BIOSERVICE

SCIENTIFIC LABORATORIES GmbH

Reverse Mutation Assay using Bacteria

(*Salmonella typhimurium* and *Escherichia coli*)

with Extracts (polar and non-polar) of

Niobium

Report

Version: Final

Date: 27 August 2009

BSL BIOSERVICE Study No.: 092573A

Sponsor:

CBMM Europe BV

WTC H-tower

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The Netherlands

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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
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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

Ritter
Leitender Gewerbedirektor

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	Desoxyribonucleic acid
EC	European Community
EPA	Environmental Protection Agency
GLP	Good Laboratory Practice
his	Histidine
ISO	International Organization of Standardization
mg/kg/bw	Miligram/kilogram/body weight
MMS	Methyl methane sulfonate
4-NOPD	4-Nitro-o-phenylene-diamine
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NaN3	Natriumazide
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:	CBMM Europe BV WTC H-tower Zuidplein 96 1077 XV Amsterdam The Netherlands
Study Monitor:	Mr. Jorge Davo CBMM Companhia Brasileira de Metalurgia e Mineração Córrego da Mata s/n 38183-903 Araxá - MG Brasil
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstrasse 6/8 82152 Planegg Germany
BSL BIOSERVICE Study No.:	092573A
Test Item:	Niobium
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium and Escherichia coli)* with Extracts (polar and non-polar) of Niobium

3.3. Project Staff

Study Director:	Dr. Barbara Wallner
Deputy Study Director:	Dipl.-Biol. Gudrun Schreib
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach
Head of Quality Assurance Unit:	Dipl.-Biol. Uwe Hamann

3.4. Schedule

Arrival of the Test Item:	20 July 2009
Date of Draft Study Plan:	05 August 2009
Date of Final Study Plan:	10 August 2009
Start of Experiment:	10 August 2009
End of Experiment:	17 August 2009
Date of Draft Report:	19 August 2009
Date of Final Report:	27 August 2009

3.5. Project Staff Signatures

Study Director: Dr. Barbara Wallner

B. Wallner
.....
Date: 27 Aug 2009

Management

.....
Print name: Dr. Wolfram Riedel

Date: 28 Aug 2009

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.

The test method is part of the BSL BIOSERVICE accreditation scope according to guideline 90/385/EWG, 93/42/EWG and DIN EN ISO/IEC 17025 for testing of medical devices.

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.

Biological evaluation of medical devices:

ISO 10993-1: 2003, "Evaluation and testing"

ISO 10993-3: 2003, "Tests for genotoxicity, carcinogenicity and reproductive toxicity"

ISO 10993-12: 2007, "Sample preparation and reference materials"

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 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 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. Unless otherwise agreed upon, remaining test item will be discarded three months after release of the report.

5. Statement of Compliance

BSL BIOSERVICE

Study No.:	092573A
Test Item:	Niobium
Study Director:	Dr. Barbara Wallner
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium and Escherichia coli)* with Extracts (polar and non-polar) of Niobium

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: Dr. Barbara Wallner

B. Wallner

Date: 27 Aug 2008

6. Statement of the Quality Assurance Unit

BSL BIOSERVICE

Study No.: 092573A

Test Item: Niobium

Study Director: Dr. Barbara Wallner

Title: Reverse Mutation Assay using Bacteria (*Salmonella typhimurium and Escherichia coli)* with Extracts (polar and non-polar) of Niobium

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

Audit	Dates of QAU Inspections	Dates of Reports to the Study Director and Management
Study Plan	10 August 2009	10 August 2009
Experimental Phase (Method)	25 June 2009	25 June 2009
Draft Report	26 August 2009	26 August 2009
Final Report	27 August 2009	27 August 2009

This report reflects the raw data.

Member of the
Quality Assurance Unit:

Anne Krabiell

Print name: Dipl.oec.troph (FH)
Anne Krabiell

Date: 27 Aug 2009

7. Summary

In order to investigate the potential of extracts (polar and non-polar) of Niobium for its ability to induce gene mutations the pre-incubation test was performed with the *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and tester strain *E. coli* WP2 uvrA.

