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RESEARCH FOR BETTER LIVING

STUDY REPORT

[redacted]

Chromosomal aberration test with [redacted] in cultured human lymphocytes

DATE	27 September 2018
AUTHOR(S)	B. Usta D. van Berlo
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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 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.

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
Authorised study plan amendment 2	27 September 2018	27 September 2018
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Cell exposure	24 January 2018	24 January 2018
Draft report and study file	20 September 2018	21 September 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

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Abbreviations

DMSO	Dimethylsulfoxide
FCS	Fetal Calf Serum
GLP	Good Laboratory Practice
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate Buffered Saline
PHA-L	Phytohemagglutinine
QA	Quality Assurance
QAU	Quality Assurance Unit

Summary

Experimental design: The test substance [redacted] was examined for its potential to induce structural chromosomal aberrations in cultured human lymphocytes, in both the absence and presence of a metabolic activation system (S9-mix). In the performed experiment, the highest achievable nominal concentration was 76% in both the pulse and continuous treatment groups, as the atmosphere in the chamber consisted of 19% O_2, 5% CO_2 and the test material (supplemented with N_2 for lower concentrations). Pulse and continuous treatment groups were exposed in one experiment; The treatment/recovery time of the pulse (in both the absence and presence of S9-mix) and continuous treatment group (in the absence of S9-mix only) was 4/20 and 24/2 hours, respectively. Negative (clean air) and positive controls were run in parallel. Duplicate cultures were used. The mitotic index was used as measurement for cytotoxicity.

Cytotoxicity: After 4 h exposure to the test substance (pulse treatment) in presence or absence of S9 mix, no cytotoxicity was seen (reduction in mitotic index <10%). Three test substance concentrations (76%, 60% and 40% (v/v%)) were selected both with and without S9-mix for analysis of induction of chromosomal aberrations. In the continuous treatment group, it was observed that the test substance induced severe cytotoxicity at the three highest concentrations resulting in an insufficient number of concentrations suitable for microscopic evaluation.

Chromosomal aberrations: In the performed experiment, in both pulse treatment groups, the numbers of cells with structural aberrations observed in the negative control (clean air) cultures were within 95% limit control of the historical control data of the test facility. Treatment with the positive controls Cyclophosphamide resulted in statistically significant increases in the numbers of metaphases containing one or more chromosomal aberrations, when compared to the numbers observed in the cultures treated with the negative control.

In both pulse treatment groups, the test substance did not show a statistically significant increase in the number of aberrant cells, at any of the concentrations when compared to the numbers found in the concurrent control (clean air) cultures. In addition, no dose related induction of aberrant cells were observed in treatment group with S9-mix. In the pulse treatment group without S9-mix, there was a non-significant trend towards a positive effect at the highest concentration of test substance (76% (v/v%)). In addition, at the highest two concentrations (76% and 60% (v/v%)) the number of aberrant cells found were outside the 95% limit control data of the test facility.

Acceptability criteria: The criteria described in OECD TG 473 for acceptability of the test (including positive and negative controls) were met for the 4 h exposure. In the continuous treatment group, the test substance induced cytotoxicity resulting in an insufficient number of concentrations suitable for microscopic evaluation. Therefore, the acceptability criterium (at least three test substance concentrations should be analysed per treatment group) was not fulfilled. In addition, at request of the sponsor, the performance (further investigation) of the *in vitro* chromosomal aberration test was discontinued. Therefore, the test was considered inconclusive.

Conclusion: From the results obtained in the performed *in vitro* chromosomal aberration test it is concluded that the acceptability criteria according to the OECD guideline 473 were not fulfilled. As a consequence, the outcome of the study is inconclusive.

Tabulated summary

Concentration [(v/v%)]	Chromosomal aberration[1,2] (%)	Cytotoxicity[2] (%)	Concentration [(v/v%)]	Chromosomal aberration[1,2] (%)	Cytotoxicity[2] (%)
Assay I					
+ Metabolic activation (4h)			**Without metabolic activation (4h)**		
Negative control (clean air)	1.00	0	Negative control (clean air)	0.67	0
10%	-	4	10%	-	0
20%	-	7	20%	-	0
40%	0.67	0	40%	1.00	3
60%	0.33	5	60%	1.67	3
76%	1.00	0	76%	2.00	9
Positive control	18.7****	41	Positive control	N.A.	N.A.

[1] 300 metaphases analyzed, [2]average of duplicate cultures, [N.A] not applicable, - not selected.

Fisher's exact probability test (one-sided); **** $p<0.001$

1 General

1.1 Study Sponsor

Sponsor:	3M Company 3M Center St. Paul, MN 55144-1000 USA
Monitor: Phone: E-mail:	[redacted]

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
Study director:	D. van Berlo, PhD
Phone:	+31 611 479 927
E-mail:	damien.vanberlo@triskelion.nl
Scientific contributor	A.A. Reus (genetic toxicology)

1.3 Time schedule

Start of experimental phase	19 January 2018
End of experimental phase	3 May 2018

2 Introduction

2.1 Objective and background

The purpose of this study was to provide data on the ability of the test substance [redacted] to induce structural chromosomal aberrations in cultured human lymphocytes after *in vitro* treatment in both the absence and presence of a metabolic activation system (S9-mix). At predetermined intervals after treatment, the cells were arrested in the metaphase stage of their cell cycle by the addition of a metaphase-arresting agent (Colcemid), harvested, fixed and dropped on to microscopic slides. After staining, the slides were analyzed microscopically for the presence of aberrant metaphases (chromosomal aberrations). In this study, the pulse treatment (4 hours exposure) both with and without S9-mix and the continuous treatment (24 hours exposure) were performed simultaneously in the one experiment.

