Physiological Sciences
Location Zeist
Utrechtseweg 48
P.O. Box 360
3700 AJ Zeist
The Netherlands

www.tno.nl

P +31 30 694 41 44
F +31 30 694 47 77
infofood@voeding.tno.nl

TNO Report

V6203/14 | draft |

Gene mutation test at the TK-locus of L5178Y cells with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol

Date	7 December, 2005
Authors	Ms. M.-J.S.T. Steenwinkel, BSc
Sponsor	Asahi Glass Co. Ltd. 10 Goikaigan Ichiharashi Chiba 290-8566 Japan
TNO project number	010.31438/01.44.05
TNO study code	6203/14
Sponsor's study code	-
Status	draft
Previous versions	-
Number of pages	30
Number of appendices	7

CONFIDENTIAL

Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete, true and accurate representation of the study and its results. All study activities performed by TNO Quality of Life were carried out in compliance with the current OECD Principles of Good Laboratory Practice.

The OECD Principles of Good Laboratory Practice are accepted by Regulatory Authorities throughout the European Community, USA and Japan.

TNO makes no GLP compliance claim for characterisation and verification of the test substance identity and properties; this is the responsibility of the sponsor.

Ms. M-J.S.T. Steenwinkel, BSc
(Study director) Date

Approved by:

Ms. Dr. Ir. A.F.M. Kardinaal
(Management, Business Unit Physiological Sciences) Date:

Quality Assurance Statement

Report title: Gene mutation test at the TK-locus of L5178Y cells with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol
Report number: V6203/14
Report date: 7 December 2005

The study plan and amendment were audited as follows:

Date of audit:	Date of audit:
29 August 2005	29 August 2005

This type of short-term study is carried out frequently and the Quality Assurance Unit does not audit the experimental phase of each individual study; the processes involved are audited at regular intervals according to a predetermined schedule. The audits of experimental phase listed below were carried out of this type of study during the period relevant to this particular study.

Date of audit:	Date of report
18 October 2005 (preparing dosing solutions)	18 October 2005

This report and study documentation was audited as follows:

Dates of audit:	Date of report:
5 December 2005 (draft)	6 December 2005

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all audits were reported to the study director and the management on the dates indicated.

M.C.T.J. Meeuwsen, MSc. Date
(Quality Assurance Auditor)

CONFIDENTIAL

Contents

Summary

1. The test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol (C6AL) was examined for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells, in both the absence and presence of a metabolic activation system (S9-mix). Two assays were conducted; in both assays nine single dose levels were tested in both the absence and presence of S9-mix; in the second assay smaller intervals were used. The test substance was diluted in dimethyl sulfoxide (DMSO) prior to testing.

2. The highest dose levels tested were based on cytotoxicity. In the absence and presence of S9-mix, the highest concentrations tested for mutagenicity were 0.18 and 0.14 mmol/l 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol, respectively.

3. In both the absence and presence of S9-mix 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-octanol was cytotoxic to the L5178Y cells. In the absence of S9-mix relative total growth (RTG) was decreased at and above 0.16 mmol/l. The RTG at the highest concentration tested for mutagenicity was 46%. In the presence of S9-mix, the RTG was decreased at and above 0.047 mmol/l. The RTG at the highest concentration used to evaluate mutagenicity was 12%.

4. In the absence of S9-mix no reproducible and/or dose related increase of the mutant frequency was observed. In the presence of S9-mix in the first assay a single positive response was observed at the highest concentration evaluated for mutagenicity; in the second assay a dose related increase was observed, although no positive responses were observed. The results in both assays were not consistent and not forceful.

5. In the presence of S9-mix at concentrations causing an increase of the mutant frequency, slightly more small than large colonies were observed. This observation might be indicative of a clastogenic potential.

6. Methyl methanesulphonate (MMS) and 3-methylcholanthrene (MCA) were used as positive control substances in the absence and presence of the S9-mix, respectively; DMSO served as negative control. The negative controls were within acceptable ranges and treatment with the positive controls yielded the expected significant increase in mutant frequency compared to the negative controls.

7. It is concluded that under the conditions used in this study, no definite judgement could be made concerning the mutagenicity of the test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol at the TK-locus of mouse lymphoma L5178Y cells. Although two experiments were conducted in which small concentration intervals were used, and sufficient toxicity was observed to perform a proper evaluation, the results remain **equivocal**.

1 General

1.1 Study sponsor and monitor

Sponsor: Asahi Glass Co. Ltd.
10 Goikaigan Ichiharashi
Chiba 290-8566
Japan

Monitor: Mr. Katsuji Ito

1.2 Testing facility

TNO Quality of Life
Business unit Physiological Sciences
P.O. Box 360, 3700 AJ ZEIST, the Netherlands
Telephone +31 30-6944144; Telefax +31 30-6957224
Visitors address: Utrechtseweg 48, Zeist, the Netherlands

1.3 Responsible personnel

Study director	: Ms. M-J.S.T. Steenwinkel, BSc
Deputy study director	: Ms. Dr. C.A.M. Krul
Technicians	: Ms. G.C.D.M. Bruyntjes-Rozier
	: R.N.C. van Meeuwen, BSc
Management	: Ms. Dr. Ir. A.F.M. Kardinaal

1.4 Time schedule

Start of the assay	: 19 September 2005
Last day of scoring plates	: 28 November 2005

2 Introduction

2.1 Objective

The objective of this *in vitro* assay was to examine the ability of 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol (C6AL) to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells in both the absence and the presence of a metabolic activation system (S9-mix).

The gene mutation assay with mammalian cells is widely applied to the toxicological evaluation of chemicals. The assay with mouse lymphoma (L5178Y) cells detects forward mutations at the thymidine kinase (TK) locus on chromosome 11. The TK mutation test detects base pair mutations, frame shift mutations, small and larger deletions, and rearrangements of the relevant chromosome.
Thymidine kinase is a cellular enzyme which phosphorylates pyrimidine thymidine for DNA synthesis. The use of a thymidine analogue such as trifluorothymidine (TFT) makes it possible to select cells with a mutated TK-locus. Cells with an intact TK locus will incorporate TFT into the DNA, which will disrupt DNA synthesis and cause cell death. Cells with a mutated TK-locus (mutant cells) will not incorporate TFT into the DNA, because DNA synthesis of these mutant cells can proceed by *de novo* pathways. Cells with a mutated TK-locus can either form small colonies (produced predominantly by chromosome rearrangements) or large colonies (produced predominantly by point mutations) in the presence of TFT. In parallel, the ability of cells to form colonies in non-selective medium was determined. The mutant frequency is expressed as the number of colonies formed in the presence of TFT per 10^6 colonies formed in the absence of TFT.

