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Safety and Health Research

Isopyrazam/Difenoconazole

Isopyrazam/Difenoconazole SC (A21295D) – *In Vitro* Skin Irritation Test in the EPISKIN™ Model

Final Report

DATA REQUIREMENT(S): OECD No. 439 (2015)
Commission Regulation (EC) No 761/2009, ANNEX III, B.46.
Guidance Document on Toxicology for Registration of Pesticides in India (2014).

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COMPLETION DATE: 09 January 2018

PERFORMING LABORATORY: Citoxlab Hungary Ltd.
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LABORATORY PROJECT ID: Report Number: 17/135-043B
Study Number: 17/135-043B
Task Number: TK0296437

SPONSOR(S): Syngenta Ltd.
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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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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.

Signature: _____ Date: 05 January 2018

Balázs Orovecz, B.Sc.
Study Director

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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Number: 17/135-043B

Study Title: Isopyrazam/Difenoconazole SC (A21295D) – *In Vitro* Skin Irritation Test in the EPISKIN™ Model

Test Item: Isopyrazam/Difenoconazole SC (A21295D)

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

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

Date of Inspection	Phase(s) Inspected/Audited	Date of report to	
		Management	Study Director
23 May 2017	Study Plan	23 May 2017	23 May 2017
24 May 2017	Treatment	24 May 2017	24 May 2017
10 August 2017	Draft Report	10 August 2017	10 August 2017
09 January 2018	Final Report	09 January 2018	09 January 2018

Signature: _____ Date: 09 January 2018

Orsolya Girst, M.Sc.
On Behalf of QA

MANAGEMENT STATEMENT

According to the conditions of the research and development agreement between Syngenta Ltd. (as Sponsor) and Citoxlab Hungary Ltd. (as Test Facility) the study titled "Isopyrazam/Difenoconazole SC (A21295D) – *In Vitro* Skin Irritation Test in the EPISKIN™ Model" was performed, in compliance with the Principles of Good Laboratory Practice.

Signature: _____ Date: 09 Jan. 2018

Jan Praslicka, DVM, PhD.
Scientific Director

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Function
Balázs Orovecz, B.Sc.	Study Director
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Nikolett Németh, B.Sc.	Quality Assurance Unit
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Study dates

Study Plan:	23 May 2017
Experimental Starting Date:	24 May 2017
Experimental Completion Date:	26 May 2017
Date of Draft Report:	17 August 2017
Date of Final Report:	09 January 2018

Deviations from the Study Plan

There was no deviation to the Study Plan.

Performing laboratory test substance reference number

170120

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 Citoxlab Hungary Ltd. 8200 Veszprém, Szabadságpuszta, 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 Isopyrazam/Difenoconazole SC (A21295D) in a reconstructed human epidermis model. The EpiSkin™ 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) assay (detailed in 3.6. section). The irritation potential of the test item was evaluated according to the OECD guideline No. 439 [1].

Disks of EpiSkin™ (three units) were treated with the test item and incubated for 15 minutes at room temperature. Exposure of the test item to the EpiSkin™ 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 RH % humidified atmosphere. After the 42 hour incubation MTT solution was added to the units and incubated for a further 3 hours 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 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 Isopyrazam/Difenoconazole SC (A21295D), the mean cell viability was 69.7% 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™ irritation assay, the results indicate that Isopyrazam/Difenoconazole SC (A21295D) 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 (OECD No. 404) [4] and is specifically approved as a replacement for the *in vivo* skin irritation test within OECD No. 439, under REACH (EC 761/2009).

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 (Faller C. et al., 2002, Mosmann T., 1983) [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 test guideline 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", 28 July 2015
- 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

- Guidance Document on Toxicology for Registration of Pesticides in India (2014). Ministry of Agriculture Department of Agriculture & co-operation Central Insecticides Board & Registration Committee Directorate of Plant Protection Quarantine & Storage. NH-IV, Faridabad

2.3 Test Facility

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

3.0 MATERIALS AND METHODS

3.1 Test Substance

Information supplied by the Sponsor:

Name:	Isopyrazam/Difenoconazole SC (A21295D)
Batch number:	JHU002-109-004
Product code:	A21295D
Other Product Code:	CGA169374/SYN520453 SC (125/125)
Appearance:	Off-white liquid
Active Ingredient Content:	CGA169374: 11.9 % w/w corresponding to 129 g/L CGA185882: 6.97 % w/w corresponding to 76.0 g/L CGA185883: 4.89 % w/w corresponding to 53.3 g/L SYN520453: 11.9 % w/w corresponding to 130 g/L SYN534969: 10.1 % w/w corresponding to 110 g/L SYN534968: 1.82 % w/w corresponding to 19.9g/L
Density:	1091 kg/m^3
Recertification date:	30 November 2018
Storage conditions:	Room temperature (<30ºC)
Safety precautions:	Routine safety precautions (gloves, goggles, face mask, lab coat) for unknown materials were applied to assure personnel health and safety.

