Isopyrazam/Difenoconazole

Isopyrazam/Difenoconazole SC (A21295D) - *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay

Final Report

TEST GUIDELINE(S): OECD 471 (2020)

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PERFORMING LABORATORY: ICCR-Roßdorf GmbH
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SPONSOR(S): Syngenta Ltd.
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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.

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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

ICCR Study Number: 2137100
Test substance: Isopyrazam/Difenoconazole SC (A21295D)
Study director: Dr. Steffi Chang
Study Title: Isopyrazam/Difenoconazole SC (A21295D) - *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay

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	04 November 2020	04 November 2020
Study Plan Amendment 1 Verification	08 March 2021	08 March 2021
Process – based Test System Preparation and Application	12 November 2020	13 November 2020
Report Audit	09 December 2020	09 December 2020

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.

H. Pilawa
Quality Assurance Auditor
ICCR-Roßdorf GmbH

11 March 2021
Date

PROJECT STAFF SIGNATURE

Study Director Dr. Steffi Chang

S.C.

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Date: 11 March 2021

GENERAL INFORMATION

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Study Dates
Study initiation date: 04 November 2020
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Experimental completion date: 25 November 2020

Deviations from the Guidelines
None

Retention of Samples
None

Performing Laboratory Test Substance Reference Number
S 2126411

Other
ICCR-Roßdorf GmbH will archive:
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 electronic and paper raw data, and report that support the reconstruction of the 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 item will not be archived.

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

Deviations from the study plan
There were no deviations (unplanned changes) from the study plan.

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

This study was performed to investigate the potential of Isopyrazam/Difenoconazole SC (A21295D) to induce gene mutations in the plate incorporation test (Experiment I) and the pre-incubation test (Experiment II) using the *Salmonella typhimurium (S. typhimurium)* strains TA1535, TA1537, TA98, and TA100, and the *Escherichia coli (E. coli)* strains WP2 *uvrA* (pKM101) and WP2 (pKM101).

1.2 Results

The plates incubated with the test item showed normal background growth up to the maximal concentration of 5000 µg/plate with and without S9 mix in all strains used.

No cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in all strains with and without metabolic activation.

No relevant increase in revertant colony numbers of any of the six tester strains was observed following treatment with Isopyrazam/Difenoconazole SC (A21295D) at any concentration, neither in the presence nor absence of metabolic activation (S9 mix). A minor increase not reaching the threshold of twice the number of the corresponding solvent control was observed in strain TA1537 in the absence of S9 mix in both experiments.

Appropriate reference mutagens were used as positive controls, which showed a distinct increase of induced revertant colonies consistent with the laboratory's historical control data, demonstrated the sensitivity of the test system and the efficacy of the S9 mix. Each batch of S9 was also tested with 2 pro-mutagens, benzo(a)pyrene and 2-aminoanthracene.

1.3 Conclusion

In conclusion, it can be stated that during the described mutagenicity tests and under the experimental conditions reported, Isopyrazam/Difenoconazole SC (A21295D) did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, Isopyrazam/Difenoconazole SC (A21295D) is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

2.0 INTRODUCTION

2.1 Purpose

These experiments were performed to assess the potential of the test substance to induce gene mutations by means of the *S. typhimurium* and *E. coli* reverse mutation assay. Experiment I was performed as a plate incorporation assay. Since a negative result was obtained in this experiment, Experiment II was performed as a pre-incubation assay.

The most widely used assays for detecting gene mutations are those using bacteria (1). They are relatively simple and rapid to perform, and give reliable data on the ability of an agent to interact with DNA and produce mutations.

Reverse mutation assays determine the frequency with which an agent reverses or suppresses the effect of the forward mutation. The genetic target presented to an agent is therefore small, specific and selective. Several bacterial strains, or a single strain with multiple markers are necessary to assure reliable detection of mutagens that may be specific to one tester strain or locus. The reversion of bacteria from growth-dependence on a particular amino acid to growth in the absence of that amino acid (reversion from auxotrophy to prototrophy) is the most widely used marker.

The S. *typhimurium* histidine (his) and the *E. coli* tryptophan (trp) reversion system measures $his^- \rightarrow his^+$ and $trp^- \rightarrow trp^+$ reversions, respectively. The *S. typhimurium* and *E. coli* strains are constructed to differentiate between base pair (TA1535, TA100, WP2 *uvrA* (pKM101), and WP2 (pKM101)) and frameshift (TA1537, TA98) mutations.

According to the direct plate incorporation and pre-incubation method the bacteria are exposed to the test substance with and without metabolic activation and plated on selective medium. After a suitable period of incubation, revertant colonies are counted.

To establish a concentration response effect at least six concentrations with adequately spaced intervals were tested. The maximum concentration was 5000 µg/plate.

To validate the test, reference mutagens were tested in parallel to the test substance.

2.2 Test Guideline(s)

This study followed the procedures indicated by the following internationally accepted guideline and recommendations:

"Ninth Addendum to OECD Guidelines for Testing of Chemicals", Section 4, No. 471: "Bacterial Reverse Mutation Test", corrected June 26, 2020

3.0 MATERIALS AND METHODS

3.1 Test Substance

Information as provided by the Sponsor.

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
Appearance:	Off-white, liquid
Recertification Date:	29 August 2023
Storage Conditions:	At room temperature
Stability in Solvent:	Not indicated by the Sponsor

The test substance concentrations were not adjusted for the content of isopyrazam or difenoconazole.

On the day of the experiment (immediately before use), the test substance was suspended in deionised water. The solvent was chosen as the most suitable solvent compared to water and ethanol, according to its solubilisation properties and its relative non-toxicity to the bacteria (2).

All formulations were prepared freshly before treatment and used within two hours of preparation. The formulation was assumed to be stable for this period unless specified otherwise by the Sponsor.

3.2 Controls

3.2.1 Negative controls

Concurrent untreated and solvent controls were performed.