2.2 Guidelines

The assay was performed in compliance with:

- the OECD guideline 476, Genetic Toxicology: *In vitro* Mammalian Cell Gene Mutation Tests, adopted 21 July 1997.

The study was conducted according to the study plan entitled: "Gene mutation test at the TK-locus of L5178Y cells with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol", which was approved by the study director on 18 August 2005.

3 Deviations from the study plan

No deviation were recorded during the performance of the study.

4 Materials and methods

4.1 Test substance

Test substance	: 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol
Other name	: C6AL
Appearance	: transparent liquid
Molecular formula	: $C_8H_5OF_{13}$
Molecular weight	: 364
CAS Reg. Nr	: 647-42-7
Batch number	: re-AL-27,28
Purity	: 98.5%
Storage conditions	: ambient temperature
Expiry date	: 26 April 2006
Supplier	: Asahi Glass Co. Ltd., Japan.
TNO Dispense no.	: 050104

Analyses for the identity and purity of the test substance were not conducted as part of this study. Characterization and verification of the test substance identity and properties are the responsibility of the sponsor. A test material information sheet with information concerning physico-chemical properties and purity of the test substance was provided by the sponsor. A certificate of analysis as provide by the sponsor is included in Appendix 6.

4.2 Other chemicals

RPMI 1640 medium (with HEPES and L-Glutamine) were purchased from Bio Whitacker, Verviers, Belgium; penicillin, streptomycin, sodium pyruvate and horse serum from Gibco BRL, Paisley, Scotland; nicotinamide-adenine dinucleotide phosphate disodium salt (NADP) from Roche, Woerden, The Netherlands; methyl methanesulphonate (MMS) from Aldrich Chemical Company, Milwaukee, WI, USA; Aroclor 1254 from Monsanto Chemical Company, St. Louis, MO, USA; D-glucose-6-phosphate disodium salt (G-6-P), dimethyl sulfoxide (DMSO), trifluorothymidine (TFT) and 3-methyl-cholanthrene (MCA) from Sigma Chemical Company, St. Louis, MO, USA.

4.3 Characterization of the test system

The mouse lymphoma L5178Y cells (L5178Y tk +/- 3.7.2C line), used in the gene mutation assay, were obtained from Dr. J. Cole, MRC Cell Mutation Unit, University of Sussex, United Kingdom. The chromosome number of these cells is 40 (stable aneuploid karyotype, 2n = 40) (see Appendix 4). The cells were stored as frozen stock cultures in liquid nitrogen. Subcultures were prepared from these stocks for experimental use. Each new stock culture is checked for mycoplasma contamination, which was absent (see Appendix 4). In this study the stock from 8 October 2004 was used.

The S9 liver homogenate used in this study was part of the batch prepared on 6 July 2005. The preparation and characterization of this batch is described in detail in Appendix 3.
Immediately before use, an aliquot of the frozen S9 liver homogenate was thawed and mixed with a NADPH-generating system. The final concentrations of the various ingredients in the S9-mix were:
$MgCl_2$ 8 mMol/l; KCl 33 mMol/l; G-6-P 5 mMol/l; NADP 4 mMol/l; 40 % (v/v) RPMI 1640 medium and 20 % (v/v) S9.

4.4 Experimental procedures

The study consisted of two assays; in both assays nine single concentrations of the test substance were tested in both the absence and presence of S9-mix. In the second assay smaller intervals were used.

4.4.1 Cell culturing

The L5178Y cells were grown in culture medium consisting of RPMI 1640 medium (with HEPES and Glutamax-I) supplemented with heat-inactivated horse serum (10 % v/v for growing in flasks, and 20 % for growing in microtiter plates), sodium pyruvate and penicillin/streptomycin.

The cells were cultured in a humidified incubator at ca. 37°C in air containing ca. 5 % CO_2. Five to seven days prior to treatment, the cells were generated from a frozen stock culture by seeding them in sterile, screw-capped tissue culture flasks (about 10,000,000 cells per flask: area ± 75 cm^2) containing 50 ml culture medium (with 10 % horse serum). Fresh cultures of L5178Y cells were harvested from a number of culture flasks and suspended in culture medium (with 10 % horse serum), and the number of cells were counted. For the cytotoxicity and gene mutation tests portions of ca. 3,000,000 and 5,000,000 L5178Y cells were used per culture in the absence and presence of S9-mix, respectively.

On the day of exposure, the growth rate (doubling time of 9-14 h) and viability (>90 %; by trypan blue exclusion) of the cells were checked. The results are shown below:

Assay No.	Growth rate (Doubling time in h)	Viability (%)
1	12.6	91
2	11.9	96

4.4.2 Preparation of the test substance solution

Just before use, the test substance was diluted in DMSO at a concentration of 40 mmol/l (14.6 mg/ml) 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol in the first assay and 20 mmol/l (7.3 mg/ml) in the second assay. This resulted in clear, colourless solutions. From these stock solutions serial dilutions in DMSO were prepared and 100 µl of each of these were added to a final volume of 10 ml culture medium. The actual concentrations of the test substance in the test solutions were not determined. The concentrations quoted in this report are therefore nominal concentrations.

4.4.3 Cell treatment without metabolic activation

In the assay without metabolic activation the cells were exposed to the test substance according to the following procedure. 100 µl Test substance, negative control or positive control and 4.9 ml culture medium (without horse serum) were added to ca. 3,000,000 L5178Y cells in 5 ml culture medium (with 10 % horse serum) to a final volume of 10 ml. Two cultures treated with the vehicle (DMSO) were used as negative controls; one single culture treated with MMS was used as positive control substance at a final concentration of 0.1 mmol/l. Duplicate cultures were used for each concentration of the test substance. The cells were exposed for 24 h at ca. 37 °C and ca. 5 % CO_2 in a humidified incubator.

In the first assay, the dose levels of the test substance ranged from 0.40 to 0.0008 mmol/l (146 to 0.3 µg/ml) 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol. In the second assay the dose levels ranged from 0.20 to 0.006 mmol/l (73 to 2.2 µg/ml). At the start and end of the treatment, all cell cultures were checked visually and selected cultures were checked for viability by trypan blue exclusion.

4.4.4 Cell treatment with metabolic activation

In the assay with metabolic activation the cells were exposed to the test substance according to the following procedure. 100 µl Test substance, negative control or positive control, and 3.9 ml culture medium (without serum), and 1 ml 20 % (v/v) S9-mix (§4.3) were added to ca. 5,000,000 L5178Y cells in 5 ml culture medium (with 10 % horse serum) to a final volume of 10 ml. Two cultures treated with the vehicle (DMSO) were used as negative controls; one single culture treated with MCA was used as positive control substance at a final concentration of 10 µg/ml. Duplicate cultures were used for each concentration of the test substance. The cells were exposed for 4 h at ca. 37 °C and ca. 5 % CO_2 in a humidified incubator.

