Benzovindiflupyr

Benzovindiflupyr EC (A15457H) - Micronucleus Test in Human Lymphocytes *In Vitro*

Final Report

TEST GUIDELINE(S): OECD 487 (2016)

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COMPLETION DATE: 10 December 2019

PERFORMING LABORATORY: ICCR-Roßdorf GmbH
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SPONSOR(S): Syngenta Ltd
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study, performed in the test facility of ICCR-Roßdorf GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version.

OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17

EC Commission Directive 2004/10/EC

These principles are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

There were no circumstances that may have affected the quality or integrity of the study.

A. S

10 December 2019

Dipl. Biol. Andrea Sokolowski
Genetic Toxicology *in vitro*

Date

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

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QUALITY ASSURANCE STATEMENT

Study Number:	1970200
Test Substance:	Benzovindiflupyr EC (A15457H)
Study Director:	Dipl. Biol. Andrea Sokolowski
Title:	Benzovindiflupyr EC (A15457H) - Micronucleus Test in Human Lymphocytes *In Vitro*

Study based activities at the Test Facility ICCR-Roßdorf GmbH were audited and inspected. The details of these audits and inspections are given below.

Type of Inspection	Date(s) of Inspection	Date Reporting to Study Director, Test Facility Management
Study Plan Verification	05 August 2019	05 August 2019
Process – based Test item preparation	07 August 2019	07 August 2019
Test item preparation, test system preparation and application	26 September 2019	26 September 2019
Report Audit	04+05 November 2019	05 November 2019

General facilities and activities where this study was conducted were inspected on an annual basis and results are reported to the relevant responsible person and Management.

The statement is to confirm, that this report reflects the raw data.

Quality Assurance

Marina Hahn

Quality Assurance Auditor
ICCR-Roßdorf GmbH

10 December 2019
Date

PROJECT STAFF SIGNATURE

Study Director — Dipl. Biol. Andrea Sokolowski

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Date: 10 December 2019

GENERAL INFORMATION

Statement
On 30.08.2019 the legal entity Envigo CRS GmbH was renamed as ICCR-Roßdorf GmbH. No other changes to the legal entity have occurred.

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

Name	Title
Dipl. Biol. Andrea Sokolowski	Study Director
Dr. Markus Schulz	Management
Frauke Hermann	Head of Quality Assurance Unit
Eva Lessmann	Syngenta Study Manager

Study dates
Study initiation date: 05 August 2019
Experimental start date: 07 August 2019
Experimental termination date: 01 October 2019

Deviations from the guidelines
None

Retention of samples
None

Performing laboratory test substance number
S 2048711

Other
Records and documentation relating to this study will be maintained in the archives of ICCR-Roßdorf GmbH for a period of 4 years from the date on which the Study Director signs the final report. This will include but may not be limited to the Study Plan, any amendments, raw data, Report and specimens generated during the course of this study.

At termination of the aforementioned period, the records and documentation will be transferred to the GLP compliant archive of Covance CRS (Switzerland) Ltd. at Füllinsdorf, Switzerland, for further archiving up to a total archiving period of 15 years.

A sample of the test item will not be archived.

ICCR-Roßdorf GmbH will retain in its archive a copy of the study plan and final report, and any amendments indefinitely.

Deviations from the study plan
None

Distribution of the report

Sponsor	2 × electronic copy (1 × pdf-file, 1 × word-file)
Study Director	1 × (original)

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

The test substance Benzovindiflupyr EC (A15457H), dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments.

In each experimental group two parallel cultures were analysed. Per culture 1000 binucleated cells were evaluated for cytogenetic damage.

The highest applied concentration in this study (2000 µg/mL of the test substance) was chosen with respect to the current OECD Guideline 487.

Concentration selection of the cytogenetic experiment was performed considering the toxicity data and phase separation in accordance with OECD Guideline 487.

1.2 Results

In Experiment I in the absence of S9 mix, cytotoxicity (46.3% cytostasis) was observed at the highest evaluated concentration. The next higher tested concentration, however, which was separated by a factor smaller than requested by the guideline was outside the recommended range of cytotoxicity (74.5% cytostasis) and therefore not evaluated for cytogenetic damage. In the presence of S9 mix, clear cytotoxicity (53.8% cytostasis) was observed at the highest evaluated concentration. In Experiment II in the absence of S9 mix after continuous treatment, clear cytotoxicity (61.7% cytostasis) was observed at the highest evaluated concentration.

In the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test item. The mean percentage of the micronuclei in all treated conditions was within the 95% historical control limits and none of the values were statistically significantly increased, when compared to the vehicle control. The outcome of the study is clearly negative.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

1.3 Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes. Therefore, Benzovindiflupyr EC (A15457H) is considered to be clearly negative and non-mutagenic in this *in vitro* micronucleus test, when tested up to cytotoxic concentrations.

2.0 INTRODUCTION

2.1 Purpose

The occurrence of micronuclei in interphase cells provides an indirect, but easy and rapid measure of structural chromosomal damage and aneugenicity in cells that have undergone cell division during or after exposure to the test substance. Micronuclei arise from chromosomal fragments or whole chromosomes and rarely occur spontaneously, but are inducible by clastogens or agents affecting the spindle apparatus (Countryman and Heddle, 1976; Obe and Beek, 1982, Rosefort *et al*, 2004).

2.2 Justification of Test System

The induction of cytogenetic damage in human lymphocytes was assessed in two independent experiments with one preparation interval (40 hours). Human lymphocytes have been widely used for this assay type as described in the OECD test guideline 487 (2016).

Micronuclei should only be evaluated in cells that have completed mitosis during exposure to the test substance or during the post-exposure period and thus a cytokinesis blocker, cytochalasin B, is added to the cell culture to ensure that there are binucleated cells to be evaluated for micronuclei (Rosefort et al, 2004).