Note: The Test Item was treated as 100% and no adjustment for purity was applied.

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 is the responsibility of the Sponsor.

3.2 Identification and Receipt

The test item of a suitable chemical purity 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 in the Pharmacy Unit of Citoxlab Hungary Ltd. on the basis of the information provided by Sponsor.

3.3 Test Item Preparation

The test item was applied in its original form.

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

Phosphate Buffered Saline:

Abbreviation:	PBS
Supplier:	Sigma-Aldrich Co.
Batch number:	BCBQ2925V
Expiry date:	January 2020

3.4.2 Positive control

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

SDS (5% w/v) 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 Co.
Batch number:	MKBV0024V
Expiry date:	19 August 2017

Distilled water:

Supplier:	Hungaro-Gal Kft
Batch number:	803 0117
Expiry date:	30 July 2017

3.5 Test System

3.5.1 Human skin

EpiSkin™ (Manufacturer: SkinEthic, France, Batch No.: 17-EKIN-021, Expiry Date: 29 May 2017) is a three-dimensional human epidermis model. Adult human-derived epidermal keratinocytes are seeded on a dermal substitute consisting of a collagen type I matrix coated with type IV collagen. A highly differentiated and stratified epidermis model is obtained after 13-day culture period comprising the main basal, supra basal, spinous and granular layers and a functional stratum corneum (Tinois et al., 1994) [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 dodecylsulphate (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 [11] 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.: 17 MAIN3 021; Exp. Date: 31 May 2017) A flask of sterile "Assay Medium" (Batch No.: 17 ESSC 020; Exp. Date: 31 May 2017)

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

The kits were found to be in good order at reception and suitable for use in the assay.

3.5.7 Storage

The EpiSkin ^{TM} kit was 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 23 May 2017) 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	Amresco	0905C424	31 December 2017
Isopropanol (2-Propanol)	VWR International Ltd.	15I250505	September 2020
Phosphate buffered saline (PBS)	Sigma-Aldrich Co.	BCBQ2925V	January 2020
Hydrochloric acid (37% HCl)	VWR International Ltd.	16E044011	May 2021

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 in a shaking water bath for 3 hours (±5minutes) protected from light, and then any colour change was recorded:

-Test items which do not react with MTT: yellow
-Test items reacting with MTT: blue or purple

After the test item, Isopyrazam/Difenoconazole SC (A21295D), had been incubated with MTT for three hours, a yellow colour of the mixture was detected in the test tube. Thus, the test item did not react with MTT and therefore the use of additional controls was not necessary.

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 (off-white liquid), the use of additional control tissues (determination of the non-specific colour percentage) were necessary to evaluate the ability of 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

In addition to the normal procedure (section 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 was filled with the pre-warmed medium (2 mL per well). The epidermis units were placed with the media below them, in contact with the epidermis into each prepared well and then incubated overnight at 37°C in an incubator with 5 % CO_2, in a >95% humidified atmosphere.

3.8.2 Application and rinsing (Day 0)

Test Item

20 µL of test item were applied evenly to the epidermal surface. If necessary, the test item was spread gently on the skin surface with a pipette tip without damaging the epidermis. The amount was sufficient to cover the epidermal surface.

Negative and positive controls

50 µL of negative control (PBS) 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 a concurrent studies (Citoxlab study codes: 17/095-043B, 17/120-043B, 17/029-043BE and 17/104-043B) 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 min) at room temperature (24.9-26.8°C).

