Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram

Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B) - Micronucleus Test in Human Lymphocytes *In Vitro*

Final Report

TEST GUIDELINE(S): OECD 487 (2016)

AUTHOR(S): Dr. Steffen Naumann

COMPLETION DATE: FINAL

PERFORMING LABORATORY: ICCR-Roßdorf GmbH
In den Leppsteinswiesen 19
64380 Rossdorf, Germany

LABORATORY PROJECT ID:
Report Number: 2190300
Study Number: 2190300
Task Number: TK0518491

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

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

The Following Statement Applies To The United States Of America:

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS
UNDER SPECIFIC 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).

Company: Syngenta Crop Protection, LLC
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419-8300 USA

Submitter: _____ Date: _____

Syngenta is the owner of this information and data. Syngenta has submitted this material to the United States Environmental Protection Agency specifically under the provisions contained in FIFRA as amended and, hereby, consents to use and disclosure of this material by EPA according to FIFRA. In submitting this material to EPA according to method and format requirements contained in PR Notice 2011-3, we do not waive any protection or right involving this material that would have been claimed by the company if this material had not been submitted to the EPA, nor do we waive any protection or right provided under FIFRA Section 3 (concerning data exclusivity and data compensation) or FIFRA Section 10(g) (prohibiting disclosure to foreign and multinational pesticide companies or their agents).

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 procedures are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHW, 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.

Dr. Steffen Naumann
Genetic Toxicology *in vitro*

.....
Date: 29 June 2022

Performing Laboratory:
ICCR-Roßdorf GmbH
In den Leppsteinswiesen 19
64380 Rossdorf, Germany

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

_____ _____
Date

Submitter/Sponsor: Syngenta Crop Protection, LLC
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419-8300 USA

FLAGGING STATEMENT

This page is intentionally left blank. It will be replaced by an appropriate Flagging statement by the Sponsor.

QUALITY ASSURANCE STATEMENT

Study Number: 2190300
Test Substance: Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B)
Study Director: Dr. Steffen Naumann
Title: Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B) - 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	17 December 2021	17 December 2021
Study – based Test system preparation & application	05 May 2022	05 May 2022
Report Audit	21 June 2022	21 June 2022

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

S. Ebert

Sabine Ebert

Quality Assurance Auditor
ICCR-Roßdorf GmbH

28 June 2022
Date

PROJECT STAFF SIGNATURE

Study Director — Dr. Steffen Naumann

.....

Date: 29 June 2022

GENERAL INFORMATION

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

Name	Title
Dr. Steffen Naumann	Study Director
Dr. Markus Schulz	Management
Frauke Hermann	Head of Quality Assurance Unit
Carolina Vaccari	Syngenta Study Manager

Study dates
Study initiation date: 22 December 2021
Experimental start date: 24 March 2022
Experimental termination date: 01 June 2022

Deviations from the guidelines
None

Retention of samples
None

Performing laboratory test substance number
S 2194111

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 Rhenus Archiv Services GmbH, Frankfurt am Main, for further archiving up to a total archiving period of 15 years.

A sample of the test substance 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
The following deviation from study plan occurred:

Culture conditions
In Experiment II blood cultures were established by preparing a 13 % mixture of whole blood in medium instead of a 5 – 12% mixture. This procedure was carried out in order to obtain a larger yield of lymphocytes at the end of the experiment.

This deviation was considered to have not affected the integrity or validity of the study.

Distribution of the report

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

TABLE OF CONTENTS

1.0 EXECUTIVE SUMMARY

1.1 Study Design

The test substance Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B), suspended (Exp. I) or dissolved (Exp. II) 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 precipitation in accordance with OECD Guideline 487.

1.2 Results

In the pulse treatment in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest evaluated concentration, which showed precipitation. In the continuous treatment, clear cytotoxicity was observed after treatment with 46.1 µg/mL (59.7 % cytostasis).

In Experiment I and II in the absence and presence of S9 mix, no relevant increases in the number 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% control limit and none of the values were statistically significantly increased, when compared with the vehicle control. There was also no concentration related increase in micronucleus formation, as judged by an appropriate trend test. The outcome of the study is clearly negative.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in binucleated cells with micronuclei demonstrating the correct performance of the assay.

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, Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B) is considered to be clearly negative (i.e. non-clastogenic and non-aneugenic) in this *in vitro* micronucleus test, when tested up to cytotoxic or precipitating 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 (28 h). 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 phytohemagglutinin (PHA) when cells were actively proliferating and the cells were prepared at approximately 1.5 – 2.0-fold of the normal cell cycle time (Whitwell *et al.*, 2019).

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.

3.0 MATERIALS AND METHODS

3.1 Test Substance

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

Identification:	Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B)		
Batch:	1200767		
Content:	Difenoconazole:	5.45% (w/w)	64.0 g/L
	Fludioxonil:	4.37% (w/w)	51.3 g/L
	Metalaxyl-M:	4.31% (w/w)	50.6 g/L
	Cyclobutrifluram:	21.0% (w/w)	247 g/L
Molecular weight:	Not relevant since the test substance is a formulation		
Physical state / Appearance:	Liquid, red		
Retest Date:	31 August 2024		
Storage Conditions:	At room temperature		
Stability in Solvent:	Not indicated by the Sponsor		

Correction for content of active ingredient was not made.