Treatments started after a 48 hour stimulation period with phytohaemagglutinine (PHA) when cells were actively proliferating and the cells were prepared at approximately 2 – 2.5 fold of the normal cell cycle time.

For validation of the test, control mutagens were tested in parallel to the test substance.

2.3 Regulatory Guidelines

This study was conducted according to the procedures indicated by the following internationally accepted guideline and recommendations:

- OECD Guideline for the Testing of Chemicals No. 487 "*In vitro* Mammalian Cell Micronucleus Test", adopted 29 July 2016.

The following alterations from the guidelines were performed:

- A series of in-house non-GLP validation experiments was performed to get distinct responses of statistical significance when using the specified positive controls (Bohnenberger et al, 2011). To achieve such response the test design, specifically for the treatment, the recovery phase and harvest time, was slightly modified comparing the current proposal given in the OECD Guideline 487. The optimum positive control micronuclei responses were found with the time schedule stated in section 3.7.1.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance and the information concerning the test substance were provided by Syngenta:

Identification:	Benzovindiflupyr EC (A15457H)
Batch:	SBM7L02095
Content of Benzovindiflupyr:	10.2% w/w corresponding to 99.1 g/l*
Molecular weight:	n.a.
Physical state / Appearance:	Liquid, yellow to light brown, clear
Retest Date:	28 February 2021
Storage Conditions:	At room temperature
Stability in Solvent:	Not indicated by the Sponsor

*Correction for content was not made.

3.2 Test Substance Preparation

On the day of the experiment (immediately before use), the test substance was dissolved in DMSO. The final concentration of DMSO in the culture medium was 0.5% (v/v). The solvent was chosen as the best suitable solvent compared to water, DMSO, acetone, and ethanol, according to its solubilisation properties and its compatibility with cell cultures (Easterbrook *et al*, 2001).

The osmolarity and pH of the test substance dissolved in DMSO and diluted in culture medium were determined by using an osmometer and a pH meter, respectively, in the pre-experiment without metabolic activation in the solvent control and the respective maximum concentration.

3.3 Controls

3.3.1 Solvent controls

Concurrent solvent controls (culture medium with 0.5 % DMSO) were performed.

Name:	DMSO
Supplier:	Fisher Chemical, 58239 Schwerte, Germany
Purity:	99.93 %
Lot No. / Expiry Date:	1871845 / July 2024 (Pre-Exp.) 1871845 / August 2024 (Exp. I & II)

3.3.2 Positive control substances

Without metabolic activation

Name:	Mitomycin C (MMC) (pulse treatment)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	108 M 4160 V
Expiry Date:	November 2020
Purity:	98 %
Dissolved in:	Deionized water
Concentration:	0.8 µg/mL

Name:	Demecolcine (continuous treatment)*
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	BCBP 6056 V
Expiry Date:	June 2020
Purity:	≥ 98 %
Dissolved in:	Deionized water
Concentration:	150 ng/mL

* OECD 487, paragraph 33 permits the use of an alternative positive control agent, if a sufficient laboratory historical data base has been established and is scientifically justified.

With metabolic activation

Name:	Cyclophosphamide (CPA)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	MKBX 1822 V
Expiry Date:	May 2020
Purity:	97 – 103 %
Dissolved in:	Saline (0.9 % NaCl [w/v])
Concentration:	17.5 µg/mL

The dilutions of the stock solutions were prepared on the day of the experiment. The stability of the positive control substance in solution is unknown but a mutagenic response in the expected range is sufficient biological evidence for chemical stability.

3.4 Experimental Design

3.4.1 Reason for the choice of human lymphocytes

Human lymphocytes are commonly used in the *in vitro* micronucleus test and have been used successfully for a long time in *in vitro* experiments. They show stable spontaneous micronucleus frequencies at a low level (Countryman and Heddle, 1976; Evans and O'Riordan, 1975).

3.4.2 Blood collection and delivery

Blood samples were obtained from healthy non-smoking donors not receiving medication. For this study, blood was collected from a male donor (22 years old) for the pre-experiment, from a male donor (21 years old) for Experiment I and from a female donor (29 years old) for Experiment II.

Blood samples were drawn by venous puncture and collected in heparinized tubes by Dr. V. Theodor (64380 Rossdorf, Germany). The tubes were sent to ICCR-Roßdorf GmbH to initiate cell cultures within 24 h after blood collection.

3.5 Mammalian Microsomal Fraction S9 Mix

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Wistar rats (RjHan:WI; Janvier Labs, 53941 Saint-Berthevin Cedex, France) induced by peroral administration of 80 mg/kg b.w. phenobarbital (Sigma-Aldrich Chemie GmbH, 82024 Taufkirchen, Germany) and by peroral administrations of ß-naphthoflavone (Acros Organics, 2440 Geel, Belgium) each, on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1+3 parts) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored in ampoules at –80 °C. Small numbers of the ampoules can be kept at –20 °C for up to one week.

Each batch of S9 is routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test (Ames et al, 1975).

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation was 30.4 mg/mL (Lot no. 210618) for the pre-experiment and 30.7 mg/mL (Lot no. 310119) for Experiment I.

3.6 Concentration Selection

Concentration selection was performed according to the current OECD Guideline 487 for the *in vitro* micronucleus test (2016). The highest test substance concentration should be 10 mM, 2 mg/mL, or 2 µL/mL, whichever is the lowest. Four test substance concentrations were evaluated for cytogenetic damage.

In case of test item induced cytotoxicity, measured by a reduced cytokinesis-block proliferation index (CBPI) and expressed as cytostasis, or precipitation / phase separation (observed at the end of test item exposure by the unaided eye) the concentration selection should reflect these properties of the test substance. Where cytotoxicity occurs, the applied concentrations should cover a range from no to approximately 55 ± 5 % cytostasis. For poorly soluble test substances, which are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analyzed should produce turbidity or visible precipitation / phase separation.