In the first assay, the dose levels of the test substance ranged from 0.40 to 0.0008 mmol/l (146 to 0.3 µg/ml) 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol. In the second assay the dose levels ranged from 0.14 to 0.0008 mmol/l (50 to 0.3 µg/ml) At the start and end of the treatment, all cell cultures were checked visually and selected cultures were checked for viability by trypan blue exclusion.

4.4.5 Assessment of cytotoxicity

The cytotoxicity of the test substance was determined by measuring the relative initial cell yield, the relative suspension growth (RSG) and the relative total growth (RTG). The relative initial cell yield is the ratio of the amount of cells after treatment to that of the vehicle control and is a measure for growth during treatment. The RSG is a measure for the cumulative growth rate of the cells 24 h and 48 h after treatment compared with untreated control cultures; the RTG is the product of the relative initial cell yield, the RSG and the relative colony-forming ability ('cloning efficiency') of the cells 48 h after treatment compared with negative control cultures, and is a measure for cytotoxicity that occurs in all phases of the assay.

After the treatment period, the cultures were checked for visibly aberrant effects (eg. flocculation/precipitation of the test substance and lysed cells), and the viability of the cells treated with the higher concentrations of test substance was checked. The medium containing the test substance, negative control or positive control was removed and the cells were washed twice with culture medium (with 10% horse serum). Finally, the cells were resuspended in culture medium (with 20% horse serum) and the number of cells was counted. The cell suspensions were diluted to 200,000 cells per ml and the cultures were incubated for about 44-48 h at ca. 37°C and ca. 5% CO_2 in a humidified incubator to allow near-optimal phenotypic expression of induced mutations (as described in §4.4.6).

After 20-24 h and 44-48 h the number of cells of all remaining cultures was counted. After 20-24 h the cell suspensions were diluted, if required, to 200,000 cells per ml and further incubated at ca. 37°C and ca. 5% CO_2 as described above. After 44-48 h a portion of the cells was diluted to 10 cells per ml for determining the cloning efficiency. The remaining cells were used for determining the frequency of TFT-resistant mutants (see §4.4.6). Portions (200 µl) of each dilution at 10 cells per ml were transferred to each well of two 96-well microtiter plates, and the plates were incubated for 10-14 days at ca. 37°C and ca. 5% CO_2 in a humidified incubator.

After this period the number of wells without growth of cells was counted and the cloning efficiency was determined using the zero term of the Poisson distribution (Cole *et al.*, 1983) as follows:

$$\textit{Cloning efficiency (CE)} = \frac{-\ln\left(\frac{\textit{number of empty wells}}{\textit{total number of wells}}\right)}{\textit{plated number of cells per well}}$$

The ratio of the cloning efficiency of cells treated with the test substance or the positive control compared to that of the vehicle control yields the relative cloning efficiency (RCE).

The suspension growth (SG) was calculated as follows:

$$\textit{Suspension growth (SG)} = \frac{\textit{cell count at 24 h}}{200{,}000^{\#}} \times \frac{\textit{cell count at 48 h}}{200{,}000^{\#}}$$

$^{\#}$ *or previous day's cell count if lower*

The ratio of the SG of treated cells to that of the vehicle control yields the relative suspension growth (RSG).

The relative total growth (RTG) is adjusted for growth during treatment to obtain a measure for cytotoxicity that occurs in all phases of the assay. The RTG is calculated as follows:

$$\textit{Relative total growth (RTG)} = \frac{\textit{cell count after treatment} \times \textit{RSG} \times \textit{RCE}}{\textit{cell count vehicle control}}$$

Reduction of the cell count after treatment, or of the RSG and of the RTG is a measure for the cytotoxicity of the test substance.

4.4.6 Gene mutation analysis

The frequency of TFT-resistant mutants and the cloning efficiency of the cells were determined 2 days after starting the test. The number of cells were counted and the cloning efficiency of the cells were determined as described in §4.4.5. To determine the frequency of TFT-resistant mutants, the cell suspensions were diluted to a density of 10,000 cells per ml in culture medium (with 20 % horse serum) containing 4 µg TFT per ml. Portions (200 µl) of each dilution were transferred to each well of two 96-wells microtiter plates, and the plates were incubated for 10-14 days at ca. 37°C and ca. 5 % CO_2 in a humidified incubator.

After this period the number of wells without growth of cells was counted and the cloning efficiency in the TFT plates (Mutant cloning efficiency) were calculated (see §4.4.5). The mutant frequency (MF) per 1,000,000 clonable cells was finally calculated as follows:

$$\textit{Mutant frequency (MF)} = \frac{\textit{Mutant Cloning efficiency (MCE)}}{\textit{Cloning efficiency (CE)}} * 1{,}000{,}000$$

The mutant colonies of the negative and positive controls and of some test substance dose levels were scored using the criteria of small and large colonies.

The following definitions were used for colony sizing:

large colony:
- covers >25% of the well area
- the edge consists of one cell layer

small colony:
- covers <25% of the well area
- the edge consists of more than one cell layer
- the diameter was 1/10 or more of the well

4.5 Analysis of results

The cloning efficiency of the cells was calculated from the total number of negative wells on the microtiter plates and the number of cells seeded per well. To assess the cytotoxic effects of the test substance or the positive controls on the cells, the initial cell yield after the treatment period, the relative suspension growth and the relative total growth to that of the vehicle negative controls were calculated. The cloning efficiency of the cells was used, together with the cloning efficiency on the TFT-containing plates, to calculate the mutant frequency. The mutant frequency was expressed as the number of TFT-resistant mutants per 1,000,000 clonable cells.

The following criteria were used to validate the data obtained in the gene mutation assay (Cole *et al.*, 1990; Aaron *et al.* 1994; Clive *et al.*, 1995):

a) the average cloning efficiency of the negative controls should not be less than 60 % or more than 140 %.
b) the average mutant frequency of the negative controls should fall within the range of 40-300 TFT-resistant mutants per 1,000,000 clonable cells.
c) the mutant frequency of the positive controls should be higher than 400 TFT-resistant mutants per 1,000,000 clonable cells, and should be at least twice that of the corresponding negative control.
d) unless the material to be tested shows no cytotoxicity at the highest possible concentration (determined by its solubility, pH and osmolar effects), the highest test substance concentration should result in a clear cytotoxic response. The RTG

value of one of the data points should be between 10 and 20%, or one data point between 1 and 10% and another between 20 and 30%.

A response was considered to be positive if the induced mutant frequency (mutant frequency of the test substance minus that of the vehicle negative control) was more than 100 mutants per 1,000,000 clonable cells (Aaron *et al*, 1994; Clive *et al.*, 1995). A response was considered to be equivocal if the induced mutant frequency was more than 50 mutants per 1,000,000 clonable cells. Any apparent increase in mutant frequency at concentrations of the test substance causing more than 90% cytotoxicity was considered to be an artefact and not indicative of genotoxicity.

