REPORT No. 2500196
Regulatory Document

DSM

Document Date:	13-Dec-2005
Authors:	Thiel A (NRD-CH), Köhl W (SCC GmbH) and Sokolowski A (RCC-CCR)
Title:	Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay with Ultrazine FG-R (Food Grade Lignosulphonate) (Study conducted at RCC-CCR; D-64380 Rossdorf, Germany. RCC-CCR study number 899101)
Project No.	6309
Compound No.	Ultrazine FG-R (Food Grade Lignosulphonate), Calcium Lignosulphonate, LS FG DP-955 FGR004

Summary

The purpose of the study was to determine the potential of Ultrazine FG-R (Food Grade Lignosulphonate) to induce gene mutations in reverse mutation experiments in bacteria.

Two independent experiments i.e. (i) plate incorporation test and (ii) pre-incubation test were performed using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, and TA 100, and the Escherichia coli strain WP2 uvrA. The experiments were performed with and without liver microsomal activation (S9-mix). Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations in both experiments: 33; 100; 333; 1000; 2500; and 5000 µg/plate.

(continued next page)

This report consists of Pages I – III and 1-56

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Approved

Name	**Signature**	**Date**
Main Author A. Thiel	Annette Thiel	11. Jan 2006
Principal Scientist / Competence Mgr J. Bausch		13/Jan 06
Research Center Head L. Pasamontes		19.1.06
Project Manager B. Mussler		16. Jan 06

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used. No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with Ultrazine FG-R (Food Grade Lignosulphonate) at any dose level, neither in the presence nor absence of metabolic activation (S9-mix). There was also no tendency of higher mutation rates with increasing concentrations (i.e. no dose-dependent increase in mutations below the biological threshold) in the range below the generally acknowledged border of biological relevance. Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

Conclusion
It can be stated that during the described reverse 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. Ultrazine FG-R (Food Grade Lignosulphonate) is considered to be non-mutagenic in this reverse mutation assay using bacteria.

Nomenclature and Structural Formula (if available)

Test Article Name:	Ultrazine FG-R (Food Grade Lignosulphonate)
Chemical Name:	Calcium Lignosulphonate
Batch No.:	FGR-004

FINAL REPORT

Study Title:

SALMONELLA TYPHIMURIUM AND ESCHERICHIA COLI REVERSE MUTATION ASSAY WITH ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

Data Requirements / Test Guidelines:

based on:

"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

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

Study Director:

Andrea Sokolowski

Study Completion Date:

December 13, 2005

Test Facility:

RCC - Cytotest Cell Research GmbH (RCC-CCR)
In den Leppsteinswiesen 19,
D-64380 Rossdorf, Germany

Sponsor:

DSM Nutritional Products AG
Wurmisweg 576
CH-4303 Kaiseraugst
Switzerland

RCC-CCR Study No.: 899101

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1 STATEMENT OF COMPLIANCE

Study Number:	899101
Test Item:	Ultrazine FG-R (Food Grade Lignosulphonate)
Study Director:	Dipl. Biol. Andrea Sokolowski
Title:	Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay with Ultrazine FG-R (Food Grade Lignosulphonate)

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.

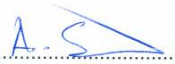
"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final].

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

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

Study Director

RCC - CCR
Dipl. Biol. Andrea Sokolowski



Date: December 13, 2005

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2 STATEMENT OF QUALITY ASSURANCE UNIT

Study Number: 899101

Test Item: Ultrazine FG-R (Food Grade Lignosulphonate)

Study Director: Dipl. Biol. Andrea Sokolowski

Title: Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay with Ultrazine FG-R (Food Grade Lignosulphonate)

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):	September 12, 2005	September 12, 2005
Study Plan:	September 15, 2005	- -
1st Amendment to Study Plan (Draft):	October 10, 2005	October 10, 2005
1st Amendment to Study Plan:	October 13, 2005	- -
Process Inspection:	October 19, 2005	October 19, 2005
Draft Report	December 05, 2005	December 05, 2005

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

Head of Quality Assurance Unit for Frauke Hermann

Date: December 13, 2005

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3 PROJECT STAFF SIGNATURES

Study Director — Dipl. Biol. Andrea Sokolowski

Date: December 13, 2005

Management — Dr. Wolfgang Völkner

Date: December 13, 2005

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5 PREFACE

5.1 General

Title:	Salmonella typhimurium and Escherichia Coli Reverse Mutation Assay with Ultrazine FG-R (Food Grade Lignosulphonate)
Sponsor:	DSM Nutritional Products AG Wurmisweg 576 CH-4303 Kaiseraugst Switzerland
Study Monitor:	SCC Scientific Consulting Company Chemisch-Wissenschaftliche Beratung GmbH Dr. Werner Köhl Mikroforum Ring 1 D-55234 Wendelsheim
SCC Project No.:	612-002
Test Facility:	R C C Cytotest Cell Reseach GmbH (RCC-CCR) In den Leppsteinswiesen 19 D-64380 Rossdorf

5.2 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

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5.3 Schedule

Date of the Study Plan:	September	13, 2004
Date of 1st Amendment to Study Plan:	October	11, 2005
Experimental Starting Date:	September	27, 2005
Experimental Completion Date:	October	31, 2005
Date of Draft Report:	November	10, 2005
Date of Final Report:	December	13, 2005

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5.4 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].

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

5.5 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

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

5.6 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.

5.7 Deviations from the Study Plan

There were no deviations from the study plan.

6 SUMMARY OF RESULTS

This study was performed to investigate the potential of Ultrazine FG-R (Food Grade Lignosulphonate) to induce gene mutations in a plate incorporation test (experiment I) and in a pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, and TA 100, and the Escherichia coli strain WP2 uvrA.