The test item extracts were tested at several concentrations. The assays were conducted **with** and **without** metabolic activation. The concentrations, including the controls, were tested in triplicate. The following concentrations of the test item extracts were prepared and used in the experiment:

10, 20, 40, 60, 80 and 100%

The 100% extract concentration corresponds to a weight/volume-ratio of 0.2 g/mL 0.9% NaCl resp. DMSO.

Toxic effects of the test item were observed only in experiment II in tester strain TA 1535 at extract concentrations of 100% (**without** metabolic activation).

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Niobium 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, the test item extracts did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, extracts (polar and non-polar) of Niobium are 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* (*S. typhimurium*) and *Escherichia coli (E. coli)* 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 a potential of extracts (polar and non-polar) of Niobium to induce gene mutations in bacteria by means of the *S. Typhimurium* and *Escherichia coli* reverse mutation assay. Due to its high sensitivity, for the performance of the experiments the pre-incubation method was selected.

The *S. typhimurium* histidine (his) reversion system and the *E. coli* tryptophan (trp) reversion system measures $his^- \rightarrow his^+$ reversions and $trp^- \rightarrow trp^+$. The *S. typhimurium* strains are constructed to differentiate between base pair (TA 100, TA 1535) and frameshift (TA 98, TA 1537) mutations (6). The *E. coli* strain WP2 uvrA detects only base substitution mutagens.

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 tumor 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.

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

At least four different concentrations of the test item extracts are examined.

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

Name:	Niobium
Product:	Niobium Metal (Nb)
CAS No.:	7440-03-1
Batch No.:	AD/4202
Chemical name:	Niobium
Density:	~ 8.5 g/cm^3
Active Components:	Nb > 98.5%
Colour:	silver grey metallic
Physical State:	solid
Storage:	room temperature
Safety Precautions:	Routine hygienic procedures were sufficient to assure personnel health and safety.

Since the test item was granulate and not soluble in a suitable solvent, it was extracted in a **polar** extraction medium [0.9% NaCl Lot No.: 25811-1C, DeltaSelect (physiological solution)] and in a **non-polar** extraction medium (DMSO Lot No.: 9K000973, AppliChem) for 72 (± 2) h at 37 (± 1) °C at a weight/volume ratio of 0.2 g/mL according to ISO 10993-3, 2003 and ISO 10993-12, 2007.

9.2. Controls

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

Negative Controls

Vehicle controls, consisting of 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:	*E. coli:* WP2 uvrA
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 *E. coli:* WP2 uvrA
Name:	2-aminoanthracene, 2-AA
Supplier:	Aldrich
Catalogue No.:	A3, 880-0
Lot No.:	S11804-114
Purity:	96%
Dissolved in:	DMSO
Concentrations:	2.5 µg/plate ; 10 µg/plate for *E. coli:* WP2 uvrA

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

9.3. Test System

9.3.1. 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

E. coli:
WP2 uvrA: trp$^-$; uvrA$^-$: base-pair substitutions and others

Tester strains TA 98, TA 100, TA 1535 and TA 1537 were obtained from MOLTOX, INC., NC 28607, USA. The *E. coli* strain was obtained from DSMZ Sales, Braunschweig, Germany. 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 and TA 100 contain the R-factor plasmide, 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 tester strain *E. coli* WP2 uvrA carries the defect in one of the genes for tryptophan biosynthesis. Tryptophan-independent 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 mutagens which substitute one base for another. Additionally, the strain is deficient in the DNA nucleotide excision repair system.

The properties of the *S. typhimurium* and *E. coli* 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.

9.3.2. 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

The *E. coli* medium (Luria Bertani) contains per litre:

10	g	Tryptone
10	g	NaCl
5	g	Yeast extract

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

9.3.3. 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.

9.3.4. Overlay Agar

The overlay agar contains per litre:

S. typhimurium:

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

E. coli:

7.0	g	Agar Agar
6.0	g	NaCl
10.2	mg	Tryptophan

Sterilisation was performed at 121 °C in an autoclave.

9.3.5. 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 is added in form of mammalian microsome enzyme activation mixture.

