**SYN545192**

# SYN545192 EC (A15457H) - Local Lymph Node Assay in the Mouse

**Final Report**

**DATA REQUIREMENT(S):** OECD Test Guideline 429 (2010)
EC 440/2008 B.42 (2008)

**AUTHOR(S):** Magdolna Török-Bathó, M.Sc.

**STUDY COMPLETION DATE:** 10 July 2012

**PERFORMING LABORATORY:** CiToxLAB Hungary Ltd.
H-8200 Veszprém, Szabadságpuszta,Hungary

**LABORATORY PROJECT ID:** Report Number: 12/063-037E
Study Number: 12/063-037E
Task Number: TK0065139

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

## STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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# GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study has been performed in accordance with the study plan agreed upon by the Sponsor, the OECD Guidelines for Testing of Chemicals No. 429. Skin Sensitisation: Local Lymph Node Assay (Adopted: 22 July 2010); Commission Regulation (EC) No 440/2008 of 30 May 2008, B.42: Skin Sensitisation: Local Lymph Node Assay (Official Journal L 142, 31/05/2008) and the Principles of Good Laboratory Practice (Hungarian GLP Regulations: 9/2001. (III. 30.) EüM-FVM joint decree of the Minister of Health and the Minister of Agriculture and Regional Development, 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: Dr. Török Magdolna Date: 10 July 2012

Magdolna Török-Bathó, M.Sc.
Study Director

Performing Laboratory: CiToxLAB Hungary Ltd.
H-8200 Veszprém, Szabadságpuszta
Hungary

## FLAGGING STATEMENT

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# QUALITY ASSURANCE STATEMENT

Study Code: 12/063-037E

Study Title: SYN545192 EC (A15457H) - Local Lymph Node Assay in the Mouse

Test Item: SYN545192 EC (A15457H)

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 are given below:

| Date of Inspection | Phase(s) Inspected/Audited | Date of report to | |
|---|---|---|---|
| | | Management | Study Director |
| 26 April 2012 | Study Plan | 26 April 2012 | 26 April 2012 |
| 14 May 2012 | Cell suspension preparation | 14 May 2012 | 14 May 2012 |
| 07 June 2012 | Draft Report | 07 June 2012 | 07 June 2012 |
| 09 July 2012 | Final Report | 09 July 2012 | 09 July 2012 |

Signature: Diana Diáno Date: 10 July 2012

Diána Czuczay, 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 "SYN545192 EC (A15457H) - Local Lymph Node Assay in the Mouse" was performed in compliance with the Principles of Good Laboratory Practice.

Signature: _____ Date: 10 July 2012

Christopher Banks, DABT
Managing Director

# GENERAL INFORMATION

## Contributors

The following contributed to this report in the capacities indicated:

| Name | Function |
|---|---|
| Magdolna Török-Bathó, M.Sc. | Study Director |
| Diána Czuczay, M.Sc. | QA Inspector |
| Claire Elliott | Syngenta Study Manager |

## Study dates

| | |
|---|---|
| Preliminary Test: | 02 – 07 May 2012 |
| Experimental Starting Date: | 09 May 2012 |
| Experimental Completion Date: | 15 May 2012 |
| Treatment: | 09 May 2012 to 11 May 2012 |
| Observation: | 09 May 2012 to 14 May 2012 |
| Termination: | 14 May 2012 |

## Deviations to the study plan

There was no deviation from the study plan.

## Retention of samples

See below under "Other".

## Performing laboratory test substance reference number

12003A

## Other

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

will be stored in the archives of CiToxLAB Hungary Ltd., 8200 Veszprém-Szabadságpuszta, Hungary according to the Hungarian GLP regulation and to test facility SOPs.

After the retention time agreed with the Sponsor has elapsed, all the archived materials listed above will be offered to the Sponsor or retained for a further period if agreed by a contract. Otherwise the materials will be discarded. No raw data or material relating to the study will be discarded without the Sponsor's prior written consent.

# TABLE OF CONTENTS

# 1.0 EXECUTIVE SUMMARY

## 1.1 Study Design

The aim of this study was to determine the skin sensitisation potential of SYN545192 EC (A15457H) following dermal exposure to CBA/J Rj mice.

For this purpose, the LLNA method was used. The test item solutions were applied on the dorsal surface of ears of experimental animals (25 µL/ear) for 3 consecutive days (Days 1, 2 and 3). There was no treatment on Days 4, 5 and 6. On Day 6, 5 hours prior to termination, animals were intravenously injected via the tail vein with tritiated methyl thymidine ($^{3}$HTdR). Cell proliferation in the local lymph nodes was measured by incorporation of $^{3}$HTdR and the values obtained were used to calculate stimulation indices (SI).

Groups of four female CBA/J Rj mice were treated with:

- 100 % (undiluted), 50 and 25 % (w/v) , (diluted in 1% Pluronic) SYN545192 EC (A15457H)
- the negative control group received 1 % Pluronic,
- the positive control group received 25 % α-Hexylcinnamaldehyde (HCA) in 1 % Pluronic.

## 1.2 Results

No mortality, systemic toxicity or local irritation was observed during the study. No treatment related effects were observed on body weight in any treated groups.

Stimulation index values of the test item were 29.4, 19.1 and 19.2 at treatment concentrations of 100 % (undiluted), 50 and 25 % (w/v), respectively.

## 1.3 Conclusion

In conclusion, under the conditions of the present assay, SYN545192 EC (A15457H) tested in a suitable vehicle, was shown to have skin sensitisation potential (sensitizer) in the Local Lymph Node Assay.

# 2.0 INTRODUCTION

## 2.1 Purpose

The purpose of this study was to determine the skin sensitisation potential of the test item SYN545192 EC (A15457H) following dermal exposure in the Local Lymph Node Assay.

