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STUDY REPORT

[redacted]

In vitro mammalian cell gene mutation test at the TK-locus of L5178Y cells with [redacted]

DATE	27 September 2018
AUTHOR(S)	B. Usta D. van Berlo
SPONSOR	[redacted]
TRISKELION PROJECT NUMBER	[redacted]
TRISKELION STUDY CODE	[redacted]
SPONSOR STUDY CODE	-
GUIDELINE	OECD 490
STATUS	Final
NUMBER OF PAGES	37

Statement of GLP compliance

I, the undersigned, hereby declare that this report constitutes a complete and accurate representation of the study and its results. All study activities performed by Triskelion B.V. were carried out in compliance with the current OECD Principles of Good Laboratory Practice (GLP)[1]. The OECD principles of Good Laboratory Practice are accepted by Regulatory Authorities throughout the European Community, USA and Japan. Chemical analysis for the verification of the test substance identity and properties was not performed in this study.

Study director

D. van Berlo

27/09/18

Date

[1] The most recent endorsement of compliance of the test facility with these principles is attached to the report as Annex 1.

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Quality Assurance Statement

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 study-based and were reported to the study director and management on the dates indicated.

Phase	Start date of audit	Date of audit report
Authorised study plan	28 December 2017	28 December 2017
Authorised study plan amendment 1	3 May 2018	3 May 2018
Cell passaging	17 January 2018	18 January 2018
Draft report and study file	24 September 2018	24 September 2018
Final report	27 September 2018	27 September 2018

M.T.A. Wolters
Quality Assurance auditor

Date : 27-09-2018

Contents

Summary

The objective of this study was to provide data on the ability of [redacted] 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). A single test was conducted. In the test, five duplicate cultures were treated for 4 hours in the presence and absence of S9-mix, and 24 hours in the absence of S9-mix. The highest nominal concentration of the test substance evaluated for mutagenicity was 76% both in the absence (4 and 24 hours) and presence (4 hours) of S9-mix. As the atmosphere in the chamber consisted of 19% O_2, 5% CO_2 and the test material (supplemented with N_2 to achieve lower concentrations), 76% (v/v) was the highest achievable concentration. Additional nominal concentrations were 60%, 40%, 20% and 10% (v/v). Nominal concentrations were analytically verified and the analytical results demonstrated that the actual exposure concentrations were acceptable (within the margin of 10% mentioned in the study plan).

Methyl methanesulphonate (MMS) and 3-methylcholanthrene (MCA) were used as positive control substances in the absence and in the presence of S9-mix, respectively and clean air served as a negative control in both tests. Negative and positive controls met all the acceptance criteria and therefore the results were considered valid.

Following 4 h exposure in the absence of S9-mix, the test material was slightly cytotoxic to the cells resulting in a reduction in the relative total growth (RTG). The mean RTG at the highest concentration evaluated (76% (v/v%)) was 59%. At the lower concentrations the RTG value fluctuated between 68% and 82%. In the presence of S9-mix after 4h treatment, the test material did not show a clear cytotoxicity to the cells. The mean RTG fluctuated between 80% and 115%. In the absence of S9-mix after 24h exposure severe cytotoxicity was observed, resulting in a reduction in RTG of more than 90% at the highest two concentrations tested (RTG of 5% and 7% following exposure to 76% and 60% test substance). As a consequence, these two concentrations were considered not suitable for assessing the mutant frequency (MF) and only the three lower concentrations were evaluated instead of four concentrations.

Following 4 h exposure in the presence of S9-mix, no increase in the mutant frequency (MF) by more than 126 mutants per 1,000,000 clonable cells compared to the negative control was observed at any concentration, i.e. no positive response.
In the absence of S9-mix, after 4h treatment, a dose-dependent response was observed; the increase in mutant frequency was 17, 32, 66, 82 and 302 per 10^6 clonable cells for target concentrations of 10%, 20%, 40%, 60% and 76% when compared to the concurrent control cultures. In addition, at the highest concentration (76% (v/v%)) an increase in the mean mutant frequency (MF) by more than 126 mutants per 1,000,000 clonable cells was observed. The increase in mutant frequency was in absence of marked cytotoxicity (41%, RTG value of 59%).

After 24 h treatment, in absence of S9-mix, no increase in the mutant frequency (MF) by more than 126 mutants per 1,000,000 clonable cells was observed at any concentration compared to the negative control, i.e. no positive response.

Exposure with the test substance for 4 h met the requirements. As the test substance is a gas, the continuous treatment (24 h without S9) was conducted in parallel with the pulse treatment

for efficiency reasons. In the 24 h exposure group, the two highest concentrations induced more than 90% cytotoxicity, which is outside the acceptability range and therefore they cannot be used for evaluation of mutagenicity. As a result, this part of the test does not meet the acceptability criteria described in OECD guideline 490; evaluation of at least four test concentrations is required.

A positive result in one experimental condition (i.e. 4 h exposure without S9-mix) is sufficient to conclude that the test substance tested positive for mutagenicity in the current experiment. Thus, even when considering that the 24 h exposure did not meet the acceptability criteria described in the guideline, the test substance is clearly positive after 4 h treatment at the highest concentration (76% (v/v/%)) in the absence of S9-mix. The clear increase in mutant frequency was observed in the absence of marked cytotoxicity. In addition, a dose-dependent response was observed.

It is concluded that under the conditions used in this study, the test substance [redacted] is **mutagenic** at the TK-locus of mouse lymphoma L5178Y cells in the absence of metabolic activation (S9-mix).

1 General

1.1 Study Sponsor

Sponsor: [redacted]

Monitor: [redacted]
Phone:
E-mail:

1.2 Test facility

Triskelion B.V.	www.triskelion.nl
Postal address:	P.O. Box 844 3700 AV Zeist The Netherlands
Location:	Utrechtseweg 48 3704 HE Zeist The Netherlands
Phone:	+31 88 866 2800

1.3 Responsible Personnel

Study director:	D. van Berlo, PhD
Phone:	+31 6 1147 9927
E-mail:	damien.vanberlo@triskelion.nl
Scientific contributor:	A.A. Reus (genetic toxicology)

1.4 Time schedule

The experiments were conducted between 9 January 2018 and 3 May 2018.