2.2 Applicable guidelines

The study plan has been drafted in accordance with the following guideline:
OECD guideline 473 Genetic Toxicology: *In vitro* mammalian chromosome aberration test; adopted 29 July 2016.

3 Study plan and deviations

3.1 Study plan

The study was conducted according to study plan [redacted] entitled: "Chromosomal aberration test with [redacted] in cultured human lymphocytes" and one amendment. The study plan was approved by the study director on 21 December 2017.

3.2 Deviations

- Section 4.6 "Slide preparation", sentence "Three slides will be prepared from each selected culture of the test substance and from the cultures of the negative and positive controls": erroneously, two slides were prepared from each selected culture of the test substance and from the cultures of the negative and positive controls. Still, the required number of cells as stated by OECD guideline 473 were evaluated.
- 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.

These deviations 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]	: 31-03-2019
Supplier	: Sponsor
Triskelion dispense number	: 170241

Characterization of the positive control substances
Indirect acting clastogenic positive control:

Name	: Cyclophosphamide
Batch number	: 5H019
Appearance	: white plaque
Storage conditions	: ambient temperature (15-25°C)
Date received	: 22 June 2016
Expiry date	: 01 August 2018
Supplier	: Baxter B.V.
Triskelion dispense no.	: 160122

Direct acting positive control (clastogen):

Name	: Mitomycin C
Batch number	: SLBN5747V
Appearance	: gray / blue powder
Storage conditions	: 2-10 ºC
Date received	: 21 February 2017
Expiry date	: 1 October 2019
Supplier	: Sigma-Aldrich
Triskelion dispense no.	: 170057

[1] Characteristics provided by the sponsor

4.2 Tissue culture media and other chemicals

Fetal calf serum; RPMI 1640 medium (with HEPES and Glutamax) and penicillin-streptomycin were purchased from Life Technologies, Paisley, U.K.; nicotinamide-adenine dinucleotide phosphate disodium salt (NADP) from Roche Diagnostics, Almere, The Netherlands; Giemsa stain and glacial acetic acid from Merck-Darmstadt, Darmstadt, Germany; methanol from Biosolve, B.V., Valkenswaard, the Netherlands; dimethylsulfoxide (DMSO), D-glucose-6-phosphate disodium salt (G-6-P), Demecolcine (Colcemid) and Mitomycin C from Sigma-Aldrich Chemie GmbH, Germany; phytohemagglutinin (PHA-L) from BioChrom AG, Germany; Cyclophosphamide from Baxter B.V., Utrecht, the Netherlands.

4.3 Characterization of the test system

A blood sample was obtained by venapuncture from a young healthy, non-smoking individual (37 years old) with no known recent exposures to genotoxic chemicals or radiation. The blood was collected in sterile, heparinized vacutainer tubes and gently mixed before use to prevent clotting. The cultures were set up within 1 hour after withdrawal of the blood.

The medium for culturing the human peripheral blood lymphocytes consisted of RPMI 1640 medium (with HEPES and Glutamax), supplemented with heat-inactivated (30 min, 56ºC) fetal calf serum (20%), penicillin (100 U/ml medium), streptomycin (100 µg/ml medium) and phytohemagglutinin (2.4 µg/ml).

4.4 Metabolic activation system

The S9-mix consisted of a liver homogenate fraction (S9) and cofactors as described by Ames et al. (1975) and Maron and Ames (1983). The S9 liver homogenate used in this study was purchased from Trinova Biochem (Giessen, Germany) and was originally from Moltox Molecular Toxicology Incorporated (Boone, USA). Annex 2 presents the quality of the used S9 batch. Immediately before use, S9-mix was prepared by mixing the thawed S9 with a NADPH-generating system. The final concentrations of the various ingredients in the S9-mix were: magnesium chloride 8 mM; potassium chloride 33 mM; G-6-P 5 mM; NADP 4 mM; sodium phosphate 100 mM (pH 7.4) and S9 40% (v/v). The final concentration of the S9 in the culture medium was 4%.

4.5 Preliminary tests / measurements

The pH and osmolality were determined in RPMI 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. Results are presented in Appendix 2 (Table 2.1).

4.6 Dose levels in the experiments

The test was carried out with five different concentrations of the test substance and appropriate negative (air without test substance) and positive controls.

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 achievable was therefore 76% (v/v%).

In the performed experiment, in the pulse and continuous treatment groups, the following test substance concentrations were used: 76%, 60%, 40%, 20% and 10% (v/v%, all ± 10%). Air consisting of 19% O_2, 5% CO_2, 76% N_2 without the test substance was used as negative control.