The test substance was considered to be mutagenic in the gene mutation test at the TK-locus if a concentration-related increase in mutant frequency was observed, or if a reproducible positive response for at least one of the test substance concentrations was observed.

The test substance was considered not to be mutagenic in the gene mutation test at the TK-locus if it produced neither a dose-related increase in the mutant frequency nor a reproducible positive response at any of the test points.

Both numerical significance and biological relevance were considered together in the evaluation. No statistical analysis was performed.

Historical data on negative and positive controls are presented in Appendix 5.

5 Results

Two assays were conducted in both the absence and presence of S9-mix. In both assays nine single concentrations of 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol were tested. In the second assay smaller intervals between the concentrations were used. The results including the test substance and the negative and positive controls are summarised in Appendix 1, Tables 1.1 to 1.4; the raw data are shown in the Appendix 2, Tables 2.1 to 2.4.

Dose levels and visual observations before and after treatment.
In the absence of S9-mix in the first assay, the dose levels of the test substance ranged from 0.40 to 0.0008 mmol/l (146 to 0.3 µg/ml) 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-octanol. At the start and end of the treatment no abnormalities were observed; the viability of the cells after treatment at and above 0.28 mmol/l was below 10%, at 0.20 mmol/l the viability was 30-40% and at lower concentrations above 90%.
In the second assay in the absence of S9-mix, the dose levels of the test substance used ranged from 0.20 to 0.006 mmol/l (73 to 2.2 µg/ml). At the start and end of the treatment no abnormalities were observed; the viability of the cells at the highest concentration was about 10%, at the next highest concentration of 0.18 mmol/l the viability was about 50% and at lower concentrations above 80%.

In the presence of S9-mix in the first assay, the dose levels ranged from 0.40 to 0.0008 mmol/l (146 to 0.3 µg/ml) 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol. At the start and end of the treatment no abnormalities were observed; the viability of the cells after treatment at the highest concentration was between 10 and 20% and at the next highest concentration of 0.28 mmol/l between 40 and 50% and at lower concentrations above 80%. In the second assay in the presence of S9-mix, the dose levels of the test substance used ranged from 0.14 to 0.0008 mmol/l (50 to 0.3 µg/ml). At the start and end of the treatment no abnormalities were observed; the viability of the cells at the highest concentration was above 80%.

Cytotoxicity
In both the absence and presence of S9-mix, 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-octanol was cytotoxic. In the absence of S9-mix the initial cell yield and the relative total growth (RTG) were decreased at concentrations above 0.14 mmol/l in both assays. The RTG at the highest concentration tested (0.20 mmol/l) was below 0.1%, at the next highest concentration of 0.18 mmol/l the RTG was 46%.
In the presence of S9-mix the initial cell yield was decreased at and above 0.096 mmol/l; the RTG was decreased at and above 0.047 mmol/l in both assays. In the first assay the RTG at the highest concentration tested (0.14) was 9%, and at the next highest concentration of 0.096 mmol/l the RTG was 25%. In the second assay the RTG at the two highest concentrations tested (0.13 and 0.12 mmol/l) was 6%, and at the next highest concentration of 0.10 mmol/l the RTG was 12%.

Mutagenicity

In the first assay in the absence of S9-mix at two concentrations the mutant frequency (MF) was increased. At 0.047 and 0.006 mmol/l the MF was increased by 135 and 59 mutants per 1,000,000 clonable cells compared to the negative control. The negative control was relatively low compared to the historical control, 86 compared to 137 ± 41. In the presence of S9-mix the mutant frequency was increased at 0.14 and 0.096 mmol/l by, respectively, 182 and 106 mutants per 1,000,000 clonable cells. However, at 0.14 mmol/l the RTG was 9%, therefore the increased MF at this concentration was not used to evaluate mutagenicity.

Summary of the results of the first assay:

Dose	absence of S9		Dose	presence of S9	
(mmol/l)	MF	RTG	(mmol/l)	MF	RTG
0.20	nd	<0.1	0.14	321	9
0.14	84	141	0.096	245	25
0.096	134	116	0.067	96	47
0.067	118	110	0.047	93	65
0.047	221	118	0.024	74	106
0.024	114	144	0.012	131	150
0.012	106	139	0.006	76	140
0.006	145	119	0.003	96	128
0.003	106	107	0.0015	159	104
0	86*	100*	0	139*	100*

* Mean of duplicate cultures

In the second assay, in the absence of S9-mix no increase of the mutant frequency by more than 50 mutants per 1,000,000 clonable cells compared to the negative control was observed. In the presence of S9-mix a dose related increase was observed as from a concentration of 0.050 mmol/l. At 0.050, 0.070 and 0.10 mmol/l the mutant frequency was increased by, respectively, 39, 64, and 88 mutants per 1,000,000 clonable cells compared to the negative control. The RTG at these concentrations ranged from 58 to 12%. The observed increases of the MF at 0.12 and 0.13 mmmol (232 and 163 mutants per 1,000,000 clonable cells) were not used to evaluate the mutagenicity, because the RTG was below 10%.

Summary of the results of the second assay:

Dose	absence of S9		Dose	presence of S9	
(mmol/l)	MF	RTG	(mmol/l)	MF	RTG
0.20	nd	<0.1			
0.18	100	46	0.13	258	6
0.16	49	61	0.12	327	6
0.14	64	93	0.10	183	12
0.13	93	137	0.070	158	37
0.12	130	111	0.050	134	58
0.10	123	106	0.026	118	98
0.070	117	103	0.013	108	92
0.050	157	102	0.006	109	98
0.026	136	112	0.003	128	97
0	144*	100*	0	95*	100*

* Mean of duplicate cultures

Colony sizing
In the presence of S9-mix at the concentration causing an increase in mutant frequency slightly more small then large colonies were formed, although the number of both small and large colonies were increased.

Positive and negative controls
Methyl methanesulphonate (MMS) and 3-methylcholanthrene (MCA) were used as positive control substances in the absence and in the presence of the S9-mix, respectively; DMSO served as negative control. The negative controls were within acceptable ranges, and treatment with the positive controls yielded the expected significant increase in mutant frequency compared to the negative controls.

