



**Propiconazole/Pydiflumetofen**

**Propiconazole/Pydiflumetofen SE (A21573C) –  
*In Vitro* Skin Irritation Test in the EPISKIN™ Model**

**Final Report**

**DATA REQUIREMENT(S):** OECD No. 439 (2015)  
Supplemental to EPA 870.2500  
EC No 761/2009,  
B.46. (2009)

**AUTHOR(S):** Barbara Varga-Kanizsai, M.Sc.

**COMPLETION DATE:** 26 April 2018

**PERFORMING LABORATORY:** Citoxlab Hungary Ltd.  
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**LABORATORY PROJECT ID:** Report Number: 17/349-043B  
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**SPONSOR(S):** Syngenta Ltd.  
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**VOLUME 1 OF 1 OF STUDY**  
**PAGE 1 OF 35**

## STATEMENT OF DATA CONFIDENTIALITY CLAIMS

### STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS UNDER SPECIFIED FIFRA PROVISIONS

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA sec. 10(d)(1)(A), (B), or (C) and which pertains to a registered or previously registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA 10(g).

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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: Varga-Kanizsai Barbara Date: 26 April 2018  
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## **FLAGGING STATEMENT**

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## QUALITY ASSURANCE STATEMENT

Study Number: 17/349-043B

Study Title: Propiconazole/Pydiflumetofen SE (A21573C) – *In Vitro* Skin Irritation Test in the EPISKIN™ Model

Test Item: Propiconazole/Pydiflumetofen SE (A21573C)

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
29 November 2017	Study Plan	29 November 2017	29 November 2017
13 December 2017	Treatment	13 December 2017	13 December 2017
25 January 2018	Draft Report	25 January 2018	25 January 2018
23 April 2018	Final Report	23 April 2018	23 April 2018

Signature: Girst Orsolya  
Orsolya Girst, M.Sc.  
On Behalf of QA

Date: 26 April 2018

## 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 “Propiconazole/Pydiflumetofen SE (A21573C) – *In Vitro* Skin Irritation Test in the EPISKIN™ Model” was performed, in compliance with the Principles of Good Laboratory Practice.

Signature:   
Alyson Leyshon, M.Sc.  
Managing Director

Date: 26 April 2018



## GENERAL INFORMATION

### Contributors

The following contributed to this report in the capacities indicated:

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### Study dates

Study Plan:	30 November 2017
Experimental Starting Date:	13 December 2017
Experimental Completion Date:	15 December 2017
Date of Draft Report:	25 January 2018
Date of Final Report	26 April 2018

### Deviations from the Study Plan

There was no deviation to the Study Plan.

### Performing laboratory test substance reference number

170356

### 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 propiconazole/pydiflumetofen SE (A21573C) 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<sub>2</sub> in a >95RH% 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<sub>living</sub>) 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 ( $\leq$ ) to 50% of the negative control, the test item is considered to be an irritant to skin.

### 1.2 Results

Following exposure to propiconazole/pydiflumetofen SE (A21573C) the mean cell viability was 78.5% 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 propiconazole/pydiflumetofen SE (A21573C) is a 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.

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 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

### 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:	Propiconazole/Pydiflumetofen SE (A21573C)
Batch number:	1007839
Active ingredient content*:	Pydiflumetofen, 13.7 % w/w corresponding to 151 g/L Propiconazole, 11.6 % w/w corresponding to 128 g/L
Density:	1,100 g/cm <sup>3</sup>
Appearance:	Beige liquid
Recertification date:	31 October 2020
Storage conditions:	Room temperature (<30°C)
Safety precautions:	Enhanced safety precautions were applied considering the supplied safety datasheet to assure personnel health and safety.
Hazards:	<b>May cause cancer.</b> May cause damage to organs through prolonged or repeated exposure.