3.2.2 Positive control substances

Without metabolic activation

Strains:	TA1535, TA100
Name:	Sodium azide, (NaN_3)
Supplier:	SERVA, 69042 Heidelberg, Germany
Batch No.:	150564
Purity:	≥ 99%
Dissolved in:	Deionised water
Concentration:	10 µg/plate

Strains:	TA1537, TA98
Name:	4-nitro-o-phenylene-diamine, (4-NOPD)
Supplier:	Sigma-Aldrich, 82024 Taufkirchen, Germany
Batch No.:	MKBM 5257V
Purity:	≥ 98%
Dissolved in:	DMSO (purity >99 %, Fisher Leics LE11 5RG, United Kingdom)
Concentration:	10 µg/plate in strain TA 98, 50 µg/plate in strain TA 1537

Strains:	WP2 *uvrA* (pKM101), WP2 (pKM101)
Name:	Methyl methane sulfonate, (MMS)
Supplier:	Sigma-Aldrich, 82024 Taufkirchen, Germany
Batch No.:	MKCG 1346
Purity:	≥ 99%
Dissolved in:	Deionised water
Concentration:	2.0 µL/plate

With metabolic activation

Strains:	TA1535, TA1537, TA98, TA100, WP2 *uvrA* (pKM101), WP2 (pKM 101)
Name:	2-aminoanthracene, (2-AA)
Supplier:	Sigma-Aldrich, 82024 Taufkirchen, Germany
Batch No.:	STBG 0630V
Purity:	≥ 96%
Dissolved in:	DMSO (purity > 99 %, Fisher Leics LE11 5RG, United Kingdom)
Concentration:	2.5 µg/plate (TA1535, TA1537, TA98, TA100), 10 µg/plate (WP2 *uvrA* (pKM101), WP2 (pKM101))

The stability of the positive control substances in solution is unknown but a mutagenic response in the expected range is sufficient evidence of biological activity.

3.3 Experimental Design

3.3.1 Characterisation of the *Salmonella typhimurium* and *E. coli* strains

The histidine dependent strains are derived from *S. typhimurium* strain LT2 through mutations in the histidine locus. Additionally, due to the "deep rough" (*rfa*$^-$) mutation they possess a faulty lipopolysaccharide envelope which enables substances to penetrate the cell wall more easily. A further mutation causes a reduction in the activity of an excision repair system. The last alteration includes mutational processes in the nitrate reductase and biotin genes produced in a UV-sensitive area of the gene named *uvrB*$^-$. In the strains TA98 and TA100 the R-factor plasmid pKM101 carries the ampicillin resistance marker (3).

Strain WP2 (4) and its derivatives all carry the same defect in one of the genes for tryptophan biosynthesis. Tryptophan-independent (*Trp*$^+$) mutants (revertants) can arise either by a base change at the site of the original alteration or by a base change elsewhere in the chromosome so that the original defect is suppressed. This second possibility can occur in several different ways so that the system seems capable of detecting all types of mutagen which substitute one base for another. Additionally, the *uvrA* derivative is deficient in the DNA repair process (excisable repair damage). Such a repair-deficient strain may be more readily mutated by agents. The *E. coli* strains WP2 *uvrA* (pKM101) and WP2 (pKM101) are constructed by introduction of the R-factor plasmid pKM101.

When summarized, the mutations of the *S. typhimurium* and *E. coli* strains used in this study can be described as follows:

Strains	Genotype	Type of mutations indicated
Salmonella typhimurium		
TA1537	*his* C 3076; *rfa*$^-$; *uvrB*$^-$	frame shift mutations
TA98	*his* D 3052; *rfa*$^-$; *uvrB*$^-$; R-factor	" "
TA1535	*his* G 46; *rfa*$^-$; *uvrB*$^-$	base-pair substitutions
TA100	*his* G 46; *rfa*$^-$; *uvrB*$^-$; R-factor	" "
Escherichia coli		
WP2 *uvrA* (pKM101)	*trp* E 56 *uvrA*$^-$; R-factor	base-pair substitutions and others
WP2 (pKM101)	*trp* E 56; R-factor	" "

Regular checking of the properties of the *S.typhimurium* and *E. coli* strains regarding the membrane permeability and ampicillin resistance; UV sensitivity, and amino acid requirement as well as normal spontaneous mutation rates is performed by ICCR-Roßdorf GmbH according to Ames *et al.* (5), Maron and Ames (3), and Mortelmans and Riccio (7). In this way it is ensured that the experimental conditions set down by Ames are fulfilled.

The bacterial strains TA1535, TA1537, TA98, TA100, WP2 *uvrA* (pKM101), and WP2 (pKM101) were obtained from Trinova Biochem GmbH (35394 Gießen, Germany).

3.3.2 Storage

The strain cultures were stored as stock cultures in ampoules with nutrient broth + 5 % DMSO (Fisher Leics, LE11 5RG, United Kingdom) in liquid nitrogen.

3.3.3 Precultures

The thawed bacterial suspension was transferred into 250 mL Erlenmeyer flasks containing nutrient medium (50 mL). A solution of ampicillin (50 µL, 25 µg/mL) was added to the strains TA98, TA100, WP2 *uvrA* (pKM101), and WP2 (pKM101). This nutrient medium contains per liter:

8 g Nutrient Broth (MERCK, 64293 Darmstadt, Germany)
5 g NaCl (MERCK, 64293 Darmstadt, Germany)

The bacterial cultures were incubated in a shaking water bath for 4 hours at 37 °C. The optical density of the bacteria was determined by absorption measurement and the obtained values indicated that the bacteria were harvested at the late exponential or early stationary phase (10^8-10^9 cells/mL).

3.3.4 Selective agar

Plates with selective agar (without Histidine/Tryptophan) were used.

3.3.5 Overlay agar

The overlay agar contained per litre:

for *Salmonella* strains:	for *Escherichia coli* strains:
7.0 g Agar Agar*	7.0 g Agar Agar*
6.0 g NaCl*	6.0 g NaCl*
10.5 mg L-Histidine×HCl×H_2O*	10.2 mg Tryptophan*
12.2 mg Biotin*	

* (MERCK, 64293 Darmstadt, Germany)

Sterilisations were performed at 121 °C in an autoclave.

3.4 Mammalian Microsomal Fraction S9 Mix

The bacteria used in this assay do not possess the enzyme systems which, in mammals, are known to convert promutagens into active DNA damaging metabolites. In order to overcome this major drawback an exogenous metabolic system is added in the form of mammalian microsome enzyme activation mixture.

3.4.1 S9 (Preparation by ICCR-Roßdorf GmbH)

Phenobarbital/ß-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Wistar rats (RjHan:WI; weight approx. 220 – 320 g,

Janvier Labs, 53941 Saint-Berthevin Cedex, France) induced by peroral administration of 80 mg/kg b.w. phenobarbital (Sigma-Aldrich Chemie GmbH, 82024 Taufkirchen, Germany) and by peroral administrations of ß-naphthoflavone (Acros Organics, 2440 Geel, Belgium) each, on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1+3 parts) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored in ampoules at –80 °C. Small numbers of the ampoules can be kept at –20 °C for up to one week. Each batch of S9 mix is routinely tested with 2-aminoanthracene as well as benzo[a]pyrene (Appendix 3).

The protein concentration in the S9 preparation was 33.0 mg/mL (lot no. 030920K) in the pre-experiment / Experiment I and 34.8 mg/mL (lot no. 030920D) in Experiment II.