2 Introduction

2.1 Objective and background

The gene mutation test with mammalian cells is widely applied for the toxicological evaluation of chemicals. The assay with mouse lymphoma (L5178Y) cells detects forward mutations at the thymidine kinase (TK) locus on chromosome 11: base pair mutations, frame shift mutations, small and larger deletions, and rearrangements of the relevant chromosome. Thymidine kinase is a cellular enzyme which phosphorylates the pyrimidine thymidine for DNA synthesis. The use of the thymidine analogue 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 proceeds 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 will be determined. The mutant frequency is expressed as the amount of colonies formed in the *presence* of TFT per 10^6 colonies that are formed in the *absence* of TFT.

2.2 Applicable guideline

The study was conducted in accordance with the following guideline:
OECD Guideline for the Testing of Chemicals, Test Guideline 490: In vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, adopted 29 July 2016.

3 Study plan and deviations

3.1 Study plan

The study was conducted according to study plan [redacted] entitled 'In vitro mammalian cell gene mutation test at the TK-locus of L5178Y cells with [redacted] ' and one amendment. The study plan was approved by the study director on 21 December 2017.

3.2 Deviations

In Section 4.1 "Characterization of the test substance" a typing error occurred the receipt date should read 29 August 2017 instead of 10 August 2017.

This deviation did not affect the validity of the study.

4 Materials and methods

4.1 Characterization of the test substance

Test material name[1]	: [redacted]
Chemical name[1]	: 2,3,3,3-tetrafluoro-2-(trifluoromethyl)propanenitrile
Identification container label[1]	: [redacted]
Molecular formula[1]	: C_4F_7N
CAS Reg No.[1]	: 42532-60-5
Molecular weight[1]	: 195.04 g/mol
Melting point[1]	: -118°C
Boiling point[1]	: -4.7°C
Solubility in water[1]	: 0.272 mg/L at 20°C
Vapor pressure[1]	: 253300 Pa at 20°C
Hygroscopy[1]	: slight
Batch number[1]	: [redacted]
Appearance	: colorless gas
Purity[1]	: >99.5%
Storage conditions[1]	: Ambient temperature (15-25°C)
Quantity	: ~450 kg
Date of receipt	: 29 August 2017 ([redacted])
Expiration date[1]	: 31 March 2019
Supplier	: Sponsor
Triskelion dispense number	: 170241

The Certificate of Analysis is included in Annex 2.

The test substance will be retained at least one month after issue of the final report and then discarded.

4.2 Culture media and other chemicals

RPMI 1640 medium (with HEPES and Glutamax-I), phosphate buffered saline (PBS), penicillin, streptomycin, sodium pyruvate and horse serum were purchased from Gibco-Life Technologies; nicotinamide-adenine dinucleotide phosphate disodium salt (NADP) from Roche, Woerden, The Netherlands; 3-Methylcholanthrene (MCA), D-glucose-6-phosphate disodium salt (G6P), dimethylsulfoxide (DMSO), Methyl methane sulfonate (MMS), trifluorothymidine (TFT) and trypan blue from Sigma-Aldrich.

4.3 Preliminary tests / measurements

The pH and osmolality were determined in cell culture medium exposed for 4 h to the test substance in modular incubator chambers. Only the top concentration of 76% (v/v%) [redacted] (in a mixture of O_2 and CO_2) was used. The pH and osmolality were determined in duplicate cultures exposed simultaneously. The pH and the osmolality values are presented in Appendix 2 (Table 2.1).

[1] Characteristics provided by the sponsor

4.4 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. The cells were stored as frozen stock cultures in liquid nitrogen. Subcultures were prepared from one of these stocks (dated 6 November 2009) for experimental use. Each new stock culture was checked for mycoplasma contamination, which was absent for the used batch. The chromosome number and mycoplasma results are presented in Annex 4.

L5178Y cells were grown in growth medium consisting of RPMI 1640 medium (with HEPES and L-Glutamine) 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.

The S9 liver homogenate was purchased from Trinova Biochem (Giessen, Germany) and was originally from Moltox Molecular Toxicology Incorporated (Boone, USA). On the day of use, aliquots of S9 liver homogenate were thawed and mixed with a NADPH generating system. The final concentrations of the various ingredients in the S9-mix will be: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose 6 phosphate, 4 mM NADP, 40% (v/v) RPMI 1640 medium and 20% S9. The S9-mix will be prepared just prior to use and kept on ice until use.

4.5 Experimental procedures

4.5.1 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% v/v for growing in microtiter plates), sodium pyruvate and penicillin/streptomycin.

Five days prior to treatment, the cells were generated from a frozen stock culture from 6 November 2009 by seeding them in sterile, screw-capped tissue culture flasks (about 10,000,000 cells per flask: area ± 75 cm²) 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 was counted. For the gene mutation tests *ca.* 6,000,000 L5178Y cells were used per culture in the absence and presence of S9-mix, respectively.

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

Experiment No.	Growth rate (Doubling time in h)	Viability* (%)
Experiment 1 (4h exposure groups)	10.0	100
Experiment 1 (24h exposure group)	12.8	97

* Mean of duplicate cultures.

4.5.2 Concentration levels of the test substance

The test was carried out with five different concentrations of the test substance and appropriate negative (clean air) and positive controls. A preliminary test to assess the toxicity of the test substance to the cells was not performed.

Since the test substance is a colorless gas , cells in culture flasks were exposed in modular incubator chambers (Billups-Rothenburg, USA) to various concentrations. The atmosphere in the chamber consisted of 19% O_2, 5% CO_2 and the test substance supplemented with N_2. The highest concentration theoretically achievable was therefore 76% (v/v). Additional concentrations of 60, 40, 20 and 10% (v/v) (all ± 10%) were used in the chambers to expose the cells. Air (19% O_2, 5% CO_2, 76% N_2) without the test substance was used as negative control.

Methyl methanesulphonate (MMS) and 3-methylcholanthrene (MCA) were used as positive control substances in the absence and presence of S9-mix, respectively. The exposure to the positive control substances was not conducted in modular incubator chambers, but by addition of 100 µl of a stock solution of the appropriate control to a final volume of 10 ml containing the cells, followed by incubation at *ca* 37 °C and *ca* 5% CO_2 in a humidified incubator.

4.5.3 Generation and monitoring of the test atmospheres

The test atmosphere was generated using Mass Flow Controllers for O_2/CO_2/N_2 and gaseous [redacted]. The MFC's were flow-calibrated prior to the experiment to determine the settings leading to the target concentrations of each compound, based on a target total flow of the mixture of 2.5 liter per minute. To ensure the stability of the test compound mixture, the liquid test substance was extracted from the cylinder, and was allowed to evaporate before entering the MFC.

