1 2

RCC - CCR STUDY NUMBER 856901

SALMONELLA TYPHIMURIUM
REVERSE MUTATION ASSAY

WITH

2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE
SULFATE (WR 23081)

FINAL REPORT

STUDY COMPLETION DATE:
March 16, 2005

1 COPY OF GLP CERTIFICATE

HESSISCHES MINISTERIUM
FÜR UMWELT, LANDWIRTSCHAFT
UND FORSTEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

RCC Cytotest Cell Research GmbH
der gleichnamigen Firma
In den Leppsteinwiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

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

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften — 2 Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo) — 3 Mutagenicity studies
8 Analytische Prüfungen an biologischen Materialien — 8 Analytical studies on biological materials
9 Virussicherheitsprüfungen — 9 Virus validation studies

11.06. bis 13.06.2001
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

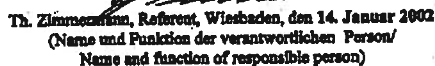
Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag



Th. Zimmermann, Referent, Wiesbaden, den 14. Januar 2002
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

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

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3 PREFACE

3.1 General

Title:	Salmonella typhimurium Reverse Mutation Assay with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081)
Sponsor:	
Study Monitor:	
Test Facility:	R C C Cytotest Cell Research GmbH (RCC-CCR) In den Leppsteinswiesen 19 D-64380 Roßdorf

3.2 Responsibilities

Study Director:	Dipl. Biol. Andrea Sokolowski
Deputy Study Director:	Dr. Hans-Eric Wollny
Management:	Dr. Wolfgang Völkner
Head of Quality Assurance Unit:	Frauke Hermann

3.3 Schedule

Date of the Study Plan:	November	17, 2004
Experimental Starting Date:	December	13, 2004
Experimental Completion Date:	January	10, 2005
Date of Draft Report:	January	11, 2005
Date of Final Report:	March	16, 2005

3.4 Project Staff Signatures

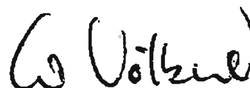
Study Director

Dipl. Biol. Andrea Sokolowski

Date: March 16, 2005

Management

Dr. Wolfgang Völkner



Date: March 16, 2005

3.5 Good Laboratory Practice

The study was performed in compliance with:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1) dated July 25, 1994 („BGBl. I 1994", pp. 1703), last revision dated June 27, 2002.

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final].

3.6 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 July 21, 1997.

"Commission Directive 2000/32/EC, L1362000, Annex 4D", dated May 19, 2000.

3.7 Archiving

RCC Cytotest Cell Research will archive the following data for 15 years:

Raw data, study plan, final report, and a sample of the test item.

No data will be discarded without the sponsor's consent.

3.8 Deviations from the Study Plan

There were no deviations from the study plan.

4 STATEMENT OF COMPLIANCE

Study Number:	856901
Test Item:	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081)
Study Director:	Dipl. Biol. Andrea Sokolowski
Title:	Salmonella Typhimurium Reverse Mutation Assay with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081)

This study performed in the test facility of RCC Cytotest Cell Research was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1) dated July 25, 1994 („BGBl. I 1994“, pp. 1703), last revision dated June 27, 2002.

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final].

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

Study Director

RCC - CCR
Dipl. Biol. Andrea Sokolowski

Date: March 16, 2005

5 STATEMENT OF QUALITY ASSURANCE UNIT

Study Number:	856901
Test Item:	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081)
Study Director:	Dipl. Biol. Andrea Sokolowski
Title:	Salmonella Typhimurium Reverse Mutation Assay with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081)

The general facilities and activities of RCC Cytotest Cell Research GmbH are inspected periodically and the results are reported to the responsible person and the management.

Study procedures were inspected periodically. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

Phases and Dates of QAU Inspections/ Audits		Dates of Reports to the Study Director and to Management
Study Plan (Draft):	November 16, 2004	November 16, 2004
Study Plan:	November 29, 2004	November 29, 2004
Process Inspection:	December 17, 2004 January 06, 2005	December 17, 2004 January 06, 2005
Draft Report:	February 03, 2005	February 03, 2005

This statement is to confirm that the present final report reflects the raw data.

Head of Quality Assurance Unit Frauke Hermann

Date: March 16, 2005

6 SUMMARY OF RESULTS

This study was performed to investigate the potential of 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081) to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations:

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

Experiment I and II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

The plates incubated with the test item showed reduced background growth at higher concentrations in nearly all strains used.

Minor toxic effects, evident as a reduction in the number of revertants, occurred with and without metabolic activation in nearly all strains used.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081) at any dose level, neither in the presence nor in the absence of metabolic activation (S9 mix).

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

6.1 Conclusion

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

Therefore, 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081) is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

7 OBJECTIVE

7.1 Aims of the Study

The experiments were performed to assess the potential of the test item to induce gene mutations by means of two independent Salmonella typhimurium reverse mutation assays. Experiment I was performed as a plate incorporation assay. Since a negative result was obtained in this experiment, experiment II was performed as a pre-incubation assay.

7.2 Reasons for the Study

The most widely used assays for detecting gene mutations are those using bacteria (3). They are relatively simple and rapid to perform, and give reliable data on the ability of an agent to interact with DNA and produce mutations.

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

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

According to the direct plate incorporation and 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.

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

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

8 MATERIALS AND METHODS

8.1 Test Item

Internal RCC-CCR Test Item Number: S 5031 11

The test item and the information concerning the test item were provided by the sponsor.

Identity:	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE(WR 23081)
Batch no.:	57
Sample no.:	R96000196
Aggregate state at room temperature:	solid
Colour:	pale grey
Molecular weight:	280.30
Purity:	99.6 area% (HPLC)
Stability in solution:	stable for up to two days in water and DMSO
Storage:	room temperature, moisture protected, light protected
Expiry date:	July, 2005

On the day of the experiment, the test item 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081) was dissolved in deionised water. The solvent was chosen because of its solubility properties (4).

No precipitation of the test item occurred up to the highest investigated dose.

8.2 Controls

8.2.1 Negative Controls

Concurrent untreated and solvent controls were performed.

8.2.2 Positive Control Substances

Without metabolic activation

Strains: TA 1535, TA 100
Name: sodium azide, NaN_3
Supplier: SERVA, D-69042 Heidelberg
Catalogue No.: 30175
Purity: at least 99 %
Dissolved in: water deionised
Concentration: 10 µg/plate

Strains: TA 1537, TA 98
Name: 4-nitro-o-phenylene-diamine, 4-NOPD
Supplier: SIGMA, D-82041 Deisenhofen
Catalogue No.: N 9504
Purity: > 99.9 %
Dissolved in: DMSO (purity >99 %, MERCK, D-64293 Darmstadt)
Concentration: 10 µg/plate in TA 98, 50 µg/plate in TA 1537

Strain: TA 102
Name: methyl methane sulfonate, MMS
Supplier: MERCK-SCHUCHARDT, D-85662 Hohenbrunn
Catalogue No.: 820775
Purity: > 99.0 %
Dissolved in: water deionised
Concentration: 4.0 µL/plate

With metabolic activation

Strains: TA 1535, TA 1537, TA 98, TA 100, TA 102
Name: 2-aminoanthracene, 2-AA
Supplier: SIGMA, D-82041 Deisenhofen
Catalogue No.: A 1381
Purity: 97.5 %
Dissolved in: DMSO (purity >99 %, MERCK, D-64293 Darmstadt)
Concentration: 2.5 µg/plate (10.0 µg/plate in TA 102)

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

8.3 Test System

8.3.1 Characterisation of the Salmonella typhimurium Strains

The histidine dependent strains are derived from S. typhimurium strain LT2 through a mutation in the histidine locus. Additionally due to the "deep rough" (rfa$^-$) mutation they possess a faulty lipopolysaccharide envelope which enables substances to penetrate the cell wall more easily. A further mutation (deletion of the uvrB gene) causes an inactivation of the excision repair system. The latter alteration also includes a deletion in the nitrate reductase and biotin genes. In the strains TA 98, TA 100, and TA 102 the R-factor plasmid pKM 101 carries umu DC analogous genes that are involved in error-prone repair and the ampicillin resistance marker. The strain TA 102 does not contain the uvrB$^-$-mutation and is excision repair proficient. Additionally, TA 102 contains the multicopy plasmid pAQ1 carrying the hisG428 mutation (ochre mutation in the hisG gene) and a tetracycline resistance gene (5).