3.2 Test Substance Preparation

On the day of the experiment (immediately before use), the test substance was suspended (Exp. I) or dissolved (Exp. II) in dimethylsulphoxide (DMSO). The final concentration of DMSO in the culture medium was 1.0 % (v/v). The solvent was chosen as the most suitable solvent compared to water, according to its solubilisation properties and its compatibility with cell cultures (Easterbrook *et al.*, 2001).

All formulations were prepared freshly before treatment and used within two hours of preparation.

Due to the short-term nature of the study, no analysis was carried out to determine the homogeneity, concentration, or stability of the test item formulation, these are not required by the OECD test guideline for the assay.

The osmolarity and pH of the test substance suspended (Exp. I) in DMSO and diluted in culture medium were determined by using an osmometer or 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 1.0 % DMSO) were performed.

Name:	DMSO
Supplier:	Gibco, Fisher Chemical, 58239 Schwerte, Germany
Purity:	≥ 99.9%
Lot No. / Expiry Date:	2179303 / March 2027 (Exp. I) 2212435 / April 2027 (Exp. II)

3.3.2 Positive control substances

Without metabolic activation

Name:	Mitomycin C (MMC) (pulse treatment, clastogen)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	207600
Expiry Date:	December 2022
Purity:	98%
Dissolved in:	Deionised water
Concentration:	0.6 µg/mL

Name:	Vinblastine (continuous treatment, aneugen)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	0000096295
Expiry Date:	March 2023
Purity:	≥ 97%
Dissolved in:	Deionised water
Concentration:	10.0 ng/mL

With metabolic activation

Name:	Cyclophosphamide (CPA, clastogen)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	MKCL2547
Expiry Date:	March 2024
Purity:	97 – 103%
Dissolved in:	Saline (0.9% NaCl [w/v])
Concentration:	10.0 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 and are recommended by the OECD 487 (2016) guideline (Countryman and Heddle, 1976; Evans and O'Riordan, 1975).

3.4.2 Blood collection and delivery

Blood samples were drawn from healthy non-smoking donors with no known illness or recent exposures to genotoxic agents (*e.g.* chemicals, ionising radiation) at levels that would increase the background incidence of micronucleate cells. For this study, blood was collected from a female donor (31 years old) for Experiment I and from a male donor (20 years old) for Experiment II. The lymphocytes of the respective donors have been shown to respond well to stimulation of proliferation with PHA and to positive control substances. All donors had a previously established low incidence of micronuclei in their peripheral blood lymphocytes. The cell cycle time for lymphocytes from each donor has been determined by BrdU previously (bromodeoxyuridine) incorporation to assess the average generation time (AGT) for the donor pool (approximately 16 hours). The cell harvest time point is at approximately 1.5 – 2.0 x AGT (Whitwell *et al.*, 2019). Any specific cell cycle time delay induced by the test item is not accounted for directly.

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 h 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). The S9 certificate is included in Appendix 3.

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 28.5 mg/mL (Lot no. 160921K).

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 for each test condition.

In case of test substance 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 substance 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 analysed should produce turbidity or visible precipitation / phase separation.

3.7 Experimental Performance Cytogenetic Experiment

3.7.1 Schedule

	Without S9 mix		With S9 mix
	Exp. II [h]	Exp. II [h]	Exp. I [h]
Stimulation period	48	48	48
Exposure period	3	28*	3
Cytochalasin B exposure	25	28*	25
Total culture period	76	76	76

* Co-treatment of test item and Cytochalasin B

3.7.2 Culture conditions

Blood cultures were established by preparing an 11% to 13 % 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 as solvent lyophilizate (Exp. 2024), 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) using 11% whole blood (for Experiment I):

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

The following volumes were added to the flasks (per 10 mL) using 13% whole blood (for Experiment II):

7.40 mL culture medium
1.00 mL fetal bovine serum
0.10 mL antibiotic solution
0.05 mL phytohemagglutinin
0.05 mL heparin
0.10 mL HEPES
1.30 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-experiment was performed with 10 concentrations of the test substance separated by no more than a factor of 2 to 3 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 3 h (with and without S9 mix). The preparation interval was 28 h after start of the exposure. Since the cultures in the presence of S9 mix fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.

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 or control. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 3 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) in the presence of Cytochalasin B (4 – 6 µg/mL) and cultured for 25 hours until preparation (Clare *et al.,* 2006, Lorge *et al.,* 2006).

Continuous exposure (without S9 mix)
About 48 h after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm^2 cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10% FBS) containing the test item and in the presence of Cytochalasin B (4 – 6 µg/mL). The cells were exposed for 28 hours until preparation (Whitwell et al, 2019).

3.7.5 Preparation of cells

The cultures were harvested by centrifugation 28 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

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

$$Cytostasis\% = 100 - 100\ [(CBPI_T - 1) / (CBPI_C - 1)]$$

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. In addition, micronuclei in mononucleate cells will be recorded when these events are seen, since aneuploid acting substances are known to increase the number of micronucleated mononucleate 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 produce a statistically significant increase in the micronucleus frequency compared with the concurrent solvent control and should be compatible with the laboratory historical positive control data range.
- Cell proliferation criteria in the solvent control are considered to be acceptable.
- All experimental conditions described in section 3.7 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

If all of the criteria are met, the test substance is 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 Laboratory's Historical Control Data

The historical control data were generated in accordance with the OECD Guideline 487 and updated annually.

