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In vitro Mammalian Chromosome Aberration Test in Chinese Hamster V79 Cells with Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf.

Report

Version: Final

Eurofins Munich Study No.: STUGC20AA0542-3

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GLP / GMP FDA approved

1. Copy of the GLP Certificate

Bayerisches Landesamt für
Gesundheit und Lebensmittelsicherheit

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/EC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Eurofins BioPharma Product
Testing Munich GmbH
Behringstraße 6-8
82152 Planegg
(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

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

Kategorie 2	**Category 2**
Kategorie 3	**Category 3**
Kategorie 8	**Category 8**
Kategorie 9*	**Category 9***
Sonstige Prüfungen: Biologische und mikrobiologische Sicherheitsprüfungen an Medizinprodukten und Arzneimitteln; Auftragsarchivierung	*other tests:* biological and microbiological safety evaluation on medical devices and pharmaceuticals; contract archiving

Datum der Inspektion/Date of Inspection
(Tag.Monat.Jahr/day.month.year)

15.03.2018

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

The above mentioned test facility/test site 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/diesem Prüfstandort 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/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Schwabach, 26.04.2018

Dr. Peter Franke
Leiter der GLP-Landesleitstelle Bayern

GLP- Landesleitstelle Bayern
Bayerisches Landesamt für Gesundheit
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4. Preface

4.1. Abbreviations

Art.	Artikel *(article)*
ATCC	American Type Culture Collection
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
bw	body weight
CA	chromosome aberration
conc.	concentration
CPA	cyclophosphamide
DNA	deoxyribonucleic acid
e.g.	exempli gratia *(for example)*
EC	European Commission
EMS	ethylmethanesulfonate
EPA	Environmental Protection Agency
Eurofins Munich	Eurofins BioPharma Product Testing Munich GmbH
FBS	fetal bovine serum
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
i.e.	id est *(that is)*
KCl	potassium chloride
MEM	minimum essential medium
NADP	nicotinamide adenine di-phosphate
No.	number
OECD	Organisation for Economic Cooperation and Development
PBS	phosphate buffered saline
QAU	Quality Assurance Unit
RICC	relative increase in cell count
S9	microsomal fraction of rat liver homogenate
SOPs	Standard Operating Procedures
v/v	volume per volume

The following abbreviations are used in the tables with structural chromosomal aberrations:

g / ig	gap/ iso-gap; gaps are achromatic lesions of chromatid or chromosome type where no dislocation of chromosomal material is visible (independent of the size of the achromatic region).
b / ib	break / iso-break
f / if	fragment / iso-fragment
d / id	deletion / iso-deletion
ma	multiple aberration is defined as a metaphase containing more than 4 events [excluding gaps]; only exchanges are recorded additionally in these cells
ex	chromatid type exchange
cx	chromosome type exchange
cd	chromosomal disintegration (pulverisation)

4.2. General

Sponsor:	Sasol Germany GmbH Paul-Baumann-Str. 1 45772 Marl Germany
Study Monitor:	Dr. Birte Dreeßen
Test Facility:	Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany
Eurofins Munich Study No.:	STUGC20AA0542-3
Test Item:	Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf.
Title:	*In vitro* Mammalian Chromosome Aberration Test in Chinese Hamster V79 Cells with Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf.

4.3. Project Staff

Study Director:	Dr. Claudia Donath
Team Leader Operational QA GLP/GCP/ISO:	Uwe Hamann

4.4. Schedule

Arrival of the Test Item:	17 February 2020
Date of Solubility test:	27 March 2020
Study Initiation Date:	28 February 2020
Experimental Starting Date:	02 April 2020
Experimental Completion Date:	10 July 2020
Study Completion Date:	Date of the study director’s signature

5. Quality Assurance

5.1. GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on July 18, 2017 (BGBl. I S. 2774) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998 [3].

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

This study was assessed for compliance with the study plan and the Standard Operating Procedures of Eurofins Munich. The study and/or the test facility are inspected periodically by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

5.2. Guidelines

This study followed the procedures indicated by internal Eurofins Munich SOPs and the following internationally accepted guidelines and recommendations:

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473, "*In vitro* Mammalian Chromosome Aberration Test", adopted 29 July, 2016 [4].

The study was based on:

Commission regulation (EU) 2017/735 B.10 "*In Vitro* Mammalian Chromosomal Aberration Test", dated February 14, 2017 [5].

5.3. Archiving

For a period of 15 years (or shorter if in compliance with the GLP regulations) Eurofins Munich will store the records, materials and specimens in their scientific archives according to the GLP regulations.

The following records have to be stored according to the GLP regulations:

The final report, the study plan and documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study. Any document relating to the study will be discarded only with the prior consent of the sponsor.

The following materials and samples have to be stored according to the period of time specified in the GLP regulations:

A retained sample of the test item will be archived according to the GLP regulations, if possible, and will be discarded without the sponsor’s prior consent.

Other materials and specimens have to be stored according to the GLP regulations and disposed of after the respective archiving period with the sponsor’s prior consent.

Unless otherwise agreed in writing, the remaining test item will be discarded three months after the release of the report.

6. Statement of Compliance

Eurofins Munich Study No.:	STUGC20AA0542-3
Test Item:	Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf.
Title:	*In vitro* Mammalian Chromosome Aberration Test in Chinese Hamster V79 Cells with Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf.
Study Director:	Dr. Claudia Donath

This study performed in the test facility Eurofins Munich was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on July 18, 2017 (BGBl. I S. 2774) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998 [3].

There were no circumstances that may have affected the quality or integrity of the study. This statement does not include the solubility test.

Study Director: Dr. Claudia Donath

Claudia Donath

Date: 07 Aug 2020

7. Statement of the Quality Assurance Unit

Eurofins Munich Study No.:	STUGC20AA0542-3
Test Item:	Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf.
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Study Director:	Dr. Claudia Donath

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates. This statement does not include the solubility test.

Phase of QAU Inspection	**Date of QAU Inspection**	**Date of Reporting to the Study Director and Management**
Audit Final Study Plan:	28 February 2020	28 February 2020
Audit Experimental Phase (process-based):	09 January 2020	09 January 2020
Audit Final Report:	07 AUG 2020	07 AUG 2020

This report reflects the raw data.

Member of the
Quality Assurance Unit:

M. Rasch
.....
Print Name: Michaela Rasch

Date: 07 AUG 2020

8. Summary

8.1. Summary Results

To investigate the potential of Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf. to induce structural chromosome aberrations in Chinese Hamster V79 cells, an *in vitro* chromosome aberration assay was carried out.

The metaphases were prepared 21 h after start of treatment with the test item. The treatment interval was 4 h **without** and **with** metabolic activation in experiment I. In experiment II, the treatment interval was 21 h **without** metabolic activation. Duplicate cultures were treated at each concentration. 150 metaphases per culture were scored for structural chromosomal aberrations (for exceptions, see Table 17, Table 18, Table 19).