\*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/Manufacturer:	Sigma-Aldrich Co.
Batch number:	BCBT9380
Expiry date:	September 2021

#### **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/Manufacturer:	Lach-ner
Batch number:	PP/2016/11637
Expiry date:	30 June 2021

Distilled water:

Supplier/Manufacturer:	Hungaro-Gal Kft.
Batch number:	8150917
Expiry date:	25 March 2018

### **3.5 Test System**

#### **3.5.1 Human skin**

EpiSkin™ (Manufacturer: SkinEthic, France, Batch No.: 17-EKIN-050, Expiry Date: 18 December 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 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 [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 <sup>2</sup> ) 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 055; Exp. Date: 20 December 2017) A flask of sterile “Assay Medium” (Batch No.: 17 ESSC 049; Exp. Date: 20 December 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™ 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 12 December 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	0646C193	28 February 2019
Isopropanol (2-Propanol)	VWR International Ltd.	17F284017	23 June 2022
Phosphate buffered saline (PBS)	Sigma-Aldrich Co.	BCBT9380	September 2021
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, propiconazole/pydiflumetofen SE (A21573C) 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 (beige 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 equipments).

### **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<sub>2</sub>, 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: 17/348-043B, 17/350-043B and 17/306-051B) 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 ( $\pm 0.5$  min) at room temperature (22.5-25.1°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 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 ( $\pm 1$ h) at 37°C in an incubator with 5% CO<sub>2</sub>, 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 ( $\pm$  5 min) at 37°C in an incubator with 5% CO<sub>2</sub> 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  $\mu$ L 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 $\times$ 200  $\mu$ L 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  $\mu$ L/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 ( $OD_{NCraw}$ ) were corrected with the mean blank OD:

$$OD \text{ Negative Control } (OD_{NC}) = OD_{NCraw} - OD_{blank \text{ mean}}$$

- 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 ( $OD_{PCraw}$ ) were corrected with the mean blank OD:

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

- 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:

$$\% \text{ Positive Control } 1 = (OD_{PC1} / \text{mean } OD_{NC}) \times 100$$

$$\% \text{ Positive Control } 2 = (OD_{PC2} / \text{mean } OD_{NC}) \times 100$$

$$\% \text{ Positive Control } 3 = (OD_{PC3} / \text{mean } OD_{NC}) \times 100$$

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

$$\text{Mean PC } \% = (\%PC1 + \%PC2 + \%PC3) / 3$$

Test item:

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

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

- 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:

$$\% \text{ Treated Tissue } 1 = (OD_{TT1} / \text{mean } OD_{NC}) \times 100$$

$$\% \text{ Treated Tissue } 2 = (OD_{TT2} / \text{mean } OD_{NC}) \times 100$$

$$\% \text{ Treated Tissue } 3 = (OD_{TT3} / \text{mean } OD_{NC}) \times 100$$

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

$$\text{Mean TT } \% = (\%TT1 + \%TT2 + \%TT3) / 3$$

### 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  $\leq 30\%$ , 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:

$$\% RV 1 = [TOD_{TT1} / \text{mean } OD_{NC}] \times 100$$

$$\% RV 2 = [TOD_{TT2} / \text{mean } OD_{NC}] \times 100$$

$$\% RV 3 = [TOD_{TT3} / \text{mean } OD_{NC}] \times 100$$

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

$$\text{Mean Relative Viability \%} = (\% RV 1 + \% RV 2 + \% RV 3) / 3$$

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 ( $NSC_{\text{living}} \%$ ):

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

$OD_{CTV}$ : test substance treated viable tissues (not incubated with MTT)

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

If  $NSC_{\text{living}} \%$  is  $\leq 5 \%$  then the normal calculation mode is used (see 3.9.1).

If  $NSC \%$  is  $> 5\%$  and  $\leq 30\%$ , then true MTT metabolic conversion ( $TOD_{TT}$ ) is undertaken as follows.

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

$OD_{TT}$ : 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:

$$\% RV 1 = [TOD_{TT1} / \text{mean } OD_{NC}] \times 100$$

$$\% RV 2 = [TOD_{TT2} / \text{mean } OD_{NC}] \times 100$$

$$\% RV 3 = [TOD_{TT3} / \text{mean } OD_{NC}] \times 100$$

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

$$\text{Mean Relative Viability \%} = (\% RV 1 + \% RV 2 + \% RV 3) / 3$$

If NSC<sub>living</sub> % 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<sub>killed</sub> %):

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

OD<sub>CTK</sub>: test substance treated killed tissues (not incubated with MTT)