For the solvent controls, data range (min-max) and data distribution (standard deviation) were calculated for each experimental part of at least 10 experiments (Appendix 1). The calculated 95% control limit of the solvent controls (realized as 95% control limit) was applied for the evaluation of acceptability and interpretation of the data (Section 3.9 and 3.10). Control charts of the corresponding experiments are added as quality control method.

For the positive controls, data range (min-max) and data distribution (standard deviation) were calculated for each experimental part of at least 10 experiments (Appendix 1). The min-max range of the positive controls was applied for the evaluation of acceptability (Section 3.9). Control charts of the corresponding experiments are added as quality control method.

3.12 Statistical Analysis

Statistical significance was confirmed by the Chi square test ($p < 0.05$), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression test was performed using a validated test script of "R", to assess a possible concentration dependent increase of micronucleus frequency. The number of micronucleated cells obtained for the groups treated with the test substance was 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 Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (A23793B), suspended (Exp. I) or dissolved (Exp. II) 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 3 h with and without S9 mix. In Experiment II, the exposure period was 28 h as well as 3 h without S9 mix. The cells were prepared 28 h after start of treatment with the test substance.

In each experimental group, two parallel cultures were analysed. 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-experiment 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 9.9 µg/mL to 2000 µg/mL (with and without S9 mix) were chosen for evaluation of cytotoxicity. In the Pre-experiment for toxicity, precipitation of the test substance was observed at the end of treatment at 286 µg/mL and above in the absence of S9 mix and at 163 µg/mL and above in the presence of S9 mix. Therefore, this was the highest concentration evaluated for micronuclei induction, in the presence of S9 mix. Since, based on the precipitation observed, the cultures in the presence of S9 mix fulfilled the requirements for cytogenetic evaluation, this test was designated Experiment I.

Due to the steep increase in cytotoxicity no evaluable concentrations in a cytotoxic range were available in the treatment without S9 mix and therefore this experiment was repeated with a modified concentration range with a top concentration of 250 µg/mL (Exp. II).

Using a reduced Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity, no cytotoxicity was observed in Experiment I after 3 h treatment in the presence of S9 mix up to the highest evaluable concentration. In the absence of S9 mix, clear cytotoxicity was observed at 163 µg/mL. Therefore, 350 µg/mL was chosen as top treatment concentration for Experiment II (without S9 mix, continuous treatment).

Precipitation was observed in Experiment II in the absence of S9 mix at 174 µg/mL in the pulse treatment and no precipitation was observed in the continuous treatment.

The applied concentrations for all experiments are presented in Table 1.

No relevant influence of the test substance on the osmolarity or pH was observed as shown below.

		Concentration [µg/mL]	Osmolarity [mOsm]	pH
Exp. I	Solvent control	-	455	7.64
	Test substance	2000	464	7.62

The osmolarity is generally high compared to the physiological level of approximately 300 mOsm. This effect, however, is based on a final concentration of 1.0% DMSO in medium. As the osmolarity is measured by freezing point reduction, 1.0% of DMSO has a substantial impact on the determination of osmolarity.

In the pulse treatment in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest evaluated concentration, which showed precipitation. In the continuous treatment, clear cytotoxicity war observed after treatment with 46.1 µg/mL (59.7 % cytostasis).

In Experiment I and II in the absence and presence of S9 mix, no relevant increases in the number 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% control limit and none of the values were statistically significantly increased, when compared with the vehicle control. There was also no concentration related increase in micronucleus formation, as judged by an appropriate trend test.

Vinblastine (10.0 ng/mL), MMC (0.6 µg/mL) or CPA (10.0 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, Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B) is considered to be clearly negative (i.e. non-clastogenic and non-aneugenic) in this *in vitro* micronucleus test, when tested up to cytotoxic or precipitating concentrations.

6.0 REFERENCES

ENVIRONMENT DIRECTORATE, ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (OECD) (2016) No. 487 In vitro Mammalian Cell Micronucleus Test. Paris: OECD Environmental Health and Safety Publications Series on Testing and Assessment.

AMES B.N., MCCANN J. and YAMASAKI E. (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. *Mutation Research*, 31, 347-363.

CLARE M.G., LORENZON G., AKHURST L.C., MARZIN D., VAN DELFT J., MONTERO R., BOTTA A., BERTENS A., CINELLI S., THYBAUD V. AND LORGE E., (2006) SFTG international collaborative study on in vitro micronucleus test II. Using human lymphocytes. *Mutation Res.,* 607, 37-60.

COUNTRYMAN P.I. and HEDDLE J.A. (1976) The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutation Research*, 41, 321-332.

EASTERBROOK J., LU C., SAKAI Y. and LI A.P. (2001) Effects of organic solvents on the activities of cytochrome P450 isoforms, UDP-dependent glucuronyl transferase, and phenol sulfotransferase in human hepatocytes. *Drug Metabolism and Disposition*, 29, 141-144.

EVANS H.J. and O`RIORDAN M.L. (1975) Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. *Mutation Research*, 31, 135-148.