3.4.2 S9 mix

Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors were added to the S9 mix to reach the following concentrations in the S9 mix:

8 mM	$MgCl_2$
33 mM	KCl
5 mM	Glucose-6-phosphate
4 mM	NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.
During the experiment the S9 mix was stored in an ice bath. The S9 mix preparation was performed according to Ames *et al.*(5).

3.5 Pre-Experiment for Cytotoxicity

To evaluate the cytotoxicity of the test substance a pre-experiment was performed with all strains. Eight concentrations were tested for cytotoxicity and mutation induction each with three replicate plates. The experimental conditions in this pre-experiment are described in section 3.7 (plate incorporation test).

Cytotoxicity of the test substance results in a reduction in the number of spontaneous revertants (below a factor of 0.5) or a clearing of the bacterial background lawn.

The pre-experiment is reported as the Main Experiment I since the criteria mentioned in Section 3.8.2 Acceptability of the Assay were met.

3.6 Concentration Selection

In the pre-experiment the concentration range of the test substance was 3 - 5000 µg/plate. The pre-experiment is reported as Experiment I. Since no cytotoxic effects were observed in Experiment I, 5000 µg/plate was chosen as the maximal concentration in Experiment II.

The concentration range included two logarithmic decades. The following concentrations were tested in Experiment II:

33; 100; 333; 1000; 2500; and 5000 µg/plate

3.7 Experimental Performance

For each strain and concentration including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

100 µL	Test solution at each concentration, solvent (negative control) or reference mutagen solution (positive control),
500 µL	S9 mix (for test with metabolic activation) or S9 mix substitution buffer* (for test without metabolic activation),
100 µL	Bacteria suspension (cf. test system, pre-culture of the strains; OD = 0.9 - 1.2; wavelength = 500 nm; approx. $8x10^8$ cells/mL),
2000 µL	Overlay agar

For the pre-incubation method test solution (100 µL) (solvent or reference mutagen solution (positive control)), S9 mix / S9 mix substitution buffer* (500 µL) and bacteria suspension (100 µL) were mixed in a test tube and incubated at 37 C ± 1.5° C for 60 minutes. After pre-incubation overlay agar (2.0 mL, 45 °C) was added to each tube. The mixture was poured on selective agar plates.

After solidification the plates were incubated upside down for 72 hours at 37 C ± 1.5° C in the dark, plates were then stored at 4 °C until counted (6).

In parallel to each test a sterile control of the test substance was performed and documented in the raw data. Therefore, stock solution (100 µL) and S9 mix / S9 mix substitution buffer* (500 µL) were mixed with overlay agar (2.0 mL) and poured on minimal agar plates.

* Substitution buffer: 7 parts of the 100 mM sodium-ortho-phosphate-buffer pH 7.4 with 3 parts of KCl solution 0.15 M

3.8 Data Evaluation

3.8.1 Data recording

The colonies were counted using a Petri Viewer with the software program Ames Study Manager (see section 3.9, Major computerized systems). The evaluation unit was connected

to a PC with printer to print out the individual values, the means from the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates (see tables of results). The print outs are kept with the raw data. Due to precipitation of the test item some test groups were scored manually (as indicated on data tables).

3.8.2 Acceptability of the assay

The *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of the historical data
- the positive control substances should produce an increase in mutant colony frequencies of at least twofold the revertant colony count of the concurrent control
- a minimum of five analysable concentrations should be present with at least four concentrations showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

3.8.3 Evaluation of results

A test substance is considered as a mutagen if a biologically relevant increase in the number of revertants of twice or above the spontaneous mutation rate of the corresponding solvent control is observed (1).

A concentration dependent increase is considered biologically relevant if the threshold is reached or exceeded at more than one concentration (6).

An increase of revertant colonies equal or above the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A concentration dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls, such an increase is not considered biologically relevant.

3.8.4 Biometry

According to the OECD guideline 471, a statistical analysis of the data is not mandatory.

3.9 Major Computerized System

Petri Viewer Sorcerer Colony Counter 3.0 (Instem, Suffolk IP33 3TA, UK) with the software program Ames Study Manager (v1.24) and Ames Archive Manager (v1.01).

4.0 RESULTS AND DISCUSSION

The test substance, Isopyrazam/Difenoconazole SC (A21295D), was assessed for its potential to induce gene mutations in the plate incorporation test (Experiment I) and the pre-incubation test (Experiment II) using *S. typhimurium* strains TA1535, TA1537, TA98, and TA100, and the *E. coli* strains WP2 (pKM101) and WP2 *uvrA* (pKM101).

In the pre-experiment the concentration range of the test substance was 3 - 5000 µg/plate. The pre-experiment is reported as Experiment I. Since no cytotoxic effects were observed in Experiment I, 5000 µg/plate was chosen as the maximal concentration in Experiment II.

The assay was performed with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The concentration range included two logarithmic decades. The test substance was tested at the following concentrations:

Pre-Experiment/Experiment I:	3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II:	33; 100; 333; 1000; 2500; and 5000 µg/plate

The test item precipitated in the overlay agar in the test tubes from 2500 to 5000 µg/plate in Experiment I and from 1000 to 5000 µg/plate in Experiment II. Precipitation of the test item in the overlay agar on the incubated agar plates was observed at 5000 µg/plate in both experiments. The undissolved particles had no influence on the data recording, a manual count was performed where required.

The plates incubated with the test item showed normal background growth up to the maximal dose of 5000 µg/plate with and without S9 mix in all strains used.

No cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

No relevant increase in revertant colony numbers of any of the six tester strains was observed following treatment with Isopyrazam/Difenoconazole SC (A21295D) at any concentration, neither in the presence nor absence of metabolic activation (S9 mix). A minor increase was observed in strain TA1537 in the absence of S9 mix in both experiments. The threshold of twice the number of the corresponding solvent control was neither reached nor exceeded. The highest increase in revertant colony count was observed at 2500 µg/plate in both experiments. In Experiment I the upper limit of the historical control data of negative and solvent control was exceeded from 1000 to 5000 µg/plate, in Experiment II the upper limit of the negative control was reached at 2500 µg/plate, the upper limit of the solvent control was slightly increased (22 versus 20 colonies) at 2500 µg/plate. It can be stated therefore, that the increase observed in Experiment I was not completely reproduced and did, therefore, not fullfill the criteria of a biological relevant increase.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies consistent with the laboratory's historical control data

and demonstrated the sensitivity of the test system and the efficacy of the S9 mix. Each batch of S9 was also tested with 2 pro-mutagens, benzo(a)pyrene and 2-aminoanthracene.

In experiment II, the data in the untreated and solvent control of strain WP2 (pKM101) in the presence of S9 mix were slightly above the laboratory historical control range. Since this deviation is rather small, this effect is considered to be based upon biologically irrelevant fluctuations in the number of colonies and had no detrimental impact on the outcome of the study.

