Thiamethoxam

Thiamethoxam SL (A23943A) – *In Vitro* Skin Irritation Test in the EPISKIN™ Model

Final Report

TEST GUIDELINE(S): OECD No. 439 (2021)
EC No 640/2012, B.46. (2012)

AUTHOR(S): Balázs Orovecz, B.Sc.

COMPLETION DATE: 21 April 2022

PERFORMING LABORATORY: Charles River Laboratories Hungary Kft.
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Hungary

LABORATORY PROJECT ID: Report Number: 21/309-043B
Study Number: 21/309-043B
Task Number: TK0599650

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

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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: 21 April 2022

Balázs Orovecz, B.Sc.
Study Director

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Representative of Submitter/Sponsor:

_____ _____
Date

Submitter/Sponsor: Syngenta Crop Protection, LLC
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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Code: 21/309-043B

Study Title: Thiamethoxam SL (A23943A) – *In Vitro* Skin Irritation Test in the EPISKIN™ Model

This Study has been audited by Quality Assurance in accordance with the applicable Good Laboratory Practice regulations. Audit reports were submitted in accordance with SOPs as follows:

Date of Inspection	Phase(s) Inspected/Audited	Date of report to	
		Management	Study Director
28 December 2021	Study Plan	28 December 2021	28 December 2021
04 January 2022	Treatment	05 January 2022	05 January 2022
07 January 2022	Measurement	13 January 2022	13 January 2022
07 January 2022	Extraction	07 January 2022	07 January 2022
14 January 2022	Formulation	14 January 2022	14 January 2022
18 February 2022	Draft Report	18 February 2022	18 February 2022
01 April 2022	Final Report	01 April 2022	01 April 2022

In addition to the above-mentioned audits, (which may include study specific inspections and/or relevant process based inspections) routine facility inspections were also conducted.

The Final Report reflects the raw data and accurately and completely describes the methods and procedures of the study.

Signature: _____ Date: 21 April 2022

Eszter Sebestyén, B.Sc.
On behalf on 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 "Thiamethoxam SL (A23943A) – *In Vitro* Skin Irritation Test in the EPISKIN™ Model" was performed, in compliance with the Principles of Good Laboratory Practice.

Signature: _____ Date: 21 April 2022

Balázs Tóth, Ph.D.
General Manager

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
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Eszter Sebestyén, B.Sc.	Quality Assurance Auditor
Carolina Vaccari, Ph.D.	Syngenta Study Manager

Study dates

Study Plan:	03 January 2022
Experimental Starting Date:	04 January 2022
Experimental Completion Date:	07 January 2022
Date of Draft Report (non-QA Audited):	28 February 2022
Date of Draft Report (QA Audited):	01 March 2022
Date of Final Report:	21 April 2022

Note: MTT-pre check was performed on 04 January 2022.

Deviations from the Study Plan

There were no deviations to the Study Plan.

Performing laboratory test substance reference number

210612

Other

The study documents and samples:

- study plan,
- all raw data,
- sample of the test item and positive control 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 thiamethoxam SL (A23943A) in a reconstructed human epidermis model. The EpiSkin^{TM} 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 EpiSkin^{TM} (three units) were treated with the test item and incubated for 15 minutes at room temperature. Exposure of the test item to the EpiSkin^{TM} surface was terminated by rinsing the units with Phosphate Buffered Saline (PBS). The epidermis units were then incubated at 37°C for 42 hours in an incubator with 5% CO_2 in a >95% relative humidified (RH) atmosphere. After the 42-hour incubation, MTT solution was added to the units and incubated for a further 3 hour-period to determine cell viability. The precipitated formazan crystals were then extracted using acidified isopropanol and quantified spectrophotometrically at 570 nm.

The negative control epidermis units were treated with PBS, 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 cell viability after 15 minutes of exposure and 42 hours post incubation is less or equal (≤) to 50% of the negative control, the test item is considered to be an irritant to skin.

1.2 Results

Following exposure to thiamethoxam SL (A23943A), the mean cell viability was 130.6% compared to the negative control. This is above the threshold of 50%, therefore the test item was considered as being non-irritant to skin under the conditions of this assay. The experiment met the validity criteria, and therefore the study was considered to be valid.

1.3 Conclusion

In conclusion, under the conditions of this *in vitro* EpiSkin ^{TM} irritation assay, the results indicate that thiamethoxam SL (A23943A) is non-irritant to skin.

