FINAL REPORT

Test Facility Study No. 787739, Report No. 30896

Alcohols, C18-22 Distn. Residues (CAS No. 1160164-88-4): Mouse Lymphoma Mutation Study

TEST FACILITY:
Charles River
Tranent
Edinburgh
EH33 2NE
UK

SPONSOR:
SASOL Germany GmbH
Anckelmannsplatz 1
D-20537 Hamburg
Germany

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 Jan 2010
Date

2 QUALITY ASSURANCE STATEMENT

Study title: Alcohols, C18-22 Distn. Residues: Mouse Lymphoma Mutation Study

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
20 October 2009	Dose Preparation/Dosing/ Protocol Compliance	20 October 2009
21 October 2009	Cell Counting and Dilutions	21 October 2009
22 October 2009	Cloning	22 October 2009
04 November 2009	Scoring	04 November 2009
29/30 December 2009	Draft Report Audit	05 January 2009
19 January 2010	Final Report Audit	19 January 2010

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.

Alison McNaughton — 25 January 2010

Alison McNaughton HNC
Quality Assurance

Date

3 RESPONSIBLE PERSONNEL

Study Director:	Colin G Riach BSc
Report Compilation:	Colin G Riach BSc
Project Leader:	Wanda C Zajac
Technical Assistance:	Pawel Jurczak
Statistician:	Marion Thom MSc
Quality Assurance:	Alison McNaughton HNC Sarah L Drake BSc

4 SUMMARY

Alcohols, C18-22 Distn. Residues was assayed for mutagenic potential in the mouse lymphoma L5178Y cell line, clone -3.7.2C, scoring for forward mutations at the thymidine kinase locus: $tk^{+}tk^{-}$ to $tk^{-}tk^{-}$. Alcohols, C18-22 Distn. Residues was prepared as a part solution/part suspension in dimethylsulphoxide. Tests were conducted both in the absence and in the presence of a post-mitochondrial supernatant fraction obtained from Aroclor 1254-induced livers of adult male rats and the co-factors required for mixed-function oxidase activity (S9 mix). The study was designed to be consistent with ICH Guidelines, OECD Guideline No. 476 and EC Directive 2000/32/EC B.17. The study also meets the requirements of the United States and Japan.

In preliminary cytotoxicity tests, Alcohols, C18-22 Distn. Residues was shown to be non-toxic at the highest practical concentration of 200 µg/mL. Precipitation of the test item occurred at this concentration.

Four independent mutation assays were conducted, as follows:

Assay No.	Presence or absence of S9	Treatment time (hours)	Final concentrations (µg/mL)
1	Absence	4	25, 50, 100, 200
2	Presence	4	25, 50, 100, 200
3	Absence	24	25, 50, 100, 200
4	Presence	4	25, 50, 100, 200

Positive control cultures were included, and the resultant mutant fractions from these provided the expected increase and proof of adequate recovery of ‘small’ type colonies. Duplicate cultures were carried through the experiments for each treatment point. Vehicle control cultures were also included and were tested in quadruplicate.

Biological relevance was given to any increase in mutant fraction greater than 126 mutants per million above the concurrent control value. In addition, the results were analysed for comparison of the log mutant fraction between the vehicle controls and each concentration of Alcohols, C18-22 Distn. Residues. In addition, all the experiments were tested for dose-related trends.

No evidence of mutagenic activity was obtained with Alcohols, C18-22 Distn. Residues in any of the 4 assays.

It was concluded that Alcohols, C18-22 Distn. Residues is not mutagenic in mouse lymphoma L5178Y cells, in either the absence or the presence of S9 mix, when tested up to and beyond its limit of solubility in the test system.

5 INTRODUCTION

The objective of the study was to determine the potential of Alcohols, C18-22 Distn. Residues to induce forward mutations at the $tk^{+}tk^{-}$ locus of mouse lymphoma L5178Y cells.

5.1 Regulatory Citations

5.1.1 Test Guideline

This study was designed to comply with ICH Guidelines, OECD Guideline No. 476 (Adopted 1997), EC Directive 2000/32/EC B.17 (Adopted 2000) and United States 40 CFR § 798.5300 (updated 2001). The study also meets Japanese requirements.

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 throughout 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 Study Location

The study was conducted at Charles River, Tranent, Edinburgh, EH33 2NE, UK.

5.3 Study Dates

Study Initiation Date:	01 October 2009
Experimental Start Date:	13 October 2009
Experimental Completion Date:	01 December 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 will be 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 Protocol

There were no deviations from the protocol.

6 MATERIALS AND METHODS

6.1 Test Item

The test item was received on 11 September 2009. An archive sample was retained. A copy of the Certificate of Analysis is shown in Appendix 1.

6.2 Test Item Characterisation

Active ingredient:	Alcohols C18-22, distn. Residues (CAS No. 1160164-88-4)
Lot No:	03585/MA (product code: 5901RN2)
Description:	Beige waxy solid
Purity:	100% by definition
Storage Conditions:	Stored in the dark at ambient room temperature
Supplier:	SASOL Germany GmbH
Expiry date:	31 August 2013

6.3 Dose Formulations

6.3.1 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. After a 1 in 100 dilution into the cell cultures, the 20 mg/mL solution gave a high concentration of 200 µg/mL.

6.3.2 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.

6.4 Test System

Specific-locus mutation tests with mammalian cells *in vitro* can be used to demonstrate and quantify genetic damage in these cells. Such tests can be used, for example, to confirm results obtained with bacterial cell tests. A positive result with the mammalian cell test as well as a bacterial cell assay increases the need for careful evaluation of the toxic potential of the test chemical. A negative result, while not reversing the interpretation of a positive result with bacteria, does reduce the value of that result and reduces the concern for the genotoxicity of the chemical.

6.5 Justification of the Test System

Since 1964, mutations have been knowingly induced in cultured mammalian cells (Fischer and Sartorelli (1964); Chu and Malling (1968); Kao and Puck (1968)). The thymidine kinase heterozygote system, where $tk^{+}tk^{-}$ is mutated to $tk^{-}tk^{-}$, was described by Clive *et al* (1972) and is based upon the L5178Y mouse lymphoma cell line established by Fischer (1958). In this assay, cells deficient in thymidine kinase (TK) due to the mutation $tk^{+}tk^{-}$ to $tk^{-}tk^{-}$ are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). Thymidine kinase proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, are not.

A more detailed description of the system as a test for mutagens was published later (Clive and Spector (1975)). Among the published validation studies of the L5178Y cell system are those of Clive *et al* (1979); Amacher *et al* (1980); Jotz and Mitchell (1981); McGregor *et al* (1987, 1988a, 1988c, 1991a, 1991b); Myhr *et al* (1990); Honma *et al* (1999a, 1999b).

6.5.1 Colony Sizing

It has been widely shown (Hozier *et al* (1981) and others) that genetic damage involving the TK locus results in 2 phenotypes. Some mutant cells divide at the normal rate, producing large colonies, while other cells divide at a distinctly slower rate, producing small colonies. A high proportion of large type colonies are associated with small chromosomal deletions or point mutations, while a large proportion of the small type colonies are associated with large chromosomal deletions. Assessment of the relative numbers of both colony types can provide information to support results obtained in bacterial mutation and chromosome aberration tests.

6.6 Cells and Cell Culture

6.6.1 Cells

The cells used were from the $tk^{+}tk^{-}$ -3.7.2C mouse lymphoma L5178Y cell line obtained from Dr D Clive, Burroughs Wellcome & Company, Research Triangle Park, NC27709, USA, in December 1982. The cells grow in suspension culture, have a generation time of about 11 h, have a stable, near-diploid chromosome number and have a high cloning efficiency in serum-enriched cloning medium.

6.6.2 Culture Medium

The basic culture medium (R_0P) was RPMI 1640 medium, supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL), sodium bicarbonate (1.125 g/L) and pluronic acid (0.05% w/v). For cell growth, heat-inactivated horse serum (10% v/v) was added to R_0P to give $R_{10}P$.

The medium used during treatment for 4 h was R_0P supplemented with 5% v/v heat-inactivated horse serum (R_5P). The medium used during treatment for 24 h was $R_{10}P$.

For colony formation, cloning medium was used, consisting of R_0P supplemented with heat-inactivated horse serum (20% v/v), sodium pyruvate (1.9 mM), and amphotericin B (fungizone) (2.5 µg/mL).

For selection of $tk^{-}tk^{-}$ cells, cloning medium was supplemented with trifluorothymidine (TFT) at 3 µg/mL.

6.6.3 Cell Culture

Cell stocks for use in the mutation assays were prepared in suitable volumes according to the procedures detailed in Appendix 2.

6.7 Experimental Design

Two principal methods of performing the mouse lymphoma assay exist: the soft agar cloning method originally developed by Clive and the microwell method (Cole *et al* (1983)). The assay performed in this laboratory is based principally on the methods of Clive, with cloning performed according to the method of Cole.

Although some guidelines *e.g.*, ICH, do not dictate an automatic repetition of experiments, it is 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 an exogenous enzyme supplement (S9 mix).

A validation study commissioned by the ICH (Honma *et al* (1999b)) has shown that the detection (by the L5178Y assay) of a significant number of substances positive in the chromosome aberration assay was enhanced by an extended exposure period in the absence of S9 mix. Such substances included nucleoside analogues, base analogues and aneuploidy inducers. The study was designed, therefore, to include a 24 h exposure period in the second experiment in the absence of S9 mix, should the results of the first experiment (using a standard 4 h exposure) be negative.

6.8 Justification of Dose Levels

Concentrations in mutation experiments should extend into the toxic range, the maximum usable limit allowing a relative total growth value of at least 10% of the concurrent vehicle control. It was necessary, therefore, to perform initial toxicity tests in the absence and presence of S9 mix.

The final concentrations of Alcohols, C18-22 Distn. Residues used in the toxicity test were as follows:

0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 200 µg/mL

The highest concentration represents the maximum attainable in the test system, when the 20 mg/mL formulation in DMSO (see Section 6.3.1) was dosed 1 in 100 into the cell cultures. Subsequently, four mutation assays were performed, as detailed on the next page.

