BIOSERVICE
SCIENTIFIC LABORATORIES GmbH

Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Glyphosate Tech.

Report

Version: Final

Study Completion Date: 17 December 2012

BSL BIOSERVICE Study No.: 126159

Sponsor:

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GLP zertifiziert

Akkreditiert durch Zentralstelle der Länder für Gesundheitsschutz bei Arzneimitteln und Medizinprodukten
ZLG-P-986.96.01

1. Copy of the GLP Certificate

BAYERISCHES LANDESAMT
FÜR GESUNDHEIT UND LEBENSMITTELSICHERHEIT,
LANDESINSTITUT FÜR ARBEITSSCHUTZ UND PRODUKTSICHERHEIT
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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/EC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

BSL Bioservice Scientific Laboratories GmbH
Behringstrasse 6 - 8
82152 Planegg

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

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

2 Prüfungen auf toxikologische Eigenschaften
3 Prüfungen auf mutagene Eigenschaften
9 Sonstige Prüfungen:
a) Mikrobiologische Sicherheitsprüfungen
b) Wirksamkeitsprüfungen an Zellkulturen

Datum der Inspektion/Date of Inspection
(Tag.Monat.Jahr/day.month.year)
16./17.09.2008

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

The above mentioned test facility/test site 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/diesem Prüfstandort 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/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

München, 06.04.2009

[redacted]

Leitender Gewerbedirektor

2. Contents

3. List of Tables

4. Preface

4.1. Abbreviations

2-AA	2-Aminoanthracene
A. dest.	Aqua destillata
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
bio	biotin
cf.	confer
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EC	European Community
EPA	Environmental Protection Agency
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
his	histidine
mg/kg/bw	milligram/kilogram/body weight
MMS	Methylmethanesulfonate
4-NOPD	4-Nitro-o-phenylene-diamine
NaCl	sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NaN_3	sodium azide
Nr.	Nummer *(number)*
OECD	Organisation for Economic Cooperation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
QAU	Quality Assurance Unit
rfa	deep rough factor
RSD	Relative Standard Deviation
S9	microsomal fraction of rat liver homogenate
SD	Standard Deviation
SOP	Standard Operating Procedures
uvrB	repair mutant, UV light sensitive
v/v	volume per volume

4.2. General

Sponsor:	Industrias Afrasa S.A. C/ Ciudad de Sevilla 53 Pol.Ind. Fuente Del Jarro E-46988 Paterna (Valencia) Spain
Study Monitor:	[redacted]
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	126159
Test Item:	Glyphosate Tech.
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Glyphosate Tech.

4.3. Project Staff

Study Director:	Dipl.-Biol. [redacted]
Management:	[redacted]
Head of Quality Assurance Unit:	Dipl.-Biol. [redacted]

4.4. Schedule

Arrival of the Test Item:	29 October 2012
Study Initiation Date:	07 November 2012
Experimental Starting Date:	13 November 2012
Experimental Completion Date:	10 December 2012

5. Project Staff Signatures

Study Director

Dipl.-Biol [redacted]

.....

Date: 17 Dec 2014

Management

.....

Print Name: [redacted]

Date: 17 Dec 2014

6. Quality Assurance

6.1. GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on July 2, 2008 (BGBl. I S. 1146 Nr. 28).

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study was assessed for compliance with the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and/or the test facility were periodically inspected by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits were carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

6.2. Guidelines

This study followed the procedures indicated by internal BSL BIOSERVICE SOPs and the following internationally accepted guidelines and recommendations:

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted 21st July, 1997

Commission Regulation (EC) No. 440/2008 B.13/14:"Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008.

EPA Health Effects Test Guidelines, OPPTS 870.5100 "Bacterial Reverse Mutation Assay" EPA 712-C-98-247, August 1998.

6.3. Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP regulations:

A copy of the final report, the study plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study.

If test item is left, a sample will be stored according to the period fixed by the GLP regulations. Material and samples that are unstable may be disposed of before that time and without sponsor's prior consent. Raw data relating to the study will be discarded only with the prior consent of the sponsor. As requested the remaining test item will be returned to the sponsor.

7. Statement of Compliance

BSL BIOSERVICE

Study No.:	126159
Test Item:	Glyphosate Tech.
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Glyphosate Tech.
Study Director:	Dipl.-Biol. [redacted]

This study performed in the test facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on July 2, 2008 (BGBl. I S. 1146 Nr. 28).

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998.

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

Study Director: Dipl.-Biol. [redacted]

..... [redacted] ....

Date: 17 Dec 2012

8. Statement of the Quality Assurance Unit

BSL BIOSERVICE

Study No.:	126159
Test Item:	Glyphosate Tech.
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Glyphosate Tech.
Study Director:	Dipl.-Biol. [redacted]

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates:

Phases of QAU Inspections	*Dates of QAU Inspections*	*Dates of Reports to the Study Director and Management*
Audit Final Study Plan:	09 November 2012	09 November 2012
Audit Experimental Phase (process-based):	24 September 2012	24 September 2012
Audit Final Report:	14 DEC 2012	14 DEC 2012

This report reflects the raw data.

Member of the
Quality Assurance Unit: [redacted]

Print Name: [redacted]

Date: 14 Dec 2012

9. Summary

9.1. Summary Results

In order to investigate the potential of Glyphosate Tech. for its ability to induce gene mutations the plate incorporation test (experiment I) and the pre-incubation test (experiment II) were performed with the *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 102.