5.0 CONCLUSIONS

In conclusion, it can be stated that during the described mutagenicity tests and under the experimental conditions reported, Isopyrazam/Difenoconazole SC (A21295D) did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, Isopyrazam/Difenoconazole SC (A21295D) is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

6.0 REFERENCES

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TABLES SECTION

TABLE 1 Summary of Results Pre-Experiment/Experiment I

Study Name: 2137100
Experiment: 2137100 VV Plate
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 11.11.2020
Date Counted: 16.11.2020

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	Deionised water		13 ± 4	16 ± 3	28 ± 6	138 ± 7	293 ± 30	384 ± 19
	Untreated		12 ± 3	9 ± 2	32 ± 4	134 ± 24	283 ± 22	364 ± 32
		3 µg	11 ± 3	12 ± 4	30 ± 5	136 ± 15	292 ± 12	354 ± 22
	Isopyrazam/	10 µg	13 ± 3	15 ± 3	27 ± 1	136 ± 4	271 ± 9	356 ± 24
	Difenoconazole	33 µg	10 ± 3	14 ± 1	33 ± 12	131 ± 1	286 ± 23	356 ± 11
	SC (A21295D)	100 µg	10 ± 1	15 ± 3	34 ± 3	138 ± 17	254 ± 6	323 ± 41
		333 µg	9 ± 3	13 ± 2	36 ± 4	148 ± 7	262 ± 13	361 ± 12
		1000 µg	13 ± 3	23 ± 4	27 ± 7	151 ± 9	283 ± 16	343 ± 14
		2500 µg	13 ± 3	29 ± 5	24 ± 6	148 ± 10	249 ± 33	341 ± 8
		5000 µg	9 ± 3 [P]	27 ± 4 [P M]	29 ± 9 [P]	142 ± 6 [P]	219 ± 13 [P]	324 ± 37 [P]
	NaN3	10 µg	1371 ± 97			1731 ± 65		
	4-NOPD	10 µg			637 ± 63			
	4-NOPD	50 µg		87 ± 8				
	MMS	2.0 µL					3053 ± 135	3092 ± 186
With Activation	Deionised water		12 ± 1	16 ± 5	39 ± 10	148 ± 9	270 ± 27	414 ± 30
	Untreated		10 ± 1	14 ± 1	45 ± 11	145 ± 23	301 ± 15	396 ± 24
		3 µg	10 ± 3	20 ± 2	38 ± 10	140 ± 19	296 ± 22	386 ± 32
	Isopyrazam/	10 µg	10 ± 1	15 ± 4	38 ± 12	132 ± 6	292 ± 6	388 ± 7
	Difenoconazole	33 µg	9 ± 2	17 ± 5	32 ± 6	141 ± 14	264 ± 15	389 ± 23
	SC (A21295D)	100 µg	10 ± 4	19 ± 5	34 ± 5	153 ± 23	229 ± 20	378 ± 12
		333 µg	10 ± 3	17 ± 6	35 ± 9	156 ± 4	272 ± 31	403 ± 49
		1000 µg	10 ± 3	19 ± 2	36 ± 4	152 ± 19	292 ± 15	373 ± 21
		2500 µg	12 ± 3	14 ± 2	41 ± 10	152 ± 9	268 ± 17	352 ± 6
		5000 µg	14 ± 3 [P]	20 ± 3 [P]	37 ± 10 [P]	147 ± 13 [P]	262 ± 23 [P]	351 ± 28 [P]
	2-AA	2.5 µg	322 ± 37	391 ± 24	3336 ± 266	4431 ± 215		
	2-AA	10.0 µg					944 ± 23	1643 ± 56

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

TABLE 2 Summary of Results Experiment II

Study Name: 2137100
Experiment: 2137100 HV2 Pre
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 19.11.2020
Date Counted: 25.11.2020

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	Deionised water		12 ± 2	13 ± 2	26 ± 3	148 ± 23	296 ± 14	377 ± 32
	Untreated		9 ± 3	13 ± 3	30 ± 8	152 ± 17	304 ± 14	350 ± 24
		33 µg	13 ± 3	13 ± 1	26 ± 8	154 ± 21	307 ± 8	407 ± 24
	Isopyrazam/	100 µg	10 ± 1	15 ± 1	21 ± 5	148 ± 21	315 ± 21	396 ± 9
	Difenoconazole	333 µg	14 ± 3	15 ± 3	28 ± 6	147 ± 7	298 ± 40	369 ± 32
	SC (A21295D)	1000 µg	11 ± 3	19 ± 3	25 ± 5	172 ± 6	301 ± 22	318 ± 26
		2500 µg	13 ± 2	22 ± 6	27 ± 10	152 ± 9	288 ± 3	313 ± 20
		5000 µg	8 ± 2 [P M]	17 ± 2 [P M]	27 ± 6 [P]	143 ± 3 [P]	255 ± 27 [P]	307 ± 21 [P]
	NaN3	10 µg	1228 ± 146			1500 ± 79		
	4-NOPD	10 µg			449 ± 21			
	4-NOPD	50 µg		95 ± 18				
	MMS	2.0 µL					3599 ± 284	3338 ± 202
With Activation	Deionised water		12 ± 3	12 ± 3	39 ± 9	142 ± 22	350 ± 12	350 ± 17
	Untreated		13 ± 4	11 ± 2	43 ± 1	139 ± 15	361 ± 25	373 ± 14
		33 µg	12 ± 3	16 ± 1	41 ± 11	143 ± 3	305 ± 15	415 ± 38
	Isopyrazam/	100 µg	11 ± 1	15 ± 5	41 ± 11	161 ± 9	281 ± 40	376 ± 27
	Difenoconazole	333 µg	15 ± 4	17 ± 5	35 ± 3	163 ± 5	276 ± 15	379 ± 41
	SC (A21295D)	1000 µg	15 ± 4	16 ± 1	38 ± 4	167 ± 9	315 ± 21	401 ± 17
		2500 µg	9 ± 3	13 ± 4	39 ± 4	151 ± 15	304 ± 21	385 ± 21
		5000 µg	7 ± 2 [P M]	11 ± 3 [P M]	31 ± 5 [P]	152 ± 16 [P]	272 ± 60 [P]	382 ± 27 [P]
	2-AA	2.5 µg	194 ± 46	263 ± 14	1881 ± 343	2812 ± 173		
	2-AA	10.0 µg					1081 ± 139	1498 ± 182

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

TABLE 3 Pre-Experiment and Experiment I: 2137100 VV Plate Incorporation Without Metabolic Activation

Study Name: 2137100
Experiment: 2137100 VV Plate
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 11.11.2020
Date Counted: 16.11.2020