In summary, the mutations of the TA strains used in this study can be described as follows:

Salmonella typhimurium		
Strains	**Genotype**	**Type of mutations indicated**
TA 1537	his C 3076; rfa$^-$; uvrB$^-$:	frame shift mutations
TA 98	his D 3052; rfa$^-$; uvrB$^-$;R-factor	" "
TA 1535	his G 46; rfa$^-$; uvrB$^-$:	base-pair substitutions
TA 102	his G 428; rfa$^-$; uvrB$^+$;R-factor	" "
TA 100	his G 46; rfa$^-$; uvrB$^-$;R-factor	" "

Regular checking of the properties of the strains regarding the membrane permeability, ampicillin- and tetracycline-resistance as well as spontaneous mutation rates is performed in the laboratory of RCC Cytotest Cell Research according to B. Ames et al. (1) and D. Maron and B. Ames (5). In this way it was ensured that the experimental conditions set down by Ames were fulfilled.

The bacterial strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 were obtained from Trinova Biochem GmbH (35394 Gießen, Germany).

8.3.2 Storage

The strain cultures were stored as stock cultures in ampoules with nutrient broth + 5 % DMSO (MERCK, D-64293 Darmstadt) in liquid nitrogen.

8.3.3 Precultures

From the thawed ampoules of the strains 0.5 mL bacterial suspension was transferred into 250 mL Erlenmeyer flasks containing 20 mL nutrient medium. A solution of 20 µL ampicillin (25 µg/mL) was added to the strains TA 98, TA 100, and TA 102. Additionally 20 µL tetracycline (2 µg/mL) was added to strain TA 102. This nutrient medium contains per litre:

8 g Merck Nutrient Broth (MERCK, D-64293 Darmstadt)
5 g NaCl (MERCK, D-64293 Darmstadt)

The bacterial cultures were incubated in a shaking water bath for 4 hours at 37° C.

8.3.4 Selective Agar

The plates with the minimal agar were obtained from E. Merck, D-64293 Darmstadt.

8.3.5 Overlay Agar

The overlay agar contains per litre:

6.0 g MERCK Agar Agar*
6.0 g NaCl*
10.5 mg L-Histidine x HCl x H_2O*
12.2 mg Biotin*
* (MERCK, D-64293 Darmstadt)

Sterilisations were performed at 121° C in an autoclave.

8.4 Mammalian Microsomal Fraction S9 Mix

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

8.4.1 S9 (Preparation by R C C - C C R)

Phenobarbital/β-Naphthoflavone induced rat liver S9 is used as the metabolic activation system. The S9 is prepared from 8 - 12 weeks old male Wistar Hanlbm rats, weight approx. 220 - 320 g induced by applications of 80 mg/kg b.w. Phenobarbital i.p. (Desitin; D-22335 Hamburg) and β-Naphthoflavone p.o. (Aldrich, D-89555 Steinheim) each on three consecutive days. The livers are prepared 24 hours after the last treatment. The S9 fractions are produced by dilution of the liver homogenate with a KCl solution (1+3) followed by centrifugation at 9000 g. Aliquots of the supernatant are frozen and stored in ampoules at -80° C. Small numbers of the ampoules can be kept at -20°C for up to one week.

The protein concentration in the S9 preparation was 27.1 mg/mL (lot no. R 041104) in all experiments.

8.4.2 S9 Mix

Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 15% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

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

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment the S9 mix was stored in an ice bath. The S9 mix preparation was performed according to Ames et al.(1).

8.5 Pre-Experiment for Toxicity

To evaluate the toxicity of the test item a pre-experiment was performed with strains TA 98 and TA 100. Eight concentrations were tested for toxicity and mutation induction with each 3 plates. The experimental conditions in this pre-experiment were the same as described for the experiment I below (plate incorporation test).

Toxicity of the test item can be evident as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn.

The pre-experiment is reported as part of the main experiment I, since the following criteria are met:

Evaluable plates (>0 colonies) at five concentrations or more in all strains used.

8.6 Dose Selection

In the pre-experiment the concentration range of the test item was 3 – 5000 µg/plate. The pre-experiment is reported as part of experiment I since no relevant toxic effects were observed and 5000 µg/plate were chosen as maximal concentration.

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

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

Experiment I and II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

8.7 Experimental Performance

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

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

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

In the pre-incubation assay 100 µL test solution, 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and incubated at 37°C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45° C) was added to each tube. The mixture was poured on minimal agar plates.

After solidification the plates were incubated upside down for at least 48 hours at 37° C in the dark (2).

8.8 Data Recording

The colonies were counted using the Petri Viewer Mk2 (Perceptive Instruments Ltd, Suffolk CB 7BN, UK) with the software program Ames Study Manager v1.2. The counter was connected to an IBM AT compatible PC with printer which printed out both, the individual and mean values of the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates (see tables of results). Due to the intense colour of the test item the colonies were counted manually at higher concentrations (cf. tables of results).

8.9 Acceptability of the Assay

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

- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of our historical data
- the positive control substances should produce a significant increase in mutant colony frequencies

8.10 Evaluation of Results

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed (3).

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration (2).

An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

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

8.11 Biometry

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

9 DISCUSSION OF RESULTS

The test item 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081) was assessed 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 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed with and without liver microsomal activation. Each concentration and the controls, were tested in triplicate. The test item was tested at the following concentrations:

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

Experiment I and II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

The plates incubated with the test item showed reduced background growth at the following concentrations (µg/plate):

Strain	Experiment I		Experiment II	
	without S9 mix	with S9 mix	without S9 mix	with S9 mix
TA 1535	5000	/	5000	/
TA 1537	5000	5000	5000	/
TA 98	5000	/	2500 - 5000	/
TA 100	5000	/	2500 - 5000	/
TA 102	5000	5000	5000	5000

/ = no reduced background growth observed

Minor toxic effects, evident as a reduction in the number of revertants were observed at the following concentrations (µg/plate):

Strain	Experiment I		Experiment II	
	without S9 mix	with S9 mix	without S9 mix	with S9 mix
TA 1535	5000	/	5000	/
TA 1537	5000	/	/	/
TA 98	/	/	5000	/
TA 100	/	5000	2500 - 5000	5000
TA 102	5000	5000	5000	5000

/ = no toxic effects observed

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081) at any concentration level, neither in the presence nor in the absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance with the exception of strain TA 98 in experiment I and II. A minor increase in revertant colony numbers was observed in experiment I at 1000 and 2500 µg/plate without S9 mix and at 1000 µg/plate with S9 mix and in experiment II at 1000 and 2500 µg/plate with S9 mix. However, the required threshold of two times the number of the corresponding solvent control was not reached. Since this increase in the number of revertant colonies is rather small, this effect is considered to be based upon biologically irrelevant fluctuations in the number of colonies.

