Cyclohexanone

Cyclohexanone - Micronucleus Test in Human Lymphocytes *In Vitro*

Final Report

TEST GUIDELINE(S):	OECD 487 (2016)
AUTHOR(S):	Dr. Steffen Naumann
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PERFORMING LABORATORY:	ICCR-Roßdorf GmbH In den Leppsteinswiesen 19 64380 Rossdorf, Germany
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SPONSOR(S):	Syngenta Ltd Jealott's Hill International Research Centre Bracknell, Berkshire RG42 6EY, United Kingdom

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This study performed in the test facility of ICCR-Roßdorf GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version

OECD Principles of Good Laboratory Practice, (as revised in 1997), ENV/MC/CHEM(98)17

EC Commission Directive 2004/10/EC

These procedures are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHW, MAFF, and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

There were no circumstances that may have affected the quality or integrity of the study.

Dr. Steffen Naumann
Genetic Toxicology *in vitro*

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Date: 14 December 2020

Performing Laboratory:
ICCR-Roßdorf GmbH
In den Leppsteinswiesen 19
64380 Rossdorf, Germany

To be completed for USA EPA submission only:
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Submitter/Sponsor: Syngenta Crop Protection, LLC
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419-8300 USA

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QUALITY ASSURANCE STATEMENT

Study Number:	1993800
Test Substance:	Cyclohexanone
Study Director:	Dr. Steffen Naumann
Title:	Cyclohexanone - Micronucleus Test in Human Lymphocytes *In Vitro*

Study based activities at the Test Facility ICCR-Roßdorf GmbH were audited and inspected. The details of these audits and inspections are given below.

Type of Inspection	**Date(s) of Inspection**	**Date Reporting to Study Director, Test Facility Management**
Study Plan Verification	09 March 2020	09 March 2020
Process – based Test item preparation Test system preparation and application	22 April 2020 13 May 2020	22 April 2020 13 May 2020
Report Audit	24 June 2020	24 June 2020

General facilities and activities where this study was conducted were inspected on an annual basis and results are reported to the relevant responsible person and Management.

The statement is to confirm that this report reflects the raw data.

Quality Assurance

Marina Hahn

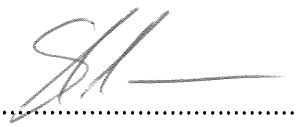
14 December 2020

Date

Quality Assurance Auditor
ICCR-Roßdorf GmbH

PROJECT STAFF SIGNATURE

Study Director — Dr. Steffen Naumann



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Date: 14 December 2020

GENERAL INFORMATION

Contributors
The following contributed to this report in the capacities indicated:

Name	Title
Dr. Steffen Naumann	Study Director
Dr. Markus Schulz	Management
Frauke Hermann	Head of Quality Assurance Unit
Eva Lessmann	Syngenta Study Manager

Study dates
Study initiation date: 11 March 2020
Experimental start date: 25 March 2020
Experimental termination date: 05 June 2020

Deviations from the guidelines
None

Retention of samples
None

Performing laboratory test substance number
S 2079611

Other
Records and documentation relating to this study will be maintained in the archives of ICCR-Roßdorf GmbH for a period of 4 years from the date on which the Study Director signs the final report. This will include but may not be limited to the Study Plan, any amendments, raw data, Report and specimens generated during the course of this study.

At termination of the aforementioned period, the records and documentation will be transferred to the GLP compliant archive of Rhenus Archiv Services GmbH, Frankfurt am Main, for further archiving up to a total archiving period of 15 years.

A sample of the test substance will not be archived.

ICCR-Roßdorf GmbH will retain in its archive a copy of the study plan and final report, and any amendments indefinitely.

Deviations from the study plan
None

Distribution of the report

Sponsor	2 × electronic copy (1 × pdf-file, 1 × word-file)
Study Director	1 × (original)

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

The test substance cyclohexanone, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments.

In each experimental group two parallel cultures were analysed. Per culture 1000 binucleated cells were evaluated for cytogenetic damage.

The highest applied concentration in this study (982 µg/mL of the test substance, approx. 10 mM) was chosen with regard to the molecular weight of the test substance and with respect to the current OECD Guideline 487.

Concentration selection of the cytogenetic experiment was performed considering the toxicity data and in accordance with OECD Guideline 487.

1.2 Results

In this study in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In both experiments in the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test substance. The mean percentage of the micronuclei in all treated conditions was within the 95% control limits of the laboratory historical vehicle control data and none of the values were statistically significantly increased when compared to the vehicle control. There was also no concentration related increase in micronucleus formation, as judged by an appropriate trend test. The outcome of the study is clearly negative.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

1.3 Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes. Therefore, cyclohexanone is considered to be non-genotoxic (non-clastogenic and non-aneugenic) in this *in vitro* micronucleus test, when tested up to the highest required concentrations.

2.0 INTRODUCTION

2.1 Purpose

The occurrence of micronuclei in interphase cells provides an indirect, but easy and rapid measure of structural chromosomal damage and aneugenicity in cells that have undergone cell division during or after exposure to the test substance. Micronuclei arise from chromosomal fragments or whole chromosomes and rarely occur spontaneously, but are inducible by clastogens or agents affecting the spindle apparatus (Countryman and Heddle, 1976; Obe and Beek, 1982, Rosefort *et al*, 2004).

2.2 Justification of Test System

The induction of cytogenetic damage in human lymphocytes was assessed in two independent experiments with one preparation interval (40 hours). Human lymphocytes have been widely used for this assay type as described in the OECD test guideline 487 (2016).

Micronuclei should only be evaluated in cells that have completed mitosis during exposure to the test substance or during the post-exposure period and thus a cytokinesis blocker, cytochalasin B, is added to the cell culture to ensure that there are binucleated cells to be evaluated for micronuclei (Rosefort et al, 2004).

Treatments started after a 48 hour stimulation period with phytohaemagglutinin (PHA) when cells were actively proliferating and the cells were prepared at approximately 2 – 2.5 fold of the normal cell cycle time (Whitwell et al, 2019).

For validation of the test, control mutagens were tested in parallel to the test substance.