Without metabolic activation

Strain	Compound	Concentration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535		3 µg	*11.0*	*2.6*	*0.8*	9, 10, 14
	Isopyrazam/	10 µg	*12.7*	*3.2*	*0.9*	15, 9, 14
	Difenoconazole	33 µg	*9.7*	*2.5*	*0.7*	7, 10, 12
	SC (A21295D)	100 µg	*10.3*	*0.6*	*0.8*	11, 10, 10
		333 µg	*9.0*	*2.6*	*0.7*	6, 11, 10
		1000 µg	*13.0*	*2.6*	*1.0*	15, 14, 10
		2500 µg	*12.7*	*3.2*	*0.9*	14, 15, 9
		5000 µg	*9.3*	*2.9*	*0.7*	11 P, 6 P, 11 P
	Deionised water		*13.3*	*3.8*		9, 15, 16
	Untreated		*12.3*	*3.2*		16, 11, 10
TA 1537		3 µg	*11.7*	*4.0*	*0.7*	7, 14, 14
	Isopyrazam/	10 µg	*14.7*	*3.2*	*0.9*	16, 17, 11
	Difenoconazole	33 µg	*14.3*	*0.6*	*0.9*	15, 14, 14
	SC (A21295D)	100 µg	*14.7*	*2.5*	*0.9*	15, 17, 12
		333 µg	*13.3*	*2.3*	*0.8*	12, 12, 16
		1000 µg	*23.0*	*3.6*	*1.4*	22, 20, 27
		2500 µg	*28.7*	*5.1*	*1.8*	33, 23, 30
		5000 µg	*26.7*	*3.5*	*1.6*	30 P M, 23 P M, 27 P M
	Deionised water		*16.3*	*2.5*		16, 19, 14
	Untreated		*8.7*	*2.3*		6, 10, 10
TA 98		3 µg	*30.3*	*4.9*	*1.1*	36, 27, 28
	Isopyrazam/	10 µg	*26.7*	*0.6*	*1.0*	27, 26, 27
	Difenoconazole	33 µg	*33.3*	*11.8*	*1.2*	27, 47, 26
	SC (A21295D)	100 µg	*34.3*	*3.1*	*1.2*	31, 37, 35
		333 µg	*36.3*	*3.5*	*1.3*	40, 36, 33
		1000 µg	*27.3*	*7.1*	*1.0*	26, 21, 35
		2500 µg	*23.7*	*5.7*	*0.9*	22, 19, 30
		5000 µg	*29.3*	*9.3*	*1.1*	19 P, 37 P, 32 P
	Deionised water		*27.7*	*5.9*		30, 21, 32
	Untreated		*32.0*	*4.0*		36, 28, 32

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

Study Name: 2137100
Experiment: 2137100 VV Plate
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 11.11.2020
Date Counted: 16.11.2020

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 100		3 µg	*136.0*	*15.4*	*1.0*	149, 119, 140
	Isopyrazam/	10 µg	*135.7*	*4.0*	*1.0*	135, 140, 132
	Difenoconazole	33 µg	*131.0*	*1.0*	*0.9*	132, 131, 130
	SC (A21295D)	100 µg	*137.7*	*16.6*	*1.0*	120, 140, 153
		333 µg	*147.7*	*7.1*	*1.1*	149, 154, 140
		1000 µg	*151.0*	*8.7*	*1.1*	156, 141, 156
		2500 µg	*148.0*	*9.5*	*1.1*	153, 154, 137
		5000 µg	*142.0*	*6.0*	*1.0*	136 P, 148 P, 142 P
	Deionised water		*138.3*	*7.4*		144, 130, 141
	Untreated		*133.7*	*23.7*		112, 159, 130
WP2		3 µg	*291.7*	*11.6*	*1.0*	305, 284, 286
pKM101	**Isopyrazam/**	10 µg	*271.3*	*9.5*	*0.9*	264, 268, 282
	Difenoconazole	33 µg	*286.0*	*22.6*	*1.0*	267, 280, 311
	SC (A21295D)	100 µg	*254.0*	*6.1*	*0.9*	257, 258, 247
		333 µg	*262.3*	*13.3*	*0.9*	277, 251, 259
		1000 µg	*283.0*	*16.1*	*1.0*	288, 265, 296
		2500 µg	*249.3*	*32.6*	*0.9*	285, 221, 242
		5000 µg	*219.3*	*13.3*	*0.7*	226 P, 228 P, 204 P
	Deionised water		*293.0*	*30.4*		278, 328, 273
	Untreated		*283.3*	*22.3*		272, 309, 269
WP2		3 µg	*354.3*	*21.5*	*0.9*	349, 378, 336
uvrA	**Isopyrazam/**	10 µg	*355.7*	*24.0*	*0.9*	331, 357, 379
pKM101	**Difenoconazole**	33 µg	*356.0*	*11.1*	*0.9*	368, 346, 354
	SC (A21295D)	100 µg	*322.7*	*41.5*	*0.8*	358, 277, 333
		333 µg	*361.3*	*11.6*	*0.9*	363, 372, 349
		1000 µg	*342.7*	*13.6*	*0.9*	341, 357, 330
		2500 µg	*340.7*	*8.4*	*0.9*	331, 345, 346
		5000 µg	*324.0*	*37.2*	*0.8*	328 P, 359 P, 285 P
	Deionised water		*384.0*	*18.7*		399, 363, 390
	Untreated		*364.0*	*32.4*		328, 373, 391
TA 1535	**NaN3**	10 µg	*1370.7*	*97.4*	*102.8*	1483, 1320, 1309
TA 1537	**4-NOPD**	50 µg	*87.3*	*8.1*	*5.3*	86, 80, 96
TA 98	**4-NOPD**	10 µg	*636.7*	*63.3*	*23.0*	688, 656, 566
TA 100	**NaN3**	10 µg	*1730.7*	*64.7*	*12.5*	1661, 1742, 1789
WP2 pKM101	**MMS**	2.0 µL	*3053.0*	*134.7*	*10.4*	3044, 2923, 3192
WP2 uvrA pKM101	**MMS**	2.0 µL	*3092.3*	*185.6*	*8.1*	2935, 3045, 3297

Key to Positive Controls

NaN3	sodium azide
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

TABLE 4 Pre-Experiment and Experiment I: 2137100 VV Plate Incorporation With Metabolic Activation

Study Name: 2137100
Experiment: 2137100 VV Plate
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 11.11.2020
Date Counted: 16.11.2020