In this experiment, Cyclophosphamide (CP) and Mitomycin C (MMC) were used as positive control substances for the pulse treatment group with S9-mix and for the continuous treatment group without S9-mix, respectively. A single positive control response (CP) was considered to demonstrate both the activity of the S9-mix and the sensitivity of the test system in both pulse treatment groups. The exposure to the positive control substances was not conducted in modular incubator chambers, but by addition of 50 µl of a stock solution of the appropriate control to a final volume of 5 ml containing cells, followed by incubation at *ca.* 37°C and *ca.* 5% CO_2 in a humidified incubator.

4.6.1 Generation and monitoring of the test atmospheres

The test atmosphere was generated using Mass Flow Controllers (MFC) 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 (PIRA; 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).

4.6.2 Study design

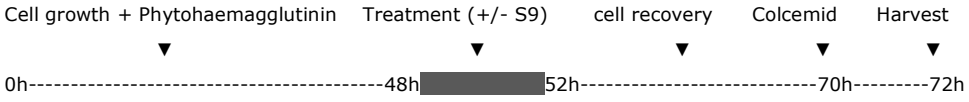
Whole blood was incubated in the presence of phytohaemagglutinin for 48 ± 2 hours at *ca.* 37ºC in humidified air containing *ca.* 5% CO_2. After this incubation period, the cells (which were then in the exponential stage of their growth) were exposed to five test substance concentrations, the negative control (clean air) or the positive control test substance.

In the performed experiment, the pulse treatment groups both with and without S9-mix and the continuous treatment group were exposed simultaneously. The cells were exposed in duplicate cultures 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 by using exposure chambers. Positive controls were run simultaneously with the test substance without the use of exposure chambers.

Accordingly to the OECD guideline 473, at least three test substance concentrations that meet the acceptability criteria (appropriate cytotoxicity, number of cells, etc.) should be evaluated in all three treatment groups. If, the acceptability criteria is not met, the outcome of the study will be inconclusive.
At the start and end of the treatment, all cell cultures were checked visually and the observed aberrant findings (when occurred) were recorded.

4.6.3 Pulse treatment groups with and without S9-mix

The schematic study design for the pulse treatment groups was as follows:



After *ca.* 48 hours incubation of the blood cultures at *ca.* 37 °C in humidified air containing *ca.* 5% CO_2, the cells were harvested by low speed centrifugation, suspended in freshly prepared tissue culture medium without foetal calf serum. The cells were transferred to culture flasks. To all cultures of the pulse treatment group in the presence of the S9-mix, 0.5 ml of S9-mix was added. In both pulse treatment groups, the total volume in each culture was 5 ml. Hereafter, the cells were treated with the test substance concentrations or with clean air (air consisting of 19% O_2, 5% CO_2, 76% N_2) during 4 hours at *ca.* 37 ºC in humidified air containing *ca.* 5% CO_2 using exposure chambers The positive control cultures in the pulse treatment group with S9-mix were exposed to 50 µL of 2.5 mg/ml Cyclophosphamide solution, at *ca.* 37 °C in humidified air containing *ca.* 5% CO_2 without using an exposure chamber, yielding a final concentration of 25 µg/ml in the cultures.

After the 4 h treatment period, the cultures were checked for visible aberrant effects (*e.g.* haemolysis of the erythrocytes. The cells were washed with PBS and subsequently supplied with 5 ml freshly prepared culture medium enriched with FCS (20%) and phytohaemagglutinin. The cells were incubated for an additional 18 h at 37ºC in humidified air containing 5% CO_2, subsequently colcemid was added to the cultures and further incubated for 2 hours at 37ºC in humidified air containing 5% CO_2. Hereafter, the cells were harvested *ca.* 72 h after initiation of the cultures (second cell-cycle), followed by the preparation of slides.

4.6.4 Continuous treatment group without S9-mix

The schematic study design for the continuous treatment group was as follows:

After 48 ± 2 hours incubation of the blood cultures at *ca.* 37°C in humidified air containing *ca.* 5% CO_2, the cells were harvested by low speed centrifugation, suspended in freshly prepared tissue culture medium enriched with 20% foetal calf serum and transferred to culture flasks. Subsequently, the cells were exposed to the test substance concentrations or clean air (air consisting of 19% O_2, 5% CO_2, 76% N_2) during 24 hours at *ca.* 37°C in humidified air containing *ca.* 5% CO_2 using exposure chambers. The positive control cultures were exposed to 50 µL of 10 µg/ml Mitomycin C solution, at *ca.* 37°C in humidified air containing *ca.* 5% CO_2 without using an exposure chamber, yielding a final concentration of 0.1 µg/ml in the cultures. After treatment period of 24 hours, colcemid was added to the cells to arrest them in the metaphase. The cultures were incubated at *ca.* 37°C in humidified air containing *ca.* 5% CO_2 for 2 hours and were harvested *ca.* 74 hours after initiation of the cultures, followed by the preparation of slides.