2.3 Regulatory Guidelines

This study was conducted according to the procedures indicated by the following internationally accepted guideline and recommendations:

- OECD Guideline for the Testing of Chemicals No. 487 "*In vitro* Mammalian Cell Micronucleus Test", adopted 29 July 2016.

The following alterations from the guidelines were performed:

- A series of in-house non-GLP validation experiments was performed to get distinct responses of statistical significance when using the specified positive controls (Bohnenberger et al, 2011). To achieve such response the test design, specifically for the treatment, the recovery phase and harvest time, was slightly modified comparing the current proposal given in the OECD Guideline 487. The optimum positive control micronuclei responses were found with the time schedule stated in section 3.7.1 and is supported by publications (Clare et al, 2006, Lorge et al, 2006, Whitwell et al, 2019).

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance and the information concerning the test substance were provided by Sigma:

Identification:	Cyclohexanone
Batch:	BCCB1352
Purity:	100.0 %
Molecular weight:	98.14 g/mol
Physical state / Appearance:	Colourless liquid
Retest Date:	30 June 2023
Storage Conditions:	At room temperature
Stability in Solvent:	Not indicated by the Sponsor

3.2 Test Substance Preparation

On the day of the experiment (immediately before use), the test substance was dissolved in DMSO. The final concentration of DMSO in the culture medium was 0.5% (v/v). The solvent was chosen as the best suitable solvent compared to water and ethanol, according to its solubilisation properties and its compatibility with cell cultures (Easterbrook *et al*, 2001).

The osmolarity and pH of the test substance dissolved in DMSO and diluted in culture medium were determined by using an osmometer or a pH meter, respectively, in the pre-experiment without metabolic activation in the solvent control and the respective maximum concentration.

3.3 Controls

3.3.1 Solvent controls

Concurrent solvent controls (culture medium with 0.5 % DMSO) were performed.

Name:	DMSO
Supplier:	Fisher Chemical, 58239 Schwerte, Germany
Purity:	≥99.9 %
Lot No. / Expiry Date:	1905836 / February 2025 (Exp. I) 1905836 / April 2025 (Exp. II)

3.3.2 Positive control substances

Without metabolic activation

Name:	Mitomycin C (MMC) (pulse treatment)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	108 M 4160 V
Expiry Date:	November 2020
Purity:	98 %
Dissolved in:	Deionised water
Concentration:	1.0 µg/mL

Name:	Demecolcine (continuous treatment)*
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	BCBX 9130
Expiry Date:	October 2020
Purity:	≥ 98 %
Dissolved in:	Deionised water
Concentration:	125 ng/mL

* OECD 487, paragraph 33 permits the use of an alternative positive control agent, if a sufficient laboratory historical data base has been established and is scientifically justified.

With metabolic activation

Name:	Cyclophosphamide (CPA)
Supplier:	Sigma Aldrich Chemie GmbH, 82024 Taufkirchen, Germany
Lot No.:	MKBX 1822 V
Expiry Date:	May 2021
Purity:	97 – 103 %
Dissolved in:	Saline (0.9 % NaCl [w/v])
Concentration:	12.5 µg/mL

The dilutions of the stock solutions were prepared on the day of the experiment. The stability of the positive control substance in solution is unknown but a mutagenic response in the expected range is sufficient biological evidence for chemical stability.

3.4 Experimental Design

3.4.1 Reason for the choice of human lymphocytes

Human lymphocytes are commonly used in the *in vitro* micronucleus test and have been used successfully for a long time in *in vitro* experiments. They show stable spontaneous micronucleus frequencies at a low level (Countryman and Heddle, 1976; Evans and O'Riordan, 1975).

3.4.2 Blood collection and delivery

Blood samples were drawn from one healthy non-smoking female donor (30 years old) not receiving medication. The donor's lymphocytes have been shown to respond well to stimulation of proliferation with PHA and to positive control substances. The donor had a previously established low incidence of micronuclei in her peripheral blood lymphocytes.

Blood samples were drawn by venous puncture and collected in heparinized tubes by Dr. V. Theodor (64380 Rossdorf, Germany). The tubes were sent to ICCR-Roßdorf GmbH to initiate cell cultures within 24 h after blood collection.

3.5 Mammalian Microsomal Fraction S9 Mix

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Wistar rats (RjHan:WI; Janvier Labs, 53941 Saint-Berthevin Cedex, France) induced by peroral administration of 80 mg/kg b.w. phenobarbital (Sigma-Aldrich Chemie GmbH, 82024 Taufkirchen, Germany) and by peroral administrations of ß-naphthoflavone (Acros Organics, 2440 Geel, Belgium) each, on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1+3 parts) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored in ampoules at –80 °C. Small numbers of the ampoules can be kept at –20 °C for up to one week.

Each batch of S9 is routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test (Ames et al, 1975).

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation was 29.0 mg/mL (Lot no. 050919D).

3.6 Concentration Selection

Concentration selection was performed according to the current OECD Guideline 487 for the *in vitro* micronucleus test (2016). The highest test substance concentration should be 10 mM, 2 mg/mL, or 2 µL/mL, whichever is the lowest. Four test substance concentrations were evaluated for cytogenetic damage.

In case of test substance induced cytotoxicity, measured by a reduced cytokinesis-block proliferation index (CBPI) and expressed as cytostasis, or precipitation / phase separation

(observed at the end of test substance exposure by the unaided eye) the concentration selection should reflect these properties of the test substance. Where cytotoxicity occurs, the applied concentrations should cover a range from no to approximately 55 ± 5 % cytostasis. For poorly soluble test substances, which are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analysed should produce turbidity or visible precipitation / phase separation.

3.7 Experimental Performance Cytogenetic Experiment

3.7.1 Schedule

	Without S9 mix		With S9 mix
	Exp. I	Exp. II	Exp. I
Stimulation period (h)	48	48	48
Exposure period (h)	4	20	4
Recovery (h)	16	—	16
Cytochalasin B exposure (h)	20	20	20
Total culture period (h)	88	88	88

3.7.2 Culture conditions

Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 h after blood collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (phytohemagglutinin) (3 µg/mL), 10 % FBS (fetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

The following volumes were added to the flasks (per 10 mL):

7.60 mL culture medium
1.00 mL fetal bovine serum
0.10 mL antibiotic solution
0.05 mL phytohemagglutinin (stock solution: 0.6 mg/mL)
0.05 mL heparin
0.10 mL HEPES
1.10 mL whole blood

All incubations were done at 37 °C with 5.5 % CO_2 in humidified air.