## 2.2 Guidelines

The study was performed according to the following guidelines:

- OECD Guidelines for Testing of Chemicals No. 429. Skin Sensitisation: Local Lymph Node Assay. Adopted: 22 July 2010.

- Commission Regulation (EC) No 440/2008 of 30 May 2008, B.42: Skin Sensitisation: Local Lymph Node Assay (Official Journal L 142, 31/05/2008)

## 2.3 Sensitivity of the Test System

The positive control group animals were treated with 25 % (w/v) α-Hexylcinnamaldehyde (HCA) solution in a relevant vehicle (1 % Pluronic) concurrent to the test item groups. The positive control substance was chosen according to the OECD guideline [1].

No mortality, cutaneous reactions or signs of toxicity were observed in the positive control group. A significant lympho-proliferative response (stimulation index value of 3.7) was noted for α-Hexylcinnamaldehyde in this experiment. The results of the positive control group demonstrated the appropriate performance of the assay.

The historical control data are given in Table 7.

| | |
|---|---|
| Name: | α-Hexylcinnamaldehyde |
| Lot No. : | MKBH9208V |
| CAS No.: | 101-86-0 |
| Manufacturer: | Sigma-Aldrich Co. |
| Appearance: | Yellow liquid |
| Purity: | 97.8 % |
| Expiry: | 30 March 2013 |
| Storage condition: | Room temperature |
| Safety precautions: | Routine safety precautions (gloves, mask, lab coat, safety glasses) were applied to assure personnel health and safety. |

## 2.4 Test Facility

The conduct of the study was approved by the Institutional Animal Care and Use Committee (IACUC) of CiToxLAB Hungary Ltd.

Dates of IACUC approval: 27 April 2012

# 3.0 MATERIALS AND METHODS

## 3.1 Test Item

The following information was provided by the Sponsor:

| | |
|---|---|
| Name: | SYN545192 EC (A15457H) |
| Batch Number: | SMU1LP001 |
| Product Code: | A15457H |
| Purity: | Content of benzovindiflupyr (SYN545192) - 10.1% (w/w) corresponding to 98.6 g/L<br>Content of water - 0.74% (w/w) |
| Appearance: | Brown liquid |
| Recertification date: | End of July 2014 |
| Storage conditions: | Room temperature (< 30 $^{o}$C) |
| Safety Precautions: | Routine safety precautions (lab coat, gloves, goggles, face mask) for unknown materials were applied to assure personnel health and safety. |

A copy of the Certificate of Analysis is presented in Appendix 1.

### 3.1.1 Identification, receipt

The test item of a suitable chemical purity was supplied by the Sponsor. All precautions required in the handling and disposal of the test item were outlined by the Sponsor. The identification of test item was made using its name, batch number, appearance and colour in the Central Dispensary Unit of CiToxLAB Hungary Ltd.

### 3.1.2 Formulation

During the Preliminary Compatibility Test, the solubility of the test item was examined in the 1 % aqueous Pluronic® PE9200 (1% Pluronic). The test item formed an appropriate formulation in this vehicle at concentrations of 50 and 25 % (w/v). The regulatory test guideline specifies 1% aqueous Pluronic as a suitable vehicle for water soluble formulations, therefore it was chosen as vehicle for the test. Pluronic PE9200 is a surfactant, which avoids immediate run-off of the aqueous test material from the ears of animals when applied topically [4, 5].

### 3.1.3 Vehicle

Materials used for the preparation of the vehicle:

Name: Pluronic PE9200

| | |
|---|---|
| Batch No.: | 57465968E0 |
| Manufacturer: | BASF |
| Expiry: | 24 October 2013 |
| Storage condition: | Room temperature |

| | |
|---|---|
| Name: | Distilled water |
| Batch No.: | 3450611 |
| Manufacturer: | TEVA Co. |
| Expiry: | June 2014 |
| Storage condition: | Room temperature |

### 3.1.4 Subsidiary materials

| | |
|---|---|
| Name | Trichloroacetic acid (TCA) |
| Batch number | BCBD9446V |
| Supplier | Sigma-Aldrich Co. |
| Expiry date | 10 November 2012 |

| | |
|---|---|
| Name | Phosphate buffered saline (PBS) |
| Batch number | 051M8422 |
| Supplier | Sigma-Aldrich Co. |
| Expiry date | 27 September 2012 |

| | |
|---|---|
| Name | (methyl-3H) Thymidine ($^{3}$HTdR) |
| Batch Number | PP011501 F / 1203013 |
| Supplier | ARC Inc. |
| Date of analysis | 13 March 2012 |

| | |
|---|---|
| Name | OptiPhase HiSafe 3 (Scintillation liquid) |
| Batch Number | 152-11291 |
| Manufacturer | Perkin Elmer |
| Expiry date | 01 February 2013 |

## 3.2 Instrument System

| | |
|---|---|
| Name: | Tri-Carb 2810 Liquid Scintillation Analyzer |
| IQ / OQ Protocol #: | 1593646-1 |
| Manufacturer: | PerkinElmer |

## 3.3 Experimental Design

### 3.3.1 Animals

Species and strain: CBA/J Rj mice

| | |
|---|---|
| Source: | Elevage Janvier<br>Route des Chènes Secs B.P. 4105<br>53940 Le Genest-ST-Isle, France |
| Hygienic level during the study: | Standard housing conditions |
| Justification of strain: | On the basis of OECD Guideline, mice of CBA/Ca or CBA/J strain can be used. Females were used because the existing database is predominantly based on females. |
| Number of animals: | 4 animals / treatment group |
| Sex: | Female, nulliparous, non pregnant |
| Age of animals at start: | 9 weeks old |
| Body weight range at start: | 20.9 – 23.7 g |
| Acclimatization time: | 13 days |