4.7 Cell fixation, slide preparation and analysis

At the end of the total incubation period, the cells were harvested by low speed centrifugation, treated for 15 min at 37 ºC with a hypotonic solution (0.075 M KCl), fixed three times with a freshly prepared 3:1 (v/v) mixture of methanol and glacial acetic acid and processed for chromosome preparations. Two slides were prepared from each selected culture. The slides were stained in a 2% solution of Giemsa, rinsed in water, air-dried and mounted with a coverslip. The slides were coded by a qualified person not involved in scoring the slides, to enable "blind" scoring.

A number of 1000 stimulated lymphocytes (500 cells per slide and two slides per culture) were examined in each culture to determine the percentage of cells in mitosis (mitotic index). Based on the results of the mitotic index scoring and the observations made with respect to the number and quality of the metaphases, at least three concentrations of the test substance together with the negative control (clean air) and positive controls were selected for chromosomal aberration analysis.

For each treatment group, 300 well-spread metaphases per concentration (150 metaphases per culture and 75 metaphases per slide), each containing 46 centromeres, were analyzed by microscopic examination for chromatid-type aberrations (gaps, breaks, fragments, interchanges),

chromosome-type aberrations (gaps, breaks, minutes, rings, dicentrics), according to the criteria recommended by Savage (1975). If heavily damaged cells or cells with numerical aberrations (such as endoreduplicated cells or polyploid cells) were observed, these cells were recorded but not counted and included in the 300 analyzed cells. The Vernier readings of all aberrant metaphases were recorded. See also Annex 3 of this report for the definition of chromosomal aberrations.

4.8 Evaluation and interpretation of the results

The study was considered valid if the positive controls demonstrated a statistically significantly increase in the number of aberrant cells (compatible to the historical data of the test facility) and the solvent controls were within the historical range.

The number of metaphases containing one or more aberrations of the test substance treated groups were compared with those of the concurrent solvent controls using Fisher's exact test (one-sided). The difference was considered statistically significant when the p-value of the Fisher's exact test was less than 0.05.

The response was considered positive if all of the following criteria are met:
- at least one of the test concentrations exhibits a statistically significant increase compared to the concurrent negative control.
- the increase is dose-related in at least in one experimental condition when evaluated with an appropriate trend test
- any of the results are outside the distribution of the historical solvent control data.

A response was considered negative if all of the following criteria are met:
- none of the test concentrations exhibits a statistically significant increase compared to the concurrent negative control.
- there is no dose-related increase when evaluated with an appropriate trend test
- all results are inside the distribution of the historical negative control data.

A test substance was considered equivocal if the response was neither positive or negative even after further investigation.

Statistical methods were used as an aid in evaluating the test results. Both biological relevance and statistical analysis were considered in evaluation of the response. Biological relevance was evaluated by comparison of the test results with the test facility's historical range of the solvent control.

5 Results and discussion

The results of the calculated and measured test substance concentrations in the pulse and continuous treatment are presented in Appendix 1 (Tables 1.1 and 1.2). No visual observations were noted during the first and second chromosomal aberration test. The effect of the test substance treatment on the pH and osmolality of the culture medium is presented in Appendix 2 (Table 2). The results of the chromosomal aberration test (both pulse treatment groups) are summarized in Appendix 3 (Tables 3.1 - 3.2 (mitotic index scoring) and in Tables 3.3 - 3.4 (analysis of chromosomal aberrations of the selected cultures). Annex 4 presents the historical data of chromosomal aberration tests in cultured human lymphocytes performed at the test facility.

5.1 Cytotoxicity (mitotic index analysis)

In the pulse treatment groups both with and without S9-mix, the cultures of the test substance concentrations (76%, 60%, 40%, 20% and 10% (v/v%)) were selected for analysis of the mitotic index together with the cultures of the concurrent negative control (clean air) and positive control (Cyclophosphamide). In the pulse treatment group with S9-mix, the mitotic indices fluctuated between 93% and 113% (7 - 0% cytotoxicity) when compared to the mitotic index of the concurrent negative control. The mitotic index of the positive control substance (Cyclophosphamide) decreased to 59% (41% cytotoxicity) when compared to the concurrent negative control cultures (Appendix 3, Table 3.1).
In the pulse treatment group without S9-mix, the mitotic indices of the three highest test substance concentrations (76%, 60% and 40% (v/v%)) fluctuated between 91% and 97% (9 - 3% cytotoxicity) when compared to the mitotic index of the concurrent negative control. At the lower concentrations (20% and 10% (v/v%)), the mitotic indices were not reduced (Appendix 3, Table 3.2).

In the continuous treatment group without metabolic activation, at the three highest test substance concentrations (76%, 60% and 40% (v/v%)) excessive cytotoxicity was observed, as demonstrated by the absence of cells on the slides. Hence, no cells were available for microscopic analysis. At the next lower concentration (20% (v/v%)), the total amount of mitotic cells was comparable to the concurrent negative control cultures, thus inducing no cytotoxicity. At the lowest concentration (10% (v/v%)), a slight reduction of the mitotic index was observed, when compared to the negative control cultures. It has to be noticed, that a variation was detected in the replicates.