The assay was performed with and without liver microsomal activation (S9-mix). 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 normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with Ultrazine FG-R (Food Grade Lignosulphonate) at any dose level, neither in the presence nor absence of metabolic activation (S9-mix). There was also no tendency of higher mutation rates with increasing concentrations (i.e. no dose-dependent increase in mutations below the biological threshold) in the range below the generally acknowledged border of biological relevance.

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.

Ultrazine FG-R (Food Grade Lignosulphonate) is considered to be non-mutagenic in this reverse mutation assay using bacteria.

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 and Escherichia coli 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) and the E. coli tryptophan (trp) reversion system measures $his^- \rightarrow his^+$ and $trp^- \rightarrow trp^+$ reversions, respectively. The S. typhimurium and Escherichia coli strains are constructed to differentiate between base pair (TA 1535, TA 100, and WP2 uvrA) 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 were tested in parallel to the test item.

8 MATERIALS AND METHODS

8.1 Test Item

Internal RCC-CCR Test Item Number: S 5644 22

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

Identity:	Ultrazine FG-R (Food Grade Lignosulphonate)
Chemical Name:	Calcium Lignosulphonate
Batch No.:	FGR-004
Aggregate State at Room Temperature:	solid
Colour:	brown
Purity:	95.5 % (dry solids)
Certificate of Analysis (date):	August 26, 2005
Stability in Solvent:	Not indicated by the sponsor
Storage:	Room temperature, moisture protected
Expiration Date:	August 26, 2007

On the day of the experiment, the test item Ultrazine FG-R (Food Grade Lignosulphonate) was dissolved in deionised water. The solvent was chosen because of its solubility (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:	WP2 uvrA
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, WP2 uvrA
Name:	2-aminoanthracene, 2-AA
Supplier:	SIGMA, D-82041 Deisenhofen
Catalogue No.:	A 1381
Purity:	97.5 %
Dissolved in:	DMSO (MERCK, D-64293 Darmstadt; purity > 99 %)
Concentration:	2.5 µg/plate (TA 1535, TA 1537, TA 98, TA 100), 10 µg/plate (WP2 uvrA)

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 and E. coli Strain

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-minus) mutation they possess a faulty lipopolysaccharide envelope which enables substances to penetrate the cell wall more easily. A further mutation causes a reduction in the activity of an excision repair system. The latter alteration includes mutational processes in the nitrate reductase and biotin genes produced in a UV-sensitive area of the gene named "uvrB-minus". In the strains TA 98 and TA 100 the R-factor plasmid pKM 101 carries the ampicillin resistance marker (6).

Strain WP2 (4) and its derivatives all carry the same defect in one of the genes for tryptophan biosynthesis. Tryptophan-independent (Trp^+) mutants (revertants) can arise either by a base change at the site of the original alteration or by a base change elsewhere in the chromosome so that the original defect is suppressed. This second possibility can occur in several different ways so that the system seems capable of detecting all types of mutations which substitute one base for another. Additionally, the uvrA derivative is deficient in the DNA repair process (excision repair damage). Such a repair-deficient strain may be more readily mutated by agents.

When summarised the mutations of the TA strains and the E. coli strain, used in this study, can be described as follows:

Table 1; Type of mutations indicated:

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	" "
Escherichia coli		
WP2 uvrA	trp^-; $uvrA^-$:	base-pair substitutions and others

Regular checking of the properties of the strains regarding the membrane permeability and ampicillin resistance as well as spontaneous mutation rates is performed in RCC Cytotest Cell Research according to B. Ames et al. (1) and D. Maron and B. Ames (6). 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 WP2 uvrA 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 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 and TA 100. 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 selective agar were obtained from E. Merck, D-64293 Darmstadt.

8.3.5 Overlay Agar

The overlay agar contains per litre:

for Salmonella strains:	for Escherichia coli:
6.0 g MERCK Agar Agar*	6.0 g MERCK Agar Agar*
6.0 g NaCl*	6.0 g NaCl*
10.5 mg L-Histidine×HCl×H_2O*	2.5 mg Tryptophan*
12.2 mg Biotin*	

* (MERCK, D-64293 Darmstadt)

Sterilisation were performed at 121° C for 15 min. at 2 bar 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 HanIbm 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 30.6 mg/mL (lot no. R 080705) 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 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. 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).

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 WP2 uvrA) 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 Ultrazine FG-R (Food Grade Lignosulphonate) was assessed for its potential to induce gene mutations in a plate incorporation test (experiment I) and a pre-incubation test (experiment II), using Salmonella typhimurium strains TA 1535, TA 1537, TA 98, and TA 100, and the Escherichia coli strain WP2 uvrA.

The assay was performed with and without liver microsomal activation (S9-mix). 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 normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with Ultrazine FG-R (Food Grade Lignosulphonate) at any dose level, neither in the presence nor absence of metabolic activation (S9-mix). There was also no tendency of higher mutation rates with increasing concentrations (i.e. no dose-dependent increase in mutations below the biological threshold) in the range below the generally acknowledged border of biological relevance.

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

In experiment II, the mean value obtained for the negative control of strain WP2 uvrA with S9 mix was slightly above our historical control range. Since this deviation is rather small and within the minimum and maximum of historical control values obtained, this effect is considered to be a biologically irrelevant fluctuation 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.

Ultrazine FG-R (Food Grade Lignosulphonate) is considered to be non-mutagenic in this reverse mutation assay using bacteria.