Also in strains TA 1537, TA 98, and TA 102 with metabolic activation the historical solvent control range was slightly exceeded at 333 and 1000 µg/plate (TA 98, exp. I), from 100 up to 2500 µg/plate (TA 98, exp. II), at 2500 and 5000 µg/plate (TA 1537, exp. II) and at 333 µg/plate (TA 102, exp. II). Due to the fact that a new image analysis system was used for the scoring of the revertant colonies the historical data are based on 80 experiments only. Since this number of experiments is rather small, colony counts that exceed the historical data range slightly can still be excepted for the controls and the test item.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

The historical control range was slightly exceeded in strains TA 1535 (negative control) and TA 102 (negative control) without S9 mix and in strains TA 1537 (negative control) and TA 102 (solvent and negative control) with S9 mix. Since this deviation is rather small, this effect is considered to be based upon biologically irrelevant fluctuations in the number of colonies.

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

10 REFERENCES

1. Ames, B.N., McCann, J. und Yamasaki, E. (1975)
   Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test.
   Mutation Res., 31, 347-363

2. de Serres F.J. and M.D. Shelby (1979)
   Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay
   Mutation Res. 64, 159-165

3. Hollstein,M., J. McCann, F.A. Angelosanto and W.W. Nichols (1979)
   Short-term tests for carcinogens and mutagens
   Mutation Res. 65, 133-226

4. Maron D.M., J. Katzenellenbogen and B.N. Ames, (1981)
   Compatibility of organic solvents with the Salmonella/Microsome Test
   Mutation Res. 88, 343-350

5. Maron, D.M., Ames, B.N. (1983)
   Revised methods for the Salmonella mutagenicity test
   Mutation Res. 113, 173-215

11 DISTRIBUTION OF THE REPORT

Sponsor	2x (2x copy)
Study Director	1x (original)

12 SUMMARY OF RESULTS

12.1 Summary of Results Pre-Experiment

Study Name: 856901
Experiment: 856901 VV Plate
Assay Conditions: Plate Incorporation

Study Code: RCC - CCR 856901
Date Plated: 13/12/2004
Date Counted: 17/12/2004

Metabolic Activation	Test Item	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) TA 98	TA 100
Without Activation	Deionised water		30 ± 5	115 ± 1
	Untreated		30 ± 7	119 ± 13
	WR 23 081	3 µg	28 ± 6	120 ± 12
		10 µg	35 ± 5	133 ± 6
		33 µg	34 ± 10	128 ± 16
		100 µg	36 ± 9	129 ± 3
		333 µg	41 ± 16	127 ± 8
		1000 µg	51 ± 5	127 ± 8
		2500 µg	51 ± 6	141 ± 20
		5000 µg	18 ± 5	92 ± 9
	4-nitro-o-phenylene-diamine	10 µg	415 ± 28	
	sodium azide	10 µg		2901 ± 71
With Activation	Deionised water		41 ± 7	112 ± 18
	Untreated		47 ± 4	110 ± 18
	WR 23 081	3 µg	47 ± 7	121 ± 11
		10 µg	50 ± 10	116 ± 3
		33 µg	48 ± 12	112 ± 24
		100 µg	42 ± 8	108 ± 10
		333 µg	63 ± 9	102 ± 21
		1000 µg	74 ± 10	119 ± 4
		2500 µg	58 ± 3	101 ± 23
		5000 µg	19 ± 1	37 ± 9
	2-aminoanthracene	2.5 µg	3528 ± 219	3569 ± 134

12.2 Summary of Results Experiment I

Study Name: 856901
Experiment: 856901 HV1
Assay Conditions: Plate Incorporation

Study Code: RCC - CCR 856901
Date Plated: 30/12/2004
Date Counted: 04/01/2005

Metabolic Activation	Test Item	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)		
			TA 1535	TA 1537	TA 102
Without Activation	Deionised water		17 ± 3	10 ± 3	357 ± 22
	Untreated		16 ± 6	9 ± 3	317 ± 41
	WR 23 081	33 µg	14 ± 6	11 ± 5	336 ± 32
		100 µg	16 ± 4	7 ± 1	367 ± 11
		333 µg	14 ± 1	21 ± 6	393 ± 20
		1000 µg	15 ± 5	9 ± 1	329 ± 20
		2500 µg	14 ± 2	12 ± 3	229 ± 30
		5000 µg	7 ± 3	3 ± 2	46 ± 10
	sodium azide	10 µg	1477 ± 77		
	4-nitro-o-phenylene-diamine	50 µg		68 ± 15	
	methyl methane sulfonate	4.0 µL			5588 ± 151
With Activation	Deionised water		15 ± 1	16 ± 3	430 ± 19
	Untreated		15 ± 2	18 ± 5	428 ± 34
	WR 23 081	33 µg	20 ± 11	21 ± 5	492 ± 57
		100 µg	16 ± 2	17 ± 4	503 ± 37
		333 µg	15 ± 2	18 ± 3	462 ± 28
		1000 µg	15 ± 1	19 ± 1	321 ± 33
		2500 µg	13 ± 2	29 ± 6	208 ± 15
		5000 µg	15 ± 4	15 ± 6	57 ± 15
	2-aminoanthracene	2.5 µg	584 ± 29	432 ± 25	
	2-aminoanthracene	10.0 µg			2445 ± 241

12.3 Summary of Results Experiment II

Study Name: 856901
Experiment: 856901 HV2
Assay Conditions: Pre-Incubation

Study Code: RCC - CCR 856901
Date Plated: 05/01/2005
Date Counted: 10/01/2005

Metabolic Activation	Test Item	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	Deionised water		26 ± 4	17 ± 2	43 ± 8	138 ± 14	411 ± 25
	Untreated		30 ± 4	16 ± 1	37 ± 8	136 ± 3	399 ± 4
	WR 23 081	33 µg	28 ± 2	19 ± 4	32 ± 4	158 ± 27	378 ± 3
		100 µg	29 ± 4	18 ± 3	42 ± 12	170 ± 18	412 ± 40
		333 µg	29 ± 2	21 ± 6	38 ± 7	161 ± 6	425 ± 24
		1000 µg	28 ± 7	20 ± 4	43 ± 6	145 ± 19	364 ± 19
		2500 µg	26 ± 5	25 ± 10	39 ± 6	20 ± 2	293 ± 30
		5000 µg	0 ± 0	n.a.	0 ± 0	0 ± 0	106 ± 8
	sodium azide	10 µg	1423 ± 13			1472 ± 167	
	4-nitro-o-phenylene-diamine	10 µg			317 ± 23		
	4-nitro-o-phenylene-diamine	50 µg		101 ± 3			
	methyl methane sulfonate	4.0 µL					2483 ± 232
With Activation	Deionised water		32 ± 7	31 ± 4	55 ± 3	196 ± 9	547 ± 43
	Untreated		34 ± 3	31 ± 4	52 ± 9	186 ± 23	557 ± 7
	WR 23 081	33 µg	34 ± 9	34 ± 8	54 ± 10	182 ± 9	589 ± 32
		100 µg	32 ± 3	33 ± 3	65 ± 8	180 ± 10	515 ± 35
		333 µg	29 ± 2	32 ± 5	64 ± 3	198 ± 15	533 ± 52
		1000 µg	37 ± 6	29 ± 4	80 ± 7	208 ± 8	430 ± 37
		2500 µg	36 ± 7	53 ± 7	87 ± 11	166 ± 17	307 ± 15
		5000 µg	37 ± 6	64 ± 13	51 ± 11	82 ± 17	172 ± 21
	2-aminoanthracene	2.5 µg	319 ± 25	280 ± 27	2261 ± 297	3060 ± 636	
	2-aminoanthracene	10.0 µg					2519 ± 109

n.a. = not analysable, due to reduced background growth

13 HISTORICAL CONTROL DATA

Due to a new evaluation unit, new historical control data are evaluated. These data represent the laboratory's historical control data since July 2004 representing 80 experiments.

