FINAL REPORT

Test Facility Study No. 787718, Report No. 30768

Alcohols, C18-22 Distn. Residues
Testing for Mutagenic Activity with *Salmonella typhimurium* TA 1535, TA 100, TA 1537 and TA 98 and *Escherichia coli* WP2*uvr*A

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

1 COMPLIANCE STATEMENT

I, the undersigned, hereby declare that this study was performed in accordance with the OECD Principles of Good Laboratory Practice as incorporated into the United Kingdom Statutory Instrument for GLP and as accepted by Regulatory Authorities throughout the European Community, United States of America (FDA and EPA) and Japan (MHLW, MAFF and METI).

The study was conducted according to the procedures herein described and this report represents a true and accurate record of the results obtained.

Colin G Riach BSc
Study Director

25 Nov 2009
Date

2 QUALITY ASSURANCE STATEMENT

Study title: Alcohols, C18-22 Distn. Residues: Testing for Mutagenic Activity with *Salmonella typhimurium* TA 1535, TA 100, TA 1537 and TA 98 and *Escherichia coli* WP2*uvr*A

The Charles River Quality Assurance Unit conducted a protocol review, process-based inspections and report audits relevant to this short-term study, as detailed below.

Date of QA Activity	Activity	Date of Report to Management
05 October 2009	Protocol Review	05 October 2009
06 October 2009	Dose Preparation/Dosing/ Protocol Compliance	06 October 2009
09 October 2009	Colony Counting	12 October 2009
07 October 2009	QC Checking	08 October 2009
02-03 November 2009	Draft Report Audit	03 November 2009
24 November 2009	Final Report Audit	24 November 2009

The protocol review and report audits were reported to the Study Director on the same date as management. The outcome of each process-based inspection is also reported to the Study Director, where relevant.

Facilities relevant to this study are included in Charles River's annual facility inspection programme. The outcome of each inspection is reported to Management.

This report is considered to describe accurately and completely the procedures used in the study and the results obtained.

Jane P Dunsire

Jane P Dunsire CBiol MSB
Quality Assurance

25 November 2009

Date

3 RESPONSIBLE PERSONNEL

Study Director:	Colin G Riach BSc
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Sponsor Representative (Study Monitor):	Hans Certa PhD

4 SUMMARY

Alcohols, C18-22 Distn. Residues was tested for mutagenic activity in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100 and in *Escherichia coli* WP2*uvr*A. Alcohols, C18-22 Distn. Residues was formulated in dimethylsulphoxide, in which it gave a part solution/part suspension.

Two independent tests were conducted on agar plates in triplicate in the absence and presence of an Aroclor 1254-induced rat liver S9 preparation and the co-factors required for mixed-function oxidase activity (S9 mix). The first test was conducted by the direct plate incorporation method, while the second test was conducted by the pre-incubation method. Alcohols, C18-22 Distn. Residues was dosed at concentrations ranging from 6 to 2000 µg per plate in both assays (2000 µg per plate was the highest practical concentration, as limited by the solubility of the test item).

Concurrent positive controls demonstrated the sensitivity of the assay and the metabolising activity of the S9 mix.

No evidence of mutagenic activity was obtained with any strain in either test.

No toxicity to the bacteria was observed. Alcohols, C18-22 Distn. Residues precipitated at the highest concentration of 2000 µg per plate.

It is concluded that Alcohols, C18-22 Distn. Residues is not mutagenic in strains of *Salmonella typhimurium* and *Escherichia coli,* when tested in the absence and presence of metabolic activation up to and beyond its limit of solubility in the test system.

The study was performed in accordance with the principles of Good Laboratory Practice.

5 INTRODUCTION

The objective of this study was to assess the mutagenic potential of the test item, Alcohols, C18-22 Distn. Residues, using *Salmonella typhimurium* and *Escherichia coli* in a bacterial mutagenicity assay, the Ames test.

5.1 Regulatory Citations

5.1.1 Test Guideline

This study was performed to comply with OECD guideline 471, the European Commission Annex V Test Method B13 and B14, ICH Guidelines CPMP/ICH/141/95 and CPMP/ICH/174/95 and USA EPA 712-C-98-247. The ICH Guidelines are recognised by Regulatory bodies of the European Community, United States of America and Japan.

5.1.2 Quality Compliance

The study was conducted in accordance with the OECD Principles of Good Laboratory Practice as set forth by the United Kingdom Department of Health and as accepted by Regulatory Authorities of the European Community, United States of America (FDA and EPA) and Japan (MHLW, MAFF and METI).

All routine activities conducted during the course of this study are detailed in Charles River's Standard Operating Procedures.

5.2 Test Facility

This report describes the methods used and the results obtained in tests carried out at the laboratories of:

Charles River
Tranent
Edinburgh
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UK

5.3 Key Dates in the Conduct of the Study:

Study Initiation:	01 October 2009
Experimental Start Date:	02 October 2009
Experimental Completion Date:	12 October 2009
Study Completion Date:	See Compliance Statement page for date of Study Director's signature.

5.4 Archiving of Data

All raw data generated and recorded during this study are stored in the Scientific Archives of Charles River Preclinical Services Edinburgh for 2 years after the issue of the final report. After the 2 year period, the Sponsor will be consulted regarding the disposal, transfer or continued storage of the raw data.

The original signed copy of the final report will be stored indefinitely in the Scientific Archives of Charles River Preclinical Services Edinburgh.

5.5 Deviations from the Study Protocol

There were no deviations from the Study Protocol.

6 MATERIALS AND METHODS

6.1 Test Item

Alcohols, C18-22 Distn. Residues (Batch 03585/MA), a beige waxy solid, was received from Sasol Germany GmbH on 11 September 2009 and was stored in the dark at ambient room temperature. The purity was quoted as 100% (being a complex distn. residue) and an expiry date of 31 August 2013 was supplied by the Sponsor. A copy of the Certificate of Analysis is shown in Appendix 1.