OD<sub>NC</sub>: negative control OD (incubated with MTT)

$$TOD_{TT} = [OD_{TT} - (OD_{KT} - OD_{KNC}) - mean OD_{CTV} + mean OD_{CTK}]$$

OD<sub>TT</sub>: test substance treated viable tissues (incubated with MTT)

OD<sub>KT</sub>: test substance treated killed tissues OD

OD<sub>KNC</sub>: negative control killed tissues OD

OD<sub>CTV</sub>: test substance treated viable tissues (not incubated with MTT)

OD<sub>CTK</sub>: 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 [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 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 exposure to the test item and 42 hours post incubation is less or equal ( $\leq$ ) 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 ( $\leq$ ) 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 exposure to the test item and 42 hours post incubation is more than ( $>$ ) to 50% of the mean viability of the negative controls.

The prediction model (PM) is described below:

Criteria for <i>In Vitro</i> interpretation	Classification
	UN GHS
Mean tissue viability % is $\leq$ 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 0.013, non-specific colour percent was calculated as 1.5% (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 78.5% 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 (0.872). Standard deviation of the viability results for negative control samples was 7.5%.

The positive control treated tissues showed 5.7% viability demonstrating the proper performance of the assay. The standard deviation of the viability results for positive control samples was 0.9%.

The standard deviation of viability values of the three test item-treated tissue samples in the MTT assay was 3.9%.

The mean OD value of the blank samples (acidified isopropanol) was 0.045.

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, propiconazole/pydiflumetofen SE (A21573C) the mean relative viability was 78.5% 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 propiconazole/pydiflumetofen SE (A21573C) the results indicate that the test item is a non-irritant to skin.

## 6.0 REFERENCES

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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
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12. 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
13. 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, July 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%) of the Additional Control Tissues**

Additional control	Optical Density (OD)		NSC% (living)
	Measured	Blank corrected	
Treated with Propiconazole/Pydiflumetofen SE (A21573C)	1	0.055	<b>1.5</b>
	2	0.061	
	mean	--	

Notes:

1. Mean blank value was 0.045.
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)
		Measured	Blank corrected	
<b>Negative Control:</b> Phosphate buffered saline	1	0.949	0.904	103.7
	2	0.960	0.915	104.9
	3	0.842	0.797	91.4
	mean	--	<b>0.872</b>	<b>100.0</b>
<b>Positive Control:</b> 5% (w/v) SDS solution	1	0.092	0.047	5.4
	2	0.088	0.043	5.0
	3	0.103	0.058	6.7
	mean	--	<b>0.050</b>	<b>5.7</b>
<b>Test Item:</b> Propiconazole/Pydiflumetofen SE (A21573C)	1	0.691	0.646	74.0
	2	0.742	0.697	79.9
	3	0.756	0.711	81.5
	mean	--	<b>0.685</b>	<b>78.5</b>

Notes:

1. Mean blank value was 0.045.
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 Crop Protection, LLC  
Analytical and Product Chemistry  
Greensboro, NC 27409

## Certificate of Analysis

A21573C
Batch ID 1007839 (GP170913)

Test Substance Name:	CGA64250/SYN545974 SE (125/150)
Common Name:	Propiconazole/Pydiflumetofen SE (125/150)
Design Code:	A21573C
Batch ID:	1007839
Other ID:	GP170913
Source:	Syngenta Crop Protection LLC.,US .410 Swing Road.Greensboro, NC 27409.

### Chemical Analysis

AI	% w/w	g/L
Pydiflumetofen	13.7	151
Propiconazole	11.6	128

Identity of the Active Ingredients: Confirmed

Methodology Used for Characterization: LC , mass spectrometry, oscillating density meter

The Active Ingredient(s) content is within the FAO limits.

### Isomer Assay

Analyte	Isomer	% w/w	g/L
CGA93590	1H-1,2,4-triazole, 1-([2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl)-, cis-	6.73	74
CGA93591	1H-1,2,4-triazole, 1-([2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl)-, trans-	4.84	53.2

COA Number: USGR170462

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## APPENDIX 1 Certificate of Analysis (continued)

### Physical Analysis

Property	Value	Units
Density	1.100	g/cm <sup>3</sup>

Appearance: Beige liquid

Storage Temperature: <30°C

Re-certification Date: End of Oct/2020

*If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.*

The stability of this test substance will be determined concurrently through reanalysis of material held in inventory under GLP conditions at Syngenta Crop Protection, LLC, Greensboro, NC.