The concentrations of Alcohols, C18-22 Distn. Residues tested were as follows (µg/mL):

Assay 1 (in the absence of S9 mix[1]): 25, 50, 100 and 200
Assay 2 (in the presence of S9 mix[1]): 25, 50, 100 and 200
Assay 3 (in the absence of S9 mix[2]): 25, 50, 100 and 200
Assay 4 (in the presence of S9 mix[1]): 25, 50, 100 and 200

[1] Experiment using a 4 h exposure period
[2] Experiment using a 24 h exposure period

Each dose range selection was based on all available results at the time the selection was made.

6.9 Administration of Test/Control Items

6.9.1 Vehicle Controls

All toxicity and mutation assays included vehicle control cultures. These cultures were subjected to the same experimental manipulations as the treated cultures. In this study, the vehicle control cultures were treated with DMSO. Single cultures were treated in the toxicity tests. Four vehicle control cultures were treated in each mutation experiment.

6.9.2 Positive Control Materials

All mutation assays included duplicate positive control cultures. The positive control materials used in the absence of S9 mix (4 h exposure period) were: 250 µg/mL ethyl methanesulphonate (EMS), a large colony inducer; and 10 µg/mL methyl methanesulphonate (MMS), which usually induces greater numbers of small colonies. When performing an experiment using the extended 24 h exposure period, these concentrations were reduced to 150 µg EMS/mL and 5 µg MMS/mL. In the presence of S9 mix, 3-methylcholanthrene (3-MC), a large and small colony inducer, was used at concentrations of 2.5 and 10 µg/mL. EMS and 3-MC were dissolved in DMSO, while MMS was dissolved in water. The positive control cultures were subjected to the same experimental manipulations as the Alcohols, C18-22 Distn. Residues-treated cultures.

6.9.3 Test Item

Single cultures were treated for each concentration in the initial toxicity tests. In the mutation assays, duplicate cultures were treated for all Alcohols, C18-22 Distn. Residues concentrations.

6.10 Experimental Procedure

All experimental procedures were conducted using aseptic technique and under amber light.

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

6.11 S9 Mix

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

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

S9 was 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 was characterised by testing selected pre-mutagens in an Ames test with *S. typhimurium* TA 1538. The results of the characterisation and preparation details of the batches used in the mutation experiments are presented in Appendix 3. S9 batches used must also have shown, within each mouse lymphoma test, a satisfactory mutagenic response in cells treated with 3-methylcholanthrene.

To prepare S9 mix, R_0P was added to pre-weighed cofactors: nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose-6-phosphate (G-6-P) disodium salt, giving final concentrations in the 'S9 mix' of:

NADP Na_2	4 mM (= 3.150 mg/mL)
G-6-P Na_2	25 mM (= 7.605 mg/mL)

This solution was immediately filter-sterilised by passage through a 0.2 µm disposable filter assembly and mixed 9:1 (v/v) with the S9.

6.12 Toxicity Test

The exposure of the cells was similar to that described below for the mutation assays with the exception that only one culture was prepared for each treatment.

The selection in the mutation experiments of the treatments for final assessment is dependent on suspension growth following treatment. The measure used to assess toxicity in the Toxicity Test was therefore relative suspension growth. The cell population densities were recorded over 2 days (following treatment) using a haemocytometer, then the total suspension

growths were expressed as percentages of the vehicle control group (= relative suspension growth, or RSG).

The toxicity test was performed using the standard 4 h exposure period in the absence and presence of S9 mix. An additional toxicity test was performed in the absence of S9 mix with 24 h exposure to Alcohols, C18-22 Distn. Residues, as a contingency against the later requirement for a full experiment using this extended exposure period.

Observations on the precipitation of Alcohols, C18-22 Distn. Residues were made after dosing and at the end of the exposure period. Observations of pH change (colour change in indicator in RPMI medium) were made and if any change was noted, pH measurements were made.

6.13 Mutation Tests

6.13.1 Treatment (4 h exposure period)

On the day of the test (Day 0), samples of cell culture (in 5 mL $R_{10}P$) were dispensed to sterile tubes containing 3.9 mL R_0P. Freshly prepared S9 mix or R_0P (1 mL) was added to each tube followed by 0.1 mL of test solution. Vehicle control cultures received 0.1 mL DMSO. Positive control cultures received 0.1 mL of the appropriate solution. The final reaction mixture in all cultures contained 10 mL of cells, at a population density of *ca* 6.0 x 10^5 cells/mL, in R_5P medium.

All tubes were incubated on a rotating drum at *ca* 37°C, 10 r.p.m. for 4 h. After this, the cells were gently sedimented by centrifugation at *ca* 200 *g* for 5 min and resuspended in $R_{10}P$ medium (20 mL). This step was repeated to give a cell density of *ca* 3 x 10^5/mL.

The cells were returned to the rotating drum and allowed to express their genetic lesions at *ca* 37°C for 2 days. Cell numbers were adjusted, after counting, to *ca* 3 x 10^5 cells/mL on Day 1.

6.13.2 Extended Treatment (24 h exposure)

An experiment is conducted using an extended 24 h exposure period, when the results of the first experiment in the absence of S9 mix are negative. The extended exposure period facilitates continuous exposure to the test item through >1 cell cycle.

On the day of the test (Day 0), samples of cell culture (in 10 mL $R_{10}P$) were dispensed to sterile tubes containing 7.8 mL R_0P. $R_{50}P$ (R_0P:serum, 50:50) (2 mL) was added to each tube followed by 0.2 mL of the test solution. Vehicle control cultures received 0.2 mL DMSO. Positive control cultures received 0.2 mL of the appropriate solution. The final reaction mixture in all cultures contained 20 mL of cells, at a population density of *ca* 3 x 10^5 cells/mL, in $R_{10}P$ medium.

(The larger volumes allow the same numbers of cells to be treated as in the experiments conducted at 4 h exposure, but at half the density. The lower density is required to allow cell growth during the exposure period. The serum concentration is not lowered, as some essential nutrients can become exhausted during the exposure period.)

All tubes were incubated on a rotating drum at *ca* 37°C, 10 r.p.m. for 24 h. After this (on Day 1), the cells were gently sedimented by centrifugation at 200 *g* for 5 min and resuspended in $R_{10}P$ medium (20 mL). This step was repeated. Cell counts were made and the densities adjusted (where higher) to give *ca* 3 x 10^5 cells/mL. The cells were returned to the rotating drum and allowed to express their genetic lesions at *ca* 37°C for 2 days. Cell numbers were adjusted, after counting, to *ca* 3 x 10^5 cells/mL on Day 2.

6.13.3 Expression of Genetic Damage

On Day 2 (4 h exposure) or Day 3 (24 h exposure), cell counts were determined. The cell counts over the 2 or 3 days of the experiments provided a measure of suspension growth. This in turn provided a measure of RSG (see Section 7.1.1). This was used when choosing dose levels to carry through to final assessment, as no other measures of toxicity were known at the time the decision was required.

In this study all treated cultures were selected for assessment. The cultures were then assessed for expression of genetic damage. This was determined by performing two parallel cloning assays: the cloning efficiency assay and the mutant selection assay.

For the cloning efficiency assay, each culture was diluted into cloning medium to give an estimated 8 cells/mL. Two 96-well dishes were filled with 200 µL cell culture per well, so giving an estimated 1.6 cells per well.

For the mutant selection assay, TFT stock solution was added to cloning medium to give a final concentration of 3 µg/mL. Into this medium, the cell cultures were diluted to give an estimated 1 x 10^4 cells/mL. Two 96-well dishes were filled with 200 µL cell culture per well, so giving an estimated 2000 cells per well.

All dishes were incubated at *ca* 37°C in an atmosphere of 5% CO_2:95% air (v/v) until the colonies were fully developed (at least 9 days for cloning efficiency assay, at least 12 days for mutant selection assay).

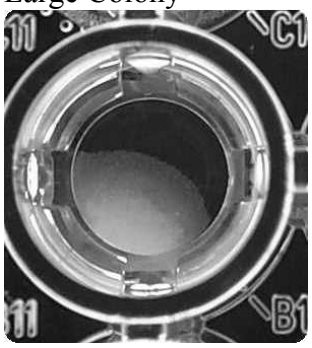
6.13.4 Plate Reading

The plates were scored using a dissecting microscope fitted to a light box with dark field illumination. The number of empty wells in each plate in the cloning efficiency assay was

counted. When scoring the mutant selection assay, separate counts were made of the numbers of wells containing large type and small type colonies. Large colonies are defined as covering greater than ¼ of the floor of the well, while small colonies cover less than ¼ of the well (Moore *et al* (2000)). In addition, there are morphological differences. Large colonies tend to be similar to those found on the cloning efficiency plates, being generally flat. Small colonies tend to look dense in comparison. See examples on next page. Any wells containing both colony types were scored as a large type. (The total number of empty wells is required for the calculation of mutant fraction, so each well can only be scored once).

Empty Well

Large Colony



Small Colony

7 CALCULATIONS AND DATA ACCEPTANCE

7.1 Calculations

Worked examples of the calculations made in the current study are given in Appendix 4.

7.1.1 Assessment of Relative Suspension Growth

For treated cultures, this value includes initial cell loss to toxicity and subsequent recovery during the expression period. The measure is used to assess the results of the preliminary toxicity test, and to determine acceptable dose levels for assessment in the mutation tests.

Total suspension growth (SG) is calculated as follows, for experiments with a 4 h exposure period:

$$\frac{\text{Day 1 Count}}{\text{Concentration on Day 0}} \times \frac{\text{Day 2 Count}}{\text{Final Concentration on Day 1}}$$

For experiments with a 24 h exposure period, the calculation is:

$$\frac{\text{Day 1 Count}}{\text{Conc. on Day 0}} \text{ x } \frac{\text{Day 2 Count}}{\text{Final Conc. on Day 1}} \text{ x } \frac{\text{Day 3 Count}}{\text{Final Conc. on Day 2}}$$

The total suspension growth values are then expressed as percentages of the vehicle control mean value to give the relative suspension growth (RSG).

