SYN549522

SYN549522 FS (A22417C) - *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay

Final Report

DATA REQUIREMENT(S): OECD 471 (1997)

AUTHOR(S): Dr. Steffi Chang

COMPLETION DATE: 05 April 2019

PERFORMING LABORATORY: Envigo CRS GmbH
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LABORATORY PROJECT ID: Report Number: 1936100
Study Number: 1936100
Task Number: TK0317072

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

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study performed in the test facility of Envigo CRS 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 [C(97)186/Final]

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. Steffi Chang
Study Director Bacterial Systems

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Date: 05 April 2019

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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Envigo Study Number: 1936100
Test substance: SYN549522 FS (A22417C)
Study director: Dr. Steffi Chang
Study Title: SYN549522 FS (A22417C) - *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay

Study based activities at the Test Facility Envigo CRS 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	20 December 2018	20 December 2018
Study Plan Amendmend 1 Verification	28 March 2019	28 March 2019
Process – based Assessment of Response	07 February 2019	07 February 2019
Report Audit	11 March 2019	11 March 2019

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

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

S. Ebert

Sabine Ebert
Quality Assurance Auditor
Envigo CRS GmbH

05 April 2019
Date

PROJECT STAFF SIGNATURE

Study Director — Dr. Steffi Chang

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Date: 05 April 2019

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
Dr. Steffi Chang	Study Director
Dr. Markus Schulz	Test Facility Management
Frauke Hermann	Head of Quality Assurance Unit
Sophie van der Kamp	Syngenta Study Manager

Study Dates

Study initiation date:	20 December 2018
Experimental start date:	16 January 2019
Experimental completion date:	20 February 2019

Deviations from the Guidelines

None

Retention of Samples

None

Performing Laboratory Test Substance Reference Number

S 2006211

Other

Envigo CRS will archive:
Records and documentation relating to this study will be maintained in the archives of Envigo CRS 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 Envigo CRS (Switzerland) Ltd. at Füllinsdorf, Switzerland, for further archiving up to a total archiving period of 15 years.

A sample of the test item will not be archived.

Envigo 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)

TABLE OF CONTENTS

1.0 EXECUTIVE SUMMARY

1.1 Study Design

This study was performed to investigate the potential of SYN549522 FS (A22417C) 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 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 any strain with and without metabolic activation.

No relevant increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN549522 FS (A22417C) at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no observed tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls, which showed a distinct increase of induced revertant colonies.

1.3 Conclusion

In conclusion, it can be stated that during the described mutagenicity tests and under the experimental conditions reported, SYN549522 FS (A22417C) did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, SYN549522 FS (A22417C) 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 Regulatory Guidelines

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", adopted July 21, 1997

3.0 MATERIALS AND METHODS

3.1 Test Substance

Information as provided by the Sponsor.

Identification:	(A22417C)
Other Product Code:	SYN549522 FS (500)
Batch:	SMU8IP001
Content of SYN549522: Content of SYN547386: Content of SYN548941:	41.4% w/w corresponding to 498 g/L 37.2 w/w corresponding to 448 g/L 4.16% w/w corresponding to 50.0 g/L
Appearance:	Red, liquid
Recertification Date:	31 October 2021
Storage Conditions:	At room temperature
Stability in Solvent:	Not indicated by the Sponsor

The test substance concentrations were not adjusted for the content of active ingredient.

In a preliminary solubility trial several solvents (deionised water, dimethyl sulphoxide (DMSO), ethanol, dimethyl formamide, and acetone) were used to find a suitable one to prepare an applicable test item preparation. Deionised water was found to be the most suitable one. On the day of the experiment, the test substance SYN549522 FS (A22417C) was suspended in deionised water. The solvent was chosen because of 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.:	MKBX 5165V (Experiment I) and MKCD 8572V (Experiment II)
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.:	STBD 3302V (Exp. I; 2.5 µg/plate) and STBG 0630V (Exp. I; 10 µg/plate, and Experiment II; 2.5 and 10 µg/plate)
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 Envigo CRS 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 Envigo CRS)

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 32.7 mg/mL (lot no. 080318K) in the pre-experiment / Experiment I and 34.4 mg/mL (lot no. 260718E) 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.

For the plate incorporation method 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 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 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 2-fold concurrent control
- a minimum of five analysable concentrations should be present with at least four 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 (Perceptive Instruments Ltd., Suffolk CB9 7BN, UK) with the software program Ames Study Manager (v1.24) and Ames Archive Manager (v1.01).

4.0 RESULTS AND DISCUSSION

The test substance, SYN549522 FS (A22417C), 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 1000 to 5000 µg/plate. Precipitation of the test item in the overlay agar on the incubated agar plates was observed from 2500 to 5000 µg/plate. The undissolved particles had no influence on the data recording.

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 substantial increase in revertant colony numbers in any of the six tester strains was observed following treatment with SYN549522 FS (A22417C) at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies demonstrating the correct performance of the assay.

In Experiment I, the data in solvent control of strain WP2 (pKM101) without S9 mix were slightly above the laboratory historical control range. Since this deviation was rather small (296 versus 289 colonies), this effect is considered to be based upon biologically irrelevant fluctuations in the number of colonies and the data are fully acceptable.