3.7.3 Pre-experiment

A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterised by the percentages of reduction in the CBPI in comparison to the controls by counting 500 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the main assay.

The pre-test was performed with 10 concentrations of the test substance separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 h (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

3.7.4 Cytogenetic experiment

Pulse exposure
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with serum-free medium containing the test substance or control. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose · H_2O, 192 mg/L Na_2HPO_4 · 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 h until preparation (Clare et al, 2006, Lorge et al, 2006).

Continuous exposure (without S9 mix)
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test substance concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test substance or control. After 20 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 h until preparation (Whitwell et al, 2019).

3.7.5 Preparation of cells

The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in saline G (approximately 5 mL) and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in KCl solution (5 mL, 0.0375 M) and incubated at 37 °C for 20 minutes. Ice-cold fixative mixture of methanol and glacial acetic acid (1 mL, 19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The mounted cells were Giemsa-stained and, after drying, covered with coverslips. All slides were labeled with a computer-generated random code to prevent scorer bias.

3.7.6 Evaluation of cytotoxicity damage

Cytotoxicity was judged in the course of a microscopical pre-check of the specimen slides for guideline requested quality and quantity criteria in a first step. Subsequently the CBPI was used as the preferred method for quantifying the effect on cell proliferation and the cytotoxic or cytostatic activity by the OECD Guideline 487. To describe cytotoxic effects the CBPI was determined in 500 cells per culture. Evaluation of the slides was performed using microscopes with 40 x objectives. Cytotoxicity is expressed as cytostasis, calculating the CBPI, and used therefore as a cut off criterion. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Under some circumstances the CBPI does not reflect the cytotoxicity accurately and concentrations may be excluded from the evaluation during the microscopic pre-check. CBPI measures proliferation and may not detect cytotoxic events like necrosis, oncosis and apoptosis. In particular mononuclear cells without cytoplasm (representing cells which undergo cell death in the treatment cell cycle) are not represented in the CBPI because those cells do not fulfil the quality criteria for evaluation (see section 3.7.7). This can result in too few cells available for scoring.

$$CBPI = \frac{(MONC \times 1) + (BINC \times 2) + (MUNC \times 3)}{n}$$

CBPI	Cytokinesis-block proliferation index
n	Total number of cells
MONC	Mononucleate cells
BINC	Binucleate cells
MUNC	Multinucleate cells

$$\text{Cytostasis \%} = 100 - 100\,[(CBPI_T - 1) / (CBPI_C - 1)]$$

T	Test substance
C	Solvent control

3.7.7 Evaluation of cytogenetic damage

Evaluation of the slides was performed using microscopes with 40 x objectives. The micronuclei were counted in binucleated cells showing a clearly visible cytoplasm area. The criteria for the evaluation of micronuclei are described in the publication of Countryman and Heddle (1976). The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not be more than one third of the area of the main nucleus. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells.

3.8 Data Recording

The data were recorded in the laboratory documentation. The results are presented in tabular form, including experimental groups with the test substance, solvent controls, and positive controls, respectively.

3.9 Acceptability Criteria

The micronucleus assay will be considered acceptable if it meets the following criteria:

- The concurrent solvent control will normally be within the 95% control limits of the laboratory's historical solvent control data.
- The concurrent positive controls should induce responses that are compatible with the laboratory historical positive control data and produce a statistically significant increase.
- Cell proliferation criteria in the solvent control are considered to be acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides must allow the evaluation of an adequate number of cells and concentrations.

The criteria for the selection of top concentration are consistent with those described in section 'Concentration selection'.

3.10 Interpretation of Results

Providing that all of the acceptability criteria are fulfilled, a test substance is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test substance concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase when assessed by a trend test
- The results in all evaluated test substance concentrations should be within the 95% control limits of the laboratory's historical solvent control data

The test substance is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test substance is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test substance concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition when assessed by a trend test
- The results are outside the range of the 95% control limit of the laboratory historical solvent control data

If all of the criteria are met, the test substance is considered able to induce chromosome breaks and/or gain or loss in this test system.

There is no requirement for verification of a clear positive or negative response.

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. narrow concentration spacing, other metabolic activation conditions, i.e. S9 concentration or S9 origin) could be useful.

However, results may remain questionable regardless of the number of times the experiment is repeated. If the data set will not allow a conclusion of positive or negative, the test substance will therefore be concluded as equivocal.

3.11 Laboratory's Historical Control Data

The historical control data were generated in accordance with the OECD Guideline 487 and updated annually.

For the solvent controls, data range (min-max) and data distribution (standard deviation) were calculated for each experimental part of at least 20 experiments (Appendix 1). The calculated 95% control limit of the solvent controls (realized as 95% confidence interval) was applied for the evaluation of acceptability and interpretation of the data (Sections 3.9 and 3.10). Control charts of the corresponding experiments are added as quality control method.

For the positive controls, data range (min-max) and data distribution (standard deviation) were calculated for each experimental part of at least 20 experiments (Appendix 1). The min-max range of the positive controls was applied for the evaluation of acceptability (Section 3.9). Control charts of the corresponding experiments are added as quality control method.

3.12 Statistical Analysis

Statistical significance was confirmed by the Chi square test ($p < 0.05$), using a validated test script of "R", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression test was performed using a validated test script of "R", to assess a possible concentration dependent increase of micronucleus frequency. The number of micronucleated cells obtained for the groups treated with the test substance was compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Both, biological and statistical significance were considered together.

4.0 RESULTS AND DISCUSSION

The test substance cyclohexanone, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure period was 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test substance.