5.2 Chromosomal aberrations

In the pulse treatment groups both with and without S9-mix, three test substance concentrations (76%, 60% and 40% (v/v%)), together with the negative control (clean air) and positive control (Cyclophosphamide) were selected for chromosomal aberration analysis.
In the group with S9-mix, the test substance did not show a statistically significant, concentration-dependent increase in the number of aberrant cells at any of the concentrations analysed compared to concurrent negative control. In addition, the number of aberrant cells found in the cultures treated with the test substance was within 95% limit control data of the test facility (Appendix 3, Table 3.3).

In the pulse treatment group without S9-mix, the test substance did not show a statistically significant increase in the number of aberrant cells at any of the concentrations analysed compared to negative control cultures. However, at the two highest concentrations (76% and 60% (v/v%)) the number of aberrant cells found were outside the 95% limit control data of the test facility (Appendix 3, Table 3.4). In addition, there was a non-significant trend towards a positive effect at the highest concentration of test substance (76 (v/v%)). As a result, the obtained response in the pulse treatment group without S9-mix could not be considered as clearly negative. The OECD guideline 473 recommends expert judgement and/or further testing if a result is neither clearly positive nor clearly negative. However, at the request of the sponsor, the *in vitro* chromosomal aberration test was discontinued.

5.3 Validity of the study

In the performed experiment, in the pulse treatment groups both with and without metabolic activation system, the negative control (clean air) was within the 95% control limit of the historical data and the positive control substance, Cyclophosphamide (in the presence of a metabolic activation system) induced the expected statistically significant increase in the incidence of structural chromosomal aberrations (Appendix 3, Tables 3.3 – 3.4).
In the continuous treatment group, during the preparation of the slides, it was observed that at the three highest test substance concentrations no cells were available for analysis. In this case, the test substance induced excessive cytotoxicity resulting in an insufficient number of concentrations suitable for microscopic evaluation. Therefore, the acceptability criterion described in OECD test guideline 473 (at least three test substance concentrations should be analysed per treatment group) was not met. As a consequence, the continuous treatment group should normally be repeated with adapted test substance concentrations, when following the guideline recommendations. However, at request of the sponsor, the conduct (further investigation) of the in vitro chromosomal aberration test was discontinued. This decision was based on the availability of additional information concerning the mutagenicity of the test substance from the *in vitro* mammalian cell gene mutation test at the TK-locus of L5178Y cells with [redacted] (Triskelion study P25103/01); the test substance was considered in this assay.
Because not all acceptability criteria as stated in the OECD guideline 473 were met, the outcome of the study is inconclusive.

5.4 Potential effect of the test substance on culture medium pH and osmolality

The OECD guideline 473 states that an effect of the test substance on pH and osmolality of the cell culture medium can cause a false-positive result (i.e. the test substance incorrectly appears clastogenic in the *in vitro* chromosomal aberration test). In the performed test, there was a non-significant trend towards a clastogenic effect at the highest concentration of test substance (76%) after 4 h exposure. To exclude any effect of the pH and the osmolality, the pH and osmolality were determined in RPMI 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).

6 Conclusion

From the results obtained in the performed *in vitro* chromosomal aberration test it is concluded that not all acceptability criteria described in OECD guideline 473 were fulfilled. As a consequence, the outcome of the study is inconclusive.

7 Documentation and retention of records, samples and specimens

The following study specific materials will be archived for 5 years:
- Raw data (or true copies if unstable)
- Correspondence
- Microscopic slides

The following study specific materials will be archived for 15 years:
- Original study plan and final report, and any amendments thereof