LORGE E., THYBAUD V., AARDEMA M.J., OLIVER J., WAKATA A., LORENZON G. and MARZIN D. (2006) SFTG international collaborative study on in vitro micronucleus test I. General conditions and overall conclusions of the study. *Mutation Res.,* 607, 13-36.

OBE G. and BEEK B. (1982) The human leukocyte test system. In: Chemical mutagens, principles and methods for their detection. Vol. 7 (De Serres, F.J., Hollander, A., eds.) Plenum Press, N.Y., London, 337-400.

ROSEFORT C., FAUTH E. and ZANKL H. (2004) Micronuclei induced by aneugens and clastogens in mononucleate and binucleate cells using the cytokinesis block assay. *Mutagenesis*, 19(4), 277-284.

WHITWELL J., SMITH R., CHIROM T., WATTERS G., HARGREAVES V., LLOYD M., PHILLIPS S. and CLEMENTS J. (2019) Inclusion of an extended treatment improves the results for the human peripheral blood lymphocyte micronucleus assay. *Mutagenesis* 34, 217-237.

TABLES SECTION

TABLE 1 Concentrations Applied in the Micronucleus Assay with Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B)

Exp.	Prep. interval (h)	Exposure period (h)	Concentrations (µg/mL)										
Without S9 mix													
I*	28	3		9.9	17.4	30.5	53.3	93.3	163	286^{P}	500^{P}	1000^{P}	2000^{P}
II	28	3	8.9	15.6	27.3	**47.8**	**83.7**	100	**121**	145	**174^{P}**	208^{P}	250^{P}
II	28	28	2.8	4.9	**8.6**	**15.0**	**26.3**	**46.1**	69.1	104	156	233	350
With S9 mix													
I	28	3		9.9	17.4	**30.5**	**53.3**	**93.3**	**163^{P}**	286^{P}	500^{P}	1000^{P}	2000^{P}

Evaluated experimental points are shown in bold characters

P Precipitation was observed at the end of treatment

* Was repeated since the cytotoxicity criteria were not met

TABLE 2 Summary of Results of the Micronucleus Assay with Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B)

Exp.	Preparation interval	Test item concentration (µg/mL)	Proliferation index CBPI	Cytostasis (%*)	Micronucleated cells (%**)	95% Ctrl limit (%)
Exposure period 3 h without S9 mix						
II	28 h	Solvent control[1]	1.87		0.55	0.01 – 0.92
		Positive control[2]	1.75	13.8	**4.95[S]**	
		47.8	1.80	7.4	0.25	
		83.7	1.72	17.5	0.55	
		121	1.79	9.1	0.45	
		174[P]	1.62	28.8	0.85	
		Trend test: p-value 0.293				
Exposure period 28 h without S9 mix						
II	28 h	Solvent control[1]	1.93		0.65	0.00 – 1.19
		Positive control[3]	1.39	57.8	**8.65[S]**	
		8.6	1.84	9.2	0.40	
		15.0	1.78	15.5	0.65	
		26.3	1.62	33.4	0.50	
		46.1	1.37	59.7	0.85	
		Trend test: p-value 0.315				
Exposure period 3 h with S9 mix						
I	28 h	Solvent control[1]	1.29		0.65	0.00 – 0.97
		Positive control[4]	1.21	29.7	**3.20[S]**	
		30.5	1.35	n.c.	0.85	
		53.3	1.36	n.c.	0.65	
		93.3	1.39	n.c.	0.60	
		163[P]	1.25	15.7	0.75	
		Trend test: p-value 0.963				

* 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

P Precipitation occurred at the end of treatment

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

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

1 DMSO 1.0 % (v/v)

2 MMC 0.6 µg/mL

3 Vinblastine 10.0 ng/mL

4 CPA 10.0 µg/mL

TABLE 3 Toxicity - Experiment I (Cytotoxicity of Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B) 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	3	28	1.46	-
9.9	3	28	1.37	19.5
17.4	3	28	1.42	9.1
30.5	3	28	1.31	32.7
53.3	3	28	1.33	28.4
93.3	3	28	1.26	43.7
163	3	28	1.12	74.0
286[P]	3	28	1.06	87.9
500[P]	3	28	n.p.	n.p.
1000[P]	3	28	n.p.	n.p.
2000[P]	3	28	n.p.	n.p.
With S9 mix				
Solvent control	3	28	1.29	-
9.9	3	28	1.37	n.c.
17.4	3	28	1.38	n.c.
30.5	3	28	1.35	n.c.
53.3	3	28	1.36	n.c.
93.3	3	28	1.39	n.c.
163[P]	3	28	1.25	15.7
286[P]	3	28	n.p.	n.p.
500[P]	3	28	n.p.	n.p.
1000[P]	3	28	n.p.	n.p.
2000[P]	3	28	n.p.	n.p.