7.1.2 Assessment of Relative Total Growth

The recommended endpoint for assessing cytotoxicity in mouse lymphoma mutation tests is Relative Total Growth (RTG) (Moore *et al* (2002)). RTG combines the suspension growth above with the cloning efficiency (CE) of the non-mutants at the end of the expression period, again expressing individual values as percentages of the vehicle control mean. The CE is calculated from the zero term of the Poisson distribution using the formula:

$$CE = \frac{-\ln(P(o))}{\text{number of cells per well}}$$

$$\text{where } P(o) = \frac{\text{empty wells}}{\text{total wells}}$$

Total Growth = SG x CE (non-mutants)

$$\text{Relative Total Growth (\%)} = \frac{\text{individual total growth}}{\text{mean vehicle control total growth}} \text{ x } \frac{100}{1}$$

7.1.3 Assessment of Mutant Fractions

The number of empty wells from the non-mutant cloning efficiency assay and the number of empty wells from the TFT-resistance assay were used to calculate the mutant fraction, as below.

The CE of both cell types was calculated as in Section 7.1.2. The mutant fraction per viable cell was calculated as below:

$$\text{Mutant fraction per viable cell} = \frac{\text{CE in medium containing TFT}}{\text{CE in non - selective medium}} = \frac{\text{CE of mutants}}{\text{CE of non - mutants}}$$

Each mutant fraction was expressed per 10^6 viable cells.

7.1.4 Colony Size Fractions

The ratio of small type mutant colonies to large type mutant colonies was expressed for each culture.

7.2 Historical Control Data

The historical ranges of vehicle and positive controls are continually updated and are used as guides in the acceptance of each experiment. The summarised historical data are presented in Appendix 5.

7.3 Analysis of Data

7.3.1 Acceptance Criteria

For 4 h experiments, the mean vehicle control suspension growth was required to be between 8 and 32 (Moore *et al* (2003)). For experiments with a 24 h exposure period, a value between 32 and 180 was required (Moore *et a*l (2007)).

Errors in cell dilution or very high mutant fractions may result in plates with no negative wells. The Poisson distribution analysis does not generate data for such scores and so any pair of plates containing 0% empty wells was rejected. Plates containing 100% empty wells may arise from either errors in cell dilution or severe toxicity. Any such data was given consideration before acceptance.

The mean vehicle control cloning efficiency was required to be greater than 65% and less than 120%. (Experience shows that the acceptance or rejection of an occasional culture giving >120% CE makes no difference to the overall results, and therefore such instances were accepted.)

Results for any one treatment/concentration were inadequate if there were less than 2 acceptable cultures. Where results were obtained from a single culture, they may have been included as supporting evidence.

Analysable results should have been obtained from at least 4 concentrations of Alcohols, C18-22 Distn. Residues in any experiment. These may have included results extending into the toxic range of less than 10% RTG.

The highest concentration of Alcohols, C18-22 Distn. Residues should have been limited by solubility or toxicity, or, in the absence of these, should have been the maximum practical concentration of test item, based on the recommendations in current guidelines, *ie* 5000 µg/mL or 10 mM, whichever is lower.

The negative control mean mutant fraction range recommended by the IWGT mouse lymphoma workgroup for the microwell assay is 50-170 mutants per million (Moore *et al* (2006)). At Charles River, the range is just slightly lower, being *ca* 40-160 mutants per million. Concern over accepting results in the range 40-50 mutants per million centres on the possibility of reduced recovery of the less viable mutants. Any experiments with a vehicle mean mutant fraction between 40 and 50 mutants per million were accepted, providing the positive control results were unequivocally acceptable.

In any experiment, MMS and at least one concentration of 3-MC should have yielded an induced mutant fraction (IMF = No. mutants per million for treated group, minus No. mutants per million for vehicle control) of at least 300 mutants per million, with a ratio of small/large colonies of greater than 0.67 (>40% small). The mean RTG values for all positive control treatments should have been >10%. EMS, a large colony inducer, was included at a low dose to monitor the sensitivity of the system to weak mutagens.

Appendix 5 reflects the current historical control database. Variations between batches of positive control chemicals, media, serum and S9 preparations can all result in large differences in positive control responses. There is therefore no requirement for the positive control mutant fractions obtained in this study to fall within the historical ranges.

There should have been an absence of confounding technical problems, *eg*, contamination, outliers, excessive toxicity, osmolality and pH changes.

8 STATISTICAL ANALYSIS AND DATA EVALUATION

8.1 Statistical Analysis

The results for each experiment were subjected to statistical analysis by the recommended UKEMS method. The methods used are detailed in Appendix 6.

8.2 Data Evaluation

8.2.1 Interpretation of Toxicity

Mutagenic responses that occur only at RTG values below 10%, while being statistically significant, are recognised as having questionable biological significance (Clive *et al* (1983), Scott *et al* (1991)). Furthermore, results arising from an initial high cell kill are also prone to high divergence and thus tend to be statistically less robust. As genetic damage naturally reduces cell viability, it is normal, however, for mutagenic responses to occur only at reduced levels of survival (*ie*, lowered RTG). If the test item was toxic, results should have been obtained from concentrations resulting in RTG values down to 20%, if a conclusion of non-

mutagenic was to be reached with confidence. It is therefore important to obtain results in the RTG range 10 to 20%, when dealing with toxic test items.

Any data obtained for treatments resulting in an RTG value below 10%, have been reported, but have not been included in the statistical analysis. Any such data were not included in the general assessment of the results unless they were required to support a conclusion of non-mutagenicity (see Section 8.2.2.2).

8.2.2 Interpretation of Mutagenic Activity

8.2.2.1 Criteria for a Positive Result

It is recognised that small, biologically irrelevant increases in mutant fraction may arise that prove statistically significant (Moore *et al* (2006)). The IWGT recommends that biological significance be attached to increases in IMF that exceed a value based on the global background mutant fraction. This value, the global evaluation factor (GEF), is defined as the mean of the global vehicle control distribution plus one standard deviation. For the microwell cloning version of the assay, this value is 126 mutants per million. Biological significance was therefore assumed to apply to treatments that gave an IMF value >126 mutants per million.

An experiment was considered positive if one or more concentrations were biologically significant and there was a significant linear trend. An experiment may also have been classed as positive in the absence of a linear trend if there was mitigating evidence. This may have been, for example, the presence of a similar level of toxicity at all concentrations assessed. In such a case, the confirmatory experiment would have been expected to assess concentrations covering different levels of toxicity, to establish a linear trend.

Additional comparisons that can aid interpretation of results include:

a comparison of the induced mutant fraction with the historical maximum for difference between vehicle controls (No Effect Maximum - see Appendix 5)

b comparison of the mutant fraction of a treated group with the historical range of vehicle control values (see Appendix 5)

A test item was positive if 2 positive experiments out of 2 were recorded within the same activation condition. Test items that gave a negative response in the standard exposure in the absence of S9 mix, but gave a positive response in the extended exposure, were liable to a confirmatory experiment with the extended exposure.

8.2.2.2 Criteria for a Negative Result

In the absence of any significant findings or other criteria for a positive response described in 8.2.2.1 above, a test item was defined as non-mutagenic, provided data were obtained in both the absence and the presence of S9 mix that accompanied one or more of the following:

- the predetermined maximum concentration of 5000 µg/mL or 10 mM, whichever is lower
- the highest practical concentration limited by the solubility or pH of the test item
- RTG in the range 10-20%

It is acknowledged (Moore *et al* (2002)) that there are some circumstances under which a chemical may be determined to be non-mutagenic when there was no treatment showing an RTG value between 10 and 20%. These situations are as follows:

a there was no evidence of mutagenic activity in a series of data points within 100% to 20% RTG and there was at least one data point between 20% and 25% RTG.

b there was no evidence of mutagenic activity in a series of data points between 100% to 25% RTG and there was also a data point between 10% and 1% RTG.

8.2.2.3 Colony Sizing

The ratio of small to large type mutant colonies was stated for all cultures. These ratios provide evidence of adequate recovery of small type colonies from control cultures, and may provide additional information regarding the type of genetic damage being induced by positive test items.

9 RESULTS AND DISCUSSION

9.1 Toxicity Tests (Tables 1 - 3)

Alcohols, C18-22 Distn. Residues was not toxic to the cells under any of the exposure conditions (4 h exposure in the absence and presence of S9 mix and 24 h exposure in the absence of S9 mix). As stated in Section 6.3.1, the highest concentration of 200 µg/mL was considered to be a precipitating dose level.

9.2 Mutation Assays (Tables 4 - 7)

The summarised results giving the mean values for the duplicate cultures (and quadruplicate vehicle controls) are presented in Tables 4 - 7. The original data obtained in the generation of the Relative Suspension Growth values for each culture in the 4 mutation assays are transcribed in Appendix 7. Similarly, the Day 2/3 Cloning Efficiencies of the non-mutants and the Relative Total Growth values are presented in Appendix 8, and the Day 2/3 Mutant Selection data are presented in Appendix 9.

9.2.1 Vehicle Control Groups

The solvent control mean mutant fractions were within the normal ranges experienced in this laboratory (Appendix 5) and reported in the literature with the L5178Y cell line (Mitchell *et al* (1997)). It was noted that the value obtained in Assay 2 (47 mutants per million) was slightly below the recommended IWGT range of 50-170. However, the concurrent positive control results were unequivocally acceptable (see Section 7.3.1) and therefore the value was accepted. All other values met the acceptance criteria in Section 7.3.1 in full.

9.2.2 Positive Control Groups

All positive control log mutant fractions were significantly higher than the vehicle controls at $P<0.05$. The IMF values and colony size ratios for MMS and 3-MC were all acceptable by the criteria stated in Section 7.3.1.

9.2.3 Alcohols, C18-22 Distn. Residues

9.2.3.1 Assays 1 (in the Absence of S9 Mix - Table 4) and 2 (in the Presence of S9 Mix - Table 5)

Alcohols, C18-22 Distn. Residues was assessed for mutagenic activity at concentrations of: 25, 50, 100 and 200 µg/mL in the absence and presence of S9 mix (4 h exposure).