5.0 CONCLUSIONS

In conclusion, it can be stated that during the described mutagenicity tests and under the experimental conditions reported, SYN549522 FS (A22417C) did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, SYN549522 FS (A22417C) is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

6.0 REFERENCES

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   Mutation Res. 455, 61-69

TABLES SECTION

TABLE 1 Summary of Results Pre-Experiment/Experiment I

Study Name: 1936100
Experiment: 1936100 VV Plate
Assay Conditions:

Study Code: Envigo 1936100
Date Plated: 16.01.2019
Date Counted: 23.01.2019

Metabolic Activation	Test Group	Concentration 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 ± 3	13 ± 1	29 ± 5	125 ± 18	296 ± 18	388 ± 5
	Untreated		8 ± 1	13 ± 3	32 ± 4	114 ± 5	289 ± 17	397 ± 15
	SYN549522	3 µg	17 ± 2	12 ± 2	36 ± 4	120 ± 26	326 ± 10	440 ± 33
	FS	10 µg	12 ± 3	13 ± 2	40 ± 6	120 ± 9	339 ± 15	441 ± 5
	(A22417C)	33 µg	12 ± 2	13 ± 4	33 ± 5	132 ± 21	331 ± 17	423 ± 18
		100 µg	11 ± 4	14 ± 5	30 ± 3	120 ± 11	342 ± 17	416 ± 32
		333 µg	14 ± 3	9 ± 1	37 ± 6	126 ± 10	321 ± 11	408 ± 43
		1000 µg	10 ± 1	11 ± 1	35 ± 8	119 ± 8	323 ± 7	395 ± 19
		2500 µg	10 ± 2 [P]	11 ± 1 [P]	28 ± 6 [P]	121 ± 14 [P]	301 ± 19 [P]	371 ± 13 [P]
		5000 µg	10 ± 0 [P]	8 ± 2 [P]	26 ± 3 [P]	97 ± 8 [P]	225 ± 28 [P]	284 ± 19 [P]
	NaN3	10 µg	1040 ± 69			1736 ± 68		
	4-NOPD	10 µg			482 ± 5			
	4-NOPD	50 µg		86 ± 10				
	MMS	2.0 µL					3589 ± 101	3988 ± 27
With Activation	Deionised water		17 ± 3	14 ± 3	51 ± 12	150 ± 2	305 ± 17	466 ± 4
	Untreated		17 ± 4	17 ± 2	48 ± 2	151 ± 11	335 ± 15	455 ± 15
	SYN549522	3 µg	12 ± 1	14 ± 2	51 ± 2	139 ± 12	326 ± 4	460 ± 21
	FS	10 µg	17 ± 3	15 ± 3	45 ± 6	163 ± 1	331 ± 11	465 ± 5
	(A22417C)	33 µg	17 ± 5	13 ± 3	53 ± 6	147 ± 32	331 ± 18	451 ± 16
		100 µg	15 ± 5	14 ± 1	44 ± 12	146 ± 17	354 ± 21	444 ± 20
		333 µg	14 ± 4	14 ± 3	41 ± 1	140 ± 9	326 ± 29	443 ± 25
		1000 µg	16 ± 4	14 ± 2	50 ± 5	151 ± 10	323 ± 17	436 ± 10
		2500 µg	15 ± 1 [P]	14 ± 4 [P]	41 ± 6 [P]	168 ± 11 [P]	331 ± 11 [P]	433 ± 17 [P]
		5000 µg	9 ± 1 [P]	9 ± 1 [P]	42 ± 10 [P]	159 ± 14 [P]	325 ± 14 [P]	377 ± 11 [P]
	2-AA	2.5 µg	260 ± 13	367 ± 20	3226 ± 323	4222 ± 118		
	2-AA	10.0 µg					965 ± 61	1730 ± 288

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

TABLE 2 Summary of Results Experiment II

Study Name: 1936100
Experiment: 1936100 HV2 Pre
Assay Conditions:

Study Code: Envigo 1936100
Date Plated: 13.02.2019
Date Counted: 20.02.2019

Metabolic Activation	Test Group	Concentration 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		9 ± 3	14 ± 4	28 ± 10	137 ± 22	276 ± 30	320 ± 40
	Untreated		12 ± 3	11 ± 4	27 ± 10	140 ± 4	269 ± 19	322 ± 16
	SYN549522 FS (A22417C)	33 µg	11 ± 4	13 ± 5	25 ± 7	138 ± 17	273 ± 9	334 ± 35
		100 µg	13 ± 5	14 ± 3	29 ± 5	132 ± 7	242 ± 47	303 ± 21
		333 µg	10 ± 2	10 ± 1	23 ± 1	138 ± 16	225 ± 7	303 ± 6
		1000 µg	13 ± 5	10 ± 1	31 ± 10	149 ± 6	258 ± 12	314 ± 29
		2500 µg	11 ± 3 P	11 ± 3 P	27 ± 7 P	150 ± 14 P	259 ± 12 P	277 ± 24 P
		5000 µg	10 ± 3 P M	9 ± 4 P	25 ± 2 P	147 ± 14 P	214 ± 20 P	254 ± 6 P
	NaN3	10 µg	989 ± 13			1534 ± 128		
	4-NOPD	10 µg			516 ± 39			
	4-NOPD	50 µg		85 ± 8				
	MMS	2.0 µL					3651 ± 132	2977 ± 150
With Activation	Deionised water		14 ± 1	14 ± 2	35 ± 11	155 ± 13	281 ± 1	353 ± 8
	Untreated		9 ± 3	12 ± 4	40 ± 5	160 ± 12	288 ± 27	356 ± 38
	SYN549522 FS (A22417C)	33 µg	13 ± 2	14 ± 3	34 ± 7	156 ± 4	300 ± 13	352 ± 13
		100 µg	15 ± 5	16 ± 5	38 ± 2	167 ± 17	286 ± 11	354 ± 11
		333 µg	13 ± 3	18 ± 3	36 ± 5	161 ± 4	294 ± 11	330 ± 8
		1000 µg	12 ± 3	17 ± 2	37 ± 10	168 ± 4	274 ± 14	350 ± 14
		2500 µg	13 ± 2 P	14 ± 2 P	38 ± 7 P	174 ± 12 P	267 ± 15 P	331 ± 14 P
		5000 µg	10 ± 1 P	15 ± 1 P	32 ± 8 P	177 ± 14 P	264 ± 50 P	297 ± 11 P
	2-AA	2.5 µg	351 ± 40	236 ± 11	3428 ± 311	3575 ± 117		
	2-AA	10.0 µg					962 ± 30	1966 ± 49

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: 1936100 VV Plate Incorporation Without Metabolic Activation

Study Name: 1936100
Experiment: 1936100 VV Plate
Assay Conditions:

Study Code: Envigo 1936100
Date Plated: 16.01.2019
Date Counted: 23.01.2019

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**SYN549522 FS**	3 µg	*16.7*	*2.1*	*1.3*	15, 19, 16
	(A22417C)	10 µg	*12.0*	*2.6*	*0.9*	11, 10, 15
		33 µg	*12.3*	*1.5*	*0.9*	12, 11, 14
		100 µg	*10.7*	*3.5*	*0.8*	11, 7, 14
		333 µg	*14.0*	*3.0*	*1.0*	11, 14, 17
		1000 µg	*10.3*	*0.6*	*0.8*	11, 10, 10
		2500 µg	*10.3*	*1.5*	*0.8*	9 P, 12 P, 10 P
		5000 µg	*10.0*	*0.0*	*0.7*	10 P, 10 P, 10 P
	Deionised water		*13.3*	*2.9*		10, 15, 15
	Untreated		*7.7*	*1.2*		7, 7, 9
TA 1537	**SYN549522 FS**	3 µg	*12.3*	*1.5*	*1.0*	14, 11, 12
	(A22417C)	10 µg	*12.7*	*2.3*	*1.0*	14, 10, 14
		33 µg	*13.3*	*4.0*	*1.1*	14, 17, 9
		100 µg	*13.7*	*4.7*	*1.1*	19, 12, 10
		333 µg	*9.3*	*0.6*	*0.7*	10, 9, 9
		1000 µg	*11.3*	*1.2*	*0.9*	12, 10, 12
		2500 µg	*11.3*	*1.2*	*0.9*	12 P, 12 P, 10 P
		5000 µg	*7.7*	*2.1*	*0.6*	10 P, 7 P, 6 P
	Deionised water		*12.7*	*1.2*		14, 12, 12
	Untreated		*12.7*	*3.1*		12, 16, 10
TA 98	**SYN549522 FS**	3 µg	*36.0*	*3.6*	*1.2*	33, 40, 35
	(A22417C)	10 µg	*40.0*	*6.2*	*1.4*	47, 35, 38
		33 µg	*33.3*	*4.7*	*1.1*	35, 37, 28
		100 µg	*30.3*	*3.1*	*1.0*	27, 31, 33
		333 µg	*37.3*	*5.5*	*1.3*	41, 40, 31
		1000 µg	*34.7*	*7.8*	*1.2*	26, 41, 37
		2500 µg	*28.3*	*6.1*	*1.0*	23 P, 35 P, 27 P
		5000 µg	*26.3*	*2.9*	*0.9*	28 P, 28 P, 23 P
	Deionised water		*29.3*	*5.1*		35, 25, 28
	Untreated		*31.7*	*4.0*		31, 36, 28

Key to Plate Postfix Codes

P Precipitate

TABLE 3 Pre-Experiment and Experiment I: 1936100 VV Plate Incorporation Without Metabolic Activation (Continued)

Study Name: 1936100 Study Code: Envigo 1936100
Experiment: 1936100 VV Plate Date Plated: 16.01.2019
Assay Conditions: Date Counted: 23.01.2019