6 Discussion and Conclusion

The highest concentrations tested and evaluated for mutagenicity in both the absence and presence of S9-mix were based on cytotoxicity. In the absence of S9-mix, the increases in mutant frequency observed in the first assay were not dose related and were not observed at the same concentrations in the second assay. Although no concentrations resulting in a RTG value between 10 and 20% could be evaluated, the small intervals used, justify a valid evaluation. This means that in the absence of S9-mix, no indication for a mutagenic potential was observed.
In the presence of S9-mix in the first assay a single increase of the mutant frequency by 106 mutants per 1,000,000 clonable cells was observed at the highest dose used to evaluate the mutagenicity. In the second assay, a dose related increase was observed, however, the maximum increase of the mutant frequency at the highest concentration was 88 mutants per 1,000,000 clonable cells; which means that no positive response was observed. Even though the responses observed in both assays are indicative for mutagenicity, they were not reproducible and not forceful. At concentrations causing an increase in mutant frequency, slightly more small then large colonies were observed; this observation might be indicative for a clastogenic potential, aswell.

It is concluded that under the conditions used in this study, no definite judgement could be made concerning the mutagenicity of the test substance 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol at the TK-locus of mouse lymphoma L5178Y cells. Although two experiments were conducted in which small concentration intervals were used, and sufficient toxicity was observed to perform a proper evaluation, the results remain **equivocal.**

7 Documentation and retention of records and test substance

Raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study have been filed in the archives of the TNO Quality of Life, Zeist, The Netherlands and will be retained for at least 15 years after submission of the final report. At the end of the fifteen year storage period, these will be discarded, unless the sponsor had indicated otherwise. The remaining test substance will be retained for at least six months after submission of the final report.

CONFIDENTIAL

8 References

- Aaron C.S., G. Bolcsfoldi, H.-R. Glatt, M. Moore, Y. Nishi, L. Stankowski, J. Theiss, and E. Thompson. Mammalian cell gene mutation assays working group report. Mutation Res. 312 (1994) 235-239.
- Ames, B.N., J. McCann and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutation Res. 31 (1975) 347-365.
- Cifone, M.A., B. Myhr, A. Eiche, and G. Bolcsfoldi. Effect of pH shifts on the mutant frequebcy at the thymidine kinase locus in mouse lymphoma L5178Y TK +/- cells. Mutation Res. 189 (1987) 39-46.
- Clive, D., G. Bolcsfoldi, J. Clements, J. Cole, M. Homna, J. Majeska, M. Moore, L. Müller, B. Myhr, T. Oberly, M.-C. Oudelkhim, C. Rudd, H. Shimada, T. Sofuni, V. Thybaud and P. Wilcox. Consensus agreement regarding protocol issues discussed during the mouse lymphoma workshop: Portland, Oregon, May 7, 1994. Environ. Molec. Mutagen. 25 (1995) 165-168.
- Cole, J., C.F. Arlett, M.H.L. Green, J. Lowe and W. Muriel. A comparison of the agar cloning and microtitration techniques for assessing cell survival and mutation frequency in L5178Y mouse lymphoma cells. Mutation Res. 111 (1983) 371-386.
- Cole, J., M. Fox, R.C. Garner, D.B. McGregor and J. Thacker. Gene mutation assays in cultured mammalian cells. In: Kirkland, D.J. (ed.). Basic mutagenicity tests: UKEMS recommended procedures. Cambridge University Press. (1990) pp. 87-114.
- Kirkland, D.J. and Dean, S.W. On the need for confirmation of negative genotoxicity results *in vitro* and on the usefulness of mammalian cell mutation tests in a core battery: experiences of a contract research laboratory. Mutagenesis, 9 (1994) 491-501.
- Maron, D.M. and B.N. Ames. Revised methods for the Salmonella mutagenicity test. Mutation Res. 113 (1983) 173-215.
- Genetic Toxicology: *In Vitro* Mammalian Cell Gene Mutation Test, OECD guidelines for the testing of chemicals, no. 476. Organization for Economic Co-Operation and Development, Paris, 21 July 1997.
- OECD Principles of Good Laboratory Practice (1997), Organisation for Economic Co-operation and Development (OECD), Paris; ENV/MC/CHEM(98)17.
- Moore, M.M., *et al.* Mouse lymphoma thymidine kinase gene mutation assay: follow-up International Workshop on Genotoxicity Test Procedures, New Orleans, Louisiana, April 2000. Environ Mol Mutagen. 2002;40(4):292-9.
- Moore, M.M., *et al.* Mouse lymphoma thymidine kinase gene mutation assay: International Workshop on Genotoxicity Tests Workgroup report--Plymouth, UK 2002. Mutat Res. 2003 Oct 7;540(2):127-40.

9 Appendices

Appendix 1: Tables of results

Table 1.1 Results of the first gene mutation assay with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol (C6AL) (summarized data) Treatment in the absence of S9-mix.

Treatment	dose (mmol/l)	initial cell yield (*10⁶)	relative[1] initial cell yield (%)	suspension growth	relative[1] suspension growth (%)	cloning efficiency	relative[1] cloning efficiency (%)	relative total growth (%)	mutant cloning efficiency (*10⁶)	mutant frequency (*10⁶)	mutant colonies[2] large (%)	mutant colonies[2] small (%)
MMS	0.1	8.83	90	9.62	97	0.41	46	41	676	1663	a	a
C6AL	0.4	0.10	1	*								
C6AL	0.28	0.11	1	*								
C6AL	0.2	0.39	4	2.60	26	0.01	1	<0.1	**			
C6AL	0.14	8.16	83	17.81	180	0.82	94	141	70	84		
C6AL	0.096	9.37	95	11.97	121	0.88	101	116	119	134		
C6AL	0.067	9.77	100	10.71	108	0.90	102	110	106	118		
C6AL	0.047	10.83	110	12.41	125	0.75	85	118	165	221		
C6AL	0.024	10.74	109	12.85	130	0.89	101	144	102	114		
C6AL	0.012	9.81	100	12.40	125	0.97	111	139	103	106		
C6AL	0.006	10.60	108	10.06	102	0.95	109	119	138	145		
C6AL	0.003	9.28	94	12.64	128	0.78	89	107	83	106		
C6AL	0.0015	9.23	94	#								
C6AL	0.0008	9.71	99	#								
DMSO	0	9.26	94	10.44	106	0.83	94	94	63	76	57	43
DMSO	0	10.38	106	9.34	94	0.93	106	106	88	95	45	55

1 values are given relative to the mean of that of the vehicle negative control

2 large and small mutant colonies are given as percentage of all mutants

cultures discarded because they were superfluous

* cultures discarded because of toxicity

a large and small colonies could not be determined.

** no value due to high toxicity

Table 1.2 Results of the first gene mutation assay with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol (C6AL) (summarized data)
Treatment in the presence of S9-mix.