Experimental groups evaluated for cytogenetic damage are shown in bold characters

* Mean value of two cultures

[P] Precipitation occurred at the end of treatment

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

n.p. Not prepared

TABLE 4 Toxicity - Experiment II (Cytotoxicity of Difenoconazole/Fludioxonil/Metalaxyl-M /Cyclobutrifluram FS (A23793B) 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	28	28	1.93	-
2.8	28	28	1.73	21.4
4.9	28	28	1.80	14.2
8.6	28	28	1.84	9.2
15.0	28	28	1.78	15.5
26.3	28	28	1.62	33.4
46.1	28	28	1.37	59.7
69.1	28	28	1.22	76.5
104	28	28	1.10	89.3
156	28	28	1.08	90.9
233	28	28	n.e.	n.e.
350	28	28	n.e.	n.e.
Without S9 mix				
Solvent control	3	28	1.87	-
8.9	3	28	1.87	n.c.
15.6	3	28	1.91	n.c.
27.3	3	28	1.88	n.c.
47.8	3	28	1.80	7.4
83.7	3	28	1.72	17.5
100	3	28	1.67	23.0
121	3	28	1.79	9.1
145	3	28	1.64	26.3
174[P]	3	28	1.62	28.8
208[P]	3	28	n.p.	n.p.
250[P]	3	28	n.p.	n.p.

Experimental groups evaluated for cytogenetic damage are shown in bold characters

* Mean value of two cultures

[P] Precipitation occurred at the end of treatment

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

n.e. Not evaluable due to strong cytotoxic effects and insufficient binucleated cells

n.p. Not prepared

TABLE 5 **Experiment I - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 3 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#	1.0 %	+	3 / 28	350	140	10	1.32	369	129	2	1.27	1.29	
Pos. control##	10.0 µg	+	3 / 28	403	93	4	1.20	399	97	4	1.21	1.21	29.7
Test item	30.5 µg	+	3 / 28	341	148	11	1.34	337	145	18	1.36	1.35	n.c.
″	53.3 µg	+	3 / 28	307	178	15	1.42	358	131	11	1.31	1.36	n.c.
″	93.3 µg	+	3 / 28	294	195	11	1.43	334	156	10	1.35	1.39	n.c.
″	163 µg	+	3 / 28	406	90	4	1.20	361	129	10	1.30	1.25	15.7

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

DMSO

CPA

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

TABLE 6 Experiment I - Number of Micronucleated Cells; Exposure Period 3 h with S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ preparation (h)	Micronucleated cells: Binucleate cells with *n* micronuclei culture 1: 1	2	>2	sum culture 1	Binucleate cells with *n* micronuclei culture 2: 1	2	>2	sum culture 2	sum in 2000 binucleate cells	[%]
Solv. control[#]	1.0 %	+	3 / 28	5	1	0	6	7	0	0	7	13	0.65
Pos. control[##]	10.0 µg	+	3 / 28	36	1	0	37	27	0	0	27	64	3.20
Test item	30.5 µg	+	3 / 28	7	1	0	8	8	1	0	9	17	0.85
″	53.3 µg	+	3 / 28	7	0	0	7	5	1	0	6	13	0.65
″	93.3 µg	+	3 / 28	4	0	0	4	8	0	0	8	12	0.60
″	163 µg	+	3 / 28	7	0	0	7	8	0	0	8	15	0.75

[#] DMSO
[##] CPA

TABLE 7 **Experiment II - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 28 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#	1.0 %	-	28 / 28	145	280	75	1.86	101	300	99	2.00	1.93	
Pos. control##	10 ng	-	28 / 28	342	124	34	1.38	337	126	37	1.40	1.39	57.8
Test item	8.6 µg	-	28 / 28	128	315	57	1.86	144	298	58	1.83	1.84	9.2
"	15.0 µg	-	28 / 28	137	311	52	1.83	173	285	42	1.74	1.78	15.5
"	26.3 µg	-	28 / 28	226	248	26	1.60	208	266	26	1.64	1.62	33.4
"	46.1 µg	-	28 / 28	314	183	3	1.38	317	181	2	1.37	1.37	59.7

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

\# DMSO

\## Vinblastine

TABLE 8 **Experiment II - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 3 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[#]	1.0 %	-	3 / 28	109	346	45	1.87	99	371	30	1.86	1.87	
Pos. control[##]	0.6 µg	-	3 / 28	111	386	3	1.78	150	345	5	1.71	1.75	13.8
Test item	47.8 µg	-	3 / 28	120	369	11	1.78	111	366	23	1.82	1.80	7.4
"	83.7 µg	-	3 / 28	141	348	11	1.74	159	337	4	1.69	1.72	17.5
"	121 µg	-	3 / 28	120	378	2	1.76	99	396	5	1.81	1.79	9.1
"	174 µg	-	3 / 28	186	312	2	1.63	204	291	5	1.60	1.62	28.8

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

[#] DMSO

[##] MMC

TABLE 9 Experiment II - Number of Micronucleated Cells; Exposure Period 28 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ preparation (h)	Micronucleated cells: Binucleate cells with *n* micronuclei culture 1: 1	2	>2	sum culture 1	Binucleate cells with *n* micronuclei culture 2: 1	2	>2	sum culture 2	sum in 2000 binucleate cells	[%]
Solv. control[#]	1.0 %	-	28 / 28	7	1	0	8	5	0	0	5	13	0.65
Pos. control[##]	10 ng	-	28 / 28	62	7	8	77	76	15	5	96	173	8.65
Test item	8.6 µg	-	28 / 28	2	0	0	2	6	0	0	6	8	0.40
″	15.0 µg	-	28 / 28	10	0	0	10	3	0	0	3	13	0.65
″	26.3 µg	-	28 / 28	4	0	0	4	6	0	0	6	10	0.50
″	46.1 µg	-	28 / 28	5	0	0	5	11	1	0	12	17	0.85