In two independent experiments several concentrations of the test item were used. Each assay was conducted **with** and **without** metabolic activation. The concentrations, including the controls, were tested in triplicate. The following concentrations of the test item were prepared and used in the experiments:

10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate

No precipitation of the test item was observed in any tester strain used in experiment I and II (**with** and **without** metabolic activation).

Toxic effects of the test item were noted in most tester strains used in experiment I and II:

- In experiment I toxic effects of the test item were observed at concentrations of 2500 µg/plate and higher (**with** and **without** metabolic activation), depending on the particular tester strain.
- In experiment II toxic effects of the test item were noted at concentrations of 2500 µg/plate and higher (**without** metabolic activation) and at concentrations of 5000 µg/plate (**with** metabolic activation), depending on the particular tester strain.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Glyphosate Tech. at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II.

The reference mutagens induced a distinct increase of revertant colonies indicating the validity of the experiments.

9.2. Conclusion

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Glyphosate Tech. did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, Glyphosate Tech. is considered to be non-mutagenic in this bacterial reverse mutation assay.

10. Introduction

10.1. Aim of the Study

Bacterial reverse mutation assays use amino acid requiring strains of *Salmonella typhimurium* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of these bacterial reversion assays is that they detect mutations which functionally reverse mutations present in the tester strains and restore the capability to synthesise an essential amino acid (1), (3), (6).

The purpose of this study is to establish the potential of the test item to induce gene mutations in bacteria by means of a *S. typhimurium* reverse mutation assay. There is no requirement for verification of a clear positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions. Negative results need to be confirmed on a case-by-case basis. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments. Study parameters that might be modified include the concentrations spacing and / or the method of treatment (pre-incubation method). In case of severe toxicity of the test item or the use of ethanol as the most appropriate solvent the confirmatory experiment is carried out according to the plate incorporation method with a different spacing between dose levels.

The *Salmonella typhimurium* histidine (his) reversion system measures $his^- \rightarrow his^+$ reversions. The *S. typhimurium* strains are constructed to differentiate between base pair (TA 100, TA 1535, TA 102) and frameshift (TA 98, TA 1537) mutations (6).

These assays directly measure heritable DNA mutations of a type which is associated with adverse effects (7), (8), (10), (11). Point mutations are the cause of many human genetic diseases and there is substantial evidence that somatic cell point mutations in oncogens and tumour suppressor genes are involved in cancer in humans and experimental systems (2).

The tester strains have several features that make them more sensitive for the detection of mutations. The specificity of the strains can provide useful information on the types of mutations that are induced by mutagenic agents.

According to the direct plate incorporation or the pre-incubation method the bacteria are exposed to the test item with and without metabolic activation and plated on selective medium. After a suitable period of incubation, revertant colonies are counted (6).

At least five different concentrations of the test item are tested with approximately half log (i.e. $\sqrt{10}$) intervals between test points for an initial test. More narrow spacing between dose levels may be appropriate when a dose response is investigated. For soluble, non-toxic test compounds the recommended maximum test concentration is 5 mg/plate or 5 µL/plate.

To validate the test, reference mutagens are tested in parallel to the test item (4).

10.2. Justification for the Selection of the Test System

The OECD Guideline for Testing of Chemicals, Section 4, No. 471 – Bacterial Reverse Mutation Test - recommends using a combination of *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 102.

11. Materials and Methods

11.1. Characterisation of the Test Item

The test item and the information concerning the test item were provided by the sponsor. All data related to the test item are the responsibility of the sponsor and have not been verified by the test facility.

Name:	Glyphosate Tech.
Chemical Name:	N-(phosphonomethyl)glycine
CAS No.:	1071-83-6
Batch No.:	20110107-2
Purity:	97%
Physical State:	solid
Storage Conditions:	at room temperature, protected from light
Expiry Date:	01.02.2013
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

11.2. Preparation of the Test Item

The test item was suspended in DMSO and diluted prior to treatment. The solvent was compatible with the survival of the bacteria and the S9 activity. According to sponsor's instructions all concentrations used were calculated based on the active component content of the test item which comprises 97%. For calculation of concentrations a correction factor of 1.03 was applied.

11.3. Controls

Negative and/or solvent as well as positive controls were included in each experiment. Strain specific positive controls were included in the assay, which demonstrated the effective performance of the test.

Negative/Solvent Controls

Negative controls (A. dest., BSL BIOSERVICE Lot No. 121029, 121022) and solvent controls (DMSO, AppliChem Lot No. 2M005967) were treated in the same way as all dose groups.

Positive Controls

Without metabolic activation

Tester Strains:	*S. typhimurium*: TA 100, TA 1535
Name:	NaN_3; sodium azide
Supplier:	Sigma
Catalogue No.:	S2002
Batch No.:	096K1266
Dissolved in:	Aqua dest.
Concentration:	10 µg/plate

Tester Strains:	*S. typhimurium*: TA 98, TA 1537
Name:	4-NOPD; 4-nitro-o-phenylene-diamine
Supplier:	Fluka
Catalogue No.:	73630
Batch No.:	MKBC3223
Dissolved in:	DMSO
Concentrations:	10 µg/plate for TA 98, 40 µg/plate for TA 1537

Tester Strain:	*S. typhimurium*: TA 102
Name:	MMS; methylmethanesulfonate
Supplier:	Sigma
Catalogue No.:	M 4016
Batch No.:	MKBG0368V, MKBJ8702V
Dissolved in:	Aqua dest.
Concentration:	1 µL/plate

