and reports pertaining to this/these study/studies are stored under the study number(s) referenced below within the archives of the GLP Testing Facility WMU at Syngenta Crop Protection AG, Switzerland.

Study number of batch characterization: CHMU200180
Study number(s) of batch recertification:

Authorization: 19-Feb-2020

Dr. Karine Heintz
Analytical Development & Product Chemistry

Page 1 of 1

APPENDIX 2 Copy of the Test Kit Quality Control

Certificate of Analysis

MatTek | IN VITRO LIFE SCIENCE LABORATORIES

Product: EpiDerm™ Reconstructed Human Epidermis

Lot Number: 30867

Part#: EPI-200, EPI-212, EPI-218

Description: Reconstructed human epidermis tissue containing normal human keratinocytes. This product is for research use only. Not for use in animals, humans or diagnostic purposes.

I. Cell source

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

Keratinocyte Strain: 00267

II. Analysis for potential biological contaminants

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

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

III. Analysis for tissue functionality and quality

Test	Specification	Acceptance criteria	Result and QA Statement	
Tissue viability	MTT QC assay, 4 hours, n=3	OD [540-570 nm] [1.0-3.0]	1.724 ± 0.01	Pass
Barrier function	ET-50 assay, 100 µL 1% Triton X-100, 4 time-points, n=3, MTT assay	ET-50 [4.77-8.72 hrs]	7.14 hrs	Pass
Sterility	Long term antibiotic and antimycotic free culture	No contamination	Sterile	Pass

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

Initials: SL

Date: 20.05.20

Paul Kearney

Paul Kearney
Quality Assurance Director

May 20, 2020
Date

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

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

www.mattek.com
information@mattek.com

APPENDIX 3 Historical Control Data

(updated 24 September 2020)

	Mean value (OD)	Standard deviation (SD)	Minimum value (OD)	Maximum value (OD)	Number of cases
	Negative control				
Year 2020	1.895	0.164	1.627	2.167	24
	Positive control				
Year 2020	0.040	0.007	0.029	0.055	24
Year 2020 viability %	2.1	0.4	1.4	2.8	

Negative control: Dulbecco's phosphate buffered saline (DPBS)
Positive control: Sodium dodecyl sulphate (SDS)
SD: Standard deviation
OD: Optical density (absorbance)
Note: All OD values (measured at 570) are background corrected values.

APPENDIX 4 GLP Certificate

OGYÉI
National Institute of
Pharmacy and Nutrition

H-1051 Budapest, Zrínyi u. 3.
1372 P.O. Box:450.
Tel: +36 1 88 69-300, Fax: +36 1 88 69 460
E-mail: ogyei@ogyei.gov.hu, Web: www.ogyei.gov.hu

Ref. no: OGYÉI/22762-5/2018
Admin.: Dr. Juhász Uzonka
Date: 03 August 2018

GOOD LABORATORY PRACTICE (GLP)
CERTIFICATE

It is hereby certified that the test facility

CiToxLAB Hungary Ltd.

H-8200 Veszprém, Szabadságpuszta

is able to carry out

physico-chemical testing, toxicity studies, mutagenicity studies, environmental toxicity studies on aquatic or terrestrial organisms, studies on behaviour in water, soil and air; bio-accumulation, analytical and clinical chemistry, pathology studies, preparation of microscopic tissue sections, reproduction toxicology, in vitro studies, inhalation toxicology, and contract archiving

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

Date of the inspection: **07-11 May 2018.**

Tarjáni Ibolya
Head of Inspectorate

Országos Gyógyszerészeti és Élelmezésegészségügyi Intézet 12.

Remark: Translation of the Stamp on the official document ("Országos Gyógyszerészeti és Élelmezésegészségügyi Intézet"): ("National Institute of Pharmacy and Nutrition").

The legal name of Citoxlab Hungary Ltd. (formerly shown as CiToxLAB Hungary Ltd.) was changed on 28 December 2019 to Charles River Laboratories Hungary Kft.