Strain		without S9 mix				with S9 mix			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA 1535	Solvent control	20	6	9	30	18	10	7	39
	Negative control	18	5	10	29	18	10	9	38
	Positive control	3042	756	1003	3618	357	111	172	476
TA1537	Solvent control	11	7	4	29	18	9	6	31
	Negative control	12	6	5	29	18	6	8	29
	Positive control	97	21	52	191	141	47	94	380
TA 98	Solvent control	24	9	14	58	37	13	21	57
	Negative control	26	10	15	52	43	15	17	64
	Positive control	379	98	137	976	1239	510	229	2566
TA 100	Solvent control	121	29	91	198	149	36	109	281
	Negative control	141	23	101	189	147	43	103	254
	Positive control	2089	408	1262	2872	921	346	546	2589
TA 102	Solvent control	338	77	242	430	426	70	332	514
	Negative control	326	53	242	390	450	60	280	556
	Positive control	2764	1479	1220	5593	2104	752	872	3052

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

14 ANNEX I: TABLES OF RESULTS (8 PAGES)

Pre-Experiment : 856901 VV Plate Incorporation (2 pages)

Experiment I: 856901 HV1 Plate Incorporation (2 pages)

Experiment II: 856901 HV2 Pre-Incubation (4 pages)

RCC Cytotest Cell Research GmbH

Study Name: 856901
Experiment: 856901 VV Plate
Assay Conditions:

Study Code: RCC - CCR 856901
Date Plated: 13/12/2004
Date Counted: 17/12/2004

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 98	**WR 23 081**	3 µg	*28.3*	*6.1*	*0.9*	23, 35, 27
		10 µg	*35.0*	*4.6*	*1.2*	34, 31, 40
		33 µg	*34.3*	*10.0*	*1.1*	44, 35, 24
		100 µg	*35.7*	*8.5*	*1.2*	44, 27, 36
		333 µg	*41.0*	*16.1*	*1.4*	28, 36, 59
		1000 µg	*51.3*	*5.1*	*1.7*	47 D, 50 D, 57 D
		2500 µg	*50.7*	*5.5*	*1.7*	45 D, 51 D, 56 D
		5000 µg	*18.3*	*4.9*	*0.6*	24 D M R, 16 D M R, 15 D M R
	Deionised water		*30.3*	*5.1*		26, 29, 36
	Untreated Control		*30.0*	*6.6*		23, 31, 36
TA 100	**WR 23 081**	3 µg	*119.7*	*12.0*	*1.0*	108, 132, 119
		10 µg	*133.0*	*6.1*	*1.2*	129, 140, 130
		33 µg	*127.7*	*16.2*	*1.1*	138, 136, 109
		100 µg	*129.3*	*3.2*	*1.1*	127, 133, 128
		333 µg	*127.3*	*7.6*	*1.1*	119, 134, 129
		1000 µg	*127.3*	*8.0*	*1.1*	128 D, 119 D, 135 D
		2500 µg	*141.3*	*19.6*	*1.2*	136 D, 125 D, 163 D
		5000 µg	*92.3*	*9.3*	*0.8*	82 D M R, 100 D M R, 95 D M R
	Deionised water		*114.7*	*1.2*		114, 114, 116
	Untreated Control		*119.0*	*13.0*		127, 126, 104
TA 98	**4-NOPD**	10 µg	*415.0*	*27.8*	*13.7*	429, 383, 433
TA 100	**NaN3**	10 µg	*2901.0*	*70.7*	*25.3*	2824, 2916, 2963

Key to Positive Controls

4-NOPD	4-nitro-o-phenylene-diamine
NaN3	sodium azide

Key to Plate Postfix Codes

D	Densely coloured plate
M	Manual count
R	Reduced background growth

Study Name: 856901
Experiment: 856901 VV Plate
Assay Conditions:

Study Code: RCC - CCR 856901
Date Plated: 13/12/2004
Date Counted: 17/12/2004

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 98	**WR 23 081**	3 µg	*47.3*	*7.0*	*1.2*	40, 54, 48
		10 µg	*50.0*	*9.5*	*1.2*	59, 51, 40
		33 µg	*48.3*	*12.1*	*1.2*	61, 47, 37
		100 µg	*42.0*	*7.5*	*1.0*	43, 49, 34
		333 µg	*63.3*	*9.0*	*1.5*	72, 54, 64
		1000 µg	*73.7*	*10.2*	*1.8*	81 D, 62 D, 78 D
		2500 µg	*58.3*	*3.1*	*1.4*	61 D, 59 D, 55 D
		5000 µg	*19.3*	*0.6*	*0.5*	20 D, 19 D, 19 D
	Deionised water		*41.0*	*6.6*		47, 34, 42
	Untreated Control		*47.3*	*3.8*		43, 49, 50
TA 100	**WR 23 081**	3 µg	*121.3*	*10.5*	*1.1*	121, 111, 132
		10 µg	*116.0*	*3.0*	*1.0*	113, 116, 119
		33 µg	*112.0*	*23.6*	*1.0*	129, 85, 122
		100 µg	*108.3*	*10.0*	*1.0*	116, 97, 112
		333 µg	*102.3*	*21.4*	*0.9*	78, 111, 118
		1000 µg	*119.0*	*4.0*	*1.1*	123 D, 119 D, 115 D
		2500 µg	*101.3*	*23.1*	*0.9*	88 D, 128 D, 88 D
		5000 µg	*37.3*	*8.5*	*0.3*	34 D, 47 D, 31 D
	Deionised water		*112.3*	*18.2*		127, 92, 118
	Untreated Control		*110.3*	*17.9*		95, 106, 130
TA 98	**2-AA**	2.5 µg	*3528.0*	*219.3*	*86.0*	3276, 3632, 3676
TA 100	**2-AA**	2.5 µg	*3569.3*	*133.8*	*31.8*	3468, 3519, 3721

Key to Positive Controls

2-AA 2-aminoanthracene

Key to Plate Postfix Codes

D Densely coloured plate

RCC Cytotest Cell Research GmbH

Study Name: 856901
Experiment: 856901 HV1 Plate
Assay Conditions:

Study Code: RCC - CCR 856901
Date Plated: 30/12/2004
Date Counted: 04/01/2005

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**WR 23 081**	33 µg	*14.0*	*6.2*	*0.8*	7, 19, 16
		100 µg	*16.0*	*4.4*	*1.0*	21, 13, 14
		333 µg	*14.0*	*1.0*	*0.8*	15, 13, 14
		1000 µg	*15.0*	*5.2*	*0.9*	12 D, 21 D, 12 D
		2500 µg	*14.0*	*1.7*	*0.8*	15 D, 12 D, 15 D
		5000 µg	*7.3*	*3.2*	*0.4*	11 D M R, 6 D M R, 5 D M R
	Deionised water		*16.7*	*3.1*		14, 16, 20
	Untreated Control		*15.7*	*5.5*		12, 22, 13
TA 1537	**WR 23 081**	33 µg	*11.0*	*4.6*	*1.1*	15, 6, 12
		100 µg	*7.3*	*0.6*	*0.7*	7, 7, 8
		333 µg	*20.7*	*5.5*	*2.1*	21, 26, 15
		1000 µg	*8.7*	*0.6*	*0.9*	8 D, 9 D, 9 D
		2500 µg	*12.0*	*3.5*	*1.2*	16 D, 10 D, 10 D
		5000 µg	*2.7*	*2.1*	*0.3*	2 D M R, 5 D M R, 1 D M R
	Deionised water		*10.0*	*3.5*		6, 12, 12
	Untreated Control		*9.0*	*3.5*		13, 7, 7
TA 102	**WR 23 081**	33 µg	*336.0*	*32.2*	*0.9*	300, 346, 362
		100 µg	*366.7*	*10.6*	*1.0*	357, 365, 378
		333 µg	*393.3*	*19.5*	*1.1*	393, 413, 374
		1000 µg	*329.0*	*20.3*	*0.9*	333 D, 347 D, 307 D
		2500 µg	*228.7*	*30.1*	*0.6*	248 D, 244 D, 194 D
		5000 µg	*46.3*	*10.0*	*0.1*	56 D R M, 47 D R M, 36 D R M
	Deionised water		*357.3*	*21.8*		364, 333, 375
	Untreated Control		*317.0*	*41.2*		272, 353, 326
TA 1535	**NaN3**	10 µg	*1477.3*	*77.3*	*88.6*	1390, 1537, 1505
TA 1537	**4-NOPD**	50 µg	*67.7*	*15.3*	*6.8*	56, 85, 62
TA 102	**MMS**	4.0 µL	*5588.0*	*151.0*	*15.6*	5649, 5699, 5416