9.3.6. 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 ß-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: 250609) was 31 mg/mL. The S9 mix preparation was performed according to Ames *et al.* (1).

9.3.7. Preparation of S9 Mix

The S9 mix preparation was performed according to Ames *et al* (1). 100 mM of ice-cold sodium-ortho-phosphate-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.

9.4. Experimental Design

9.4.1. Pre-Experiment for Toxicity

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

9.4.2. Exposure Concentrations

The test item was tested with the following extract concentrations in the experiments:

10, 20, 40, 60, 80 and 100%

9.4.3. Experimental Performance

The following materials were mixed in a test tube and incubated for 60 min. at 37 °C (pre-incubation method):

100 µL	Test extract at each dose level, extract vehicle 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),

After the incubation period (60 min.), the overlay agar (2000 µL) was added and poured 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.

9.5. Data Recording

The colonies were counted using a ProtoCOL counter (Meintrup DWS Laborgeräte GmbH). If precipitation of the test item precludes 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.

9.6. 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 extract vehicle control.

9.7. Criteria of Validity

A test is considered acceptable if for each strain:

- the bacteria demonstrate their typical responses to ampicillin (TA 98, TA 100)
- the control plates 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 - 61
TA 100:	75 - 159	81 - 165
TA 1535:	5 - 29	5 - 31
TA 1537:	5 - 30	5 - 36
E. coli WP2 uvrA	35 - 92	38 - 101

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

9.8. Evaluation of Mutagenicity

The Mutation Factor is calculated by dividing the mean value of the revertant counts through the mean values of the extract vehicle 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 *E. coli* WP2 uvrA 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 extract vehicle 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. Deviation from the Study Plan

There were no deviations from the Study Plan.

11. Results

11.1. Experiment I (polar extraction medium: 0.9% NaCl)

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.		20 20 27	**22**	4.0	35 20 28	**28**	7.5	**0.8**	**1.0**
0.9% NaCl		30 29 30	**30**	0.6	24 32 30	**29**	4.2	**1.0**	**1.0**
Test Item	10 %	23 23 20	**22**	1.7	23 27 31	**27**	4.0	**0.7**	**0.9**
Test Item	20 %	14 21 14	**16**	4.0	28 35 27	**30**	4.4	**0.6**	**1.0**
Test Item	40 %	13 24 32	**23**	9.5	26 24 24	**25**	1.2	**0.8**	**0.9**
Test Item	60 %	32 15 30	**26**	9.3	30 39 43	**37**	6.7	**0.9**	**1.3**
Test Item	80 %	25 16 24	**22**	4.9	26 18 37	**27**	9.5	**0.7**	**0.9**
Test Item	100 %	23 25 22	**23**	1.5	31 35 24	**30**	5.6	**0.8**	**1.0**
4-NOPD	10 µg	529 544 516	**530**	14.0	/ / /	**/**	/	**17.9**	**/**
2-AA	2.5 µg	/ / /	**/**	/	384 300 790	**491**	262.0	**/**	**17.1**

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

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

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.		107 113 104	**108**	4.6	125 118 101	**115**	12.3	**1.1**	**1.1**
0.9% NaCl		89 101 103	**98**	7.6	96 104 101	**100**	4.0	**1.0**	**1.0**
Test Item	10 %	98 109 125	**111**	13.6	96 95 104	**98**	4.9	**1.1**	**1.0**
Test Item	20 %	102 104 83	**96**	11.6	105 101 109	**105**	4.0	**1.0**	**1.0**
Test Item	40 %	100 120 105	**108**	10.4	104 93 104	**100**	6.4	**1.1**	**1.0**
Test Item	60 %	113 93 109	**105**	10.6	99 110 92	**100**	9.1	**1.1**	**1.0**
Test Item	80 %	100 96 121	**106**	13.4	107 98 127	**111**	14.8	**1.1**	**1.1**
Test Item	100 %	124 108 105	**112**	10.2	102 99 115	**105**	8.5	**1.2**	**1.0**
NaN_3	10 µg	721 754 838	**771**	60.3	/ / /	/	/	**7.9**	/
2-AA	2.5 µg	/ / /	/	/	454 321 1080	**618**	405.3	/	**6.2**