With metabolic activation

Strain	Compound	Concentration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535		3 µg	*10.3*	*2.9*	*0.9*	7, 12, 12
	Isopyrazam/	10 µg	*10.3*	*0.6*	*0.9*	11, 10, 10
	Difenoconazole	33 µg	*8.7*	*1.5*	*0.7*	7, 9, 10
	SC (A21295D)	100 µg	*10.3*	*3.5*	*0.9*	14, 10, 7
		333 µg	*10.0*	*2.6*	*0.9*	12, 7, 11
		1000 µg	*9.7*	*3.2*	*0.8*	6, 11, 12
		2500 µg	*12.3*	*2.9*	*1.1*	14, 9, 14
		5000 µg	*13.7*	*2.5*	*1.2*	16 P, 14 P, 11 P
	Deionised water		*11.7*	*0.6*		12, 12, 11
	Untreated		*9.7*	*0.6*		9, 10, 10
TA 1537		3 µg	*19.7*	*2.3*	*1.2*	17, 21, 21
	Isopyrazam/	10 µg	*15.0*	*4.0*	*0.9*	19, 11, 15
	Difenoconazole	33 µg	*16.7*	*4.5*	*1.0*	21, 12, 17
	SC (A21295D)	100 µg	*19.3*	*4.7*	*1.2*	14, 21, 23
		333 µg	*17.3*	*5.5*	*1.1*	17, 23, 12
		1000 µg	*18.7*	*2.3*	*1.2*	16, 20, 20
		2500 µg	*13.7*	*1.5*	*0.9*	12, 15, 14
		5000 µg	*19.7*	*3.2*	*1.2*	22 P, 21 P, 16 P
	Deionised water		*16.0*	*5.0*		21, 11, 16
	Untreated		*14.3*	*0.6*		14, 15, 14
TA 98		3 µg	*38.0*	*9.8*	*1.0*	27, 41, 46
	Isopyrazam/	10 µg	*38.3*	*11.6*	*1.0*	40, 26, 49
	Difenoconazole	33 µg	*32.3*	*6.0*	*0.8*	26, 33, 38
	SC (A21295D)	100 µg	*34.0*	*5.2*	*0.9*	40, 31, 31
		333 µg	*35.0*	*8.5*	*0.9*	36, 43, 26
		1000 µg	*36.3*	*4.2*	*0.9*	33, 41, 35
		2500 µg	*40.7*	*9.7*	*1.0*	49, 30, 43
		5000 µg	*37.3*	*10.0*	*1.0*	27 P, 38 P, 47 P
	Deionised water		*39.0*	*10.1*		41, 28, 48
	Untreated		*45.3*	*10.7*		33, 52, 51

Key to Plate Postfix Codes

P	Precipitate

Study Name: 2137100
Experiment: 2137100 VV Plate
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 11.11.2020
Date Counted: 16.11.2020

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 100		3 µg	*139.7*	*18.5*	*0.9*	128, 161, 130
	Isopyrazam/	10 µg	*131.7*	*6.1*	*0.9*	137, 133, 125
	Difenoconazole	33 µg	*141.3*	*14.0*	*1.0*	156, 140, 128
	SC (A21295D)	100 µg	*153.3*	*23.0*	*1.0*	128, 159, 173
		333 µg	*156.0*	*4.4*	*1.1*	153, 161, 154
		1000 µg	*152.3*	*18.5*	*1.0*	162, 131, 164
		2500 µg	*151.7*	*9.3*	*1.0*	156, 141, 158
		5000 µg	*147.0*	*12.8*	*1.0*	144 P, 136 P, 161 P
	Deionised water		*147.7*	*9.3*		154, 137, 152
	Untreated		*145.3*	*22.5*		153, 163, 120
WP2		3 µg	*295.7*	*21.5*	*1.1*	320, 279, 288
pKM101	**Isopyrazam/**	10 µg	*291.7*	*5.9*	*1.1*	296, 294, 285
	Difenoconazole	33 µg	*264.3*	*14.8*	*1.0*	277, 268, 248
	SC (A21295D)	100 µg	*229.0*	*19.5*	*0.8*	228, 210, 249
		333 µg	*272.0*	*31.5*	*1.0*	263, 246, 307
		1000 µg	*292.0*	*14.9*	*1.1*	275, 298, 303
		2500 µg	*268.0*	*16.5*	*1.0*	251, 284, 269
		5000 µg	*262.3*	*23.3*	*1.0*	246 P, 252 P, 289 P
	Deionised water		*270.3*	*27.5*		301, 262, 248
	Untreated		*300.7*	*14.7*		312, 306, 284
WP2		3 µg	*386.3*	*31.5*	*0.9*	393, 352, 414
uvrA	**Isopyrazam/**	10 µg	*388.0*	*7.0*	*0.9*	393, 380, 391
pKM101	**Difenoconazole**	33 µg	*389.3*	*22.9*	*0.9*	363, 405, 400
	SC (A21295D)	100 µg	*377.7*	*12.1*	*0.9*	387, 364, 382
		333 µg	*402.7*	*48.8*	*1.0*	448, 409, 351
		1000 µg	*373.0*	*20.7*	*0.9*	396, 367, 356
		2500 µg	*352.0*	*5.6*	*0.8*	347, 358, 351
		5000 µg	*351.0*	*28.1*	*0.8*	380 P, 349 P, 324 P
	Deionised water		*414.3*	*30.0*		408, 447, 388
	Untreated		*396.3*	*23.7*		403, 416, 370
TA 1535	**2-AA**	2.5 µg	*322.0*	*37.3*	*27.6*	279, 342, 345
TA 1537	**2-AA**	2.5 µg	*390.7*	*24.4*	*24.4*	364, 412, 396
TA 98	**2-AA**	2.5 µg	*3336.0*	*266.0*	*85.5*	3080, 3317, 3611
TA 100	**2-AA**	2.5 µg	*4431.0*	*215.3*	*30.0*	4512, 4187, 4594
WP2 pKM101	**2-AA**	10.0 µg	*944.3*	*23.0*	*3.5*	964, 919, 950
WP2 uvrA pKM101	**2-AA**	10.0 µg	*1643.3*	*56.3*	*4.0*	1623, 1600, 1707

Key to Positive Controls

2-AA 2-aminoanthracene

Key to Plate Postfix Codes

P Precipitate

TABLE 5 Experiment II: 2137100 HV2 Pre Incubation Without Metabolic Activation

Study Name: 2137100
Experiment: 2137100 HV2 Pre
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 19.11.2020
Date Counted: 25.11.2020