### 3.3.2 Husbandry

| | |
|---|---|
| Animal health: | Only healthy animals were used for the study. Health status was certified by the veterinarian. |
| Housing / Enrichment: | Group caging / mice were provided with glass tunnel-tubes |
| Cage type: | Type II. polypropylene/ polycarbonate |
| Bedding: | Bedding was available to animals during the study |
| Light: | 12 hours daily, from 6.00 a.m. to 6.00 p.m. |
| Temperature: | 22 ± 3 °C |
| Relative humidity: | 30 - 70% |
| Ventilation: | 15-20 air exchange/hour |

The temperature and relative humidity were recorded twice every day during the acclimatisation and experimental phases.

| | |
|---|---|
| Room/Cabinet (non-radioactive phase): | 243 |
| Room/Cabinet (radioactive phase): | 139 – 140 |

### 3.3.3 Food and feeding

Animals received ssniff SM R/M-Z+H "Autoclavable complete diet for rats and mice – breeding and maintenance" (Batch number: 719 6627 Expiry Date: May 2012) produced by ssniff Spezialdiäten GmbH (Ferdinand-Gabriel-Weg 16, D-59494 Soest, Germany), *ad libitum*.

### 3.3.4 Water supply

Animals received tap water from the municipal supply from a 500 mL bottle, *ad libitum*.

Water quality control analysis was performed once every three months and microbiological assessment was performed monthly, by Veszprém County Institute of State Public Health and Medical Officer Service (ÁNTSZ, H-8201 Veszprém, József A.u.36., Hungary). Copies of the relevant Certificates of Analysis are retained in the Archive at CiToxLAB Hungary Ltd.

### 3.3.5 Bedding

Lignocel® Hygienic Animal Bedding produced by J. Rettenmaier & Söhne GmbH+Co.KG (D-73494 Rosenberger (Germany) Holzmühle 1) was available to animals during the study.

### 3.3.6 Animal welfare

Euthanasia for all animals was by asphyxiation with ascending doses of carbon dioxide, followed by confirmation of death before discarding carcasses. At scheduled termination (with excision of lymph nodes); deep anaesthesia was confirmed before making an incision. Death was confirmed by cervical dislocation or by cutting through major cervical blood vessels.

### 3.3.7 Identification, randomisation

A unique number written on the tail with a permanent marker identified each animal. The animal number was assigned on the basis of CiToxLAB Hungary Ltd's master file. The cages were marked with identity cards with information including study code, cage number, and dose group, sex and individual animal number. The animals were randomised and allocated to the experimental groups. The randomisation was checked by computer software according to the actual body weights, verifying the homogeneity and variability between the groups.

## 3.4 Administration of the Test Item

### 3.4.1 Dose selection and justification of dose selection

A Preliminary Irritation/Toxicity Test was performed on CBA/J Rj mice using two doses, at test item concentrations of 100 % (undiluted) and 50 % (w/v) %, respectively. This preliminary experiment was conducted in a similar experimental manner to the main study, and was terminated on Day 6 with a body weight measurement but without the radioactive proliferation assay.

During the Preliminary Irritation / Toxicity Test no mortality, systemic toxicity or visible local irritation were observed. No treatment related effect on mean body weights was observed in any treated groups.

Ear thickness of the animals was measured using by a thickness gauge on Days 1, 3 and 6. Additional quantification of the ear thickness was performed by ear punch weight determination after the euthanasia of the experimental animals. The ear thickness data and the revealing ear punch weights were within the historical control range.

Based on the observations recorded in this preliminary test, the 100 % (undiluted) formulation is considered to be suitable maximum dose level for the main study. The application of the material and the local effects on the animals are considered acceptable for a valid LLNA.

Results of the Preliminary Irritation/Toxicity Test are summarised in Tables 1, 2 and 3.

Based on the results of the preliminary experiments the following groups were tested in the main assay:

| **Groups** | **Test item concentration** (w/v) % | **No. of animals** |
|---|---|---|
| Negative Control (1% Pluronic) | - | 4 |
| SYN545192 EC (A15457H) | 100 (undiluted) | 4 |
| SYN545192 EC (A15457H) | 50 | 4 |
| SYN545192 EC (A15457H) | 25 | 4 |
| Positive Control (25 % HCA in 1% Pluronic) | - | 4 |

### 3.4.2 Topical application

During the assay each mouse was topically dosed on the dorsal surface of each ear with 25 µL of the appropriate test item applied using a pipette. Each animal was dosed once a day for 3 consecutive days (Days 1, 2 and 3). There was no treatment on Days 4, 5 and 6.

## 3.5 Proliferation Assay

### 3.5.1 Injection of tritiated thymidine ($^3$HTdR)

On Day 6, animals were taken to the radioactive suite and each mouse was intravenously injected via the tail vein with 250 µL of sterile PBS (phosphate buffered saline) containing approximately 20 µCi of $^3$HTdR. Once injected, the mice were left for 5 hours.

### 3.5.2 Removal and preparation of draining auricular lymph nodes

Five hours after intravenous injection, the mice were euthanized by asphyxiation with ascending doses of carbon dioxide (deep anaesthesia was confirmed before making the incision, death was confirmed before discarding carcasses). The draining auricular lymph nodes were excised by making a small incision on the skin between the jaw and sternum, pulling the skin gently back towards the ears and exposing the lymph nodes. The nodes were then removed using forceps. The carcasses were discarded after cervical dislocation or after cutting through major cervical blood vessels. Once removed, the nodes were collected in separate Petri dishes containing a small amount (1-2 mL) of PBS to keep the nodes wet before processing. The nodes of each animal were processed individually.