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

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

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.		3 7 10	**7**	3.5	9 12 9	**10**	1.7	**1.0**	**1.9**
0.9% NaCl		5 7 9	**7**	2.0	3 5 8	**5**	2.5	**1.0**	**1.0**
Test Item	10 %	14 7 9	**10**	3.6	8 C 5 3	**5**	2.5	**1.4**	**1.0**
Test Item	20 %	7 9 7	**8**	1.2	15 11 8	**11**	3.5	**1.1**	**2.1**
Test Item	40 %	7 7 12	**9**	2.9	6 11 10	**9**	2.6	**1.2**	**1.7**
Test Item	60 %	8 6 9	**8**	1.5	10 13 10	**11**	1.7	**1.1**	**2.1**
Test Item	80 %	14 10 11	**12**	2.1	9 7 11	**9**	2.0	**1.7**	**1.7**
Test Item	100 %	6 9 11	**9**	2.5	10 9 10	**10**	0.6	**1.2**	**1.8**
NaN_3	10 µg	1136 1334 1364	**1278**	123.9	/ / /	**/**	/	**182.6**	/
2-AA	2.5 µg	/ / /	**/**	/	55 71 102	**76**	23.9	/	**14.3**

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

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

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.		10 4 5	**6**	3.2	6 9 3	**6**	3.0	**1.5**	**1.0**
0.9% NaCl		4 5 4	**4**	0.6	8 6 4	**6**	2.0	**1.0**	**1.0**
Test Item	10 %	4 8 15	**9**	5.6	6 7 9	**7**	1.5	**2.1**	**1.2**
Test Item	20 %	8 7 6	**7**	1.0	7 5 5	**6**	1.2	**1.6**	**0.9**
Test Item	40 %	5 8 6	**6**	1.5	7 5 3	**5**	2.0	**1.5**	**0.8**
Test Item	60 %	9 4 2	**5**	3.6	5 5 11	**7**	3.5	**1.2**	**1.2**
Test Item	80 %	8 7 6	**7**	1.0	3 10 6	**6**	3.5	**1.6**	**1.1**
Test Item	100 %	4 6 7	**6**	1.5	2 11 7	**7**	4.5	**1.3**	**1.1**
4-NOPD	40 µg	104 165 102	**124**	35.8	/ / /	**/**	/	**28.5**	**/**
2-AA	2.5 µg	/ / /	**/**	/	95 82 110	**96**	14.0	**/**	**15.9**

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

P: Precipitation
C: Contamination

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

Tester Strain: WP2 uvrA

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.		56 72 49	**59**	11.8	80 77 60	**72**	10.8	**1.0**	**1.2**
0.9% NaCl		58 75 46	**60**	14.6	52 66 69	**62**	9.1	**1.0**	**1.0**
Test Item	10 %	44 45 51	**47**	3.8	67 64 68	**66**	2.1	**0.8**	**1.1**
Test Item	20 %	42 29 54	**42**	12.5	51 57 67	**58**	8.1	**0.7**	**0.9**
Test Item	40 %	89 48 39	**59**	26.7	68 59 52	**60**	8.0	**1.0**	**1.0**
Test Item	60 %	43 67 58	**56**	12.1	44 63 50	**52**	9.7	**0.9**	**0.8**
Test Item	80 %	80 73 62	**72**	9.1	54 58 59	**57**	2.6	**1.2**	**0.9**
Test Item	100 %	74 49 54	**59**	13.2	51 58 55	**55**	3.5	**1.0**	**0.9**
MMS	1 µL	345 376 447	**389**	52.3	/ / /	/	/	**6.5**	/
2-AA	10 µg	/ / /	/	/	144 134 126	**135**	9.0	/	**2.2**