3.7 Experimental Performance Cytogenetic Experiment

3.7.1 Schedule

	Without S9 mix		With S9 mix
	Pre-exp. & Exp. I	Exp. II	Pre-exp. & Exp. I
Stimulation period (h)	48	48	48
Exposure period (h)	4	20	4
Recovery (h)	16	—	16
Cytochalasin B exposure (h)	20	20	20
Total culture period (h)	88	88	88

3.7.2 Culture conditions

Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 h after blood collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (phytohemagglutinin) (3 µg/mL), 10 % FBS (fetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

The following volumes were added to the flasks (per 10 mL):

7.60 mL culture medium
1.00 mL fetal bovine serum
0.10 mL antibiotic solution
0.05 mL phytohemagglutinin (stock solution: 0.6 mg/mL)
0.05 mL heparin
0.10 mL HEPES
1.10 mL whole blood

All incubations were done at 37 °C with 5.5 % CO_2 in humidified air.

3.7.3 Pre-experiment

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison to the controls by counting 500 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the main assay.

The pre-test was performed with 10 concentrations of the test substance separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 h (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

3.7.4 Cytogenetic experiment

Pulse exposure
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with serum-free medium containing the test substance. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose · H_2O, 192 mg/L Na_2HPO_4 · 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 hours until preparation.

Continuous exposure (without S9 mix)
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test substance. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 hours until preparation.

3.7.5 Preparation of cells

The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in saline G (approximately 5 mL) and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in KCl solution (5 mL, 0.0375 M) and incubated at 37 °C for 20 minutes. Ice-cold fixative mixture of methanol and glacial acetic acid (1 mL, 19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by

centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The mounted cells were Giemsa-stained and, after drying, covered with coverslips. All slides were labeled with a computer-generated random code to prevent scorer bias.

3.7.6 Evaluation of cytotoxicity damage

The cytotoxicity was judged in the course of a microscopical pre-check of the specimen slides for guideline requested quality and quantity criteria in a first step. Subsequently the CBPI was used as the preferred method for quantifying the effect on cell proliferation and the cytotoxic or cytostatic activity by the OECD Guideline 487. To describe cytotoxic effects the CBPI was determined in 500 cells per culture. Evaluation of the slides was performed using microscopes with 40 x objectives. Cytotoxicity is expressed as cytostasis, calculating the CBPI, and used therefore as a cut off criterion. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Under some circumstances the CBPI does not reflect the cytotoxicity accurately and concentrations may be excluded from the evaluation during the microscopic pre-check. CBPI measures proliferation and may not detect cytotoxic events like necrosis, oncosis and apoptosis. In particular mononuclear cells without cytoplasm (representing cells which undergo cell death in the treatment cell cycle) are not represented in the CBPI because those cells do not fulfil the quality criteria for evaluation (see section 3.7.7). This can result in too few cells available for scoring.

$$CBPI = \frac{(MONC \times 1) + (BINC \times 2) + (MUNC \times 3)}{n}$$

CBPI	Cytokinesis-block proliferation index
n	Total number of cells
MONC	Mononucleate cells
BINC	Binucleate cells
MUNC	Multinucleate cells

$$\text{Cytostasis \%} = 100 - 100\,[(CBPI_T - 1) / (CBPI_C - 1)]$$

T	Test substance
C	Solvent control

3.7.7 Evaluation of cytogenetic damage

Evaluation of the slides was performed using microscopes with 40 x objectives. The micronuclei were counted in binucleated cells showing a clearly visible cytoplasm area. The criteria for the evaluation of micronuclei are described in the publication of Countryman and Heddle (1976). The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not be more than one third of the area of the main nucleus. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells.

3.8 Data Recording

The data were recorded in the laboratory documentation. The results are presented in tabular form, including experimental groups with the test substance, solvent controls, and positive controls, respectively.

3.9 Acceptability Criteria

The micronucleus assay will be considered acceptable if it meets the following criteria:

- The concurrent solvent control will normally be within the 95% control limits of the laboratory's historical solvent control data.
- The concurrent positive controls should induce responses that are compatible with the laboratory historical positive control data and produce a statistically significant increase.
- Cell proliferation criteria in the solvent control are considered to be acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides must allow the evaluation of an adequate number of cells and concentrations.

The criteria for the selection of top concentration are consistent with those described in section 'Concentration selection'.

3.10 Interpretation of Results

Providing that all of the acceptability criteria are fulfilled, a test substance is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test substance concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase when assessed by a trend test
- The results in all evaluated test substance concentrations should be within the 95% control limits of the laboratory's historical solvent control data

The test substance is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test substance is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test substance concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition when assessed by a trend test
- The results are outside the range of the 95% control limit of the laboratory historical solvent control data

When all of the criteria are met, the test substance is then considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions, i.e. S9 concentration or S9 origin) could be useful.

However, results may remain questionable regardless of the number of times the experiment is repeated. If the data set will not allow a conclusion of positive or negative, the test substance will therefore be concluded as equivocal.

3.11 Statistical Analysis

Statistical significance was confirmed by the Chi-squared test ($p < 0.05$) using a validated "R" Script for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R", to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells, obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

4.0 RESULTS AND DISCUSSION

The test substance Benzovindiflupyr EC (A15457H), dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure period was 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test substance.

In each experimental group two parallel cultures were analyzed. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides making a total of 2000 binucleated cells per test substance concentration. To assess cytotoxicity, the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 cells per culture. Percentage of cytostasis (inhibition of cell growth) is also reported.