[#] DMSO
[##] Vinblastine

TABLE 10 Experiment II - Number of Micronucleated Cells; Exposure Period 3 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ preparation (h)	Micronucleated cells: Binucleate cells with *n* micronuclei culture 1: 1	2	>2	sum culture 1	Binucleate cells with *n* micronuclei culture 2: 1	2	>2	sum culture 2	sum in 2000 binucleate cells	[%]
Solv. control[#]	1.0 %	-	3 / 28	1	0	0	1	10	0	0	10	11	0.55
Pos. control[##]	0.6 µg	-	3 / 28	40	2	1	43	54	2	0	56	99	4.95
Test item	47.8 µg	-	3 / 28	1	1	0	2	3	0	0	3	5	0.25
″	83.7 µg	-	3 / 28	4	0	0	4	7	0	0	7	11	0.55
″	121 µg	-	3 / 28	5	1	0	6	2	1	0	3	9	0.45
″	174 µg	-	3 / 28	7	0	0	7	10	0	0	10	17	0.85

[#] DMSO
[##] MMC

TABLE 11 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	30.5	28	3	+	0.537	0.464
"	53.3	28	3	+	n.c.	n.c.
"	93.3	28	3	+	n.c.	n.c.
"	163	28	3	+	0.144	0.705
Positive control versus solvent control (µg/mL)						
CPA	10.0	28	3	+	34.442	4.391×10^{-9} 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	8.6	28	28	-	n.c.	n.c.
"	15.0	28	28	-	n.c.	n.c.
"	26.3	28	28	-	n.c.	n.c.
"	46.1	28	28	-	0.537	0.464
"	47.8	28	3	-	n.c.	n.c.
"	83.7	28	3	-	n.c.	n.c.
"	121	28	3	-	n.c.	n.c.
"	174	28	3	-	1.295	0.255
Positive control versus solvent control (µg/mL)						
Vinblastine	0.010	28	28	-	144.35	$< 2.2\times10^{-16}$ s
MMC	0.6	28	3	-	72.391	$< 2.2\times10^{-16}$ 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 concentration dependency in the rates of micronucleated cells. The number of micronucleated cells, obtained for the groups treated with the test substance 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 3 h with S9 mix	0.963
Experiment II, exposure period 28 h without S9 mix	0.315
Experiment II, exposure period 3 h without S9 mix	0.293

APPENDICES SECTION

APPENDIX 1 Historical Control Data

Micronucleus Test in Human Lymphocytes - Historical Control Data (2021)

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 (3/28)	Continuous treatment (28/28)
Number of experiments	19*	20**
Min – Max	0.15 – 1.15	0.10 – 1.35
Mean Value	0.46	0.59
Standard Deviation	0.23	0.30
95% Control Limit	**0.01 – 0.92**	**0.00 – 1.19**

* Aqueous solvents – 11 Experiments; Organic solvents – 8 Experiments
** Aqueous solvents – 11 Experiments; Organic solvents – 9 Experiments

Solvent Control with S9	
Micronucleated cells in%	
	Pulse treatment (3/28)
Number of experiments	21*
Min – Max	0.05 – 1.15
Mean Value	0.43
Standard Deviation	0.27
95% Control Limit	**0.00 – 0.97**

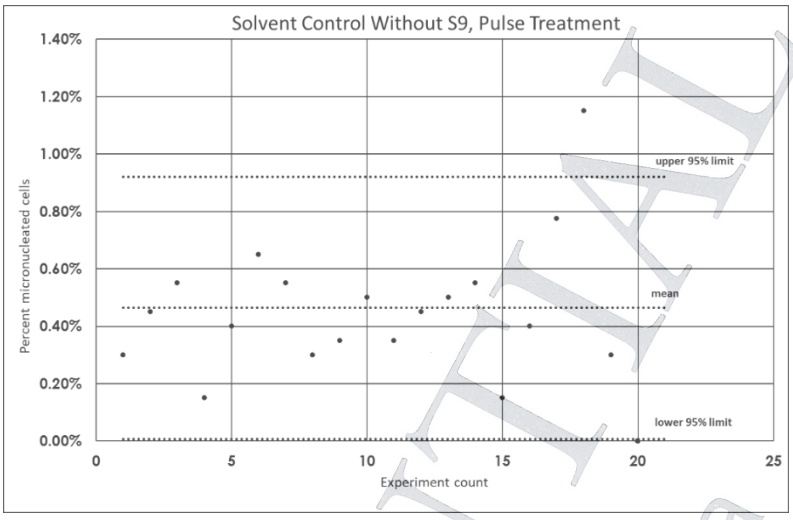
* Aqueous solvents – 12 Experiments; Organic solvents – 9 Experiments

