BIOSERVICE
SCIENTIFIC LABORATORIES GmbH

Reverse Mutation Assay using Bacteria

(*Salmonella typhimurium*)

with

Glyphosate TC

Report

Version: Final

Date: 08 April 2010

BSL BIOSERVICE Study No.: 101268

Sponsor:

Helm AG

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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

L.S.

2. Contents

3. Preface

3.1. Abbreviations

2-AA	2-Aminoanthracene
A. dest.	Aqua destillata
BGBl.	Bundesgesetzblatt
bio	Biotin
cf.	confer
chl	Nitrate reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EC	European Community
EPA	Environmental Protection Agency
GLP	Good Laboratory Practice
his	Histidine
mg/kg/bw	Milligram/kilogram/body weight
MMS	Methyl methane sulfonate
4-NOPD	4-Nitro-o-phenylene-diamine
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NaN_3	Sodium azide
OECD	Organisation for Economic Co-operation 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
uvrB	Repair mutant, UV light sensitive

3.2. General

Sponsor:	Helm AG Nordkanalstraße 28 20097 Hamburg Germany
Study Monitor:	Dr. rer. nat. [redacted]
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstraße 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	101268
Test Item:	Glyphosate TC
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Glyphosate TC

3.3. Project Staff

Study Director: Deputy Study Director: Management: Head of Quality Assurance Unit:	[redacted]

3.4. Schedule

Arrival of the Test Item:	24 March 2010
Date of Draft Study Plan:	24 March 2010
Date of Final Study Plan:	24 March 2010
Start of Experiment:	25 March 2010
End of Experiment:	06 April 2010
Date of Draft Report:	06 April 2010
Date of Final Report:	08 April 2010

3.5. Project Staff Signatures

Study Director:

[redacted]

.....

Date: 08 Apr 2010

Management

[redacted]

.....

Print name: [redacted]

Date: 08 Apr 2010

4. Quality Assurance

4.1. 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 June 20, 2002 (BGBl. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

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 are periodically inspected by the Quality Assurance unit according to the corresponding SOPs. These inspections and audits are 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.

4.2. Guidelines

This study followed the procedures indicated by 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.

4.3. 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 over a sample will be stored according to the period fixed by the GLP-Regulations. Samples that are unstable may be disposed of before that time. No raw data or material relating to the study will be discarded without the sponsor's prior consent. Remaining test item will be returned to the sponsor, as requested.

5. Statement of Compliance

BSL BIOSERVICE
Study No.: 101268

Test Item: Glyphosate TC

Study Director: [redacted]

Title: Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Glyphosate TC

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 June 20, 2002 (BGBl. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

"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: [redacted]

[redacted] .....

Date: 12 Apr 2010

6. Statement of the Quality Assurance Unit

BSL BIOSERVICE
Study No.: 101268

Test Item: Glyphosate TC

Study Director: [redacted]

Title: Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Glyphosate TC

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:	25 March 2010	25 March 2010
Audit Experimental Phase (Method Audit):	01 December 2009	01 December 2009
Audit Draft Report:	07 April 2010	07 April 2010
Audit Final Report:	09 April 2010	09 April 2010

This report reflects the raw data.

Member of the
Quality Assurance Unit:

[redacted]
Print name: [redacted]

Date: 09 Apr 2010

7. Summary

In order to investigate the potential of Glyphosate TC 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:

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 some 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 a concentration of 5000 µg/plate (**with** and **without** 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 TC 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.

Conclusion

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

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

8. Purpose 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 two independent *S. typhimurium* reverse mutation assays. For the confirmatory experiment modifications are carried out which include the performance of the liquid pre-incubation assay if a clearly negative result is obtained in the plate incorporation study or a more narrow spacing between dose levels in order to investigate a dose-response if a positive result is obtained. In case of a positive result, 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 amounts 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).

Justification for 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 *S. typhimurium* TA 102 or *E. coli* WP2 uvrA.