2.0 INTRODUCTION

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.

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 EpiSkin™ and parameters related to skin irritation.

EpiSkin™ 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 [5, 6]. 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", 14 June 2021
- 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, B.46.

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:	Thiamethoxam SL (A23943A)
Other names:	TMX 75 SL, EXF23867A
Batch number:	NSI001-085-017
Design code:	A23943A
Active ingredient content*:	6.63 % w/w corresponding to 75.42 g/L
Appearance:	Dark orange homogeneous and translucent liquid
Recertification date:	10 September 2023
Storage conditions:	Room temperature (< 30°C)
Safety precautions:	Enhanced safety precautions (nitrile gloves, goggles, face mask (ABEK-P3-filter), lab coat) for unknown materials were applied to ensure 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 (undiluted).

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 3.7.2.).

3.4.1 Negative control

Phosphate Buffered Saline:

Abbreviation:	PBS
Supplier:	Life Technologies Corporation
Batch number:	2276740
Expiry date:	30 March 2023

3.4.2 Positive control

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

SDS (5% w/v aqueous solution) was freshly prepared in the testing laboratory.

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

Sodium Dodecyl Sulphate:

Supplier:	Sigma-Aldrich
Batch number:	STBJ9692
Expiry date:	31 October 2026

Distilled water:

Supplier:	B. Braun Pharmaceuticals SA
Batch number:	11511Y25-2
Expiry date:	31 March 2024

3.5 Test System

3.5.1 Human skin

EpiSkin™ (Manufacturer: SkinEthic, France, Batch No.: 22-EKIN-001, Expiry Date: 10 January 2022) is a three-dimensional human epidermis model. Adult human-derived epidermal keratinocytes are seeded on a dermal substitute consisting of a collagen type I matrix coated with type IV collagen. A highly differentiated and stratified epidermis model is obtained after 13-day culture period comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum [7]. 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

EpiSkin™ 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 a MTT cell viability test and a cytotoxicity test with sodium dodecyl sulphate (SDS). These quality control experiments were conducted at SkinEthic laboratories (supplier of the EpiSkin™ test kits used in the present study) and are documented in Appendix 2.

3.5.3 Justification for selection of the test system

The EpiSkin™ 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

Units:	EpiSkin™ 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™ biopsy punch for easy sampling of epidermis
Medium:	A flask of sterile “Maintenance Medium” (Batch No.: 22-MAIN3-001; Exp. Date: 10 January 2022) A flask of sterile “Assay Medium” (Batch No.: 22-ESSC-001; Exp. Date: 10 January 2022)

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 pH of the agar medium used for transport was checked by checking the colour of the medium:

- 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 colour change is irreversible, independent of the length of the period above 40 °C):

- white colour = good
- grey or black colour = not acceptable

3.5.7 Storage

The EpiSkin ^{TM} kits were kept in their packaging at 37°C, the Assay Medium and Maintenance Medium supplied with the kits were stored at 2-8°C until the initiation of the test.

3.6 Additional Materials

3.6.1 MTT solution

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1] was diluted in phosphate buffered saline (PBS) at a final concentration of 3 mg/mL (MTT stock solution). The obtained stock solution (prepared on 04 January 2022) was stored in a refrigerator (2-8°C) protected from light. It was diluted with pre-warmed (37°C) Assay Medium to a final concentration of 0.3 mg/mL (MTT working solution) immediately before use.

3.6.2 Acidified isopropanol

Isopropanol was acidified with HCl acid to achieve a final concentration of 0.04M HCl (1.8 mL of 12M HCl acid was diluted in 500 mL isopropanol, or similar ratio was applied). The solution was prepared on the day of use.

3.6.3 Chemicals used in the experiment

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

Chemical	Supplier	Batch Number	Expiry date
MTT	Thermo Fisher	2406587	31 December 2022
Isopropanol (2-Propanol)	VWR International Ltd.	19D244010	23 April 2024
Hydrochloric acid (37% HCl)	VWR International Ltd.	18J094018	03 October 2023
Phosphate buffered saline (PBS)	Life Technologies Corporation	2215013	31 December 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

10 µL of test item was added to 2 mL MTT working solution and mixed. The mixture was incubated at 37°C for 3 hours protected from light, and then any colour change was recorded:

-Test items which directly react with MTT:	blue or purple
-Test items which do not react with MTT:	another colour

After the test item thiamethoxam SL (A23943A) had been incubated with MTT for three hours, yellow 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 and/or isopropanol** (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) was necessary to evaluate the ability of the test item to stain the epidermis.