10 REFERENCES

1. Ames, B.N., J. McCann, and E. Yamasaki (1977)
   Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test
   In: B.J. Kilbey et al. (Eds.)"Handbook of Mutagenicity Test Procedures" Elsevier, Amsterdam, 1-17

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

Study Monitor	3 x (1 x duplicate bounded, 2 x copy unbounded, one complete pdf-file)
Study Director	1 x (original)

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12 SUMMARY OF RESULTS

12.1 Summary of Results Pre-Experiment

Study Name: 899101
Experiment: 899101 VV Plate
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 27/09/2005
Date Counted: 30/09/2005

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)	
			TA 98	TA 100
Without Activation (S9-mix)	Deionised water		25 ± 4	136 ± 10
	Untreated		31 ± 2	131 ± 10
	Ultrazine FG-R	3 µg	31 ± 2	134 ± 3
		10 µg	29 ± 7	138 ± 16
		33 µg	33 ± 3	144 ± 7
		100 µg	31 ± 11	142 ± 4
		333 µg	28 ± 6	125 ± 13
		1000 µg	30 ± 4	147 ± 13
		2500 µg	29 ± 7	145 ± 5
		5000 µg	22 ± 7	165 ± 12
	4-NOPD	10 µg	461 ± 21	
	NaN3	10 µg		2268 ± 101
With Activation (S9-mix)	Deionised water		38 ± 9	149 ± 10
	Untreated		34 ± 5	151 ± 12
	Ultrazine FG-R	3 µg	45 ± 6	150 ± 2
		10 µg	37 ± 8	141 ± 5
		33 µg	39 ± 6	152 ± 6
		100 µg	34 ± 8	173 ± 2
		333 µg	36 ± 2	134 ± 6
		1000 µg	42 ± 6	137 ± 4
		2500 µg	40 ± 4	170 ± 9
		5000 µg	40 ± 3	144 ± 12
	2-AA	2.5 µg	1728 ± 225	1939 ± 83

Key to Positive Controls

NaN3 sodium azide
2-AA 2-aminoanthracene
4-NOPD 4-nitro-o-phenylene-diamine

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12.2 Summary of Results Experiment I

Study Name: 899101 Study Code: RCC - CCR 899101
Experiment: 899101 HV1 Plate Date Plated: 20/10/2005
Assay Conditions: Date Counted: 25/10/2005

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)		
			TA 1535	TA 1537	WP2 uvrA
Without Activation (S9-mix)	Deionised water		18 ± 4	27 ± 4	55 ± 10
	Untreated		20 ± 3	24 ± 1	50 ± 5
	Ultrazine FG-R	33 µg	26 ± 4	25 ± 5	53 ± 10
		100 µg	23 ± 1	19 ± 3	57 ± 10
		333 µg	21 ± 2	25 ± 8	62 ± 7
		1000 µg	21 ± 1	19 ± 4	45 ± 6
		2500 µg	27 ± 5	19 ± 2	50 ± 7
		5000 µg	16 ± 1	18 ± 3	57 ± 7
	NaN3	10 µg	1260 ± 50		
	4-NOPD	50 µg		118 ± 7	
	MMS	4.0 µL			1467 ± 79
With Activation (S9-mix)	Deionised water		30 ± 4	29 ± 4	62 ± 13
	Untreated		35 ± 2	33 ± 6	61 ± 4
	Ultrazine FG-R	33 µg	27 ± 8	19 ± 2	58 ± 4
		100 µg	25 ± 9	27 ± 3	61 ± 4
		333 µg	24 ± 2	24 ± 4	68 ± 3
		1000 µg	31 ± 6	26 ± 3	62 ± 11
		2500 µg	24 ± 10	31 ± 13	64 ± 13
		5000 µg	32 ± 4	23 ± 7	69 ± 4
	2-AA	2.5 µg	302 ± 35	191 ± 6	
	2-AA	10.0 µg			338 ± 45

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

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12.3 Summary of Results Experiment II

Study Name: 899101
Experiment: 899101 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 28/10/2005
Date Counted: 31/10/2005

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	WP2 uvrA
Without Activation (S9-mix)	Deionised water		26 ± 14	22 ± 5	41 ± 3	160 ± 21	34 ± 4
	Untreated		24 ± 9	25 ± 2	30 ± 8	152 ± 4	50 ± 1
	Ultrazine FG-R	33 µg	25 ± 7	30 ± 4	35 ± 2	158 ± 12	45 ± 9
		100 µg	22 ± 7	20 ± 4	32 ± 13	166 ± 24	43 ± 7
		333 µg	27 ± 2	23 ± 6	29 ± 2	160 ± 11	43 ± 14
		1000 µg	20 ± 7	22 ± 7	32 ± 4	146 ± 10	53 ± 4
		2500 µg	25 ± 2	25 ± 3	31 ± 2	168 ± 7	53 ± 5
		5000 µg	24 ± 2	28 ± 6	35 ± 6	163 ± 8	49 ± 5
	NaN3	10 µg	1393 ± 27			1903 ± 98	
	4-NOPD	10 µg			382 ± 9		
	4-NOPD	50 µg		107 ± 13			
	MMS	4.0 µL					587 ± 62
With Activation (S9-mix)	Deionised water		21 ± 4	26 ± 1	40 ± 1	186 ± 8	64 ± 3
	Untreated		28 ± 1	26 ± 2	41 ± 1	168 ± 27	70 ± 8
	Ultrazine FG-R	33 µg	21 ± 2	25 ± 1	41 ± 8	176 ± 10	59 ± 10
		100 µg	31 ± 5	30 ± 9	31 ± 14	186 ± 13	55 ± 11
		333 µg	26 ± 2	27 ± 7	37 ± 7	176 ± 8	61 ± 3
		1000 µg	25 ± 3	22 ± 7	38 ± 6	179 ± 16	61 ± 5
		2500 µg	26 ± 4	28 ± 4	33 ± 3	189 ± 11	66 ± 10
		5000 µg	29 ± 4	26 ± 8	34 ± 1	185 ± 11	60 ± 11
	2-AA	2.5 µg	219 ± 3	183 ± 11	1071 ± 56	1505 ± 185	
	2-AA	10.0 µg					347 ± 30

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

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13 HISTORICAL CONTROL DATA

Due to a new evaluation unit, new historical control data are being established. These data represent the laboratory´s historical control data since July 2004 representing 150 experiments.