### 3.5.3 Preparation of single cell suspension of lymph node cells

A single cell suspension (SCS) of lymph node cells (LNCs) was prepared and collected in disposable tubes by gentle mechanical disaggregating of the lymph nodes through a cell strainer using the plunger of a disposable syringe. The cell strainer was washed with PBS (up to 10 mL). LNCs was pelleted with a relative centrifugal force (RCF) of 190 x g (approximately) for 10 minutes at 4 °C. After centrifugation supernatants were discarded. Pellets were gently resuspended and 10 mL of PBS was added to the tubes. The washing step was repeated twice. This procedure was repeated for each group of lymph nodes of each individual animal.

### 3.5.4 Determination of incorporated $^{3}$HTdR

After the final washing step, the suspensions were centrifuged and the supernatants were removed leaving a small volume (<0.5 mL) of supernatant above each pellet. Each pellet was gently agitated before suspending the LNCs in 3 mL of 5% trichloroacetic acid (TCA) for precipitation of macromolecules. After incubation with 5% TCA at 2-8 °C overnight (approximately 18 hours) precipitate was recovered by centrifugation at 190 x g for 10 minutes at 4 °C), and supernatants were removed and pellets were resuspended in 1 mL of 5% TCA solution and dispersed using an ultrasonic water bath. Each precipitate was transferred to a suitable sized scintillation vial with 10 mL of scintillation liquid and thoroughly mixed. The vials were loaded into a β-scintillation counter and $^{3}$HTdR incorporation was measured for up to 10 minutes per sample.

The β-counter expressed the $^{3}$HTdR incorporation as the number of radioactive disintegrations per minute (DPM). Background radiation levels were measured in two 1 mL aliquots of 5% TCA.

## 3.6 Observations

### 3.6.1 Clinical observations

During the study (Days 1 to 6), all animals were observed at least once daily for any clinical signs, including local irritation and systemic toxicity. Individual records were maintained.

### 3.6.2 Measurement of body weight

Individual body weights were recorded on Day 1 (beginning of the assay) and at Day 6 (prior to $^{3}$HTdR injection) with a precision of +/- 0.1 g.

## 3.7 Evaluation of the Results

Disintegrations per minute (DPM) were measured for each animal of nodes (correcting for background radioactivity). The results were expressed as disintegrations per node (DPN) by dividing the DPM by the number of lymph nodes.

Stimulation index (SI =mean DPN of treated group divided by mean DPN of the appropriate control group) for each treatment group was calculated. A stimulation index of 3 or greater is an indication of a positive result.

### 3.7.1 Interpretation of results

The test item is regarded as a sensitizer if both of the following criteria are fulfilled:

- That exposure to at least one concentration resulted in an incorporation of $^{3}$HTdR at least 3-fold or greater than recorded in control mice, as indicated by the stimulation index.
- The data are compatible with a conventional dose response, although allowance must be made (especially at high topical concentrations) for either local toxicity or immunological suppression.

### 3.7.2 Acceptability of the test

The Local Lymph Node Assay is considered valid if it meets the following criteria:

- the DPN value of the negative (vehicle) control group falls within the range of historical laboratory control data,
- the positive control substance produces a significant lymphoproliferative response increases (SI>3),
- each treated and control group includes at least 4 animals,
- the test item does not cause serious systemic or local toxicity.

## 4.0 RESULTS AND DISCUSSION

### 4.1 Clinical Observation

No mortality or signs of systemic toxicity were observed during the main study. No clinical signs were observed. The data are summarized in Table 6.

### 4.2 Body Weight Measurement

No treatment related effects were observed on body weight. Individual and mean body weights are given in Table 4.

### 4.3 Proliferation Assay

The results of the proliferation assay are summarized in Table 5 and Figure 1. The stimulation index was higher than the control in all test item treated groups and in the positive control group.

### 4.4 Interpretation of Observations

The test item was a brown liquid, which was prepared in 1 % Pluronic. Since there were no confounding effects of irritation at any dose level and no systemic toxicity at the applied concentrations, the proliferation values obtained are considered to reflect the real potential of the test item to cause lymphoproliferation in the Local Lymph Node Assay.

Stimulation index values of the test item were 29.4, 19.1 and 19.2 at treatment concentrations of 100 % (undiluted), 50 and 25 % (w/v), respectively.

A significant lymphoproliferative response (SI ≥ 3) was noted for SYN545192 EC (A15457H) at concentrations of 100 % (undiluted), 50 % and 25 % (w/v). Stimulation index values of the test item were 29.4, 19.1 and 19.2 at treatment concentrations of 100 % (undiluted), 50 and 25 % (w/v), respectively. The stimulation index values indicate a clear positive response with the undiluted material giving a larger response than the 25 and 50% dilutions (Figure 1). The positive control substance gave the expected positive response with a stimulation index of 3.7 %.