**Note: Water is the environment during exposure, isopropanol is the extracting solution.

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 Maintenance Medium was pre-warmed to 37°C. The appropriate number of wells in an assay plate were filled with the pre-warmed medium (2 mL per well). The epidermis units

were placed with the media below them, in contact with the epidermis into each prepared well and then incubated overnight (at least 18 hours) at 37°C in an incubator with 5 % CO_2, in a >95 RH % humidified atmosphere.

3.8.2 Application and rinsing (Day 0)

Test Item

20 µL of test item was applied evenly to each of three test units and each additional control skin unit. The 10 µL test item was not sufficient to cover the epidermal surface.

Negative and positive controls

10 µL of negative control (PBS) or positive control (5% (w/v) SDS solution) was 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 Kft. study codes: 21/300-043B and 21/301-043B) performed in the same experimental period using the same batch of chemicals and same batch of skin units.

Incubation with Test Item

The plates with the treated epidermis units were incubated for the exposure time of 15 minutes (± 0.5 minute) at room temperature (22.9-25.7°C).

Rinsing

After the 15 minutes incubation time, the EpiSkin ™ units were removed and rinsed thoroughly with PBS (25mL) to remove as much of the remaining test material as possible from the epidermal surface. The remaining PBS was removed from the epidermal surface with a pipette (without touching the epidermis).

After rinsing the units were placed into the plate wells with fresh pre-warmed Maintenance Medium (2 mL/well) and then incubated for 42 hours (±1 hour) at 37°C in an incubator with 5% CO_2, in a >95 RH % humidified atmosphere.

3.8.3 MTT test (Day 2)

After the 42 hours incubation, all EpiSkin ™ units (except the two colour control units, which were incubated with Assay Medium) were transferred into the MTT working solution filled wells (2 mL of 0.3 mg/mL MTT per well). Then, all transferred EpiSkin ™ units were incubated for 3 hours at 37°C in an incubator with 5% CO_2 protected from light, in a >95 RH % humidified atmosphere.

3.8.4 Formazan extraction (Day 2)

After the incubation with MTT, a formazan extraction was undertaken. A uniform-size disk of epidermis was cut from each skin unit (this involved 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 containing 500 µL acidified isopropanol (one tube corresponded to one well of the assay plate).

The capped tubes were thoroughly mixed by using a vortex mixer to achieve a good contact of all of the material and the acidified isopropanol, and then incubated for about two hours at room temperature protected from light with gentle agitation (~150 rpm) for formazan extraction.

A blank sample consisting of an empty tube filled with 2 mL of acidified isopropanol was processed in parallel.

3.8.5 Cell viability measurements (Day 2)

Following the formazan extraction, 2×200 µL samples 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 6 wells of acidified isopropanol solution (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: 14 October 2020, calibration is valid until October 2022) 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 six 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 % = (PC1 % +PC2 % +PC3 %) / 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 % = (TT1 %+TT2 %+TT3 %) / 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}] × 100
RV 3 % = [TOD_{TT3} / mean OD_{NC}] × 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}*) ×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}*) ×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 0.6 and 1.5 and the standard deviation value (SD) of the % viability values should be ≤ 18.

The acceptable mean percentage viability range for the three positive controls is 0-40% and the standard deviation value (SD) of the % viability values should be ≤ 18.

The SD calculated from individual % tissue viability values of the three test item 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 items can be classified according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals [9], and a similar system is used in CLP [11,12]. In the present study, the irritancy potential of test items 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 15 minutes of 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 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 15 minutes of 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 2 or Category 1
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

Yellow 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, 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 0.015, non-specific colour percent was calculated as 1.8% (see Table 1). This value was below 5%, therefore an additional data calculation to account for non-specific colouring 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 (RV%) 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 130.6%.

4.3 Validity of the Test

After receipt, the two indicators included in the package of the delivered kits (reflecting the storage temperature history and the pH) were checked. 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 (0.838). Standard deviation of the viability results for negative control samples was 4.7%.

The positive control treated tissues showed 15.9% viability demonstrating the proper performance of the assay. The standard deviation of the viability results for positive control samples was 3.3%.