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

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

11.2. Experiment II (non-polar extraction medium: DMSO)

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.		25 26 27	**26**	1.0	29 33 C 34	**32**	2.6	**1.2**	**0.9**
DMSO		22 19 24	**22**	2.5	51 29 30	**37**	12.4	**1.0**	**1.0**
Test Item	10 %	21 20 19	**20**	1.0	40 30 42	**37**	6.4	**0.9**	**1.0**
Test Item	20 %	24 20 27	**24**	3.5	33 38 21	**31**	8.7	**1.1**	**0.8**
Test Item	40 %	25 17 17	**20**	4.6	39 41 12	**31**	16.2	**0.9**	**0.8**
Test Item	60 %	20 35 17	**24**	9.6	40 37 34	**37**	3.0	**1.1**	**1.0**
Test Item	80 %	18 25 18	**20**	4.0	32 27 45	**35**	9.3	**0.9**	**0.9**
Test Item	100 %	34 17 19	**23**	9.3	34 45 36	**38**	5.9	**1.1**	**1.0**
4-NOPD	10 µg	375 333 509	**406**	91.9	/ / /	**/**	/	**18.7**	**/**
2-AA	2.5 µg	/ / /	**/**	/	927 1954 1890	**1590**	575.4	**/**	**43.4**

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

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

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.		101 120 111	**111**	9.5	122 114 105	**114**	8.5	**1.3**	**1.3**
DMSO		81 103 81	**88**	12.7	92 103 70	**88**	16.8	**1.0**	**1.0**
Test Item	10 %	97 100 103	**100**	3.0	85 103 96	**95**	9.1	**1.1**	**1.1**
Test Item	20 %	85 113 92	**97**	14.6	93 101 80	**91**	10.6	**1.1**	**1.0**
Test Item	40 %	80 83 85	**83**	2.5	96 76 78	**83**	11.0	**0.9**	**0.9**
Test Item	60 %	97 79 72	**83**	12.9	101 78 84	**88**	11.9	**0.9**	**1.0**
Test Item	80 %	70 63 80	**71**	8.5	84 80 67	**77**	8.9	**0.8**	**0.9**
Test Item	100 %	82 82 114	**93**	18.5	92 91 103	**95**	6.7	**1.0**	**1.1**
NaN_3	10 µg	704 820 1009	**844**	153.9	/ / /	/	/	**9.6**	/
2-AA	2.5 µg	/ / /	/	/	1292 1622 2254	**1723**	488.8	/	**19.5**

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

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

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 9 6	**9**	2.5	18 8 5	**10**	6.8	**0.7**	**1.8**
DMSO		10 7 18	**12**	5.7	6 2 9	**6**	3.5	**1.0**	**1.0**
Test Item	10 %	10 11 7	**9**	2.1	6 11 9	**9**	2.5	**0.8**	**1.5**
Test Item	20 %	7 9 11	**9**	2.0	8 4 7	**6**	2.1	**0.8**	**1.1**
Test Item	40 %	10 9 6	**8**	2.1	5 8 5	**6**	1.7	**0.7**	**1.1**
Test Item	60 %	6 4 7	**6**	1.5	5 9 5	**6**	2.3	**0.5**	**1.1**
Test Item	80 %	8 9 5	**7**	2.1	12 11 7	**10**	2.6	**0.6**	**1.8**
Test Item	100 %	5 6 6	**6**	0.6	8 7 6	**7**	1.0	**0.5**	**1.2**
NaN_3	10 µg	1208 1008 1322	**1179**	159.0	/ / /	/	/	**101.1**	/
2-AA	2.5 µg	/ / /	/	/	145 112 102	**120**	22.5	/	**21.1**

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

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

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.		7 6 8	**7**	1.0	7 3 6	**5**	2.1	**1.4**	**0.9**
DMSO		5 7 3	**5**	2.0	8 5 5	**6**	1.7	**1.0**	**1.0**
Test Item	10 %	8 11 3	**7**	4.0	10 11 12	**11**	1.0	**1.5**	**1.8**
Test Item	20 %	7 12 3	**7**	4.5	7 8 9	**8**	1.0	**1.5**	**1.3**
Test Item	40 %	8 5 7	**7**	1.5	4 7 9	**7**	2.5	**1.3**	**1.1**
Test Item	60 %	13 5 6	**8**	4.4	4 4 2	**3**	1.2	**1.6**	**0.6**
Test Item	80 %	10 2 4	**5**	4.2	8 10 8	**9**	1.2	**1.1**	**1.4**
Test Item	100 %	4 6 8	**6**	2.0	9 7 7	**8**	1.2	**1.2**	**1.3**
4-NOPD	40 µg	89 154 136	**126**	33.6	/ / /	/	/	**25.3**	/
2-AA	2.5 µg	/ / /	/	/	110 82 176	**123**	48.3	/	**20.4**