9. Materials and Methods

9.1. 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 TC
Batch No.:	20090305
Sponsor's Sample No.:	37/125/08
Active Ingredient/Content:	Declared: min. 950 g/kg
	Analysed: 958 g/kg
	(cf. Manufacturer's Certificate of Analysis attached in the Annex)
	Authenticated: 982 g/kg
	(cf. Charles River Study 215602)
Formulation Type:	Technical grade active ingredient
Type:	Herbicide
Physical State at RT:	Solid
Colour:	White
Production Date:	March 26, 2009
Date of Receipt/Condition at the Receipt at the Test Facility:	March 24, 2010 The test item was received in proper conditions
Expiry Date:	March 26, 2011
Storage:	At room temperature
Safety Precautions:	Routine hygienic procedures were sufficient to assure personnel health and safety.

9.2. Preparation of the Test Item

Due to low solubility of the test item, two stock solutions of a concentration of 50 and 25 mg/ml, respectively, were prepared by suspending the test item in DMSO and processing by ultrasound for 30 minutes at 37°C. The stock solution of a concentration of 25 mg/ml was used for preparation of the dilution series. The solvent was compatible with the survival of the bacteria and the S9 activity.

9.3. Controls

Positive and negative controls were included in each experiment. Strain specific positive controls were included in the assay, which demonstrated the effective performance of the test.

Negative Controls

Solvent controls, consisting of solvent or vehicle alone as well as untreated controls were treated in the same way as the treatment groups.

Positive Controls

Without metabolic activation

Tester Strains:	*S. typhimurium*: TA 100, TA 1535
Name:	Sodium azide, NaN_3
Supplier:	Merck
Catalogue No.:	106688
Lot No.:	K 28585088
Purity:	at least 99%
Dissolved in:	Aqua dest.
Concentration:	10 µg/plate
Tester Strains:	*S. typhimurium*: TA 98, TA 1537
Name:	4-nitro-o-phenylene-diamine, 4-NOPD
Supplier:	Fluka
Catalogue No.:	73630
Lot No.:	1364330
Purity:	> 97%
Dissolved in:	DMSO
Concentrations:	10 µg/plate for TA 98, 40 µg/plate for TA 1537
Tester Strain:	*S. typhimurium*: TA 102
Name:	Methyl methane sulfonate, MMS
Supplier:	Sigma
Catalogue No.:	M4016
Lot No.:	76296KJ
Purity:	99.0%
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-aminoanthracene, 2-AA
Supplier:	Aldrich
Catalogue No.:	A3, 880-0
Lot No.:	S34773-337
Purity:	96%
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.

9.4. *Test System*

9.4.1. *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

The Salmonella tester strains TA 98, TA 100, TA 1535, TA 1537 and TA 102 were obtained from MOLTOX, INC, NC 28607, USA. 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.

9.4.2. *Preparation of Bacteria*

Samples of each tester strain were grown by culturing for 12 h at 38.5°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.

9.4.3. 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 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 at 121°C in an autoclave.

9.4.4. 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 at 121°C in an autoclave.

9.4.5. 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.

9.4.6. S9 Homogenate

The S9 liver microsomal fraction was prepared at BSL BIOSERVICE GmbH. 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.

The following quality control determinations are performed:

a) Biological activity in the *Salmonella typhimurium* assay
b) Sterility Test

A stock of the supernatant containing the microsomes was frozen in ampoules of 2 and 4.5 mL and stored at ≤-75°C.

The protein concentration in the S9 preparation (Lot: 091009) was 33 mg/mL. The S9 mix preparation was performed according to Ames *et al.* (1).

9.4.7. Preparation of S9 Mix

100 mM of ice-cold sodium-ortho-phosphat-buffer, pH 7.4, was 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.

9.5. Experimental Design

9.5.1. Pre-Experiment for Toxicity

The performance of a pre-experiment for toxicity was not regarded as necessary.

9.5.2. Exposure Concentrations

5000 µg/plate was selected as the maximum concentration. Two independent experiments were performed with the following concentrations:

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

The concentration range covered two logarithmic decades.

9.5.3. 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 minutes 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 were used.

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

9.6. 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.

9.7. 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.