The highest treatment concentration in the pre-test for toxicity, 2000 µg/mL was chosen with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

Test substance concentrations ranging from 13.0 µg/mL to 2000 µg/mL (with and without S9 mix) were chosen for evaluation of cytotoxicity. In the pre-test for toxicity, phase separation of the test substance was observed at the end of treatment at 213 µg/mL and above in the absence of S9 mix and at 122 µg/mL and above in the presence of S9 mix.
The experiment was repeated due to strong cytotoxicity. Test substance concentrations ranging from 0.19 µg/mL to 30.0 µg/mL (with and without S9 mix) were chosen for evaluation of cytotoxicity. No phase separation of the test substance was observed at the end of treatment. Since the cultures fulfilled the requirements for cytogenetic evaluation, this test was designated Experiment I.

Using a reduced Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity, cytotoxicity was observed in Experiment I after 4 hours treatment in the absence and presence of S9 mix at the highest evaluated concentration (5.6 µg/mL).

Considering the phase separation and cytotoxicity data, 20.0 µg/mL (without S9 mix) was chosen as top treatment concentration for Experiment II.

The applied concentrations for each experiment are presented in Table 1.

No relevant influence on the osmolarity and pH was observed as shown below.

		Concentration [µg/mL]	Osmolarity [mOsm]	pH
Pre-exp.	Solvent control	-	347	7.60
	Benzovindiflupyr EC (A15457H)	2000	342	7.55

In Experiment I in the absence of S9 mix, cytotoxicity (46.3% cytostasis) was observed at the highest evaluated concentration. The next higher tested concentration, however, which was separated by a factor smaller than requested by the guideline was outside the recommended range of cytotoxicity (74.5% cytostasis) and therefore not evaluated for cytogenetic damage. In the presence of S9 mix, clear cytotoxicity (53.8% cytostasis) was observed at the highest evaluated concentration. In Experiment II in the absence of S9 mix after continuous treatment, clear cytotoxicity (61.7% cytostasis) was observed at the highest evaluated concentration.

In the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test item. The mean percentage of the micronuclei in all treated conditions was within the 95% historical control limits and none of the values were statistically significantly increased, when compared to the vehicle control. The outcome of the study is clearly negative.

Demecolcine (150 ng/mL), MMC (0.8 µg/mL) or CPA (17.5 µg/mL) were used as appropriate positive control chemicals and showed statistically significant increases in binucleated cells with micronuclei demonstrating the correct performance of the assay.

5.0 CONCLUSIONS

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes. Therefore, Benzovindiflupyr EC (A15457H) is considered to be clearly negative and non-mutagenic in this *in vitro* micronucleus test, when tested up to cytotoxic concentrations.

6.0 REFERENCES

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TABLES SECTION

TABLE 1 Concentrations Applied in the Micronucleus Assay with Benzovindiflupyr EC (A15457H)

Exp.	Prep. interval (h)	Exposure period (h)	Concentrations (µg/mL)										
Without S9 mix													
Pre-*	40	4		13.0	22.7	39.8	69.6	122	213[PS]	373[PS]	653[PS]	1143[PS]	2000[PS]
I	40	4		0.19	0.34	**0.60**	1.0	**1.8**	**3.2**	**5.6**	9.8	17.1	30.0
II	40	20	0.12	0.21	0.36	0.63	**1.1**	**1.9**	3.4	**5.1**	**7.6**	11.4	20.0
With S9 mix													
Pre-*	40	4		13.0	22.7	39.8	69.6	122[PS]	213[PS]	373[PS]	653[PS]	1143[PS]	2000[PS]
I	40	4		0.19	0.34	**0.60**	**1.0**	**1.8**	3.2	**5.6**	9.8	17.1	30.0

Evaluated experimental points are shown in bold characters

PS Phase separation was observed at the end of treatment

* Was repeated due to strong cytotoxicity

TABLE 2 Summary of Results of the Micronucleus Assay with Benzovindiflupyr EC (A15457H)

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	95% Ctrl limit
Exposure period 4 h without S9 mix						
I	40 h	Solvent control[1]	1.99		0.65	0.01 – 1.20
		Positive control[2]	1.92	6.7	**6.55[S]**	2.66 – 22.74
		0.60	1.94	5.1	0.55	
		1.8	1.82	16.9	0.60	
		3.2	1.75	24.0	1.15	
		5.6	1.53	46.3	0.65	
		Trend test: p-value 0.619				
Exposure period 20 h without S9 mix						
II	40 h	Solvent control[1]	1.94		0.45	0.00 – 1.14
		Positive control[3]	1.41	56.8	**3.75[S]**	1.15 – 6.44
		1.1	1.88	5.9	0.35	
		1.9	1.80	15.1	0.40	
		5.1	1.62	33.8	0.40	
		7.6	1.36	61.7	0.20	
		Trend test: p-value 0.115				
Exposure period 4 h with S9 mix						
I	40 h	Solvent control[1]	1.95		0.50	0.00 – 1.24
		Positive control[4]	1.82	13.1	**2.25[S]**	1.01 – 7.34
		0.60	1.80	15.9	0.10	
		1.0	1.77	18.8	0.30	
		1.8	1.62	34.6	0.40	
		5.6	1.44	53.8	0.55	
		Trend test: p-value 0.367				

* For the positive control groups and the test item treatment groups the values are related to the solvent controls

** The number of micronucleated cells was determined in a sample of 2000 binucleated cells

s The number of micronucleated cells is statistically significantly higher than corresponding control values

1 DMSO 0.5 % (v/v)

2 MMC 0.8 µg/mL

3 Demecolcine 150 ng/mL

4 CPA 17.5 µg/mL

TABLE 3 Toxicity – Pre-Experiment (Cytotoxicity of Benzovindiflupyr EC (A15457H) to the Cultures of Human Lymphocytes)

