Isopyrazam/Difenoconazole

Isopyrazam/Difenoconazole SC (A21295D) - Micronucleus Test in Human Lymphocytes *In Vitro*

Final Report

TEST GUIDELINE(S): OECD 487 (2016)

AUTHOR(S): Dr. Steffen Naumann

COMPLETION DATE: 04 May 2021

PERFORMING LABORATORY: ICCR-Roßdorf GmbH
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SPONSOR(S): Syngenta Ltd
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Bracknell, Berkshire RG42 6EY, United Kingdom

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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*

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Date: 04 May 2021

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QUALITY ASSURANCE STATEMENT

Study Number: 2137000
Test Substance: Isopyrazam/Difenoconazole SC (A21295D)
Study Director: Dr. Steffen Naumann
Title: Isopyrazam/Difenoconazole SC (A21295D) - 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	13 November 2020	13 November 2020
Process – based Test item preparation Test system preparation and application Test performance	 09 December 2020 06 January 2021 15 February 2021	 09 December 2020 06 January 2021 15 February 2021
Report Audit	06 April 2021	07 April 2021

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

H. Pilawa

04 May 2021
Date

Quality Assurance Auditor
ICCR-Roßdorf GmbH

PROJECT STAFF SIGNATURE

Study Director Dr. Steffen Naumann

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Date: 04 May 2021

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
Merielen Pontes	Syngenta Study Manager

Study dates
Study initiation date: 13 November 2020
Experimental start date: 02 December 2020
Experimental termination date: 09 March 2021

Deviations from the guidelines
None

Retention of samples
None

Performing laboratory test substance number
S 2126411

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
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 isopyrazam/difenoconazole SC (A21295D), suspended in deionised water, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in three 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 regard to the molecular weight of the test substance and 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

For the pulse treatment in the absence of S9 mix, three independent experiments (Experiments I, II and III) were performed. Due to the cytotoxic effects, in none of the three experiments was the optimal range of 55 ± 5% cytostasis met, two of the experiments (Experiment I and II) were chosen for evaluation. The selection for evaluation was based on the highest evaluable concentration (104 µg/ml in Experiment I) and the highest observed cytostasis, which was seen in Experiment II (35.6% at 44.7 µg/ml). Furthermore, the cytotoxicity data of Experiment III showed a steep cytotoxic gradient in a small concentration range, which does not support further testing (Table 5).

In Experiment I in the absence of S9 mix, after pulse treatment at the highest evaluated concentration of 104 µg/mL, a cytostasis of 24.5% was observed. This was accompanied by a reduced number of suitable binucleated cells in the pre-check, which indicates marked cytotoxic effects. In Experiment II in the absence of S9 mix after pulse treatment, the desired cytotoxic range of 55 ± 5% cytostasis was again not met (35.6% cytostasis at 44.7 µg/ml) due to the steep increase of cytotoxicity. Higher concentrations were not available for evaluation.
In Experiment II in the absence of S9 mix, after continuous treatment, clear cytotoxicity (48.1% cytostasis) was observed at the highest evaluated concentration, which showed precipitation.
In Experiment I in the presence of S9 mix, after pulse treatment, no cytotoxicity was observed up to the highest evaluated concentration, which showed precipitation.

In this study in the absence and presence of S9 mix, no relevant increases in the number of micronucleated cells were observed after treatment with the test substance. 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 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, Isopyrazam/Difenoconazole SC (A21295D) is considered to be non-genotoxic (i.e. non-clastogenic and non-aneugenic) in this *in vitro* micronucleus test, when tested up to cytotoxic and/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 three independent experiments with one preparation interval (40 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 phytohaemagglutinin (PHA) when cells were actively proliferating and the cells were prepared at approximately 2 – 2.5 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.

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 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 and is supported by publications (Clare et al, 2006, Lorge et al, 2006, Whitwell et al, 2019).

3.0 MATERIALS AND METHODS

3.1 Test Substance

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

Identification:	Isopyrazam/Difenoconazole SC (A21295D)
Batch:	SG40038
Content of difenoconazole (CGA169374):	11.93% w/w corresponding to 130 g/L
Content of isopyrazam (SYN520453):	12.105% w/w corresponding to 132 g/L
Physical state / Appearance:	Liquid, off-white
Retest Date:	29 August 2023
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 suspended in deionised water. The final concentration of deionised water in the culture medium was 10.0% (v/v). The solvent was chosen as the best suitable solvent compared to DMSO, and ethanol, according to its solubilisation properties and its compatibility with cell cultures.

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 dissolved in deionised water 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 10.0 % deionised water (local tap water deionised at ICCR-Roßdorf GmbH)) were performed.