A schematic diagram of the generation and exposure system is presented in Figure 1.

After the flow calibration of the MFC's a photoacoustic infrared analyzer (Lumasense INNOVA 1412i Multigas monitoring instrument) was calibrated at concentrations comprising the concentrations during the experiment. The analyzer was calibrated by sampling a known volume (respectively 5, 4, 3 and 5 ml) of test atmosphere (respectively 0, 10, 20 and 20 (v/v%)) with a gas-tight syringe and injecting the sample in a gas sample bag filled with 10 Ln of air (Ln = litre under normal conditions, i.e. at 273.15K and 1013 hPa).

The diluted concentrations were calculated to be respectively 0, 38.1, 56.9 and 94.9 ppm. The response Y (in % recorder reading) of the PIRA was related to the concentration C (in ppm) in the sample bags: $Y = 5.74^{e+2} * C + 3.06^{e+1}$, with a coefficient of determination (R^2) = 1.000. The dilution step was necessary because the sample volume (flow x time) necessary for the infrared analyzer to obtain a stable output would be too large to extract from the relatively small incubator chamber.

The above mentioned relation was used to convert the reading of the photoacoustic infrared analyzer to the test atmosphere concentration of test substance in the gas sample bag. The concentrations inside the incubator chamber were calculated using the sample volume and the volume of diluted air in the gas sample bag.

For the exposure, the MFC's were used at the settings calibrated. The resulting gas mixture was lead to the container/incubator for 10 minutes. Assuming the mixing of the gasses inside the container/incubator is ideal, flushing the container during 10 minutes would lead to an end concentration of >99% of the target concentration (T_{99} = 4.6 * V / Flow, V (volume incubator)= 5,3 L, Flow = 2.5 L/min, hence T_{99} = 9.75 min).
Directly after flushing the container/incubator, a sample of the atmosphere inside the container was taken using the gas-tight syringe (Hamilton gastight 5 ml) and injected into a gas sample bag filled with 10 Ln of air. The concentration of the diluted test atmosphere in the sample bag was measured with the photoacoustic infrared analyzer. The response of the analyzer was recorded with a chart recorder.
After exposure (4 or 24 hours later) the atmosphere inside the container was measured again using the method described to ensure that the container/incubator was not leaking. The results are presented in Appendix 1, Tables 1.1 - 1.2).

4.5.4 Study design

In both the absence and the presence of S9-mix, duplicate cultures were used for each concentration of the test substance and the negative (vehicle) control. Single cultures were used for the positive control.
To 6,000,000 cells in 5 ml growth medium (with 10% horse serum), growth medium (with 0% horse serum) was added for treatment without the S9-mix to a final volume of 10 ml. For treatment with S9-mix, 1 ml 20% (v/v) S9 mix (§ 4.4) was also added. In this study, the first (pulse treatment groups) and second test (continuous treatment group) were performed simultaneously in one experiment. The cells were exposed to the test substance or the negative (clean air) control for 4 and 24 h in the absence of S9-mix and for 4 h in the presence of S9-mix, at *ca.* 37°C and *ca.* 5% CO_2 in a humidified incubator. In the absence of S9-mix, one single culture treated with 100 µl MMS for 24 h was used as a positive control substance at a final concentration of 0.1 mmol/l. In the presence of S9-mix, one single culture treated with 100 µl MCA was used as a positive control substance at a final concentration of 10 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.

4.5.5 Assessment of cytotoxicity

The cytotoxicity of the test substance was determined by counting the cells after exposure and by measuring the relative suspension growth (RSG) and the relative total growth (RTG) of the cells. The RSG is a measure for the cumulative growth rate of the cells 24 hours and

48 hours after treatment compared with untreated control cultures and the RTG is the product of the relative initial cell yield after treatment, the RSG and the relative colony-forming ability ('cloning efficiency') of the cells 48 hours after treatment compared with untreated control cultures.

After 20-24 hours and 44-48 hours after treatment, the number of cells in all remaining cultures was counted. After 20-24 hours the cell suspensions were diluted, if required, to *ca.* 300,000 cells per ml and further incubated as described above. For determining the cloning efficiency, after *ca.* 48 hours after treatment a portion of the cells was diluted to a density of 10 cells per ml in growth medium (with 20% serum). The remaining cells were used for determining the frequency of TFT-resistant mutants (see section 4.4.4). 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 10-12 days at *ca* 37 °C and *ca* 5% CO_2 in a humidified incubator.

At the end of the 10-12 days incubation, the number of wells without growth of cells were counted and the cloning efficiency was determined using the zero term of the Poisson distribution (Cole *et al.*, 1983) as follows:

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

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:

$$\text{Suspension growth (SG)} = \frac{\text{cell count at 24 h}}{300{,}000^{\#}} \times \frac{\text{cell count at 48 h}}{300{,}000^{\#}}$$

 # or previous day's cell count if lower

The ratio of the SG of treated cells to that of the vehicle control yields the 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 was calculated as follows:

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

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.

4.5.6 Gene mutation analysis

A decision for the concentration levels to be tested for mutation induction was made after data on viability and growth rate were available. At least four cultures were selected for gene mutation analysis.

The frequency of TFT-resistant mutants was determined after *ca.* 48 hours after treatment. The cell suspensions were diluted to a density of *ca.* 10,000 cells per ml in growth 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. The plates were incubated for 10-12 days at *ca.* 37 °C and *ca.* 5% CO_2 in a humidified incubator.

For determination of the cloning efficiency in the TFT plates after 10-12 days of incubation, the number of wells with growth of cells was counted. The mutant frequency (MF) per 1,000,000 clonable cells was finally calculated as follows:

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

For the negative (solvent) and positive control cultures, the mutant colonies were scored using the criteria of small and large colonies.