6.2 Dose Formulations

6.2.1 Preparation of Dose Formulations

In preliminary tests, Alcohols, C18-22 Distn. Residues was found to have limited solubility in all solvents compatible with the test system. Dimethylsulphoxide (DMSO) was chosen as the solvent giving the best solubility/dispersal characteristics. A partial solution was obtained using the following technique.

DMSO was added to a pre-weighed sample of Alcohols, C18-22 Distn. Residues to give a concentration of 20 mg/mL. The mixture was vortex-mixed for several minutes, and then placed in an incubator at 37°C for *ca* 45 min. After this, the mixture was vortex-mixed for a further *ca* 10 minutes. This process produced a milky suspension with fine particles that could be drawn up a wide-bore pipette tip.

To check whether the DMSO was carrying dissolved test item above the saturation limit in the aqueous test conditions, 0.1 mL of the above 20 mg/mL preparation was added to 2.5 mL samples of water and DMSO and the level of precipitate compared. There was a greater level of precipitate/undissolved material present in the water sample than in the DMSO sample and

it was therefore concluded that a concentration of 20 mg/mL in DMSO (being greater than the saturation level in aqueous conditions) was a suitable maximum concentration to use in the study.

6.2.2 Analysis of Dose Formulations

The test item formulations were prepared immediately prior to dosing (within 1 h). Detailed records of preparation of the dosing solutions were maintained to allow checking of procedures. Chemical analysis of the test item formulations was not conducted; however Alcohols, C18-22 Distn. Residues was tested up to and beyond its limit of solubility in the test system in all experiments.

6.3 Test System

The bacterial reverse mutation assay uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base-pairs. The principle of this bacterial reverse mutation assay is that it detects reverse mutations in histidine or tryptophan loci of the test strains that restore the functional capability of the bacteria to synthesise an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the essential amino acid required by the parent test strain.

6.4 Justification of Test System

Chemicals that react with DNA may cause different types of mutations and many of these can be detected with the use of different strains of bacteria.

It is well recognised that many chemicals that may be reactive in mammalian cells, following metabolic activation, are quite inactive in bacterial cells. S9 mix (homogenate prepared from liver of Aroclor 1254-treated rats, along with cofactors necessary for enzymic activity) provides an exogenous metabolic activation system. S9 mix is combined with the test item and bacterial cells to increase the relevance of the test in assessing the mutagenicity of chemicals to humans.

The relevant testing guidelines recommend that a concentration limit of 5 mg per plate should be used if no physicochemical property prevents this. (In the event, the limited solubility of the test item restricted the highest concentration to 2 mg per plate.)

6.5 Bacteria

Four strains of *Salmonella typhimurium* were used:

S. typhimurium TA 1535
S. typhimurium TA 100
S. typhimurium TA 1537
S. typhimurium TA 98

These *S. typhimurium* strains were obtained in 1976 from Professor B N Ames, Department of Biochemistry, University of California, USA, and have been maintained in liquid nitrogen storage since receipt.

All these *S. typhimurium* strains contain mutations in the histidine operon, thereby imposing the essential requirement for histidine in the growth medium. Three mutations in the histidine operon are involved:

his G 46 in TA 1535 and TA 100
his C 3076 in TA 1537
his D 3052 in TA 98

his G 46 is a mis-sense mutation that is reverted to prototrophy by mutagens that cause base-pair substitutions.

his C 3076 contains a frameshift mutation that appears to have added a $\begin{matrix} -G- \\ -C- \end{matrix}$ base-pair, resulting in $\begin{matrix} -GGGG- \\ -CCCC- \end{matrix}$. This mutation is reverted to prototrophy by 9-aminoacridine, ICR-191 and epoxides of polycyclic hydrocarbons.

his D 3052 also contains a frameshift mutation with the sequence $\begin{matrix} -CGCGCG- \\ -GCGCGC- \end{matrix}$ that is reverted with the deletion of 2 base-pairs, $\begin{matrix} -CG- \\ -GC- \end{matrix}$. It is readily reverted by aromatic amines and derivatives.

All 4 strains contain the deep rough (rfa) mutation, which results in deletion of the polysaccharide side chain of the lipopolysaccharide coat of the bacterial cell surface. This deletion increases cell permeability to more hydrophobic substances and, furthermore, greatly decreases the pathogenicity of these organisms. A second deletion, through *uvr*B, renders the

organisms incapable of DNA excision repair and thus more susceptible to mutagenicity. These 2 deletions also include the nitrate reductase (*chl*) and biotin (*bio*) genes.

TA 100 and TA 98 strains contain a plasmid (R-Utrecht) that was originally shown to increase the sensitivity of the *his* G 46 mutation in *S. typhimurium* to methyl methanesulphonate and trimethyl phosphate. The particular R-factor (pKM101) in TA 100 and TA 98 carries resistance to ampicillin.

One strain of *Escherichia coli* was used:

Escherichia coli WP2*uvr*A

This *Escherichia coli* strain was obtained in 1976 from the National Collection of Industrial Bacteria, Aberdeen, Scotland, and has been maintained in liquid nitrogen storage since receipt.

The strain contains an ochre mutation in the *trp*E locus and can be mutated to tryptophan-independence either by a base-pair reversion of an A-T base-pair in the *trp*E locus, or more commonly, by a base-pair substitution within a number of transfer RNA loci elsewhere in the chromosome. The latter mutation causes the original defect to be suppressed (ochre suppression) and involves only base-pair substitutions at G-C base-pairs.

Thus, while trp^+ reversion systems can detect mutations resulting from chemical attack at both A-T and G-C base-pairs, it does not detect frameshift mutagens. The *uvr*A mutation causes this bacterial strain to be deficient in the excision of bulky lesions from the DNA and thus readily mutated by certain agents (*eg* ultraviolet radiation, polycyclic hydrocarbons).

6.5.1 Bacterial Cultures

Samples of each bacterial strain were grown up by culturing for 16 h at *ca* 37°C in nutrient broth (25 g Oxoid Nutrient Broth No. 2 per litre). Fresh overnight cultures were used in the tests for this study. Samples from the cultures were kept for up to 7 days at *ca* 4°C to allow relevant checks to be performed.