In each experimental group two parallel cultures were analysed. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides making a total of 2000 binucleated cells per test substance concentration. To assess cytotoxicity, the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 cells per culture. Percentage of cytostasis (inhibition of cell growth) is also reported.

The highest treatment concentration in the pre-test for toxicity, 982 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight of the test substance and with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

Test substance concentrations ranging from 6.4 µg/mL to 982 µg/mL (with and without S9 mix) were chosen for evaluation of cytotoxicity. In the pre-test for toxicity, no precipitation of the test substance was observed at the end of treatment. Since the cultures fulfilled the requirements for cytogenetic evaluation, this test was designated Experiment I.

Using a reduced Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity, no cytotoxicity was observed in Experiment I after 4 hours treatment in the absence and presence of S9 mix up to the highest applied concentrations.
Therefore, the same concentration (982 µg/mL) was chosen as top treatment concentration for Experiment II. No precipitation of the test substance was observed at the end of treatment.

The applied concentrations for all experiments are presented in Table 1.

No relevant influence on the osmolarity and pH was observed as shown below.

		Concentration [µg/mL]	Osmolarity [mOsm]	pH
Exp. I	Solvent control	-	364	7.56
	Cyclohexanone	982	394	7.51

In this study in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In both experiments in the absence and presence of S9 mix, no biologically relevant increases in the number of micronucleate cells were observed after treatment with the test substance. The mean percentages of the micronuclei in all treated conditions were within the 95% control limit of the laboratory historical vehicle control data and none of the values were statistically significantly increased, when compared with the vehicle control. There was also no concentration related increase in micronucleus formation, as judged by an appropriate trend test. The outcome of the study is clearly negative.

Demecolcine (125 ng/mL), MMC (1.0 µg/mL) or CPA (12.5 µg/mL) were used as appropriate positive control chemicals and showed statistically significant increases in binucleated cells with micronuclei demonstrating the correct performance of the assay.

5.0 CONCLUSIONS

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes. Therefore, cyclohexanone is considered to be non-genotoxic (non-clastogenic and non-aneugenic) in this *in vitro* micronucleus test, when tested up to the highest required concentrations.

6.0 REFERENCES

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TABLES SECTION

TABLE 1 Concentrations Applied in the Micronucleus Assay with Cyclohexanone

Exp.	Prep. interval (h)	Exposure period (h)	Concentrations (µg/mL)									
Without S9 mix												
I	40	4	6.4	11.2	19.5	34.2	59.8	105	**183**	**321**	**561**	**982**
II	40	20					59.8	105	**183**	**321**	**561**	**982**
With S9 mix												
I	40	4	6.4	11.2	19.5	34.2	59.8	105	**183**	**321**	**561**	**982**

Evaluated experimental points are shown in bold characters

TABLE 2 Summary of Results of the Micronucleus Assay with Cyclohexanone

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	95% Ctrl limit in %
Exposure period 4 h without S9 mix						
I	40 h	Solvent control[1]	1.73		0.70	0.00 – 1.04
		Positive control[2]	1.45	38.4	**13.65[S]**	
		183	1.69	5.9	0.35	
		321	1.72	1.5	0.50	
		561	1.71	3.7	0.50	
		982	1.65	12.0	0.60	
Trend test: p-value 0.940						
Exposure period 20 h without S9 mix						
II	40 h	Solvent control[1]	1.95		0.40	0.00 – 0.86
		Positive control[3]	1.70	25.8	**4.70[S]**	
		183	1.88	6.9	0.40	
		321	1.95	n.c.	0.45	
		561	1.98	n.c.	0.35	
		982	1.89	5.8	0.65	
Trend test: p-value 0.156						
Exposure period 4 h with S9 mix						
I	40 h	Solvent control[1]	1.65		0.60	0.00 – 1.03
		Positive control[4]	1.41	37.0	**3.50[S]**	
		183	1.65	n.c.	0.95	
		321	1.59	10.6	0.90	
		561	1.71	n.c.	0.65	
		982	1.65	1.1	0.80	
Trend test: p-value 0.910						

* For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c. Not calculated as the CBPI is equal or higher than the solvent control value
[1] DMSO 0.5 % (v/v)
[2] MMC 1.0 µg/mL
[3] Demecolcine 125 ng/mL
[4] CPA 12.5 µg/mL

TABLE 3 Toxicity - Experiment I (Cytotoxicity of Cyclohexanone to the Cultures of Human Lymphocytes)

Concentration (µg/mL)	Exposure time (h)	Preparation interval (h)	CBPI per 500 cells*	Cytostasis (%)
Without S9 mix				
Solvent control	4	40	1.73	-
6.4	4	40	n.p.	n.p.
11.2	4	40	n.p.	n.p.
19.5	4	40	n.p.	n.p.
34.2	4	40	n.p.	n.p.
59.8	4	40	1.72	1.6
105	4	40	1.71	3.4
183	4	40	1.69	5.9
321	4	40	1.72	1.5
561	4	40	1.71	3.7
982	4	40	1.65	12.0
With S9 mix				
Solvent control	4	40	1.65	-
6.4	4	40	n.p.	n.p.
11.2	4	40	n.p.	n.p.
19.5	4	40	n.p.	n.p.
34.2	4	40	n.p.	n.p.
59.8	4	40	1.71	n.c.
105	4	40	1.67	n.c.
183	4	40	1.65	n.c.
321	4	40	1.59	10.6
561	4	40	1.71	n.c.
982	4	40	1.65	1.1

Experimental groups evaluated for cytogenetic damage are shown in bold characters

* Mean value of two cultures

n.p. Not prepared

n.c. Not calculated as the CBPI was equal or higher than solvent control value

TABLE 4 Toxicity - Experiment II (Cytotoxicity of Cyclohexanone to the Cultures of Human Lymphocytes)

Concentration (µg/mL)	Exposure time (h)	Preparation interval (h)	CBPI per 500 cells*	Cytostasis (%)
Without S9 mix				
Solvent control	20	40	1.95	-
59.8	20	40	1.96	n.c.
105	20	40	1.91	3.9
183	20	40	1.88	6.9
321	20	40	1.95	n.c.
561	20	40	1.98	n.c.
982	20	40	1.89	5.8