Table 2; Historical Control Data:

Strain		without S9 mix				with S9 mix			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA 1535	Solvent control	19	6	9	35	21	7	7	41
	Negative control	18	5	10	30	21	6	9	38
	Positive control	1681	789	1003	4900	387	126	172	695
TA1537	Solvent control	12	3	4	29	18	6	6	36
	Negative control	11	3	5	29	19	6	8	33
	Positive control	87	18	52	191	337	191	94	746
TA 98	Solvent control	26	6	14	58	39	9	21	57
	Negative control	26	6	15	60	41	9	17	64
	Positive control	361	204	176	1818	2386	1195	296	4854
TA 100	Solvent control	131	24	91	198	147	25	109	281
	Negative control	140	21	101	189	154	23	103	254
	Positive control	2030	340	1178	2872	2629	1326	546	5230
WP2uvrA	Solvent control	52	8	31	67	55	10	34	75
	Negative control	50	8	36	64	52	8	33	64
	Positive control	998	515	320	1976	342	134	221	930

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

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14 ANNEX I: TABLES OF RESULTS (8 PAGES)

Pre-Experiment: 899101 VV Plate Incorporation (2 pages)

Experiment I: 899101 HV1 Plate Incorporation (2 pages)

Experiment II: 899101 HV2 Pre-Incubation (4 pages)

Study Name: 899101
Experiment: 899101 VV Plate
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 27/09/2005
Date Counted: 30/09/2005

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 98	**Ultrazine FG-R**	3 µg	*31.3*	*2.3*	*1.3*	34, 30, 30
		10 µg	*29.3*	*6.8*	*1.2*	37, 24, 27
		33 µg	*33.3*	*2.5*	*1.4*	31, 33, 36
		100 µg	*31.0*	*11.0*	*1.3*	20, 31, 42
		333 µg	*27.7*	*5.7*	*1.1*	26, 34, 23
		1000 µg	*30.3*	*4.2*	*1.2*	29, 27, 35
		2500 µg	*29.3*	*7.2*	*1.2*	34, 33, 21
		5000 µg	*22.0*	*7.0*	*0.9*	22, 15, 29
	Deionised water		*24.7*	*4.2*		28, 20, 26
	Untreated Control		*30.7*	*2.1*		30, 29, 33
TA 100	**Ultrazine FG-R**	3 µg	*134.3*	*2.5*	*1.0*	132, 137, 134
		10 µg	*137.7*	*16.3*	*1.0*	149, 119, 145
		33 µg	*144.0*	*6.9*	*1.1*	140, 152, 140
		100 µg	*142.3*	*3.8*	*1.0*	144, 145, 138
		333 µg	*125.0*	*13.0*	*0.9*	112, 138, 125
		1000 µg	*146.7*	*13.2*	*1.1*	135, 144, 161
		2500 µg	*145.3*	*5.1*	*1.1*	151, 144, 141
		5000 µg	*165.0*	*12.5*	*1.2*	155, 161, 179
	Deionised water		*135.7*	*9.8*		147, 130, 130
	Untreated Control		*131.0*	*10.4*		137, 119, 137
TA 98	**4-NOPD**	10 µg	*461.3*	*20.8*	*18.7*	438, 468, 478
TA 100	**NaN3**	10 µg	*2268.0*	*101.0*	*16.7*	2268, 2167, 2369

Key to Positive Controls

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

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Study Name: 899101
Experiment: 899101 VV Plate
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 27/09/2005
Date Counted: 30/09/2005

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 98	**Ultrazine FG-R**	3 µg	*44.7*	*5.9*	*1.2*	47, 49, 38
		10 µg	*37.3*	*8.1*	*1.0*	41, 43, 28
		33 µg	*39.0*	*5.6*	*1.0*	40, 44, 33
		100 µg	*34.3*	*8.0*	*0.9*	42, 26, 35
		333 µg	*35.7*	*1.5*	*0.9*	37, 36, 34
		1000 µg	*42.0*	*6.0*	*1.1*	48, 36, 42
		2500 µg	*39.7*	*3.8*	*1.0*	44, 37, 38
		5000 µg	*40.0*	*2.6*	*1.0*	42, 41, 37
	Deionised water		*38.3*	*9.5*		35, 31, 49
	Untreated Control		*34.3*	*4.7*		38, 36, 29
TA 100	**Ultrazine FG-R**	3 µg	*150.0*	*2.0*	*1.0*	152, 150, 148
		10 µg	*140.7*	*4.5*	*0.9*	136, 145, 141
		33 µg	*152.3*	*6.4*	*1.0*	145, 156, 156
		100 µg	*173.3*	*1.5*	*1.2*	172, 175, 173
		333 µg	*134.3*	*6.0*	*0.9*	128, 135, 140
		1000 µg	*137.0*	*3.6*	*0.9*	133, 140, 138
		2500 µg	*169.7*	*9.0*	*1.1*	169, 179, 161
		5000 µg	*144.3*	*11.5*	*1.0*	144, 156, 133
	Deionised water		*149.0*	*9.5*		140, 159, 148
	Untreated Control		*151.0*	*12.5*		147, 141, 165
TA 98	**2-AA**	2.5 µg	*1727.7*	*225.2*	*45.1*	1490, 1755, 1938
TA 100	**2-AA**	2.5 µg	*1939.0*	*82.5*	*13.0*	2009, 1960, 1848

Key to Positive Controls

2-AA 2-aminoanthracene

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Study Name: 899101
Experiment: 899101 HV1 Plate
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 20/10/2005
Date Counted: 25/10/2005