## 5.0 CONCLUSIONS

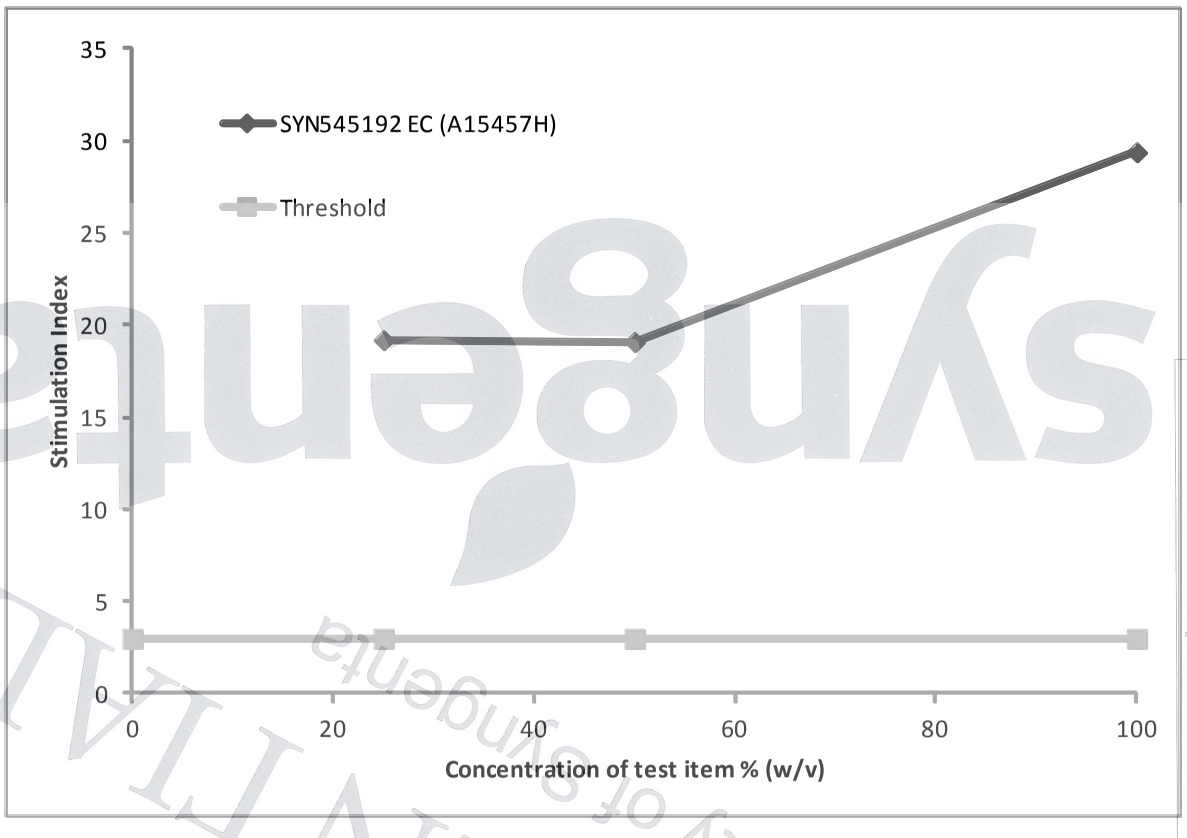
In conclusion, under the conditions of the present assay, SYN545192 EC (A15457H) tested in a suitable vehicle, was shown to have skin sensitisation potential (sensitizer) in the Local Lymph Node Assay.

## 6.0 REFERENCES


1. OECD Guidelines for Testing of Chemicals No.404. “Acute Dermal Irritation / Corrosion”, Adopted: 24 April 2002
2. Hungarian GLP Regulations: 9/2001 (III.30.) EüM-FVM joint decree of the Minister of Health and the Minister of Agriculture and Regional Development, which corresponds to the OECD GLP, ENV/MC/CHEM (98) 17
3. Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).
4. Boverhof, D.R., Wiescinski, C.M., Botham, P., Lees, D., Debruyne, E., Repetto-Larsay, M., Ladics, G., Hoban, D., Gamer, A., Remmele, M., Wang-Fan, W., Ullmann, L.G., Mehta, J., Billington, R., Woolhiser, M.R.: Interlaboratory validation of 1 % Pluronic L92 surfactant as a suitable , aqueous vehicle for testing pesticide formulations using the murine local lymph node assay. Toxicological Sciences 105 (2008), 79-85
5. Ryan, C.A., Cruse, L.W., Skinner, R.A., Dearman, R.J., Kimber, I., Gerberick, G.F.: Examination of a vehicle for use with water soluble materials in the murine local lymph node assay. Food and Chem. Tox. 40 (2002), 1719-1725

## FIGURES SECTION

**FIGURE 1 Test Item Stimulation Index Values**



## TABLES SECTION

## TABLE 1 Individual Body Weights for all Animals with Group Means (Preliminary Irritation/Toxicity Test)

Test item: SYN545192 EC (A15457H);
Vehicle: 1 % Pluronic

| Animal Number | Test Group Name | Initial Body Weight (g) | Terminal Body Weight* (g) |
|---|---|---|---|
| 9244 | 100 % (undiluted) | 18.1 | 18.2 |
| 9248 | 100 % (undiluted) | 18.4 | 18.4 |
| | **Mean:** | **18.3** | **18.3** |
| 9253 | 50 % (w/v) in 1% Pluronic | 19.9 | 20.9 |
| 9274 | 50 % (w/v) in 1% Pluronic | 18.2 | 18.7 |
| | **Mean:** | **19.1** | **19.8** |

*Terminal body weights were measured on Day 6.

## TABLE 2 Individual ear thickness for all animals (Preliminary Irritation/Toxicity Test)

| Animal Number | Test Group Name | Ear Thickness on Day 1 (mm) | Ear Thickness on Day 3 (mm) | Ear Thickness on Day 6 (mm) | Ear Weight** on Day 6 (mg) |
|---|---|---|---|---|---|
| 9244 | 100 % | 0.22 / 0.22 | 0.23 / 0.22 | 0.23 / 0.23 | 19.8 |
| 9248 | 100 % | 0.22 / 0.22 | 0.21 / 0.23 | 0.22 / 0.24 | 21.3 |
| 9253 | 50 (w/v) % | 0.23 / 0.22 | 0.23 / 0.22 | 0.25 / 0.24 | 19.9 |
| 9274 | 50 (w/v) % | 0.23 / 0.23 | 0.23 / 0.23 | 0.23 / 0.23 | 19.6 |