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

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

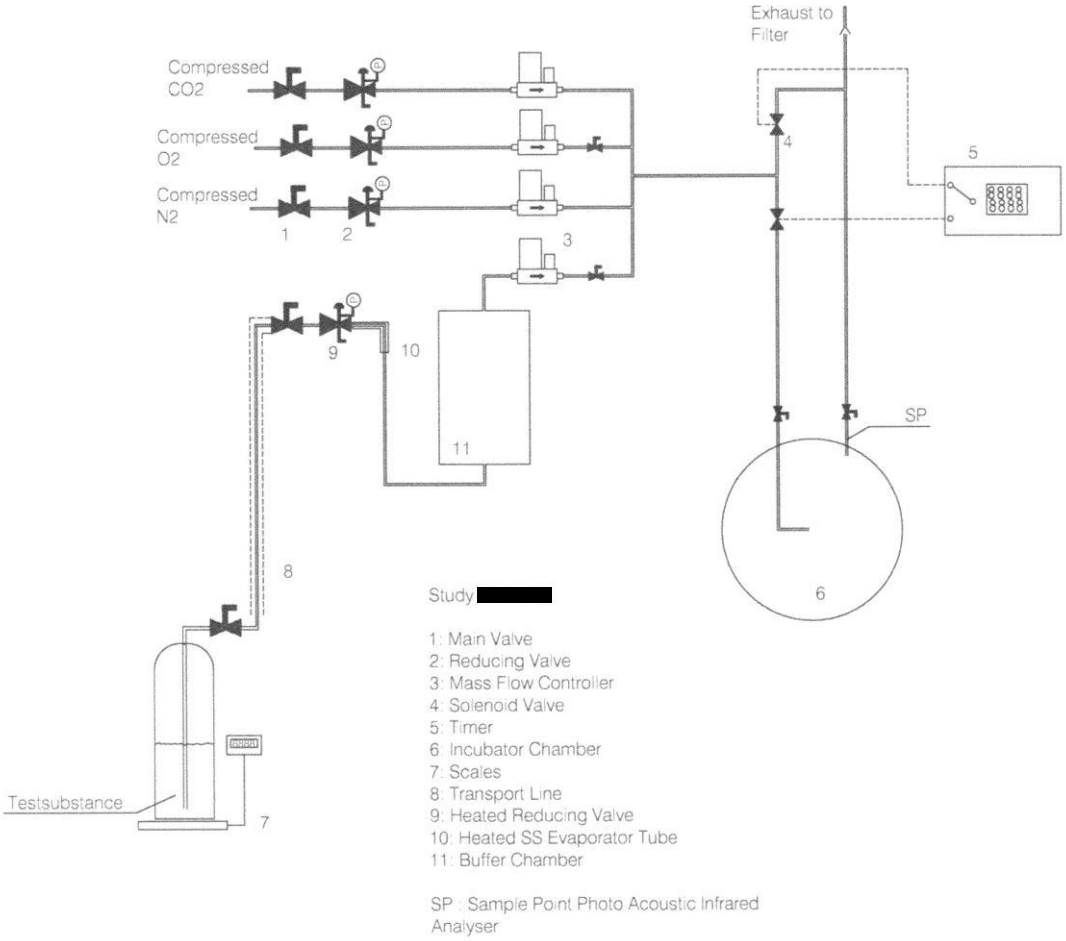
All materials will be retained in the archives of TNO, Utrechtseweg 48, 3704 HE Zeist, The Netherlands. The archiving period for starts on the cover date of the final report.

8 References

- OECD guidelines for the Testing of Chemicals, no. 473: *In vitro* mammalian chromosome aberration test; adopted 26 September 2014.
- OECD Principles of Good Laboratory Practice (as revised in 1997), Organisation for Economic Co-operation and Development (OECD), Paris; ENV/MC/CHEM(98)17.
- Ames, B.N., J. McCann and E. Yamasaki (1975) "Methods for detecting carcinogens and mutagens with the *Salmonella* mammalian microsome mutagenicity test". Mutation Res. 31, 347 - 365.
- Galloway, S.M., Aardema, M.J., Ishidate, M.Jr., Ivett, J.L., Kirkland, D.J. Morita, T., Mosesso, P. and Sofuni, T.: International workshop on standardisation of genotoxicity test procedures. Report from working group on *in vitro* tests for chromosomal aberrations. Mutation Res., 312 (1994) 241-261.
- Kao, F.T. and T.T. Puck. Genetics of somatic mammalian cells. VII. Proc. Nat. Acad. Sci. (USA) 60 (1968) 1275-1281.
- Triskelion report [redacted], D. van Berlo. In vitro mammalian cell gene mutation test at the TK-locus of L5178Y cells with [redacted] (2018).

Figures

Figure 1: Schematic diagram of the generation and exposure system



Appendices

Appendix 1: Test substance concentrations

Table 1.1: Calculated and measured test substance concentrations in the pulse treatment groups. January 24, 2018

Group	Calculated concentrations (v/v%) derived from the settings of the mass flow controller			Measured concentrations (v/v%) 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	0.1	0.1
10	19.0	5.0	10.0	9.7	8.3
20	19.0	5.0	19.9	19.4	19.0
40	19.0	5.0	40.2	39.6	37.8
60	19.0	5.0	60.1	59.7	59.0
76	19.0	5.0	76.0	73.7	73.7

Table 1.2: Calculated and measured test substance concentrations in the continuous treatment groups. January 24 and 25, 2018

Group	Calculated concentrations (v/v%) derived from the settings of the mass flow controller			Measured concentrations (v/v%) 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	0.1	0.1
10	19.0	5.0	10.0	9.1	8.8
20	19.0	5.0	19.9	18.6	18.8
40	19.0	5.0	40.2	39.4	38.9
60	19.0	5.0	60.1	60.8	60.2
76	19.0	5.0	76.0	76.5	66.8

Appendix 2: Osmolality and pH measurements

Table 2: Osmolality and pH measurements

Target 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: Tables of results

Table 3.1: Mitotic index analysis

Test 1►*Pulse treatment method with metabolic activation (S9-mix)*							
Treatment time: Harvesting time:			4 h 24 h				
Treatment	Nominal dose level (v/v/%)	Number of cells scored	Mitotic index				
			% of cells in mitosis	Mean % of cells in mitosis	Relative mitotic index (%)	Cytotox (%)	Selected for chromosomal aberration scoring
Clean Air	0	1000	8.4	8.2	100	0	+
		1000	8.0				+
[redacted]	76%	1000	8.0	8.8	107	0	+
		1000	9.5				+
	60%	1000	8.0	7.8	95	5	+
		1000	7.6				+
	40%	1000	9.7	9.3	113	0	+
		1000	8.8				+
	20%	1000	8.6	7.6	93	7	-
		1000	6.6				-
	10%	1000	7.6	7.9	96	4	-
		1000	8.1				-
CP	25 µg/ml	1000	4.9	4.8	59	41	+
		1000	4.6				+