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 100	**SYN549522 FS**	3 µg	*120.3*	*25.8*	*1.0*	103, 108, 150
	(A22417C)	10 µg	*120.3*	*9.2*	*1.0*	115, 115, 131
		33 µg	*131.7*	*21.2*	*1.1*	109, 135, 151
		100 µg	*120.0*	*10.5*	*1.0*	121, 130, 109
		333 µg	*126.0*	*10.4*	*1.0*	132, 132, 114
		1000 µg	*119.0*	*7.5*	*1.0*	120, 111, 126
		2500 µg	*121.3*	*14.2*	*1.0*	129 P, 130 P, 105 P
		5000 µg	*97.0*	*7.5*	*0.8*	89 P, 98 P, 104 P
	Deionised water		*125.0*	*18.2*		134, 137, 104
	Untreated		*114.3*	*5.0*		115, 119, 109
WP2	**SYN549522 FS**	3 µg	*326.0*	*9.8*	*1.1*	329, 334, 315
pKM101	**(A22417C)**	10 µg	*338.7*	*15.2*	*1.1*	325, 355, 336
		33 µg	*331.0*	*16.7*	*1.1*	313, 334, 346
		100 µg	*341.7*	*17.0*	*1.2*	341, 359, 325
		333 µg	*320.7*	*11.0*	*1.1*	328, 308, 326
		1000 µg	*322.7*	*6.8*	*1.1*	315, 325, 328
		2500 µg	*301.3*	*18.8*	*1.0*	318 P, 305 P, 281 P
		5000 µg	*225.0*	*27.5*	*0.8*	198 P, 253 P, 224 P
	Deionised water		*296.3*	*17.8*		312, 300, 277
	Untreated		*289.3*	*17.2*		309, 277, 282
WP2	**SYN549522 FS**	3 µg	*440.3*	*33.2*	*1.1*	402, 460, 459
uvrA	**(A22417C)**	10 µg	*440.7*	*4.5*	*1.1*	445, 436, 441
pKM101		33 µg	*422.7*	*17.9*	*1.1*	427, 438, 403
		100 µg	*415.7*	*32.0*	*1.1*	448, 415, 384
		333 µg	*408.0*	*42.8*	*1.1*	389, 457, 378
		1000 µg	*394.7*	*18.9*	*1.0*	373, 408, 403
		2500 µg	*371.3*	*13.3*	*1.0*	380 P, 378 P, 356 P
		5000 µg	*284.0*	*19.0*	*0.7*	284 P, 265 P, 303 P
	Deionised water		*388.0*	*5.0*		393, 383, 388
	Untreated		*397.0*	*15.4*		414, 393, 384
TA 1535	**NaN3**	10 µg	*1040.0*	*69.4*	*78.0*	1118, 1017, 985
TA 1537	**4-NOPD**	50 µg	*86.0*	*9.8*	*6.8*	75, 94, 89
TA 98	**4-NOPD**	10 µg	*482.0*	*5.2*	*16.4*	476, 485, 485
TA 100	**NaN3**	10 µg	*1736.0*	*68.0*	*13.9*	1791, 1757, 1660
WP2 pKM101	**MMS**	2.0 µL	*3589.0*	*100.7*	*12.1*	3552, 3512, 3703
WP2 uvrA pKM101	**MMS**	2.0 µL	*3987.7*	*26.5*	*10.3*	3988, 4014, 3961

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

TABLE 4 Pre-Experiment and Experiment I: 1936100 VV Plate Incorporation With Metabolic Activation

Study Name: 1936100 Study Code: Envigo 1936100
Experiment: 1936100 VV Plate Date Plated: 16.01.2019
Assay Conditions: Date Counted: 23.01.2019

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**SYN549522 FS**	3 µg	*11.7*	*0.6*	*0.7*	11, 12, 12
	(A22417C)	10 µg	*16.7*	*2.9*	*1.0*	15, 15, 20
		33 µg	*16.7*	*4.9*	*1.0*	11, 19, 20
		100 µg	*15.3*	*5.0*	*0.9*	16, 10, 20
		333 µg	*14.0*	*4.4*	*0.8*	19, 12, 11
		1000 µg	*15.7*	*3.5*	*0.9*	12, 19, 16
		2500 µg	*14.7*	*1.2*	*0.9*	14 P, 14 P, 16 P
		5000 µg	*9.3*	*0.6*	*0.6*	9 P, 10 P, 9 P
	Deionised water		*16.7*	*2.5*		14, 17, 19
	Untreated		*17.0*	*4.4*		15, 14, 22
TA 1537	**SYN549522 FS**	3 µg	*14.0*	*2.0*	*1.0*	12, 16, 14
	(A22417C)	10 µg	*15.3*	*2.9*	*1.1*	17, 17, 12
		33 µg	*13.3*	*3.2*	*1.0*	17, 11, 12
		100 µg	*14.3*	*0.6*	*1.0*	14, 15, 14
		333 µg	*14.3*	*2.5*	*1.0*	14, 17, 12
		1000 µg	*13.7*	*1.5*	*1.0*	15, 12, 14
		2500 µg	*14.3*	*4.0*	*1.0*	12 P, 19 P, 12 P
		5000 µg	*9.3*	*0.6*	*0.7*	10 P, 9 P, 9 P
	Deionised water		*14.0*	*3.5*		10, 16, 16
	Untreated		*17.0*	*2.0*		15, 19, 17
TA 98	**SYN549522 FS**	3 µg	*50.7*	*1.5*	*1.0*	52, 49, 51
	(A22417C)	10 µg	*44.7*	*5.5*	*0.9*	41, 51, 42
		33 µg	*53.3*	*6.0*	*1.0*	59, 47, 54
		100 µg	*44.0*	*11.5*	*0.9*	57, 40, 35
		333 µg	*41.3*	*0.6*	*0.8*	41, 42, 41
		1000 µg	*50.3*	*4.9*	*1.0*	56, 48, 47
		2500 µg	*41.3*	*5.7*	*0.8*	35 P, 46 P, 43 P
		5000 µg	*41.7*	*10.1*	*0.8*	48 P, 47 P, 30 P
	Deionised water		*51.0*	*12.1*		49, 64, 40
	Untreated		*48.3*	*2.3*		51, 47, 47