Concentration (µg/mL)	Exposure time (h)	Preparation interval (h)	CBPI per 500 cells*	Cytostasis (%)
Without S9 mix				
Solvent control	4	40	1.83	-
13.0	4	40	1.22	73.4
22.7	4	40	1.12	85.9
39.8#	4	40	1.03	96.1
69.6#	4	40	1.07	91.9
122#	4	40	1.03	96.9
213PS #	4	40	n.e.	n.e.
373	4	40	n.p.	n.p.
653	4	40	n.p.	n.p.
1143	4	40	n.p.	n.p.
2000	4	40	n.p.	n.p.
With S9 mix				
Solvent control	4	40	1.81	-
13.0	4	40	1.09	88.3
22.7#	4	40	1.06	92.0
39.8#	4	40	1.04	95.3
69.6#	4	40	n.e.	n.e.
122PS #	4	40	n.e.	n.e.
213	4	40	n.p.	n.p.
373	4	40	n.p.	n.p.
653	4	40	n.p.	n.p.
1143	4	40	n.p.	n.p.
2000	4	40	n.p.	n.p.

* Mean value of two cultures
\# Due to strong cytotoxic effects, there were no 1000 evaluable cells per culture
PS Phase separation occurred at the end of treatment
n.e. Not evaluable due to strong cytotoxic effects and insufficient binucleated cells
n.p. Not prepared

TABLE 4 Toxicity - Experiment I (Cytotoxicity of Benzovindiflupyr EC (A15457H) to the Cultures of Human Lymphocytes)

Concentration (µg/mL)	Exposure time (h)	Preparation interval (h)	CBPI per 500 cells*	Cytostasis (%)
Without S9 mix				
Solvent control	4	40	1.99	-
0.19	4	40	2.00	n.c.
0.34	4	40	1.96	2.5
0.60	4	40	1.94	5.1
1.0	4	40	1.83	16.4
1.8	4	40	1.82	16.9
3.2	4	40	1.75	24.0
5.6	4	40	1.53	46.3
9.8	4	40	1.25	74.5
17.1	4	40	1.15	85.2
30.0	4	40	1.23	76.9
With S9 mix				
Solvent control	4	40	1.95	-
0.19	4	40	1.91	4.0
0.34	4	40	1.81	14.9
0.60	4	40	1.80	15.9
1.0	4	40	1.77	18.8
1.8	4	40	1.62	34.6
3.2	4	40	1.55	42.1
5.6	4	40	1.44	53.8
9.8	4	40	1.20	78.9
17.1	4	40	1.06	93.8
30.0	4	40	1.03	97.1

Experimental groups evaluated for cytogenetic damage are shown in bold characters

* Mean value of two cultures

n.c. Not calculated as the CBPI was equal or higher than solvent control value

TABLE 5 Toxicity - Experiment II (Cytotoxicity of Benzovindiflupyr EC (A15457H) to the Cultures of Human Lymphocytes)

Concentration (µg/mL)	Exposure time (h)	Preparation interval (h)	CBPI per 500 cells*	Cytostasis (%)
Without S9 mix				
Solvent control	20	40	1.94	-
0.12	20	40	1.88	6.1
0.21	20	40	1.87	7.9
0.36	20	40	1.94	0.3
0.63	20	40	1.91	3.4
1.1	20	40	1.88	5.9
1.9	20	40	1.80	15.1
3.4	20	40	1.66	29.9
5.1	20	40	1.62	33.8
7.6	20	40	1.36	61.7
11.4	20	40	1.18	80.4
20.0	20	40	n.e.	n.e.

Experimental groups evaluated for cytogenetic damage are shown in bold characters
* Mean value of two cultures
n.e. Not evaluable due to strong cytotoxic effects and insufficient binucleated cells

TABLE 6 **Experiment I - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 4 h without S9 Mix**

Treatment group	Conc. per mL	S9 mix	Exposure / preparation (h)	Cell proliferation culture 1* c1	c2	c4-c8	Proliferation Index CBPI	Cell proliferation culture 2* c1	c2	c4-c8	Proliferation Index CBPI	CBPI mean	Cytostasis [%]
Solv. control#	0.5 %	-	4 / 40	41	430	29	1.98	32	437	31	2.00	1.99	
Pos. control##	0.8 µg	-	4 / 40	39	442	19	1.96	74	411	15	1.88	1.92	6.7
Test item	0.60 µg	-	4 / 40	41	439	20	1.96	51	440	9	1.92	1.94	5.1
″	1.8 µg	-	4 / 40	85	412	3	1.84	105	388	7	1.80	1.82	16.9
″	3.2 µg	-	4 / 40	162	335	3	1.68	96	399	5	1.82	1.75	24.0
″	5.6 µg	-	4 / 40	234	264	2	1.54	239	260	1	1.52	1.53	46.3

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

DMSO

MMC

TABLE 7 **Experiment I - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 4 h with S9 Mix**

Treatment group	Conc. per mL	S9 mix	Exposure / preparation (h)	Cell proliferation culture 1* c1	c2	c4-c8	Proliferation Index CBPI	Cell proliferation culture 2* c1	c2	c4-c8	Proliferation Index CBPI	CBPI mean	Cytostasis [%]
Solv. control#	0.5 %	+	4 / 40	81	368	51	1.94	76	372	52	1.95	1.95	
Pos. control##	17.5 µg	+	4 / 40	122	353	25	1.81	124	333	43	1.84	1.82	13.1
Test item	0.60 µg	+	4 / 40	120	375	5	1.77	97	395	8	1.82	1.80	15.9
″	1.0 µg	+	4 / 40	126	371	3	1.75	118	373	9	1.78	1.77	18.8
″	1.8 µg	+	4 / 40	187	312	1	1.63	198	299	3	1.61	1.62	34.6
″	5.6 µg	+	4 / 40	269	229	2	1.47	296	204	0	1.41	1.44	53.8