9.8. 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:	18 - 46	18 - 57
TA 100:	77 - 163	78 - 165
TA 1535:	5 - 29	5- 27
TA 1537:	5 - 30	5 - 36
TA 102:	164 - 390	163 - 472

- 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

9.9. 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 100 and TA 102 the number of reversions is at least twice as high
- if in tester strains TA 98, 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.

10. Deviations from the Study Plan

There were no deviations from the Study Plan.

11. Results

11.1. Experiment I (Plate-incorporation Test)

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.		22 22 18	**21**	2.3	29 18 20	**22**	5.9	**1.0**	**1.1**
DMSO		23 25 17	**22**	4.2	22 21 20	**21**	1.0	**1.0**	**1.0**
Test Item	31.6 µg	24 22 22	**23**	1.2	31 37 21	**30**	8.1	**1.0**	**1.4**
Test Item	100 µg	24 19 25	**23**	3.2	26 29 33	**29**	3.5	**1.0**	**1.4**
Test Item	316 µg	23 17 17	**19**	3.5	32 29 28	**30**	2.1	**0.9**	**1.4**
Test Item	1000 µg	17 17 21	**18**	2.3	29 25 31	**28**	3.1	**0.8**	**1.3**
Test Item	2500 µg	21 22 22	**22**	0.6	35 28 30	**31**	3.6	**1.0**	**1.5**
Test Item	5000 µg	11 14 19	**15**	4.0	21 16 25	**21**	4.5	**0.7**	**1.0**
4-NOPD	10 µg	374 350 360	**361**	12.1	/ / /	/	/	**16.7**	/
2-AA	2.5 µg	/ / /	/	/	1661 1704 2348	**1904**	384.8	/	**90.7**

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

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

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.		119 96 98	**104**	12.7	99 115 114	**109**	9.0	**1.4**	**1.1**
DMSO		74 85 57	**72**	14.1	103 108 95	**102**	6.6	**1.0**	**1.0**
Test Item	31.6 µg	86 77 99	**87**	11.1	134 120 114	**123**	10.3	**1.2**	**1.2**
Test Item	100 µg	85 69 88	**81**	10.2	94 118 93	**102**	14.2	**1.1**	**1.0**
Test Item	316 µg	73 93 77	**81**	10.6	92 100 107	**100**	7.5	**1.1**	**1.0**
Test Item	1000 µg	78 58 89	**75**	15.7	104 116 138	**119**	17.2	**1.0**	**1.2**
Test Item	2500 µg	86 62 69	**72**	12.3	96 108 91	**98**	8.7	**1.0**	**1.0**
Test Item	5000 µg	12 15 8	**12**	3.5	56 62 36	**51**	13.6	**0.2**	**0.5**
NaN_3	10 µg	351 698 852	**634**	256.6	/ / /	/	/	**8.8**	/
2-AA	2.5 µg	/ / /	/	/	2258 2430 v 2431	**2373**	99.6	/	**23.3**

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.		7 11 8	**9**	2.1	8 9 10	**9**	1.0	**1.2**	**1.4**
DMSO		6 10 6	**7**	2.3	8 5 6	**6**	1.5	**1.0**	**1.0**
Test Item	31.6 µg	9 3 8	**7**	3.2	4 5 8	**6**	2.1	**0.9**	**0.9**
Test Item	100 µg	9 8 7	**8**	1.0	5 3 12	**7**	4.7	**1.1**	**1.1**
Test Item	316 µg	7 5 7	**6**	1.2	8 2 4	**5**	3.1	**0.9**	**0.7**
Test Item	1000 µg	5 4 5	**5**	0.6	6 4 9	**6**	2.5	**0.6**	**1.0**
Test Item	2500 µg	1 2 0	**1**	1.0	1 4 5	**3**	2.1	**0.1**	**0.5**
Test Item	5000 µg	0 B 0 B 0 B	**0**	0.0	0 B 2 B 0 B	**1**	1.2	**0.0**	**0.1**
NaN_3	10 µg	212 553 865	**543**	326.6	/ / /	/	/	**74.1**	/
2-AA	2.5 µg	/ / /	/	/	109 124 163	**132**	27.9	/	**20.8**