The following definitions will be used for colony sizing:
large colony: covers >25% of the well area
small colony: covers <25% of the well area

4.6 Evaluation and interpretation of the results

The cloning efficiency of the cells was calculated from the total number of negative wells on two microtiter plates and the number of cells seeded per well. To assess the cytotoxic effects of the test substance or the positive control on the cells, the initial cell yield after the treatment period, the relative suspension growth and the relative total growth to that of the concomitant negative (solvent) controls was 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 (according to OECD Test Guideline 490):

a) the average cloning efficiency of the negative controls should not be less than 65% or more than 120%;
b) the average suspension growth of the negative controls should be between 8 and 32 (4 hours treatments) and between 32 and 180 (24 hours treatment, if applicable);
c) the average mutant frequency of the negative controls should fall within the range of 50-170 TFT-resistant mutants per 1,000,000 clonable cells;
d) The mutant frequency of the positive controls should meet at least one of the two following acceptance criteria:
   1) The positive control should demonstrate an absolute increase in total MF, that is, an increase above the spontaneous background MF [an induced MF (IMF)] of at least 300 X 10^{-6}. At least 40% of the IMF should be reflected in the small colony MF.
   2) The positive control has an increase in the small colony MF of at least 150 X 10^{-6} above that seen in the concurrent untreated/solvent control (a small colony IMF of 150 X 10^{-6}).

   The upper limit of cytotoxicity observed in the positive control culture should be the same as of the experimental cultures. That is, the RTG should not be less than 10%. It is sufficient to use a single concentration to demonstrate that the acceptance criteria for the positive control

have been satisfied. Further, the MF of the positive control must be within the acceptable range established for the laboratory;

e) unless the test substance 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 aimed to be between 10 and 20%.

Providing that all acceptability criteria were fulfilled, a test substance was considered to be clearly positive if, in any of the experimental conditions examined, the increase in MF above the concurrent background exceeds the Global Evaluation Factor (= 126 mutants per 1,000,000 clonable cells and the increase is concentration related. The test substance was then considered able to induce mutation in this test system.

If any apparent increase in MF was observed at concentrations of the test substance causing more than 90% cytotoxicity, it was considered to be an artefact and not biological relevant if at least one of the two following conditions is fulfilled:

- at least one test concentration with an RTG between 10% and 20% is tested with no evidence of mutagenicity
- no evidence of mutagenicity is observed at all other test concentrations with a RTG > 10%

The mutagenicity assay was regarded inconclusive if

- less than four analysable concentrations are obtained
- the highest concentration (if limited by cytotoxicity) results in insufficient cytotoxicity (see point e)
- a positive response is obtained at only one concentration other than the highest concentration and/or in absence of excessive cytotoxicity

Providing that all acceptability criteria are fulfilled, a test substance was considered to be clearly negative if there is no concentration related response in any of the experimental conditions examined or if there was an increase in MF, it did not exceed the GEF. The test substance was then considered unable to induce mutations in this test system.

If there was no culture showing an RTG value between 10-20%, while determining a test substance as not mutagenic, the following consensus should be taken in to account:

- there was no evidence of mutagenicity (*e.g.* no dose response, no mutant frequencies above those seen in the concurrent negative control or historical background ranges, etc.) in a series of data points within 100% to 20% RTG and there was at least one data point between 20 and 25% RTG.
- there was no evidence of mutagenicity (*e.g.* no dose response, no mutant frequencies above those seen in the concurrent negative control or historical background ranges, etc.) in a series of data points between 100% to 25% RTG and there was also a negative data point slightly below 10% RTG.

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 Annex 5.

5 Results and discussion

In the current study, duplicate cell cultures were treated for 4 hours and 24 hours in the absence of S9-mix, and for 4 hours in the presence of S9-mix, to five concentrations of [redacted]. The measured concentrations at the start and at the end are presented in Appendix 1 (Tables 1.1 and 1.2). All five concentrations were evaluated for mutagenicity in both the absence and presence of S9-mix.

The summarized results are presented in Appendix 3 (Tables 3.1 to 3.3) and the individual data are shown in Appendix 4 (Tables 4.1 to 4.3). The results are summarized below.

Positive and negative controls
MMS and MCA were used as positive control substances in the absence and in the presence of S9-mix, respectively; an atmosphere of 76% N_2, 19% O_2, and 5% CO_2 served as a negative control.
Results for the negative controls were within the acceptance criteria and treatment with the positive controls yielded the expected significant increase in mutant frequency compared to the negative controls for all treatment conditions tested.

Concentration levels and visual observations before and after treatment
Since [redacted] is a gas, cultures were exposed to the test material in modular incubator chambers at *ca.* 37°C. The atmosphere in the chamber consisted of 19% O_2, 5% CO_2 and the test material supplemented with nitrogen (N_2). The nominal concentrations of the test material were 76%, 60%, 40%, 20% and 10%. The actual concentrations were measured at start and end of exposure using photoacoustic infrared analysis. Results demonstrate that target concentrations were met, taking into account the margin of 10% mentioned in the study plan.
At the start and end of the 4 hour treatment in the absence and presence of S9-mix, no visual abnormalities were observed. Only after 24 hour treatment at the highest concentration of 76%, slight flocculation was observed within the cultures.
At the end of the treatment period, the viability of the cells (as determined with trypan blue dye exclusion) at 76% [redacted] was 91 and 94%, for 4 h exposure in the absence of S9-mix; 91% and 96%, for the 4 h exposure in the presence of S9-mix; and 59% and 60%, for the 24 h exposure.

Cytotoxicity
Following 4 hours exposure in the absence of S9-mix, the test material was mildly cytotoxic to the cells resulting in a reduction in the RTG. The mean RTG at the highest treatment concentration was 59%. At the lower concentrations, the RTG value fluctuated between 68% and 82%.
After 4h treatment in the presence of S9-mix, the test material did not induce cytotoxicity. The mean RTG value at the concentrations evaluated (76%, 60%, 40%, 20% and 10% (v/v%)) fluctuated between 80% and 115%.
After 24h treatment (in absence of S9-mix) severe cytotoxicity was observed, resulting in a marked reduction in RTG at the highest two concentrations. The mean RTG at the concentrations evaluated (76%, 60%, 40%, 20% and 10% [redacted]) was 5%, 7%, 14%, 48% and 76%.

Mutagenicity

Following 4 h exposure in the presence of S9-mix, no increase in the mutant frequency (MF) by more than 126 mutants per 1,000,000 clonable cells, i.e. no positive response, compared to the negative control was observed at any concentration.

After 4h treatment in the absence of S9-mix, a slight dose-dependent response was observed; the increase in mutant frequency was 17, 32, 66, 82 and 302 per 10^6 clonable cells for target concentrations of 10%, 20%, 40%, 60% and 76% when compared to the concurrent control cultures. Moreover, at the highest concentration (76% (v/v%)) an increase in the mean mutant frequency (MF) by more than 126 mutants per 1,000,000 clonable cells was observed. The increase in mutant frequency occurred in absence of marked cytotoxicity.