The standard deviation of viability values of the three test item-treated tissue samples in the MTT assay was 6.4%.

The mean OD value of the blank samples (acidified isopropanol) was 0.047.

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 of the EpiSkin™ model to the test item, thiamethoxam SL (A23943A), the mean relative viability was 130.6% compared to the negative control value. This is above the threshold of 50%, therefore under the condition of this assay the test item was considered to be non-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* EpiSkin™ irritation assay conducted on thiamethoxam SL (A23943A), the results indicate that the test item is non-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", 14 June 2021
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. EpiSkin™ SOP, Version 1.8 (February 2009), ECVAM Skin Irritation Validation Study: Validation of the EpiSkin™ test method 15 min - 42 hours for the prediction of acute skin irritation of chemicals
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. Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application toproliferation and cytotoxicity assays. *J. Immunological Methods* 65, 55-62

7. Tinois E., Gaetani Q., Gayraud, B., Dupond D., Rougier A. and Pouradier-Duteil X. (1994) The Episkin model: Successful reconstruction of a human epidermis *in vitro*. In: Rougier, A., Goldberg M., Maibach H.I. Eds. *In Vitro* Skin Toxicology. Mary Ann Liebert, New York, pp. 133-140

8. 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

9. UN (2021), United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Ninth revised edition, UN New York and Geneva.

10. 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

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

12. 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

7.0 DISTRIBUTION OF THE FINAL REPORT

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	0.055	0.008	**1.8**
Thiamethoxam SL (A23943A)	2	0.069	0.022	
	mean	--	**0.015**	

Notes:

1. Mean blank value was 0.047.
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 (RV%)	Standard Deviation (SD)
		Measured	Blank corrected		
Negative Control:	1	0.840	0.792	94.6	--
Phosphate buffered saline	2	0.904	0.856	102.2	--
	3	0.912	0.864	103.2	--
	mean	--	**0.838**	**100.0**	**4.7**
Positive Control:	1	0.177	0.130	15.5	--
5% (w/v) SDS solution	2	0.155	0.107	12.8	--
	3	0.210	0.163	19.4	--
	mean	--	**0.133**	**15.9**	**3.3**
Test Item:	1	1.190	1.143	136.4	--
Thiamethoxam SL (A23943A)	2	1.084	1.036	123.7	--
	3	1.151	1.103	131.7	--
	mean	--	**1.094**	**130.6**	**6.4**

Notes:

1. Mean blank value was 0.047.
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

ALS Laboratórios LS Ltda.
Rua Fábia, 59 – CEP: 05051-030
São Paulo, SP - Brazil

SYNGENTA PROTEÇÃO DE CULTIVOS Ltda.
Rua Doutor Rubens Gomes Bueno nº 691,
11º andar, Torre Sigma
CEP 04730-000 – Bairro Várzea de Baixo
São Paulo-SP – Brazil

Certificate of Analysis

A23943A
Thiamethoxam SL (075)
NSI001-085-017

Batch Identification	NSI001-085-017
Product Code	A23943A
Other Product Code(s)	A23943A; EXF23867A; CGA293343 SL (075); Thiamethoxam SL (075)
EUP number:	739/2021 Expiry date: 26/04/2024
Received on:	28 September 2021
Source	Syngenta Proteção de Cultivos Ltda. Rodovia Professor Zeferino Vaz, SP 332, s/nº, km 127,5 – Bairro Santa Terezinha, CEP 13148-915 – Paulínia-SP – Brasil

Chemical Analysis
(Active Ingredients Content)

– **Content of Thiamethoxam ***	**6.63 % w/w corresponding to 75.42 g/L**

The Active Ingredient content is within the FAO limits.
Methodology used for Characterization: HPLC (SF-1151/1)

Physical Analysis

– **Appearance**	Homogeneous and translucent
– **Color**	6/12 – 5YR (Dark Orange)
– **Physical state**	Liquid
– **Density ***	1.1378 g/cm³

Stability:

– **Storage Temperature**	**<30°C**
– **Recertification Date**	**10 September 2023**

If stored under the conditions given above, this test item 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. All original raw data, including any storage medium for electronically recorded data, documentation, the signed study plan, the protocol amendments, the final report and a sample of the test item will be retained in the GLP Archives at ALS Laboratórios LS Ltda.