Key to Positive Controls

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

Key to Plate Postfix Codes

D	Densely coloured plate
R	Reduced background growth
M	Manual count

RCC Cytotest Cell Research GmbH

Study Name: 856901
Experiment: 856901 HV1 Plate
Assay Conditions:

Study Code: RCC - CCR 856901
Date Plated: 30/12/2004
Date Counted: 04/01/2005

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**WR 23 081**	33 µg	*19.7*	*11.0*	*1.3*	26, 26, 7
		100 µg	*16.0*	*1.7*	*1.1*	14, 17, 17
		333 µg	*14.7*	*2.3*	*1.0*	12, 16, 16
		1000 µg	*15.0*	*1.0*	*1.0*	16 D, 14 D, 15 D
		2500 µg	*13.3*	*2.3*	*0.9*	12 D, 12 D, 16 D
		5000 µg	*15.3*	*4.0*	*1.0*	13 D, 20 D, 13 D
	Deionised water		*15.0*	*1.0*		16, 14, 15
	Untreated Control		*14.7*	*1.5*		16, 15, 13
TA 1537	**WR 23 081**	33 µg	*20.7*	*4.9*	*1.3*	23, 15, 24
		100 µg	*17.0*	*4.4*	*1.0*	19, 12, 20
		333 µg	*18.3*	*3.1*	*1.1*	21, 19, 15
		1000 µg	*19.3*	*0.6*	*1.2*	19 D, 19 D, 20 D
		2500 µg	*28.7*	*6.1*	*1.8*	30 D, 34 D, 22 D
		5000 µg	*15.3*	*6.1*	*0.9*	10 D M R, 14 D M R, 22 D M R
	Deionised water		*16.3*	*3.2*		14, 20, 15
	Untreated Control		*18.3*	*4.7*		22, 13, 20
TA 102	**WR 23 081**	33 µg	*492.3*	*56.8*	*1.1*	431, 503, 543
		100 µg	*503.3*	*37.0*	*1.2*	502, 541, 467
		333 µg	*461.7*	*28.3*	*1.1*	478, 478, 429
		1000 µg	*321.3*	*32.7*	*0.7*	291 D, 356 D, 317 D
		2500 µg	*207.7*	*14.5*	*0.5*	222 D, 193 D, 208 D
		5000 µg	*56.7*	*14.5*	*0.1*	57 D M R, 71 D M R, 42 D M R
	Deionised water		*430.3*	*19.3*		408, 442, 441
	Untreated Control		*427.7*	*33.7*		392, 459, 432
TA 1535	**2-AA**	2.5 µg	*584.0*	*29.2*	*38.9*	588, 553, 611
TA 1537	**2-AA**	2.5 µg	*432.3*	*25.1*	*26.5*	426, 460, 411
TA 102	**2-AA**	10.0 µg	*2444.7*	*241.0*	*5.7*	2378, 2712, 2244

Key to Positive Controls

2-AA	2-aminoanthracene

Key to Plate Postfix Codes

D	Densely coloured plate
M	Manual count
R	Reduced background growth

RCC Cytotest Cell Research GmbH

Study Name: 856901
Experiment: 856901 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 856901
Date Plated: 05/01/2005
Date Counted: 10/01/2005

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**WR 23 081**	33 µg	*28.3*	*1.5*	*1.1*	27, 28, 30
		100 µg	*29.3*	*3.5*	*1.1*	29, 26, 33
		333 µg	*28.7*	*2.1*	*1.1*	31, 28, 27
		1000 µg	*28.0*	*6.6*	*1.1*	27 D, 35 D, 22 D
		2500 µg	*25.7*	*4.9*	*1.0*	20 D, 29 D, 28 D
		5000 µg	*0.0*	*0.0*	*0.0*	0 D R, 0 D R, 0 D R
	Deionised water		*25.7*	*4.2*		21, 27, 29
	Untreated Control		*30.0*	*3.6*		29, 34, 27
TA 1537	**WR 23 081**	33 µg	*19.3*	*3.5*	*1.1*	19, 23, 16
		100 µg	*17.7*	*3.2*	*1.0*	20, 19, 14
		333 µg	*21.3*	*5.7*	*1.2*	15, 26, 23
		1000 µg	*19.7*	*4.0*	*1.1*	24 D, 16 D, 19 D
		2500 µg	*24.7*	*10.3*	*1.4*	22 D, 16 D, 36 D
		5000 µg				D N R, D N R, D N R
	Deionised water		*17.3*	*1.5*		19, 16, 17
	Untreated Control		*15.7*	*1.2*		17, 15, 15
TA 98	**WR 23 081**	33 µg	*32.3*	*4.2*	*0.7*	29, 37, 31
		100 µg	*42.0*	*11.8*	*1.0*	29, 45, 52
		333 µg	*37.7*	*6.5*	*0.9*	44, 31, 38
		1000 µg	*43.3*	*5.5*	*1.0*	49 D, 43 D, 38 D
		2500 µg	*39.0*	*6.2*	*0.9*	41 D M R, 44 D M R, 32 D M R
		5000 µg	*0.0*	*0.0*	*0.0*	0 D R, 0 D R, 0 D R
	Deionised water		*43.3*	*7.8*		41, 37, 52
	Untreated Control		*36.7*	*7.5*		29, 37, 44
TA 100	**WR 23 081**	33 µg	*157.7*	*27.4*	*1.1*	163, 128, 182
		100 µg	*169.7*	*18.5*	*1.2*	191, 159, 159
		333 µg	*161.3*	*5.5*	*1.2*	165, 155, 164
		1000 µg	*144.7*	*18.5*	*1.0*	166 D, 133 D, 135 D
		2500 µg	*19.7*	*1.5*	*0.1*	21 D M R, 18 D M R, 20 D M R
		5000 µg	*0.0*	*0.0*	*0.0*	0 D R, 0 D R, 0 D R
	Deionised water		*138.3*	*13.5*		152, 125, 138
	Untreated Control		*136.0*	*3.5*		140, 134, 134
TA 102	**WR 23 081**	33 µg	*378.0*	*3.0*	*0.9*	375, 378, 381
		100 µg	*412.3*	*39.7*	*1.0*	375, 454, 408
		333 µg	*425.3*	*24.1*	*1.0*	400, 428, 448
		1000 µg	*363.7*	*18.9*	*0.9*	349 D, 385 D, 357 D
		2500 µg	*292.7*	*30.3*	*0.7*	314 D, 258 D, 306 D
		5000 µg	*105.7*	*8.0*	*0.3*	114 D M R, 98 D M R, 105 D M R
	Deionised water		*410.7*	*24.8*		393, 400, 439
	Untreated Control		*399.0*	*4.4*		396, 397, 404

Key to Plate Postfix Codes

D	Densely coloured plate
M	Manual count
R	Reduced background growth
N	Analysis not possible

RCC Cytotest Cell Research GmbH

Study Name: 856901
Experiment: 856901 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 856901
Date Plated: 05/01/2005
Date Counted: 10/01/2005