After 24h treatment (in absence of S9-mix), no increase in the mutant frequency (MF) by more than 126 mutants per 1,000,000 clonable cells, i.e. no positive response, compared to the negative control was observed at any concentration. However, it should be noted that this treatment was very cytotoxic to the cells resulting in a severe reduction in the RTG (RTG was 5%, 7% and 14% for target concentrations of 76%, 60% and 40%, respectively). As a result, the number of concentrations showing acceptable cytotoxicity for assessment of the mutagenic response after 24 h treatment was 3 instead of the 4 recommended concentrations.

Table 1: Summary of the results of the 4 h exposures in absence and presence of S9-mix:

Conc. (%)	absence of S9-mix (4h) MF* x10^6	ΔMF* X10^6	RTG* %	Conc. (%)	presence of S9-mix (4h) MF* x10^6	ΔMF* X10^6	RTG* %
76	370	302	59	76	74	8	115
60	151	82	82	60	89	22	108
40	134	66	82	40	82	15	100
20	101	32	68	20	67	0	86
10	85	17	76	10	49	-17	80
0	68	0	100	0	66	0	100

Table 2: Summary of the results of the 24 h exposure in the absence of S9-mix:

Conc. (%)	absence of S9-mix (24h) MF* x10^6	ΔMF* X10^6	RTG* %
76	110	41	5
60	83	13	7
40	92	22	14
20	57	-13	48
10	57	-13	76
0	70	0	100

* Mean of duplicate cultures

Values were increased by more than 126 mutants per 1,000,000 clonable cells compared to the negative control.

MF = mutant frequency; ΔMF = increase in mutant frequency; RTG = relative total growth; conc. = concentration of the test substance

Colony sizing
Colony sizing was performed as a positive response was observed in cultures treated with the highest concentration (76% (v/v%) [redacted]) for 4 hours in the absence of S9-mix. At this concentration, the relative contribution of small colonies was 70% (mean of duplicate cultures), while 30% (mean of duplicate cultures) were large colonies. The induction of mainly small colonies can indicate clastogenicity, providing some insight into the mechanism of mutagenicity.

pH and osmolality measurements
In OECD guideline 490, it is described that an effect of the test substance on pH and osmolality of the cell culture medium may cause a false-positive result in the mouse lymphoma assay. In the current mouse lymphoma assay [redacted] tested positive for mutagenicity at the highest concentration of test substance (76%) after 4 h exposure in the absence of S9-mix. To exclude the possibility that this is a false-positive effect due to the test substance affecting pH and/or osmolality of the culture medium, the pH and osmolality were determined in cell culture medium exposed for 4 h to the test substance in modular incubator chambers. Only the top concentration of 76% (v/v%) [redacted] (in a mixture of O_2 and CO_2) was used. The measured pH and osmolality values were comparable to the concurrent control (Appendix 2, Table 2.1); thus, a potential false-positive effect caused by changes in pH and/or osmolality is excluded.

Discussion
OECD guideline 490 states that at least four test concentrations that meet the acceptability criteria (appropriate cytotoxicity, number of cells, etc) should be evaluated. In case of the 4 h exposures with and without S9-mix, these conditions were met. Exposure with the test substance for 4 h was adequate and because of the observed positive effect, the performance of the longer treatment group was not required according to the guideline recommendations. However, longer treatment group (24 h without S9-mix) was conducted in parallel with the pulse treatment groups for practical reasons. In the 24 h exposure group, the two highest concentrations induced more than 90% cytotoxicity, which is beyond the acceptability range and therefore cannot be used for evaluation of mutagenicity due to the severity of the cytotoxicity. In summary, according to OECD guideline 490, it is concluded that sufficient data were obtained in the pulse and long treatment groups to justify evaluation of mutagenicity.

6 Conclusions

It is concluded that under the conditions used in this study, the test substance [redacted] is **mutagenic** at the TK-locus of mouse lymphoma L5178Y cells in the absence of metabolic activation (S9-mix).

7 Archiving

The following study specific materials will be archived for 5 years:
- Raw data (or true copies if unstable)
- Correspondence
- All other information related to the study

Master copies of the approved study plan, any amendments thereof and the final report will be retained for at least 15 years.

At the end of the archiving period the sponsor will be asked whether the study plan, final report, amendments, raw data, and correspondence should be discarded, retained for an additional period, or transferred to the archives of the sponsor.

General raw data will be retained for at least 25 years, after which they may be destroyed without further notice. These may include, but are not necessarily limited to:
- Facility-based documents
- Calibration and quality control data
- General registrations potentially used for more than one study

All materials will be retained in the archives of TNO, Utrechtseweg 48, 3704 HE Zeist, The Netherlands. The archiving period for starts on the cover date of the final report.