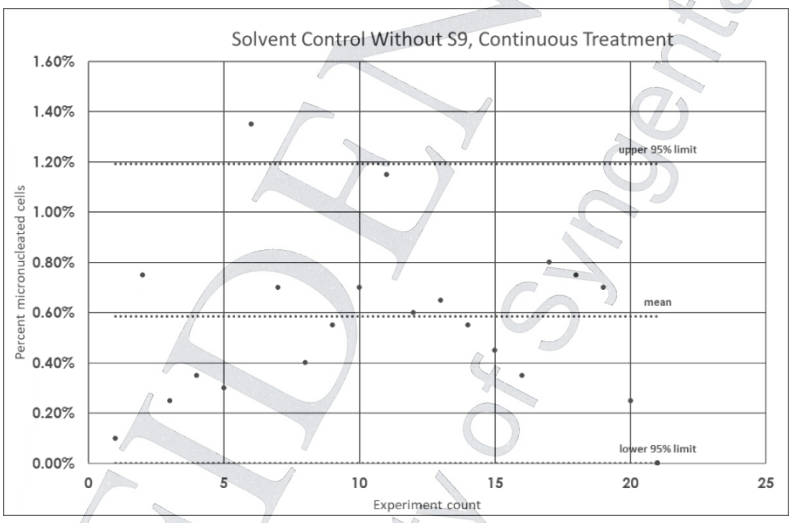
Positive Control without S9		
Micronucleated cells in%		
	Pulse treatment (3/28)	Continuous treatment (28/28)
	MMC	Vinblastine
Number of experiments	19	21
Min – Max	**3.55 – 14.10**	**2.75 – 9.65**
Mean Value	8.57	5.12
Standard Deviation	3.10	1.75
95% Control Limit	2.37 – 14.77	1.62 – 8.63

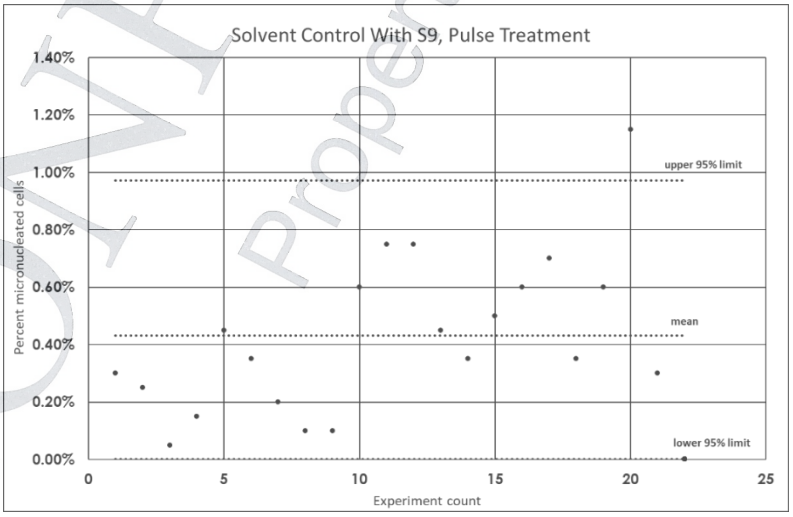
Positive Control with S9	
Micronucleated cells in%	
	Pulse treatment (3/28)
	CPA
Number of experiments	18
Min – Max	**1.60 – 4.85**
Mean value	2.90
Standard Deviation	0.77
95% Control limit	1.36 – 4.44

The 95% Control Limit is derived from the mean value plus/minus 2 times the standard deviation.

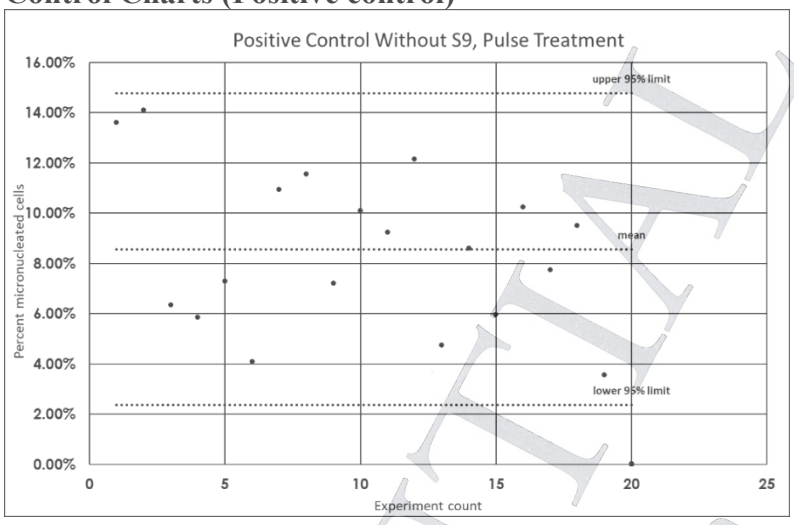
Micronucleus Test in Human Lymphocytes - Historical Control Data (2021)
Control Charts (Solvent control)