**: Based on the historical control data, the maximum normal weight is 21.1 mg. Positive response is over 26.3 mg.

## TABLE 3 Summarized Clinical Observations (Preliminary Irritation/Toxicity Test)

| Period | Group | Animal No. | Clinical observations |
|---|---|---|---|
| **DAY 1** | 100% (undiluted) | 9244 | Before treatment: symptom-free<br>After treatment: symptom-free, E.S.:0 |
| | 100% (undiluted) | 9248 | Before treatment: symptom-free<br>After treatment: symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9253 | Before treatment: symptom-free<br>After treatment: symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9274 | Before treatment: symptom-free<br>After treatment: symptom-free, E.S.:0 |
| **DAY 2** | 100% (undiluted) | 9244 | Before treatment: symptom-free, E.S.:0<br>After treatment: symptom-free, E.S.:0 |
| | 100% (undiluted) | 9248 | Before treatment: symptom-free, E.S.:0<br>After treatment: symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9253 | Before treatment: symptom-free, E.S.:0<br>After treatment: symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9274 | Before treatment: symptom-free, E.S.:0<br>After treatment: symptom-free, E.S.:0 |
| **DAY 3** | 100% (undiluted) | 9244 | Before treatment: symptom-free, E.S.:0<br>After treatment: symptom-free, E.S.:0 |
| | 100% (undiluted) | 9248 | Before treatment: symptom-free, E.S.:0<br>After treatment: symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9253 | Before treatment: symptom-free, E.S.:0<br>After treatment: symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9274 | Before treatment: symptom-free, E.S.:0<br>After treatment: symptom-free, E.S.:0 |
| **DAY 4** | 100% (undiluted) | 9244 | symptom-free, E.S.:0 |
| | 100% (undiluted) | 9248 | symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9253 | symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9274 | symptom-free, E.S.:0 |

**TABLE 3 Summarized Clinical Observations (Preliminary Irritation/Toxicity Test) (continued)**

| | | | |
|---|---|---|---|
| **DAY 5** | 100% (undiluted) | 9244 | symptom-free, E.S.:0 |
| | 100% (undiluted) | 9248 | symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9253 | symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9274 | symptom-free, E.S.:0 |
| **DAY 6** | 100% (undiluted) | 9244 | symptom-free, E.S.:0 |
| | 100% (undiluted) | 9248 | symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9253 | symptom-free, E.S.:0 |
| | 50 % (w/v) in 1% Pluronic | 9274 | symptom-free, E.S.:0 |

E.S.: erythema score

## TABLE 4 Individual Body Weights for all Animals with Group Means

| Identity Number | Animal Number | Test Group Name | Day 1 | Day 6 |
|---|---|---|---|---|
| 1 | 9286 | Negative control | 21.4 | 20.7 |
| 2 | 9356 | | 21.5 | 21.3 |
| 3 | 9334 | 1 % Pluronic | 21.9 | 22.0 |
| 4 | 9293 | | 23.7 | 23.6 |
| | **Mean** | | **22.1** | **21.9** |
| 5 | 9357 | SYN545192 EC (A15457H) 100 % (undiluted) | 21.4 | 22.1 |
| 6 | 9296 | | 21.0 | 20.8 |
| 7 | 9337 | | 22.0 | 21.7 |
| 8 | 9299 | | 23.0 | 21.9 |
| | **Mean** | | **21.9** | **21.6** |
| 9 | 9374 | SYN545192 EC (A15457H) 50 % (w/v) in 1 % Pluronic | 21.7 | 21.1 |
| 10 | 9326 | | 21.0 | 21.4 |
| 11 | 9364 | | 22.2 | 21.6 |
| 12 | 9302 | | 23.2 | 22.0 |
| | **Mean** | | **22.0** | **21.5** |
| 13 | 9370 | SYN545192 EC (A15457H) 25 % (w/v) in 1 % Pluronic | 21.6 | 22.7 |
| 14 | 9324 | | 21.3 | 20.8 |
| 15 | 9308 | | 21.7 | 21.9 |
| 16 | 9318 | | 22.8 | 21.9 |
| | **Mean** | | **21.9** | **21.8** |
| 17 | 9372 | Positive control 25 % HCA in 1 % Pluronic | 21.9 | 21.4 |
| 18 | 9312 | | 20.9 | 20.4 |
| 19 | 9366 | | 22.2 | 22.1 |
| 20 | 9316 | | 23.4 | 22.3 |
| | **Mean** | | **22.1** | **21.6** |

## TABLE 5 DPM, DPN and Stimulation Index Values for all Groups

| Test Group Name | Animal Number | Measured DPM | DPM | No. of Nodes | DPN | Group DPN | SI |
|---|---|---|---|---|---|---|---|
| Background 5% (w/v) TCA | - | 35 | - | - | - | - | - |
| Negative control 1% Pluronic | 9286 | 267 | 232.0 | 2 | 116.0 | 97.4 | 1.0 |
| | 9356 | 269 | 234.0 | 2 | 117.0 | | |
| | 9334 | 266 | 231.0 | 2 | 115.5 | | |
| | 9293 | 117 | 82.0 | 2 | 41.0 | | |
| SYN545192 EC (A15457H) 100 % (undiluted) | 9357 | 4701 | 4666.0 | 2 | 2333.0 | 2866.8 | 29.4 |
| | 9296 | 8271 | 8236.0 | 2 | 4118.0 | | |
| | 9337 | 5356 | 5321.0 | 2 | 2660.5 | | |
| | 9299 | 4746 | 4711.0 | 2 | 2355.5 | | |
| SYN545192 EC (A15457H) 50 % (w/v) in 1% Pluronic | 9374 | 3838 | 3803.0 | 2 | 1901.5 | 1864.6 | 19.1 |
| | 9326 | 3360 | 3325.0 | 2 | 1662.5 | | |
| | 9364 | 3386 | 3351.0 | 2 | 1675.5 | | |
| | 9302 | 4473 | 4438.0 | 2 | 2219.0 | | |
| SYN545192 EC (A15457H) 25 % (w/v) in 1% Pluronic | 9370 | 4065 | 4030.0 | 2 | 2015.0 | 1866.6 | 19.2 |
| | 9324 | 3864 | 3829.0 | 2 | 1914.5 | | |
| | 9308 | 3842 | 3807.0 | 2 | 1903.5 | | |
| | 9318 | 3302 | 3267.0 | 2 | 1633.5 | | |
| Positive control 25 % HCA in 1% Pluronic | 9372 | 844 | 809.0 | 2 | 404.5 | 356.3 | 3.7 |
| | 9312 | 1036 | 1001.0 | 2 | 500.5 | | |
| | 9366 | 798 | 763.0 | 2 | 381.5 | | |
| | 9316 | 312 | 277.0 | 2 | 138.5 | | |