8 Literature

- Aaron *et al.,* Mammalian cell gene mutation assays working group report. Mutation Res. 312 (1994) 235-239.
- Ames *et al.,* Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutation Res. 31 (1975) 347-365.
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- Kirkland and Dean, 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.
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- Moore *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, 40 (2002) 292-299.
- Moore *et al.,* Mouse lymphoma thymidine kinase gene mutation assay: International Workshop on Genotoxicity Tests Workgroup report--Plymouth, UK 2002. Mutat Res. 540 (2003) 127-140.
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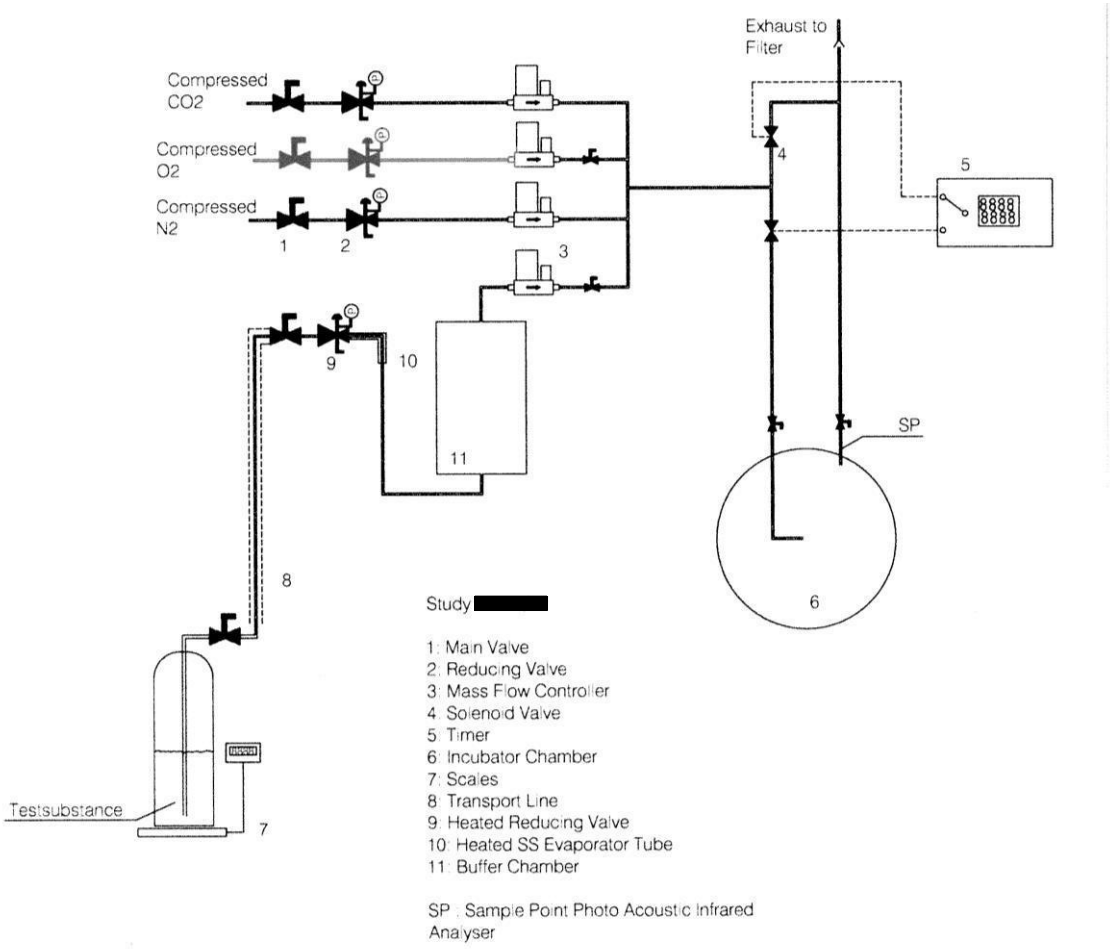
Figures

Figure 1: Schematic diagram of the generation and exposure system



Appendix 1: Test substance concentrations

Table 1.1: Calculated and measured test substance concentrations in the pulse treatment groups. January 17, 2018

Group	Calculated concentrations (vol%) derived from the settings of the mass flow controller			Measured concentrations (vol% of [redacted])	
[redacted]	$[O_2]$	$[CO_2]$	[redacted]	start exp	end exp
Target vol %	vol %	vol %	vol %	vol %	vol %
0	19,0	5,0	0,0	0,0	0,0
10	19,0	5,0	10,0	9,4	9,2
20	19,0	5,0	20,0	18,9	18,7
40	19,0	5,0	40,0	38,5	37,6
60	19,0	5,0	60,0	57,0	58,1
76	19,0	5,0	76,0	71,8	69,4

Table 1.2: Calculated and measured test substance concentrations in the continuous treatment groups. January 16 and 17, 2018

Group	Calculated concentrations (vol%) derived from the settings of the mass flow controller			Measured concentrations (vol% of [redacted])	
[redacted]	$[O_2]$	$[CO_2]$	[redacted]	start exp	end exp
vol %	vol %	vol %	vol %	vol %	vol %
0	19,0	5,0	0,0	0,0	0,0
10	19,0	5,0	10,0	9,3	9,1
20	19,0	5,0	20,0	18,6	18,4
40	19,0	5,0	40,0	37,1	37,8
60	19,0	5,0	60,0	55,8	56,3
76	19,0	5,0	76,0	71,2	63,1

Appendix 2: Osmolality and pH measurements

Table 2.1: Osmolality and pH measurements

Final concentrations (v/v%) in culture medium	pH measurements	Osmolality measurements (mOsmol/kg)
NC	7.30	290
76%	7.37	298
76%	7.39	297

NC: negative control (Clean Air)

Appendix 3 – Summarized results of the gene mutation test

Table 3.1: 4 hours treatment with [redacted] in the absence of S9-mix

Dose (v/v%)	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 (%)
76	4.55	75	15.13	105	0.85	76	60	306	360	33	67
76	4.61	76	13.58	94	0.91	81	58	344	380	28	72
60	4.78	79	16.97	118	0.94	84	78	132	140		
60	4.04	67	15.83	110	1.32	118	87	212	161		
40	5.06	84	15.29	106	1.11	100	88	138	124		
40	4.93	82	16.22	112	0.92	83	76	133	144		
20	5.16	85	13.78	95	0.92	83	67	94	102		
20	4.78	79	13.76	95	1.03	93	70	103	100		
10	5.74	95	14.25	99	0.94	84	79	66	71		
10	5.45	90	13.48	93	0.97	87	73	97	100		
NC	5.83	96	14.71	102	1.00	90	88	71	71	57	43
NC	6.27	104	14.18	98	1.23	110	112	81	66	63	38

[1] values are given relative to the mean of the negative control (clean air)

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

NC: negative control (clean air); No entry: not determined

Appendix 3 – continued

Table 3.2: 4 hours treatment with [redacted] in the presence of S9-mix (summarized data)

Dose (v/v%)	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	5.38	96	14.46	106	0.89	86	87	657	742	48	52
76	5.29	94	17.07	125	0.89	86	102	70	78		
76	5.10	91	17.31	127	1.15	111	128	81	71		
60	5.05	90	17.09	125	0.91	88	98	72	80		
60	5.18	92	17.19	126	1.05	101	117	102	98		
40	5.20	93	19.34	141	0.83	80	105	67	81		
40	4.67	83	18.24	133	0.88	85	94	73	83		
20	4.85	86	14.12	103	1.12	108	96	64	58		
20	4.34	77	13.36	98	1.05	101	76	79	76		
10	4.79	85	12.34	90	1.01	98	75	57	57		
10	4.52	80	13.20	97	1.12	108	84	47	42		
NC	5.46	97	12.91	94	1.05	101	93	61	59	59	41
NC	5.77	103	14.43	106	1.02	99	107	76	74	70	30

[1] values are given relative to the mean of the solvent control dimethyl sulfoxide (DMSO)