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Ultrazine FG-R**	33 µg	*25.7*	*4.2*	*1.5*	29, 27, 21
		100 µg	*23.0*	*1.0*	*1.3*	24, 23, 22
		333 µg	*21.3*	*1.5*	*1.2*	20, 21, 23
		1000 µg	*21.0*	*1.0*	*1.2*	20, 21, 22
		2500 µg	*27.3*	*4.6*	*1.5*	22, 30, 30
		5000 µg	*15.7*	*0.6*	*0.9*	16, 16, 15
	Deionised water		*17.7*	*3.8*		16, 15, 22
	Untreated Control		*20.3*	*3.1*		17, 23, 21
TA 1537	**Ultrazine FG-R**	33 µg	*25.3*	*5.1*	*0.9*	24, 21, 31
		100 µg	*19.3*	*2.9*	*0.7*	21, 16, 21
		333 µg	*24.7*	*8.0*	*0.9*	24, 33, 17
		1000 µg	*19.3*	*3.5*	*0.7*	16, 19, 23
		2500 µg	*18.7*	*2.3*	*0.7*	16, 20, 20
		5000 µg	*18.3*	*3.1*	*0.7*	15, 19, 21
	Deionised water		*27.0*	*4.0*		31, 27, 23
	Untreated Control		*23.7*	*0.6*		24, 23, 24
WP2 uvrA	**Ultrazine FG-R**	33 µg	*53.3*	*10.4*	*1.0*	45, 65, 50
		100 µg	*57.3*	*10.0*	*1.0*	58, 47, 67
		333 µg	*62.0*	*7.0*	*1.1*	69, 55, 62
		1000 µg	*44.7*	*6.4*	*0.8*	52, 42, 40
		2500 µg	*50.0*	*7.0*	*0.9*	58, 45, 47
		5000 µg	*57.3*	*7.1*	*1.0*	51, 65, 56
	Deionised water		*55.0*	*10.4*		48, 67, 50
	Untreated Control		*50.3*	*5.0*		51, 45, 55
TA 1535	**NaN3**	10 µg	*1259.7*	*50.1*	*71.3*	1202, 1293, 1284
TA 1537	**4-NOPD**	50 µg	*117.7*	*6.7*	*4.4*	125, 116, 112
WP2 uvrA	**MMS**	4.0 µL	*1466.7*	*78.6*	*26.7*	1378, 1494, 1528

Key to Positive Controls

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

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Study Name: 899101
Experiment: 899101 HV1 Plate
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 20/10/2005
Date Counted: 25/10/2005

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Ultrazine FG-R**	33 µg	*27.3*	*7.6*	*0.9*	36, 22, 24
		100 µg	*25.3*	*9.3*	*0.8*	15, 33, 28
		333 µg	*24.0*	*2.0*	*0.8*	26, 22, 24
		1000 µg	*31.3*	*6.4*	*1.0*	36, 34, 24
		2500 µg	*24.3*	*9.6*	*0.8*	14, 26, 33
		5000 µg	*32.3*	*4.2*	*1.1*	31, 37, 29
	Deionised water		*30.0*	*3.6*		31, 33, 26
	Untreated Control		*35.3*	*1.5*		37, 34, 35
TA 1537	**Ultrazine FG-R**	33 µg	*19.0*	*1.7*	*0.6*	17, 20, 20
		100 µg	*27.0*	*2.6*	*0.9*	29, 24, 28
		333 µg	*24.0*	*3.6*	*0.8*	28, 21, 23
		1000 µg	*26.3*	*3.1*	*0.9*	23, 29, 27
		2500 µg	*30.7*	*13.2*	*1.0*	28, 19, 45
		5000 µg	*22.7*	*6.7*	*0.8*	30, 17, 21
	Deionised water		*29.3*	*4.0*		27, 27, 34
	Untreated Control		*32.7*	*6.1*		38, 34, 26
WP2 uvrA	**Ultrazine FG-R**	33 µg	*57.7*	*3.5*	*0.9*	58, 61, 54
		100 µg	*61.0*	*4.4*	*1.0*	56, 64, 63
		333 µg	*67.7*	*2.9*	*1.1*	71, 66, 66
		1000 µg	*61.7*	*10.5*	*1.0*	72, 62, 51
		2500 µg	*63.7*	*13.1*	*1.0*	65, 76, 50
		5000 µg	*69.0*	*4.4*	*1.1*	74, 66, 67
	Deionised water		*61.7*	*13.4*		77, 56, 52
	Untreated Control		*60.7*	*3.8*		59, 65, 58
TA 1535	**2-AA**	2.5 µg	*302.3*	*35.1*	*10.1*	299, 269, 339
TA 1537	**2-AA**	2.5 µg	*191.3*	*5.7*	*6.5*	185, 196, 193
WP2 uvrA	**2-AA**	10.0 µg	*337.7*	*44.7*	*5.5*	317, 307, 389

Key to Positive Controls

2-AA 2-aminoanthracene

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Study Name: 899101
Experiment: 899101 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 28/10/2005
Date Counted: 31/10/2005