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

DMSO

CPA

TABLE 8 **Experiment I - Number of Micronucleated Cells; Exposure Period 4 h without S9 Mix**

Treatment group	Conc. per mL	S9 mix	Exposure/ Preparation (h)	Micronucleated cells									
				Binucleate cells with *n* micronuclei culture 1			sum culture 1	Binucleate cells with *n* micronuclei culture 2			sum culture 2	sum in 2000 binucleate cells	[%]
				1	2	>2		1	2	>2			
Solv. control[#]	0.5 %	-	4 / 40	6	1	0	7	6	0	0	6	13	0.65
Pos. control[##]	0.8 µg	-	4 / 40	56	5	2	63	59	8	1	68	131	6.55
Test item	0.60 µg	-	4 / 40	6	1	0	7	4	0	0	4	11	0.55
″	1.8 µg	-	4 / 40	7	0	0	7	5	0	0	5	12	0.60
″	3.2 µg	-	4 / 40	7	0	0	7	14	2	0	16	23	1.15
″	5.6 µg	-	4 / 40	7	0	0	7	6	0	0	6	13	0.65

[#] DMSO
[##] MMC

TABLE 9 **Experiment I - Number of Micronucleated Cells; Exposure Period 4 h with S9 Mix**

Treatment group	Conc. per mL	S9 mix	Exposure/ Preparation (h)	Micronucleated cells									
				Binucleate cells with *n* micronuclei culture 1			sum culture 1	Binucleate cells with *n* micronuclei culture 2			sum culture 2	sum in 2000 binucleate cells	[%]
				1	2	>2		1	2	>2			
Solv. control[#]	0.5 %	+	4 / 40	1	0	0	1	9	0	0	9	10	0.50
Pos. control[##]	17.5 µg	+	4 / 40	17	0	0	17	28	0	0	28	45	2.25
Test item	0.60 µg	+	4 / 40	1	0	0	1	1	0	0	1	2	0.10
″	1.0 µg	+	4 / 40	2	0	0	2	4	0	0	4	6	0.30
″	1.8 µg	+	4 / 40	3	0	0	3	5	0	0	5	8	0.40
″	5.6 µg	+	4 / 40	7	0	0	7	4	0	0	4	11	0.55

[#] DMSO
[##] CPA

TABLE 10 **Experiment II - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 20 h without S9 Mix**

Treatment group	Conc. per mL	S9 mix	Exposure / preparation (h)	Cell proliferation culture 1* c1	c2	c4-c8	Proliferation Index CBPI	Cell proliferation culture 2* c1	c2	c4-c8	Proliferation Index CBPI	CBPI mean	Cytostasis [%]
Solv. control#	0.5 %	-	20 / 40	65	391	44	1.96	73	394	33	1.92	1.94	
Pos. control##	150 ng	-	20 / 40	306	188	6	1.40	302	190	8	1.41	1.41	56.8
Test item	1.1 µg	-	20 / 40	87	376	37	1.90	92	382	26	1.87	1.88	5.9
″	1.9 µg	-	20 / 40	112	375	13	1.80	115	374	11	1.79	1.80	15.1
″	5.1 µg	-	20 / 40	199	296	5	1.61	187	310	3	1.63	1.62	33.8
″	7.6 µg	-	20 / 40	303	196	1	1.40	339	160	1	1.32	1.36	61.7

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

\# DMSO

\## Demecolcine

TABLE 11 Experiment II - Number of Micronucleated Cells; Exposure Period 20 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ Preparation (h)	Micronucleated cells									
				Binucleate cells with *n* micronuclei culture 1			sum culture 1	Binucleate cells with *n* micronuclei culture 2			sum culture 2	sum in 2000 binucleate cells	[%]
				1	2	>2		1	2	>2			
Solv. control[#]	0.5 %	-	20 / 40	6	1	0	7	2	0	0	2	9	0.45
Pos. control[##]	150 ng	-	20 / 40	33	0	2	35	38	1	1	40	75	3.75
Test item	1.1 µg	-	20 / 40	4	1	0	5	2	0	0	2	7	0.35
″	1.9 µg	-	20 / 40	2	0	0	2	6	0	0	6	8	0.40
″	5.1 µg	-	20 / 40	5	2	0	7	1	0	0	1	8	0.40
″	7.6 µg	-	20 / 40	3	0	0	3	1	0	0	1	4	0.20

[#] DMSO
[##] Demecolcine

TABLE 12 Biometry

Statistical significance was confirmed by using the Chi-squared test ($\alpha < 0.05$) using a validated R Script for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

Biometry of Experiment I (Chi-squared test)

Test substance versus solvent control (µg/mL)		Preparation interval (h)	Exposure period (h)	S9 mix	Chi^2	p-value
Test substance	0.60	40	4	-	n.c.	n.c.
"	1.8	40	4	-	n.c.	n.c.
"	3.2	40	4	-	2.803	0.094
"	5.6	40	4	-	n.c.	n.c.
"	0.60	40	4	+	n.c.	n.c.
"	1.0	40	4	+	n.c.	n.c.
"	1.8	40	4	+	n.c.	n.c.
"	5.6	40	4	+	0.048	0.827
Positive control versus solvent control (µg/mL)						
MMC	0.8	40	4	-	100.305	2.2×10^{-16} s
CPA	17.5	40	4	+	22.583	2.0×10^{-6} s

n.c. Not calculated as the micronucleus rate is equal or lower than the control rate
s Micronucleus rate is statistically significantly higher than the control rate

Biometry of Experiment II (Chi-squared test)