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.		12 9 14	**12**	2.5	7 13 15	**12**	4.2	**1.3**	**1.6**
DMSO		8 5 15	**9**	5.1	8 5 9	**7**	2.1	**1.0**	**1.0**
Test Item	31.6 µg	15 9 8	**11**	3.8	13 10 9	**11**	2.1	**1.1**	**1.5**
Test Item	100 µg	5 6 11	**7**	3.2	14 9 14	**12**	2.9	**0.8**	**1.7**
Test Item	316 µg	9 10 11	**10**	1.0	7 10 11	**9**	2.1	**1.1**	**1.3**
Test Item	1000 µg	10 15 10	**12**	2.9	12 8 10	**10**	2.0	**1.3**	**1.4**
Test Item	2500 µg	10 7 4	**7**	3.0	11 10 6	**9**	2.6	**0.8**	**1.2**
Test Item	5000 µg	7 8 1	**5**	3.8	8 9 7	**8**	1.0	**0.6**	**1.1**
4-NOPD	40 µg	96 108 125	**110**	14.6	/ / /	/	/	**11.8**	/
2-AA	2.5 µg	/ / /	/	/	342 337 361	**347**	12.7	/	**47.3**

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.		291 281 298	**290**	8.5	187 190 180	**186**	5.1	**1.2**	**1.5**
DMSO		256 239 236	**244**	10.8	126 112 142	**127**	15.0	**1.0**	**1.0**
Test Item	31.6 µg	223 240 241	**235**	10.1	134 138 152	**141**	9.5	**1.0**	**1.1**
Test Item	100 µg	246 236 233	**238**	6.8	142 157 157	**152**	8.7	**1.0**	**1.2**
Test Item	316 µg	212 217 226	**218**	7.1	136 142 128	**135**	7.0	**0.9**	**1.1**
Test Item	1000 µg	222 221 244	**229**	13.0	175 172 155	**167**	10.8	**0.9**	**1.3**
Test Item	2500 µg	163 197 189	**183**	17.8	149 118 124	**130**	16.4	**0.8**	**1.0**
Test Item	5000 µg	135 139 150	**141**	7.8	64 69 88	**74**	12.7	**0.6**	**0.6**
MMS	1 µL	1738 1403 1639	**1593**	172.1	/ / /	**/**	/	**6.5**	**/**
2-AA	10 µg	/ / /	**/**	/	1097 1298 1086	**1160**	119.3	**/**	**9.2**

SD: Standard deviation
B: Background lawn reduced
N: No background lawn

P: Precipitation
C: Contamination

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

11.2. Experiment II (Pre-incubation Test)

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.		29 18 20	**22**	5.9	34 27 25	**29**	4.7	**1.0**	**1.1**
DMSO		25 28 15	**23**	6.8	24 27 28	**26**	2.1	**1.0**	**1.0**
Test Item	31.6 µg	22 22 17	**20**	2.9	36 25 24	**28**	6.7	**0.9**	**1.1**
Test Item	100 µg	16 19 20	**18**	2.1	31 25 23	**26**	4.2	**0.8**	**1.0**
Test Item	316 µg	26 26 16	**23**	5.8	33 30 25	**29**	4.0	**1.0**	**1.1**
Test Item	1000 µg	16 16 15	**16**	0.6	32 41 29	**34**	6.2	**0.7**	**1.3**
Test Item	2500 µg	10 27 18	**18**	8.5	29 30 36	**32**	3.8	**0.8**	**1.2**
Test Item	5000 µg	20 10 23	**18**	6.8	33 19 16	**23**	9.1	**0.8**	**0.9**
4-NOPD	10 µg	537 580 668	**595**	66.8	/ / /	/	/	**26.3**	/
2-AA	2.5 µg	/ / /	/	/	2025 2142 2350	**2172**	164.6	/	**82.5**