The following concentrations were selected for the microscopic analysis of chromosomal aberrations:

Experiment I:

Without and **with** metabolic activation, 4 h treatment, 21 h preparation interval:

25, 50 and 100 µg/mL

Experiment II

Without metabolic activation, 21 h treatment, 21 h preparation interval:

5, 10 and 25 µg/mL

In experiment I, precipitation of the test item was noted **without** and **with** metabolic activation at concentrations of 100 µg/mL and above. In experiment II, precipitation of the test item was seen **without** metabolic activation at concentrations of 25 µg/mL and above.

No decrease in cell count (decrease below 70% RICC) was noted in the concentration groups evaluated in experiment I **without** and **with** metabolic activation and in experiment II **without** metabolic activation (Table 7 and Table 9).

In both experiments, no biologically relevant increase of the aberration rates was noted after treatment with the test item **without** and **with** metabolic activation compared to the concurrent solvent control (Table 8 and Table 10).

In the experiments I and II **without** and **with** metabolic activation no biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item as compared to the solvent controls.

The Fisher´s exact test was performed to verify the results in the experiment. No statistically significant increase ($p < 0.05$) of cells with chromosomal aberrations was noted in the dose groups of the test item evaluated in experiment I and II **without** metabolic activation. A statistically significant increase ($p < 0.05$) of cells with chromosomal aberrations was noted at a concentration of 25 µg/mL in experiment I **with** metabolic activation that was considered not biologically relevant since no concentration related increase was observed (Table 12).

The χ^2 Test for trend was performed to test whether there is a concentration-related increase in chromosomal aberrations. No statistically significant increase was observed in all experimental conditions.

EMS (400 and 900 µg/mL) and CPA (1.11 µg/mL) were used as positive controls and induced distinct and biologically relevant increases of chromosomal aberrations, thus proving the efficiency of the test system to indicate potential clastogenic effects.

Table 1: Summary: Experiment I, without and with metabolic activation

	Dose Group	Concentration [µg/mL]	RICC [%]	Mean % Aberrant Cells		Historical Laboratory Negative Control Range	Precipi-tation[a]	Statistical Signifi-cance[b]
				incl. Gaps	excl. Gaps			
without 4 h treatment, 21 h preparation interval	C	0	112	**3.7**	**1.3**	**-0.28% - 3.49% aberrant cells excl. gaps**	/	/
	S	0	100	**5.8**	**3.1**		/	/
	3	25	79	**5.3**	**5.3**		-	-
	4	50	84	**4.3**	**2.7**		-	-
	5	100	90	**4.0**	**2.0**		+	-
	EMS	900	83	**10.7**	**8.7**		-	+
with 4 h treatment, 21 h preparation interval	C	0	94	**2.7**	**1.0**	**0.10% - 3.70% aberrant cells excl. gaps**	/	/
	S	0	100	**2.3**	**1.3**		/	/
	3	25	92	**6.0**	**4.3**		-	+
	4	50	97	**4.7**	**2.3**		-	-
	5	100	76	**4.0**	**3.3**		+	-
	CPA	1.11	85	**18.4**	**16.8**		-	+

C: Negative Control (Culture Medium)
S: Solvent Control (0.5% TFH; v/v)
EMS: Ethylmethanesulfonate
CPA: Cyclophosphamide
RICC: Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control groups. The cell count was determined by a cell counter per culture for each test group.
a: - without precipitation, + with precipitation
b: statistical significant increase compared to solvent controls (Fisher's exact test, $p < 0.05$), +: significant; -not significant;

Table 2: Summary: Experiment II, without metabolic activation

	Dose Group	Concentration [µg/mL]	RICC [%]	Mean % Aberrant Cells		Historical Laboratory Negative Control Range	Precipi-tation[a]	Statistical Signifi-cance[b]
				incl. Gaps	excl. Gaps			
Experiment II 21 h treatment, 21 h preparation interval	C	0	105	**4.3**	**1.7**	**-0.43% - 3.01% aberrant cells excl. gaps**	/	/
	S	0	100	**2.3**	**1.3**		/	/
	1	5	103	**3.3**	**1.0**		-	-
	2	10	100	**4.0**	**3.7**		-	-
	3	25	98	**3.7**	**2.0**		+	-
	EMS	400	88	**9.6**	**6.4**		-	+

C: Negative Control (Culture Medium)
S: Solvent Control (0.5% TFH; v/v)
EMS: Ethylmethanesulfonate
RICC: Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control groups. The cell count was determined by a cell counter per culture for each test group.
a: - without precipitation, + with precipitation
b: statistical significant increase compared to solvent controls (Fishers exact test, $p< 0.05$), +: significant; -not significant

8.2. Conclusion

In conclusion, it can be stated that during the described *in vitro* chromosome aberration test and under the experimental conditions reported, the test item Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf. did not induce structural chromosomal aberrations in the V79 Chinese Hamster cell line.

Therefore, the test item Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf. is considered to be non-clastogenic in this chromosome aberration test.

9. Introduction

9.1. Aim of the Study

The *in vitro* chromosomal aberration (CA) test is a genotoxicity test method for the detection of chromosomal aberration in cultured mammalian cells [7][8][9]. This assay is carried out using the Chinese Hamster V79 cell line.

Chromosome aberration assays aim to detect the induction of chromosome breakage (clastogenesis). Although mutagenic substances produce structural chromosome aberrations by a variety of mechanisms, the endpoint is a discontinuity in the chromosomal DNA which is left unrejoined or rejoined inaccurately, thus producing a mutated chromosome. Many of these changes are lethal to the cells during the first few cell cycles after their induction but are used as indicators of the presence of non-lethal changes such as reciprocal translocations, inversions and small deletions. These more subtle changes may have important consequences in both germ and somatic cells. Chromosomal mutations and related events are the cause of many human genetic diseases and there is substantial evidence that these changes, including oncogenes and tumour suppressor genes, are involved in carcinogenesis in humans and experimental systems. CAs are generally evaluated in first post treatment mitosis.

For treatment an asynchronous population of V79 cells in exponential growth should be used. A fixation time of around 20 h after treatment is appropriate since the guidelines recommend fixation times of about 1.5-fold of the normal cell cycle and the normal cell cycle of the used V79 cell line is 12 - 14 h. However, because there may be substances which induce very extensive mitotic delay at clastogenic concentrations or may display their clastogenicity only when cells have passed through more than one cell cycle since the beginning of treatment, an additional later sampling time (28 h) should be included in the second experiment, when indicated.

For soluble, non-toxic test items the highest concentration should correspond to 2 mg/mL, 2 µL/mL or 10 mM, whichever is the lowest. When the test chemical is not of defined composition, e.g. substance of unknown or variable composition, the top concentration may need to be higher (e.g. 5 mg/mL) in the absence of sufficient cytotoxicity. If the highest concentration is based on cytotoxicity the highest concentration chosen for evaluation should show a reduction of the mitotic index or relative increase in cell count to 45 ± 5%, which equals 55 ± 5% cytotoxicity. The lowest concentration should be in the range of the negative control.