Test substance versus solvent control (µg/mL)		Preparation interval (h)	Exposure period (h)	S9 mix	Chi^2	p-value
Test substance	1.1	40	20	-	n.c.	n.c.
"	1.9	40	20	-	n.c.	n.c.
"	5.1	40	20	-	n.c.	n.c.
"	7.6	40	20	-	n.c.	n.c.
Positive control versus solvent control per mL						
Demecolcine	150 ng	40	20	-	52.970	3.4×10^{-13} s

n.c. Not calculated as the micronucleus rate is equal or lower than the control rate
s Micronucleus rate is statistically significantly higher than the control rate

A linear regression was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells, obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Linear regression (Trend test)

Experimental groups	p-value
Experiment I, exposure period 4 hrs without S9 mix	0.619
Experiment I, exposure period 4 hrs with S9 mix	0.367
Experiment II, exposure period 4 hrs without S9 mix	0.115

APPENDICES SECTION

APPENDIX 1 Historical Control Data

Percentage of micronucleated cells in human lymphocyte cultures (2018)

Aqueous solvents: DMEM/Ham's F12, Deionised water (10 % v/v)
Organic solvents: DMSO (0.5 or 1.0 %), Acetone, Ethanol and THF (0.5 %)

Solvent Control without S9

	Micronucleated cells in %	
	Pulse treatment (4/40)	Continuous treatment (20/40)
No. of experiments	69*	69**
Mean	0.60	0.53
95 % Ctrl limit	**0.01 – 1.20**	**0.00 – 1.14**
1x SD (2x SD)	0.30 (0.60)	0.31 (0.61)
Min – Max	0.00 – 1.55	0.10 – 1.30

* Aqueous solvents – 16 Experiments; Organic solvents – 53 Experiments
** Aqueous solvents – 15 Experiments; Organic solvents – 54 Experiments

Solvent Control with S9

	Micronucleated cells in %
	Pulse treatment (4/40)
No. of experiments	70*
Mean	0.62
95 % Ctrl limit	**0.00 – 1.24**
1x SD (2x SD)	0.31 (0.62)
Min – Max	0.05 – 1.60

* Aqueous solvents – 16 Experiments; Organic solvents – 54 Experiments

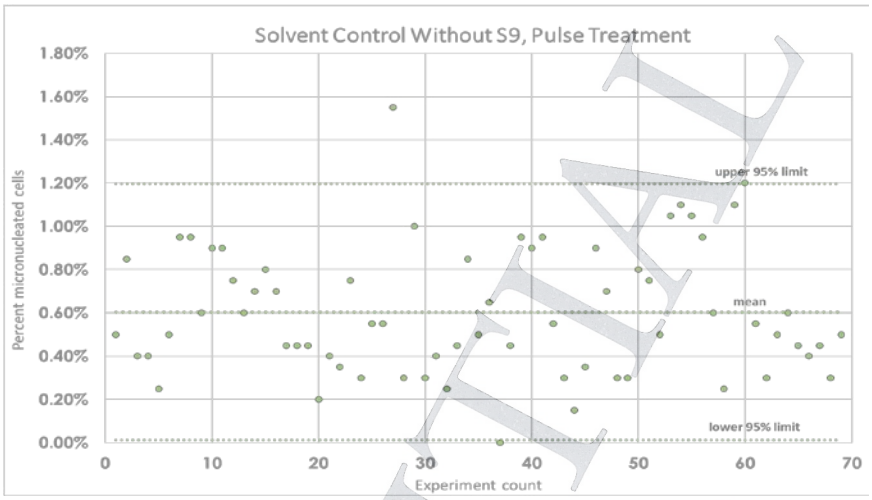
Positive Control without S9

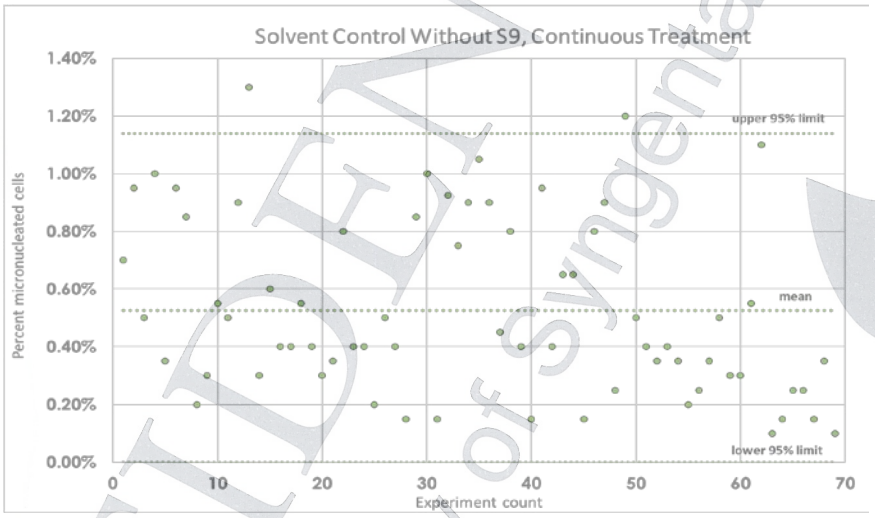
	Micronucleated cells in %	
	Pulse treatment (4/40)	Continuous treatment (20/40)
	MMC	Demecolcin
No. of experiments	69	79
Mean	12.70	3.80
95 % Ctrl limit	**2.66 – 22.74**	**1.15 – 6.44**
1x SD (2x SD)	5.02 (10.04)	1.32 (2.64)
Min – Max	3.95 – 28.60	1.95 – 8.80