Experimental groups evaluated for cytogenetic damage are shown in bold characters
* Mean value of two cultures
n.c. Not calculated as the CBPI was equal or higher than solvent control value

TABLE 5 Experiment I - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 4 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure / preparation (h)	Cell proliferation culture 1* c1	c2	c4-c8	Proliferation Index CBPI	Cell proliferation culture 2* c1	c2	c4-c8	Proliferation Index CBPI	CBPI mean	Cytostasis [%]
Solv. control[#]	0.5 %	-	4 / 40	148	316	36	1.78	197	260	43	1.69	1.73	
Pos. control[##]	1.0 µg	-	4 / 40	295	186	19	1.45	291	190	19	1.46	1.45	38.4
Test item	183 µg	-	4 / 40	173	291	36	1.73	197	278	25	1.66	1.69	5.9
″	321 µg	-	4 / 40	154	312	34	1.76	195	267	38	1.69	1.72	1.5
″	561 µg	-	4 / 40	199	263	38	1.68	161	310	29	1.74	1.71	3.7
″	982 µg	-	4 / 40	188	283	29	1.68	222	251	27	1.61	1.65	12.0

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

[#] DMSO

[##] MMC

TABLE 6 Experiment I - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 4 h with S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure / preparation (h)	Cell proliferation culture 1* c1	c2	c4-c8	Proliferation Index CBPI	Cell proliferation culture 2* c1	c2	c4-c8	Proliferation Index CBPI	CBPI mean	Cytostasis [%]
Solv. control[#]	0.5 %	+	4 / 40	196	269	35	1.68	215	255	30	1.63	1.65	
Pos. control[##]	12.5 µg	+	4 / 40	329	150	21	1.38	304	172	24	1.44	1.41	37.0
Test item	183 µg	+	4 / 40	208	259	33	1.65	204	263	33	1.66	1.65	n.c.
″	321 µg	+	4 / 40	246	230	24	1.56	230	233	37	1.61	1.59	10.6
″	561 µg	+	4 / 40	188	275	37	1.70	172	296	32	1.72	1.71	n.c.
″	982 µg	+	4 / 40	222	250	28	1.61	190	279	31	1.68	1.65	1.1

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

[#] DMSO

[##] CPA

n.c. Not calculated as the CBPI is equal or higher than the solvent control value

TABLE 7 Experiment I - Number of Micronucleated Cells; Exposure Period 4 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ preparation (h)	Micronucleated cells: Binucleate cells with *n* micronuclei culture 1: 1	2	>2	sum culture 1	Binucleate cells with *n* micronuclei culture 2: 1	2	>2	sum culture 2	sum in 2000 binucleate cells	[%]
Solv. control[#]	0.5 %	-	4 / 40	7	0	0	7	7	0	0	7	14	0.70
Pos. control[##]	1.0 µg	-	4 / 40	89	15	2	106	149	15	3	167	273	13.65
Test item	183 µg	-	4 / 40	5	0	0	5	2	0	0	2	7	0.35
″	321 µg	-	4 / 40	5	0	0	5	4	1	0	5	10	0.50
″	561 µg	-	4 / 40	2	2	0	4	6	0	0	6	10	0.50
″	982 µg	-	4 / 40	3	0	0	3	9	0	0	9	12	0.60

[#] DMSO
[##] MMC

TABLE 8 Experiment I - Number of Micronucleated Cells; Exposure Period 4 h with S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ preparation (h)	Micronucleated cells: Binucleate cells with *n* micronuclei culture 1: 1	2	>2	sum culture 1	Binucleate cells with *n* micronuclei culture 2: 1	2	>2	sum culture 2	sum in 2000 binucleate cells	[%]
Solv. control[#]	0.5 %	+	4 / 40	6	0	0	6	6	0	0	6	12	0.60
Pos. control[##]	12.5 µg	+	4 / 40	32	4	0	36	31	2	1	34	70	3.50
Test item	183 µg	+	4 / 40	7	1	0	8	10	0	1	11	19	0.95
″	321 µg	+	4 / 40	5	0	0	5	12	1	0	13	18	0.90
″	561 µg	+	4 / 40	6	0	0	6	5	2	0	7	13	0.65
″	982 µg	+	4 / 40	8	0	1	9	7	0	0	7	16	0.80

[#] DMSO
[##] CPA

TABLE 9 Experiment II - Cytotoxicity Indicated as Cytokinesis-block Proliferation Index and Cytostasis; Exposure Period 20 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure / preparation (h)	Cell proliferation culture 1* c1	c2	c4-c8	Proliferation Index CBPI	Cell proliferation culture 2* c1	c2	c4-c8	Proliferation Index CBPI	CBPI mean	Cytostasis [%]
Solv. control[#]	0.5 %	-	20 / 40	50	430	20	1.94	54	417	29	1.95	1.95	
Pos. control[##]	125 ng	-	20 / 40	148	347	5	1.71	159	338	3	1.69	1.70	25.8
Test item	183 µg	-	20 / 40	97	394	9	1.82	50	432	18	1.94	1.88	6.9
″	321 µg	-	20 / 40	44	431	25	1.96	60	408	32	1.94	1.95	n.c.
″	561 µg	-	20 / 40	24	446	30	2.01	50	425	25	1.95	1.98	n.c.
″	982 µg	-	20 / 40	68	420	12	1.89	65	424	11	1.89	1.89	5.8

* c1: mononucleate cells; c2: binucleate cells; c4-c8: multinucleate cells

[#] DMSO

[##] Demecolcine

n.c. Not calculated as the CBPI is equal or higher than the solvent control value

TABLE 10 Experiment II - Number of Micronucleated Cells; Exposure Period 20 h without S9 Mix