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.:	197987
Expiry Date:	June 2022
Purity:	98 %
Dissolved in:	Deionised water
Concentration:	0.8 µg/mL

Name:	Demecolcine (continuous treatment, aneugen)*
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	BCBX 9130
Expiry Date:	October 2021
Purity:	≥ 98 %
Dissolved in:	Deionised water
Concentration:	125 ng/mL

* The reference mutagen demecolcine, which is a well-known aneugen, was used as an alternative positive control for aneugenicity in the absence of S9 mix, as allowed by the guideline. The laboratory historical control data provide sufficient evidence for the responsiveness of the substance used in this system (cf. OECD 487 (2016), section 33).

With metabolic activation

Name:	Cyclophosphamide (CPA, clastogen)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	MKCG5464
Expiry Date:	August 2023
Purity:	97 – 103 %
Dissolved in:	Saline (0.9 % NaCl [w/v])
Concentration:	20.0 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 male donor (29 years old) for Experiment I and from a male donor (21 years old) for Experiment II and III. 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 (bromodeoxyuridine) incorporation to assess the average generation time (AGT) for the donor pool (approximately 16 hours). The cell harvest time point is at approximately 2 – 2.5 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 31.0 mg/mL (Lot no. 100920).

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. I, II and III	Exp. II	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) 1.5% (v/v) as extract, 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):

7.50 mL culture medium
1.00 mL fetal bovine serum
0.10 mL antibiotic solution
0.15 mL phytohemagglutinin
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 characterised 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 √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. Since the cultures 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 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 h 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 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test substance or control. After 20 h 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 h until preparation (Whitwell et al, 2019).

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

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 20 experiments (cf. Appendix 1). The calculated 95% control limit of the solvent controls (realized as 95% confidence interval) was applied for the evaluation of acceptability and interpretation of the data (cf. 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 20 experiments (cf. Appendix 1). The min-max range of the positive controls was applied for the evaluation of acceptability (cf. 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 Isopyrazam/Difenoconazole SC (A21295D), suspended in deionised water, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Three independent experiments were performed. In Experiment I, the exposure period was 4 h with and without S9 mix. In Experiment II, the exposure periods were 4 h and 20 h without S9 mix. In Experiment III, the exposure period was 4 h without S9 mix. The cells were prepared 40 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 regard to the molecular weight of the test substance and with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

Test substance concentrations ranging from 6.4 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 183 µg/mL and above in the absence of S9 mix and at 104 µg/mL and above in the presence of S9 mix. Due to a technical error in the part without S9 mix, the first concentration showing precipitation (183 µg/mL) was discarded and not prepared. Nevertheless, the next lower concentration (104 µg/mL) with a CBPI mediated cytostasis value of 24.5 % showed a reduced number of suitable binucleated cells during the pre-check, which indicates cytotoxic effects. Since the cultures fulfilled the requirements for cytogenetic evaluation, this test was designated Experiment I.

The experimental part without S9 mix and 4 h treatment was repeated in Experiment II with a top concentration of 350 µg/mL and narrower concentration spacing to meet the optimal range of cytotoxicity of 55 ± 5% using the CBPI parameter. Precipitation of the test substance was observed at the end of treatment at 93.8 µg/mL and above.

Considering the precipitation data of Experiment I, 350 µg/mL was chosen as top treatment concentration for the experimental part without S9 mix and 20 h treatment in Experiment II. In this experimental part, precipitation was observed at 78.1 µg/mL and above at the end of treatment.

The experimental part without S9 mix and 4 h treatment was repeated again in Experiment III with a top concentration of 110 µg/mL and narrower concentration spacing because the optimal range of cytotoxicity of 55 ± 5% was not met in Experiment II. Precipitation of the test substance was observed at the end of treatment at 110 µg/mL.

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	-	291	7.69
	Test substance	2000	295	7.61

For the pulse treatment in the absence of S9 mix, three independent experiments (Experiments I, II and III) were performed. Due to the cytotoxic effects, in none of the three experiments was the optimal range of 55 ± 5% cytostasis met, two of the experiments (Experiment I and II) were chosen for evaluation. The selection for evaluation was based on the highest evaluable concentration (104 µg/ml in Experiment I) and the highest observed cytostasis, which was seen in Experiment II (35.6% at 44.7 µg/ml). Furthermore, the cytotoxicity data of Experiment III showed a steep cytotoxic gradient in a small concentration range, which does not support further testing (Table 5).