For poorly soluble test chemicals that are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. Even if cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration producing turbidity or with a visible precipitate because artifactual effects may result from the precipitate. At the concentration producing a precipitate, care should be taken to assure that the precipitate does not interfere with the conduct of the test.

At least three concentrations of the test item with concentration intervals of approximately 2 to 3 fold should be used at fixation time of 20 ± 2 h.

Though the purpose of the assay is to detect structural chromosome aberrations, it is important to report polyploidy and/or endoreduplication when this is seen.

Reference mutagens are tested concurrently with the test item in order to demonstrate the sensitivity of the test system.

The assay is considered as acceptable, when all three experimental conditions are conducted: short term treatment **with** and **without** metabolic activation and long term treatment **without** metabolic activation. There is no requirement for verification of a clearly negative or positive result. In case the response is neither clearly negative nor clearly positive 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 or performing a repeat experiment could be useful.

9.2. Justification for the Selection of the Test System

The OECD Guideline for Testing of Chemicals Section 4, No 473 [4] – "*In vitro* Mammalian Chromosome Aberration Test", adopted 29 July, 2016 – recommends using a variety of cell lines or primary cell cultures (e.g. Chinese Hamster fibroblasts, human or other mammalian peripheral blood lymphocytes).

9.3. Justification for the Selection of the Test Method

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473 [4] – "*In vitro* Mammalian Chromosome Aberration Test", adopted 29 July, 2016 – recommends the treatment of proliferating cells in the presence and absence of a metabolic activation system.

10. Materials and Methods

10.1. Characterisation of the Test Item

The identity of the test item was inspected upon delivery at the test facility (e.g. test item name, batch no. and additional data were compared with the label) based on the following specifications provided by the sponsor. The following listed information applies to the sample as received.

Name:	Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf.
Product (Common Name/Code):	Alfol 20+
CAS No.:	1190630-03-5
Batch No.:	05513/MA
Physical State:	waxy
Colour:	yellow
Density:	0.8 g/cm³ (80 °C); 0.85 g/cm³ (15.6 °C)
Active Components:	100% (UVCB)
Storage Conditions:	room temperature, protected from light
Expiry Date:	01 June 2021
Safety Precautions:	The routine hygienic procedures were sufficient to assure personnel health and safety.

A certificate of analysis was provided by the sponsor, accepted by Eurofins Munich and can be found in the appendix (Appendix 3: Certificate of Analysis).

10.2. Preparation of the Test Item

A solubility test was performed with different solvents and vehicles up to the maximum recommended concentration of 5 mg/mL. Based on the results of the solubility test THF was used as solvent. To reach a final concentration of 0.05% THF v/v in cell culture medium, the test item stock solution in THF (200 fold concentrated) was rediluted in MEM + 0% FBS for short-term exposure or MEM+ 10% FBS for long-term exposure. The solvent was compatible with the survival of the cells and the S9 activity.
The osmolality was 347 mOsmol/kg in the highest tested concentration of 500 µg/mL (solvent control: 366 mOsmol/kg). The pH was within the physiological range of 7.0±0.4.

10.3. Controls

Negative and solvent as well as positive controls were included in each experiment.

Negative and Solvent Control

Negative controls (treatment medium) and solvent controls (THF, AppliChem Lot No. 10984207) were treated in the same way as all dose groups.

Positive Controls

Without metabolic activation

Name:	EMS; ethylmethanesulfonate
CAS No.:	62-50-0
Supplier:	Sigma-Aldrich
Catalogue No.:	M0880
Batch No.:	BCBZ8402
Expiry date:	March 2021
Dissolved in:	nutrient medium
Final concentrations:	400 and 900 µg/mL

The stability of the positive control substance in solution was proven by the clastogenic response in the expected range. The solution was prepared on the day of experiment.

Given that a high amount of historical control data was established at Eurofins Munich with EMS this substance was used instead of MMS (OECD Guideline for Testing of Chemicals No 473 [4]) as positive control.

With metabolic activation

Name:	CPA; cyclophosphamide
CAS No.:	50-18-0
Supplier:	Sigma-Aldrich
Catalogue No.:	C0768
Batch No.:	MKCF1756
Expiry date:	October 2020
Dissolved in:	nutrient medium
Final concentration:	0.83 and 1.11 µg/mL

CPA displays a good stability at room temperature. At 25 °C only 3.5% of its potency is lost after 24 h [10]. The solution was aliquoted and stored at ≤ -15 °C. Additionally, the stability of CPA in solution was proven by the clastogenic response in the expected range.

10.4. Test System

10.4.1. The Cells

V79 cells *in vitro* are widely used to examine the ability of chemicals to induce cytogenetic changes and thus identify potential carcinogens or mutagens. These cells are chosen because of their relatively small number of chromosomes (diploid number, 2n = 22), their high proliferation rate (doubling time of the Eurofins Munich V79 in stock cultures: 12 - 14 h) and a high plating efficiency of untreated cells (normally more than 50%). These facts are necessary for the appropriate performance of the study.

The V79 cells (ATCC, CCL-93) were stored over liquid nitrogen (vapour phase) in the cell bank of Eurofins Munich, as large stock cultures allowing the repeated use of the same cell culture batch in experiments. Routine checking of mycoplasma infections was carried out before freezing.

For the experiment thawed cultures were set up in 75 cm^2 cell culture plastic flasks at 37 °C in a 5% carbon dioxide atmosphere (95% air). 5 x 10^5 cells per flask were seeded in 15 mL of MEM (minimum essential medium) supplemented with 10% FBS (fetal bovine serum) and subcultures were made 3-4 days after seeding.

10.4.2. Culture Medium

Complete Culture Medium

MEM medium supplemented with:

10	% (v/v)	fetal bovine serum (FBS)
100 U/100	µg/mL	penicillin/streptomycin solution
2	mM	L-glutamine
2.5	µg/mL	amphotericin
25	mM	HEPES

Also used for the long-term treatment and the post incubation.

Treatment Medium (short-term exposure)

Complete culture medium without FBS.

10.4.3. Mammalian Microsomal Fraction S9 Homogenate

An advantage of using *in vitro* cell cultures is the accurate control of the concentration and exposure time of cells to the test item under study. However, due to the limited capacity of cells growing *in vitro* for metabolic activation of potential mutagens an exogenous metabolic activation system is necessary. Many substances only develop mutagenic potential when they are metabolized by the mammalian organism. Metabolic activation of substances can be achieved by supplementing the cell cultures with liver microsome preparations (S9 mix).

The S9 liver microsomal fraction was obtained from Trinova Biochem GmbH, Giessen, Germany. Male Sprague Dawley rats were induced with phenobarbital / β-naphthoflavone.

The following quality control determinations were performed by Trinova Biochem GmbH:

a) Alkoxyresorufin-0-dealkylase activities
b) Test for the presence of adventitious agents
c) Promutagen activation (including biological activity in the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene)

A stock of the supernatant containing the microsomes was frozen in aliquots of 5 mL and stored at ≤ -75 °C.