6.6 Experimental Design

Although some testing guidelines, *eg* ICH, do not dictate an automatic repetition of experiments, it was considered appropriate to prove the reproducibility of all findings in this assay system. Consequently, 2 experiments were conducted in both the absence and the presence of S9 mix.

The first test was performed using the Direct Plate Incorporation Method. If the first test was negative, the repeat test was performed using the Pre-incubation Method. If the first test was positive, the second test was also performed using the Direct Plate Incorporation Method to confirm the response.

Triplicate plates were poured for each exposure level (n = 6) and bacterial strain (n = 5) in the absence or presence of the exogenous activation system. Each plate was labelled with a number/colour code to identify the study, bacterial strain, treatment and activation type.

6.7 Justification of Dose Levels

The relevant testing guidelines recommend that a concentration limit of 5000 µg per plate should be used if no physicochemical property prevents this. As described in Section 6.2.1 above, the limited solubility of Alcohols, C18-22 Distn. Residues restricted the maximum concentration to 2000 µg per plate.

A toxicity test was performed to establish the range of concentrations to be used in the first mutation test. The concentrations tested were:

6, 20, 60, 200, 600 and 2000 µg per plate

No toxicity was observed in the preliminary toxicity test, therefore the concentrations tested in the first mutation experiment were as follows for all 5 bacterial strains, in both the absence and the presence of S9 mix:

6, 20, 60, 200, 600 and 2000 µg per plate

As neither toxicity nor mutagenic activity was observed in the first mutation assay, the concentrations tested in the second mutation experiment were as follows for all 5 bacterial strains, in both the absence and the presence of S9 mix:

6, 20, 60, 200, 600 and 2000 µg per plate

6.8 Administration of Test/Control Items

6.8.1 Vehicle Controls

DMSO was used as the vehicle control and was plated in triplicate with each strain used, both in the absence and in the presence of S9 mix.

6.8.2 Positive Controls

The following positive control concentrations were plated in triplicate:

With S9 mix:

2-Aminoanthracene (2AAN): 2 µg per plate with *S. typhimurium* TA 1535 and TA 1537, 0.5 µg per plate with *S. typhimurium* TA 98 and TA 100 and 20 µg per plate with *E. coli* WP2*uvr*A

Without S9 mix:

Sodium azide (NaN_3): 1 µg per plate with *S. typhimurium* TA 1535 and TA 100
9-Aminoacridine (9-AA): 80 µg per plate with *S. typhimurium* TA 1537
2-Nitrofluorene (2-NF): 1 µg per plate with *S. typhimurium* TA 98
N-Ethyl-N-nitro-N-nitrosoguanidine (ENNG): 2 µg per plate with *E. coli* WP2*uvr*A

6.8.3 Test Item

All test item concentrations were plated in triplicate.

6.9 Experimental Procedure

The procedures used are based on the method of Ames *et al* (1975). Aseptic techniques, conducted under amber light, were used throughout this study.

All water used in the preparation of reagents was produced (in-house), by reverse osmosis followed by mixed-bed deionisation and sterilisation by autoclaving.

6.9.1 S9 Mix

For a general reference, see McGregor *et al* (1988).

S9 enzymes (supernatant post-mitochondrial fraction obtained after centrifugation at 9000 *g*) were prepared in-house from the livers of Aroclor 1254-treated adult, male Fischer rats, as described by Ames *et al* (1975).

S9 enzymes were stored in sterile plastic tubes immersed in liquid nitrogen (*ca* -196°C) and used within 6 months of preparation.

The enzymic activity of each batch of S9 enzymes was characterised by testing selected pre-mutagens in an Ames test with *S. typhimurium* TA 1538. (Although strain TA 1538 is not used in the study, a considerable amount of historical data exists on enzymic activity with this

S. typhimurium strain, and these data were used for comparison with current batches of S9 enzymes.) The results for the characterisation of the batch of S9 enzymes used in the mutation tests of the present study are shown in Appendix 2. S9 enzyme batches used must also have demonstrated, within each test, a satisfactory mutagenic response in cultures treated with 2-aminoanthracene (positive control).

To prepare S9 mix, 0.05 M phosphate buffer pH 7.4 was added to pre-weighed cofactors: nicotinamide adenine dinucleotide phosphate (NADP) disodium salt, glucose-6-phosphate (G-6-P) disodium salt, magnesium ions as $MgCl_2.6H_2O$ and potassium ions as KCl, giving final concentrations in the 'S9 mix' of:

NADP Na_2	4 mM	(= 3.150 mg/mL)
Glucose-6-phosphate Na_2	25 mM	(= 7.605 mg/mL)
$MgCl_2.6H_2O$	8 mM	(= 1.626 mg/mL)
KCl	33 mM	(= 2.460 mg/mL)

This solution was immediately filter sterilised by passage through a 0.45 µm disposable filter assembly and mixed 9:1 (v/v) with the S9 enzymes.

6.9.2 Toxicity Tests

To establish suitable exposure levels for the first mutation test it was necessary to conduct an initial dose-finding test in the presence and absence of S9 mix. A single strain of bacteria, *S. typhimurium* TA 100 was used, and one plate per exposure level of test item was prepared. The test method was similar to that described below for the direct plate mutation assay (Section 6.9.3.1).

Plates were incubated at *ca* 37°C for 2 or 3 days. The numbers of revertant colonies were noted and the plates carefully examined, microscopically, for thinning of the background lawn of microcolonies. Condition of background lawn was assessed as normal, slightly thin lawn (ST), thin lawn (TL), very thin lawn (VT) or lawn absent (A). Any precipitation of the test item on the plates was noted.

6.9.3 Mutation Tests

Diluted agar (0.6% Difco Bacto-agar, 0.6% NaCl) was autoclaved and, just before use, supplemented as follows:

For *S. typhimurium*, sterile 1.0 mM L-histidine.HCl, 1.0 mM biotin solution was added at 50 mL per litre of soft agar. For *E. coli*, sterile 1.35 mM L-tryptophan solution was added at 10 mL per litre of soft agar. These soft agars were thoroughly mixed and kept in a water bath at *ca* 45°C.