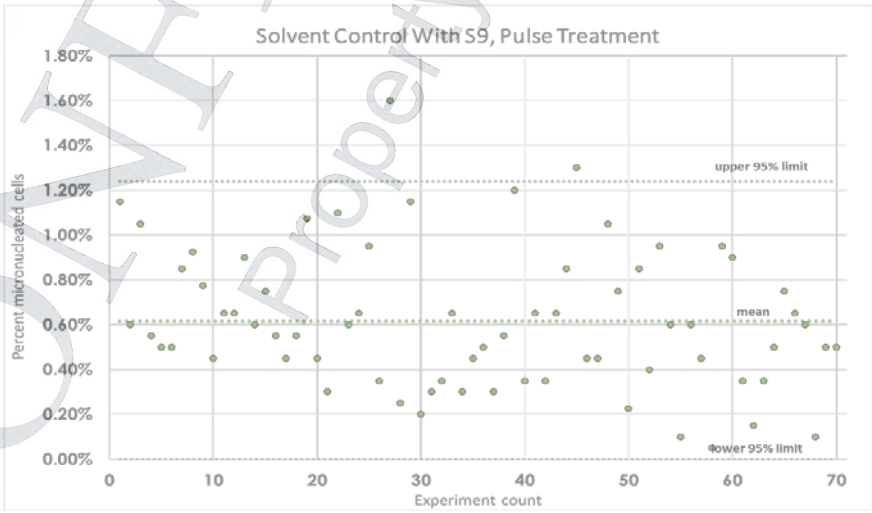
Positive Control with S9

	Micronucleated cells in %
	Pulse treatment (4/40)
	CPA
No. of experiments	80
Mean	4.18
95 % Ctrl limit	**1.01 – 7.34**
1x SD (2x SD)	1.58 (3.17)
Min – Max	1.80 – 8.85

Control Charts







APPENDIX 2 Copy of GLP Certificate

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

ICCR-Roßdorf GmbH
Institute for Competent Contract Research
In den Leppsteinswiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**2** Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**3** Mutagenicity studies
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical and clinical chemistry testing

22.11.2018, 21.02.2019, 12. bis 14.03.2019
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Brandt

Dr. Astrid Brandt, Referentin, Wiesbaden, den **23. Oktober 2019**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hessisches Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80, D 65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority)

English name and address of the GLP Monitoring Authority:
Hessian Ministry for Environment, Energy, Agriculture and Consumer Protection;
Department II 10; P.O. Box 31 09; 65189 Wiesbaden
Translation of the seal inscription:
Hessian Ministry for Environment, Rural Regions and Consumer Protection

APPENDIX 3 Certificate of S9

ENVIGO

CERTIFICATE

ENVIGO CRS S9 PREPARATION LOT NO. 210618

Date of preparation: June 21, 2018
Recertification date: December 12, 2018
Second Recertification date: July 19, 2019

Protein assay: 30.4 mg protein / ml S9

Sterility: 0 colonies / ml S9 on glucose-minimal-agar

Salmonella typhimurium assay (AMES-test)

Treatment	µl S9 / plate	number of revertants in TA 98	number of revertants in TA 98 (second Recertification)
negative	0	28	27
control	100	35	28
10 µg/plate	0	68	47
2-Aminoanthracene	100	2132	2019
10 µg/plate	0	25	27
Benzo(a)pyrene	100	91	84

The S9 was obtained from the livers of male Wistar rats which received triple treatments of 80 mg / kg body weight Phenobarbital and ß-Naphthoflavone orally on consecutive days. The livers were prepared 24 hours after the last treatment.

Quality Assurance Auditor
Envigo CRS GmbH H. Pilawa — Date 26. JULI 2019

Dr. Steffen Naumann
Study Director
Envigo CRS GmbH — Date 12. AUG. 2019

Envigo CRS GmbH
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SOP Origin TS-SOP S9_20

CERTIFICATE

ENVIGO

ENVIGO CRS S9 PREPARATION LOT NO. 310119

Date of preparation: January 31, 2019
Recertification date: August 23, 2019

Protein assay: 30.7 mg protein / ml S9

Sterility: 0 colonies / ml S9 on glucose-minimal-agar

Salmonella typhimurium assay (AMES-test)

Treatment	µl S9 / plate	number of revertants in TA 98	number of revertants in TA 98 (Recertification)
negative	0	29	32
control	100	35	32
10 µg/plate 2-Aminoanthracene	0	81	52
	100	2997	2790
10 µg/plate Benzo(a)pyrene	0	29	26
	100	97	100

The S9 was obtained from the livers of male Wistar rats which received triple treatments of 80 mg / kg body weight Phenobarbital and ß-Naphthoflavone orally on consecutive days. The livers were prepared 24 hours after the last treatment.

H Pilawa — 29. AUG. 2019

Quality Assurance Auditor
Envigo CRS GmbH — H. Pilawa — Date

29. AUG. 2019

Dr. Steffen Naumann
Study Director
Envigo CRS GmbH — Date

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SOP Origin TS-SOP S9_20

APPENDIX 4 Certificate of Analysis

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

Certificate of Analysis

A15457H
Benzovindiflupyr EC (100)
SBM7L02095

Batch Identification	**SBM7L02095**
Other Batch ID	1023902
Product Code	**A15457H**
Other Product Code(s)	benzovindiflupyr EC (100)
Chemical Analysis (Active Ingredient content)	
– **Identity of the Active Ingredient(s)***	**confirmed**
– **Content of benzovindiflupyr***	**10.2 % w/w corresponding to 99.1 g/l** The Active Ingredient(s) content is within the FAO limits.
Methodology used for Characterization / Recertification	HPLC, oscillating density meter
Physical Analysis	
– **Appearance**	**light brown liquid**
– **Density***	**972 kg/m³**
Stability:	
– **Storage Temperature**	**< 30 °C**
– **Recertification Date**	**End of February 2021**

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

Study number of batch characterization: CHMU180071
Study number(s) of batch recertification: —

Authorization: February 19, 2018

Elke Ebi
Analytical Development & Product Chemistry