Key to Plate Postfix Codes

P Precipitate

TABLE 4 Pre-Experiment and Experiment I: 1936100 VV Plate Incorporation With Metabolic Activation (Continued)

Study Name: 1936100
Experiment: 1936100 VV Plate
Assay Conditions:

Study Code: Envigo 1936100
Date Plated: 16.01.2019
Date Counted: 23.01.2019

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 100	**SYN549522 FS**	3 µg	*138.7*	*12.1*	*0.9*	126, 140, 150
	(A22417C)	10 µg	*163.0*	*1.0*	*1.1*	164, 162, 163
		33 µg	*146.7*	*31.6*	*1.0*	158, 171, 111
		100 µg	*146.3*	*17.1*	*1.0*	166, 135, 138
		333 µg	*140.3*	*8.6*	*0.9*	131, 142, 148
		1000 µg	*151.0*	*10.0*	*1.0*	141, 151, 161
		2500 µg	*168.0*	*10.6*	*1.1*	176 P, 156 P, 172 P
		5000 µg	*158.7*	*13.6*	*1.1*	143 P, 167 P, 166 P
	Deionised water		*150.3*	*2.1*		148, 151, 152
	Untreated		*151.0*	*10.8*		148, 163, 142
WP2 pKM101	**SYN549522 FS (A22417C)**	3 µg	*326.0*	*4.4*	*1.1*	323, 331, 324
		10 µg	*331.3*	*10.5*	*1.1*	331, 321, 342
		33 µg	*330.7*	*18.4*	*1.1*	315, 326, 351
		100 µg	*353.7*	*20.5*	*1.2*	371, 359, 331
		333 µg	*326.0*	*28.7*	*1.1*	307, 359, 312
		1000 µg	*322.7*	*17.2*	*1.1*	338, 326, 304
		2500 µg	*331.0*	*11.0*	*1.1*	320 P, 342 P, 331 P
		5000 µg	*324.7*	*13.9*	*1.1*	340 P, 321 P, 313 P
	Deionised water		*305.3*	*16.8*		316, 286, 314
	Untreated Control		*334.7*	*14.5*		335, 349, 320
WP2 uvrA pKM101	**SYN549522 FS (A22417C)**	3 µg	*459.7*	*21.0*	*1.0*	480, 461, 438
		10 µg	*465.0*	*5.3*	*1.0*	459, 467, 469
		33 µg	*451.3*	*16.0*	*1.0*	435, 467, 452
		100 µg	*444.0*	*20.3*	*1.0*	448, 462, 422
		333 µg	*442.7*	*24.6*	*0.9*	471, 427, 430
		1000 µg	*435.7*	*10.2*	*0.9*	443, 440, 424
		2500 µg	*432.7*	*16.8*	*0.9*	429 P, 451 P, 418 P
		5000 µg	*377.3*	*10.7*	*0.8*	368 P, 389 P, 375 P
	Deionised water		*466.3*	*4.0*		462, 470, 467
	Untreated Control		*454.7*	*15.0*		446, 446, 472
TA 1535	**2-AA**	2.5 µg	*260.0*	*13.2*	*15.6*	270, 245, 265
TA 1537	**2-AA**	2.5 µg	*366.7*	*19.6*	*26.2*	345, 383, 372
TA 98	**2-AA**	2.5 µg	*3226.0*	*323.0*	*63.3*	3040, 3039, 3599
TA 100	**2-AA**	2.5 µg	*4221.7*	*118.5*	*28.1*	4085, 4295, 4285
WP2 pKM101	**2-AA**	10.0 µg	*964.7*	*61.0*	*3.2*	926, 1035, 933
WP2 uvrA pKM101	**2-AA**	10.0 µg	*1729.7*	*288.5*	*3.7*	1411, 1973, 1805

Key to Positive Controls

2-AA 2-aminoanthracene

Key to Plate Postfix Codes

P Precipitate

TABLE 5 Experiment II: 1936100 HV2 Pre Incubation Without Metabolic Activation

Study Name: 1936100
Experiment: 1936100 HV2 Pre
Assay Conditions:

Study Code: Envigo 1936100
Date Plated: 13.02.2019
Date Counted: 20.02.2019

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**SYN549522 FS**	33 µg	*11.3*	*4.0*	*1.2*	16, 9, 9
	(A22417C)	100 µg	*13.0*	*5.2*	*1.4*	19, 10, 10
		333 µg	*10.0*	*1.7*	*1.1*	9, 9, 12
		1000 µg	*13.0*	*5.2*	*1.4*	19, 10, 10
		2500 µg	*10.7*	*2.9*	*1.1*	14 P, 9 P, 9 P
		5000 µg	*10.3*	*3.2*	*1.1*	14 P M, 9 P M, 8 P M
	Deionised water		*9.3*	*2.5*		9, 12, 7
	Untreated		*11.7*	*2.5*		9, 14, 12
TA 1537	**SYN549522 FS**	33 µg	*13.0*	*5.3*	*0.9*	19, 9, 11
	(A22417C)	100 µg	*14.3*	*3.1*	*1.0*	15, 17, 11
		333 µg	*10.0*	*1.0*	*0.7*	9, 11, 10
		1000 µg	*10.0*	*1.0*	*0.7*	9, 11, 10
		2500 µg	*11.3*	*2.5*	*0.8*	14 P, 9 P, 11 P
		5000 µg	*9.3*	*4.0*	*0.7*	14 P, 7 P, 7 P
	Deionised water		*14.3*	*4.0*		19, 12, 12
	Untreated		*10.7*	*3.5*		11, 7, 14
TA 98	**SYN549522 FS**	33 µg	*25.0*	*7.2*	*0.9*	23, 33, 19
	(A22417C)	100 µg	*29.0*	*5.3*	*1.0*	31, 23, 33
		333 µg	*22.7*	*0.6*	*0.8*	23, 22, 23
		1000 µg	*31.3*	*9.9*	*1.1*	38, 36, 20
		2500 µg	*26.7*	*7.1*	*0.9*	33 P, 19 P, 28 P
		5000 µg	*25.0*	*2.0*	*0.9*	23 P, 25 P, 27 P
	Deionised water		*28.3*	*10.4*		25, 20, 40
	Untreated		*26.7*	*9.9*		38, 20, 22
TA 100	**SYN549522 FS**	33 µg	*138.0*	*17.0*	*1.0*	155, 121, 138
	(A22417C)	100 µg	*132.3*	*7.1*	*1.0*	131, 126, 140
		333 µg	*138.0*	*15.6*	*1.0*	120, 146, 148
		1000 µg	*148.7*	*5.9*	*1.1*	142, 151, 153
		2500 µg	*150.0*	*14.2*	*1.1*	155 P, 134 P, 161 P
		5000 µg	*147.0*	*13.5*	*1.1*	146 P, 134 P, 161 P
	Deionised water		*136.7*	*22.1*		162, 121, 127
	Untreated		*139.7*	*4.0*		142, 135, 142

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

TABLE 5 Experiment II: 1936100 HV2 Pre Incubation Without Metabolic Activation (Continued)

Study Name: 1936100 Study Code: Envigo 1936100
Experiment: 1936100 HV2 Pre Date Plated: 13.02.2019
Assay Conditions: Date Counted: 20.02.2019

Without metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
WP2 pKM101	**SYN549522 FS (A22417C)**	33 µg	*273.3*	*9.1*	*1.0*	272, 283, 265
		100 µg	*242.0*	*46.5*	*0.9*	284, 192, 250
		333 µg	*225.0*	*6.6*	*0.8*	226, 218, 231
		1000 µg	*258.0*	*11.5*	*0.9*	267, 245, 262
		2500 µg	*259.3*	*12.1*	*0.9*	272 P, 248 P, 258 P
		5000 µg	*214.3*	*20.4*	*0.8*	219 P, 232 P, 192 P
	Deionised water		*276.0*	*29.5*		252, 309, 267
	Untreated		*269.0*	*19.3*		255, 261, 291
WP2 uvrA pKM101	**SYN549522 FS (A22417C)**	33 µg	*333.7*	*34.7*	*1.0*	338, 366, 297
		100 µg	*303.0*	*20.5*	*0.9*	323, 304, 282
		333 µg	*303.3*	*6.1*	*0.9*	298, 310, 302
		1000 µg	*314.0*	*29.1*	*1.0*	342, 284, 316
		2500 µg	*277.3*	*24.1*	*0.9*	304 P, 271 P, 257 P
		5000 µg	*254.3*	*5.9*	*0.8*	261 P, 252 P, 250 P
	Deionised water		*320.0*	*40.4*		308, 287, 365
	Untreated		*322.0*	*15.9*		304, 334, 328
TA 1535	**NaN3**	10 µg	*989.3*	*13.4*	*106.0*	999, 974, 995
TA 1537	**4-NOPD**	50 µg	*85.3*	*8.1*	*6.0*	84, 78, 94
TA 98	**4-NOPD**	10 µg	*516.0*	*39.1*	*18.2*	542, 535, 471
TA 100	**NaN3**	10 µg	*1534.0*	*128.3*	*11.2*	1675, 1424, 1503
WP2 pKM101	**MMS**	2.0 µL	*3651.3*	*132.3*	*13.2*	3514, 3778, 3662
WP2 uvrA pKM101	**MMS**	2.0 µL	*2977.0*	*149.6*	*9.3*	3026, 2809, 3096

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: 1936100 HV2 Pre Incubation With Metabolic Activation

Study Name: 1936100
Experiment: 1936100 HV2 Pre
Assay Conditions:

Study Code: Envigo 1936100
Date Plated: 13.02.2019
Date Counted: 20.02.2019

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**SYN549522 FS**	33 µg	*12.7*	*2.1*	*0.9*	11, 15, 12
	(A22417C)	100 µg	*15.0*	*5.2*	*1.0*	21, 12, 12
		333 µg	*13.0*	*2.6*	*0.9*	16, 12, 11
		1000 µg	*12.3*	*2.5*	*0.9*	12, 15, 10
		2500 µg	*13.3*	*2.3*	*0.9*	16 P, 12 P, 12 P
		5000 µg	*10.3*	*0.6*	*0.7*	10 P, 11 P, 10 P
	Deionised water		*14.3*	*0.6*		14, 15, 14
	Untreated		*9.0*	*2.6*		11, 10, 6
TA 1537	**SYN549522 FS**	33 µg	*14.0*	*3.5*	*1.0*	16, 10, 16
	(A22417C)	100 µg	*15.7*	*5.0*	*1.1*	15, 11, 21
		333 µg	*18.3*	*3.2*	*1.3*	16, 22, 17
		1000 µg	*16.7*	*2.1*	*1.2*	15, 19, 16
		2500 µg	*13.7*	*1.5*	*1.0*	15 P, 14 P, 12 P
		5000 µg	*14.7*	*1.2*	*1.1*	16 P, 14 P, 14 P
	Deionised water		*13.7*	*2.3*		15, 11, 15
	Untreated		*11.7*	*3.8*		9, 16, 10
TA 98	**SYN549522 FS**	33 µg	*34.0*	*7.2*	*1.0*	40, 26, 36
	(A22417C)	100 µg	*38.0*	*2.0*	*1.1*	40, 38, 36
		333 µg	*35.7*	*5.0*	*1.0*	31, 41, 35
		1000 µg	*36.7*	*9.8*	*1.0*	48, 31, 31
		2500 µg	*38.3*	*7.2*	*1.1*	43 P, 42 P, 30 P
		5000 µg	*32.3*	*8.1*	*0.9*	23 P, 36 P, 38 P
	Deionised water		*35.0*	*10.8*		32, 47, 26
	Untreated		*40.0*	*5.3*		38, 36, 46
TA 100	**SYN549522 FS**	33 µg	*156.3*	*4.2*	*1.0*	153, 155, 161
	(A22417C)	100 µg	*167.0*	*16.6*	*1.1*	179, 148, 174
		333 µg	*160.7*	*3.5*	*1.0*	157, 164, 161
		1000 µg	*167.7*	*3.5*	*1.1*	164, 171, 168
		2500 µg	*173.7*	*11.5*	*1.1*	174 P, 185 P, 162 P
		5000 µg	*176.7*	*13.7*	*1.1*	179 P, 162 P, 189 P
	Deionised water		*154.7*	*13.2*		143, 152, 169
	Untreated		*160.3*	*12.0*		148, 172, 161

Key to Plate Postfix Codes

P Precipitate

TABLE 6 Experiment II: 1936100 HV2 Pre Incubation With Metabolic Activation (Continued)

Study Name: 1936100 Study Code: Envigo 1936100
Experiment: 1936100 HV2 Pre Date Plated: 13.02.2019
Assay Conditions: Date Counted: 20.02.2019

With metabolic activation

Strain	Compound	Concen-tration per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
WP2 pKM101	**SYN549522 FS (A22417C)**	33 µg	*300.3*	*13.2*	*1.1*	303, 286, 312
		100 µg	*285.7*	*11.0*	*1.0*	292, 273, 292
		333 µg	*294.3*	*11.2*	*1.0*	282, 297, 304
		1000 µg	*273.7*	*13.8*	*1.0*	258, 284, 279
		2500 µg	*266.7*	*15.0*	*0.9*	282 P, 252 P, 266 P
		5000 µg	*264.3*	*50.3*	*0.9*	225 P, 247 P, 321 P
	Deionised water		*281.3*	*0.6*		282, 281, 281
	Untreated		*288.3*	*26.7*		270, 276, 319
WP2 uvrA pKM101	**SYN549522 FS (A22417C)**	33 µg	*351.7*	*12.6*	*1.0*	340, 350, 365
		100 µg	*354.3*	*11.0*	*1.0*	349, 367, 347
		333 µg	*330.0*	*7.8*	*0.9*	339, 325, 326
		1000 µg	*349.7*	*13.9*	*1.0*	338, 346, 365
		2500 µg	*331.0*	*14.0*	*0.9*	347 P, 321 P, 325 P
		5000 µg	*297.0*	*10.8*	*0.8*	309 P, 294 P, 288 P
	Deionised water		*353.3*	*7.6*		345, 360, 355
	Untreated		*356.0*	*38.3*		389, 314, 365
TA 1535	**2-AA**	2.5 µg	*351.3*	*39.7*	*24.5*	360, 386, 308
TA 1537	**2-AA**	2.5 µg	*236.0*	*11.0*	*17.3*	247, 225, 236
TA 98	**2-AA**	2.5 µg	*3428.3*	*310.8*	*98.0*	3260, 3238, 3787
TA 100	**2-AA**	2.5 µg	*3574.7*	*117.1*	*23.1*	3667, 3614, 3443
WP2 pKM101	**2-AA**	10.0 µg	*962.0*	*30.4*	*3.4*	977, 927, 982
WP2 uvrA pKM101	**2-AA**	10.0 µg	*1966.0*	*49.3*	*5.6*	1920, 1960, 2018