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 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.		108 126 121	**118**	9.3	124 116 135	**125**	9.5	**1.3**	**1.2**
DMSO		90 96 96	**94**	3.5	122 100 82	**101**	20.0	**1.0**	**1.0**
Test Item	31.6 µg	103 118 108	**110**	7.6	134 102 99	**112**	19.4	**1.2**	**1.1**
Test Item	100 µg	114 103 104	**107**	6.1	109 109 91	**103**	10.4	**1.1**	**1.0**
Test Item	316 µg	91 100 111	**101**	10.0	113 96 117	**109**	11.2	**1.1**	**1.1**
Test Item	1000 µg	93 86 74	**84**	9.6	97 89 102	**96**	6.6	**0.9**	**0.9**
Test Item	2500 µg	86 76 101	**88**	12.6	98 109 81	**96**	14.1	**0.9**	**0.9**
Test Item	5000 µg	36 48 45	**43**	6.2	85 83 79	**82**	3.1	**0.5**	**0.8**
NaN_3	10 µg	672 728 767	**722**	47.8	/ / /	**/**	/	**7.7**	**/**
2-AA	2.5 µg	/ / /	**/**	/	1989 2163 1922	**2025**	124.4	**/**	**20.0**

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.		5 11 8	**8**	3.0	12 8 12	**11**	2.3	**1.0**	**1.1**
DMSO		8 11 5	**8**	3.0	8 12 8	**9**	2.3	**1.0**	**1.0**
Test Item	31.6 µg	12 11 18	**14**	3.8	8 10 3	**7**	3.6	**1.7**	**0.8**
Test Item	100 µg	15 8 7	**10**	4.4	5 10 11	**9**	3.2	**1.3**	**0.9**
Test Item	316 µg	8 8 6	**7**	1.2	5 13 6	**8**	4.4	**0.9**	**0.9**
Test Item	1000 µg	7 10 11	**9**	2.1	10 4 15	**10**	5.5	**1.2**	**1.0**
Test Item	2500 µg	3 7 5	**5**	2.0	6 10 10	**9**	2.3	**0.6**	**0.9**
Test Item	5000 µg	5 0 5	**3**	2.9	3 5 5	**4**	1.2	**0.4**	**0.5**
NaN_3	10 µg	804 911 720	**812**	95.7	/ / /	**/**	/	**101.5**	**/**
2-AA	2.5 µg	/ / /	**/**	/	100 113 85	**99**	14.0	**/**	**10.6**

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 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.		14 8 11	**11**	3.0	12 7 7	**9**	2.9	**1.1**	**0.9**
DMSO		6 16 8	**10**	5.3	8 18 2	**9**	8.1	**1.0**	**1.0**
Test Item	31.6 µg	7 10 6	**8**	2.1	9 9 10	**9**	0.6	**0.8**	**1.0**
Test Item	100 µg	6 5 9	**7**	2.1	5 8 9	**7**	2.1	**0.7**	**0.8**
Test Item	316 µg	14 9 9	**11**	2.9	11 11 15	**12**	2.3	**1.1**	**1.3**
Test Item	1000 µg	9 7 7	**8**	1.2	11 5 9	**8**	3.1	**0.8**	**0.9**
Test Item	2500 µg	3 11 5	**6**	4.2	10 6 8	**8**	2.0	**0.6**	**0.9**
Test Item	5000 µg	10 9 7	**9**	1.5	14 10 12	**12**	2.0	**0.9**	**1.3**
4-NOPD	40 µg	136 163 132	**144**	16.9	/ / /	**/**	/	**14.4**	**/**
2-AA	2.5 µg	/ / /	**/**	/	241 206 274	**240**	34.0	**/**	**25.8**

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.		357 343 352	**351**	7.1	261 291 262	**271**	17.0	**1.2**	**1.3**
DMSO		298 287 291	**292**	5.6	203 202 226	**210**	13.6	**1.0**	**1.0**
Test Item	31.6 µg	216 247 237	**233**	15.8	189 187 209	**195**	12.2	**0.8**	**0.9**
Test Item	100 µg	231 244 246	**240**	8.1	177 198 176	**184**	12.4	**0.8**	**0.9**
Test Item	316 µg	285 276 274	**278**	5.9	189 204 222	**205**	16.5	**1.0**	**1.0**
Test Item	1000 µg	253 251 251	**252**	1.2	138 139 175	**151**	21.1	**0.9**	**0.7**
Test Item	2500 µg	166 177 204	**182**	19.6	154 170 130	**151**	20.1	**0.6**	**0.7**
Test Item	5000 µg	147 199 206	**184**	32.2	114 120 114	**116**	3.5	**0.6**	**0.6**
MMS	1 µL	1761 1785 1694	**1747**	47.2	/ / /	/	/	**6.0**	/
2-AA	10 µg	/ / /	/	/	1321 1343 968	**1211**	210.4	/	**5.8**