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

No entry: not determined

NC: negative control (clean air); MCA: 3-Methylcholanthrene (10 µg/ml)

Appendix 3 – continued

Table 3.3: 24 hours treatment with [redacted] in the absence of S9-mix (summarized data)

Dose (v/v%)	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	13.76	75	60.61	80	0.56	49	30	760	1364	41	59
76	5.95	33	16.75	22	0.82	73	5	93	113		
76	5.72	31	16.05	21	0.77	68	5	83	108		
60	5.98	33	21.41	28	0.94	83	8	70	75		
60	5.85	32	19.11	25	0.85	76	6	78	91		
40	6.56	36	29.38	39	1.15	102	14	90	79		
40	6.59	36	30.99	41	1.03	92	14	109	105		
20	13.89	76	55.37	73	0.97	86	48	50	51		
20	12.24	67	53.97	71	1.16	103	49	73	63		
10	17.87	98	69.64	92	1.12	99	89	70	63		
10	16.36	90	60.58	80	0.99	88	63	50	50		
NC	18.19	100	73.90	97	1.05	93	91	86	82	63	37
NC	18.26	100	77.71	103	1.20	107	110	69	58	48	52

[1] values are given relative to the mean of the solvent control dimethyl sulfoxide (DMSO)

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

No entry: not determined

NC: negative control (clean air); MMS: Methyl methane sulfonate (0.1 mM)

Appendix 4 – Individual results of the gene mutation test

Table 4.1: 4 hours treatment with [redacted] in the absence of S9-mix

Dose (µg/ml)	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[3]			
											large colonies		small colonies	
76	11351	11410	27256	27226	31537	30978	15	20	48	40	16	13	32	27
76	11529	11538	25181	24989	30709	30266	15	16	54	42	17	10	37	33
60	11990	11915	30080	30097	31767	31920	13	17	25	19				
60	10155	10048	28973	28438	31021	30998	6	8	30	36				
40	12587	12732	27596	27731	31202	31033	8	13	22	24				
40	12296	12363	28863	29193	31928	31025	11	19	21	24				
20	12947	12845	28630	28411	27137	27367	9	21	14	19				
20	11978	11899	28503	28548	27361	27041	14	10	20	16				
10	14409	14309	28670	28834	28126	27874	14	15	8	16				
10	13731	13542	28261	28794	26762	26281	17	10	23	11				
NC	14390	14742	28190	28028	29685	29057	14	12	12	13	7	10	5	8
NC	15624	15730	28172	28469	28369	27831	8	8	11	18	5	10	6	3

[1] cell suspensions were counted twice

[2] cells seeded for analysis: 2/well

[3] cells seeded for analysis: 2000/well

NC: negative control (clean air); No entry: not determined

Appendix 4 – continued

Table 4.2: First experiment: 4 hours treatment with [redacted] in the presence of S9-mix

Dose (µg/ml)	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[3]			
											large colonies		small colonies	
MCA	13433	13453	26183	26281	31415	30731	15	18	67	73	34	34	35	39
76	13192	13246	32788	32488	29517	29500	13	19	9	16				
76	12804	12703	32964	32734	29542	29599	12	7	15	14				
60	13199	12069	31365	31216	29522	29717	18	13	14	12				
60	13036	12841	31722	31454	30977	30540	14	9	20	16				
40	13158	12832	32206	31613	34189	34202	20	17	11	13				
40	11742	11596	29989	29614	34552	34649	16	17	5	21				
20	12054	12183	29535	29880	27272	26357	7	14	9	14				
20	10902	10816	28026	27759	27190	26534	10	14	15	13				
10	11989	11984	26050	26133	26366	26737	8	17	14	7				
10	11260	11353	28953	28154	26339	25829	11	10	11	6				
NC	13796	13524	28547	28532	25385	25481	9	15	11	11	6	7	5	4
NC	14486	14376	30151	29814	27112	26938	10	15	13	14	10	9	3	5

[1] cell suspensions were counted twice

[2] cells seeded for analysis: 2/well

[3] cells seeded for analysis: 2000/well

No entry: not determined

NC: negative control (clean air); MCA: 3-Methylcholanthrene (10 µg/ml)

Appendix 4 – continued

Table 4.3: Second experiment: 24 hours treatment with [redacted] in the absence of S9-mix

Dose (µg/ml)	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[3]			
											large colonies		small colonies	
MMS	34216	34566	28252	28144	26542	26104	30	33	78	72	33	30	47	44
76	14728	15002	18574	18607	25661	25721	39	35	40	25				
76	14195	14388	19139	18772	24977	25079	41	41	26	33				
60	15021	14887	21574	21293	28356	28209	17	13	12	13				
60	14579	14667	19718	19558	28221	28056	20	14	15	13				
40	16228	16578	24838	24498	30803	30317	8	11	19	13				
40	16264	16684	25917	25325	31016	30593	8	17	16	21				
20	34349	35081	24320	24295	27615	27582	15	13	11	7				
20	30550	30662	27077	26652	27744	27712	11	8	15	11				
10	44928	44446	22383	22799	29303	28914	8	13	14	11				
10	40852	40963	23487	23063	26751	26924	16	11	8	10				
NC	45296	45630	24875	25024	27493	27345	9	15	16	14	12	7	4	7
NC	45811	45491	25875	25722	27866	28099	6	11	8	17	4	8	4	9

[1] cell suspensions were counted twice

[2] cells seeded for analysis: 2/well

[3] cells seeded for analysis: 2000/well

No entry: not determined

NC: negative control (clean air); MMS: Methyl methane sulfonate (0.1 mM)

Annex 1 – GLP compliance monitoring unit statement

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 17-20 October 2017, 7 December 2017 and 31 January 2018 at

Triskelion BV
Utrechtseweg 48, 3704 HE Zeist
PO Box 844, 3700 AV Zeist

It is herewith confirmed that the afore-mentioned test facility is currently operating in compliance with the OECD Principles of Good Laboratory Practice in the following areas of expertise: Toxicity, mutagenicity, analytical and clinical chemistry, safety pharmacology, kinetics, metabolism and in-vitro studies.