Treatment group	Conc. per mL	S9 mix	Exposure/ preparation (h)	Micronucleated cells: Binucleate cells with *n* micronuclei culture 1: 1	2	>2	sum culture 1	Binucleate cells with *n* micronuclei culture 2: 1	2	>2	sum culture 2	sum in 2000 binucleate cells	[%]
Solv. control[#]	0.5 %	-	20 / 40	2	1	0	3	3	2	0	5	8	0.40
Pos. control[##]	125 ng	-	20 / 40	40	7	4	51	31	10	2	43	94	4.70
Test item	183 µg	-	20 / 40	1	0	0	1	4	3	0	7	8	0.40
″	321 µg	-	20 / 40	5	0	0	5	4	0	0	4	9	0.45
″	561 µg	-	20 / 40	0	1	0	1	5	1	0	6	7	0.35
″	982 µg	-	20 / 40	7	0	0	7	6	0	0	6	13	0.65

[#] DMSO
[##] Demecolcine

TABLE 11 Biometry

Statistical significance was confirmed by using the Chi-squared test ($\alpha < 0.05$) using a validated R Script for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

Biometry of Experiment I (Chi-squared test)

Test substance versus solvent control [µg/mL]		Preparation interval (h)	Exposure period (h)	S9 mix	Chi^2	p-value
Test substance	183	40	4	-	n.c.	n.c.
"	321	40	4	-	n.c.	n.c.
"	561	40	4	-	n.c.	n.c.
"	982	40	4	-	n.c.	n.c.
"	183	40	4	+	1.593	0.207
"	321	40	4	+	1.209	0.272
"	561	40	4	+	0.040	0.841
"	982	40	4	+	0.576	0.448
Positive control versus solvent control [µg/mL]						
MMC	1.0	40	4	-	251.798	$< 2.2\times10^{-16}$ **s**
CPA	12.5	40	4	+	41.883	9.69×10^{-11} **s**

n.c. Not calculated as the micronucleus rate is equal or lower than the control rate
s Micronucleus rate is statistically significantly higher than the control rate

Biometry of Experiment II (Chi-squared test)

Test substance versus solvent control [µg/mL)		Preparation interval (h)	Exposure period (h)	S9 mix	Chi^2	p-value
Test substance	183	40	20	-	n.c.	n.c.
"	321	40	20	-	0.059	0.808
"	561	40	20	-	n.c.	n.c.
"	982	40	20	-	1.197	0.274
Positive control versus solvent control per [ng/mL]						
Demecolcine	125	40	20	-	74.407	$< 2.2\times10^{-16}$ **s**

n.c. Not calculated as the micronucleus rate is equal or lower than the control rate
s Micronucleus rate is statistically significantly higher than the control rate

A linear regression was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to assess a possible dose dependency in the rates of micronucleated cells. The number of micronucleated cells, obtained for the groups treated with the test substance were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

Linear regression (Trend test)

Experimental groups	p-value
Experiment I, exposure period 4 hrs without S9 mix	0.940
Experiment I, exposure period 4 hrs with S9 mix	0.910
Experiment II, exposure period 4 hrs without S9 mix	0.156

APPENDICES SECTION

APPENDIX 1 Historical Control Data

Percentage of micronucleated cells in human lymphocyte cultures (2019)

Aqueous solvents: DMEM/Ham's F12, Deionised water (10 % v/v)
Organic solvents: DMSO (0.5 or 1.0 %), Acetone, Ethanol and THF (0.5 %)

Solvent Control without S9		
Micronucleated cells in %		
	Pulse treatment (4/40)	Continuous treatment (20/40)
No. of experiments	50*	43**
Mean	0.46	0.43
95 % Ctrl limit	**0.00 – 1.04**	**0.00 – 0.86**
1x SD	0.29	0.21
2x SD	0.58	0.43
Min – Max	0.05 – 1.20	0.05 – 1.00

* Aqueous solvents – 17 Experiments; Organic solvents – 33 Experiments
** Aqueous solvents – 13 Experiments; Organic solvents – 30 Experiments

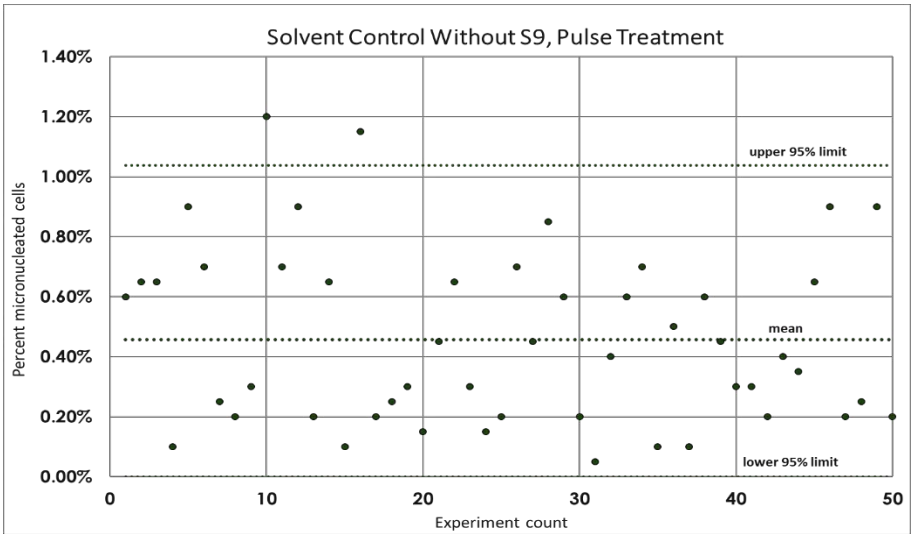
Solvent Control with S9	
Micronucleated cells in %	
	Pulse treatment (4/40)
No. of experiments	52*
Mean	0.48
95 % Ctrl limit	**0.00 – 1.03**
1x SD	0.27
2x SD	0.55
Min – Max	0.05 – 1.25