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**NaN3**	10 µg	*1422.7*	*13.4*	*55.4*	1417, 1413, 1438
TA 1537	**4-NOPD**	50 µg	*101.3*	*3.2*	*5.8*	99, 100, 105
TA 98	**4-NOPD**	10 µg	*317.0*	*23.1*	*7.3*	339, 293, 319
TA 100	**NaN3**	10 µg	*1472.3*	*166.9*	*10.6*	1374, 1665, 1378
TA 102	**MMS**	4.0 µL	*2483.0*	*232.4*	*6.0*	2629, 2605, 2215

Key to Positive Controls

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

Key to Plate Postfix Codes

D Densely coloured plate
M Manual count
R Reduced background growth
N Analysis not possible

RCC Cytotest Cell Research GmbH

Study Name: 856901
Experiment: 856901 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 856901
Date Plated: 05/01/2005
Date Counted: 10/01/2005

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**WR 23 081**	33 µg	*34.3*	*9.3*	*1.1*	28, 30, 45
		100 µg	*32.0*	*2.6*	*1.0*	34, 29, 33
		333 µg	*28.7*	*2.3*	*0.9*	30, 26, 30
		1000 µg	*37.3*	*5.5*	*1.2*	41 D, 40 D, 31 D
		2500 µg	*35.7*	*6.7*	*1.1*	34 D, 30 D, 43 D
		5000 µg	*36.7*	*6.0*	*1.1*	31 D, 43 D, 36 D
	Deionised water		*32.3*	*6.8*		27, 40, 30
	Untreated Control		*33.7*	*3.2*		35, 30, 36
TA 1537	**WR 23 081**	33 µg	*34.3*	*7.6*	*1.1*	43, 31, 29
		100 µg	*32.7*	*2.5*	*1.0*	30, 35, 33
		333 µg	*31.7*	*5.0*	*1.0*	27, 37, 31
		1000 µg	*29.0*	*4.4*	*0.9*	27 D, 34 D, 26 D
		2500 µg	*53.3*	*6.7*	*1.7*	50 D, 49 D, 61 D
		5000 µg	*64.3*	*12.6*	*2.1*	51 D, 76 D, 66 D
	Deionised water		*31.3*	*4.0*		36, 29, 29
	Untreated Control		*30.7*	*3.8*		29, 35, 28
TA 98	**WR 23 081**	33 µg	*53.7*	*9.9*	*1.0*	65, 47, 49
		100 µg	*65.0*	*7.9*	*1.2*	74, 62, 59
		333 µg	*64.0*	*2.6*	*1.2*	65, 61, 66
		1000 µg	*80.3*	*7.2*	*1.5*	84 D, 72 D, 85 D
		2500 µg	*87.3*	*10.7*	*1.6*	78 D, 99 D, 85 D
		5000 µg	*51.0*	*11.4*	*0.9*	38 D, 56 D, 59 D
	Deionised water		*55.3*	*3.1*		56, 58, 52
	Untreated Control		*52.3*	*8.5*		61, 52, 44
TA 100	**WR 23 081**	33 µg	*182.0*	*8.7*	*0.9*	172, 187, 187
		100 µg	*180.0*	*9.6*	*0.9*	169, 187, 184
		333 µg	*198.3*	*15.0*	*1.0*	213, 199, 183
		1000 µg	*207.7*	*8.0*	*1.1*	216 D, 207 D, 200 D
		2500 µg	*165.7*	*16.8*	*0.8*	184 D, 162 D, 151 D
		5000 µg	*82.0*	*16.8*	*0.4*	88 D, 63 D, 95 D
	Deionised water		*195.7*	*8.5*		186, 199, 202
	Untreated Control		*186.0*	*23.1*		212, 178, 168
TA 102	**WR 23 081**	33 µg	*589.3*	*32.5*	*1.1*	552, 605, 611
		100 µg	*515.0*	*34.7*	*0.9*	497, 555, 493
		333 µg	*533.3*	*52.3*	*1.0*	576, 549, 475
		1000 µg	*430.3*	*37.1*	*0.8*	392 D, 433 D, 466 D
		2500 µg	*306.7*	*14.7*	*0.6*	290 D, 318 D, 312 D
		5000 µg	*172.0*	*21.0*	*0.3*	172 D M R, 151 D M R, 193 D M R
	Deionised water		*546.7*	*43.4*		584, 557, 499
	Untreated Control		*557.3*	*7.2*		549, 561, 562

Key to Plate Postfix Codes

D	Densely coloured plate
M	Manual count
R	Reduced background growth

RCC Cytotest Cell Research GmbH

Study Name: 856901
Experiment: 856901 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 856901
Date Plated: 05/01/2005
Date Counted: 10/01/2005

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**2-AA**	2.5 µg	*319.0*	*24.6*	*9.9*	346, 313, 298
TA 1537	**2-AA**	2.5 µg	*279.7*	*26.5*	*8.9*	306, 280, 253
TA 98	**2-AA**	2.5 µg	*2261.0*	*297.1*	*40.9*	2437, 2428, 1918
TA 100	**2-AA**	2.5 µg	*3060.0*	*635.9*	*15.6*	2326, 3445, 3409
TA 102	**2-AA**	10.0 µg	*2519.3*	*109.1*	*4.6*	2395, 2564, 2599

Key to Positive Controls

2-AA	2-aminoanthracene

Key to Plate Postfix Codes

D	Densely coloured plate
M	Manual count
R	Reduced background growth

15 ANNEX I: CERTIFICATE OF ANALYSIS (2 PAGES)

COSMITAL SA

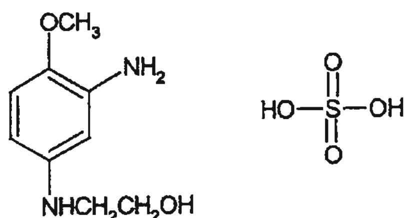
Marly, 19.02.2001 LSY/BR/DOU/bs
modified 03.08.2004

CERTIFICATE OF ANALYSIS

raw material no.: 23081

Code: A000157

Structure:



Molecular formula: $C_9H_{14}N_2O_2.H_2O_4S$

Molecular weight: 280.30
Molecular weight of free acid: 182.22

Wella name: LEHMANN BLAU

Trade name: HC BLAU AC (ROBINSON). HC BLUE AC (CLARIANT)

Chemical name: 2-Amino-4-(2-hydroxyethyl)amino-anisole-sulfate

Name (INCI): 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE

CAS-No: 83763-48-8

EINECS/ELINCS-No: 280-734-8

Testing material

Sample name:

Sample no: R96000196

Batch: 57

Study no.: A9803/086 and A2004/237

Date of entry: 08.01.96

Expiry date: July, 2005

Results

Aspect: pale grey powder

Identity: The ^{1}H-NMR spectra confirmed the chemical identity of the test substance.

Purity: 99.6 area% (by HPLC)

Content: 99.1 weight% ($C_9H_{14}N_2O_2xH_2SO_4xH_2O$) } by
93.5 weight% ($C_9H_{14}N_2O_2xH_2SO_4$) } NMR

By-products: 0.03 weight% 4-methoxy-1,3-phenylenediaminesulfate (2,4-diaminoanisole)
4-methoxyaniline was not detectable
4-methoxy-3-nitroaniline was not detectable
2-methoxy-5-nitroaniline was not detectable
10.6 weight% of water
28 ppm of sodium

Solubility: 10g/l in water pH 2.8 (>5 weight% pH 8)
1 weight% in acetone/water 1:1 (pH 2.1)
9-10 weight% in DMSO
0.2 weight% in ethanol

Stability: The substance on storage in dryness and darkness to be stable → July 2005.