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Ultrazine FG-R**	33 µg	*25.0*	*7.0*	*1.0*	33, 20, 22
		100 µg	*22.3*	*6.8*	*0.9*	17, 30, 20
		333 µg	*26.7*	*2.3*	*1.0*	24, 28, 28
		1000 µg	*20.3*	*7.4*	*0.8*	23, 12, 26
		2500 µg	*25.0*	*1.7*	*1.0*	24, 27, 24
		5000 µg	*23.7*	*2.1*	*0.9*	26, 22, 23
	Deionised water		*26.0*	*13.9*		33, 35, 10
	Untreated Control		*23.7*	*9.3*		16, 21, 34
TA 1537	**Ultrazine FG-R**	33 µg	*30.0*	*3.6*	*1.3*	34, 29, 27
		100 µg	*20.3*	*3.8*	*0.9*	22, 16, 23
		333 µg	*22.7*	*6.1*	*1.0*	24, 16, 28
		1000 µg	*22.3*	*7.2*	*1.0*	27, 14, 26
		2500 µg	*25.3*	*2.9*	*1.1*	27, 27, 22
		5000 µg	*28.0*	*5.6*	*1.3*	33, 29, 22
	Deionised water		*22.3*	*4.9*		19, 20, 28
	Untreated Control		*25.3*	*2.3*		28, 24, 24
TA 98	**Ultrazine FG-R**	33 µg	*35.0*	*2.0*	*0.9*	37, 33, 35
		100 µg	*32.0*	*13.1*	*0.8*	41, 17, 38
		333 µg	*29.0*	*2.0*	*0.7*	31, 29, 27
		1000 µg	*32.0*	*4.4*	*0.8*	29, 30, 37
		2500 µg	*31.3*	*1.5*	*0.8*	33, 30, 31
		5000 µg	*35.0*	*6.2*	*0.9*	40, 37, 28
	Deionised water		*40.7*	*2.5*		38, 43, 41
	Untreated Control		*30.3*	*8.0*		22, 31, 38
TA 100	**Ultrazine FG-R**	33 µg	*158.3*	*12.4*	*1.0*	166, 144, 165
		100 µg	*165.7*	*23.7*	*1.0*	144, 162, 191
		333 µg	*160.3*	*10.7*	*1.0*	172, 158, 151
		1000 µg	*145.7*	*10.1*	*0.9*	155, 135, 147
		2500 µg	*168.3*	*6.7*	*1.1*	165, 176, 164
		5000 µg	*162.7*	*7.6*	*1.0*	166, 168, 154
	Deionised water		*160.0*	*21.0*		137, 178, 165
	Untreated Control		*152.3*	*3.5*		156, 149, 152
WP2 uvrA	**Ultrazine FG-R**	33 µg	*44.7*	*8.5*	*1.3*	51, 35, 48
		100 µg	*42.7*	*7.1*	*1.3*	44, 49, 35
		333 µg	*42.7*	*14.0*	*1.3*	57, 42, 29
		1000 µg	*52.7*	*4.0*	*1.6*	55, 48, 55
		2500 µg	*52.7*	*5.1*	*1.6*	57, 54, 47
		5000 µg	*49.3*	*5.1*	*1.5*	55, 45, 48
	Deionised water		*33.7*	*4.0*		29, 36, 36
	Untreated Control		*49.7*	*0.6*		50, 49, 50

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Study Name: 899101
Experiment: 899101 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 28/10/2005
Date Counted: 31/10/2005

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	*1393.3*	*26.6*	*53.6*	1424, 1377, 1379
TA 1537	**4-NOPD**	50 µg	*107.0*	*13.0*	*4.8*	94, 120, 107
TA 98	**4-NOPD**	10 µg	*382.3*	*8.5*	*9.4*	392, 376, 379
TA 100	**NaN3**	10 µg	*1903.3*	*97.6*	*11.9*	1967, 1791, 1952
WP2 uvrA	**MMS**	4.0 µL	*587.0*	*61.6*	*17.4*	603, 519, 639

Key to Positive Controls

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

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Study Name: 899101
Experiment: 899101 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 28/10/2005
Date Counted: 31/10/2005

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Ultrazine FG-R**	33 µg	*21.0*	*1.7*	*1.0*	23, 20, 20
		100 µg	*30.7*	*4.5*	*1.5*	35, 31, 26
		333 µg	*25.7*	*1.5*	*1.2*	26, 24, 27
		1000 µg	*24.7*	*2.9*	*1.2*	28, 23, 23
		2500 µg	*26.0*	*3.6*	*1.3*	29, 22, 27
		5000 µg	*29.0*	*3.6*	*1.4*	28, 33, 26
	Deionised water		*20.7*	*3.5*		17, 24, 21
	Untreated Control		*28.0*	*1.0*		28, 29, 27
TA 1537	**Ultrazine FG-R**	33 µg	*24.7*	*1.2*	*0.9*	24, 24, 26
		100 µg	*30.3*	*9.3*	*1.2*	33, 38, 20
		333 µg	*27.0*	*7.2*	*1.0*	19, 33, 29
		1000 µg	*22.0*	*6.6*	*0.8*	16, 21, 29
		2500 µg	*28.0*	*4.4*	*1.1*	23, 30, 31
		5000 µg	*25.7*	*7.5*	*1.0*	30, 17, 30
	Deionised water		*26.3*	*0.6*		27, 26, 26
	Untreated Control		*25.7*	*1.5*		24, 26, 27
TA 98	**Ultrazine FG-R**	33 µg	*41.0*	*7.8*	*1.0*	36, 50, 37
		100 µg	*31.3*	*14.0*	*0.8*	47, 27, 20
		333 µg	*37.3*	*6.5*	*0.9*	44, 31, 37
		1000 µg	*37.7*	*6.4*	*0.9*	45, 35, 33
		2500 µg	*33.3*	*2.5*	*0.8*	31, 36, 33
		5000 µg	*34.3*	*0.6*	*0.9*	34, 35, 34
	Deionised water		*40.3*	*0.6*		40, 40, 41
	Untreated Control		*41.0*	*1.0*		42, 41, 40
TA 100	**Ultrazine FG-R**	33 µg	*176.0*	*9.6*	*0.9*	165, 180, 183
		100 µg	*186.0*	*13.0*	*1.0*	194, 171, 193
		333 µg	*176.0*	*8.0*	*0.9*	184, 176, 168
		1000 µg	*178.7*	*15.9*	*1.0*	169, 197, 170
		2500 µg	*189.3*	*11.0*	*1.0*	198, 177, 193
		5000 µg	*185.0*	*11.1*	*1.0*	183, 197, 175
	Deionised water		*186.0*	*7.5*		178, 187, 193
	Untreated Control		*168.0*	*26.9*		138, 190, 176
WP2 uvrA	**Ultrazine FG-R**	33 µg	*58.7*	*9.6*	*0.9*	69, 50, 57
		100 µg	*55.0*	*10.8*	*0.9*	43, 64, 58
		333 µg	*60.7*	*2.5*	*1.0*	63, 58, 61
		1000 µg	*61.0*	*5.0*	*1.0*	66, 61, 56
		2500 µg	*66.0*	*9.8*	*1.0*	77, 63, 58
		5000 µg	*59.7*	*11.4*	*0.9*	47, 69, 63
	Deionised water		*63.7*	*3.1*		67, 63, 61
	Untreated Control		*69.7*	*7.8*		72, 76, 61