In Experiment I in the absence of S9 mix, after pulse treatment at the highest evaluated concentration of 104 µg/mL, a cytostasis of 24.5% was observed. This was accompanied by a reduced number of suitable binucleated cells in the pre-check, which indicates marked cytotoxic effects. In Experiment II in the absence of S9 mix after pulse treatment, the desired cytotoxic range of 55 ± 5% cytostasis was again not met (35.6% cytostasis at 44.7 µg/ml) due to the steep increase of cytotoxicity. Higher concentrations were not available for evaluation.
In Experiment II in the absence of S9 mix, after continuous treatment, clear cytotoxicity (48.1% cytostasis) was observed at the highest evaluated concentration, which showed precipitation.
In Experiment I in the presence of S9 mix, after pulse treatment, no cytotoxicity was observed up to the highest evaluated concentration, which showed precipitation.

In this study in the absence and presence of S9 mix, no relevant increases in the number of micronucleated cells were observed after treatment with the test substance. 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 (125 ng/mL), MMC (0.8 µg/mL) or CPA (20.0 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, Isopyrazam/Difenoconazole SC (A21295D) is considered to be non-genotoxic (i.e. non-clastogenic and non-aneugenic) in this *in vitro* micronucleus test, when tested up to cytotoxic and/or precipitating concentrations.

6.0 REFERENCES

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TABLES SECTION

TABLE 1 Concentrations Applied in the Micronucleus Assay with Isopyrazam/Difenoconazole SC (A21295D)

Exp.	Prep. interval (h)	Exposure period (h)	Concentrations (µg/mL)										
Without S9 mix													
I*	40	4		6.4	11.1	**19.5**	**34.1**	**59.7**	**104**	183[P]	320[P]	800[P]	2000[P]
II*	40	4	**14.6**	**25.5**	**44.7**	78.1	93.8[P]	113[P]	135[P]	162[P]	194[P]	233[P]	350[P]
II	40	20	8.3	**14.6**	**25.5**	**44.7**	**78.1**[P]	93.8[P]	113[P]	135[P]	162[P]	194[P]	233[P]
													350[P]
III	40	4	6.8	11.9	20.9	36.6	42.1	48.4	55.6	64.0	73.6	84.6	110[P]
With S9 mix													
I	40	4		6.4	11.1	**19.5**	**34.1**	**59.7**	**104**[P]	183[P]	320[P]	800[P]	2000[P]

Evaluated experimental points are shown in bold characters

[P] Precipitation was observed at the end of treatment

* Was repeated because the optimal range of cytotoxicity of 55 ± 5% could not be met

TABLE 2 Summary of Results of the Micronucleus Assay with Isopyrazam/Difenoconazole SC (A21295D)

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	95% Ctrl limit in %
Exposure period 4 h without S9 mix						
I	40 h	Solvent control[1]	1.78		0.45	0.00 – 1.04
		Positive control[2]	1.76	2.1	**11.55[S]**	
		19.5	1.75	3.7	0.25	
		34.1	1.77	1.4	0.15	
		59.7	1.69	11.8	0.30	
		104	1.59	24.5	0.50	
		Trend test: p-value 0.560				
II	40 h	Solvent control[1]	1.93		0.45	0.00 – 1.04
		Positive control[2]	1.46	50.3	**7.50[S]**	
		14.6	1.80	13.9	0.30	
		25.5	1.75	19.4	0.20	
		44.7	1.60	35.6	0.20	
		Trend test: p-value 0.107				
Exposure period 20 h without S9 mix						
II	40 h	Solvent control[1]	1.86		0.20	0.00 – 0.86
		Positive control[3]	1.44	48.9	**5.80[S]**	
		14.6	1.82	5.0	0.45	
		25.5	1.74	13.9	0.10	
		44.7	1.60	30.8	0.35	
		78.1[P]	1.45	48.1	0.50	
		Trend test: p-value 0.310				

* 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

1 Deion. water 10.0 % (v/v)

2 MMC 0.8 µg/mL

3 Demecolcine 125 ng/mL

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	95% Ctrl limit in %
Exposure period 4 h with S9 mix						
I	40 h	Solvent control[1]	1.94		0.35	0.00 – 1.03
		Positive control[4]	1.81	13.8	**3.85**[S]	
		19.5	1.85	10.1	0.30	
		34.1	1.84	11.0	0.30	
		59.7	1.78	16.9	0.20	
		104[P]	1.64	31.8	0.30	
Trend test: p-value 0.469						

* 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

1 Deion. water 10.0 % (v/v)

4 CPA 20.0 µg/mL

TABLE 3 Toxicity - Experiment I (Cytotoxicity of Isopyrazam/Difenoconazole SC (A21295D) 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	1.78	-
6.4	4	40	1.80	n.c.
11.1	4	40	1.80	n.c.
19.5	4	40	1.75	3.7
34.1	4	40	1.77	1.4
59.7	4	40	1.69	11.8
104[#]	4	40	1.59	24.5
183[P]	4	40	n.p.	n.p.
320[P]	4	40	n.p.	n.p.
800[P]	4	40	n.p.	n.p.
2000[P]	4	40	n.p.	n.p.
With S9 mix				
Solvent control	4	40	1.94	-
6.4	4	40	1.85	9.6
11.1	4	40	1.84	10.4
19.5	4	40	1.85	10.1
34.1	4	40	1.84	11.0
59.7	4	40	1.78	16.9
104[P]	4	40	1.64	31.8
183[P]	4	40	n.p.	n.p.
320[P]	4	40	n.p.	n.p.
800[P]	4	40	n.p.	n.p.
2000[P]	4	40	n.p.	n.p.