CP: Cyclophosphamide

$$\%\ of\ cells\ in\ mitosis\ (\%\ MI) = \frac{\text{Number of mitotic cells}}{\text{Total number of cells scored}} x100$$

$$Relative\ mitotic\ index\ (\%) = \frac{\%\ \text{MI treatment}}{\%\ \text{MI control}} x100$$

$$Cytotox\ (\%) = 100 - \text{relative mitotic index (\%)}$$

Appendix 3 - continued

Table 3.2: Mitotic index analysis

Test 1► *Pulse treatment method without metabolic activation (S9-mix)*							
Treatment time: Harvesting time:			4 h 24 h				
Treatment	Nominal dose level (v/v%)	Number of cells scored	Mitotic index				
			% of cells in mitosis	Mean % of cells in mitosis	Relative mitotic index (%)	Cytotox (%)	Selected for chromosomal aberration scoring
Clean Air	0	1000	8.1	7.7	100	0	+
		1000	7.3				+
[redacted]	76%	1000	6.3	7.0	91	9	+
		1000	7.7				+
	60%	1000	7.3	7.5	97	3	+
		1000	7.6				+
	40%	1000	6.7	7.5	97	3	+
		1000	8.3				+
	20%	1000	8.7	9.5	123	0	-
		1000	10.2				-
	10%	1000	8.6	9.7	126	0	-
		1000	10.8				-

Formulas: see Table 3.1

Appendix 3 - continued

Table 3.3: Mitotic index analysis

Test 1► *Continuous treatment method without metabolic activation (S9-mix)*							
Treatment time: Harvesting time:			4 h 24 h				
Treatment	Nominal dose level (v/v%)	Number of cells scored	Mitotic index				
			% of cells in mitosis	Mean % of cells in mitosis	Relative mitotic index (%)	Cytotox (%)	Selected for chromosomal aberration scoring
Clean Air	0	1000	8.7	8.2	100	0	-
		1000	7.6				-
[redacted]	76%	1000	*	-	-	-	-
		1000	*				-
	60%	1000	*	-	-	-	-
		1000	*				-
	40%	1000	*	-	-	-	-
		1000	*				-
	20%	1000	8.3	8.5	103	-3	-
		1000	8.6				-
	10%	1000	7.8	6.7	82	18	-
		1000	5.5				-
	MMC 0.1	1000	4.3	4.6	56	44	-
		1000	4.8				-

MMC: MitomycinC

Formulas: see Table 3.1

Appendix 3 - continued

Table 3.4: Chromosomal aberration analysis

Test 1 ►Pulse treatment method with metabolic activation (S9-mix)

Treatment / harvest time (h)	Nominal dose level (v/v%)	Number of cells showing structural chromosome aberrations							Statistics [2] (p-value)	Number of cells with only gaps [1]	Cytotox (%)
		Cells observed	Chromatid break	Chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)			
4/24 (+ S9)	NC	150	2	0	0	0	0	2	-	0	0
		150	0	0	1	0	0	1		1	
		300	2	0	1	0	0	3 (1.00)		1	
	76%	150	1	0	2	0	0	2	n.s.	0	0
		150	1	0	0	0	0	1		0	
		300	2	0	2	0	0	3 (1.00)		0	
	60%	150	0	0	1	0	0	1	n.s.	0	5
		150	0	0	0	0	0	0		0	
		300	0	0	1	0	0	1 (0.33)		0	
	40%	150	2	0	0	0	0	2	n.s.	1	0
		150	0	0	0	0	0	0		0	
		300	2	0	0	0	0	2 (0.67)		1	
	CP 25.0 µg/ml	150	11	10	6	0	0	27	**** p<0.0001	2	41
		150	10	14	5	0	0	29		0	
		300	21	24	11	0	0	56 (18.7)		2	

[1] Total number of cells showing only (chromatid-type and chromosome-type) gaps
[2] Fisher's exact probability test (one-sided); **** p<0.0001
NC: Clean Air, CP: Cyclophosphamide, n.s.: not significant

Appendix 3 - continued

Table 3.5: Chromosomal aberration analysis

Test 1►Pulse treatment method without metabolic activation (S9-mix)

Treatment / harvest time (h)	Nominal dose level (v/v%)	Number of cells showing structural chromosome aberrations								Number of cells with only gaps [1]	Cytotox (%)
		Cells observed	Chromatid break	Chromatid exchange	Chromo-some break	Chromo-some exchange	Others	Number of cells showing aberrations (%)	Statistics [2] (p-value)		
4/24 (- S9)	NC	150	1	0	0	0	0	1	-	0	0
		150	0	1	0	0	0	1		0	
		300	1	1	0	0	0	2 (0.67)		0	
	76%	150	2	1	0	0	0	3	n.s.	0	9
		150	3	0	0	0	0	3		4	
		300	5	1	0	0	0	6 (2.00)		4	
	60%	150	0	0	1	0	0	1	n.s.	2	3
		150	2	2	0	0	0	4		0	
		300	2	2	1	0	0	5 (1.67)		2	
	40%	150	0	0	1	0	0	1	n.s.	0	3
		150	2	0	0	0	0	2		0	
		300	2	0	1	0	0	3 (1.00)		0	