6.9.3.1 Treatment Methods

Direct Plate Method: For this assay method, 2 mL of soft agar were dispensed into a small, plastic, sterile tube. S9 mix or 0.05 M phosphate buffer, pH 7.4 (0.5 mL) was added, followed by 0.1 mL of bacteria and, finally, the test item solution (0.1 mL). The tube contents (which were continually cooling) were mixed, then poured on to minimal medium plates prepared in-house. The minimal medium plates contained 20 mL of 1.5% BBL Purified Agar in Vogel-Bonner Medium E (Vogel and Bonner, 1956) with 2% glucose.

Pre-incubation Method: For this assay method, 0.5 mL of S9 mix or 0.05 M phosphate buffer, pH 7.4 was dispensed into a small, plastic, sterile tube followed by 0.1 mL of bacteria and, finally, the test solution (0.1 mL). The tubes were then placed in a shaking incubator at *ca* 37°C for 20 min. After incubation, 2 mL of soft agar was added to each tube. The tube contents were then mixed and poured onto minimal medium plates (preparation described above).

When the soft agar had set, the plates were inverted and incubated at *ca* 37°C for 2 or 3 days and then examined. The numbers of mutant colonies on each plate were determined using a Sorcerer Colony Counter and captured electronically in a validated software system Ames Study Manager (both from Perceptive Instruments). The plates were also examined microscopically for precipitates and for microcolony growth (condition of background lawn was assessed as described in Section 6.9.2).

6.10 Quality Control of Bacterial Strains

All bacterial strains were plated (one plate per strain) onto complete medium and tested for ampicillin resistance (indicating the presence of pKM101) and crystal violet sensitivity (indicating persistence of the rfa mutation: the *E. coli* strain typically gives a +/- response to crystal violet). All bacterial strains were also checked for ultraviolet radiation sensitivity (indicating persistence of the *uvr*B mutation) and for essential amino acid requirement.

6.11 Calculations and Data Acceptance

6.11.1 Calculations

The mean number of mutant colonies, plus standard deviation, was calculated for each set of 3 plates. In addition, the fold-increase over the vehicle control was calculated for all test item and positive control treatments.

6.11.2 Historical Control Data

Historical vehicle and positive control values were used to assess the acceptability of the results. The current historical control data are shown in Appendix 3.

6.11.3 Acceptance Criteria

A test was acceptable if the following occurred:

Each bacterial strain demonstrated typical responses to crystal violet, ampicillin and ultraviolet radiation.

At least 2 of the 3 vehicle control plates were within the following ranges for mean number of revertant colonies: *S. typhimurium* TA 1535: 4-30; TA 100: 60-200; TA 1537: 1-30; TA 98: 10-60 and *E. coli* WP2*uvrA*: 1-60.

There were at least 2-fold increases over the mean vehicle control values in at least 2 of the 3 positive control plates for each strain and activation state (in the case of TA 100, at least 1.5-fold was required).

No toxicity or contamination was observed in at least 4 concentration levels.

In cases where a mutagenic response is observed, no more than one exposure level is discarded below the concentration that gives the highest mean colony number.

6.11.4 Interpretation of Mutagenicity

For *S. typhimurium* strains TA 1535, TA 1537, and TA 98 and for *E. coli* WP2*uvr*A, at least a doubling of the mean concurrent vehicle control value was required before mutagenic activity was suspected. For *S. typhimurium* strain TA 100, a 1.5-fold increase over the control value was considered indicative of a mutagenic effect.

If the mean colony count on the vehicle control plates was less than 10, then a value of 10 was assumed for assessment purposes. In such cases, a minimum count of 20 (representing a 2-fold increase over 10) was required before a response was registered.

A concentration-related response was also required for identification of a mutagenic effect. At high concentrations, this relationship may be reversed, because of, for example, toxicity of the test item to the bacteria, specific toxicity of the test item to the mutants, or inhibition of S9 enzymes (where a mutagen requires metabolic activation by the S9 mix).

A response should be reproducible in an independent test.

No statistical analysis was performed in this study.

7 RESULTS

7.1 Toxicity Tests

The results of the toxicity test using Alcohols, C18-22 Distn. Residues are shown in Table 1.

No toxicity to the bacteria was observed at the highest concentration of 2000 µg per plate in either the absence or the presence of S9 mix. Precipitated/undissolved test item was observed on the plates at 2000 µg per plate in both the absence and the presence of S9 mix (see Section 6.2.1).

7.2 Mutation Tests

The average numbers of *his*$^+$ and *trp*$^+$ revertant colonies, the individual plate counts, and the fold-increases over vehicle control value per dose level obtained in the mutation tests (conducted using both direct plate incorporation and pre-incubation methods) are shown in Table 2 and Table 3. Condition of background lawn is also noted.

7.2.1 Quality Control

The results of the Quality Control checks are shown in Appendix 4. All *Salmonella* strains were sensitive to crystal violet, whereas only the plasmid-containing *Salmonella* strains, TA 98 and TA 100, were resistant to ampicillin. All strains were also tested for sensitivity to ultraviolet radiation emitted over a period of 5-10 s from a source delivering a wavelength of 254 nm. Increased sensitivity to ultraviolet radiation was demonstrated. These results are consistent with the known properties of these strains of bacteria.

7.2.2 Vehicle Control Groups

The vehicle control values were within the normal/historical ranges recorded in this laboratory (Appendix 3) and reported in the literature with these strains of *S. typhimurium* and *E. coli* (Ames *et al*, 1975; Gatehouse *et al*, 1994).

7.2.3 Positive Control Groups

The positive control values were within the normal/historical ranges recorded in this laboratory for each bacterial strain and activation condition (Appendix 3).

7.2.4 Alcohols, C18-22 Distn. Residues

No evidence of mutagenic activity was obtained with any strain in either test.

There was no toxicity to any of the strains of bacteria in either test.

Precipitated/undissolved test item was observed on the plates at 2000 µg per plate in both the absence and the presence of S9 mix in both tests (see Section 6.2.1).