The protein concentration in the S9 preparation (Lot: 4180) was 39.2 mg/mL.

10.4.4. S9 Mix

An appropriate quantity of the 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. The final percentage of S9 mix in cell culture medium is 5% (v/v).

Cofactors were added to the S9 mix to reach the following concentrations:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	Glucose-6-phosphate
5	mM	NADP

in 100 mM sodium-phosphate-buffer pH 7.4. During the experiment the S9 mix was stored on ice.

10.5. Experimental Design

Table 3: Schematic presentation of the test procedure (V79 cells)

Preparation day 1	Seeding of the cells in 25 cm² culture flasks
1. Day of the Test: Incubation (approx. 48 h after seeding of the cells)	The culture medium was replaced by serum-free medium (short-term incubation) or by 10% serum-containing medium (long-term treatment) containing different concentrations of the test item and S9 mix (only with metabolic activation). Beginning of the treatment Incubation for 4 h for short-term treatment and 21 h for long-term treatment. Additional negative and/or solvent and positive controls were treated in the same way. Cytotoxicity and precipitation was determined after the treatment period of the cultures.
2. Day of the Test: Preparation of the Cultures	Colcemid was added to the cultures of approximately 2 h before the preparation. The cultures were harvested 21 h after beginning of treatment. After centrifugation, the supernatant was discarded and cells were resuspended in approximately 7 mL hypotonic solution (0.4 % KCl). After removal of the hypotonic solution by centrifugation cells were fixed with methanol/glacial acetic acid (3:1, v/v). The fixation procedure was done at least two times and afterwards cells were spread onto slides.
3. Day of the Test: Staining of the Cells with Giemsa	The air dried slides were stained with Giemsa solution.
Analysis of Metaphase Cells	All structural chromosome aberrations such as breaks, fragments, deletions, exchanges and chromosomal disintegration were recorded. Gaps were recorded as well but are not included in the calculation of the aberration rates.
Relative Increase in Cell Count	Cytotoxicity was assessed by the relative increase in cell count. Values were compared with negative/solvent controls.

10.5.1. Seeding of the Cultures

Three or four days old stock cultures (in exponential growth) more than 50% confluent were rinsed with Ca-Mg-free PBS solution prior to the trypsin treatment. Cells subsequently were trypsinised with a solution of 0.05% trypsin in Ca-Mg-free PBS at 37 °C for about 5 min. By adding complete culture medium the detachment was stopped and a single cell suspension was prepared. About 1 x 10^4 cells/mL were seeded into cell culture flasks with complete culture medium.

10.5.2. Pre-Experiment for Toxicity

A pre-experiment was conducted under identical conditions as described for the main experiment. The following concentrations were tested **without** and **with** S9 mix:

10, 25, 50, 100, 250, 500, 750, 1000, 1250 µg/mL

The concentration of 1250 µg/mL was considered to be the highest test concentration used in this test system following the recommendation of the corresponding OECD testing guideline 487 [4] and based on the physical-chemical properties of the test item. Since the organic solvent THF (Merck; Charge: 109842078118; MHD: March 2021) was used, which can only be applied at a final concentration of 0.5% (v/v) in cell culture, the maximum technically feasible concentration used in this study was determined to be 1250 µg/mL.

10.5.3. Exposure Concentrations

On the basis of the data and the observations from the pre-experiment and taking into account the recommendations of the guidelines, the following concentrations were selected for the main experiments I and II.

The dose group selection for microscopic analyses of chromosomal aberrations was based in accordance with the recommendations of the guidelines.

Table 4: Exposure concentrations

S9 Mix	Exp. interval	Prep. interval	Concentrations in µg/mL										
Experiment I													
-	4 h	21 h	-	-	-	-	5	10	**25**	**50**	**100P**	250P	500P
+	4 h	21 h	-	-	-	-	5	10	**25**	**50**	**100P**	250P	500P
Experiment II													
-	21 h	21 h	-	-	-	-	**5**	**10**	**25P**	50P	100P	250P	500P

Evaluated experimental points are shown in bold letters
P Precipitation was observed at the end of treatment

10.6. Experimental Performance

10.6.1. Treatment

Experiment I: Short-term exposure 4 h (without and with S9 mix)

Two days after seeding of the cells, the culture medium was replaced with serum-free medium containing the different concentrations of the test item and S9 mix (only with metabolic activation). Additional negative and positive controls were performed **without** and **with** metabolic activation.4 h after the treatment the cultures were washed twice with PBS and then the cells were cultured in complete culture medium (see 10.4.2) until preparation of the cells (21 h after short term treatment).

Experiment II: Long-term exposure 21 h (without S9 mix)

For the 21 h treatment time two days after seeding of the cells the culture medium is replaced with complete medium containing the different concentrations of the test item. This medium is not changed until preparation of the cells (see 10.4.2).

All cultures were incubated at 37 ± 1 °C in a humidified atmosphere with 5.0% CO_2 (95.0% air).

Table 5: Study Design

	without S9 mix		*with S9 mix*
	Exp. I	**Exp. II**	**Exp. I**
Treatment period	4 h	21 h	4 h
Recovery time	17 h	-	17 h
Preparation interval	21 h	21 h	21 h

10.6.2. Preparation of the Cultures

Colcemid (0.2 µg/mL culture medium) was added to the cultures around 17.5 h after the start of the treatment. About 2.5 h later preparation was started. At first cells were trypsinated and resuspended in about 9 mL complete culture medium. An aliquot of each culture was removed to determine the cell count by a cell counter (AL-Systems).Then cultures were transferred into tubes and incubated with hypotonic solution (0.4% KCl) for 15-20 min. After hypotonic treatment the cells were fixed at least two times with 3 + 1 methanol + glacial acetic acid and spread onto the slides. After the fixation steps the slides were dried and stained with Giemsa. The slides were coverslipped using 2-3 drops of Eukitt$^{(R)}$. Afterwards they were air dried.

10.6.3. Analysis of Metaphase Cells

All slides, including those of positive, negative and (if necessary) solvent controls were independently coded before microscopic analysis. Evaluation of the cultures was performed according to the standard protocol of the "Arbeitsgruppe der Industrie, Cytogenetik" [11] using microscopes with 100x oil immersion objectives. If observed, structural chromosomal aberrations, including breaks, fragments, deletions, exchanges and chromosomal disintegration were recorded. Gaps were recorded as well but not included in the calculation of the aberration rates. The definition of a gap is as follows: an achromatic region (occurring in one or both chromatids) independent of its width. The remaining visible chromosome regions should not be dislocated either longitudinally or laterally. If available, 300 well spread metaphases (containing 22 ± 1 centromeres) per concentration and validity controls were scored for cytogenetic damage. The number of metaphases scored can be reduced when high numbers of cells with chromosome aberration were observed and the chemical was considered as clearly positive [4].