Stability in solution:
The stability over a total period of seven days was tested by HPLC. The test stock solutions (approx. 5 weight%) were stored at room temperature and in the absence of light.
Water solution: the results (t = 0h: 100.0%; 6h: 93.8%; 2d: 95.1%; 7d: 79.7%) confirm a low degradation (G2000/003)
DMSO solution: the results (t = 0h: 100.0%; 6h: 98.8%; 2d: 92.0%; 7d: 80.5%) confirm a low degradation (G2000/003)

P. Dougoud

RCC - CCR STUDY NUMBER 856901

SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY

WITH

2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081)

STUDY PLAN

1 SIGNATURES

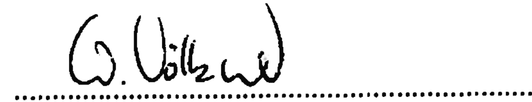
Study Director

Dipl. Biol. Andrea Sokolowski

Date: November 17, 2004

Management

Dr. Wolfgang Völkner



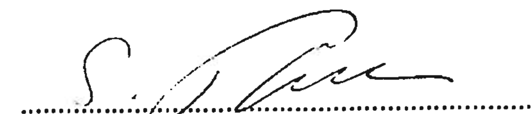
Date: November 17, 2004

Head of Quality Assurance Unit

Frauke Hermann

Date: November 29, 2004

Sponsor



Date: Dec 15, 2004

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3 PREFACE

3.1 General

Title:	Salmonella typhimurium Reverse Mutation Assay with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE (WR 23081)
Sponsor:	
Study Monitor:	
Test Facility:	R C C Cytotest Cell Reseach GmbH (RCC-CCR) In den Leppsteinswiesen 19 D-64380 Rossdorf

3.2 Responsibilities

Study Director:	Dipl. Biol. Andrea Sokolowski
Deputy Study Director:	Dr. Hans-Eric Wollny
Management:	Dr. Wolfgang Völkner
Head of Quality Assurance Unit:	Frauke Hermann

3.3 Schedule

Date of the Study Plan:	November 17, 2004
Proposed Experimental Starting Date:	November, 2004
Proposed Experimental Completion Date:	December, 2004
Proposed Date of Draft Report:	January, 2005

3.4 Good Laboratory Practice

The study will be performed in compliance with:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1) dated July 25, 1994 ("BGBl. I 1994", pp. 1703), last revision: dated June 27, 2002.

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final]

3.5 Guidelines

This study will be conducted according to 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 July 21, 1997

"Commission Directive 2000/32/EC, L1362000, Annex 4D", dated May 19, 2000

3.6 Amendment and Deviation Procedures

Amendments (planned changes) to the study plan will be issued and signed by the Study Director. The sponsor will receive the original and a copy of the amendment. The original is to be countersigned upon agreement and returned to RCC-CCR. The amendment will be distributed (see Distribution) and added to all copies of the study plan.

Deviations (unplanned changes) to the study plan will be documented and maintained with the raw data. The report will reflect any deviations. The sponsor will be promptly informed of any relevant deviations from the study plan.

3.7 Archiving

RCC Cytotest Cell Research will archive the following data for 15 years:

Raw data, study plan, final report, and a sample of the test item.

No data will be discarded without the sponsor´s consent.

4 OBJECTIVE

4.1 Aims of the Study

The experiment will be performed to assess the potential of the test item to induce gene mutations in the Salmonella typhimurium reverse mutation assay. The experiment will be performed as a plate incorporation assay. If a negative or equivocal result is obtained in this experiment, a second experiment will be performed as pre-incubation assay. In case of a clear positive response, a second experiment is not required.

4.2 Reasons for the Study

The most widely used assays for detecting gene mutations are those using bacteria (3). They are relatively simple and rapid to perform, and give reliable data on the ability of an agent to interact with DNA and produce mutations.

Reverse mutation assays determine the frequency at which an agent abolishes or suppresses the effect of the forward mutation. The genetic target presented to an agent is therefore small, specific and selective. Several bacterial strains, or a single strain with multiple markers are necessary to overcome the effects of mutagen specificity. The reversion of bacteria from growth-dependence on a particular amino acid to growth in the absence of that amino acid (reversion from auxotrophy to prototrophy) is the most widely used marker.

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

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.

To establish a dose response effect at least 5 dose levels with adequately spaced intervals are tested. The maximum dose level will be 5000 µg/plate, unless limited by toxicity of the test item.

To validate the test, reference mutagens will be tested in parallel to the test item.

5 MATERIALS AND METHODS

5.1 Test Item

Internal RCC-CCR Test Item Number: S 5031 11

The test item and the information concerning the test item were provided by the sponsor.

Identity:	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE SULFATE(WR 23081)
Batch no.:	57
Sample no.:	R96000196
Aggregate state at room temperature:	solid
Colour:	pale grey
Molecular weight:	280.30
Purity:	99.6 area% (HPLC)
Stability in solution:	stable for up to two days in water and DMSO
Storage:	room temperature, moisture protected, light protected
Expiry date:	July, 2005

On the day of the experiment, the test item will be dissolved in water or an appropriate solvent (e.g. DMSO, DMF, ethanol, acetone). The solvent will be chosen according to its solubility properties and its relative nontoxicity for the bacteria (4).

5.2 Safety precautions

Routine hygienic procedures will be sufficient to ensure personnel health and safety. Additional measurements will be provided according to the material safety data sheet.

5.3 Controls

5.3.1 Negative Controls

Concurrent untreated and solvent controls will be performed.

5.3.2 Positive Control Substances

Without metabolic activation

Strains: TA 1535, TA 100
Name: sodium azide, NaN_3
Supplier: SERVA, D-69042 Heidelberg
Catalogue No.: 30175
Purity: at least 99 %
Dissolved in: water deionised
Concentration: 10 µg/plate

Strains: TA 1537, TA 98
Name: 4-nitro-o-phenylene-diamine, 4-NOPD
Supplier: SIGMA, D-82041 Deisenhofen
Catalogue No.: N 9504
Purity: > 99.9 %
Dissolved in: DMSO (purity >99 %, MERCK, D-64293 Darmstadt)
Concentration: 10 µg/plate in strain TA 98, 50 µg/plate in strain TA 1537

Strain: TA 102
Name: methyl methane sulfonate, MMS
Supplier: MERCK-SCHUCHARDT, D-85662 Hohenbrunn
Catalogue No.: 820775
Purity: > 99.0 %
Dissolved in: water deionised
Concentration: 4.0 µL/plate

With metabolic activation

Strains: TA 1535, TA 1537, TA 98, TA 100, TA 102
Name: 2-aminoanthracene, 2-AA
Supplier: SIGMA, D-82041 Deisenhofen
Catalogue No.: A 1381
Purity: 97.5 %
Dissolved in: DMSO (purity >99 %, MERCK, D-64293 Darmstadt)
Concentration: 2.5 µg/plate (10.0 µg/plate in TA 102)

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

5.4 Test System

5.4.1 Characterisation of the Salmonella typhimurium Strains

The histidine dependent strains are derived from S. typhimurium strain LT2 through mutations in the histidine locus. Additionally due to the "deep rough" (rfa^-) mutation they possess a faulty lipopolysaccharide envelope, which enables substances to penetrate the cell wall more easily. A further mutation (deletion of the uvrB gene) causes an inactivation of the excision repair system. The latter alteration also includes a deletion in the nitrate reductase and biotin genes. In the strains TA 98, TA 100, and TA 102 the R-factor plasmid pKM 101 carries umu DC analogous genes that are involved in error-prone repair and the ampicillin resistance marker. The strain TA 102 does not contain the $uvrB^-$-mutation. Additionally TA 102 contains the multicopy plasmid pAQ1, which carries the hisG428 mutation and a tetracycline resistance gene. TA 102 contains the ochre mutation in the hisG gene (5).