None of the assessed concentrations tested significant for increase in log mutant fraction in either experiment. In the absence of S9 mix, the test for linear trend was not reported as the

slope was negative, while in the presence of S9 mix, the test for linear trend was not significant (P= 0.79). There was no toxicity at any concentration in either experiment. The highest concentration of 200 µg/mL was considered to be a precipitating dose level (See Section 6.3.1).

Both experiments were classed negative, and therefore the second experiment in the absence of S9 mix was conducted with the extended, 24 h exposure period.

9.2.3.2 Assays 3 (in the Absence of S9 Mix - Table 6) and 4 (in the Presence of S9 Mix - Table 7)

Alcohols, C18-22 Distn. Residues was assessed for mutagenic activity at concentrations of: 25, 50, 100 and 200 µg/mL in the absence of S9 mix (24 h exposure) and in the presence of S9 mix (4 h exposure).

None of the assessed concentrations tested significant for increase in log mutant fraction in either experiment. In the absence of S9 mix, the test for linear trend was not reported as the slope was negative, while in the presence of S9 mix, the test for linear trend was not significant (P= 0.36). There was no toxicity at any concentration in either experiment. The highest concentration of 200 µg/mL was considered to be a precipitating dose level (See Section 6.3.1).

Both experiments were classed negative.

9.2.3.3 Combined Statistical Analysis

A combined statistical analysis of Assays 2 and 4 (where all treatment conditions and concentrations of test item were identical) was made. There were no statistically significant differences between the Alcohols, C18-22 Distn. Residues-treated groups and the vehicle controls. The test for linear trend was not significant (P = 0.51).

10 CONCLUSION

It was concluded that Alcohols, C18-22 Distn. Residues is not mutagenic in mouse lymphoma L5178Y cells, in either the absence or the presence of S9 mix, when tested in dimethylsulphoxide up to and beyond its limit of solubility in the test system.

11 REFERENCES

Amacher D E, Paillet S C, Turner G N, Ray V A and Salzburg D S (1980). Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. II. Test Validation and Interpretation. Mutation Research, 72, 447-474.

Ames B N, McCann J and Yamasaki E (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/microsome test: assay of 300 chemicals. Mutation Research, 31, 347-364.

Chu E H Y and Malling H V (1968). Mammalian cell genetics. II. Chemical induction of specific locus mutations in Chinese hamster cells *in vitro*. Proceedings of the National Academy of Sciences of the USA, 61, 1306-12.

Clive D, Flamm W G, Machesko M R and Bernheim N J (1972). A mutational assay system using the thymidine kinase locus in mouse lymphoma cells. Mutation Research, 16, 77-87.

Clive D and Spector J F S (1975). Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Research, 31, 17-29.

Clive D, Johnson K O, Spector J F S, Batson A G and Brown M M M (1979). Validation and characterisation of the L5178Y/TK$^{+/-}$ mouse lymphoma mutagen assay system. Mutation Research, 59, 61-108.

Clive D, McCuen R, Spector J F S, Piper C and Mavournin K H (1983). Specific gene mutations in L5178Y cells in culture. Mutation Research, 115, 225-251.

Cole J, Arlett C F, Green M H L, Lowe J and Muriel W (1983). A comparison of the agar cloning and microtitration techniques for assaying cell survival and mutation frequency in L5178Y mouse lymphoma cells. Mutation Research, 111, 371-386.

Fischer G A (1958). Studies of the culture of leukemic cells *in vitro*. Annals of the New York Academy of Sciences, 76, 673-680.

Fischer G A and Sartorelli A C (1964). Development, Maintenance and Assay of Drug Resistance. Methods in Medical Research, 10, 247-267.

Honma M, Hayashi M, Shimada H, Tanaka N, Wakuri S, Awogi T, Yamamoto K I, Noriko-Ushio K, Nishi Y, Nakadate M and Sofuni T (1999a). Evaluation of the mouse lymphoma *tk*

assay (microwell method) as an alternative to the *in vitro* chromosomal aberration test. Mutagenesis, 14, 5-22.

Honma M, Zhang L-S, Sakamoto H, Ozaki M, Takeshita K, Momose M, Hayashi M and Sofuni T (1999b). The need for long-term treatment in the mouse lymphoma assay. Mutagenesis, 14, 23-29.

Hozier J, Sawyer J, Moore, M, Howard B and Clive D (1981). Cytogenetic analysis of the L5178Y/$TK^{+/-}$ leads to $TK^{-/-}$ mouse lymphoma mutagenesis assay system. Mutation Research, 84, 169-181.

Jotz M M and Mitchell A D (1981). In, 'Evaluation of Short-Term Tests for Carcinogens', de Serres F J and Ashby J (Eds). Elsevier, Amsterdam, 580-593.

Kao F T and Puck T T (1968). Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proceedings of the National Academy of Sciences of the USA, 60, 1275-1281.

McGregor D B, Martin R, Cattanach P, Edwards I, McBride D and Caspary W J (1987). Responses of the L5178Y tk^{+}/tk^{-} Mouse Lymphoma Cell Forward Mutation Assay to Coded Chemicals. I: Results for Nine Compounds. Environmental Mutagenesis, 9, 143-160.

McGregor D B, Brown A, Cattanach P, Edwards I, McBride D and Caspary W J (1988a). Responses of the L5178Y tk^{+}/tk^{-} Mouse Lymphoma Cell Forward Mutation Assay II: 18 Coded Chemicals. Environmental and Molecular Mutagenesis, 11, 91-118.

McGregor D B, Edwards I, Riach C G, Cattanach P, Martin R, Mitchell A and Caspary W J (1988b). Studies of an S9-based metabolic activation system used in the mouse lymphoma L5178Y cell mutation assay. Mutagenesis, 3, 485-490.

McGregor D B, Brown A, Cattanach P, Edwards I, McBride D, Riach C and Caspary W (1988c). Responses of the L5178Y tk^{+}/tk^{-} Mouse Lymphoma Cell Forward Mutation Assay: III. 72 Coded Chemicals. Environmental and Molecular Mutagenesis, 12, 85-154.

McGregor D B, Brown A G, Howgate S, McBride D, Riach C G and Caspary W J (1991a). Responses of the L5178Y Mouse Lymphoma Cell Forward Mutation Assay. V: 27 Coded Chemicals. Environmental and Molecular Mutagenesis, 17, 196-219.

McGregor D B, Brown A, Cattanach P, Edwards I, McBride D, Riach C G, Shepherd W and Caspary W J (1991b). Responses of the L5178Y Mouse Lymphoma Forward Mutation Assay: V. Gases and Vapors. Environmental and Molecular Mutagenesis, 17, 122-129.

Mitchell A D, Auletta A E, Clive D, Kirby P E, Moore M M and Myhr B C (1997). The L5178Y/tk$^{+/-}$ mouse lymphoma specific gene and chromosomal mutation assay. A phase III report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Research, 394, 177-303.

Moore M M, Honma M, Clements J, Awogi T, Bolcsfoldi G, Cole J, Gollapudi B, Harrington-Brock K, Mitchell A, Muster W, Myhr B, O'Donovan M, Ouldelhkim M-C, San R, Shimada H and Stankowski L F (2000). Mouse Lymphoma Thymidine Kinase Locus Gene Mutation Assay: International Workshop on Genotoxicity Test Procedures Workgroup Report. Environmental and Molecular Mutagenesis, 35, 185-190.

Moore M M, Honma M, Clements J, Harrington-Brock K, Awogi T, Bolcsfoldi G, Cifone M, Collard D, Fellows M, Flanders K, Gollapudi B, Jenkinson P, Kirby P, Kirchner S, Kraycer J, McEnaney S, Muster W, Myhr B, O'Donovan M, Oliver J, Ouldelhkim M-C, Pant K, Preston R, Riach C, San R, Shimada H, Stankowski Jr. L F (2002). Mouse lymphoma thymidine kinase gene mutation assay: Follow-up International Workshop on Genotoxicity Test Procedures, New Orleans, Louisiana, April 2000. Environmental and Molecular Mutagenesis, 40, Issue 4, 292-299.

Moore M M, Honma M, Clements J, Bolcsfoldi G, Cifone M, Delongchamp R, Fellows M, Gollapudi B, Jenkinson P, Kirby P, Kirchner S, Muster W, Myhr B, O'Donovan M, Oliver J, Ornori T, Ouldelhkim M-C, Pant K, Preston R, Riach C, San R, Stankowski Jr L F, Thakur A, Wakuri S, Yoshimura I (2003). Mouse Lymphoma Thymidine Kinase Gene Mutation Assay: International Workshop on Genotoxicity Tests Workgroup Report—Plymouth, UK 2002. Mutation Research, 540, 127-140.

Moore M M, Honma M, Clements J, Bolcsfoldi G, Burlinson B, Cifone M, Clarke J, Delongchamp R, Durward R, Fellows M, Gollapudi B, Hou S, Jenkinson P, Lloyd M, Majeska J, Myhr B, O'Donovan M, Omori T, Riach C, San R, Stankowski Jr. L F, Thakur A K, Van Goethem F, Wakuri S, Yoshimura I (2006). Mouse lymphoma thymidine kinase gene mutation assay: Follow-up meeting of the international workshop on Genotoxicity testing - Aberdeen, Scotland, 2003 - Assay acceptance criteria, positive controls, and data evaluation. Environmental and Molecular Mutagenesis, 47, 1-5.

Moore M M, Honma M, Clements J, Bolcsfoldi G, Burlinson B, Cifone M, Clarke J, Clay P, Doppalapudi R, Fellows M, Gollapudi B, Hou S, Jenkinson P, Muster W, Pant K, Kidd D A, Lorge E, Lloyd M, Myhr B, O'Donovan M, Riach C, Stankowski Jr. L F, Thakur A K, Van Goethem F.. Mouse lymphoma thymidine kinase gene mutation assay: Meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment. Mutation Research 627 (2007) 36-40.

Myhr B, McGregor D, Bowers L, Riach C G, Brown A G, Edwards I, McBride D, Martin R and Caspary W J (1990). L5178Y Mouse Lymphoma Cell Mutation Assay Results With 41 Compounds. Environmental and Molecular Mutagenesis, 16 (Suppl 18), 138-167.