With metabolic activation

Tester Strains:	*S. typhimurium*: TA 98, TA 100, TA 1535, TA 1537 and TA 102
Name:	2-AA; 2-aminoanthracene
Supplier:	Aldrich
Catalogue No.:	A3, 880-0
Batch No.:	STBB1901V
Dissolved in:	DMSO
Concentrations:	2.5 µg/plate; 10 µg/plate for TA 102

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

11.4. Test System

11.4.1. Bacteria

Five strains of *S. typhimurium* with the following characteristics were used:

TA 98: his D 3052; rfa$^-$; uvrB$^-$; R-factor:	frame shift mutations
TA 100: his G 46; rfa$^-$; uvrB$^-$; R-factor:	base-pair substitutions
TA 1535: his G 46; rfa$^-$; uvrB$^-$:	base-pair substitutions
TA 1537: his C 3076; rfa$^-$; uvrB$^-$:	frame shift mutations
TA 102: his G 428 (pAQ1); rfa$^-$; R-factor:	base-pair substitutions

Tester strains TA 98, TA 1535 and TA 102 are obtained from MOLTOX, INC., NC 28607, USA. Tester strains TA 100 and TA 1537 are obtained from Xenometrix AG, Switzerland. They are stored as stock cultures in ampoules with nutrient broth (OXOID) supplemented with DMSO (approx. 8% v/v) over liquid nitrogen.

All Salmonella strains contain mutations in the histidine operon, thereby imposing a requirement for histidine in the growth medium. They contain the deep rough (rfa) mutation, which deletes the polysaccharide side chain of the lipopolysaccharides of the bacterial cell surface. This increases cell permeability of larger substances. The other mutation is a deletion of the uvrB gene coding for a protein of the DNA nucleotide excision repair system resulting in an increased sensitivity in detecting many mutagens. This deletion also includes the nitrate reductase (chl) and biotin (bio) genes (bacteria require biotin for growth).

The tester strains TA 98, TA 100 and TA 102 contain the R-factor plasmid, pkM101. These strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains. pkM101 increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present in these organisms (6), (9).

The properties of the *S. typhimurium* strains with regard to membrane permeability, ampicillin- and tetracycline-resistance as well as normal spontaneous mutation rates are checked regularly according to Ames *et al.* (1). In this way it is ensured that the experimental conditions set up by Ames are fulfilled.

11.4.2. Preparation of Bacteria

Samples of each tester strain were grown by culturing for 12 h at 37 °C in Nutrient Broth to the late exponential or early stationary phase of growth (approx. 10^9 cells/mL). The nutrient medium consists per litre:

8 g	Nutrient Broth
5 g	NaCl

A solution of 125 µL ampicillin (10 mg/mL) (TA 98, TA 100, TA 102) was added in order to retain the phenotypic characteristics of the strain.

11.4.3. Agar Plates

The Vogel-Bonner Medium E agar plates with 2% glucose used in the Ames Test were prepared by BSL BIOSERVICE GmbH or provided by an appropriate supplier. Quality controls were performed.

Vogel-Bonner-salts contain per litre:

10	g	$MgSO_4 \times 7\ H_2O$
100	g	citric acid
175	g	$NaNH_4HPO_4 \times 4\ H_2O$
500	g	K_2HPO_4

Sterilisation was performed for 20 min at 121 °C in an autoclave.

Vogel-Bonner Medium E agar plates contain per litre:

15	g	Agar Agar
20	mL	Vogel-Bonner salts
50	mL	glucose-solvent (40%)

Sterilisation was performed for 20 min at 121 °C in an autoclave.

11.4.4. Overlay Agar

The overlay agar contains per litre:

7.0	g	Agar Agar
6.0	g	NaCl
10.5	mg	L-histidine x HCl x H_2O
12.2	mg	biotin

Sterilisation was performed for 20 min at 121 °C in an autoclave.

11.4.5. Mammalian Microsomal Fraction S9 Mix

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

11.4.6. S9 Homogenate

The S9 liver microsomal fraction was prepared at BSL BIOSERVICE GmbH (Experiment I) and obtained from Trinova Biochem GmbH, Giessen, Germany (Experiment II). Male Wistar rats were induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days by oral route (BSL) and male Sprague Dawley rats were induced with phenobarbital / β-naphthoflavone (Trinova).

The following quality control determinations were performed by BSL BIOSERVICE GmbH:

a) Biological activity in:
   - the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene
   - the mouse lymphoma assay using benzo[*a*]pyrene
   - the chromosome aberration assay using cyclophosphamide.

b) Sterility Test

A stock of the supernatant containing the microsomes was frozen in aliquots of 2 and 4 mL and stored at ≤-75 °C.

The protein concentration in the S9 preparation (Lot: 190712) was 37 mg/mL.

The following quality control determinations were performed by Trinova Biochem GmbH:

a) Alkoxyresorufin-0-dealkylase activities
b) Test for the presence of adventitious agents
c) Promutagen activation (including biological activity in the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene)

A stock of the supernatant containing the microsomes is frozen in aliquots of 5 mL and stored at ≤ -75 °C.

The protein concentration in the S9 preparation (Lot: 2996) was 47.7 mg/mL.

The protein concentrations in both S9 preparations were adjusted to 35 mg/mL.

11.4.7. Preparation of S9 Mix

The S9 mix preparation was performed according to Ames *et al.* (1).