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

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

Tester Strain: WP2 uvrA 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.		64 71 70	**68**	3.8	81 74 73	**76**	4.4	**1.3**	**1.2**
DMSO		60 51 43	**51**	8.5	71 59 60	**63**	6.7	**1.0**	**1.0**
Test Item	10 %	64 33 35	**44**	17.3	62 58 76	**65**	9.5	**0.9**	**1.0**
Test Item	20 %	57 43 64	**55**	10.7	54 48 58	**53**	5.0	**1.1**	**0.8**
Test Item	40 %	48 55 68	**57**	10.1	70 72 64	**69**	4.2	**1.1**	**1.1**
Test Item	60 %	42 44 45	**44**	1.5	63 62 78	**68**	9.0	**0.9**	**1.1**
Test Item	80 %	54 51 49	**51**	2.5	58 69 52	**60**	8.6	**1.0**	**0.9**
Test Item	100 %	60 49 56	**55**	5.6	55 50 66	**57**	8.2	**1.1**	**0.9**
MMS	1 µL	402 479 560	**480**	79.0	/ / /	/	/	**9.4**	/
2-AA	10 µg	/ / /	/	/	144 134 126	**135**	9.0	/	**2.1**

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

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

12. Discussion

Extracts (polar and non-polar) of Niobium were investigated for their potential to induce gene mutations according to the pre-incubation method using *Salmonella typhimurium* tester strains TA 98, TA 100, TA 1535, TA 1537 and tester strain *E. coli* WP2 uvrA.

Several concentrations of the test item extracts 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 extracts were prepared and used in the experiments:

10, 20, 40, 60, 80 and 100%

The 100% extract concentration corresponds to a weight/volume-ratio of 0.2 g/mL 0.9% NaCl resp. DMSO.

Toxic effect of the test item extract was observed only in experiment II in tester strain TA 1535 at extract concentrations of 100% (**without** metabolic activation). The reduction in the number of revertants down to a mutation factor of 0.5 found in experiment II tester strain TA 1535 at an extract concentration of 60% (**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 extracts (polar and non-polar) of Niobium at any concentration level, neither in the presence nor absence of metabolic activation in both experiments.

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, the test item extracts did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, extracts (polar and non-polar) of Niobium are considered to be non-mutagenic in this bacterial reverse mutation assay.

13. Distribution of the Report

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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

Table 1: Historical Laboratory Control Data of the Negative Control (in 2006 - 2008) without S9 (-S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	24.9	111.7	14.9	11.7	239.3
SD	4.8	15.7	4.7	4.0	50.0
Min	18	75	5	5	165
Max	46	159	29	30	390
RSD [%]	19.3	14.0	31.3	34.1	20.9
n =	894	905	873	856	601

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 2006 - 2008) without S9 (-S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	557.8	976.0	1048.9	150.6	1718.6
SD	168.7	244.9	252.8	34.0	359.4
Min	250	235	234	48	221
Max	1543	2307	1827	453	2825
RSD [%]	30.3	25.1	24.1	22.6	20.9
n =	885	895	863	845	595

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 2006 - 2008) with S9 (+S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	33.8	113.1	11.3	12.8	293.1
SD	6.9	15.3	3.2	4.2	65.7
Min	18	81	5	5	163
Max	61	165	31	36	541
RSD [%]	20.4	13.5	28.3	32.8	22.4
n =	893	910	872	856	600

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 2006 - 2008) with S9 (+S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	2276.5	2064.3	138.9	279.2	1161.5
SD	609.2	561.5	62.0	84.6	332.0
Min	255	253	29	44	117
Max	3599	3314	387	509	2540
RSD [%]	26.8	27.2	44.6	30.3	28.6
n =	884	900	858	846	593

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