Robinson W D, Green M H L, Cole J, Healy M J R, Garner R C and Gatehouse D (1989). In 'Statistical Evaluation of Mutagenicity Test Data', Kirkland D (Ed). CUP, Cambridge, 102-140.

Scott D, Galloway S M, Marshall R R, Ishidate M, Brusick D, Ashby J and Myhr B C (1991). International Commission for Protection Against Environmental Mutagens and Carcinogens. Genotoxicity under extreme culture conditions. [A report from ICPEMC Task Group 9.] Mutation Research, 257, 147-205.

12 TABLES

Table 1 Toxicity Test in the Absence of S9 Mix (4 h Exposure)

Chemical	Concentration (µg/mL)	Observations	Suspension Count (x 10^5/mL)		Total Suspension Growth	Relative Suspension Growth %
			Day 1	Day 2		
DMSO	(100 µL added)	-	15.0	18.9	31.5	100.0
Alcohols, C18-22 Distn. Residues	0.78	-	18.8	15.0	31.3	99.5
	1.56	-	19.1	13.6	28.9	91.6
	3.13	-	18.1	13.0	26.1	83.0
	6.25	-	17.7	14.3	28.1	89.3
	12.50	-	17.6	14.0	27.4	86.9
	25.00	-	16.8	15.4	28.7	91.3
	50.00	-	18.4	15.2	31.1	98.7
	100.00	-	13.6	16.9	25.5	81.1
	200.00	pptn	14.2	14.5	22.9	72.6

pptn = Precipitation

Table 2 Toxicity Test in the Presence of S9 Mix (4 h Exposure)

Chemical	Concentration (µg/mL)	Observations	Suspension Count (x 10^5/mL)		Total Suspension Growth	Relative Suspension Growth %
			Day 1	Day 2		
DMSO	(100 µL added)	-	15.8	14.0	24.6	100.0
Alcohols, C18-22 Distn. Residues	0.78	-	14.0	14.2	22.1	89.9
	1.56	-	15.0	12.0	20.0	81.4
	3.13	-	16.7	12.6	23.4	95.1
	6.25	-	14.2	14.7	23.2	94.4
	12.50	-	14.7	14.7	24.0	97.7
	25.00	-	15.6	12.9	22.4	91.0
	50.00	-	12.3	15.7	21.5	87.3
	100.00	-	15.6	12.0	20.8	84.6
	200.00	pptn	14.3	13.6	21.6	87.9

pptn = Precipitation

Table 3 Toxicity Test in the Absence of S9 Mix (24 h Exposure)

Chemical	Concentration (µg/mL)	Observations	Suspension Count (x 10^5/mL)			Total Suspension Growth	Relative Suspension Growth %
			Day 1	Day 2	Day 3		
DMSO	(200 µL added)	-	11.2	17.7	13.0	95.4	100.0
Alcohols, C18-22 Distn. Residues	0.78	-	11.2	18.7	12.9	100.1	104.8
	1.56	-	11.0	15.9	13.5	87.5	91.6
	3.13	-	11.7	13.6	16.0	94.3	98.8
	6.25	-	12.2	14.1	13.3	84.7	88.8
	12.50	-	11.9	17.7	11.0	85.8	89.9
	25.00	-	12.8	13.9	13.9	91.6	96.0
	50.00	-	13.1	15.4	13.5	100.9	105.7
	100.00	-	14.2	15.0	13.0	102.6	107.4
	200.00	pptn	14.1	15.1	11.2	88.3	92.5

pptn = Precipitation

Table 4 Mutation Test in the Absence of S9 Mix (4 h Exposure)
Summary of Means of Data (Assay 1)

Chemical	Concentration (µg/mL)	Relative Total Growth %	Mutant Fraction (x 10^{-6})	IMF (Induced Mutant Fraction x10^{-6})	Ratio of Small to Large Colonies	Statistical Comparison
DMSO	(100 µL added)	100	54	N/A	1.28	N/A
EMS	250	66	539	484	0.47	*
MMS	10	37	708	654	2.55	*
Alcohols, C18-22 Distn. Residues	25	111	52	-	1.72	NS
	50	98	58	3	1.93	NS
	100	107	37	-	2.17	NS
	pptn 200	114	35	-	1.25	NS

IMF = Mutant fraction of treatment minus mutant fraction of vehicle control group
N/A = Not Applicable
* = Significant difference in log mutant fraction compared with vehicle control ($P<0.05$)
NS = Not Significant
Test for linear trend of mutant fraction with concn. of Alcohols, C18-22 Distn. Residues = not reported – slope negative
- = IMF ≤ 0
pptn = Precipitation

Table 5 Mutation Test in the Presence of S9 Mix (4 h Exposure)
Summary of Means of Data (Assay 2)

Chemical	Concentration (µg/mL)	Relative Total Growth %	Mutant Fraction (x 10^{-6})	IMF (Induced Mutant Fraction x10^{-6})	Ratio of Small to Large Colonies	Statistical Comparison
DMSO	(100 µL added)	100	47	N/A	1.18	N/A
3-MC	2.5	71	403	355	1.08	*
	10	65	576	528	1.20	*
Alcohols, C18-22 Distn. Residues	25	104	66	19	2.43	NS
	50	101	56	9	1.24	NS
	100	92	60	13	0.61	NS
	pptn 200	122	53	6	1.78	NS

IMF = Mutant fraction of treatment minus mutant fraction of vehicle control group
N/A = Not Applicable
* = Significant difference in log mutant fraction compared with vehicle control (P<0.05)
NS = Not Significant
Test for linear trend of mutant fraction with concn. of Alcohols, C18-22 Distn. Residues = not significant (P = 0.79)
pptn = Precipitation

Table 6 Mutation Test in the Absence of S9 Mix (24 h Exposure) Summary of Means of Data (Assay 3)

Chemical	Concentration (µg/mL)	Relative Total Growth %	Mutant Fraction (x 10^{-6})	IMF (Induced Mutant Fraction x10^{-6})	Ratio of Small to Large Colonies	Statistical Comparison
DMSO	(200 µL added)	100	134	N/A	2.94	N/A
EMS	150	11	3741	3608	0.32	*
MMS	5	31	1912	1778	1.53	*
Alcohols, C18-22 Distn. Residues	25	86	107	-	3.29	NS
	50	78	141	8	3.94	NS
	100	86	144	10	2.82	NS
	pptn 200	101	120	-	2.15	NS

IMF = Mutant fraction of treatment minus mutant fraction of vehicle control group
N/A = Not Applicable
* = Significant difference in log mutant fraction compared with vehicle control ($P<0.05$)
NS = Not Significant
Test for linear trend of mutant fraction with concn. of Alcohols, C18-22 Distn. Residues = not reported – slope negative
- = IMF ≤ 0
pptn = Precipitation

Table 7 Mutation Test in the Presence of S9 Mix (4 h Exposure)
Summary of Means of Data (Assay 4)

Chemical	Concentration (µg/mL)	Relative Total Growth %	Mutant Fraction (x 10^{-6})	IMF (Induced Mutant Fraction x10^{-6})	Ratio of Small to Large Colonies	Statistical Comparison
DMSO	(100 µL added)	100	114	N/A	1.51	N/A
3-MC	2.5	73	935	822	1.29	*
	10	66	1143	1029	1.21	*
Alcohols, C18-22 Distn. Residues	25	94	149	36	1.35	NS
	50	117	117	4	1.56	NS
	100	91	143	29	2.34	NS
	pptn 200	89	135	22	2.21	NS

IMF = Mutant fraction of treatment minus mutant fraction of vehicle control group
N/A = Not Applicable

* = Significant difference in log mutant fraction compared with vehicle control ($P<0.05$)
NS = Not Significant
Test for linear trend of mutant fraction with concn. of Alcohols, C18-22 Distn. Residues = not significant ($P = 0.36$)
pptn = Precipitation

13 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 Cell Culture

Cell Storage

Cells in logarithmic phase of growth were collected by centrifugation (1000 r.p.m. x 5 min (200 *g*)) and resuspended in freezing medium (10% DMSO in culture medium) to a final cell density of *ca* 10 x 10^6 cells/mL. Samples (2 mL) were frozen slowly before being stored in liquid nitrogen. New cultures are established from frozen stocks every 3 months.

Initially, a new culture was seeded into a T-75 flask containing 20 mL $R_{10}P$ gassed with 5% CO_2 in air. The cell suspension was gently layered on the bottom of the flask without disturbing the cells and incubated for *ca* 24 h at *ca* 37°C. When the cells were determined to be growing well (3-5 generations in 24 h) a stock culture was established by seeding 3 x 10^5 cells/mL in fresh $R_{10}P$ into a polypropylene centrifuge tube (Corning 250 mL), where it was maintained on a 10 r.p.m. roller at *ca* 37°C. Stock cultures were examined for mycoplasma and shown to be uninfected.

Cell Dilution

With a generation time of 10-12 h, there is a 5-fold increase in cell number in 24 h and 25-fold in 48 h. Cultures were subcultured daily (except at weekends) to a density of approximately 3 x 10^5 cells/mL to maintain the cells in logarithmic growth. For weekend maintenance, the cultures were diluted back to *ca* 1 x 10^4 cells/mL.

Cell 'Cleansing'

The tk^+tk^- heterozygote cells grown in suspension spontaneously mutate to tk^-tk^- at a rate of 2 x 10^{-6} mutations/generation. These homozygous mutants were removed before testing began.

The week before an experiment, Thymidine/Hypoxanthine/Glycine/Methotrexate (THGM) stock solution (1 mL) was added to a sub-population of the stock cell suspension (100 mL). The following day the culture was pelleted and resuspended in $R_{10}P$ supplemented with THG (THGM without methotrexate). This treatment of the cells was carried out once only before their use in an experiment. The culture was ready for use the following week. Unused cells were disposed of at the end of that week.

Mechanism: Methotrexate blocks folate metabolism by binding strongly to dihydrofolate reductase, hence thymidylate synthetase-mediated thymidylate production (TMP) stops. TMP can be maintained by TK phosphorylation of exogenous thymidine (***T***HG). Purine

Appendix 2
(continued)

metabolism is similarly affected and this block is surmounted by added hypoxanthine. Glycine is added to provide a single carbon source to replace the blocked folate metabolic pathway. Hence $tk^{+}tk^{-}$ cells can survive the methotrexate treatment while $tk^{-}tk^{-}$ cells die.