100 mM of sodium-ortho-phosphat-buffer, pH 7.4, was ice-cold added to the following pre-weighed sterilised reagents to give final concentrations in the S9 mix of:

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

This solution was mixed with the liver 9000 x g supernatant fluid in the following proportion:

co-factor solution	9.5 parts
liver preparation	0.5 parts

During the experiment the S9 mix was stored on ice.

11.4.8. S9 Mix Substitution Buffer

The S9 mix substitution buffer was used in the study as a replacement of S9 mix, without metabolic activation (-S9).

Phosphate-buffer (0.2 M) contains per litre:

0.2 M $NaH_2PO_4 \times H_2O$	120 mL
0.2 M Na_2HPO_4	880 mL

The two solutions were mixed and the pH was adjusted to 7.4. Sterilisation was performed for 20 min at 121 °C in an autoclave.

This 0.2 M phosphate-buffer was mixed with 0.15 M KCl solution (sterile) in the following proportion:

0.2 M phosphate-buffer	9.5 parts
0.15 M KCl solution	0.5 parts

This S9 mix substitution buffer was stored at 4 °C.

11.5. Experimental Design

11.5.1. Pre-Experiment for Toxicity

The toxicity of the test item was determined with tester strains TA 98 and TA 100 in a pre-experiment. Eight concentrations were tested for toxicity and induction of mutations with three plates each. The experimental conditions in this pre-experiment were the same as described below for the main experiment I (plate incorporation test).

Toxicity may be detected by a clearing or rather diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control.

The test item was tested in the pre-experiment with the following concentrations:

3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate

11.5.2. Exposure Concentrations

The test item concentrations to be applied in the main experiments were chosen according to the results of the pre-experiment (see chapter 13.1 Pre-Experiment). 5000 µg/plate was selected as the maximum concentration. The concentration range covered two logarithmic decades. Two independent experiments were performed with the following concentrations:

10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate

As the results of the pre-experiment were in accordance with the criteria described above, these were reported as a part of the main experiment I.

11.5.3. Experimental Performance

For the plate incorporation method the following materials were mixed in a test tube and poured over the surface of a minimal agar plate:

100 µL	Test solution at each dose level, solvent control, negative control or reference mutagen solution (positive control),
500 µL	S9 mix (for testing with metabolic activation) or S9 mix substitution buffer (for testing without metabolic activation),
100 µL	Bacteria suspension (cf. Preparation of Bacteria, pre-culture of the strain),
2000 µL	Overlay agar.

For the pre-incubation method 100 µL of the test item preparation was pre-incubated with the tester strains (100 µL) and sterile buffer or the metabolic activation system

(500 µL) for 60 min at 37 °C prior to adding the overlay agar (2000 µL) and pouring onto the surface of a minimal agar plate.

For each strain and dose level, including the controls, three plates (in one case only two plates were evaluated, see tables experiment II) were used.

After solidification the plates were inverted and incubated at 37 °C for at least 48 h in the dark.

11.6. Data Recording

The colonies were counted using a ProtoCOL counter (Meintrup DWS Laborgeräte GmbH). If precipitation of the test item precluded automatic counting the revertant colonies were counted by hand. In addition, tester strains with a low spontaneous mutation frequency like TA 1535 and TA 1537 were counted manually.

11.7. Evaluation of Cytotoxicity

Cytotoxicity can be detected by a clearing or rather diminution of the background lawn (indicated as "B" in the result tables) or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control.

11.8. Criteria of Validity

A test is considered acceptable if for each strain:

- the bacteria demonstrate their typical responses to ampicillin (TA 98, TA 100, TA 102)
- the control plates with and without S9 mix are within the following ranges (mean values of the spontaneous reversion frequency are within the historical control data range):

	-S9	+S9
TA 98:	16 - 46	18 - 53
TA 100:	77 - 174	79 - 162
TA 1535:	5 - 27	5 - 26
TA 1537:	5 - 28	5 - 32
TA 102	164 - 399	169 - 467

- corresponding background growth on negative control, solvent control and test plates is observed
- the positive controls show a distinct enhancement of revertant rates over the control plate

11.9. Evaluation of Mutagenicity

The Mutation Factor is calculated by dividing the mean value of the revertant counts through the mean values of the solvent control (the exact and not the rounded values are used for calculation).

A test item is considered as mutagenic if:

- a clear and dose-related increase in the number of revertants occurs and/or
- a biologically relevant positive response for at least one of the dose groups occurs

in at least one tester strain with or without metabolic activation.

A biologically relevant increase is described as follows:

- if in tester strains TA 98, TA 100 and TA 102 the number of reversions is at least twice as high
- if in tester strains TA 1535 and TA 1537 the number of reversions is at least three times higher

than the reversion rate of the solvent control (5).

According to OECD guidelines, the biological relevance of the results is the criterion for the interpretation of results, a statistical evaluation of the results is not regarded as necessary.

A test item producing neither a dose related increase in the number of revertants nor a reproducible biologically relevant positive response at any of the dose groups is considered to be non-mutagenic in this system.

12. Deviations from the Study Plan

There were the following deviations from the study plan:

Concerning:

Study Director, study plan, p. 2, 6

Before:

Study Director: Dr. [redacted]

New:

Study Director: Dipl.-Biol. [redacted]

Reason:

Intended handover due to absence of the study director

Concerning:

S9 Homogenate, study plan, p. 13, 14

Before:

The S9 liver microsomal fraction is prepared at BSL BIOSERVICE GmbH. Male Wistar rats are induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days by oral route.