Rinsing

After the 15 minutes incubation time, the EpiSkin ™ units were removed and rinsed thoroughly with PBS to remove as much of the remaining 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 (± 1h) 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 of two colour control units) 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 (± 5 min) 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 well 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 sample from each tube was 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: 22 August 2016, calibration is valid until August 2018) 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 were 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 were 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 were 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%):

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

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:

$$\%\ RV\ 1 = [TOD_{TT1} / mean\ OD_{NC}] \times 100$$
$$\%\ RV\ 2 = [TOD_{TT2} / mean\ OD_{NC}] \times 100$$
$$\%\ RV\ 3 = [TOD_{TT3} / mean\ OD_{NC}] \times 100$$

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 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 % 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) is also require a third set of controls 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 0.6 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 (UN GHS) of Classification and Labelling of Chemicals [10], and a similar system is used in CLP [12,13]. 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 item considered to be irritant to skin (GHS category 1 or category 2), if the mean relative viability after 15 minutes exposure and 42 hours post incubation is less or equal (≤) to 50% of the negative control.

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

The test materials did not react with MTT as no colour change (yellow colour) was observed after three hours of incubation of the test item in MTT working solution. 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 were 0.026, non-specific colour percent was calculated as 3.8% (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 for the test item treated skin samples showed 69.7% relative viability.

4.3 Validity of the Test

After receipt, the two indicators of fitness for the delivered kit (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 (0.681). Standard deviation of the viability results for negative control samples was 5.5%.

The three positive control treated tissues showed 4.3% cell viability demonstrating the proper performance of the assay. The standard deviation of the viability results for positive control samples was 0.7%.

Note: The OD data of two replicate tissues at the positive control in this study was lower (0.024 and 0.030) than the minimum OD of the historical control range (0.032). This fact has no impact on the results or integrity of the study since the positive control material showed severe effect.

The standard deviation of viability values of the three test item-treated tissue samples in the MTT assay was 5.1%.

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^{TM} model to the test item, Isopyrazam/Difenoconazole SC (A21295D), the mean relative viability was 69.7% 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 Isopyrazam/Difenoconazole SC (A21295D), the results indicate that the test item is a non-irritant to skin.

6.0 REFERENCES

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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%) of the Additional Control Tissues

Additional control		Optical Density (OD)		NSC%
		Measured	Blank corrected	
Treated with Isopyrazam/Difenoconazole SC (A21295D)	1	0.067	0.019	**3.8**
	2	0.079	0.032	
	mean	--	**0.026**	

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
		Measured	Blank corrected	(% RV)
Negative Control:	1	0.694	0.647	95.0
Phosphate buffered saline	2	0.768	0.721	105.9
	3	0.722	0.674	99.1
	mean	--	**0.681**	**100.0**
Positive Control:	1	0.077	0.030	4.4
5% (w/v) SDS solution	2	0.071	0.024	3.5
	3	0.081	0.033	4.9
	mean	--	**0.029**	**4.3**
Test Item:	1	0.532	0.485	71.2
Isopyrazam/Difenoconazole SC (A21295D)	2	0.551	0.503	73.9
	3	0.483	0.436	64.0
	mean	--	**0.475**	**69.7**

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

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GLP Testing Facility WMU
Analytical Development &
Product Chemistry GS2131

Syngenta Crop Protection Münchwilen AG
Im Breitenloh 5
4333 Münchwilen, Switzerland

Certificate of Analysis

A21295D
CGA169374/SYN520453 SC (125/125)
JHU002-109-004

Batch Identification	**JHU002-109-004**
Product Code	**A21295D**
Other Product Code(s)	CGA169374/SYN520453 SC (125/125)

Chemical Analysis
(Active Ingredient Content)

– **Identity of the Active Ingredients***	**Confirmed**
– **Content of CGA169374 (difenoconazole)***	**11.9 % w/w corresponding to 129 g/l**
– **Content of CGA185882 (cis isomer of difenoconazole)***	**6.97 % w/w corresponding to 76.0 g/l**
– **Content of CGA185883 (trans isomer of difenoconazole)***	**4.89 % w/w corresponding to 53.3 g/l**
– **Content of SYN520453 (isopyrazam)***	**11.9 % w/w corresponding to 130 g/l**
– **Content of SYN534969 (syn isomer of isopyrazam)***	**10.1 % w/w corresponding to 110 g/l**
– **Content of SYN534968 (anti isomer of isopyrazam)***	**1.82 % w/w corresponding to 19.9 g/l**

The Active Ingredient(s) content is within the FAO limits.
Methodology used for Characterization HPLC, OECD 109 (oscillating density meter)

Physical Analysis

– **Appearance**	**Off-white liquid**
– **Density***	**1091 kg/m^3**

Stability:

– **Storage Temperature**	**< 30 °C**
– **Recertification Date**	**End of November 2018**

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 Münchwilen AG, Switzerland.

syngenta

GLP Testing Facility WMU
Analytical Development &
Product Chemistry GS2131

Syngenta Crop Protection
Münchwilen AG
Im Breitenloh 5
4333 Münchwilen, Switzerland

Certificate of Analysis

Study number of batch characterization:	CHMU151002
Study number(s) of batch recetification:	---
Authorisation:	14-Dec-2015

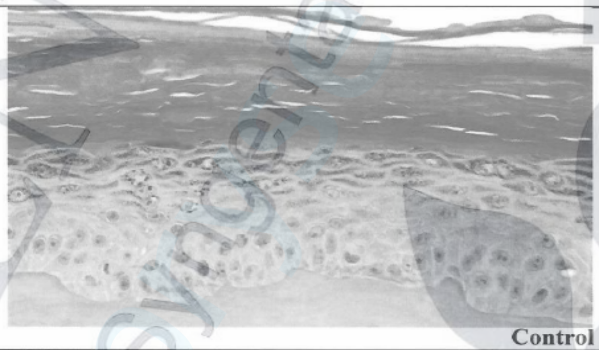
Elke Ebi
Analytical Development & Product Chemistry

APPENDIX 2 Copy of the Test Kit Quality Control

EPISKIN

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

CCE-091-SM D13-S/02

Description:	**Episkin Small Model** 0.38 cm² reconstructed epidermis of normal human keratinocytes. Cells are grown on a collagen matrix, for 13 days.		
Usage:	**FOR SCIENTIFIC USE ONLY - PRODUCT OF HUMAN ORIGIN**		
Storage:	This product was prepared and packaged using aseptic techniques. Store in an incubator at 37°C, 5% CO_2 with saturated humidity.		
Passage:	Second (Strains n° : 09-KERA-009 + 11-KERA-002)		
Batch N°:	**17-EKIN-021**		
Origin:	Adult donors.		
Histology:	 **Control n° E170674**		
Quality Controls:	**Test**	**Specification**	**Result**
	Histology scoring (HES stained vertical paraffin sections)	≥ 19.5	**22.7 ± 0.3** (CV = 1.1 %)
		Well-differentiated epidermis consisting of a basal layer, several spinous and granular layers and a thick stratum corneum	Satisfactory
	IC 50 determination (SDS concentration, MTT test)	1.5 mg/mL ≤ IC50 ≤ 3 mg/mL	**1.8 mg/mL**
	Statistical Analysis: → Histology : probability 0.95 that 100 % of the batch > 20 → IC 50 : probability 0.95 that IC 50 ≥ 1.7 mg/mL (threshold value)		
Biological safety:	On blood of the same donors, we have verified: . the absence of HIV1 and 2 antibodies . the absence of hepatitis C antibodies . the absence of hepatitis B antigen HBs On epidermal cells of the same donors, we have verified: . the absence of bacteria, fungus and mycoplasma		
Expiration date	May 29, 2017.		

"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"

Lyon, May 23, 2017.
Certified and released by
Anaïs JENSEN, Quality Control Manager
Manufactured in accordance to the ISO9001 quality system of Episkin.

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

Mod. 005C

APPENDIX 3 Historical Control Data

(updated 11 August 2016)

	Negative control (PBS)	Positive control (5% (w/v) SDS solution)
Mean optical density (OD)	0.802	0.094
Standard deviation	0.157	0.048
Minimum optical density (OD)	0.573	0.032
Maximum optical density (OD)	1.362	0.354
Number of cases	111	102

PBS: Phosphate buffered saline
SDS: Sodium dodecyl sulphate
OD: Optical density (absorbance)
Note: All OD values (measured at 570 ±30 nm) are background corrected values.

APPENDIX 4 GLP Certificate

OGYÉI
National Institute of
Pharmacy and Nutrition

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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: OGYI/19440-7/2015
Admin.: Szatmári Andrea
Date: 22 September, 2015

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, in vitro studies and mutagenicity studies, environmental toxicity studies on aquatic or terrestrial organisms, studies on behaviour in water, soil and air; bio-accumulation, reproduction toxicology, inhalation toxicology, analytical chemistry 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: **02-04. June 2015.**

Dr. József Reiter
Deputy Director-General

Note: Translation of the Stamp on the official document ("Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet"): ("National Institute of Pharmacy and Nutrition")