8 CONCLUSION

It is concluded that Alcohols, C18-22 Distn. Residues is not mutagenic in strains of *Salmonella typhimurium* and *Escherichia coli,* when tested in the absence and presence of metabolic activation up to and beyond its limit of solubility in the test system.

9 REFERENCES

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10 TABLES

Table 1 Toxicity Test

Without metabolic activation

Strain	Compound	Dose level per plate	Ratio treated / solvent	Individual revertant colony counts
TA 100	**Alcohols, C18-22**	6 µg	*0.8*	65
	Distn. Residues	20 µg	*1.1*	95
		60 µg	*1.4*	116
		200 µg	*1.0*	88
		600 µg	*1.1*	95
		2000 µg	*0.9*	79 P
	DMSO	-	-	84

Key to Plate Postfix Codes

P Precipitate

With metabolic activation

Strain	Compound	Dose level per plate	Ratio treated / solvent	Individual revertant colony counts
TA 100	**Alcohols, C18-22**	6 µg	*0.8*	92
	Distn. Residues	20 µg	*0.9*	99
		60 µg	*0.9*	103
		200 µg	*0.8*	87
		600 µg	*0.8*	84
		2000 µg	*0.9*	104 P
	DMSO	-	-	110

Key to Plate Postfix Codes

P Precipitate

Table 2 First Mutation Assay (Direct Plate Incorporation Method)

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Alcohols, C18-22 Distn. Residues**	6 µg	*15.0*	*3.5*	*0.8*	11, 17, 17
		20 µg	*12.3*	*3.1*	*0.7*	9, 13, 15
		60 µg	*17.7*	*6.7*	*1.0*	22, 10, 21
		200 µg	*15.3*	*2.5*	*0.8*	18, 13, 15
		600 µg	*23.3*	*4.0*	*1.3*	28, 21, 21
		2000 µg	*15.7*	*2.3*	*0.9*	13 P, 17 P, 17 P
	DMSO	-	*18.3*	*8.1*	-	9, 24, 22
TA 1537	**Alcohols, C18-22 Distn. Residues**	6 µg	*10.3*	*5.1*	*0.7*	16, 9, 6
		20 µg	*11.0*	*1.0*	*0.7*	10, 11, 12
		60 µg	*9.0*	*2.6*	*0.6*	6, 10, 11
		200 µg	*13.3*	*7.5*	*0.9*	9, 22, 9
		600 µg	*10.0*	*3.0*	*0.7*	13, 7, 10
		2000 µg	*12.3*	*2.3*	*0.8*	15 P, 11 P, 11 P
	DMSO	-	*15.3*	*8.6*	-	6, 17, 23
TA 98	**Alcohols, C18-22 Distn. Residues**	6 µg	*27.0*	*5.6*	*1.0*	22, 26, 33
		20 µg	*22.7*	*1.5*	*0.8*	23, 24, 21
		60 µg	*25.3*	*5.1*	*0.9*	24, 31, 21
		200 µg	*27.7*	*2.9*	*1.0*	31, 26, 26
		600 µg	*26.0*	*4.4*	*0.9*	28, 29, 21
		2000 µg	*23.5*	*10.6*	*0.8*	16 P, 31 P, P C
	DMSO	-	*28.3*	*11.9*	-	23, 20, 42
TA 100	**Alcohols, C18-22 Distn. Residues**	6 µg	*95.3*	*19.6*	*1.1*	77, 93, 116
		20 µg	*102.0*	*3.5*	*1.2*	100, 100, 106
		60 µg	*103.0*	*2.0*	*1.2*	101, 103, 105
		200 µg	*100.7*	*3.5*	*1.1*	101, 97, 104
		600 µg	*93.3*	*7.0*	*1.1*	86, 94, 100
		2000 µg	*104.0*	*19.7*	*1.2*	120 P, 82 P, 110 P
	DMSO	-	*88.0*	*11.1*	-	76, 90, 98
WP2uvrA	**Alcohols, C18-22 Distn. Residues**	6 µg	*10.0*	*3.0*	*1.4*	13, 7, 10
		20 µg	*10.3*	*3.1*	*1.5*	13, 7, 11
		60 µg	*6.3*	*2.5*	*0.9*	9, 4, 6
		200 µg	*5.3*	*1.5*	*0.8*	4, 5, 7
		600 µg	*10.7*	*4.7*	*1.5*	7, 16, 9
		2000 µg	*3.3*	*2.3*	*0.5*	6 P, 2 P, 2 P
	DMSO	-	*7.0*	*3.0*	-	10, 4, 7

Key to Plate Postfix Codes

P	Precipitate
C	Contaminated

Table 2 First Mutation Assay (Direct Plate Incorporation Method) (continued)

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**NaN_3**	1 µg	*554.3*	*35.0*	*30.2*	594, 541, 528
TA 1537	**9AA**	80 µg	*4833.7*	*346.1*	*315.2*	4484, 4841, 5176
TA 98	**2NF**	1 µg	*880.0*	*19.7*	*31.1*	859, 898, 883
TA 100	**NaN_3**	1 µg	*989.7*	*45.1*	*11.2*	938, 1021, 1010
WP2uvrA	**ENNG**	2 µg	*90.3*	*17.9*	*12.9*	111, 79, 81

Key to Positive Controls

NaN_3	Sodium Azide
9AA	9-Aminoacridine
2NF	2-Nitrofluorene
ENNG	N-Ethyl-N-Nitro-N-nitrosoguanidine

Table 2 First Mutation Assay (Direct Plate Incorporation Method) (continued)