The following quality control determinations are performed:

a) Biological activity in:
  - the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene
  - the mouse lymphoma assay using benzo[*a*]pyrene
  - the chromosome aberration assay using cyclophosphamide.

b) Sterility Test

A stock of the supernatant containing the microsomes is frozen in aliquots of 2 and 4 mL and stored at ≤ -75 °C.

The protein concentration in the S9 preparation is usually between 20 and 45 mg/mL.

New:

The S9 liver microsomal fraction was prepared at BSL BIOSERVICE GmbH (Experiment I) and obtained from Trinova Biochem GmbH, Giessen, Germany (Experiment II). Male Wistar rats were induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days by oral route (BSL) and male Sprague Dawley rats were induced with phenobarbital / β-naphthoflavone (Trinova).

The following quality control determinations were performed by BSL BIOSERVICE GmbH:

a) Biological activity in:
- the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene
- the mouse lymphoma assay using benzo[*a*]pyrene
- the chromosome aberration assay using cyclophosphamide.

b) Sterility Test

A stock of the supernatant containing the microsomes was frozen in aliquots of 2 and 4 mL and stored at ≤-75 °C.

The protein concentration in the S9 preparation (Lot: 190712) was 37 mg/mL.

The following quality control determinations were performed by Trinova Biochem GmbH:

a) Alkoxyresorufin-0-dealkylase activities
b) Test for the presence of adventitious agents
c) Promutagen activation (including biological activity in the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene)

A stock of the supernatant containing the microsomes is frozen in aliquots of 5 mL and stored at ≤ -75 °C.

The protein concentration in the S9 preparation (Lot: 2996) was 47.7 mg/mL.

The protein concentrations in both S9 preparations were adjusted to 35 mg/mL.

Reason:

Additional Supplier of the S9 Homogenate.

These deviations did not influence the quality or integrity of the present study.

13. Results

13.1. Pre-Experiment

Toxicity may be detected by a clearing or rather diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control.

Table 1: Results Pre-Experiment

Substance	Dose (µg/plate)	TA 98 Mutation Factor [toxicity]*		TA 100 Mutation Factor [toxicity]*	
		without S9	with S9	without S9	with S9
Solvent Control (DMSO)		1.0	1.0	1.0	1.0
4-NOPD	10.0	19.5	-	-	-
NaN_3	10.0	-	-	4.8	-
2-AA	2.50	-	70.6	-	17.2
Test Item	3.16	1.1	0.9	0.9	1.0
	10.0	0.7	0.8	1.0	0.9
	31.6	1.3	0.9	1.0	1.0
	100	0.9	0.8	1.1	1.0
	316	1.0	0.9	0.9	1.1
	1000	0.9	0.8	0.9	1.0
	2500	0.7	0.8	0.6 [B]	1.0 [B]
	5000	0.8	0.5	0.2 [B]	0.6 [B]

* *[toxicity parameter]: B= Background lawn reduced;*

13.2. Experiment I (Plate-incorporation Test)

Table 2: Results Experiment I

Tester Strain: TA 98 Experiment: 1

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		25 22 19	**22**	3.0	19 35 25	**26**	8.1	**1.4**	**1.0**
DMSO		19 10 17	**15**	4.7	24 29 28	**27**	2.6	**1.0**	**1.0**
Test Item	10.0 µg	12 11 10	**11**	1.0	22 20 26	**23**	3.1	**0.7**	**0.8**
Test Item	31.6 µg	16 24 20	**20**	4.0	24 26 25	**25**	1.0	**1.3**	**0.9**
Test Item	100 µg	14 16 13	**14**	1.5	21 21 22	**21**	0.6	**0.9**	**0.8**
Test Item	316 µg	14 15 17	**16**	1.5	19 27 26	**24**	4.4	**1.0**	**0.9**
Test Item	1000 µg	16 12 13	**14**	2.1	18 21 25	**21**	3.5	**0.9**	**0.8**
Test Item	2500 µg	9 11 10	**10**	1.0	13 24 24	**20**	6.4	**0.7**	**0.8**
Test Item	5000 µg	18 9 8	**12**	5.5	7 16 21	**15**	7.1	**0.8**	**0.5**
4-NOPD	10 µg	299 324 273	**299**	25.5	/ / /	/	/	**19.5**	/
2-AA	2.5 µg	/ / /	/	/	1825 1739 2154	**1906**	219.0	/	**70.6**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Tester Strain: TA 100 Experiment: 1

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		85 83 95	**88**	6.4	109 130 105	**115**	13.4	**1.1**	**1.3**
DMSO		74 87 82	**81**	6.6	103 82 83	**89**	11.8	**1.0**	**1.0**
Test Item	10.0 µg	84 98 72	**85**	13.0	84 78 87	**83**	4.6	**1.0**	**0.9**
Test Item	31.6 µg	76 83 72	**77**	5.6	88 92 90	**90**	2.0	**1.0**	**1.0**
Test Item	100 µg	93 88 77	**86**	8.2	84 102 94	**93**	9.0	**1.1**	**1.0**
Test Item	316 µg	84 70 72	**75**	7.6	91 97 120	**103**	15.3	**0.9**	**1.1**
Test Item	1000 µg	67 81 70	**73**	7.4	85 97 82	**88**	7.9	**0.9**	**1.0**
Test Item	2500 µg	34 B 64 B 48 B	**49**	15.0	67 B 83 B 105 B	**85**	19.1	**0.6**	**1.0**
Test Item	5000 µg	19 B 28 B 11 B	**19**	8.5	36 B 56 B 66 B	**53**	15.3	**0.2**	**0.6**
NaN_3	10 µg	415 307 447	**390**	73.4	/ / /	/	/	**4.8**	/
2-AA	2.5 µg	/ / /	/	/	1742 1106 1751	**1533**	369.8	/	**17.2**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Tester Strain: TA 1535 Experiment: 1