Appendix 3 *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
Strain: *S. typhimurium* TA 1538
Date plated: 17 July 2009
Culture batch: G169
Batch No. (plates): 000766
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 4 Worked Examples of Calculations

The calculations involved in the result analysis for the first methyl methanesulphonate-treated culture in Assay 1 are described below. The calculations may contain rounding errors.

Method of calculating the Suspension Growth (Appendix 7, page 51)

Suspension growth (SG) represents the potential of the culture to multiply itself over the 2 day expression period (or 3 day, in the case of experiments with 24 h exposure periods), by accounting for the concentration to which the cells are diluted after counting:

> On Day 1, after counting, the cells were diluted to 3.0×10^5/mL. On Day 2, the cells had reached 11.0×10^5/mL.

The calculation for SG from Day 1 to Day 2 would be:

$$\frac{11.0}{3.0} = 3.7$$

The cells therefore had multiplied 3.7-fold between Day 1 and Day 2.

The calculation for the full period therefore, is:

$$\frac{\text{Day 1 Count}}{\text{Concentration on Day 0 *}} \times \frac{\text{Day 2 Count}}{\text{Final Concentration on Day 1}}$$

* Routinely 3.0×10^5/mL for all cultures

In this instance,

$$\frac{12.0}{3.0} \times \frac{11.0}{3.0} = 14.7$$

Note: if toxicity causes the cell density to be below 3.0×10^5/mL on any day, the culture is not diluted. In such an instance, the denominator in the above calculations for the day in question will be the actual cell density, rather than the more usual 3.0×10^5/mL.

The cells therefore had multiplied 14.7-fold over the 2 day expression period.

This figure is then expressed as a percentage of the mean vehicle control value to give the Relative Suspension Growth (RSG).

Appendix 4
(continued) Worked Examples of Calculations

In this instance,

$$\frac{14.7}{25.7} \text{ x } \frac{100}{1} = 57.1\%$$

Assessment of Cloning Efficiency and Relative Total Growth (Appendix 8, page 55)

The cloning efficiency (CE) is calculated from the zero term of the Poisson distribution using the formula:

$$CE = \frac{-\ln(P(o))}{\text{number of cells per well}}$$

$$\text{where } P(o) = \frac{\text{empty wells}}{\text{total wells}}$$

In this instance,

$$P(o) = \frac{41+36}{192} = 0.40$$

$$CE = \frac{-\ln(0.40)}{1.6} = 0.57$$

Thus 57% of the cells in the MMS-treated culture were clonable at the end of the expression period.

Relative Total Growth (RTG) combines SG with CE.

Total Growth = SG x CE (non-mutants)

In this instance,

14.7 x 0.57 = 8.38

$$\text{Relative Total Growth (\%)} = \frac{\text{individual total growth}}{\text{mean vehicle control total growth}} \text{ x } \frac{100}{1}$$

Appendix 4
(continued) Worked Examples of Calculations

In this instance,

$$\frac{8.38}{25.12} \times \frac{100}{1} = 33$$

The total growth of the culture therefore was 33% of the vehicle control mean value.

Method of calculating the Mutant Fraction (Appendix 9, page 59)

This figure gives the number of mutant cells per million clonable cells. The number of empty wells is obtained by subtracting the small colony wells and the large colony wells from the total wells plated.

In this instance,

$$192 - (12 + 45 + 17 + 36) = 82$$

The CE of the mutant cells is calculated as for the non-mutants.

In this instance,

$$P(o) = \frac{82}{192} = 0.43$$

$$CE = \frac{-\ln(0.43)}{2000} = 425.39$$

This figure is finally adjusted to compensate for the CE of the whole population, thus:

$$\text{Mutant fraction per viable cell} = \frac{\text{CE of mutants}}{\text{CE of non - mutants}}$$

In this instance:

$$\text{Mutant fraction per viable cell} = \frac{425.39 \times 10^{-6}}{0.57} = 745 \times 10^{-6}$$

Appendix 5 Mouse Lymphoma L5178Y Cell Historical Control Data from July 2003 to December 2008

Vehicle Control Data

Vehicle Control	S9	Exposure Time	$n^{\dagger}$	Mutant Fraction x 10^{-6}			No-effect Maximum (Induced Mutant Fraction)	Mean Colony Size Ratio (Small/Large)		
				Mean	SD	Range		Mean	SD	Range
All, pooled	-	4 h	71	71	16	49-121	42	1.28	0.47	0.45-2.73
All, pooled	-	24 h	50	78	21	51-129	49	1.09	0.43	0.35-2.11
All, pooled	+	4 h	120	78	22	45-148	70	1.24	0.44	0.35-2.75

† = Each value is the mean of 4 replicate cultures
The No-effect Maximum represents the maximum difference recorded between the 2 pairs of vehicle control cultures in any experiment. That is, the lower mean mutant fraction (x 10^{-6}) is subtracted from the higher. This difference, when applied to the response from a mutagen, is termed the induced mutant fraction (IMF).

Positive Control Data

Positive Control	S9	Concen-tration (µg/mL)	Exposure Time	$n^{\dagger}$	Mutant Fraction x 10^{-6}			RTG %		Mean Colony Size Ratio (Small/Large)		
					Mean	SD	Range	Mean	Range	Mean	SD	Range
EMS	-	250	4 h	69	601	127	431-910	64	38-94	0.51	0.13	0.25-0.85
MMS	-	10	4 h	71	908	238	429-1659	40	22-78	2.34	0.55	1.46-3.65
EMS	-	150	24 h	50	2096	608	1324-3801	31	15-99	0.38	0.10	0.22-0.63
MMS	-	5	24 h	50	1880	471	1026-3023	31	17-82	1.78	0.41	1.07-3.07
3-MC	+	2.5	4 h	120	1061	338	570-2277	49	16-96	1.37	0.37	0.93-3.61

EMS = Ethyl methanesulphonate
MMS = Methyl methanesulphonate
3-MC = 3-Methylcholanthrene

† = Each value is the mean of 2 replicate cultures

Audited by: Alison McNaughton (Quality Assurance)
09 February 2009

Appendix 6 Statistical Method

Survival and mutant data were available for 4 experiments, two without S9 mix (Assays 1 and 3) and two with S9 (Assays 2 and 4). Assays were measured over an exposure period of 4 hours or 24 hours. Data were analysed using methods outlined in Robinson *et al* (1989). The analyses comprised the following:

1. Determination of the heterogeneity factor for each dose level.
2. Comparison of the heterogeneity factor with the historical control. Any dose levels with heterogeneity factor statistically higher than the historical control were excluded from all statistical analysis.
3. Determination of the heterogeneity factor for the experiment.
4. Calculation of a new historical control heterogeneity factor.
5. Calculation of the log mutant fraction.
6. Comparison of the log mutant fraction between the control and each treatment dose (at $P<0.05$).
7. Test for linear increasing trend of mutant fraction with increasing dose of test item (at $P<0.05$).

Unless otherwise stated, the term 'heterogeneity factors' is defined separately for survival (Hs) and mutant (Hm) data.

When each assay was analysed, a new value for the heterogeneity factor was calculated and compared with the historical value. As such, assays were analysed in chronological order. If the new value was exceptionally large (as determined by significance testing below), the experimental results were considered unacceptably variable and were discarded. Otherwise, the new heterogeneity factors were calculated for use in the analysis and the historical value was updated.

Beginning with Assay 1, the data were analysed using the methods outlined in Robinson *et al* (1989). The dose-specific heterogeneity factors for each dose level were determined.

The dose-specific heterogeneity factors were compared with the historical controls for consistency using a one-sided F-test (Pearson and Hartley (1989)) at the 0.1% level. Any dose levels where either the mutant or the survival heterogeneity factors were significantly higher than their respective historical controls were excluded from all of the analyses for the assay.

The overall heterogeneity factors for the assay were determined.

The heterogeneity factors were compared with the historical controls for consistency using a one-sided F-test at the 1% level. If either the mutant or the survival heterogeneity factors

Appendix 6
(continued)

were significantly higher than their respective historical controls the assay was discarded and the old historical controls remained in place. Otherwise, new historical control heterogeneity factors were calculated, using weighting of 1/20 of the current estimate and 19/20 of the historical estimate.

For each assay not discarded under the above, the following were performed:

The log mutant fraction and its variance were calculated for each dose level.

Log mutant fraction was compared between the control and each treatment dose at the 5% level using a one-sided Dunnett's test (Dunnett (1955)) for an increase in mutant fraction, if and only if the treatment dose mutant fraction was greater than the control.

A one sided χ^2 test (Pearson and Hartley (1989)) with 1 degree of freedom was performed at the 5 % level to test for linear increasing trend of mutant fraction with increasing dose of test item, if and only if the direction of the slope parameter was positive.

In addition, Assay 2 and Assay 4 had equal exposure periods and equal dose levels were used. A two-sided χ^2 test (Pearson and Hartley (1976)), with (M-1) degrees of freedom (M = number of assays) was therefore used to test for consistency at each dose level between the assays at the 1% level. At each dose level the assays were considered consistent and therefore it was appropriate to report a combined result.

In Assays 2 and 4, the combined differences in log mutant frequency between the control and each remaining treatment dose were tested at the 5% level using a one-sided Dunnett's test (Dunnett (1955)) for an increase in mutant frequency. (This was only performed if the treatment dose mutant frequency was greater than the control.)

A one-sided χ^2 test with 1 degree of freedom was performed for the combined data at the 5% level to test for linear increasing trend of mutant frequency with increasing dose of test material. (This was only performed if the direction of the combined slope parameter was positive.)

Appendix 6
(continued)

References:

Robinson W D, Green M H L, Healy M J R, Garner R C and Gatehouse D: (1989). Statistical Evaluation of Bacterial/Mammalian Fluctuation Tests, in: Kirkland D J (Ed.), Statistical Evaluation of Mutagenicity Test Data, Cambridge University Press, Cambridge.