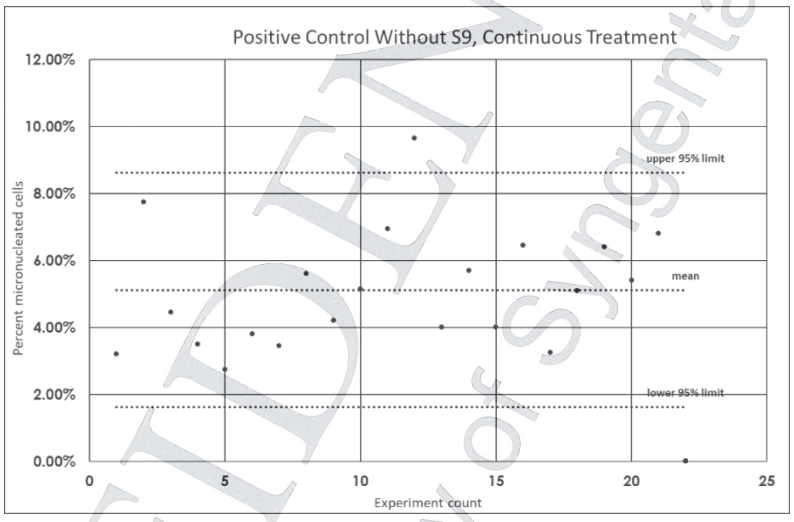


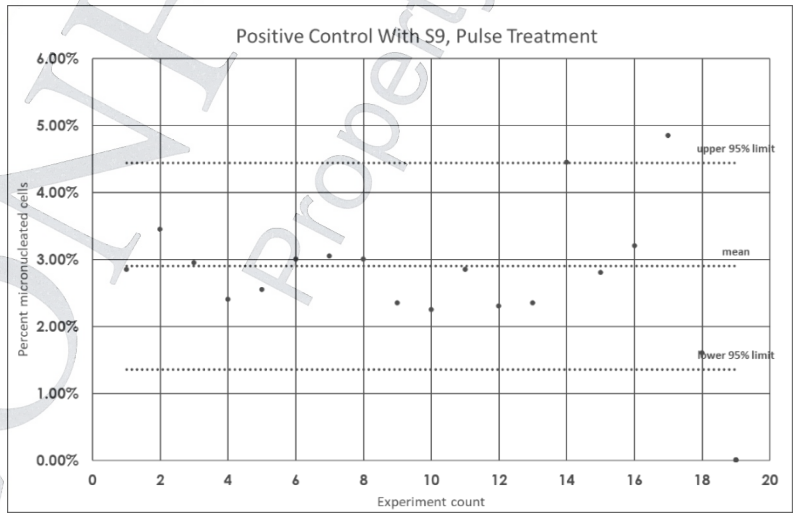




Micronucleus Test in Human Lymphocytes - Historical Control Data (2021)
Control Charts (Positive control)







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, Climate Protection, Agriculture and Consumer Protection;
Department II 10; P.O. Box 31 09; 65189 Wiesbaden
Translation of the seal inscription:
Hessian Ministry for Environment, Climate Protection, Agriculture and Consumer Protection

APPENDIX 3 Certificate of S9

ICCR ROßDORF

CERTIFICATE

ICCR-Roßdorf S9 Preparation Lot No. 160921K

Date of preparation: September 16, 2021
Release date: October 1, 2021

Protein assay: 28.5 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
negative	0	30
control	100	41
10 µg/plate	0	79
2-Aminoanthracene	100	3593
10 µg/plate	0	34
Benzo(a)pyrene	100	106

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.

S. Ebert Sabine Ebert — 11. OKT. 2021

Quality Assurance Auditor
ICCR-Roßdorf GmbH — Date

S. Chang — 19. OKT. 2021

Dr. Steffi Chang
Study Director
ICCR-Roßdorf GmbH — Date

ICCR-Roßdorf GmbH
In den Leppsteinswiesen 19, 64380 Roßdorf, Deutschland
T +49 6154 8070 F +49 6154 83399
Registergericht Darmstadt, HRB 6837, USt.-ID DE812333696
Geschäftsführer: Dr. Markus Schulz

SOP Origin TS-SOP S9_23

APPENDIX 4 Certificate of Analysis

syngenta

Syngenta Crop Protection, LLC
Analytical and Product Chemistry
Greensboro, NC 27409

Certificate of Analysis

A23793B

Batch ID 1200767 (GP210610)

Test Substance Name:	CGA169374/CGA173506/CGA329351/SYN549522 FS (062.51/049.93/050.05/250.08)
Common Name:	Difenoconazole/Fludioxonil/Metalaxyl-M/Cyclobutrifluram FS (062.51/049.93/050.05/250.08)
Material ID:	A23793B
Batch ID:	1200767
Other ID:	GP210610
Source:	Syngenta Crop Protection LLC., 410 Swing Road, Greensboro, NC 27409, US

Chemical Analysis

AI	% w/w	g/L
Difenoconazole	5.45	64.0
Fludioxonil	4.37	51.3
Metalaxyl-M	4.31	50.6
Cyclobutrifluram	21.0	247

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
CGA329351	D-alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, Methyl Ester	4.15
CGA351920	L-alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, Methyl Ester	0.15

COA Number: USGR210208

Physical Analysis

Analyte	Value	Units
Density	1.174	g/cm3

Appearance: red liquid

Storage Temperature: <30°C

Re-certification Date:End of Aug/2024

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: USGR210208

Authorization: Sherry Perine

Sherry C Perine

Sherry Perine

Analytical and Product Chemistry Department

Aug 24, 2021

Date

COA Number: USGR210208