To describe a cytotoxic effect the relative increase in cell count (RICC) was determined.

The RICC was calculated as follows:

$$\text{RICC (\%)} = \frac{\text{N-N}_0\text{ (treated)}}{\text{N-N}_0\text{ (untreated)}} \times 100$$

N_0: initial cell number; N: cell number at end of treatment

Additionally the number of polyploid cells is scored. Polyploid means a near tetraploid karyotype in the case of this aneuploid cell line.

10.7. Data Recording

The data generated were recorded in the raw data. The results are presented in tables, including experimental groups with the test item, positive, negative and (if necessary) solvent controls. The experimental unit is the cell, and therefore the percentage of cells with structural aberrations is evaluated. Different types of chromosome aberrations are listed with their numbers of frequencies for experimental and control groups. Gaps are recorded separately and reported but generally not included in the aberration frequency. Concurrent measurements of cytotoxicity were also recorded.

10.8. Acceptability of the Assay

The chromosomal aberration assay is considered acceptable if it meets the following criteria:

- the number of aberration found in the negative and/or solvent controls falls within the range of historical laboratory control data / is considered acceptable for addition to the laboratory historical negative control database.
- concurrent positive controls should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control;
- the proliferation criteria in the solvent control should be similar to the corresponding negative control (where applicable);
- All three experimental conditions were tested unless one resulted in positive results;
- Adequate number of cells and concentrations are analyzable;
- The criteria for the selection of top concentration are consistent with those described earlier (10.5.3)

10.9. Evaluation of Results

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

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control;

b) the increase is dose-related when evaluated with an appropriate trend test;

c) any of the results are outside the 95% control limits of the historical negative control data.

When all of these criteria are met, the test chemical is then considered able to induce chromosomal aberrations in cultured mammalian cells in this test system.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control;

b) there is no concentration-related increase when evaluated with an appropriate trend test;

c) all results are inside the 95% control limits of the historical negative control data.

The test chemical is then considered unable to induce chromosomal aberrations in cultured mammalian cells in this test system.

11. Deviations from the Study Plan

There was the following deviation from the study plan:

- **Concerning:**

Study Director, study plan, p. 2 and 7

Study Plan:

Dr. Ewa Schmidt

Report:

Dr. Claudia Donath

Reason:

Project handover due to maternity leave.

This deviation did not influence the quality or integrity of the present study.

12. Results and Discussion

12.1. Results

12.1.1. Pre-Experiment for Toxicity

According to the guidelines the highest recommended concentration was 5000 µg/mL. Based on the physical-chemical properties of the test item THF which can only be used at a final concentration of 0.5% (v/v) in cell culture, the maximum technically feasible concentration used in this study was determined to be 1250 µg/mL. The test item was dissolved in THF and re-diluted in cell culture medium. Precipitation of the test item was noted at concentrations of 100 µg/mL and higher. The highest dose group evaluated in the pre-experiment was 1250 µg/mL. The relative increase in cell count (RICC) was used as parameter for toxicity. The concentrations tested in the main experiment were based on the results obtained in the pre-experiment (Table 6).

Table 6: Test for Cytotoxicity

Dose Group	Concentration [µg/mL]	Cell Count		RICC	Precipitate (+/-)
		Culture 1	Mean	[%]	
without metabolic activation					
C	0	178.12	89.06	86	-
S	0	203.68	101.84	100	-
1	10	153.50	76.75	73	-
2	25	117.52	58.76	53	-
3	50	173.38	86.69	84	-
4	100	108.05	54.02	48	+
5	250	106.15	53.08	47	+
6	500	12.32	6.16	-4	+
7	750	123.20	61.60	56	+
8	1000	110.89	55.44	49	+
9	1250	84.28	42.14	35	+
with metabolic activation					
C	0	154.45	77.22	72	-
S	0	207.47	103.74	100	-
1	10	211.26	105.63	102	-
2	25	133.61	66.81	61	-
3	50	182.85	91.43	87	-
4	100	188.53	94.27	90	+
5	250	190.43	95.21	91	+
6	500	100.47	50.24	43	+
7	750	120.36	60.18	54	+
8	1000	103.31	51.66	44	+
9	1250	159.18	79.59	74	+

RICC: Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control. The cell count was determined by a cell counter per culture for each test group.
C: Negative Control (Culture Medium)
S: Solvent Control (0.5% THF; v/v)

12.1.2. Summary of Experiment I and Experiment II

Table 7: Experiment I - Summary of Cytotoxicity Data

Dose Group	Concentration [µg/mL]	Cell Count Culture 1	Cell Count Culture 2	Cell Count Mean	RICC [%]	Precipitate (+/-)
without metabolic activation						
C	0	198.95	189.48	194.22	112	-
S	0	168.65	180.01	174.33	100	-
3	25	134.56	144.98	139.77	79	-
4	50	133.61	161.07	147.34	84	-
5	100	142.14	173.38	157.76	90	+
EMS	900	143.08	148.76	145.92	83	-
with metabolic activation						
C	0	154.45	195.16	174.80	94	-
S	0	176.22	194.22	185.22	100	-
3	25	162.02	180.96	171.49	92	-
4	50	136.45	222.62	179.54	97	-
5	100	132.67	152.55	142.61	76	+
CPA	1.11	134.56	158.23	146.40	78	-

RICC: Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control. The cell count was determined by a cell counter per culture for each test group.

C: Negative Control (Culture Medium)
S: Solvent Control (0.5% THF; v/v)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

Table 8: Experiment I – Summary of Aberration Rates

Dose Group	Concentration [µg/mL]	Treatment Time	Fixation Interval	mean % aberrant cells		Precipitation
				incl. Gaps	excl. Gaps	
without metabolic activation						
C	0	4	21	3.7	1.3	-
S	0	4	21	5.8	3.1	-
3	25	4	21	5.3	5.3	-
4	50	4	21	4.3	2.7	-
5	100	4	21	4.0	2.0	+
EMS	900	4	21	10.7	8.7	-
with metabolic activation						
C	0	4	21	2.7	1.0	-
S	0	4	21	2.3	1.3	-
3	25	4	21	6.0	4.3	-
4	50	4	21	4.7	2.3	-
5	100	4	21	4.0	3.3	+
CPA	1.11	4	21	18.4	16.8	-

300 cells evaluated for each concentration, except for the positive control with metabolic activation (CPA: 125 cells) due to a clearly positive increase in chromosomal aberrations.