Key to Positive Controls

2-AA 2-aminoanthracene

Key to Plate Postfix Codes

P Precipitate

APPENDICES SECTION

APPENDIX 1 Historical Control Data

These data represent the laboratory´s historical control data from November 2016 until August 2018 representing approx. 600 experiments (WP2 pKM101, WP2 uvrA pKM101 the historical data are based on approx. 60 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	11	2.3	6	22	12	2.3	7	22
	Untreated control	11	2.9	6	28	12	2.8	7	23
	Positive control	1245	161.4	367	1791	398	61.0	183	613
TA 1537	Solvent control	10	2.2	6	19	13	3.2	7	30
	Untreated control	10	2.7	5	21	14	3.6	6	29
	Positive control	94	30.0	48	231	170	64.8	81	421
TA 98	Solvent control	26	4.2	13	43	36	6.1	12	56
	Untreated control	27	4.7	14	40	39	6.4	12	59
	Positive control	421	176.8	196	2068	3908	815.0	223	5918
TA 100	Solvent control	160	29.3	79	214	148	31.3	76	216
	Untreated control	181	26.1	80	235	171	27.7	87	218
	Positive control	2074	262.7	511	2850	3626	981.9	553	5860
WP2 pKM101	Solvent control	205	27.6	171	289	233	29.3	194	310
	Untreated control	220	32.2	167	297	259	33.4	204	338
	Positive control	3894	522.5	2576	5458	1277	403.5	950	4063
WP2 uvrA pKM101	Solvent control	324	39.5	243	389	378	48.0	277	469
	Untreated control	339	32.3	279	402	389	39.2	302	476
	Positive control	3706	589.3	2754	5261	2191	252.7	1720	2731

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

ENVIGO CRS GmbH
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 studies on biological materials

13. – 16. Juli 2015
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

Th. Zimmermann, Referatsleiter, Wiesbaden, den **14. September 2015**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hess. Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority

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

APPENDIX 3 Certificate of S9

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ENVIGO

CERTIFICATE

ENVIGO CRS S9 PREPARATION LOT NO. 080318K

Date of preparation: 08 March 2018
Recertification date: 10 October, 2018

Protein assay: 32.7 mg protein / ml S9

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

Salmonella typhimurium assay (AMES-test)

Treatment	µl S9 / plate	number of revertants in TA 98	number of revertants in TA 98 (Recertification)
negative	0	23	37
control	100	31	33
10 µg/plate	0	94	47
2-Aminoanthracene	100	3489	1795
10 µg/plate	0	27	30
Benzo(a)pyrene	100	105	77 (93*)

* value of 150 µL S9/plate

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.

11. OKT. 2018

Dipl. Biol. Andrea Sokolowski
Study Director
Envigo CRS GmbH

Date

11. OKT. 2018

Quality Assurance Auditor
Envigo CRS GmbH H. Pilawa

Date

Envigo CRS GmbH
In den Leppsteinswiesen 19, 64380 Rossdorf, Deutschland
T +49 6154 8070 F +49 6154 83399

envigo.com

SOP Origin TS-SOP S9_20

ENVIGO

CERTIFICATE

ENVIGO CRS S9 PREPARATION LOT NO. 260718E

Date of preparation: July 26, 2018
Recertification date: February 04, 2019

Protein assay: 34.4 mg protein / ml S9

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

Salmonella typhimurium assay (AMES-test)

Treatment	µl S9 / plate	number of revertants in TA 98	number of revertants in TA 98 (Recertification)
negative	0	35	42
control	100	37	30
10 µg/plate	0	107	34
2-Aminoanthracene	100	3537	2412
10 µg/plate	0	33	23
Benzo(a)pyrene	100	174	75

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 — 04. FEB. 2019

Quality Assurance Auditor
Envigo CRS GmbH
H. Pilawa

Date

05. FEB. 2019

Dr. Steffen Naumann
Study Director
Envigo CRS GmbH

Date

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SOP Origin TS-SOP S9_20

APPENDIX 4 Certificate of Analysis

syngenta

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

Certificate of Analysis

A22417C
SYN549522 FS (500)
SMU8IP001

Batch Identification	**SMU8IP001**
Other Batch ID	1058463
Product Code	**A22417C**
Other Product Code(s)	SYN549522 FS (500)
Chemical Analysis (Active Ingredient content)	
– **Identity of the Active Ingredient(s)***	**confirmed**
– **Content of SYN549522***	**41.4 % w/w corresponding to 498 g/l**
– **Content of SYN547386***	**37.2 % w/w corresponding to 448 g/l**
– **Content of SYN548941***	**4.16 % w/w corresponding to 50.0 g/l**
	The Active Ingredient(s) content is within the FAO limits.
Methodology used for Characterization / Recertification	HPLC, chiral HPLC, oscillating density meter
Physical Analysis	
– **Appearance**	**red liquid**
– **Density***	**1203 kg/m^3**
Stability:	
– **Storage Temperature**	**< 30 °C**
– **Recertification Date**	**End of October 2021**

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

Study number of batch characterization:	CHMU180671
Study number(s) of batch recertification:	---

Authorization: 17-Oct-2018

Daniel Jenniches
Analytical Development & Product Chemistry

Page 1 of 1