When summarised the mutations of the bacterial strains used in this study can be described as follows:

Salmonella typhimurium		
Strains	**Genotype**	**Type of mutations indicated**
TA 1537	his C 3076; rfa^-; $uvrB^-$:	frame shift mutations
TA 98	his D 3052; rfa^-; $uvrB^-$;R-factor	" "
TA 1535	his G 46; rfa^-; $uvrB^-$:	base-pair substitutions
TA 100	his G 46; rfa^-; $uvrB^-$;R-factor	" "
TA 102	his G 428; rfa^-; $uvrB^+$;R-factor:	" "

Regular checking of the properties of the Salmonella typhimurium strains regarding the membrane permeability and ampicillin and tetracycline resistance as well as normal spontaneous mutation rates is performed in RCC Cytotest Cell according to B. Ames et al. (1) and D. Maron and B. Ames (5). In this way it is ensured that the experimental conditions set down by Ames are fulfilled.

The bacterial strains TA 1535, TA 1537 TA 98, TA 100, and TA 102 were obtained from Trinova Biochem GmbH (35394 Gießen, Germany).

5.4.2 Storage

The strain cultures are stored as stock cultures in ampoules with nutrient broth + 5 % DMSO (MERCK, D-64293 Darmstadt) in liquid nitrogen.

5.4.3 Precultures

From the thawed ampoules of the strains 0.5 mL bacterial suspension will be transferred into 250 mL Erlenmeyer flasks containing 20 mL nutrient medium. A solution of 20 µL ampicillin (25 µg/mL) will be added to the strains TA 98, TA 100, and TA 102. 20 µL tetracycline (2 µg/mL) will be added to strain TA 102. This nutrient medium contains per litre:

8 g Merck Nutrient Broth (MERCK, D-64293 Darmstadt)
5 g NaCl (MERCK, D-64293 Darmstadt)

The bacterial culture will be incubated in a shaking water bath for up to 8 hours at 37° C.

5.4.4 Selective Agar

The plates with the selective agar will be obtained from E. Merck, D-64293 Darmstadt

5.4.5 Overlay Agar

The overlay agar contains per litre:

6.0 g MERCK Agar Agar*
6.0 g NaCl*
10.5 mg L-Histidine x HCl x H_2O*
12.2 mg Biotin*

* (MERCK, D-64293 Darmstadt)

Sterilisations will be performed at 121 °C in an autoclave.

5.5 Mammalian Microsomal Fraction S9 Mix

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

5.5.1 S9 (Preparation by RCC Cytotest Cell Research)

Phenobarbital/β-Naphthoflavone induced rat liver S9 is used as the metabolic activation system. The S9 is prepared from 8 - 12 weeks old male Wistar Hanlbm rats, weight approx. 220 - 320 g induced by applications of 80 mg/kg b.w. Phenobarbital i.p. (Desitin; D-22335 Hamburg) and β-Naphthoflavone p.o. (Aldrich, D-89555 Steinheim) each on three consecutive days. The livers are prepared 24 hours after the last treatment. The S9 fractions are produced by dilution of the liver homogenate with a KCl solution (1+3) followed by centrifugation at 9000 g. Aliquotes of the supernatant are frozen and stored in ampoules at -80° C. Small numbers of the ampoules can be kept at -20°C for up to one week. The protein concentration in the S9 preparation is usually between 20 and 45 mg/mL.

5.5.2 S9 Mix

An appropriate quantity of S9 supernatant is thawed and mixed with S9 cofactor solution, to result in a final concentration of approx. 15 % v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

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

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment, the S9 mix is stored in an ice bath. The S9 mix preparation is performed according to Ames et al.(1).

5.6 Pre-Experiment for Toxicity

To evaluate the toxicity of the test item a prestudy will be performed with strains TA 98 and TA 100. Eight concentrations will be tested for toxicity and mutation induction with each 3 plates. The experimental conditions in this pre-experiment will be the same as described below for the experiment I (plate incorporation test).

Toxicity of the test item results in a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn.

The pre-experiment will be reported as part of the main experiment I if the following criteria are met:

Evaluable plates (>0 colonies) at five concentrations or more.

5.7 Dose Selection

According to the results of this pre-experiment the concentrations to be applied in the main experiments will be chosen.

The maximum concentration is 5000 µg/plate, unless limited by toxicity of the test item. The concentration range covers at least two logarithmic decades. In this study at least five adequately spaced concentrations are tested. In case of a negative or equivocal result a second experiment will be performed.

5.8 Experimental Performance

For each strain and dose level, including the controls, three plates will be used.

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

100 µL Test solution at each dose level, solvent (negative control) or reference mutagen solution (positive control),

500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),

100 µL Bacteria suspension (cf. test system, pre-culture of the strains),

2000 µL Overlay agar

If the pre-incubation method will be performed 100 µL test solution, 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacteria suspension will be mixed in a test tube and incubated at 37°C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45°) will be added to each tube. The mixture will be poured on selective agar plates.

After solidification the plates will be incubated upside down for at least 48 hours at 37° C in the dark (2).

5.9 Data Recording

The colonies are counted using the Petri Viewer Mk2 (Perceptive Instruments Ltd, Suffolk CB 7BN, UK) with the software program Ames Study Manager v1.2. The counter is connected to an IBM AT compatible PC with printer to print out the individual values and the means from the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates (see tables of results). If precipitation of the test item precludes automatic counting the revertant colonies are counted manually.

5.10 Acceptability of the Assay

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

- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of our historical data
- the positive control substances should produce a significant increase in mutant colony frequencies

5.11 Evaluation of Results

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed (3).

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration (2).

An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

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

6 REPORTING

A GLP-compliant draft report will be submitted to the sponsor for scientific review. Following receipt of the sponsor's comments, a QA-audited final report will be issued.

7 DISTRIBUTION

Sponsor	2×(1x original, to be returned to RCC-CCR, 1x copy)
Study Director	1×(copy)
QAU	1×(copy)

8 REFERENCES

1. Ames, B.N., McCann, J. und Yamasaki, E. (1975)
   Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test.
   Mutation Res., 31, 347-363

2. de Serres F.J. and M.D. Shelby (1979)
   Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay
   Mutation Res. 64, 159-165

3. Hollstein,M., J. McCann, F.A. Angelosanto and W.W. Nichols (1979)
   Short-term tests for carcinogens and mutagens
   Mutation Res. 65, 133-226

4. Maron D.M., J. Katzenellenbogen and B.N. Ames, (1981)
   Compatibility of organic solvents with the Salmonella/Microsome Test
   Mutation Res. 88, 343-350

5. Maron D.M., Ames, B.N. (1983)
   Revised methods for the Salmonella mutagenicity test
   Mutation Res. 113, 173-215

9 HISTORICAL CONTROL DATA

Due to a new evaluation unit, new historical control data are evaluated. These data represent the laboratory's historical control data since July 2004 representing 80 experiments.

Strain		without S9 mix				with S9 mix			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA 1535	Solvent control	20	6	9	30	18	10	7	39
	Negativecontrol	18	5	10	29	18	10	9	38
	Positive control	3042	756	1003	3618	357	111	172	476
TA1537	Solvent control	11	7	4	29	18	9	6	31
	Negative control	12	6	5	29	18	6	8	29
	Positive control	97	21	52	191	141	47	94	380
TA 98	Solvent control	24	9	14	58	37	13	21	57
	Negative control	26	10	15	52	43	15	17	64
	Positive control	379	98	137	976	1239	510	229	2566
TA 100	Solvent control	121	29	91	198	149	36	109	281
	Negative control	141	23	101	189	147	43	103	254
	Positive control	2089	408	1262	2872	921	346	546	2589
TA 102	Solvent control	338	77	242	430	426	70	332	514
	Negative control	326	53	242	390	450	60	280	556
	Positive control	2764	1479	1220	5593	2104	752	872	3052

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