Experimental groups evaluated for cytogenetic damage are shown in bold characters

* Mean value of two cultures

[#] Observation during the pre-check: Indication of cytotoxic effects, as there were reduced number of suitable (binuclear) cells on the specimen slides.

[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 Isopyrazam/Difenoconazole SC (A21295D) 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	1.93	-
14.6	4	40	1.80	13.9
25.5	4	40	1.75	19.4
44.7	4	40	1.60	35.6
78.1	4	40	1.30	67.5
93.8[P]	4	40	1.30	67.5
113[P]	4	40	n.p.	n.p.
135[P]	4	40	n.p.	n.p.
162[P]	4	40	n.p.	n.p.
194[P]	4	40	n.p.	n.p.
233[P]	4	40	n.p.	n.p.
350[P]	4	40	n.p.	n.p.
Solvent control	20	40	1.86	-
8.3	20	40	1.84	2.2
14.6	20	40	1.82	5.0
25.5	20	40	1.74	13.9
44.7	20	40	1.60	30.8
78.1[P]	20	40	1.45	48.1
93.8[P]	20	40	n.p.	n.p.
113[P]	20	40	n.p.	n.p.
135[P]	20	40	n.p.	n.p.
162[P]	20	40	n.p.	n.p.
194[P]	20	40	n.p.	n.p.
233[P]	20	40	n.p.	n.p.
350[P]	20	40	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.p. Not prepared

TABLE 5 Toxicity - Experiment III (Cytotoxicity of Isopyrazam/Difenoconazole SC (A21295D) 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	2.08	-
6.8	4	40	1.92	14.8
11.9	4	40	2.03	4.8
20.9	4	40	2.05	2.7
36.6	4	40	1.85	21.7
42.1	4	40	1.04	96.7
48.4	4	40	1.02	98.2
55.6	4	40	1.01	99.2
64.0	4	40	1.01	98.8
73.6	4	40	1.01	99.3
84.6	4	40	1.01	99.2
110[P]	4	40	1.03	97.5

* Mean value of two cultures
[P] Precipitation occurred at the end of treatment

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#	10 %	-	4 / 40	126	364	10	1.77	117	371	12	1.79	1.78	
Pos. control##	0.8 µg	-	4 / 40	105	388	7	1.80	141	357	2	1.72	1.76	2.1
Test item	19.5 µg	-	4 / 40	140	359	1	1.72	111	389	0	1.78	1.75	3.7
″	34.1 µg	-	4 / 40	111	387	2	1.78	124	375	1	1.75	1.77	1.4
″	59.7 µg	-	4 / 40	167	330	3	1.67	150	349	1	1.70	1.69	11.8
″	104 µg	-	4 / 40	232	267	1	1.54	182	317	1	1.64	1.59	24.5

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

Deion. water

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#	10.0 %	+	4 / 40	66	400	34	1.94	67	392	41	1.95	1.94	
Pos. control##	20.0 µg	+	4 / 40	112	384	4	1.78	95	390	15	1.84	1.81	13.8
Test item	19.5 µg	+	4 / 40	105	383	12	1.81	80	400	20	1.88	1.85	10.1
″	34.1 µg	+	4 / 40	77	413	10	1.87	102	391	7	1.81	1.84	11.0
″	59.7 µg	+	4 / 40	97	401	2	1.81	124	374	2	1.76	1.78	16.9
″	104 µg	+	4 / 40	188	312	0	1.62	175	320	5	1.66	1.64	31.8

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

\# Deion. water

\## 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: 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[#]	10 %	-	4 / 40	3	1	0	4	4	1	0	5	9	0.45
Pos. control[##]	0.8 µg	-	4 / 40	91	7	0	98	125	7	1	133	231	11.55
Test item	19.5 µg	-	4 / 40	3	0	0	3	2	0	0	2	5	0.25
″	34.1 µg	-	4 / 40	1	0	0	1	2	0	0	2	3	0.15
″	59.7 µg	-	4 / 40	3	1	0	4	2	0	0	2	6	0.30
″	104 µg	-	4 / 40	6	0	0	6	4	0	0	4	10	0.50