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Study Name: 899101
Experiment: 899101 HV2 Pre
Assay Conditions:

Study Code: RCC - CCR 899101
Date Plated: 28/10/2005
Date Counted: 31/10/2005

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	*218.7*	*3.2*	*10.6*	215, 220, 221
TA 1537	**2-AA**	2.5 µg	*183.3*	*11.0*	*7.0*	172, 184, 194
TA 98	**2-AA**	2.5 µg	*1070.7*	*55.8*	*26.5*	1035, 1042, 1135
TA 100	**2-AA**	2.5 µg	*1505.0*	*185.4*	*8.1*	1292, 1593, 1630
WP2 uvrA	**2-AA**	10.0 µg	*347.3*	*29.7*	*5.5*	357, 314, 371

Key to Positive Controls

2-AA	2-aminoanthracene

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15 ANNEX II: COPY OF GLP CERTIFICATE

Hessisches Ministerium für Umwelt,
ländlichen Raum und Verbraucherschutz

HESSEN

Gute Laborpraxis/Good Laboratory Practice

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

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

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

☒ Prüfeinrichtung/Test facillity ☐ Prüfstandort/Test site

RCC Cytotest Cell Research GmbH
RCC Cytotest Cell Research GmbH
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

03.06.; 19.07.-22.07.2004
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 **Wiesbaden, den 06. Januar 200**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

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

D-65189 Wiesbaden, Mainzer Straße 80
Telefon: 0611. 81 50
Telefax: 0611. 81 51 94 1
E-Mail: poststelle@hmulv.hessen.de

D-65187 Wiesbaden, Hölderlinstraße 1-3
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16 ANNEX III: COPY OF STUDY PLAN

(16 pages)

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RCC - CCR STUDY NUMBER 899101

SALMONELLA TYPHIMURIUM AND ESCHERICHIA COLI REVERSE MUTATION ASSAY WITH ULTRAZINE FG-R (FOOD GRADE LIGNOSULPHONATE)

STUDY PLAN

1 SIGNATURES

Study Director

Dipl. Biol. Andrea Sokolowski

Date: September 13, 2005

Management

Dr. Wolfgang Völkner

Date: September 13, 2005

Head of Quality
Assurance Unit

Frauke Hermann

Date: September 15, 2005

Study Monitor
(on behalf of the Sponsor)

DR. WERNER KÖHL

Date: 19. Sep. 2005

SCC Scientific Consulting
Chemisch-Wissenschaftliche Beratung GmbH
Mikroforum Ring 1 D-55234 Wendelsheim
Tel. 06734-919-0 Fax 06734-919-199

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3 PREFACE

3.1 General

Title:	Salmonella typhimurium and Escherichai coli Reverse Mutation Assay with Ligninsulfonat
Sponsor:	DSM Nutritional Products AG Wurmisweg 576 CH-4303 Kaiseraugst Switzerland
Study Monitor:	Dr. Werner Köhl SCC Scientific Consulting Company Mikroforum Ring 1 D-55234 Wendelsheim
SCC Project No.:	612-002
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:	September 13, 2005
Proposed Experimental Starting Date:	September, 2005
Proposed Experimental Completion Date:	October, 2005
Proposed Date of Draft Report:	November, 2005

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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].

Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

3.4 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.

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

3.5 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.

3.6 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.

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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. Before initiating the second experiment, concentrations to be used and study design will be confirmed by the sponsor. 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, WP2 uvrA) 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 5644 22

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

Identity:	Ultrazine FG-R (Food Grade Lignosulphonate)
Chemical Name:	Calcium Lignosulphonate
Batch No.:	FGR-004
Aggregate State at Room Temperature:	solid
Colour:	brown
Purity:	95.5 % (dry solids)
Certificate of Analysis (date):	August 26, 2005
Stability in Solvent:	Not indicated by the sponsor
Storage:	Room temperature, moisture protected
Expiration Date:	August 26, 2007

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.

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:	WP2 uvrA
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, WP2 uvrA
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 WP2 uvrA)

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 and E. coli Strain

The histidine dependent strains are derived from S. typhimurium strain LT2 through mutations in the histidine locus. Additionally due to the "deep rough" (rfa^-) mutation they possess a faulty lipopolysaccharide envelope which enables substances to penetrate the cell wall more easily. A further mutation causes a reduction in the activity of an excision repair system. The latter alteration includes mutational processes in the nitrate reductase and biotin genes produced in a UV-sensitive area of the gene named $uvrB^-$. In the strains TA 98 and TA 100 the R-factor plasmid pKM 101 carries the ampicillin resistance marker (6).