## TABLE 6 Clinical Observations

| Group | Animal No. | CLINICAL OBSERVATIONS | | | | | |
|---|---|---|---|---|---|---|---|
| | | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 | DAY 6 |
| Neg. control (1 % Pluronic) | 9286 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9356 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9334 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9293 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| 100 % (undiluted) | 9357 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9296 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9337 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9299 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| 50 % (w/v) in 1 % Pluronic | 9374 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9326 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9364 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9302 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |

Note: B.T.: Before treatment; A.T.: After treatment;

## TABLE 6 Clinical Observations (Continued)

| Group | Animal No. | CLINICAL OBSERVATIONS | | | | | |
|---|---|---|---|---|---|---|---|
| | | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 | DAY 6 |
| 25 % (w/v) in 1 % Pluronic | 9370 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9324 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9308 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9318 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| Positive control<br><br>25 % HCA in 1 % Pluronic | 9372 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9312 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9366 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |
| | 9316 | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | B. T.: symptom-free<br>A. T: symptom-free | symptom-free | symptom-free | symptom-free |

Note: B.T.: Before treatment; A.T.: After treatment;

## TABLE 7 Historical Control Data of the Positive Control Substance

| | Solvents | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Acetone- Olive oil (AOO) | | | 1% Pluronic PE9200 in water (1%Plu) | | | Methyl ethyl ketone (MEK) | | |
| | DPN values | | SI value | DPN values | | SI value | DPN values | | SI value |
| | Control | HCA 25% | HCA 25% | Control | HCA 25% | HCA 25% | Control | HCA 25% | HCA 25% |
| ***Average*** | 171.9 | 1536.6 | 11.3 | 131.8 | 919.8 | 7.9 | 118.8 | 727.6 | 8.5 |
| ***Range:*** *min* | 36.4 | 401.8 | 3.2 | 21.4 | 22.2 | 3.1 | 72.2 | 412.6 | 4.9 |
| *max* | 586.9 | 3300.5 | 29.2 | 469.6 | 2157.5 | 28.8 | 232.5 | 1042.6 | 12.0 |
| ***Number of cases*** | 79 | 53 | 53 | 39 | 25 | 25 | 4 | 2 | 2 |

| | Solvents | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *N,N*-Dimethylformamide (DMF) | | | Dimethyl sulfoxide (DMSO) | | | n-Hexane:Olive oil (HOO) | | |
| | DPN values | | SI value | DPN values | | SI value | DPN values | | SI value |
| | Control | HCA 25% | HCA 25% | Control | HCA 25% | HCA 25% | Control | HCA 25% | HCA 25% |
| ***Average*** | 152.1 | 2097.9 | 15.6 | 263.3 | 2178.6 | 9.3 | 125.9 | 1056.9 | 8.7 |
| ***Range: min*** | 20.8 | 350.9 | 3.8 | 133.3 | 1052.8 | 4.2 | 81.1 | 490.0 | 5.0 |
| *max* | 423.1 | 4438.9 | 75.7 | 553.3 | 5291.3 | 24.1 | 165.9 | 1296.4 | 14.0 |
| ***Number of cases*** | 84 | 43 | 46 | 24 | 19 | 19 | 10 | 10 | 10 |

| | Solvents | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Propylene glycol (PG) | | | Absolute ethanol: Distilled water 70:30 mixture (EtOH) | | | | |
| | DPN values | | SI value | DPN values | | | SI values | |
| | Control | HCA 25% | HCA 25% | Control | HCA 10% | HCA 25% | HCA 10% | HCA 25% |
| ***Average*** | 135.4 | 1245.4 | 9.2 | 123.1 | 1264.2 | 3805.0 | 17.3 | 36.8 |
| ***Range:*** *min* | 50.6 | 510.4 | 3.7 | 52.6 | 1214.8 | 2178.8 | 17.1 | 25.7 |
| *max* | 288.8 | 3231.3 | 27.9 | 357.6 | 1313.5 | 9207.1 | 17.4 | 54.3 |
| ***Number of cases*** | 17 | 10 | 10 | 6 | 2 | 5 | 2 | 5 |

HCA = alpha-Hexylcinnamaldehyde
SI (Stimulation Index) = DPN of a treated group divided by DPN of the appropriate control group.
DPN (Disintegrations Per Node) = DPM (Disintegrations Per Minute) divided by the number of lymph nodes.
In case of individual approach, SI values were calculated from the mean DPN values of the group.

# APPENDICES SECTION

## APPENDIX 1 Certificate of Analysis

syngenta

GLP Testing Facility WMU
Analytical Development &
Product Chemistry GS2131

Syngenta Crop Protection
Münchwilen AG
Breitenloh 5
CH-4333 Münchwilen

### Certificate of Analysis

**A15457H**
**benzovindiflupyr EC (100)**
**SMU1LP001**

| | |
|---|---|
| **Batch Identification** | **SMU1LP001** |
| **Product Code** | **A15457H** |
| Other Product Code(s) | benzovindiflupyr EC (100)<br>SYN545192 EC (100) |
| **Chemical Analysis (Active Ingredient Content)** | |
| – **Identity of the Active Ingredient(s)*** | **confirmed** |
| – **Content of benzovindiflupyr (SYN545192) *** | **10.1 % w/w corresponding to 98.6 g/l** |
| – **Content of water *** | **0.74 % w/w** |
| The Active Ingredient(s) content is within the FAO limits. | |
| Methodology used for Characterization | HPLC, Karl Fischer titration, OECD 109 (oscillating density meter) |
| **Physical Analysis** | |
| – **Appearance** | **brown liquid** |
| – **Density *** | **976 kg/m$^3$** |
| **Stability:** | |
| – **Storage Temperature** | **< 30°C** |
| – **Recertification Date** | **End of July 2014** |

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 Muenchwilen AG.