Pearson, E S & Hartley, H O (1989) *Biometrika Tables for Statisticians* Volume 1 3rd edition, Griffin, London

Dunnett, C W (1955). A multiple comparison procedure for comparing several treatments with a control. *Journal of American Statistical Association*, 1096-1121.

Appendix 7 Individual Suspension Growth Data

Mutation Test in the Absence of S9 Mix (4 h Exposure)
Suspension Growth (Assay 1)

Chemical	Concentration (µg/mL)	Observations	Suspension Count (x 10^5/mL)		Total Suspension Growth (%)	Relative Suspension Growth
			Day 1	Day 2		
Vehicle Control Mean	→	-	-	-	*25.7*	-
DMSO	(100 µL added)	-	12.5	18.8	26.1	101.6
			13.7	18.0	27.4	106.6
			14.9	14.0	23.2	90.2
			14.7	16.0	26.1	101.7
EMS	250	-	11.9	14.4	19.0	74.1
			12.7	12.4	17.5	68.1
MMS	10	-	12.0	11.0	14.7	57.1
			12.0	14.0	18.7	72.6
Alcohols, C18-22 Distn. Residues	25	-	12.0	16.0	21.3	83.0
			13.8	18.8	28.8	112.1
	50	-	15.8	17.6	30.9	120.2
			15.7	14.4	25.1	97.7
	100	-	12.1	17.0	22.9	88.9
			13.1	17.8	25.9	100.8
	200	-	13.7	18.4	28.0	109.0
			13.2	18.6	27.3	106.1

Appendix 7
(continued)

Mutation Test in the Presence of S9 Mix (4 h Exposure)
Suspension Growth (Assay 2)

Chemical	Concentration (µg/mL)	Observations	Suspension Count (x 10^5/mL)		Total Suspension Growth (%)	Relative Suspension Growth
			Day 1	Day 2		
Vehicle Control Mean	→	-	-	-	*25.5*	-
DMSO	(100 µL added)	-	14.3	20.6	32.7	128.5
			12.0	17.6	23.5	92.1
			13.7	15.0	22.8	89.6
			13.2	15.6	22.9	89.8
3-MC	2.5	-	11.3	15.2	19.1	74.9
			9.9	18.0	19.8	77.7
	10	-	9.9	14.6	16.1	63.0
			11.3	14.0	17.6	69.0
Alcohols, C18-22 Distn. Residues	25	-	14.9	18.0	29.8	117.0
			15.5	16.2	27.9	109.5
	50	-	15.6	16.0	27.7	108.9
			12.4	18.0	24.8	97.3
	100	-	13.9	16.0	24.7	97.0
			12.4	15.0	20.7	81.1
	200	-	15.1	16.0	26.8	105.4
			13.0	15.0	21.7	85.0

Appendix 7
(continued)

Mutation Test in the Absence of S9 Mix (24 h Exposure)
Suspension Growth (Assay 3)

Chemical	Concentration (µg/mL)	Observations	Suspension Count (x 10^5/mL)			Total Suspension Growth (%)	Relative Suspension Growth
			Day 1	Day 2	Day 3		
Vehicle Control Mean	→	-	-	-	-	*55.8*	-
DMSO	(200 µL added)	-	8.2	11.4	17.0	58.9	105.5
			8.0	13.1	16.0	62.1	111.4
			8.1	10.8	16.2	52.5	94.1
			7.9	10.6	16.0	49.6	89.0
EMS	150	-	5.8	8.8	7.5	14.2	25.4
			6.0	6.3	7.0	9.8	17.6
MMS	5	-	6.9	10.1	9.4	24.3	43.5
			6.0	11.1	9.0	22.2	39.8
Alcohols, C18-22 Distn. Residues	25	-	6.8	12.0	17.0	51.4	92.1
			5.6	14.3	14.6	43.3	77.6
	50	-	5.9	14.1	15.4	47.4	85.1
			5.7	14.7	17.4	54.0	96.8
	100	-	6.0	15.0	14.6	48.7	87.3
			5.7	15.7	14.6	48.4	86.8
	200	-	6.2	13.9	16.6	53.0	95.0
			6.1	11.0	16.6	41.3	74.0

Appendix 7
(continued)

Mutation Test in the Presence of S9 Mix (4 h Exposure)
Suspension Growth (Assay 4)

Chemical	Concentration (µg/mL)	Observations	Suspension Count (x 10^5/mL)		Total Suspension Growth (%)	Relative Suspension Growth
			Day 1	Day 2		
Vehicle Control Mean	→	-	-	-	*17.8*	-
DMSO	(100 µL added)	-	10.0	14.8	16.4	92.4
			10.4	17.6	20.3	114.3
			9.0	17.6	17.6	98.9
			9.1	16.6	16.8	94.3
3-MC	2.5	-	8.1	13.6	12.2	68.8
			9.1	12.6	12.7	71.6
	10	-	7.2	15.0	12.0	67.4
			7.7	14.0	12.0	67.3
Alcohols, C18-22 Distn. Residues	25	-	10.2	14.0	15.9	89.2
			11.0	15.0	18.3	103.0
	50	-	10.2	14.0	15.9	89.2
			11.1	15.0	18.5	104.0
	100	-	10.0	18.0	20.0	112.4
			8.9	18.0	17.8	100.0
	200	-	7.7	17.8	15.2	85.6
			9.3	17.6	18.2	102.2

Appendix 8 Individual Cloning Efficiency Data (Non-mutants)

Mutation Test in the Absence of S9 Mix (4 h Exposure)
Day 2 Cloning Efficiency (Non-mutants) and Relative Total Growth (Assay 1)

Chemical	Concentration (µg/mL)	Day 2 Cloning Efficiency Plates		Wells Lost	Total Wells Scored	Cloning Efficiency (non-mutants)	Total Growth	Relative Total Growth %
		Plate 1 Empty Wells	Plate 2 Empty Wells					
Vehicle Control Mean	→	-	-	-	-	*0.98*	*25.12*	-
DMSO	(100 µL added)	30	15	0	192	0.91	23.68	94
		23	22	0	192	0.91	24.85	99
		16	17	0	192	1.10	25.51	102
		20	18	0	192	1.01	26.46	105
EMS	250	27	25	0	192	0.82	15.54	62
		15	24	0	192	1.00	17.43	69
MMS	10	41	36	0	192	0.57	8.38	33
		38	43	0	192	0.54	10.07	40
Alcohols, C18-22 Distn. Residues	25	14	10	0	192	1.30	27.73	110
		23	17	0	192	0.98	28.26	112
	50	25	20	0	192	0.91	28.02	112
		28	21	0	192	0.85	21.44	85
	100	13	19	0	192	1.12	25.59	102
		14	20	0	192	1.08	28.03	112
	200	20	20	0	192	0.98	27.46	109
		20	13	0	192	1.10	30.02	120

Appendix 8
(continued)

Mutation Test in the Presence of S9 Mix (4 h Exposure)
Day 2 Cloning Efficiency (Non-mutants) and Relative Total Growth (Assay 2)

Chemical	Concentration (µg/mL)	Day 2 Cloning Efficiency Plates		Wells Lost	Total Wells Scored	Cloning Efficiency (non-mutants)	Total Growth	Relative Total Growth %
		Plate 1 Empty Wells	Plate 2 Empty Wells					
Vehicle Control Mean	→	-	-	-	-	*1.05*	*26.16*	-
DMSO	(100 µL added)	23	31	0	192	0.79	25.95	99
		17	17	0	192	1.08	25.39	97
		13	13	0	192	1.25	28.53	109
		17	17	0	192	1.08	24.76	95
3-MC	2.5	20	21	0	192	0.96	18.42	70
		26	16	0	192	0.95	18.81	72
	10	22	16	0	192	1.01	16.26	62
		17	22	0	192	1.00	17.51	67
Alcohols, C18-22 Distn. Residues	25	20	16	0	192	1.05	31.18	119
		28	22	1	191	0.84	23.37	89
	50	20	19	0	192	1.00	27.63	106
		19	19	0	192	1.01	25.11	96
	100	15	21	0	192	1.05	25.85	99
		18	16	2	190	1.08	22.23	85
	200	13	9	0	192	1.35	36.35	139
		9	16	0	192	1.27	27.61	106

Appendix 8
(continued)

Mutation Test in the Absence of S9 Mix (24 h Exposure)
Day 3 Cloning Efficiency (Non-mutants) and Relative Total Growth (Assay 3)

Chemical	Concentration (µg/mL)	Day 3 Cloning Efficiency Plates		Wells Lost	Total Wells Scored	Cloning Efficiency (non-mutants)	Total Growth	Relative Total Growth %
		Plate 1 Empty Wells	Plate 2 Empty Wells					
Vehicle Control Mean	→	-	-	-	-	*0.94*	*52.29*	-
DMSO	(200 µL added)	24	26	0	192	0.84	49.49	95
		19	26	0	192	0.91	56.31	108
		18	21	0	192	1.00	52.29	100
		22	15	0	192	1.03	51.07	98
EMS	150	37	43	0	192	0.55	7.76	15
		52	51	0	192	0.39	3.81	7
MMS	5	41	29	0	192	0.63	15.30	29
		28	29	0	192	0.76	16.85	32
Alcohols, C18-22 Distn. Residues	25	23	18	0	192	0.96	49.58	95
		23	21	0	192	0.92	39.87	76
	50	29	22	0	192	0.83	39.31	75
		35	20	0	192	0.78	42.19	81
	100	28	18	0	192	0.89	43.46	83
		24	17	0	192	0.96	46.69	89
	200	16	23	0	192	1.00	52.78	101
		9	16	0	192	1.27	52.56	101

Appendix 8
(continued)

Day 2 Cloning Efficiency (Non-mutants) and Relative Total Growth (Assay 4)