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535		33 µg	*12.7*	*3.2*	*1.1*	14, 15, 9
	Isopyrazam/	100 µg	*9.7*	*0.6*	*0.8*	10, 9, 10
	Difenoconazole	333 µg	*13.7*	*2.5*	*1.1*	14, 16, 11
	SC (A21295D)	1000 µg	*11.3*	*3.2*	*0.9*	10, 9, 15
		2500 µg	*13.3*	*2.1*	*1.1*	14, 11, 15
		5000 µg	*7.7*	*1.5*	*0.6*	6 P M, 8 P M, 9 P M
	Deionised water		*12.0*	*1.7*		14, 11, 11
	Untreated		*9.0*	*3.0*		9, 6, 12
TA 1537		33 µg	*13.3*	*1.2*	*1.0*	14, 12, 14
	Isopyrazam/	100 µg	*15.3*	*0.6*	*1.1*	16, 15, 15
	Difenoconazole	333 µg	*14.7*	*3.2*	*1.1*	16, 17, 11
	SC (A21295D)	1000 µg	*18.7*	*3.2*	*1.4*	15, 20, 21
		2500 µg	*22.0*	*5.6*	*1.6*	21, 28, 17
		5000 µg	*17.3*	*1.5*	*1.3*	16 P M, 19 P M, 17 P M
	Deionised water		*13.3*	*2.1*		15, 14, 11
	Untreated		*12.7*	*2.9*		16, 11, 11
TA 98		33 µg	*26.0*	*7.9*	*1.0*	20, 35, 23
	Isopyrazam/	100 µg	*21.0*	*5.3*	*0.8*	19, 17, 27
	Difenoconazole	333 µg	*27.7*	*5.5*	*1.1*	33, 28, 22
	SC (A21295D)	1000 µg	*25.0*	*5.3*	*1.0*	31, 23, 21
		2500 µg	*26.7*	*9.8*	*1.0*	38, 21, 21
		5000 µg	*27.3*	*5.5*	*1.1*	33 P, 27 P, 22 P
	Deionised water		*26.0*	*2.6*		23, 27, 28
	Untreated		*30.3*	*8.1*		35, 21, 35
TA 100		33 µg	*154.0*	*21.0*	*1.0*	172, 159, 131
	Isopyrazam/	100 µg	*147.7*	*21.2*	*1.0*	138, 133, 172
	Difenoconazole	333 µg	*147.0*	*6.6*	*1.0*	146, 141, 154
	SC (A21295D)	1000 µg	*172.3*	*6.4*	*1.2*	165, 177, 175
		2500 µg	*151.7*	*9.0*	*1.0*	151, 161, 143
		5000 µg	*143.3*	*3.1*	*1.0*	144 P, 140 P, 146 P
	Deionised water		*147.7*	*22.9*		132, 137, 174
	Untreated		*152.0*	*17.3*		132, 162, 162

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

Study Name: 2137100
Experiment: 2137100 HV2 Pre
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 19.11.2020
Date Counted: 25.11.2020

Without metabolic activation

Strain	Compound	Concentration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
WP2		33 µg	*306.7*	*8.1*	*1.0*	301, 316, 303
pKM101	**Isopyrazam/**	100 µg	*314.7*	*21.0*	*1.1*	336, 314, 294
	Difenoconazole	333 µg	*298.3*	*39.8*	*1.0*	256, 335, 304
	SC (A21295D)	1000 µg	*300.7*	*22.2*	*1.0*	321, 304, 277
		2500 µg	*287.7*	*2.5*	*1.0*	285, 290, 288
		5000 µg	*254.7*	*27.4*	*0.9*	243 P, 235 P, 286 P
	Deionised water		*296.0*	*13.7*		311, 293, 284
	Untreated		*303.7*	*13.8*		288, 309, 314
WP2		33 µg	*406.7*	*23.6*	*1.1*	380, 415, 425
uvrA	**Isopyrazam/**	100 µg	*396.0*	*8.9*	*1.1*	389, 393, 406
pKM101	**Difenoconazole**	333 µg	*369.0*	*32.1*	*1.0*	352, 349, 406
	SC (A21295D)	1000 µg	*318.3*	*26.2*	*0.8*	346, 294, 315
		2500 µg	*312.7*	*20.2*	*0.8*	301, 301, 336
		5000 µg	*306.7*	*20.5*	*0.8*	286 P, 307 P, 327 P
	Deionised water		*377.0*	*32.1*		384, 342, 405
	Untreated		*349.7*	*23.7*		346, 375, 328
TA 1535	**NaN3**	10 µg	*1228.3*	*146.3*	*102.4*	1246, 1074, 1365
TA 1537	**4-NOPD**	50 µg	*94.7*	*18.3*	*7.1*	74, 101, 109
TA 98	**4-NOPD**	10 µg	*448.7*	*21.5*	*17.3*	424, 459, 463
TA 100	**NaN3**	10 µg	*1500.0*	*78.5*	*10.2*	1420, 1503, 1577
WP2 pKM101	**MMS**	2.0 µL	*3599.3*	*284.2*	*12.2*	3495, 3382, 3921
WP2 uvrA pKM101	**MMS**	2.0 µL	*3338.3*	*202.1*	*8.9*	3139, 3333, 3543

Key to Positive Controls	
NaN3	sodium azide
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes	
P	Precipitate
M	Manual count

TABLE 6 Experiment II: 2137100 HV2 Pre Incubation With Metabolic Activation

Study Name: 2137100
Experiment: 2137100 HV2 Pre
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 19.11.2020
Date Counted: 25.11.2020

With metabolic activation

Strain	Compound	Concentration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535		33 µg	*11.7*	*3.1*	*1.0*	11, 15, 9
	Isopyrazam/	100 µg	*10.7*	*0.6*	*0.9*	10, 11, 11
	Difenoconazole	333 µg	*14.7*	*4.0*	*1.3*	17, 10, 17
	SC (A21295D)	1000 µg	*15.3*	*4.0*	*1.3*	19, 16, 11
		2500 µg	*9.3*	*2.9*	*0.8*	11, 6, 11
		5000 µg	*7.0*	*1.7*	*0.6*	6 P M, 6 P M, 9 P M
	Deionised water		*11.7*	*3.1*		15, 9, 11
	Untreated		*13.0*	*3.6*		12, 10, 17
TA 1537		33 µg	*16.0*	*1.0*	*1.3*	17, 16, 15
	Isopyrazam/	100 µg	*15.3*	*5.1*	*1.2*	21, 14, 11
	Difenoconazole	333 µg	*16.7*	*4.9*	*1.4*	20, 11, 19
	SC (A21295D)	1000 µg	*15.7*	*1.2*	*1.3*	15, 17, 15
		2500 µg	*12.7*	*4.0*	*1.0*	9, 17, 12
		5000 µg	*10.7*	*3.2*	*0.9*	7 P M, 12 P M, 13 P M
	Deionised water		*12.3*	*2.5*		12, 15, 10
	Untreated		*11.0*	*1.7*		12, 12, 9
TA 98		33 µg	*41.0*	*11.4*	*1.1*	33, 36, 54
	Isopyrazam/	100 µg	*41.0*	*10.8*	*1.1*	38, 32, 53
	Difenoconazole	333 µg	*35.3*	*2.9*	*0.9*	32, 37, 37
	SC (A21295D)	1000 µg	*38.0*	*4.4*	*1.0*	41, 40, 33
		2500 µg	*39.3*	*4.0*	*1.0*	35, 40, 43
		5000 µg	*30.7*	*4.5*	*0.8*	35 P, 26 P, 31 P
	Deionised water		*39.0*	*8.7*		49, 35, 33
	Untreated		*43.3*	*0.6*		44, 43, 43
TA 100		33 µg	*143.0*	*3.5*	*1.0*	141, 147, 141
	Isopyrazam/	100 µg	*161.3*	*9.1*	*1.1*	168, 151, 165
	Difenoconazole	333 µg	*163.3*	*5.1*	*1.1*	159, 169, 162
	SC (A21295D)	1000 µg	*167.0*	*9.2*	*1.2*	159, 165, 177
		2500 µg	*150.7*	*15.4*	*1.1*	158, 161, 133
		5000 µg	*152.3*	*15.5*	*1.1*	165 P, 157 P, 135 P
	Deionised water		*142.3*	*22.3*		151, 117, 159
	Untreated		*139.0*	*14.7*		131, 130, 156