Strain WP2 (4) and its derivatives all carry the same defect in one of the genes for tryptophan biosynthesis. Tryptophan-independent (Trp^+) mutants (revertants) can arise either by a base change at the site of the original alteration or by a base change elsewhere in the chromosome so that the original defect is suppressed. This second possibility can occur in several different ways so that the system seems capable of detecting all types of mutagen which substitute one base for another. Additionally, the uvrA derivative is deficient in the DNA repair process (excisable repair damage). Such a repair-deficient strain may be more readily mutated by agents.

When summarised, the mutations of the TA strains and the E. coli strain used in this study can be described as follows:

Table 1; Type of mutations indicated:

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	" "
Escherichia coli		
WP2 uvrA	trp^-; $uvrA^-$:	base-pair substitutions and others

Regular checking of the properties of the Salmonella typhimurium and E. coli strains regarding the membrane permeability and ampicillin resistance as well as normal spontaneous mutation rates is performed in RCC Cytotest Cell Research GmbH according to B. Ames et al. (1) and D. Maron and B. Ames (6). 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 WP2 uvrA 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. 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 for at least 15 min. at 2 bar 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 HanIbm 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 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. Before initiating the main experiments, concentrations to be used will be confirmed by the monitor.

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

Two independent experiments will be performed. Before initiating the second experiment, concentrations to be used and study design will be confirmed by the sponsor. For each strain and dose level, including the controls, three plates will be used.

For the plate incorporation method 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

For the pre-incubation method 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).

*Since some solvents (i.e. ethanol, acetone, THF) are toxic to the bacteria a lower amount of test solution will be applied if one of these solvents will be used.

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. 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. 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 WP2 uvrA) 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.

The report will contain all relevant information about the test item (e.g. Certificate of Analysis, etc) as well as the study protocol.

The report will be in line with EPA-format requirements.

The report will cover all information required by the relevant OECD and EU guidelines.

The sponsor will receive 1 Original (bound) and two copies (unbound) of the report and one complete pdf-file of the report.

7 DISTRIBUTION

Sponsor	4 x (1 x original to be returned to RCC-CCR, 1 x duplicate, and 1 x copy, and 1 x pdf-file)
Study Director	1 x (copy)
QAU	1 x (copy)

J:\ames\899101\SCC Ames Test Study Plan

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 being established. These data represent the laboratory's historical control data since July 2004 representing 150 experiments.

Table 2; Historical Control Data:

Strain		without S9 mix				with S9 mix			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA 1535	Solvent control	19	6	9	35	21	7	7	41
	Negative control	18	5	10	30	21	6	9	38
	Positive control	1681	789	1003	4900	387	126	172	695
TA1537	Solvent control	12	3	4	29	18	6	6	36
	Negative control	11	3	5	29	19	6	8	33
	Positive control	87	18	52	191	337	191	94	746
TA 98	Solvent control	26	6	14	58	39	9	21	57
	Negative control	26	6	15	60	41	9	17	64
	Positive control	361	204	176	1818	2386	1195	296	4854
TA 100	Solvent control	131	24	91	198	147	25	109	281
	Negative control	140	21	101	189	154	23	103	254
	Positive control	2030	340	1178	2872	2629	1326	546	5230
WP2uvrA	Solvent control	52	8	31	67	55	10	34	75
	Negative control	50	8	36	64	52	8	33	64
	Positive control	998	515	320	1976	342	134	221	930

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

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17 ANNEX IV: COPY OF THE 1ST AMENDMENT TO STUDY PLAN

(4 pages)

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RCC - CCR STUDY NUMBER 899101

1ST AMENDMENT TO STUDY PLAN

(4 Pages)

TITLE:	**SALMONELLA TYPHIMURIUM AND ESCHERICHIA COLI REVERSE MUTATION ASSAY WITH Ultrazine FG-R (Food Grade Lignosulphonate)**
SPONSOR:	**DSM Nutritional Products AG Wurmisweg 576 CH-4303 Kaiseraugst Switzerland**
STUDY MONITOR:	**Dr. Werner Köhl SCC Scientific Consulting Company Mikroforum Ring 1 D-55234 Wendelsheim**
DATE OF AMENDMENT:	**October 11, 2005**

RCC

Concerning: **5.4.5 Overlay Agar**
page 10

Present:

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)

New:

The overlay agar contains per litre:

for Salmonella strains:

6.0 g MERCK Agar Agar*
6.0 g NaCl*
10.5 mg L-Histidin×HCl×H2O*
12.2 mg Biotin*

for Escherichia coli:

6.0 g MERCK Agar Agar*
6.0 g NaCl*
2.5 mg Tryptophan*

* (MERCK, D-64293 Darmstadt)

Concerning: **5.7 Dose Selection**
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Present:

According to the results of this pre-experiment the concentrations to be applied in the main experiments will be chosen. Before initiating the main experiments, concentrations to be used will be confirmed by the monitor.

Additional:

The test item was dissolved in deionised water. Neither in the stock solution nor in the top agar with and without metabolic activation precipitation was observed.

To evaluate the toxicity of the test item a pre-study (plate incorporation) was performed with strains TA 98 and TA 100. The following eight concentrations were tested for toxicity and mutation induction with each 3 plates (plate incorporation test):

3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Since neither mutagenic nor toxic effects were observed the following concentrations will be tested in experiment I:

33; 100; 333; 1000; 2500; and 5000 µg/plate

Reason for the Alteration:

Dose selection based on the solubility of the test item and a concentration range finding study and in agreement with the Study monitor.

Signatures

Study Director — Dipl. Biol. Andrea Sokolowski

Date: October 11, 2005

Management — Dr. Wolfgang Völkner

Date: October 11, 2005

Head of Quality Assurance Unit — Frauke Hermann

Date: October 13, 2005

Sponsor

Date: October 17, 2005

Distribution

Sponsor	4 x (1 x original to be returned to RCC-CCR, 1 x duplicate, 1 x copy, and 1 x pdf-file)
Study Director	1 x (copy)
QAU	1 x (copy)