C: Negative Control (Culture Medium)
S: Solvent Control (0.5% THF; v/v)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

Table 9: Experiment II - Summary of Cytotoxicity Data

Dose Group	Concentration [µg/mL]	Cell Count			RICC	Precipitate (+/-)
		Culture 1	Culture 2	Mean	[%]	
without metabolic activation						
C	0	250.08	243.45	246.77	105	-
S	0	237.77	234.93	236.35	100	-
1	5	233.04	253.87	243.45	103	-
2	10	227.36	244.40	235.88	100	-
3	25	241.56	220.73	231.14	98	+
EMS	400	208.42	208.42	208.42	88	-

RICC: Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control. The cell count was determined by a cell counter per culture for each test group.
C: Negative Control (Culture Medium)
S: Solvent Control (0.5% THF; v/v)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)

Table 10: Experiment II - Summary of Aberration Rates

Dose Group	Concentration [µg/mL]	Treatment Time	Fixation Interval	mean % aberrant cells		
				incl. Gaps	excl. Gaps	Precipitation
without metabolic activation						
C	0	21	21	4.3	1.7	-
S	0	21	21	2.3	1.3	-
1	5	21	21	3.3	1.0	-
2	10	21	21	4.0	3.7	-
3	25	21	21	3.7	2.0	+
EMS	400	21	21	9.6	6.4	-

300 cells evaluated for each concentration, except for the positive control (EMS: 450 cells) to support the outcome for the positive controls.

C: Negative Control (Culture Medium)
S: Solvent Control (0.5% THF; v/v)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)

12.2. Biometry

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the Fischer´s exact test. The p value was used as a limit in judging for significance levels in comparison with the concurrent solvent control. Aberrant cells without gaps were only used for the calculation. Gaps are recorded separately and reported but generally not included in the total aberration frequency calculation according to the guideline.

Table 11: Biometry - Experiment I, without metabolic activation

solvent control versus test group	Concentration [µg/mL]	Treatment Time [h]	Aberrant Cells (excl. gap)	Significance	p Value
S	0	4	3.1	/	/
3	25	4	5.3	-	0.1330
4	50	4	2.7	-	0.8271
5	100	4	2.0	-	0.4887
EMS	900	4	8.7	+	0.0014

+: significantly increased
-: not significant
EMS: Positive Control (Ethylmethanesulfonate)

Table 12: Biometry - Experiment I, with metabolic activation

#4 Control versus Test Group	Concentration [µg/mL]	Treatment Time [h]	Aberrant Cells (excl. gap)	Significance	p Value
S	0	4	1.3	/	/
3	25	4	4.3	+	0.0459
4	50	4	2.3	-	0.5450
5	100	4	3.3	-	0.1744
CPA	1.11	4	16.8	+	<0.0001

+: significantly increased
-: not significant
CPA: Positive Control (Cyclophosphamide)

Table 13: Biometry - Experiment II, without metabolic activation

Solvent control versus Test Group	Concentration [µg/mL]	Treatment Time [h]	Aberrant Cells (excl. gap)	Significance	p Value
S	0	21	1.3	/	/
1	5	21	1.0	-	1.0000
2	10	21	3.7	-	0.1139
3	25	21	2.0	-	0.7518
EMS	400	21	6.4	+	0.0008

+: significantly increased
-: not significant
EMS: Positive Control (Ethylmethanesulfonate)

Table 14: Biometry – Trend test

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the χ^2 test for trend. The p value was used as a limit in judging for significance levels.

Experiment	Treatment Time [h]	Significance	P Value
Exp. I without metabolic activation	4	+*	0.0229
Exp. I with metabolic activation	4	-	0.4951
Exp. II without metabolic activation	21	-	0.0816

+: significant
-: not significant
+*: statistical significance was noted but not considered for further evaluation since a decrease was detected.

Statistical significance: statistical significant concentration-related increase in cells with chromosomal aberrations (χ^2 test for trend, $p < 0.05$).

12.3. Discussion

In an *in vitro* chromosome aberration assay, the test item Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf. was investigated for the potential to induce structural chromosomal aberrations in Chinese Hamster V79 cells in the absence and presence of metabolic activation with S9 homogenate.

The selection of the concentrations used in experiment I and II was based on data from the solubility test and the pre-experiment, which were performed according to the guidelines.

In experiment I **without** and **with** metabolic activation 100 µg/mL were selected as highest dose groups for the microscopic analysis of chromosomal aberrations. In experiment II **without** metabolic activation 25 µg/mL was selected as highest dose group for the microscopic analysis of chromosomal aberrations.

The chromosomes were prepared 21 h after start of treatment with the test item. The treatment intervals were 4 h **without** and **with** metabolic activation (experiment I) and 21 h **without** metabolic activation (experiment II). Duplicate cultures were set up. 150 metaphases per culture were scored for structural chromosomal aberrations (for exceptions see Table 17, Table 18 and Table 19).

As an exception the number of metaphases scored was reduced to 125 metaphases for the positive control in experiment I with metabolic activation (1.11 µg/mL CPA; Table 18) due to high numbers of cells with chromosome aberrations reported. In experiment II the number of metaphases scored for the positive control was 450 metaphases (400 µg/mL EMS; Table 19).

The following concentrations were selected for microscopic analysis:

Experiment I:

without metabolic activation:	25, 50 and 100 µg/mL
with metabolic activation:	25, 50 and 100 µg/mL

Experiment II:

without metabolic activation:	5, 10 and 25 µg/mL

12.3.1. Precipitation

The test item was dissolved in THF and rediluted in cell culture medium (MEM medium) at a ratio of 1:200 to achieve the final test item concentrations and a final THF concentration of 0.5% (v/v). Precipitation of the test item was noted at 100 µg/mL and above **without** and **with** metabolic activation in experiment I. In experiment II precipitation was observed at a concentration of 25 µg/mL and above.

12.3.2. Toxicity

All concentrations evaluated in experiment I **with** and **without** metabolic activation showed no biologically relevant decrease in cell count (decrease below 70% RICC, Table 7).

In experiment II **without** metabolic activation no biologically relevant decrease of the relative mitotic index (decrease below 70% RICC) was noted in all dose groups evaluated (Table 9).

These results indicated that the test item has no cytotoxic effects in this test system up to the highest concentration evaluated.

12.3.3. Clastogenicity

There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells with chromosome aberrations for at least one of the dose groups, which is higher than the laboratory negative control range.

In experiment I **without** metabolic activation (Table 8) the aberration rates of the negative control (1.3%) and solvent control (3.1%) were within the historical control limits of the negative control (-0.28 to 3.49% aberrant cells exclusive gaps; Table 15). The concentration of 25 µg/mL showed a number of aberrant cells (5.3%) that lay above the historical control data of the testing facility (-0.28 to 3.49% aberrant cells exclusive gaps; Table 15). However, the increase was not statistically significant compared to the concurrent solvent control and no concentration related increase was observed. Thus, this increase was considered as not biologically relevant. All other concentrations evaluated (50 µg/mL (2.7%) and 100 µg/mL (2.0%)) were within the historical control data of the testing facility.

In experiment I **with** metabolic activation (Table 8), the aberration rates of the negative control (1.0%), the solvent control (1.3%) and the dose groups 50 µg/mL (2.3%) and 100 µg/mL (3.3%) were within the historical control data of the testing facility (0.10 to 3.70% aberrant cells exclusive gaps, Table 15). The lowest test concentration of 25 µg/mL showed an aberration rate of 4.3% which lay above the historical control data of the testing facility and was marginally statistically significant compared to the concurrent solvent control. Since no concentration related increase was observed, this increase was considered as not biologically relevant.