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

Study Name: 2137100
Experiment: 2137100 HV2 Pre
Assay Conditions:

Study Code: ICCR 2137100
Date Plated: 19.11.2020
Date Counted: 25.11.2020

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
WP2		33 µg	*305.3*	*14.6*	*0.9*	322, 295, 299
pKM101	**Isopyrazam/**	100 µg	*280.7*	*39.5*	*0.8*	288, 316, 238
	Difenoconazole	333 µg	*276.0*	*14.9*	*0.8*	265, 293, 270
	SC (A21295D)	1000 µg	*314.7*	*21.4*	*0.9*	326, 290, 328
		2500 µg	*303.7*	*21.4*	*0.9*	279, 315, 317
		5000 µg	*272.3*	*60.0*	*0.8*	203 P, 307 P, 307 P
	Deionised water		*350.0*	*12.3*		345, 341, 364
	Untreated		*361.0*	*25.2*		354, 389, 340
WP2		33 µg	*414.7*	*38.4*	*1.2*	456, 380, 408
uvrA	**Isopyrazam/**	100 µg	*375.7*	*26.6*	*1.1*	389, 345, 393
pKM101	**Difenoconazole**	333 µg	*379.0*	*41.3*	*1.1*	367, 425, 345
	SC (A21295D)	1000 µg	*401.0*	*16.7*	*1.1*	404, 416, 383
		2500 µg	*385.3*	*20.5*	*1.1*	409, 374, 373
		5000 µg	*381.7*	*26.8*	*1.1*	361 P, 372 P, 412 P
	Deionised water		*350.0*	*17.4*		362, 330, 358
	Untreated		*373.3*	*14.0*		374, 387, 359
TA 1535	**2-AA**	2.5 µg	*193.7*	*45.7*	*16.6*	198, 146, 237
TA 1537	**2-AA**	2.5 µg	*263.3*	*14.0*	*21.4*	249, 264, 277
TA 98	**2-AA**	2.5 µg	*1881.0*	*343.1*	*48.2*	1555, 1849, 2239
TA 100	**2-AA**	2.5 µg	*2812.3*	*173.3*	*19.8*	2645, 2801, 2991
WP2 pKM101	**2-AA**	10.0 µg	*1081.0*	*138.8*	*3.1*	1240, 1019, 984
WP2 uvrA pKM101	**2-AA**	10.0 µg	*1498.0*	*182.1*	*4.3*	1436, 1355, 1703

Key to Positive Controls

2-AA	2-aminoanthracene

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

APPENDICES SECTION

APPENDIX 1 Historical Control Data

These data represent the laboratory´s historical control data from July 2018 until July 2020 representing approx. 600 experiments (WP2 pKM101, WP2 uvrA pKM101 the historical data are based on approx. 80 experiments).

The positive controls that used to compile the historical positive control data correspond to the positive control substances described in Methods; section 3.2.2 (Positive control substances).

Strain		without S9 mix				with S9 mix			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA 1535	Solvent control	12	2.6	7	22	13	2.5	7	24
	Untreated control	12	2.9	6	26	13	2.8	7	23
	Positive control	1116	141.3	340	1612	346	72.1	170	736
TA1537	Solvent control	11	2.4	6	20	14	2.8	7	28
	Untreated control	11	2.8	5	22	14	3.2	7	30
	Positive control	83	22.1	48	400	286	98.7	82	630
TA 98	Solvent control	28	4.9	13	46	38	6.4	12	62
	Untreated control	29	5.0	14	48	41	6.8	14	64
	Positive control	421	91.2	216	1218	3275	774.9	322	5699
TA 100	Solvent control	127	30.7	63	214	131	30.0	72	214
	Untreated control	135	35.7	64	233	140	34.4	68	217
	Positive control	1759	273.4	511	2588	3566	837.6	553	5444
WP2 pKM 101	Solvent control	248	31.7	171	299	266	33.0	205	315
	Untreated control	269	26.6	212	346	299	28.2	233	345
	Positive control	3343	428.4	2332	4653	1092	257.8	933	2781
WP2uvrA pKM 101	Solvent control	322	31.6	248	388	375	38.5	287	466
	Untreated control	346	28.2	279	403	393	32.6	313	480
	Positive control	3176	468.5	2021	4717	1897	183.2	1270	2464

Mean = mean value of revertants/plate
SD = standard deviation
Min = minimal value
Max = maximal value

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 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. 030920K

Date of preparation: September 03, 2020
Release date: September 14, 2020

Protein assay: 33 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	28
control	100	33
10 µg/plate	0	69
2-Aminoanthracene	100	2608
10 µg/plate	0	30
Benzo(a)pyrene	100	99

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

Quality Assurance Auditor
ICCR-Roßdorf GmbH

30. SEP. 2020
Date

Dr. Steffen Naumann
Study Director
ICCR-Roßdorf GmbH

30. SEP. 2020
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_21

ICCR ROßDORF

CERTIFICATE

ICCR-Roßdorf S9 Preparation Lot No. 030920D

Date of preparation: September 03, 2020
Release date: November 11, 2020

Protein assay: 34.8 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	27
control	100	34
10 µg/plate	0	87
2-Aminoanthracene	50	1732
10 µg/plate	0	29
Benzo(a)pyrene	100	97

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 — 17. NOV. 2020

Quality Assurance Auditor
ICCR-Roßdorf GmbH

Date

18. NOV. 2020

Dr. Steffen Naumann
Study Director
ICCR-Roßdorf GmbH

Date

ICCR-Roßdorf GmbH
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Geschäftsführer: Dr. Markus Schulz

SOP Origin TS-SOP S9_23

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/m³**

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