[#] Deion. water
[##] 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: 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[#]	10.0 %	+	4 / 40	2	0	0	2	5	0	0	5	7	0.35
Pos. control[##]	20.0 µg	+	4 / 40	38	4	0	42	35	0	0	35	77	3.85
Test item	19.5 µg	+	4 / 40	0	1	0	1	5	0	0	5	6	0.30
″	34.1 µg	+	4 / 40	2	0	0	2	4	0	0	4	6	0.30
″	59.7 µg	+	4 / 40	4	0	0	4	0	0	0	0	4	0.20
″	104 µg	+	4 / 40	3	0	0	3	3	0	0	3	6	0.30

[#] Deion. water
[##] CPA

TABLE 10 **Experiment II - 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[#]	10 %	-	4 / 40	70	400	30	1.92	71	391	38	1.93	1.93	
Pos. control[##]	0.8 µg	-	4 / 40	307	192	1	1.39	253	227	20	1.53	1.46	50.3
Test item	14.6 µg	-	4 / 40	119	373	8	1.78	100	391	9	1.82	1.80	13.9
″	25.5 µg	-	4 / 40	148	344	8	1.72	121	371	8	1.77	1.75	19.4
″	44.7 µg	-	4 / 40	201	297	2	1.60	206	292	2	1.59	1.60	35.6

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

[#] Deion. water

[##] MMC

TABLE 11 **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#	10.0 %	-	20 / 40	80	393	27	1.89	112	362	26	1.83	1.86	
Pos. control##	125 ng	-	20 / 40	306	185	9	1.41	272	219	9	1.47	1.44	48.9
Test item	14.6 µg	-	20 / 40	107	372	21	1.83	107	382	11	1.81	1.82	5.0
″	25.5 µg	-	20 / 40	128	360	12	1.77	146	351	3	1.71	1.74	13.9
″	44.7 µg	-	20 / 40	212	287	1	1.58	195	303	2	1.61	1.60	30.8
″	78.1 µg	-	20 / 40	268	232	0	1.46	285	215	0	1.43	1.45	48.1

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

\# Deion. water

\## Demecolcine

TABLE 12 Experiment II - 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: 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[#]	10 %	-	4 / 40	4	0	0	4	5	0	0	5	9	0.45
Pos. control[##]	0.8 µg	-	4 / 40	81	11	0	92	55	3	0	58	150	7.50
Test item	14.6 µg	-	4 / 40	2	0	0	2	4	0	0	4	6	0.30
″	25.5 µg	-	4 / 40	2	0	0	2	2	0	0	2	4	0.20
″	44.7 µg	-	4 / 40	2	0	0	2	2	0	0	2	4	0.20

[#] Deion. water
[##] MMC

TABLE 13 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: 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[#]	10.0 %	-	20 / 40	1	0	0	1	3	0	0	3	4	0.20
Pos. control[##]	125 ng	-	20 / 40	38	12	6	56	45	9	6	60	116	5.80
Test item	14.6 µg	-	20 / 40	1	0	0	1	8	0	0	8	9	0.45
″	25.5 µg	-	20 / 40	0	1	0	1	1	0	0	1	2	0.10
″	44.7 µg	-	20 / 40	4	0	0	4	3	0	0	3	7	0.35
″	78.1 µg	-	20 / 40	6	0	1	7	3	0	0	3	10	0.50

[#] Deion. water
[##] Demecolcine

TABLE 14 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	19.5	40	4	-	n.c.	n.c.
"	34.1	40	4	-	n.c.	n.c.
"	59.7	40	4	-	n.c.	n.c.
"	104	40	4	-	0.053	0.818
"	19.5	40	4	+	n.c.	n.c.
"	34.1	40	4	+	n.c.	n.c.
"	59.7	40	4	+	n.c.	n.c.
"	104	40	4	+	n.c.	n.c.
Positive control versus solvent control (µg/mL)						
MMC	0.8	40	4	-	218.457	$< 2.2\times10^{-16}$ s
CPA	20.0	40	4	+	59.585	1.2×10^{-14} 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	14.6	40	4	-	n.c.	n.c.
"	25.5	40	4	-	n.c.	n.c.
"	44.7	40	4	-	n.c.	n.c.
"	14.6	40	20	-	1.929	0.165
"	25.5	40	20	-	n.c.	n.c.
"	44.7	40	20	-	0.820	0.365
"	78.1	40	20	-	2.581	0.108
Positive control versus solvent control per mL						
MMC	0.8 µg	40	4	-	130.214	$< 2.2\times10^{-16}$ s
Demecolcine	125 ng	40	20	-	107.766	$< 2.2\times10^{-16}$ 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 4 h without S9 mix	0.560
Experiment I, exposure period 4 h with S9 mix	0.469
Experiment II, exposure period 4 h without S9 mix	0.107
Experiment II, exposure period 20 h without S9 mix	0.310