In experiment II **without** metabolic activation (Table 10), the aberration rates of the negative control (1.7%), the solvent control (1.3%) and the dose groups 5 µg/mL(1.0%) and 25 µg/mL (2.0%) treated with the test item, were within the historical control data of the testing facility (-0.43 to 3.01% aberrant cells exclusive gaps, Table 15). At a concentration of 10 µg/mL (3.7%) the aberration rate lay above the historical control data of the testing facility (-0.43 to 3.01% aberrant cells exclusive gaps, Table 15). However, this increase was not statistically significant compared to the concurrent solvent control and no concentration related increase was observed. Thus, it was considered as not biologically relevant.

The Fisher´s exact test was performed to verify the results in the experiment (Table 11, Table 12 and Table 13). A statistically significant increase ($p < 0.05$) of cells with chromosomal aberrations was noted at a concentration of 25 µg/mL in experiment I **with** metabolic activation. As no concentration related increase was observed, this was considered as not biologically relevant. All other concentrations evaluated in experiment I **without** and **with** metabolic activation and experiment II **without** metabolic activation showed no statistically significant increase.

The χ^2 Test for trend was performed to test whether there is a concentration-related increase in chromosomal aberrations (Table 14). No statistically significant increase was observed in experiment I **without** and **with** metabolic activation and in experiment II **without** metabolic activation.

EMS (400 and 900 µg/mL) and CPA (1.11 µg/mL) were used as positive controls and induced distinct and biologically relevant increases in cells with structural chromosomal aberrations, thus proving the ability of the test system to indicate potential clastogenic effects.

12.3.4. Polyploid Cells

Table 17, Table 18 and Table 19 show the number of polyploid metaphases. No biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item.

13. Conclusion

In conclusion, it can be stated that during the described *in vitro* chromosome aberration test and under the experimental conditions reported, the test item Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf. did not induce structural chromosomal aberrations in the V79 Chinese Hamster cell line.

Therefore, the test item Ethene, homopolymer, oxidized, hydrolyzed, distn. residues, from C16-18 alcs. manuf. is considered to be non-clastogenic in this chromosome aberration test.

14. Distribution of the Report

Original:	Eurofins Munich
Copy:	sponsor

15. References

15.1. Guidelines

[1] Chemikaliengesetz (“Chemicals Act”) of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on July 18, 2017 (BGBl. I S. 2774)

[2] Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis (“Consensus Document of the National and Länder Working Party on Good Laboratory Practice“) on the archiving and storage of records and materials, 5 May 1998

[3] OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998

[4] Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473, "In vitro Mammalian Chromosome Aberration Test", adopted 29 July, 2016.

[5] Commission regulation (EU) 2017/735 B.10 “*In Vitro* Mammalian Chromosomal Aberration Test”, dated February 14, 2017.

15.2. Literature

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15.3. Internal Eurofins Munich SOPs

Standard Operating Procedures (SOPs), No. 15-1-2, No. 15-2-6, No. 15-2-8

16. Appendix

16.1. Appendix 1: Historical Laboratory Control Data

Table 15: Historical Laboratory Control Data of the negative control (2014 - 2019)

	NC **Number of aberrant cells** *metabolic activation*					
	with (4h)		without (4h)		without (21 h)	
	+gaps	-gaps	+gaps	-gaps	+gaps	-gaps
mean [%]	3.5	1.9	3.2	1.6	2.7	1.3
SD	1.46	0.90	1.52	0.94	1.29	0.86
RSD [%]	41.9	47.3	47.8	58.9	48.7	66.8
min [%]	0.3	0.0	0.0	0.0	0.3	0.0
max [%]	7.0	4.0	9.0	4.0	5.5	3.0
n	89	89	73	73	59	59
LCL	0.56	0.10	0.14	-0.28	0.07	-0.43
UCL	6.38	3.70	6.22	3.49	5.24	3.01

NC: Negative Control (cell culture medium)
mean: mean number of aberrant cells
SD: Standard Deviation
RSD: relative Standard Deviation
min.: minimum number of aberrant cells
max.: maximum number of aberrant cells
n: Number of assays
LCL: Lower control limit (95%, mean-2SD)
UCL: Upper control limit (95%, mean+2SD)

Table 16: Historical Laboratory Control Data of the positive control (2014 - 2019)

	PC **Number of aberrant cells** *metabolic activation*			
	with (CPA)		without (EMS)	
	+gaps	-gaps	+gaps	-gaps
mean [%]	12.0	9.5	13.4	11.1
SD	3.70	3.30	5.78	5.65
RSD [%]	30.9	34.6	43.2	51.0
min [%]	7.0	4.5	6.7	5.3
max [%]	26.0	24.0	34.4	32.0
n	80	80	121	121
LCL	4.56	2.93	1.82	-0.22
UCL	19.36	16.13	24.95	22.37

PC: Positive Control (EMS **without** metabolic activation, CPA **with** metabolic activation)
mean: mean number of aberrant cells
SD: Standard Deviation
RSD: relative Standard Deviation
min.: minimum number of aberrant cells
n: Number of assays
LCL: Lower control limit (95%, mean-2SD)
UCL: Upper control limit (95%, mean+2SD)
max.: maximum number of aberrant cells
The historical data **without** metabolic activation comprise the 4 h and 21 h treatment interval.

16.2. Appendix 2: Raw Data

16.2.1. Main Experiment I

Table 17: Experiment I - Structural Chromosomal Aberrations, *without* metabolic activation: 4 h treatment, 21 h fixation period.

Dose Group	Concentration [µg/mL]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps g	Gaps ig	Types of Aberrations Found: Chromatid Types b	f	d	ex	Chromosome Types ib	if	id	cx	Other ma	cd
C	0	1	150	0	6	3	2	1	2	1	0	1	0	0	0	0	0	0
		2	150	0	5	1	3	1	1	0	0	0	0	0	0	0	0	0
		total	**300**	**0**	**11**	**4**	**5**	**2**	**3**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**
S	0	1	150	0	5	3	1	1	3	0	0	0	0	0	0	0	0	0
		2	300	2	21	11	13	1	5	0	0	2	0	2	0	2	0	0
		total	**450**	**2**	**26**	**14**	**14**	**2**	**8**	**0**	**0**	**2**	**0**	**2**	**0**	**2**	**0**	**0**
3	25	1	150	0	8	8	0	0	6	0	0	3	0	2	0	0	0	0
		2	150	0	8	8	0	0	5	0	0	0	0	3	0	0	0	0
		total	**300**	**0**	**16**	**16**	**0**	**0**	**11**	**0**	**0**	**3**	**0**	**5**	**0**	**0**	**0**	**0**
4	50	1	150	0	10	7	4	0	4	2	0	1	0	1	0	1	0	0
		2	150	2	3	1	3	0	1	0	0	0	0	0	0	0	0	0
		total	**300**	**2**	**13**	**8**	**7**	**0**	**5**	**2**	**0**	**1**	**0**	**1**	**0**	**1**	**0**	**0**
5	100	1	150	1	6	4	1	1	2	1	0	0	0	0	0	0	0	1
		2	150	0	6	2	3	1	1	0	0	0	0	1	0	0	0	0
		total	**300**	**1**	**12**	**6**	**4**	**2**	**3**	**1**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**1**
EMS	900	1	150	0	15	14	1	1	8	2	0	3	0	1	0	1	0	0
		2	150	1	17	12	7	0	7	1	0	5	0	0	0	0	0	0
		total	**300**	**1**	**32**	**26**	**8**	**1**	**15**	**3**	**0**	**8**	**0**	**1**	**0**	**1**	**0**	**0**