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		20 24 26	**23**	3.1	12 17 13	**14**	2.6	**1.3**	**1.3**
DMSO		14 18 22	**18**	4.0	5 12 16	**11**	5.6	**1.0**	**1.0**
Test Item	10.0 µg	21 15 22	**19**	3.8	15 19 21	**18**	3.1	**1.1**	**1.7**
Test Item	31.6 µg	18 23 23	**21**	2.9	9 11 13	**11**	2.0	**1.2**	**1.0**
Test Item	100 µg	16 17 20	**18**	2.1	8 9 11	**9**	1.5	**1.0**	**0.8**
Test Item	316 µg	6 18 21	**15**	7.9	7 14 11	**11**	3.5	**0.8**	**1.0**
Test Item	1000 µg	12 19 17	**16**	3.6	9 10 15	**11**	3.2	**0.9**	**1.0**
Test Item	2500 µg	16 19 20	**18**	2.1	10 13 19	**14**	4.6	**1.0**	**1.3**
Test Item	5000 µg	5 B 8 B 12 B	**9**	2.3	7 14 15	**12**	4.4	**0.5**	**1.1**
NaN_3	10 µg	1397 1451 1420	**1423**	27.1	/ / /	/	/	**79.0**	/
2-AA	2.5 µg	/ / /	/	/	194 197 219	**203**	13.7	/	**18.5**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Tester Strain: TA 1537 Experiment: 1

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		4 4 9	**6**	2.9	5 11 10	**9**	3.2	**0.9**	**1.0**
DMSO		3 7 8	**6**	2.6	6 9 10	**8**	2.1	**1.0**	**1.0**
Test Item	10.0 µg	3 6 10	**6**	3.5	4 10 8	**7**	3.1	**1.1**	**0.9**
Test Item	31.6 µg	5 6 9	**7**	2.1	5 7 7	**6**	1.2	**1.1**	**0.8**
Test Item	100 µg	6 6 1	**4**	2.9	3 3 5	**4**	1.2	**0.7**	**0.4**
Test Item	316 µg	7 7 8	**7**	0.6	5 6 9	**7**	2.1	**1.2**	**0.8**
Test Item	1000 µg	3 7 7	**6**	2.3	4 6 8	**6**	2.0	**0.9**	**0.7**
Test Item	2500 µg	4 5 9	**6**	2.6	4 7 10	**7**	3.0	**1.0**	**0.8**
Test Item	5000 µg	5 6 0	**4**	3.2	5 6 7	**6**	1.0	**0.6**	**0.7**
4-NOPD	40 µg	52 60 43	**52**	8.5	/ / /	**/**	/	**8.6**	**/**
2-AA	2.5 µg	/ / /	**/**	/	100 154 137	**130**	27.6	**/**	**15.6**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Tester Strain: TA 102 Experiment: 1

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		232 236 229	**232**	3.5	293 368 337	**333**	37.7	**1.2**	**1.2**
DMSO		204 190 211	**202**	10.7	269 288 305	**287**	18.0	**1.0**	**1.0**
Test item	10.0 µg	243 230 203	**225**	20.4	337 322 354	**338**	16.0	**1.1**	**1.2**
Test item	31.6 µg	207 261 188	**219**	37.9	330 309 304	**314**	13.8	**1.1**	**1.1**
Test item	100 µg	241 216 217	**225**	14.2	278 290 295	**288**	8.7	**1.1**	**1.0**
Test item	316 µg	193 200 198	**197**	3.6	235 256 261	**251**	13.8	**1.0**	**0.9**
Test item	1000 µg	178 188 195	**187**	8.5	222 277 262	**254**	28.4	**0.9**	**0.9**
Test item	2500 µg	205 199 204	**203**	3.2	358 327 308	**331**	25.2	**1.0**	**1.2**
Test item	5000 µg	28 99 104	**77**	42.5	254 246 264	**255**	9.0	**0.4**	**0.9**
MMS	1 µL	1246 1133 1635	**1338**	263.3	/ / /	/	/	**6.6**	/
2-AA	10 µg	/ / /	/	/	747 844 717	**769**	66.4	/	**2.7**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

13.3. Experiment II (Pre-incubation Test)

Table 3: Results Experiment II

Tester Strain: TA 98 Experiment: 2

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		21 23 19	**21**	2.0	34 18 34	**29**	9.2	**1.0**	**1.2**
DMSO		28 20 13	**20**	7.5	22 20 27	**23**	3.6	**1.0**	**1.0**
Test item	10.0 µg	22 29 17	**23**	6.0	30 22 37	**30**	7.5	**1.1**	**1.3**
Test item	31.6 µg	26 23 24	**24**	1.5	31 35 21	**29**	7.2	**1.2**	**1.3**
Test item	100 µg	15 14 24	**18**	5.5	27 28 22	**26**	3.2	**0.9**	**1.1**
Test item	316 µg	23 20 16	**20**	3.5	16 26 24	**22**	5.3	**1.0**	**1.0**
Test item	1000 µg	20 24 31	**25**	5.6	27 26 43	**32**	9.5	**1.2**	**1.4**
Test item	2500 µg	16 27 24	**22**	5.7	38 18 21	**26**	10.8	**1.1**	**1.1**
Test item	5000 µg	5 6 11	**7**	3.2	22 28 29	**26**	3.8	**0.4**	**1.1**
4-NOPD	10 µg	448 524 683	**552**	119.9	/ / /	/	/	**27.1**	/
2-AA	2.5 µg	/ / /	/	/	1511 1629 2733	**1958**	674.0	/	**85.1**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Tester Strain: TA 100 Experiment: 2