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

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

12. Discussion

The test item Glyphosate TC 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:

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 several tester strains evaluated in experiment I and II.

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

In experiment II toxic effects of the test item were noted in tester strain TA 100 at a dose of 5000 µg/plate (**without** metabolic activation). In tester strain TA 1535 toxic effects of the test item were noted at a dose of 5000 µg/plate (**with** and **without** metabolic activation).

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Glyphosate TC 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.

Conclusion

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

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

13. Distribution of the Report

1 original (paper)	Sponsor
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1 copy (electronic)	Sponsor

14. References

14.1. Literature

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14.2. 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)

15. Annex

15.1. Historical Laboratory Control Data

Table 1: Historical Laboratory Control Data of the Negative Control (in 2007 - 2009) without S9 (-S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	24.0	113.9	13.3	11.0	234.4
SD	4.3	16.2	4.7	3.9	50.4
Min	18	77	5	5	164
Max	46	163	29	30	390
RSD [%]	18.0	14.3	35.4	35.7	21.5
n =	909	921	863	859	588

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 2: Historical Laboratory Control Data of the Positive Control (in 2007 - 2009) without S9 (-S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	522.5	1002.8	1099.2	140.4	1601.4
SD	145.1	240.2	246.7	34.3	308
Min	250	240	389	43	550
Max	1508	2307	1827	453	2407
RSD [%]	27.8	24.0	22.4	24.4	19.2
n =	871	888	825	821	566

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 3: Historical Laboratory Control Data of the Negative Control (in 2007 - 2009) with S9 (+S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	32.1	114.5	10.4	12.1	283.2
SD	6.1	16.7	3.0	4.2	61.6
Min	18	78	5	5	163
Max	57	165	27	36	472
RSD [%]	19.0	14.6	28.8	34.5	21.8
n =	910	921	863	859	588

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 4: Historical Laboratory Control Data of the Positive Control (in 2007 - 2009) with S9 (+S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	2378.0	2083.8	148.3	278.4	1154.1
SD	536.1	528.0	63.0	81.8	316.4
Min	260	500	31	58	419
Max	3599	3341	387	502	2102
RSD [%]	22.5	25.3	42.5	29.4	27.4
n =	872	892	825	821	565

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

15.2. Certificate of Analysis

南通江山农药化工股份有限公司
中国江苏
NANTONG JIANGSHAN AGROCHEMICAL & CHEMICALS LIMITED LIABILITY CO.
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Technological Development Zone, Nantong, Jiangsu, China 传真：Fax: 86-513-83527783

CERTIFICATE OF ANALYSIS

Product:	Glyphosate TC	***Producer:***	Nantong Jiangshan Agrochemical & Chemicals Limited Liability Co.
Helm Sample No.:	37/125/08	***Helm Product No.:***	P-000124
Spec No.:	GB12686-90	***Batch No.:***	20090305
Date of Production:	26 Mar. 2009	***Date of Expiry:***	26 Mar. 2011
Date of Analysis:	26 Mar. 2009	***Quantity:***	250g

Parameter	***Specification***	***Test Result***	***Test Method***
Appearance	White powder	White powder	Visual
Glyphosate acid content	950 g/kg Min.	958 g/kg	GB 12686-2004
formaldehyde	0.8 g/kg Max.	ND	GB 12686-2004
N-nitrosoglyphosate	1 mg/kg Max.	ND	GB 12686-2004
Insoluble in 1 M NaOH	0.2 g/kg Max.	0.1 g/kg	GB 12686-2004

Nantong Jiangshan Agrochemical & Chemicals Limited Liability Co

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