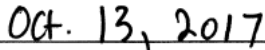
This Certificate of Analysis is summarizing data from a study that has been performed in compliance with Good Laboratory Practices per 40 CFR Part 160. Raw data, documentation, protocols, any amendments to study protocols and reports pertaining to this study are maintained in the Syngenta Crop Protection Archives in Greensboro, NC.

Study Number: USGR170462

Authorization: Kirt Durand

  
\_\_\_\_\_

Kirt Durand  
Analytical and Product Chemistry Department

  
\_\_\_\_\_

Date

COA Number: USGR170462

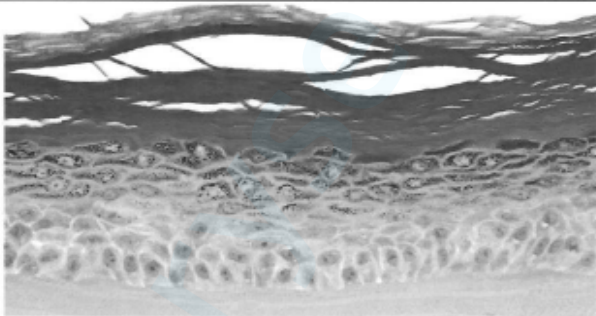
Page 2 of 2

## 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-S03

<b>Description:</b>	<b>Episkin Small Model</b> 0.38 cm <sup>2</sup> reconstructed epidermis of normal human keratinocytes. Cells are grown on a collagen matrix, for 13 days.		
<b>Usage:</b>	<b>FOR SCIENTIFIC USE ONLY - PRODUCT OF HUMAN ORIGIN</b>		
<b>Storage:</b>	This product was prepared and packaged using aseptic techniques. Store in an incubator at 37°C, 5% CO <sub>2</sub> with saturated humidity.		
<b>Passage:</b>	Second (Strains n° : 09-KERA-007 + 09-KERA-010)		
<b>Batch N°:</b>	<b>17-EKIN-050</b>		
<b>Origin:</b>	Adult donors.		
<b>Histology:</b>	 <p style="text-align: right;">Control n° E171567</p>		
<b>Quality Controls:</b>	<b>Test</b>	<b>Specification</b>	<b>Result</b>
	<b>Histology scoring</b> (HES stained vertical paraffin sections)	≥ 19.5	<b>22.5 ± 0.3</b> (CV = 1.4 %)
		Well-differentiated epidermis consisting of a basal layer, several spinous and granular layers and a thick stratum corneum	Satisfactory
<b>IC 50 determination</b> (SDS concentration, MTT test)	1.5 mg/mL ≤ IC50 ≤ 3.0 mg/mL	<b>2.1 mg/mL</b>	
<b>Biological safety:</b>	On blood of the same donors, we have verified: <ul style="list-style-type: none"> <li>. the absence of HIV1 and 2 antibodies</li> <li>. the absence of hepatitis C antibodies</li> <li>. the absence of hepatitis B antigen HBs</li> </ul> On epidermal cells of the same donors, we have verified: <ul style="list-style-type: none"> <li>. the absence of bacteria, fungus and mycoplasma</li> </ul>		
<b>Expiration date</b>	December 18, 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, December 12, 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



### APPENDIX 3 Historical Control Data

(updated 18 January 2018)

	<b>Negative control (PBS)</b>	<b>Positive control (5% (w/v) SDS solution)</b>
Mean optical density (OD)	0.788	0.065
Standard deviation	0.129	0.041
Minimum optical density (OD)	0.573	0.019
Maximum optical density (OD)	1.362	0.354
Number of cases	251	246

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



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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

The official stamp is circular with a blue border containing the text 'Országos Gyógyszerészeti és Élelmezés-egészségügyi Intézet'. In the center, there is a stylized graphic of a building and a scale. Below the stamp, the name 'Dr. József Reiter' and his title 'Deputy Director-General' are printed.

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