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		112 112 106	**110**	3.5	80 109 120	**103**	20.7	**1.1**	**1.1**
DMSO		98 111 104	**104**	6.5	100 86 97	**94**	7.4	**1.0**	**1.0**
Test Item	10.0 µg	98 134 124	**119**	18.6	83 96 116	**98**	16.6	**1.1**	**1.0**
Test Item	31.6 µg	121 104 118	**114**	9.1	111 110 125	**115**	8.4	**1.1**	**1.2**
Test Item	100 µg	106 108 99	**104**	4.7	112 123 111	**115**	6.7	**1.0**	**1.2**
Test Item	316 µg	113 109 118	**113**	4.5	90 109 107	**102**	10.4	**1.1**	**1.1**
Test Item	1000 µg	104 120 168	**131**	33.3	124 98 101	**108**	14.2	**1.3**	**1.1**
Test Item	2500 µg	121 119 101	**114**	11.0	97 80 90	**89**	8.5	**1.1**	**0.9**
Test Item	5000 µg	4 B 23 B 37 B	**21**	16.6	102 98 99	**100**	2.1	**0.2**	**1.1**
NaN_3	10 µg	1023 1110 1172	**1102**	74.8	/ / /	/	/	**10.6**	/
2-AA	2.5 µg	/ / /	/	/	2409 1875 1722	**2002**	360.7	/	**21.2**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Tester Strain: TA 1535 Experiment: 2

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		11 13 17	**14**	3.1	7 10 19	**12**	6.2	**1.3**	**1.0**
DMSO		8 8 16	**11**	4.6	7 12 16	**12**	4.5	**1.0**	**1.0**
Test item	10.0 µg	14 17 19	**17**	2.5	13 10 15	**13**	2.5	**1.6**	**1.1**
Test item	31.6 µg	13 18 20	**17**	3.6	9 12 15	**12**	3.0	**1.6**	**1.0**
Test item	100 µg	19 19 19	**19**	0.0	6 9 10	**8**	2.1	**1.8**	**0.7**
Test item	316 µg	14 15 17	**15**	1.5	7 10 16	**11**	4.6	**1.4**	**0.9**
Test item	1000 µg	11 11 16	**13**	2.9	12 13 13	**13**	0.6	**1.2**	**1.1**
Test item	2500 µg	21 13 11	**15**	5.3	7 9 16	**11**	4.7	**1.4**	**0.9**
Test item	5000 µg	10 7 6	**8**	2.1	12 10 15	**12**	2.5	**0.7**	**1.1**
NaN_3	10 µg	1350 1136 1175	**1220**	114.0	/ / /	/	/	**114.4**	/
2-AA	2.5 µg	/ / /	/	/	164 213 213	**197**	28.3	/	**16.9**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Tester Strain: TA 1537 Experiment: 2

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		2 7 8	**6**	3.2	5 7 8	**7**	1.5	**0.8**	**1.4**
DMSO		7 8 /	**8**	0.7	4 3 7	**5**	2.1	**1.0**	**1.0**
Test item	10.0 µg	6 11 12	**10**	3.2	2 4 6	**4**	2.0	**1.3**	**0.9**
Test item	31.6 µg	6 5 7	**6**	1.0	3 8 6	**5**	1.7	**0.8**	**1.1**
Test item	100 µg	5 1 2	**3**	2.1	3 5 7	**5**	2.0	**0.4**	**1.1**
Test item	316 µg	3 3 7	**4**	2.3	5 6 7	**6**	1.0	**0.6**	**1.3**
Test item	1000 µg	2 4 12	**6**	5.3	1 2 5	**3**	2.1	**0.8**	**0.6**
Test item	2500 µg	1 5 6	**4**	2.6	12 6 15	**11**	4.6	**0.5**	**2.4**
Test item	5000 µg	1 2 2	**2**	0.6	2 6 10	**6**	4.0	**0.2**	**1.3**
4-NOPD	40 µg	95 111 144	**117**	25.0	/ / /	**/**	/	**23.3**	**/**
2-AA	2.5 µg	/ / /	**/**	/	132 147 175	**151**	21.8	**/**	**32.4**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Tester Strain: TA 102 Experiment: 2