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Alcohols,**	6 µg	*14.0*	*2.6*	*0.9*	13, 12, 17
	C18-22	20 µg	*16.3*	*6.0*	*1.0*	17, 10, 22
	Distn. Residues	60 µg	*15.7*	*4.0*	*1.0*	20, 12, 15
		200 µg	*11.7*	*6.5*	*0.7*	12, 18, 5
		600 µg	*18.7*	*8.1*	*1.1*	10, 26, 20
		2000 µg	*17.0*	*2.6*	*1.0*	20 P, 16 P, 15 P
	DMSO	-	*16.3*	*0.6*	-	16, 17, 16
TA 1537	**Alcohols,**	6 µg	*8.3*	*1.2*	*0.5*	7, 9, 9
	C18-22	20 µg	*17.0*	*10.0*	*1.0*	27, 17, 7
	Distn. Residues	60 µg	*16.0*	*5.0*	*1.0*	21, 11, 16
		200 µg	*15.0*	*2.0*	*0.9*	15, 17, 13
		600 µg	*15.7*	*2.3*	*0.9*	13, 17, 17
		2000 µg	*13.0*	*5.2*	*0.8*	19 P, 10 P, 10 P
	DMSO	-	*16.7*	*1.5*	-	17, 18, 15
TA 98	**Alcohols,**	6 µg	*37.3*	*11.5*	*1.2*	24, 44, 44
	C18-22	20 µg	*37.3*	*7.8*	*1.2*	46, 31, 35
	Distn. Residues	60 µg	*42.3*	*10.4*	*1.3*	34, 54, 39
		200 µg	*33.3*	*9.5*	*1.0*	43, 24, 33
		600 µg	*44.7*	*1.5*	*1.4*	45, 43, 46
		2000 µg	*41.7*	*2.1*	*1.3*	44 P, 40 P, 41 P
	DMSO	-	*32.0*	*6.9*	-	28, 28, 40
TA 100	**Alcohols,**	6 µg	*95.0*	*9.8*	*1.0*	106, 87, 92
	C18-22	20 µg	*102.3*	*12.5*	*1.1*	108, 88, 111
	Distn. Residues	60 µg	*106.3*	*12.9*	*1.1*	92, 110, 117
		200 µg	*103.0*	*5.3*	*1.1*	101, 99, 109
		600 µg	*114.3*	*10.3*	*1.2*	123, 103, 117
		2000 µg	*104.7*	*6.4*	*1.1*	101 P, 112 P, 101 P
	DMSO	-	*96.7*	*8.1*	-	104, 98, 88
WP2uvrA	**Alcohols,**	6 µg	*13.7*	*2.9*	*1.0*	12, 12, 17
	C18-22	20 µg	*5.0*	*0.0*	*0.4*	5, 5, 5
	Distn. Residues	60 µg	*10.7*	*2.1*	*0.8*	9, 10, 13
		200 µg	*7.0*	*2.0*	*0.5*	7, 9, 5
		600 µg	*10.0*	*1.0*	*0.8*	9, 11, 10
		2000 µg	*6.3*	*2.3*	*0.5*	5 P, 5 P, 9 P
	DMSO	-	*13.3*	*1.5*	-	15, 13, 12

Key to Plate Postfix Codes

P Precipitate

Table 2 First Mutation Assay (Direct Plate Incorporation Method) (continued)

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**2AAN**	2 µg	*629.0*	*28.2*	*38.5*	597, 640, 650
TA 1537	**2AAN**	2 µg	*434.7*	*45.2*	*26.1*	486, 417, 401
TA 98	**2AAN**	0.5 µg	*509.3*	*66.5*	*15.9*	563, 530, 435
TA 100	**2AAN**	0.5 µg	*1093.3*	*72.2*	*11.3*	1137, 1010, 1133
WP2uvrA	**2AAN**	20 µg	*519.7*	*13.6*	*39.0*	518, 534, 507

Key to Positive Controls

2AAN 2-Aminoanthracene

Table 3 Second Mutation Assay (Pre-incubation Method)

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Alcohols, C18-22 Distn. Residues**	6 µg	*12.0*	*4.6*	*1.2*	16, 7, 13
		20 µg	*10.3*	*0.6*	*1.0*	11, 10, 10
		60 µg	*13.3*	*3.2*	*1.3*	12, 11, 17
		200 µg	*15.7*	*2.5*	*1.5*	16, 18, 13
		600 µg	*13.7*	*2.9*	*1.3*	12, 17, 12
		2000 µg	*14.0*	*6.1*	*1.4*	18 P, 7 P, 17 P
	DMSO	-	*10.3*	*2.9*	-	12, 12, 7
TA 1537	**Alcohols, C18-22 Distn. Residues**	6 µg	*10.3*	*1.2*	*0.8*	11, 11, 9
		20 µg	*10.7*	*4.0*	*0.8*	15, 7, 10
		60 µg	*10.3*	*1.2*	*0.8*	9, 11, 11
		200 µg	*11.0*	*2.0*	*0.9*	9, 13, 11
		600 µg	*12.0*	*6.6*	*0.9*	13, 5, 18
		2000 µg	*15.0*	*3.0*	*1.2*	18 P, 15 P, 12 P
	DMSO	-	*12.7*	*2.5*	-	15, 13, 10
TA 98	**Alcohols, C18-22 Distn. Residues**	6 µg	*25.3*	*3.8*	*1.0*	27, 21, 28
		20 µg	*18.7*	*4.7*	*0.7*	17, 24, 15
		60 µg	*26.7*	*2.5*	*1.0*	29, 24, 27
		200 µg	*28.3*	*8.5*	*1.1*	20, 37, 28
		600 µg	*22.7*	*8.3*	*0.9*	20, 32, 16
		2000 µg	*21.0*	*2.6*	*0.8*	19 P, 20 P, 24 P
	DMSO	-	*25.7*	*1.5*	-	24, 27, 26
TA 100	**Alcohols, C18-22 Distn. Residues**	6 µg	*76.3*	*11.9*	*0.9*	90, 71, 68
		20 µg	*89.0*	*19.3*	*1.0*	103, 67, 97
		60 µg	*97.3*	*25.7*	*1.1*	84, 127, 81
		200 µg	*91.7*	*5.5*	*1.0*	98, 89, 88
		600 µg	*101.3*	*14.6*	*1.1*	117, 88, 99
		2000 µg	*90.0*	*9.8*	*1.0*	101 P, 87 P, 82 P
	DMSO	-	*88.3*	*2.1*	-	86, 89, 90
WP2uvrA	**Alcohols, C18-22 Distn. Residues**	6 µg	*8.3*	*1.2*	*1.8*	7, 9, 9
		20 µg	*8.7*	*3.2*	*1.9*	10, 5, 11
		60 µg	*13.3*	*3.8*	*2.9*	15, 9, 16
		200 µg	*6.0*	*2.6*	*1.3*	9, 4, 5
		600 µg	*6.0*	*1.0*	*1.3*	6, 7, 5
		2000 µg	*9.7*	*0.6*	*2.1*	9 P, 10 P, 10 P
	DMSO	-	*4.7*	*4.0*	-	1, 4, 9