C: Negative Control (Culture Medium)
S: Solvent Control (0.5% THF; v/v)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

Table 18: Experiment I - Structural Chromosomal Aberrations, *with* metabolic activation: 4 h treatment, 21 h fixation period.

Dose Group	Concentration [µg/mL]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps g	Gaps ig	Types of Aberrations Found: Chromatid Types b	f	d	ex	Chromosome Types ib	if	id	cx	Other ma	cd
		1	150	0	5	2	2	1	2	0	0	0	0	0	0	0	0	0
C	**0**	2	150	1	3	1	2	0	1	0	0	0	0	0	0	0	0	0
		total	**300**	**1**	**8**	**3**	**4**	**1**	**3**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
		1	150	1	4	3	2	0	1	0	0	0	0	2	0	0	0	0
S	**0**	2	150	0	3	1	2	0	1	0	0	0	0	0	0	0	0	0
		total	**300**	**1**	**7**	**4**	**4**	**0**	**2**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**
		1	150	0	10	8	2	0	3	1	0	1	0	4	0	0	0	0
3	**25**	2	150	1	8	5	3	0	1	2	0	1	0	1	0	0	0	0
		total	**300**	**1**	**18**	**13**	**5**	**0**	**4**	**3**	**0**	**2**	**0**	**5**	**0**	**0**	**0**	**0**
		1	150	0	9	5	3	1	3	0	0	4	0	0	0	0	0	0
4	**50**	2	150	0	5	2	3	0	1	0	0	0	1	0	0	0	0	0
		total	**300**	**0**	**14**	**7**	**6**	**1**	**4**	**0**	**0**	**4**	**1**	**0**	**0**	**0**	**0**	**0**
		1	150	0	7	7	1	0	4	1	0	3	0	0	0	0	0	0
5	**100**	2	150	0	5	3	2	0	3	0	0	0	0	0	0	0	0	0
		total	**300**	**0**	**12**	**10**	**3**	**0**	**7**	**1**	**0**	**3**	**0**	**0**	**0**	**0**	**0**	**0**
		1	50	0	11	10	1	0	5	0	1	1	1	2	0	0	0	0
CPA	**1.11**	2	75	1	12	11	6	0	2	0	0	11	0	0	0	0	0	0
		total	**125**	**1**	**23**	**21**	**7**	**0**	**7**	**0**	**1**	**12**	**1**	**2**	**0**	**0**	**0**	**0**

C: Negative Control (Culture Medium)
S: Solvent Control (0.5% THF; v/v)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

16.2.2. Main Experiment II

Table 19: Experiment II - Structural Chromosomal Aberrations, *without* metabolic activation: 21 h treatment, 21 h fixation period.

Dose Group	Concentration [µg/mL]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps g	Gaps ig	Types of Aberrations Found: Chromatid Types b	f	d	ex	Chromosome Types ib	if	id	cx	Other ma	cd
		1	150	1	5	1	3	1	0	0	0	0	0	0	0	1	0	0
C	**0**	2	150	0	8	4	4	1	1	2	0	0	0	1	0	0	0	0
		total	**300**	**1**	**13**	**5**	**7**	**2**	**1**	**2**	**0**	**0**	**0**	**1**	**0**	**1**	**0**	**0**
		1	150	1	2	1	1	0	0	0	0	0	0	1	0	0	0	0
S	**0**	2	150	0	5	3	1	1	1	1	0	0	0	1	0	0	0	0
		total	**300**	**1**	**7**	**4**	**2**	**1**	**1**	**1**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**
		1	150	0	7	3	4	0	0	1	1	0	0	1	0	1	0	0
1	**5**	2	150	1	3	0	2	1	0	0	0	0	0	0	0	0	0	0
		total	**300**	**1**	**10**	**3**	**6**	**1**	**0**	**1**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**
		1	150	1	5	4	2	0	1	0	0	0	0	0	0	3	0	0
2	**10**	2	150	0	7	7	1	0	5	1	0	1	0	0	0	0	0	0
		total	**300**	**1**	**12**	**11**	**3**	**0**	**6**	**1**	**0**	**1**	**0**	**0**	**0**	**3**	**0**	**0**
		1	150	1	5	2	3	0	1	0	0	0	0	0	0	1	0	0
3	**25**	2	150	0	6	4	2	0	1	1	0	1	0	1	0	0	0	0
		total	**300**	**1**	**11**	**6**	**5**	**0**	**2**	**1**	**0**	**1**	**0**	**1**	**0**	**1**	**0**	**0**
		1	300	1	27	13	12	3	4	0	0	4	1	2	0	0	2	0
EMS	**400**	2	150	1	16	16	0	0	11	0	0	3	0	3	0	1	0	0
		total	**450**	**2**	**43**	**29**	**12**	**3**	**15**	**0**	**0**	**7**	**1**	**5**	**0**	**1**	**2**	**0**

C: Negative Control (Culture Medium)
S: Solvent Control (0.5% THF; v/v)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

16.3. Appendix 3: Certificate of Analysis

Brunsbüttel, 10.02.2020

ANALYSENZERTIFIKAT

Produkt: ALFOL 20+ (E20+0003)
Lotnummer: 05513/MA

Herstellungsdatum 20.06.2019
Haltbarkeitsdatum: 6/2021

Tests	Ergebnisse	Norm
Gehalt an C 18-OH [%]	4.9	(GC)
Gehalt an C 20-OH [%]	45.3	(GC)
Gehalt an C 22-OH [%]	26.1	(GC)
Gehalt an C 24-OH [%]	15.2	(GC)
Gehalt an C 26-OH [%]	8.5	(GC)
Hazen - Farbzahl [HAZEN]	1700	(EN ISO 6271)
Esterzahl [mgKOH/g]	4.08	(600-33)
Säurezahl [mgKOH/g]	0.08	(600-31)
Wasser [%]	0.02	(DIN 51777)
Iodzahl [mgI/100mg]	11.86	(600-39)

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Mit freundlichen Grüßen
Abnahmebeauftragter
Manfred Sprung

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