Treatment	dose (mmol/l)	initial cell yield (*10⁶)	relative[1] initial cell yield (%)	suspension growth	relative[1] suspension growth (%)	cloning efficiency	relative[1] cloning efficiency (%)	relative total growth (%)	mutant cloning efficiency (*10⁶)	mutant frequency (*10⁶)	mutant colonies[2] large (%)	mutant colonies[2] small (%)
MCA	10 µg/ml	5.22	97	13.84	48	0.43	67	31	683	1600	58	42
C6AL	0.4	0.38	7	*								
C6AL	0.28	1.06	20	*								
C6AL	0.2	3.15	59	1.29	4	*						
C6AL	0.14	4.25	79	3.77	13	0.56	88	9	181	321	36	64
C6AL	0.096	4.23	78	8.99	31	0.64	101	25	158	245	37	63
C6AL	0.067	4.89	91	16.74	58	0.57	89	47	55	96		
C6AL	0.047	4.59	85	21.41	74	0.66	103	65	61	93		
C6AL	0.024	5.19	96	27.27	95	0.74	116	106	54	74		
C6AL	0.012	5.19	96	28.26	98	1.02	159	150	133	131		
C6AL	0.006	5.41	101	28.86	100	0.89	139	140	67	76		
C6AL	0.003	5.06	94	29.15	101	0.86	134	128	83	96		
C6AL	0.0015	5.23	97	30.05	104	0.66	102	104	104	159		
C6AL	0.0008	5.21	97	#								
DMSO	0	5.58	104	28.60	99	0.57	89	92	91	159	53	47
DMSO	0	5.19	96	29.06	101	0.71	111	108	84	119	70	30

1 values are given relative to the mean of that of the vehicle negative control

2 large and small mutant colonies are given as percentage of all mutants

cultures discarded because they were superfluous.

* cultures discarded because of toxicity

Table 1.3 Results of the second gene mutation assay with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol (C6AL) (summarized data)
Treatment in the absence of S9-mix.

Treatment	dose (mmol/l)	initial cell yield (*10^6)	relative[1] initial cell yield (%)	suspension growth	relative[1] suspension growth (%)	cloning efficiency	relative[1] cloning efficiency (%)	relative total growth (%)	mutant cloning efficiency (*10^6)	mutant frequency (*10^6)	mutant colonies[2] large (%)	mutant colonies[2] small (%)
MMS	0.1	6.43	65	4.42	39	0.07	7	2	257	3839	30	70
C6AL	0.2	0.72	7	1.29	11	0.004	0.4	< 0.1	*			
C6AL	0.18	3.68	37	12.55	110	1.11	111	46	111	100		
C6AL	0.16	4.48	46	14.45	127	1.05	105	61	52	49		
C6AL	0.14	6.09	62	14.97	132	1.14	114	93	73	64		
C6AL	0.13	9.60	98	17.05	150	0.93	93	137	87	93		
C6AL	0.12	9.24	94	15.77	139	0.85	85	111	110	130		
C6AL	0.1	9.18	93	14.30	126	0.90	90	106	111	123		
C6AL	0.07	10.49	107	11.93	105	0.92	92	103	108	117		
C6AL	0.05	10.50	107	15.01	132	0.73	73	102	114	157		
C6AL	0.026	10.29	105	12.51	110	0.97	97	112	133	136		
C6AL	0.013	9.12	93	#								
C6AL	0.006	9.76	99	#								
DMSO	0	9.94	101	11.00	97	0.94	94	92	136	145	32	68
DMSO	0	9.74	99	11.76	103	1.06	106	108	151	142	56	44

1 values are given relative to the mean of that of the vehicle negative control
2 large and small mutant colonies are given as percentage of all mutants
cultures discarded because they were superfluous
* no value due to high toxicity

Table 1.4 Results of the second gene mutation assay with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol (C6AL) (summarized data)
Treatment in the presence of S9-mix.

Treatment	dose (mmol/l)	initial cell yield (*10⁶)	relative[1] initial cell yield (%)	suspension growth	relative[1] suspension growth (%)	cloning efficiency	relative[1] cloning efficiency (%)	relative total growth (%)	mutant cloning efficiency (*10⁶)	mutant frequency (*10⁶)	mutant colonies[2] large (%)	mutant colonies[2] small (%)
MCA	10 µg/ml	4.90	97	18.97	60	0.65	74	43	538	830	55	45
C6AL	0.14	4.34	86	2.82	9	*						
C6AL	0.13	4.27	85	3.02	10	0.60	68	6	154	258	42	58
C6AL	0.12	4.19	83	3.53	11	0.55	63	6	180	327	40	60
C6AL	0.10	4.42	88	4.91	16	0.78	89	12	143	183	40	60
C6AL	0.07	4.52	90	11.46	36	1.01	115	37	160	158	32	68
C6AL	0.05	4.81	96	19.03	60	0.89	101	58	119	134	61	39
C6AL	0.026	4.75	94	28.23	89	1.02	116	98	120	118	49	51
C6AL	0.013	5.18	103	31.46	99	0.79	90	92	85	108	45	55
C6AL	0.006	5.07	101	32.20	102	0.84	95	98	91	109	47	53
C6AL	0.003	4.85	96	33.19	105	0.84	96	97	107	128	51	49
C6AL	0.002	4.80	95	#								
C6AL	0.0008	5.16	103	#								
DMSO	0	5.24	104	31.19	99	0.87	99	101	91	105	47	53
DMSO	0	4.82	96	32.05	101	0.89	101	98	75	85	52	48

1 values are given relative to the mean of that of the vehicle negative control

2 large and small mutant colonies are given as percentage of all mutants

cultures discarded because they were superfluous

* cultures discarded because of toxicity

Appendix 2: Raw data

Table 2.1 Results of the first gene mutation assay with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol(C6AL) (individual data)
Treatment in the absence of S9-mix.

Treatment	dose (mmol/l)	initial cell yield[1] (cells per 25 µl)		cell yield[1] after 24 h. (cells per 25 µl)		cell yield[2] after 48 h. (cells per 25 µl)		cloning efficiency[2] (wells with no colonies)		mutant cloning efficiency[3] (wells with colonies)		number of mutant colonies: large colonies		number of mutant colonies: small colonies	
MMS	0.1	22047	22118	11894	11596	20213	20718	45	40	68	73[c]	a	a	a	a
C6AL	0.4	303	185	*											
C6AL	0.28	344	216	*											
C6AL	0.2	994	949	912	766	2571	2478	96	94	0[b]	0				
C6AL	0.14	20259	20527	17500	17236	25385	25831	21	16	12[e]	12[d]				
C6AL	0.096	23518	23335	13785	13248	22449	21866	18	15	16[d]	23[e]				
C6AL	0.067	24527	24340	11721	11315	22979	23512	13	19	19[e]	16[e]				
C6AL	0.047	27122	27028	12501	12200	25128	25046	22	21	26	28				
C6AL	0.024	26879	26821	11648	11687	27692	27319	17	16	15	20				
C6AL	0.012	24781	24290	12152	11895	25956	25651	14	13	19	17				
C6AL	0.006	26228	26747	10711	10088	24196	24102	17	12	25	21				
C6AL	0.003	23264	23129	11658	11241	27263	27832	19	22	16	13				
C6AL	0.0015	23123	23025	10909	10724	#									
C6AL	0.0008	24311	24221	9919	9700	#									
DMSO	0	23148	23150	10925	10722	24111	24108	20	16	12	11	5	8	7	3
DMSO	0	26219	25674	9075	9113	26707	24662	19	11	14	17	6	8	8	9