Chemical	Concentration (µg/mL)	Day 2 Cloning Efficiency Plates		Wells Lost	Total Wells Scored	Cloning Efficiency (non-mutants)	Total Growth	Relative Total Growth %
		Plate 1 Empty Wells	Plate 2 Empty Wells					
Vehicle Control Mean	→	-	-	-	-	*1.04*	*18.42*	-
DMSO	(100 µL added)	12	18	0	192	1.16	19.08	104
		23	18	0	192	0.96	19.62	107
		20	14	0	192	1.08	19.04	103
		19	23	0	192	0.95	15.94	87
3-MC	2.5	23	22	0	192	0.91	11.10	60
		11	16	0	192	1.23	15.62	85
	10	14	26	0	192	0.98	11.76	64
		18	18	0	192	1.05	12.53	68
Alcohols, C18-22 Distn. Residues	25	18	14	0	192	1.12	17.77	96
		21	23	0	192	0.92	16.88	92
	50	13	14	0	192	1.23	19.45	106
		11	14	0	192	1.27	23.57	128
	100	27	30	0	192	0.76	15.18	82
		23	14	0	192	1.03	18.32	99
	200	17	17	0	192	1.08	16.48	89
		29	17	0	192	0.89	16.24	88

Appendix 9 Individual Mutant Selection Data

Mutation Test in the Absence of S9 Mix (4 h Exposure)
Day 2 Mutant Selection (Assay 1)

Chemical	Concentration (µg/mL)	Day 2 Mutant Colonies				Wells Lost	Ratio of Small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($x10^{-6}$)	Mutant Fraction ($x10^{-6}$)
		Plate 1		Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells						
Vehicle Control Mean	→	-	-	-	-	-	-	-	-	-	*54*
DMSO	(100 µL added)	4	3	4	5	0	1.00	192	176	43.51	48
		3	3	3	3	0	1.00	192	180	32.27	36
		4	9	6	11	0	2.00	192	162	84.95	77
		4	6	6	5	0	1.10	192	171	57.92	57
EMS	250	42	20	38	19	0	0.49	192	73	483.52	592
		37	22	45	15	0	0.45	192	73	483.52	485
MMS	10	12	45	17	36	0	2.79	192	82	425.39	745
		13	35	17	34	0	2.30	192	93	362.45	672
Alcohols, C18-22 Distn. Residues	25	3	3	3	7	0	1.67	192	176	43.51	33
		4	7	5	9	0	1.78	192	167	69.75	71
	50	1	4	1	2	0	3.00	192	184	21.28	23
		8	6	7	7	0	0.87	192	164	78.81	92

Appendix 9
(continued)

Day 2 Mutant Selection (Assay 1) Continued

Chemical	Concentration (µg/mL)	Day 2 Mutant Colonies				Wells Lost	Ratio of Small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($x10^{-6}$)	Mutant Fraction ($x10^{-6}$)
		Plate 1		Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells						
Alcohols, C18-22 Distn. Residues	100	4	3	2	5	0	1.33	192	178	37.86	34
		3	5	1	7	0	3.00	192	176	43.51	40
	200	3	5	3	4	0	1.50	192	177	40.67	41
		2	1	4	5	0	1.00	192	180	32.27	29

Appendix 9
(continued)

Mutation Test in the Presence of S9 Mix (4 h Exposure)
Day 2 Mutant Selection (Assay 2)

Chemical	Concentration (µg/mL)	Day 2 Mutant Colonies				Wells Lost	Ratio of Small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($x10^{-6}$)	Mutant Fraction ($x10^{-6}$)
		Plate 1		Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells						
Vehicle Control Mean	→	-	-	-	-	-	-	-	-	-	*47*
DMSO	(100 µL added)	2	3	5	4	0	1.00	192	178	37.86	48
		5	6	5	8	0	1.40	192	168	66.77	62
		5	7	5	3	0	1.00	192	172	55.00	44
		2	5	4	3	0	1.33	192	178	37.86	35
3-MC	2.5	25	28	22	23	0	1.09	192	94	357.10	370
		21	34	31	22	0	1.08	192	84	413.34	435
	10	29	43	29	34	0	1.33	192	57	607.22	600
		31	32	31	34	0	1.06	192	64	549.31	551
Alcohols, C18-22 Distn. Residues	25	4	9	5	6	0	1.67	192	168	66.77	64
		2	8	3	8	0	3.20	192	171	57.92	69
	50	6	6	2	8	0	1.75	192	170	60.85	61
		5	4	6	4	0	0.73	192	173	52.10	51

Appendix 9
(continued)

Day 2 Mutant Selection (Assay 2) Continued

Chemical	Concentration (µg/mL)	Day 2 Mutant Colonies				Wells Lost	Ratio of Small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($x10^{-6}$)	Mutant Fraction ($x10^{-6}$)
		Plate 1		Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells						
Alcohols, C18-22 Distn. Residues	100	5	5	6	3	0	0.73	192	173	52.10	50
		6	5	12	4	0	0.50	192	165	75.77	70
	200	2	9	7	6	0	1.67	192	168	66.77	49
		3	11	6	6	0	1.89	192	166	72.75	57

Appendix 9
(continued)

Mutation Test in the Absence of S9 Mix (24 h Exposure)
Day 3 Mutant Selection (Assay 3)

Chemical	Concentration (µg/mL)	Day 3 Mutant Colonies				Wells Lost	Ratio of Small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($x10^{-6}$)	Mutant Fraction ($x10^{-6}$)
		Plate 1		Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells						
Vehicle Control Mean	→	-	-	-	-	-	-	-	-	-	*134*
DMSO	(200 µL added)	10	13	3	18	0	2.38	192	148	130.14	155
		7	18	6	16	0	2.62	192	145	140.38	155
		2	10	5	15	0	3.57	192	160	91.16	92
		6	19	5	16	0	3.18	192	146	136.94	133
EMS	150	73	21	68	21	0	0.30	192	9	1530.14	2796
		70	23	69	25	0	0.35	192	5	1824.03	4686
MMS	5	35	51	36	52	0	1.45	192	18	1183.56	1877
		39	53	31	59	0	1.60	192	10	1477.46	1947
Alcohols, C18-22 Distn. Residues	25	5	16	2	14	0	4.29	192	155	107.04	111
		3	10	7	13	0	2.30	192	159	94.30	102
	50	2	17	7	14	0	3.44	192	152	116.81	141
		5	17	2	14	0	4.43	192	154	110.27	141

Appendix 9
(continued)

Day 3 Mutant Selection (Assay 3) Continued

Chemical	Concentration (µg/mL)	Day 3 Mutant Colonies				Wells Lost	Ratio of Small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($x10^{-6}$)	Mutant Fraction ($x10^{-6}$)
		Plate 1		Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells						
Alcohols, C18-22 Distn. Residues	100	6	15	3	21	0	4.00	192	147	133.53	150
		7	18	10	10	0	1.65	192	147	133.53	138
	200	4	9	9	16	0	1.92	192	154	110.27	111
		7	20	9	18	0	2.38	192	138	165.12	130

Appendix 9
(continued)

Mutation Test in the Presence of S9 Mix (4 h Exposure)
Day 2 Mutant Selection (Assay 4)

Chemical	Concentration (µg/mL)	Day 2 Mutant Colonies				Wells Lost	Ratio of Small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($x10^{-6}$)	Mutant Fraction ($x10^{-6}$)
		Plate 1		Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells						
Vehicle Control Mean	→	-	-	-	-	-	-	-	-	-	*114*
DMSO	(100 µL added)	10	12	8	11	0	1.28	192	151	120.11	104
		8	10	5	15	0	1.92	192	154	110.27	114
		10	13	9	12	0	1.32	192	148	130.14	120
		9	11	6	12	0	1.53	192	154	110.27	116
3-MC	2.5	40	40	31	50	0	1.27	192	31	911.75	1005
		38	43	35	53	0	1.32	192	23	1061.00	865
	10	42	44	48	39	0	0.92	192	19	1156.53	1180
		33	53	36	51	0	1.51	192	19	1156.53	1105
Alcohols, C18-22 Distn. Residues	25	15	18	10	15	0	1.32	192	134	179.83	161
		8	9	10	16	0	1.39	192	149	126.77	138
	50	7	10	7	12	0	1.57	192	156	103.82	85
		9	19	15	18	0	1.54	192	131	191.15	150

Appendix 9
(continued)

Day 2 Mutant Selection (Assay 4) Continued

Chemical	Concentration (µg/mL)	Day 2 Mutant Colonies				Wells Lost	Ratio of Small to Large Colonies	Total Wells Scored	Total Empty Wells	Cloning Efficiency (mutants) ($x10^{-6}$)	Mutant Fraction ($x10^{-6}$)
		Plate 1		Plate 2							
		Large Colony Wells	Small Colony Wells	Large Colony Wells	Small Colony Wells						
Alcohols, C18-22 Distn. Residues	100	7	16	4	12	0	2.55	192	153	113.53	150
		8	16	7	16	0	2.13	192	145	140.38	136
	200	9	22	6	12	0	2.27	192	143	147.33	136
		6	16	7	12	0	2.15	192	151	120.11	134

Appendix 10 List of Abbreviations

3-MC	3-methylcholanthrene
CCF	Cell Count Factor
CE	Cloning Efficiency
DMSO	dimethylsulphoxide
EMS	ethyl methanesulphonate
GEF	global evaluation factor
G-6-P	glucose 6-phosphate
ICH	International Committee on Harmonisation
IMF	Induced Mutant Fraction
IWGT	International Workshop on Genetic Toxicology
MMS	methyl methanesulphonate
NADP	nicotinamide adenine dinucleotide phosphate
NPS	Not plated, as surplus to requirement
NPT	Not plated, as too toxic for assessment
OECD	Organisation for Economic Co-operation and Development
P (0)	Zero term of the Poisson distribution
RPMI	Roswell Park Memorial Institute (developers of RPMI 1640 medium)
R_0P	RPMI 1640 medium, supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL), sodium bicarbonate (1.125 g/L) and pluronic acid (0.05% w/v)
$R_{10}P$	as R_0P with Heat –inactivated horse serum (10% v/v)
$R_{50}P$	as R_0P with Heat –inactivated horse serum (50% v/v)
R_5P	as R_0P with Heat –inactivated horse serum (5% v/v)
RSG	Relative Suspension Growth
RTG	Relative Total Growth
S	survival
SG	Suspension Growth
TFT	trifluorothymidine
TK	thymidine kinase
UKEMS	United Kingdom Environmental Mutagen Society