APPENDICES SECTION

APPENDIX 1 Historical Control Data

Percentage of micronucleated cells in human lymphocyte cultures (2019)

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	50*	43**
Mean	0.46	0.43
95 % Ctrl limit	**0.00 – 1.04**	**0.00 – 0.86**
1x SD	0.29	0.21
2x SD	0.58	0.43
Min – Max	0.05 – 1.20	0.05 – 1.00

* Aqueous solvents – 17 Experiments; Organic solvents – 33 Experiments
** Aqueous solvents – 13 Experiments; Organic solvents – 30 Experiments

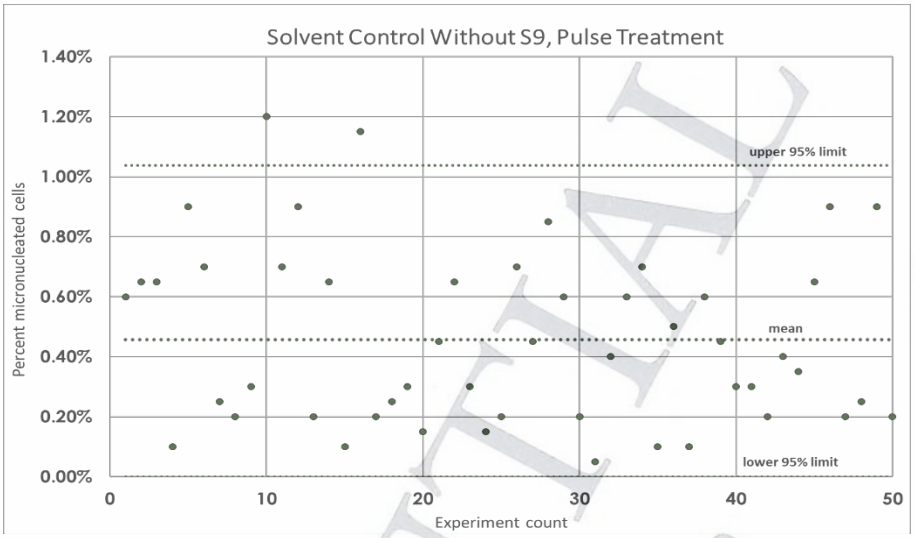
Solvent Control with S9	
Micronucleated cells in %	
	Pulse treatment (4/40)
No. of experiments	52*
Mean	0.48
95 % Ctrl limit	**0.00 – 1.03**
1x SD	0.27
2x SD	0.55
Min – Max	0.05 – 1.25

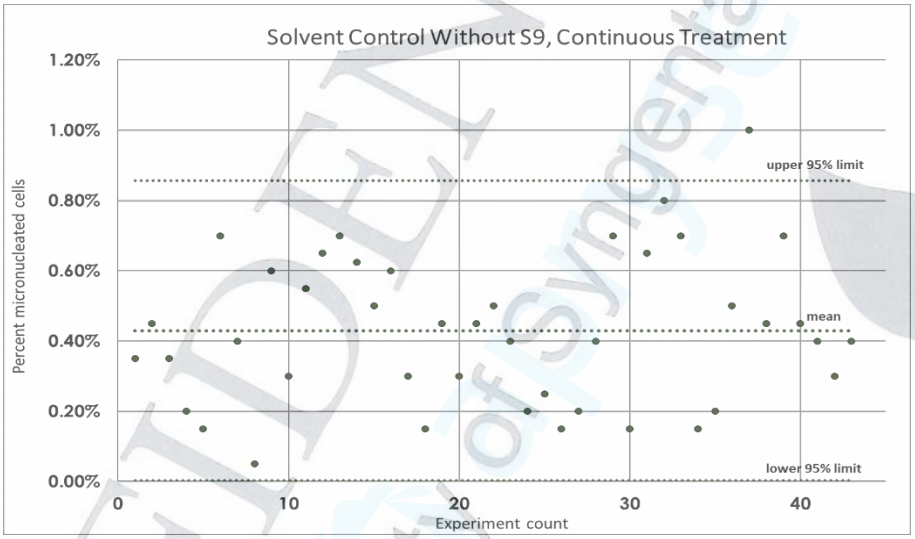
* Aqueous solvents – 17 Experiments; Organic solvents – 35 Experiments