1 cell suspensions were counted twice
2 cells seeded for analysis: 2/well
3 cells seeded for analysis: 2000/well

cultures discarded because they were superfluous
* cultures discarded because of toxicity

a large and small colonies could not be determined, since the plates were fallen.
b 95 wells scored instead of 96
c 94 wells scored instead of 96
d 93 wells scored instead of 96
e 92 wells scored instead of 96

Table 2.2 Results of the first gene mutation assay with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol(C6AL) (individual data)
Treatment in the presence of S9-mix.

Treatment	dose (mmol/l)	initial cell yield[1] (cells per 25 µl)		cell yield[1] after 24 h. (cells per 25 µl)		cell yield[1] after 48 h. (cells per 25 µl)		cloning efficiency[2] (wells with no colonies)		mutant cloning efficiency[3] (wells with colonies)		number of mutant colonies: large colonies		number of mutant colonies: small colonies	
MCA	10 µg/ml	13258	12846	16997	17177	20298	20120	43	38	68	72[a]	41	42	28	31
C6AL	0.4	1080	831	844	675	*									
C6AL	0.28	2759	2544	1824	1695	*									
C6AL	0.2	8004	7749	4388	4324	6655	6229	*							
C6AL	0.14	10732	10538	6854	6698	13971	13912	69[b]	55[b]	52[b]	65[b]	18	25	36	42
C6AL	0.096	10533	10592	10648	10375	21240	21337	26	27	20	32	8	11	12	21
C6AL	0.067	12345	12128	17005	16921	24760	24345	31	30	9	11				
C6AL	0.047	11576	11362	20678	20359	26176	26037	25	27	11	11				
C6AL	0.024	13016	12925	25142	24959	27096	27535	15	28	10	10				
C6AL	0.012	13144	12821	26155	26083	27315	26870	10	15	25	20				
C6AL	0.006	13835	13231	26917	26685	26895	26806	17	16	13	11				
C6AL	0.003	12647	12669	27244	26917	26852	26678	19	16	19	10				
C6AL	0.0015	13143	13007	27240	27108	27807	27578	29	23	16	20				
C6AL	0.0008	13134	12900	26546	26133	#									
DMSO	0	14034	13850	26272	26265	27227	27083	32	29	14	18	8	9	6	9
DMSO	0	12914	13042	26773	26601	27357	27175	27	19	16	14	13	8	3	6

1 cell suspensions were counted twice
2 cells seeded for analysis: 2/well
3 cells seeded for analysis: 2000/well

cultures discarded because they were superfluous.
* cultures discarded because of toxicity

a 91 wells scored instead of 96
b 192 wells scored instead of 96

Table 2.3 Results of the second gene mutation assay with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol(C6AL) (individual data)
Treatment in the absence of S9-mix.

Treatment	dose (mmol/l)	initial cell yield[1] (cells per 25 µl)		cell yield[1] after 24 h. (cells per 25 µl)		cell yield[1] after 48 h. (cells per 25 µl)		cloning efficiency[2] (wells with no colonies)		mutant cloning efficiency[3] (wells with colonies)		number of mutant colonies: large colonies		number of mutant colonies: small colonies	
MMS	0.1	16130	16025	11652	11656	9593	9370	86	82	40	37	11	12	29	25
C6AL	0.2	1866	1734	2180	1927	2408	2249	95	95	0	0				
C6AL	0.18	9076	9326	17859	17976	16380	16879	9	12	18	20[a]				
C6AL	0.16	11158	11239	18657	18612	19375	19320	8	15	11	8				
C6AL	0.14	15316	15125	20320	19801	18830	18615	12	8	11	15				
C6AL	0.13	24404	23571	19313	19515	21870	22088	17	12	13	18				
C6AL	0.12	23148	23065	18846	19020	20779	20859	21	14	13	25				
C6AL	0.10	23090	22828	16830	16987	21419	20934	15	17	19	19				
C6AL	0.070	26269	26162	15765	15577	19177	18996	18	13	19	18				
C6AL	0.050	26486	26016	18476	18416	20496	20305	22	23	22	17				
C6AL	0.026	26053	25383	16232	16093	19277	19477	12	15	20	25				
C6AL	0.013	22989	22599	16829	16382	#									
C6AL	0.006	24547	24273	#											
DMSO	0	24750	24952	14737	14780	18666	18516	13	16	21	25	6	9	15	17
DMSO	0	24255	24436	16261	15996	18154	18271	16	7	22	28	11	17	11	11

1 cell suspensions were counted twice

2 cells seeded for analysis: 2/well

3 cells seeded for analysis: 2000/well

cultures discarded because they were superfluous

a 95 wells scored instead of 96

Table 2.4 Results of the second gene mutation assay with 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctanol(C6AL) (individual data)
Treatment in the presence of S9-mix.

Treatment	dose (mmol/l)	initial cell yield[1] (cells per 25 µl)		cell yield[1] after 24 h. (cells per 25 µl)		cell yield[1] after 48 h. (cells per 25 µl)		cloning efficiency[2] (wells with no colonies)		mutant cloning efficiency[3] (wells with colonies)		number of mutant colonies: large colonies		number of mutant colonies: small colonies	
MCA	10 µg/ml	12403	12073	18093	18028	26361	25900	22	31	66	60	36	38	36	25
C6AL	0.14	10883	10835	6649	6455	10631	10949	*							
C6AL	0.13	10788	10582	6329	6298	11835	12029	28	30	26	25	10	12	17	13
C6AL	0.12	10744	10215	6638	6615	13410	13183	32	32	33	25	13	10	20	15
C6AL	0.10	11098	10987	7619	7517	16446	15894	25	15	24	24	12	7	12	17
C6AL	0.070	11330	11285	11289	11347	25267	25218	14	11	29	24	8	9	21	15
C6AL	0.050	12042	12019	16082	16064	29677	29587	16	16	20	21	11	14	9	7
C6AL	0.026	11959	11779	22616	22620	31087	31445	11	14	21	20	10	10	11	10
C6AL	0.013	13002	12900	24551	24736	32096	32099	18	22	17	13	10	4	8	9
C6AL	0.006	12785	12560	25947	25838	31253	31163	18	18	16	16	8	7	8	9
C6AL	0.003	12178	12089	26840	26588	31121	31144	15	21	19	18	6	13	13	5
C6AL	0.002	11977	12028	26749	26647	#									
C6AL	0.0008	12921	12864	25490	25677	#									
DMSO	0	13107	13075	25944	25895	30215	30223	18	16	19	13	11	4	8	9
DMSO	0	12036	12087	26324	26403	30497	30439	19	13	14	13	9	6	6	8

1 cell suspensions were counted twice
2 cells seeded for analysis: 2/well,
3 cells seeded for analysis: 2000/well

cultures discarded because they were superfluous
* cultures discarded because of toxicity

Appendix 3: Preparation and characterization of Aroclor 1254-induced rat liver homogenate (batche 6 July 2005)

The batche of S9 dated 6 July 2005 was prepared according to Ames et al. (1975) and Maron and Ames (1983) as follows.