Utrecht, 12 February 2018

Dr.R.M.A. Jaspers
Coordinating/specialist senior inspector

Health and Youth Care Inspectorate, Ministry of Health, Welfare and Sport
Stadsplateau 1, 3521 AZ Utrecht
P.O. Box 2518, 6401 DA Heerlen, The Netherlands

Annex 2 – Certificate of Analysis for the test substance

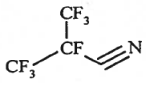
[redacted] Analytical Laboratory

An ISO9001/2015 Certified Laboratory

Certificate of Analysis

PSB-en

0 8 NOV. 2017

Nominal Product: 

Dispense nr.: 170241

Product Code: [redacted] Mfg. date 03/2017

Product Name: 2,3,3,3-tetrafluoro-2-(trifluoromethyl)propanenitrile

Physical State: Clear and colorless liquid at approximately -17 °C

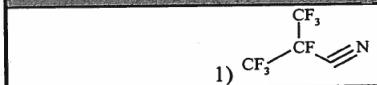
Issue Date: May 1, 2017

The sample of [redacted] was subjected to low temperature $^{1}H/^{19}F$-NMR spectral analyses to determine the purity of the nominal product and to characterize as many impurity components as possible. The qualitative and quantitative compositional results that were derived from the combined $^{1}H/^{19}F$-NMR spectral analyses are summarized below.

TABLE-1

[redacted] Sample: [redacted] mfg. date 03/2017

Compositional Results by Low Temperature $^{1}H/^{19}F$-NMR Cross Integration Spectral Analysis

Components [1]	$^{1}H/^{19}F$-NMR **Relative Wt.%** Concentrations
1) 	98.95%
2) CF_3-CFH-CF_3	0.78%
3) $CF_3CF_2CF_2$-CN	0.25%
4) Acetone	0.0079%
5) CH_3-CF_2-CN	0.0025%
6) Water	0.0021%
7) C_nH_{2n+2} saturated aliphatic hydrocarbons	0.0005%

1) Trace amounts of a couple other unassigned impurity components are also detected in the NMR spectra.

Analytical Chemist:
[redacted] (NMR)

[redacted] Analytical Laboratory [redacted]

Annex 3 – Quality Control and Production Certificate of Aroclor 1254-induced rat liver homogenate

MOLTOX®
Molecular Toxicology, Inc.

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information
SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
AGE: 5 – 6 weeks
WEIGHT: 175 – 199 g
TISSUE: Liver

Part Number Information
LOT NO.: 3853
PART NO.: 11-101
VOLUME: 1 & 5 mL
BUFFER: 0.15 M KCl
STORAGE: At or below -70°C

PREP: September 07, 2017
EXPIRY: September 07, 2019
INDUCING AGENT: Aroclor 1254, (Monsanto KL615), 500 mg/kg i.p.

REFERENCE: Maron, D & Ames, B., *Mutat Res*, **113**: 173, 1983. **For Research Purposes Only**

BIOCHEMISTRY:
- PROTEIN: 38.2 mg/ml — Assayed according to the method of Lowry et al., *JBC* **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
BROD	2B1, 2B2	84.3
EROD	1A1, 1A2	110.1
MROD	1A1, 1A2	98.9
PROD	2B1, 2B2	38.5

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 131.1, 131.6, 56, & 55 for BROD, EROD, MROD and PROD, respectively.

BIOASSAY:
- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS
Samples of S-9 were assayed for the presence of contaminating microorganisms by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Duplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
TA98	TA1535
153.6	1038

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**: 299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 – 10% in S9 mix, were tested for their ability to activate benzo(α)pyrene (BP) and 2-aminoanthracene (2-AA) to metabolites mutagenic to TA100. Assays were conducted as described by Maron & Ames, (*Mutat Res* **113**: 173, 1983).

	µl S9 per plate/number *his*⁺ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	98	178	290	366	464	840
2-AA (2.5 µg)	93	484	916	1623	1881	1581

Approved: Kathy Pelletier 09/12/17

MOLECULAR TOXICOLOGY, INC. www.moltox.com (828) 264-9099

Annex 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 6 November 2009.

The mycoplasma determination in the stock from 6 November 2009 of the L5178Y cells used was carried out on 11 December 2009. The determination was carried out by BaseClear Labservices, Leiden, The Netherlands. Samples were tested with a PCR-based test using primers specific to the highly conserved, multicopy rRNA operon, or more specifically, the 16S rRNA coding region in the mycoplasma genome.

Results

The L5178Y cells used in the present assay were mycoplasma-negative.

Annex 5 – Historical data of the gene mutation test

Historical negative (solvent) and positive control data from studies performed at the test facility: summarized data 2008 – 2016.

Historical negative controls

Treatment time	Mutant frequency per 10^6 clonable cells mean ± standard deviation; range (number of assays)								
	Culture medium			DMSO			Air		
4 h (-S9)	79 ± 15	56-102	(8)	65 ± 14	44-93	(15)	73 ± 20	45-104	(8)
4 h (+S9)	79 ± 20	43-121	(36)	75 ± 23	41-144	(54)	67 ± 15	38-89	(8)
24 h (-S9)	80 ± 24	30-134	(33)	72 ± 22	37-133	(49)	59 ± 14	35-76	(8)

Historical positive controls

Treatment time	Positive control substance	Mutant frequency per 10^6 clonable cells mean ± standard deviation; range (number of assays)		
4 h (-S9)	MMS 100 µM	556 ± 134	383-985	(21)
4 h (+S9)	MCA 10 µg/ml	818 ± 219	391-1496	(64)
24 h (-S9)	MMS 100 µM	1195 ± 320	470-1861	(62)

Historical data on sizes of mutant colonies

Treatment time	% Mutant colonies mean ± standard deviation; range (number of assays)					
	large colonies			small colonies		
4 or 24 h (-S9)						
Culture medium [1]	53 ± 13	28-91	(43)	47 ± 13	9-72	(43)
DMSO [1]	58 ± 13	32-89	(59)	42 ± 13	11-68	(59)
Air	66 ± 11	44-81	(14)	34 ± 11	19-56	(14)
MMS 100 µM	41 ± 8	20-60	(80)	59 ± 8	40-80	(80)
4 h (+S9)						
Culture medium [1]	55 ± 11	33-84	(38)	45 ± 11	16-67	(38)
DMSO [1]	54 ± 11	26-84	(50)	47 ± 11	16-74	(50)
Air	60 ± 4	52-63	(5)	41 ± 4	38-48	(5)
MCA 10 µg/ml	51 ± 9	36-96	(65)	49 ± 9	04-64	(65)

DMSO: Dimethyl sulfoxide
MMS = methyl methane sulfonate
MCA = 3-methylcholanthrene
[1] Mean values of duplicate cultures per assay