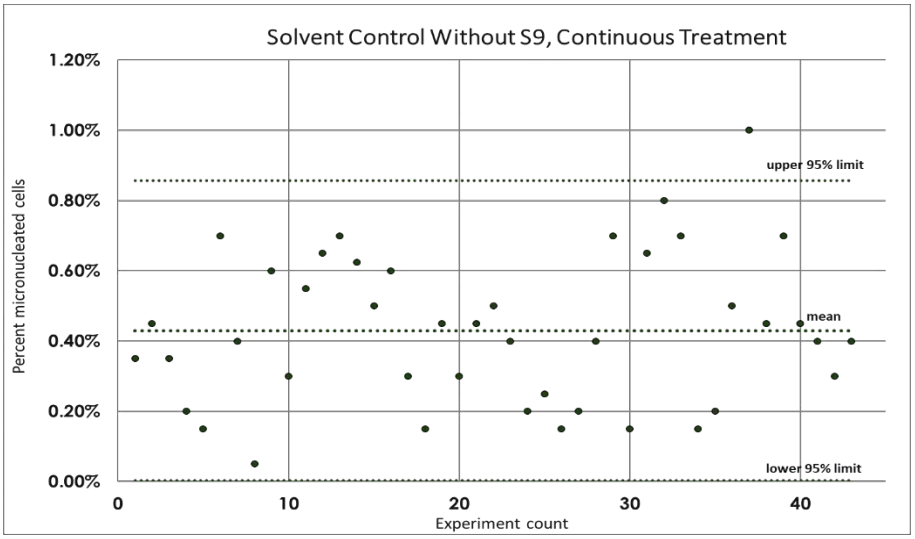
* Aqueous solvents – 17 Experiments; Organic solvents – 35 Experiments

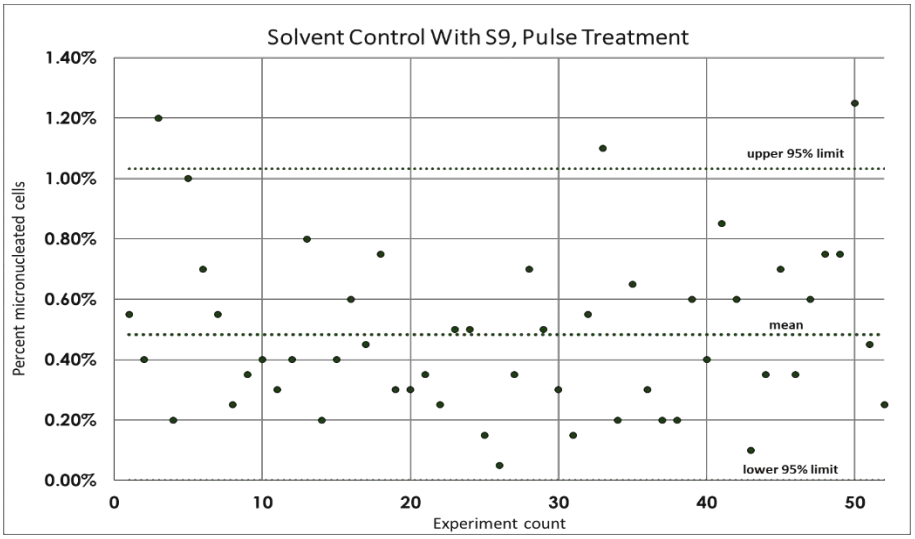
Positive Control without S9		
Micronucleated cells in %		
	Pulse treatment (4/40)	Continuous treatment (20/40)
	MMC	Demecolcin
No. of experiments	50	43
Mean	10.18	4.56
Min – Max	**4.70 – 19.10**	**2.50 – 7.45**
1x SD	3.31	1.23

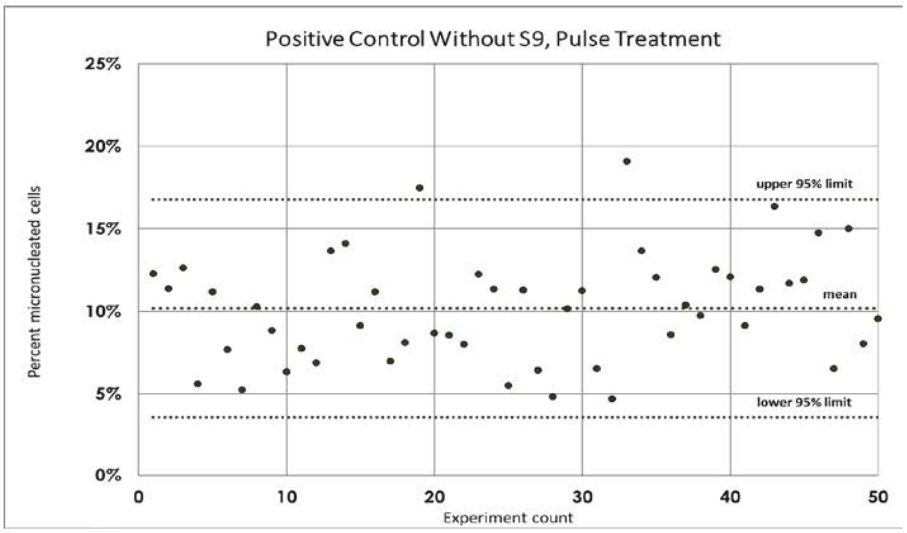
Positive Control with S9	
Micronucleated cells in %	
	Pulse treatment (4/40)
	CPA
No. of experiments	50
Mean	3.67
Min – Max	**2.15 – 6.90**
1x SD	1.19

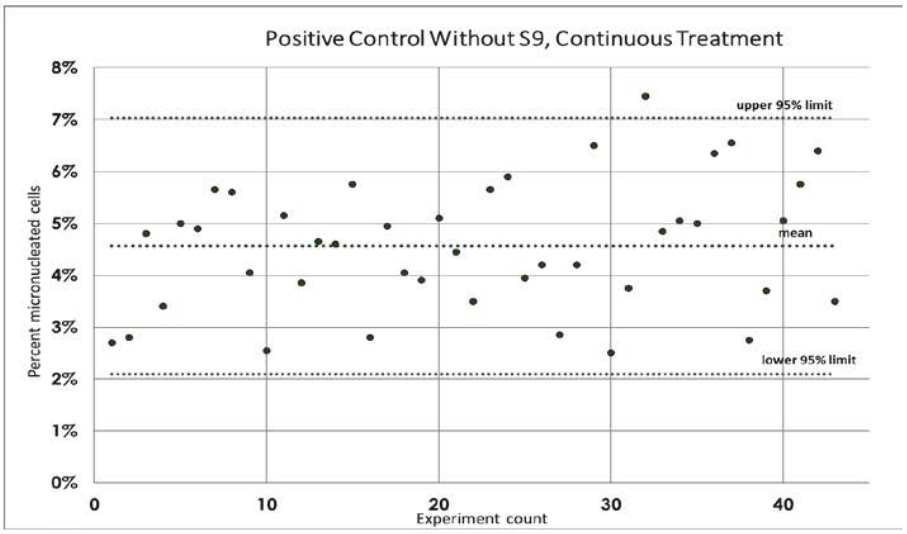
Historical Laboratory Control Data - Control Charts