[1] Total number of cells showing only (chromatid-type and chromosome-type) gaps
[2] Fisher's exact probability test (one-sided)
NC: Clean Air, n.s: not significant

Annexes

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: The quality certificate of S9

The batch of S9 used obtained from Trinova Biochem (Giessen. Germany) and were originally from Moltox Molecular Toxicology Incorporated (Boone. USA). The quality certificate was provided by the supplier.

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 Peckham 09/12/17

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

Annex 3: Definition of chromosomal aberrations

Chromatid gap:	An achromatic lesion smaller than the width of one chromatid, and with minimal misalignment of the chromatid.
Chromatid break:	A breakage of one chromatid larger than the width of one chromatid, or a clear misalignment of a chromatid.
Chromatid exchange:	A breakage and reunion between chromatids from different chromosomes (interchange) or within a chromosome (intrachange; including interstitial deletion).
Chromosome gap:	An achromatic lesion at an identical site in both chromatids smaller than the width of one chromatid, and with minimal misalignment of the chromatids.
Chromosome break:	A breakage at an identical site of both chromatids larger than the width of one chromatid, or a clear misalignment of the chromatids (misalignment of the chromatids can result in cases where only the acentric fragment but not the shortened monocentric chromosome can be identified).
Chromosome exchange:	A breakage of both chromatids with a reunion between chromatids from different chromosomes (dicentric) or within a chromosome (ring).
Multiple aberrations:	A cell containing more than 10 chromosomal aberrations.
Polyploidy:	A cell containing a multiple of the haploid chromosome number (n) other than the diploid number (i.e., 3n, etc.).
Endoreduplication:	A cell in which after an S (synthesis) period of DNA replication, the nucleus did not go into mitosis but started another S period. The result is chromosomes with 4, 8, 16 or more chromatids.

References:

- Savage, Annotation (1975), Classification and relationships of induced chromosome structural change. J. Med. Gen. 13, 103 - 122.
- Scott, D. Dean, B.J., Danford, N.D., and Kirkland, D.J. Metaphase chromosome aberration assays *in vitro*. In: Basic Mutagenicity Tests.
- UKEMS Recommended Procedures, editor D.J. Kirkland, Cambridge University Press (1990), Report. Part 1 revised, pp. 62 - 86..

Annex 4: Historical data of the *in vitro* chromosomal aberration tests

Historical negative control data of chromosomal aberration tests performed with cultured human lymphocytes
Summarized data from 2000 – 2016

		Percentage of metaphases with aberrations (excluding metaphases with only gaps)			
Treatment / harvest time (± S9)	Solvent	Mean ± S.D.	95% control limit	Range	N
4 / 24 (-S9)	All[#]	0.25 ± 0.37	0.00 - 1.00	0.00 – 1.50	43
4 / 24 (+S9)	All[#]	0.25 ± 0.36	0.00 - 1.00	0.00 – 1.50	74
24 / 24 (-S9)	All[#]	0.14 ± 0.30	0.0 0- 1.00	0.00 – 1.50	41

[#] Culture medium, saline and 1% DMSO
N = number of treatment groups

Historical positive control (Cyclophosphamide) data of chromosomal aberration tests performed with cultured human lymphocytes:
Summarized data from 2000 – 2016

		Percentage of metaphases with aberrations (excluding metaphases with only gaps)			
Treatment / harvest time (h) (+S9)	Dose level (µg/ml)	Mean ± SD	95% control limit	Range	N
4 / 24	25	25.2 ± 7.3	14.5 - 37.5	10.3 – 44.5	50

N = number of treatment groups

Historical positive control (Mitomycin C) data of chromosomal aberrations tests performed with cultured human lymphocytes:
Summarized data from 2000 – 2016

		Percentage of metaphases with aberrations (excluding metaphases with only gaps)			
Treatment / harvest time (h) (-S9)	Dose level (µg/ml)	Mean ± SD	95% control limit	Range	N
4 / 24	0.4	31.6 ± 4.8		22.0 – 39.5	35
24 / 24	0.1 0.2	18.0 ± 8.06 27.8 ± 7.9	13.1 - 25.9 15.5 - 42.9	13.0 – 27.3 14.0 – 47.0	3 35

N = number of treatment groups

Annex 5: Certificate of Analysis

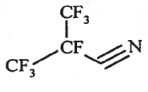
[redacted] Analytical Laboratory

An ISO9001/2015 Certified Laboratory

Certificate of Analysis

PSB-en

08 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

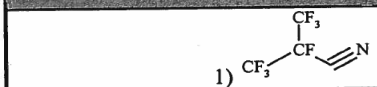
Issue Date: May 1, 2017

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]

[redacted] Analytical Laboratory [redacted]

Page 1 of 1
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