Study number of batch characterization: 26785/2021CF and 26787/2021CC

Authorization: 10 November 2021

Victor F. G. da Silva
Victor Ferreira Gomes da Silva
ALS Laboratórios LS Ltda.

CA2144073 -B

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APPENDIX 2 Copy of the Test Kit Quality Control

EPISKIN

TECHNICAL DATA, SAFETY SHEET AND CERTIFICATE OF ANALYSIS

CCE-091-SM D13-S/04

NAME

EpiSkin™ Small / Human Epidermis (SM/13)

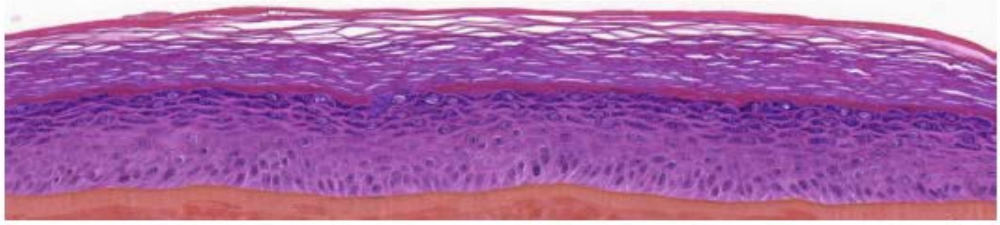
DESCRIPTION

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

BATCH : 22-EKIN-001
ORIGIN : Adult donors
USAGE : FOR SCIENTIFIC USE ONLY - PRODUCT OF HUMAN ORIGIN
STORAGE : This product was prepared and packaged using aseptic techniques. Store in an incubator at 37°C, 5% CO2 with saturated humidity

QUALITY CONTROLS

Control # E220002

	Process	Specification	Result
HISTOLOGY	HES stained paraffin section	Multi-layered, highly differentiated epidermis consisting of organized basal, spinous and granular layers, and a multilayered *stratum corneum*	Satisfactory
		Number of cell layers ≥ 4	8 cell layers
			
IC50 DETERMINATION	SDS concentration, MTT test.	1.5 mg/mL ≤ IC50 ≤ 3.0 mg/mL	2.1 mg/mL

BIOLOGICAL SAFETY:

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

SUGGESTED EXPIRATION DATE:

January 10, 2022

Lyon, January 4, 2022
Certified and released by **Anaïs JENSEN**, Quality Control Manager

Manufactured in accordance to the ISO9001 quality system of Episkin.

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

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

APPENDIX 3 Historical Control Data

(updated: 25 August 2021)

	Mean value (OD)	Standard deviation (SD)	Minimum value (OD)	Maximum value (OD)	Number of cases
	Negative control				
Year 2014-2020	0.811	0.127	0.573	1.381	630
	Positive control				
Year 2014-2020	0.062	0.045	0.007	0.354	624
Year 2014-2020 viability %	7.6	5.2	0.8	29.7	

Note: The HC data of the last 7 years are shown in the table. All OD values (measured at 570 ±30 nm) are background corrected values. Before 27 February 2019 the treatment volume was 50µl. After 27 February 2019 the treatment volume was 20µl. After 08 July 2020 the treatment volume is 10µl due to scientific reasons.
Negative control: Phosphate buffered saline (PBS)
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 Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet

Hatósági Ellenőrzési Főosztály

1051 Budapest, Zrínyi utca 3.
Levélcím: 1372 Postafiók 450
Tel.: +36 1 886 9300, Fax: +36 1 886 9460
E-mail: ogyei@ogyei.gov.hu
Web: www.ogyei.gov.hu

Ref. no: OGYÉI/-29520-2/2021
Admin.: Dr. Szaller Zoltán

GOOD LABORATORY PRACTICE (GLP)
CERTIFICATE

It is hereby certified that the test facility

Charles River Laboratories Hungary Kft.

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.**

This certificate is valid up to 11th of May, 2022.

Dr. Lukács Ferenc József
Digitálisan aláírta: Dr. Lukács Ferenc József
Dátum: 2021.05.06 13:04:14 +02'00'

Dr. Ferenc Lukács
Head of Inspectorate

Note: Translation of the text of the certificate in the header: ("Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet") - ("National Institute of Pharmacy and Nutrition"); (“Hatósági Ellenőrzési Főosztály”) - (Inspectorate Division) and at the signature: (“Digitálisan aláírta”) - (Digitally signed); (“Dátum”) - (“Date”).