Methods

Male Wistar rats (n =12; obtained from Charles River Deutschland, Sulzfeld, Germany) were injected intraperitoneally with a single dose of Aroclor 1254 (nominal dose of 500 mg/kg body weight) in soy bean oil (20% w/v). The rats were provided with tap water and the Institute's stock diet ad libitum. Five days after the injection of Aroclor 1254 the rats were killed by CO_2 asphyxiation. The livers were removed aseptically and immediately put into a cold, sterile 0.15 M KCl solution. After washing in the KCl solution, the livers were weighed, cut into pieces and homogenized in 3 volumes of 0.15 M KCl solution in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged for 10 minutes at 9,000 g. The supernatant, which is called S9, was collected and divided into small aliquots in sterile polypropylene vials. The vials were quickly frozen on dry ice and subsequently stored in a freezer at <-60 °C.
The S9 was checked for sterility. The protein and cytochrome P-450 content of the S9 fraction were determined according to the methods published by Rutten et al. (1987).

Results for batch of 6 July 2005

The protein content of the batch was 21.2 g/litre.
The cytochrome P450 content of the batch was 19.4 µmol/litre.
The batch contained 0.91 µmol cytochrome P450 per gram protein.
The sterility check of the batch resulted in 0 colonies per 100 µl S9.

Conclusion

The batch of S9 of 6 July 2005 meets all of the in-house quality criteria.

References

- Ames, B.N., J. McCann and E. Yamasaki "Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian microsome mutagenicity test." Mutation Res. 31 (1975) 347-365.
- Maron, D.M. and B.N. Ames "Revised methods for the Salmonella mutagenicity test." Mutation Res. 113 (1983) 173-215.
- Rutten, A.A.J.J.L., H.E. Falke, J.F. Catsburg, R. Topp, B.J. Blaauboer, I. van Holstein, L. Doorn and F.X.R. van Leeuwen "Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver microsomes. Reinvestigation of assay conditions." Arch. Toxicol. 61 (1987) 27-33.

Appendix 4: Characteristics of the test system

1. Determination of the modal chromosome number.

The modal chromosome number of the L5178Y cells was determined by counting the number of chromosomes in 150 metaphases. The analysis was carried out on 19-23 September 1995.

Results

Five metaphases contained 39 chromosomes, 132 metaphases contained 40 chromosomes, 12 metaphases contained 41 chromosomes and 1 metaphase contained 42 chromosomes. The mean chromosome number of these L5178Y cells was 40.06.

2. Check for the absence of mycoplasma contamination in the stock from 8 October 2004.

The mycoplasma determination in the stock from 8 October 2004 of the L5178Y cells used in the present assay was carried out between 23 and 29 August 2005. The determination was carried out by BaseClear Labservices, Leiden, The Netherlands with the Mycoplasma-PCR-Detection Kit VenorgeM, Minerva Biolabs GmbH, Berlin, Germany.

Results

The L5178Y cells used in the present assay were negative for the most common mycoplasmas: *M. orale, M. hyorhinis, M. arginine, M. fermentans, M. saivarium, M. hominis, M. pneumonia, Acholeplasma laidlawii and M synoviae.*

Appendix 5: Historical Data

Negative controls

Historical negative control (vehicle) data from studies in 2000-2004.

Metabolic activation	Mutant frequency per 10^6 clonable cells mean ± standard deviation; range (number of assays)	
	medium	DMSO
without S9-mix (4 h)	97 ± 34 52-158 (20)	111 ± 35 50-177 (30)
without S9-mix (24 h)	147 ± 40 87-271 (48)	137 ± 41 59-235 (54)
with S9-mix (4 h)	111 ± 31 45-193 (46)	109 ± 27 53-182 (56)

Positive controls

Historical positive control data from studies in 2000-2004.

Metabolic activation	Compound	Mutant frequency per 10^6 clonable cells mean ± standard deviation; range (number of assays)
without S9-mix (4h)	MMS# 200 µM	952 ± 410 329-2302 (23)
without S9-mix (24h)	MMS 100 µM	1782 ± 339 704-2458 (55)
with S9-mix (4h)	MCA# 10 µg/ml	905 ± 265 441-1697 (54)

#MMS = methyl methanesulphonate
MCA = 3-methylcholanthrene

Colony sizes

Historical data on sizes of mutant colonies from studies in 2000-2004.

Treatment	% Mutant colonies mean ± standard deviation; range (number of assays)	
	large colonies	small colonies
without S9-mix (4 or 24 h)		
medium*	53 ± 9 40-71 (24)	47 ± 9 29-60 (24)
DMSO*	54 ± 7 41-73 (27)	46 ± 7 27-60 (27)
MMS# 100 µM	43 ± 6 27-59 (56)	57 ± 6 41-73 (56)
with S9-mix (4 h)		
medium*	52 ± 7 42-69 (23)	48 ± 7 31-58 (23)
DMSO*	56 ± 7 43-70 (26)	44 ± 7 31-57 (26)
MCA# 10 µg/ml	54 ± 7 36-67 (54)	46 ± 7 33-64 (54)

#MMS = methyl methanesulphonate
MCA = 3-methylcholanthrene
*mean values of duplicate cultures per assay were used.

Appendix 6: Certificate of Analysis (provided by the sponsor)

Appendix 7: GLP compliance monitoring unit statement

voedsel en waren autoriteit

ENDORSEMENT OF COMPLIANCE

WITH THE OECD PRINCIPLES OF
GOOD LABORATORY PRACTICE

Pursuant to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC the conformity with the OECD Principles of GLP was assessed on 7-11 June 2004 at

TNO Nutrition and Food Research
[illegible] 48, P.O. Box 360
3700 AJ ZEIST

it is herewith confirmed that the above-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity, mutagenicity, [illegible], residues, analytical and clinical chemistry, [illegible], and [illegible].

[illegible] Hague, 19 August 2004

Dr. [illegible]
GLP Compliance Monitoring Department

CONFIDENTIAL