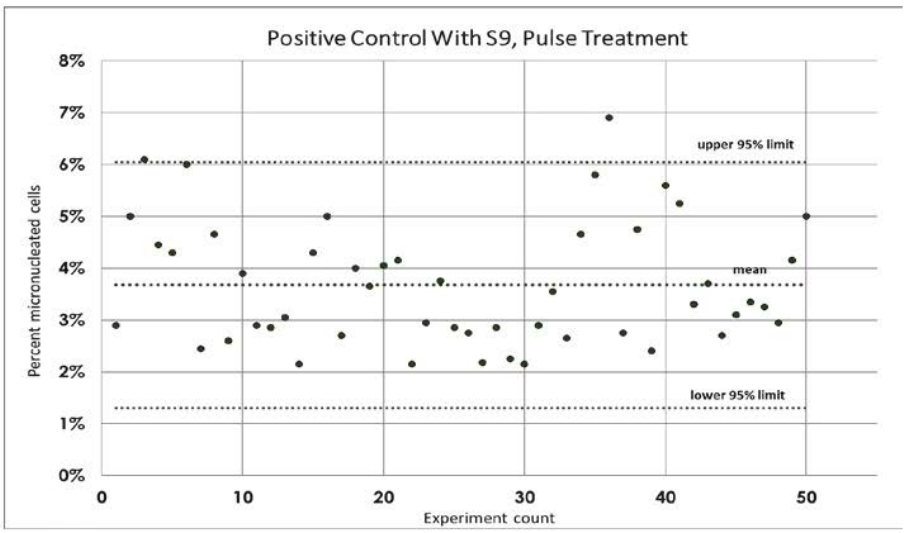







Positive Control Without S9, Pulse Treatment
Percent micronucleated cells
25%
20%
15%
10%
5%
0%
upper 95% limit
mean
lower 95% limit
0
10
20
30
40
50
Experiment count


Positive Control Without S9, Continuous Treatment
Percent micronucleated cells
8%
7%
6%
5%
4%
3%
2%
1%
0%
upper 95% limit
mean
lower 95% limit
0
10
20
30
40
Experiment count


Positive Control With S9, Pulse Treatment
Percent micronucleated cells
8%
7%
6%
5%
4%
3%
2%
1%
0%
upper 95% limit
mean
lower 95% limit
0
10
20
30
40
50
Experiment count

APPENDIX 2 Copy of GLP Certificate

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

ICCR-Roßdorf GmbH
Institute for Competent Contract Research
In den Leppsteinswiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**2** Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**3** Mutagenicity studies
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical and clinical chemistry testing

22.11.2018, 21.02.2019, 12. bis 14.03.2019
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Brandt

Dr. Astrid Brandt, Referentin, Wiesbaden, den **23. Oktober 2019**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hessisches Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80, D 65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority)

English name and address of the GLP Monitoring Authority:
Hessian Ministry for Environment, Energy, Agriculture and Consumer Protection;
Department II 10; P.O. Box 31 09; 65189 Wiesbaden
Translation of the seal inscription:
Hessian Ministry for Environment, Rural Regions and Consumer Protection

APPENDIX 3 Certificate of S9

ICCR-Roßdorf

CERTIFICATE

ENVIGO CRS S9 PREPARATION LOT NO. 050919D

Date of preparation: September 05, 2019
Release date: September 16, 2019

Protein assay: 29 mg protein / ml S9

Sterility: 0 colonies / ml S9 on glucose-minimal-agar

Salmonella typhimurium assay (AMES-test)

Treatment	µl S9 / plate	number of revertants in TA 98
negative	0	33
control	100	36
10 µg/plate	0	42
2-Aminoanthracene	100	2302
10 µg/plate	0	34
Benzo(a)pyrene	100	130

The S9 was obtained from the livers of male Wistar rats which received triple treatments of 80 mg / kg body weight Phenobarbital and ß-Naphthoflavone orally on consecutive days. The livers were prepared 24 hours after the last treatment.

H Pilawa

Quality Assurance Auditor
ICCR-Roßdorf GmbH H. Pilawa

18. SEP. 2019
Date

Dr. Steffen Naumann
Study Director
ICCR-Roßdorf GmbH

18. SEP. 2019
Date

ICCR-Roßdorf GmbH
In den Leppsteinswiesen 19
64380 Roßdorf, Germany
T +49 6154 8070 F +49 6154 89933

APPENDIX 4 Certificate of Analysis

SIGMA-ALDRICH®

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	CYCLOHEXANONE Selectophore®, >= 99.5 %
Product Number:	29135
Batch Number:	BCCB1352
Brand:	Sigma-Aldrich
CAS Number:	108-94-1
Formula:	$C_6H_{10}(=O)$
Formula Weight:	98.14
Quality Release Date:	18 FEB 2019
Recommended Retest Date:	JUL 2023

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	COLORLESS	COLORLESS
APPEARANCE (FORM)	LIQUID	LIQUID
PURITY (GC AREA %)	≥ 99.5 %	100.0 %
REFRACTIVE INDEX N20/D	1.450 - 1.452	1.451
WATER (COULOMETR.)	≤ 0.1 %	< 0.1 %
RESIDUE (EVAPORATION)	≤ 0.05 %	< 0.01 %
RESIDUE (FILTER TEST)	NO RESIDUE	NO RESIDUE

Dr. Reinhold Schwenninger
Quality Assurance
Buchs, Switzerland