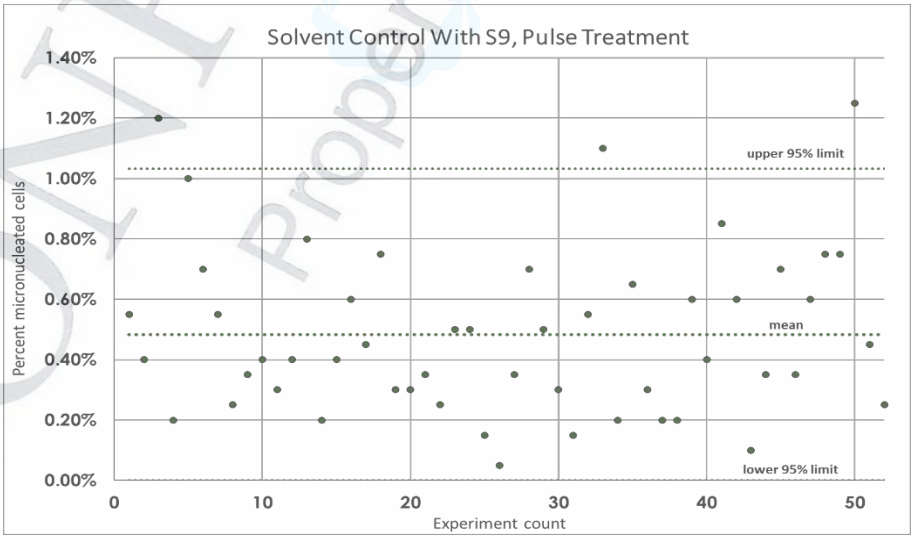
Positive Control without S9		
Micronucleated cells in %		
	Pulse treatment (4/40)	Continuous treatment (20/40)
	MMC	Demecolcin
No. of experiments	50	43
Mean	10.18	4.56
Min – Max	**4.70 – 19.10**	**2.50 – 7.45**
1x SD	3.31	1.23
95 % Ctrl limit	3.57 – 16.79	2.09 – 7.03

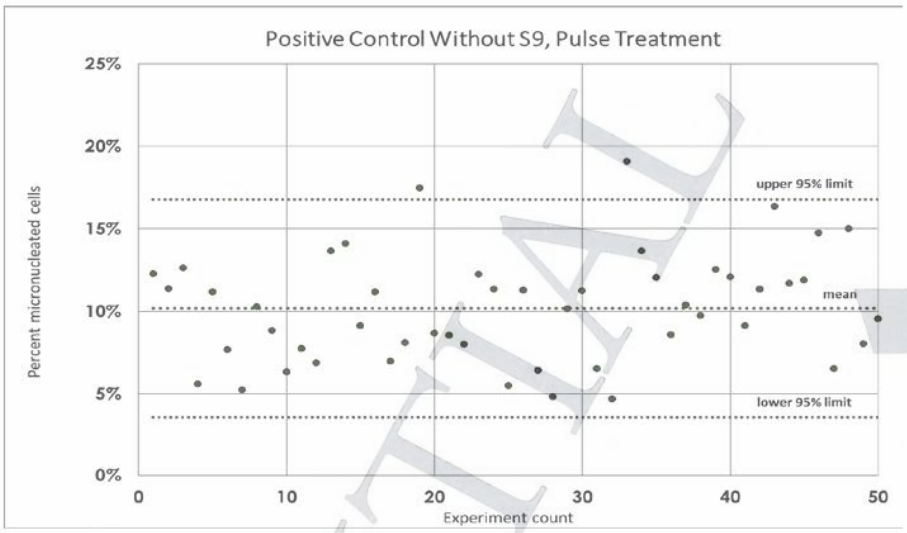
Positive Control with S9	
Micronucleated cells in %	
	Pulse treatment (4/40)
	CPA
No. of experiments	50
Mean	3.67
Min – Max	**2.15 – 6.90**
1x SD	1.19
95 % Ctrl limit	1.30 – 6.05

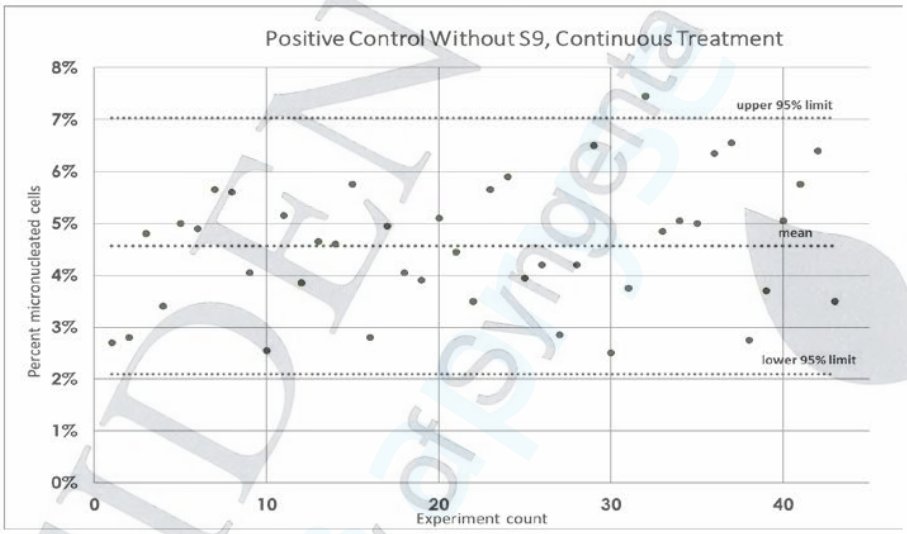
Historical Laboratory Control Data - Control Charts