Key to Plate Postfix Codes

P Precipitate

Table 3 (continued) Second Mutation Assay (Pre-incubation Method)

Without metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**NaN_3**	1 µg	*513.7*	*37.8*	*49.7*	555, 481, 505
TA 1537	**9AA**	80 µg	*4223.3*	*423.4*	*333.4*	4308, 3764, 4598
TA 98	**2NF**	1 µg	*608.0*	*90.8*	*23.7*	710, 536, 578
TA 100	**NaN_3**	1 µg	*1082.0*	*55.1*	*12.2*	1139, 1078, 1029
WP2uvrA	**ENNG**	2 µg	*153.3*	*23.0*	*32.9*	176, 154, 130

Key to Positive Controls

NaN_3	Sodium Azide
9AA	9-Aminoacridine
2NF	2-Nitrofluorene
ENNG	N-Ethyl-N-Nitro-N-nitrosoguanidine

Table 3 (continued) Second Mutation Assay (Pre-incubation Method)

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**Alcohols, C18-22 Distn. Residues**	6 µg	*10.3*	*1.5*	*0.9*	9, 10, 12
		20 µg	*11.7*	*0.6*	*1.0*	11, 12, 12
		60 µg	*18.0*	*1.7*	*1.5*	20, 17, 17
		200 µg	*15.7*	*4.5*	*1.3*	20, 11, 16
		600 µg	*15.7*	*6.0*	*1.3*	22, 15, 10
		2000 µg	*18.0*	*2.0*	*1.5*	20 P, 18 P, 16 P
	DMSO	-	*11.7*	*1.5*	-	12, 10, 13
TA 1537	**Alcohols, C18-22 Distn. Residues**	6 µg	*10.0*	*5.2*	*1.0*	13, 13, 4
		20 µg	*10.3*	*4.5*	*1.0*	15, 6, 10
		60 µg	*18.0*	*2.6*	*1.7*	16, 21, 17
		200 µg	*12.0*	*5.6*	*1.2*	11, 7, 18
		600 µg	*10.3*	*1.5*	*1.0*	10, 9, 12
		2000 µg	*11.7*	*5.7*	*1.1*	10 P, 7 P, 18 P
	DMSO	-	*10.3*	*2.3*	-	9, 13, 9
TA 98	**Alcohols, C18-22 Distn. Residues**	6 µg	*23.7*	*3.1*	*0.8*	21, 27, 23
		20 µg	*24.0*	*2.0*	*0.8*	22, 24, 26
		60 µg	*37.7*	*4.5*	*1.3*	38, 42, 33
		200 µg	*23.7*	*2.1*	*0.8*	26, 23, 22
		600 µg	*32.3*	*4.6*	*1.1*	27, 35, 35
		2000 µg	*31.3*	*2.9*	*1.0*	33 P, 33 P, 28 P
	DMSO	-	*30.0*	*3.6*	-	26, 33, 31
TA 100	**Alcohols, C18-22 Distn. Residues**	6 µg	*96.0*	*15.6*	*0.9*	78, 104, 106
		20 µg	*96.7*	*8.6*	*0.9*	106, 89, 95
		60 µg	*104.0*	*6.0*	*1.0*	110, 104, 98
		200 µg	*103.0*	*6.1*	*0.9*	100, 99, 110
		600 µg	*100.0*	*5.6*	*0.9*	94, 101, 105
		2000 µg	*96.0*	*3.5*	*0.9*	98 P, 92 P, 98 P
	DMSO	-	*109.3*	*12.0*	-	110, 121, 97
WP2uvrA	**Alcohols, C18-22 Distn. Residues**	6 µg	*13.7*	*4.2*	*1.5*	9, 15, 17
		20 µg	*8.0*	*6.1*	*0.9*	11, 12, 1
		60 µg	*10.3*	*1.5*	*1.1*	12, 9, 10
		200 µg	*13.0*	*2.6*	*1.4*	11, 16, 12
		600 µg	*15.7*	*6.0*	*1.7*	22, 10, 15
		2000 µg	*9.7*	*1.5*	*1.0*	10 P, 8 P, 11 P
	DMSO	-	*9.3*	*3.1*	-	6, 10, 12

Key to Plate Postfix Codes

P Precipitate

Table 3 (continued) Second Mutation Assay (Pre-incubation)

With metabolic activation

Strain	Compound	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts
TA 1535	**2AAN**	2 µg	*368.7*	*25.3*	*31.6*	396, 346, 364
TA 1537	**2AAN**	2 µg	*224.3*	*40.7*	*21.7*	270, 192, 211
TA 98	**2AAN**	0.5 µg	*697.7*	*33.3*	*23.3*	676, 681, 736
TA 100	**2AAN**	0.5 µg	*843.7*	*67.5*	*7.7*	920, 792, 819
WP2uvrA	**2AAN**	20 µg	*469.3*	*26.3*	*50.3*	483, 486, 439

Key to Positive Controls

2AAN 2-Aminoanthracene

11 APPENDICES

Appendix 1 Certificate of Analysis

sasol
reaching new frontiers

Brunsbuettel, 9/09/2009

C E R T I F I C A T E O F A N A L Y S I S

Product:	Alcohols, C18-22, distn. Residues	(59B1RN2)
Lot No.:	03585/MA	
Manufacturing date:	25/08/2009	
Expiry date:	08/2013	