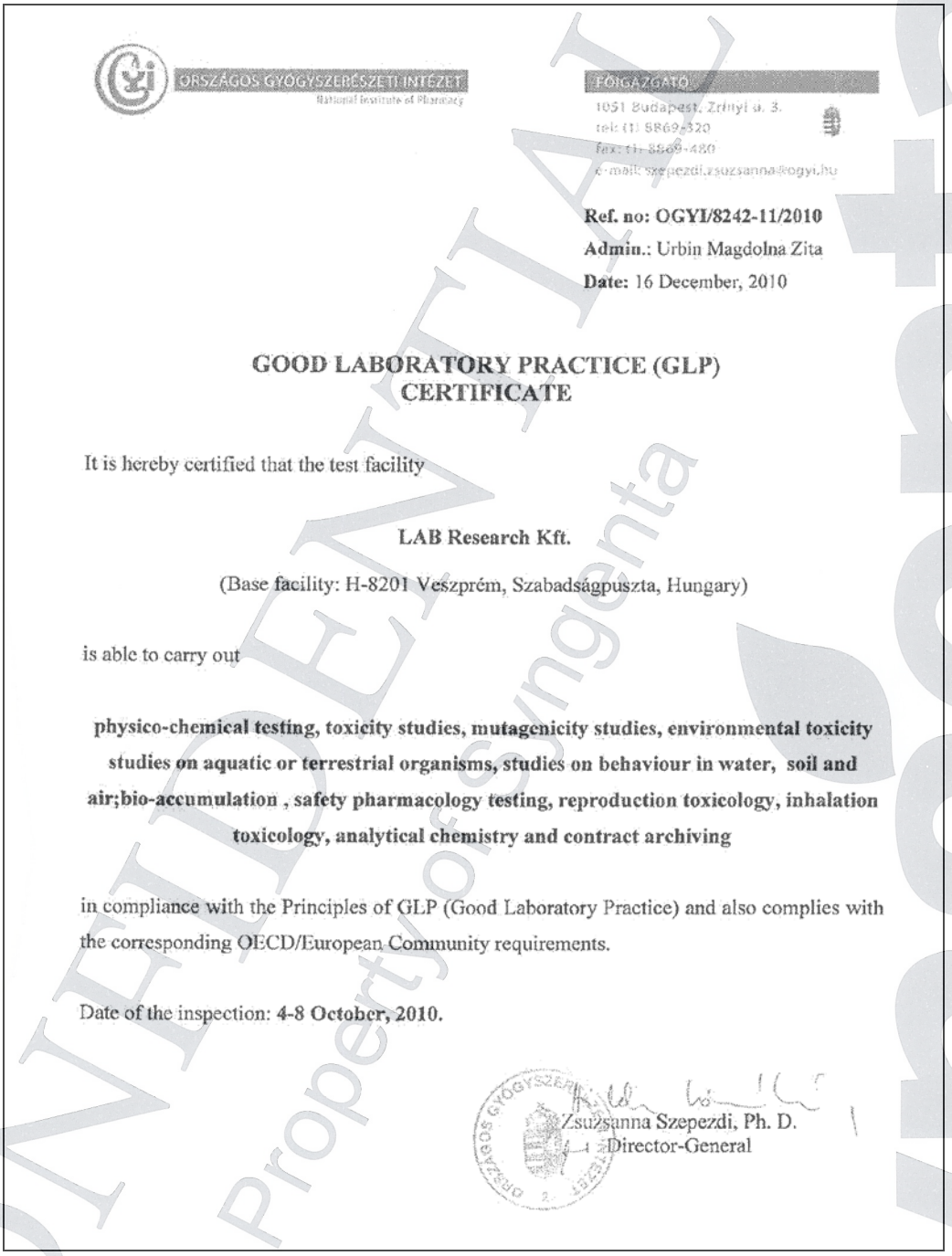
Study number of batch characterization: 124140

Authorisation:

06 February 2012

E. Ebi
Analytical Development & Product Chemistry

## APPENDIX 2 GLP Certificate



ORSZÁGOS GYÓGYSZERÉSZETI INTÉZET
National Institute of Pharmacy

FŐIGAZGATÓ
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**Ref. no: OGYI/8242-11/2010**
**Admin.:** Urbin Magdolna Zita
**Date:** 16 December, 2010

**GOOD LABORATORY PRACTICE (GLP) CERTIFICATE**

It is hereby certified that the test facility

**LAB Research Kft.**

(Base facility: H-8201 Veszprém, Szabadságpuszta, Hungary)

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 , safety pharmacology testing, 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: **4-8 October, 2010.**

ORSZÁGOS GYÓGYSZERÉSZETI INTÉZET

Zsuzsanna Szepezdi, Ph. D.
Director-General

*Translation (from Hungarian to English):*

*Stamp Translation = Országos Gyógyszerészeti Intézet (OGYI) = National Institute of Pharmacy*
*Főigazgató = Director-Genera*

*The facility name was LAB Research Ltd until 1st September 2011. From this date, the registered name is now CiToxLAB Hungary Ltd.,this information has been transmitted to the GLP competent authority. The above GLP certificate is valid for this facility (now known as CiToxLAB Hungary Ltd.) until the certificate expires (16 December 2012).*