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		232 201 211	**215**	15.8	213 220 212	**215**	4.4	**1.3**	**1.2**
DMSO		155 190 168	**171**	17.7	172 185 195	**184**	11.5	**1.0**	**1.0**
Test Item	10.0 µg	159 175 155	**163**	10.6	236 238 225	**233**	7.0	**1.0**	**1.3**
Test Item	31.6 µg	176 149 177	**167**	15.9	235 240 223	**233**	8.7	**1.0**	**1.3**
Test Item	100 µg	140 139 161	**147**	12.4	196 224 210	**210**	14.0	**0.9**	**1.1**
Test Item	316 µg	146 157 161	**155**	7.8	202 206 221	**210**	10.0	**0.9**	**1.1**
Test Item	1000 µg	172 159 180	**170**	10.6	212 223 254	**230**	21.8	**1.0**	**1.2**
Test Item	2500 µg	176 176 182	**178**	3.5	252 232 203	**229**	24.6	**1.0**	**1.2**
Test Item	5000 µg	20 37 53	**37**	16.5	88 56 38	**61**	25.3	**0.2**	**0.3**
MMS	1 µL	1993 2162 1760	**1972**	201.8	/ / /	/	/	**11.5**	/
2-AA	10 µg	/ / /	/	/	560 458 573	**530**	63.0	/	**2.9**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn
P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

14. Discussion

The test item Glyphosate Tech. was investigated for its potential to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 102.

In two independent experiments several concentrations of the test item were used. Each assay was conducted **with** and **without** metabolic activation. The concentrations, including the controls, were tested in triplicate. The following concentrations of the test item were prepared and used in the experiments:

10.0, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate

No precipitation of the test item was observed in any tester strain used in experiment I and II (**with** and **without** metabolic activation).

Toxic effects of the test item were noted in all tester strains evaluated in experiment I and II.

In experiment I toxic effects of the test item were observed in tester strain TA 98 at a concentration of 5000 µg/plate (**with** metabolic activation). In tester strain TA 100 toxic effects of the test item were noted at concentrations of 2500 µg/plate and higher (**with** and **without** metabolic activation). In tester strains TA 1535 and TA 102 toxic effects of the test item were observed at a concentration of 5000 µg/plate (**without** metabolic activation).

In experiment II toxic effects of the test item were noted in tester strains TA 98 and TA 100 at a concentration of 5000 µg/plate (**without** metabolic activation). In tester strain TA 1537 toxic effects of the test item were noted at concentrations of 2500 µg/plate and higher (**without** metabolic activation). In tester strain TA 102 toxic effects of the test item were observed at a concentration of 5000 µg/plate (**with** and **without** metabolic activation). The reduction in the number of revertants down to a mutation factor of 0.4 found in tester strain TA 1537 at a concentration of 100 µg/plate (**without** metabolic activation) was regarded as not biologically relevant due to lack of a dose-response relationship.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Glyphosate Tech. at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II.

The reference mutagens induced a distinct increase of revertant colonies indicating the validity of the experiments.

14.1. Conclusion

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Glyphosate Tech. did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, Glyphosate Tech. is considered to be non-mutagenic in this bacterial reverse mutation assay.

15. Distribution of the Report

1 original (paper):	Sponsor
1 copy (paper):	BSL BIOSERVICE
1 copy (electronic):	Sponsor

16. References

16.1. Guidelines

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted 21st July, 1997.

Commission Regulation (EC) No. 440/2008 B.13/14: "Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008.

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16.2. Literature

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16.3. Internal BSL BIOSERVICE SOPs

Stammhaltung und Prüfung des Genotyps der Ames Teststämme (SOP 15-2-2)

Salmonella typhimurium / Escherichia coli – Rückmutationstest (SOP 15-2-3)

Bedienung und Kontrolle des ProtoCOL-Counters SR (SOP 4-6-6)

Validierung des ProtoCOL-Counters SR (SOP 4-6-7)

17. Historical Laboratory Control Data

Table 4: Historical Laboratory Control Data of the Negative Control (in 2009 - 2011) without S9 (-S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	23.8	113.3	10.0	8.8	252.1
SD	5.8	16.2	2.9	2.9	46.6
Min	16	77	5	5	164
Max	46	174	27	28	399
RSD [%]	21.6	14.3	29.1	32.7	18.5
n =	1016	1057	959	959	631

S9: metabolic activation
Mean: mean of revertants/plate
Min.: minimum of revertants/plate
Max.: maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

Table 5: Historical Laboratory Control Data of the Positive Control (in 2009 - 2011) without S9 (-S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	491.8	933.9	1031.9	125.2	1516.9
SD	154.9	263.7	287.1	30.6	335.0
Min	205	279	67	34	391
Max	2613	1876	1850	275	2902
RSD [%]	31.5	28.2	27.8	24.4	22.1
n =	1002	1045	946	946	622

S9: metabolic activation
Mean: mean of revertants/plate
Min.: minimum of revertants/plate
Max.: maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

Table 6: Historical Laboratory Control Data of the Negative Control (in 2009 - 2011) with S9 (+S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	30.6	114.8	8.7	9.1	288.0
SD	5.1	16.2	2.3	3.0	58.0
Min	18	79	5	5	169
Max	53	162	26	32	467
RSD [%]	18.9	14.1	26.6	33.4	20.1
n =	1017	1058	959	959	631

S9: metabolic activation
Mean: mean of revertants/plate
Min.: minimum of revertants/plate
Max.: maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

Table 7: Historical Laboratory Control Data of the Positive Control (in 2009 - 2011) with S9 (+S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	2283.1	1759.0	115.7	237.6	1043.6
SD	651.4	508.4	59.4	91.7	305.9
Min	313	462	27	32	371
Max	3587	3204	732	474	2422
RSD [%]	28.5	28.9	51.4	38.6	29.3
n =	1003	1046	469	946	621

S9: metabolic activation
Mean: mean of revertants/plate
Min.: minimum of revertants/plate
Max.: maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values