Tests	Unit	Result
Hydroxyl - Number	[mg KOH/g]	50,
Ester No.	[mg KOH/g]	41,17
Acid No.	[mg KOH/g]	0,09
Water	[wt.%]	0,02
Iodine No.	[mg I/100mg]	5,34

Sasol Germany GmbH
P.O. Box 1160
D-25534 Brunsbuettel
Phone: 049 (0)4852/392-0 Fax: 049 (0)4852/3285

Best regards
Works inspector
M. Sprung

Appendix 2 *In vitro* Activation Preparation Form

In vitro Activation Preparation Form
Activation Batch: FLI 112

PREPARATION		Date Prepared:	15 July 2009
Operators:	Wanda Zajac/Colin Riach/ Pawel Jurczak	Date of Expiry:	15 January 2010
Animal:	Rat	Number of animals:	20
Sex:	Male	Average animal weight (g):	232g
Strain:	Fischer 344	Date induced:	10 July 2009
Supplier:	Harlan UK	Inducer:	Aroclor 1254
Organ:	Liver	Supplier:	Monsanto (UK) Limited
Total grams of organ:	194 g	Preparation solution:	0.15 M KCl
Details of spin:	9,000 g supernatant	Number of vials prepared:	74 x 5 mL
Total volume prepared:	577 mL		41 x 3 mL
Storage temperature:	-196°C		42 x 2 mL
Sterility check:	Sterile		

METABOLIC ACTIVATION

Operators:	Wanda Zajac/Pawel Jurczak	Culture batch:	G169
Strain:	*S. typhimurium* TA 1538	Batch No. (plates):	000766
Date plated:	17 July 2009	Date counted:	20 July 2009

Substance	Quantity per Plate	Revertant Colonies per Plate			
					Mean
Dimethylsulphoxide	100 µL	21	24	22	22
2-Aminoanthracene	0.5 µg	931	897	899	909
2-Acetylaminofluorene	10 µg	1700	1805	1901	1802
4-Acetylaminofluorene	1 mg	116	137	121	125
Benzo(α)pyrene	5 µg	457	422	428	436
Dimethylaminoazobenzene	100 µg	71	88	83	81

QUALITY ASSURANCE

Auditor:	Alison McNaughton HNC
Date Audited:	27 July 2009
Date Signed-off:	04 August 2009

Appendix 3 Historical Positive and Negative Control Data

Ames Test Historical Vehicle and Positive Control Data 2005-2009

Vehicle Controls (Pooled) - Presence of S9 Mix						
Strain		TA 1535	TA 1537	TA 98	TA 100	WP2*uvr*A
Mean		15	17	32	94	9
Standard Deviation		6	7	9	17	4
Range	Min	4	2	13	59	1
	Max	31	35	62	160	24
No. of Plates		227	225	225	264	222

Vehicle Controls (Pooled) - Absence of S9 Mix						
Strain		TA 1535	TA 1537	TA 98	TA 100	WP2*uvr*A
Mean		15	13	23	92	9
Standard Deviation		5	6	7	19	4
Range	Min	5	1	10	60	1
	Max	30	31	44	163	23
No. of Plates		224	222	225	264	222

Positive Controls - Presence of S9 Mix						
Strain		TA 1535	TA 1537	TA 98	TA 100	WP2*uvr*A
Substance		2AAN	2AAN	2AAN	2AAN	2AAN
Concentration (µg per plate)		2	2	0.5	0.5	20
Mean		428	288	510	735	557
Standard Deviation		133	127	218	337	171
Range	Min	92	66	165	331	125
	Max	821	765	1646	2468	1103
No. of Plates		222	222	222	225	216

Positive Controls - Absence of S9 Mix						
Strain		TA 1535	TA 1537	TA 98	TA 100	WP2*uvr*A
Substance		NaN_3	9AA	2NF	NaN_3	ENNG
Concentration (µg per plate)		1	80	1	1	2
Mean		495	5727	958	1116	227
Standard Deviation		106	2102	474	397	122
Range	Min	263	152	331	551	45
	Max	882	10354	7084	3553	673
No. of Plates		222	222	225	225	222

Audited: Alison McNaughton HNC
Date audited: 21 April 2009
Date signed off: 21 April 2009

Appendix 4 Quality Control Checks of Bacterial Cultures

First mutation assay (Direct Plate Incorporation Method)

Cell Type	No. of Bacteria Added per Plate in Mutation Test ($x10^8$)	Ultraviolet Sensitivity		Resistant to Ampicillin	Zone (mm)	Sensitive to Crystal Violet	Zone (mm)	Amino Acid Requirement
		Treated (Colonies)	Untreated (Colonies)					
S. typhimurium TA 1535	3.13	0	313	-	20	+	11	Yes
S. typhimurium TA 1537	2.41	0	241	-	21	+	17	Yes
S. typhimurium TA 98	1.38	0	138	+	0	+	13	Yes
S. typhimurium TA 100	2.04	0	204	+	0	+	16	Yes
E. coli WP2*uvr*A	2.14	0	214	-	18	+/-	8	Yes
S. typhimurium (Wild Type)	N/A	71	382	N/A	N/A	N/A	N/A	N/A

Second mutation assay (Pre-incubation Method)

Cell Type	No. of Bacteria Added per Plate in Mutation Test ($x10^8$)	Ultraviolet Sensitivity		Resistant to Ampicillin	Zone (mm)	Sensitive to Crystal Violet	Zone (mm)	Amino Acid Requirement
		Treated (Colonies)	Untreated (Colonies)					
S. typhimurium TA 1535	2.18	0	214	-	21	+	18	Yes
S. typhimurium TA 1537	1.81	0	181	-	22	+	18	Yes
S. typhimurium TA 98	1.00	0	100	+	0	+	15	Yes
S. typhimurium TA 100	1.56	0	156	+	0	+	15	Yes
E. coli WP2*uvr*A	2.30	0	230	-	19	+/-	10	Yes
S. typhimurium (Wild Type)	N/A	32	205	N/A	N/A	N/A	N/A	N/A

N/A Not Applicable
\+ Positive
\- Negative