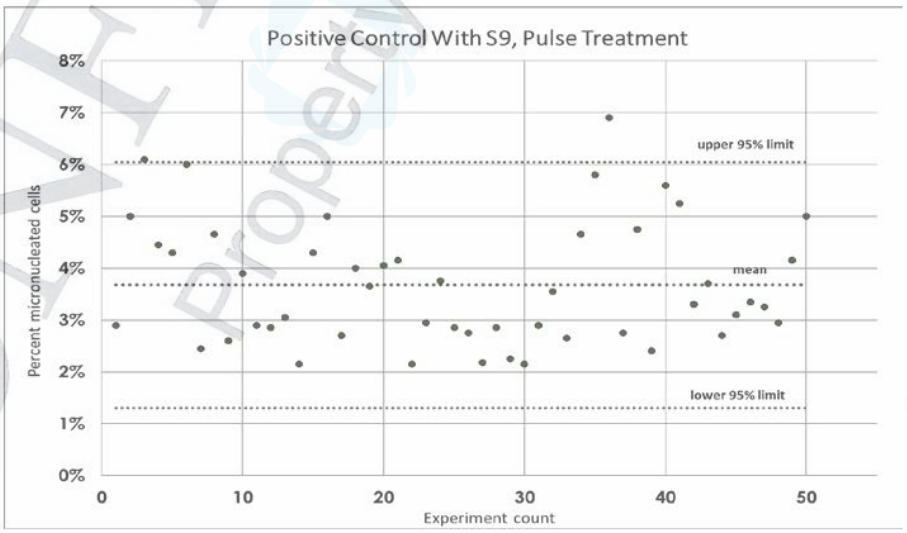












APPENDIX 2 Copy of GLP Certificate

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, 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. 100920

Date of preparation: September 10, 2020
Release date: September 28, 2020

Protein assay: 31 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	34
control	100	40
10 µg/plate	0	92
2-Aminoanthracene	100	2847
10 µg/plate	0	32
Benzo(a)pyrene	150	118

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 — 05. OKT. 2020

Quality Assurance Auditor
ICCR-Roßdorf GmbH — Date

06. OKT. 2020

Dr. Steffen Naumann — Date
Study Director
ICCR-Roßdorf GmbH

ICCR-Roßdorf GmbH
In den Leppsteinswiesen 19, 64380 Roßdorf, Deutschland
T +49 6154 8070 F +49 6154 83399
Registergericht Darmstadt, HRB 6837, USt.-ID DE812933696
Geschäftsführer: Dr. Markus Schulz

SOP Origin TS-SOP S9_22

APPENDIX 4 Certificate of Analysis

syngenta

GLP Testing Facility GOA
Analytical & Product Chemistry

Syngenta Biosciences Pvt. Ltd.
Santa Monica Works,
Corlim, Ilhas Goa 403 110
India

Certificate of Analysis

A21295D
isopyrazam/difenoconazole SC (125/125)
SG40038

Batch Identification	**SG40038**
Other Batch ID	1156078
Product Code	**A21295D**
Other Product Code(s)	CGA169374/SYN520453 SC (125/125)

Chemical Analysis
(Active Ingredient content)

– **Identity of the Active Ingredients***	**Confirmed**
– **Content of difenoconazole (CGA169374)***	**11.93 % w/w corresponding to 130 g/l**
– **Content of isopyrazam (SYN520453) ***	**12.105 % w/w corresponding to 132 g/l**

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

Methodology used for Characterization	HPLC, oscillating density meter

Physical Analysis

– **Appearance**	**Off-white liquid**
– **Density***	**1093 kg/m^3**

Stability:

– **Storage Temperature**	**< 30 °C**
– **Recertification Date**	**End of August 2023**

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

This Certificate of Analysis is summarizing data which originate 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 study(ies) are stored under the study number(s) referenced below within the archives of the GLP Testing Facility Goa at Syngenta Biosciences Pvt. Ltd., Santa Monica Works, Corlim, Ilhas, Goa 403110.

Study number of batch characterization:	SMG16520
Study number(s) of batch recertification:	---

Authorization: 16-Sep-2020

Sunil B. Khot
Analytical and Product Chemistry, Goa

Page 1 of 1

Mod. 005C