BASF
We create chemistry

Corresponds to study #15 in Attachment A of transmittal memo on CBI
HERO ID:4731536

Does Not Contain Confidential Business Information

[redacted]

GENE MUTATION ASSAY IN CHINESE HAMSTER V79 CELLS *IN VITRO* (V79 / HPRT) WITH PALIOGEN VIOLET 5011

[redacted] Study Number: 1443105

Test Item: Paliogen Violet 5011

BASF Project Number: 50M0223/11X116

REPORT

Author: [redacted]

Test Facility: [redacted]

Test Guidelines: OECD 476
Commission Regulation (EC) No. 440/2008; B. 17
US EPA OPPTS 870.5300

Final Report Issued: January 17, 2012

Sponsor: BASF SE
[redacted]

1 PAGE RESERVED FOR PROPRIETARY INFORMATION OR STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

2 STATEMENT OF COMPLIANCE

This study performed in the test facility of [redacted] 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 [C(97)186/Final].

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

Study Director [redacted]

.....

Date: January 17, 2012

To be filled for USA EPA submission only:

Sponsor	.....	.....
		Date
Submitter	.....	.....
		Date

3 PAGE RESERVED FOR FLAGGING STATEMENTS

4 SIGNATURE

Study Director [redacted]

[redacted] .....

Date: January 17, 2012

5 STATEMENT OF QUALITY ASSURANCE UNIT

[redacted] Study: 1443105

Test Item: Paliogen Violet 5011

Study Director: [redacted]

Title: Gene Mutation Assay
in Chinese Hamster V79 Cells
in vitro (V79/HPRT)
with Paliogen Violet 5011

The general facilities and activities of [redacted] are inspected periodically and the results are reported to the responsible person and the Management.

Study procedures were inspected periodically. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

The General Study Plan 476.HPRT.BASF.01 was reviewed for compliance on September 09, 2011 and September 12, 2011.

The General Study Plan 476.HPRT.BASF.02 was reviewed for compliance on December 27, 2011.

Phases and Dates of QAU Inspections/ Audits		Dates of Reports to the Study Director and to Management
Study Specific Supplement:	September 22, 2011	September 22, 2011
First Amendment to Study Specific Supplement:	November 02, 2011	November 02, 2011
Second Amendment to Study Specific Supplement:	January 11, 2012	January 11, 2012
<u>Process Inspection</u> Preparation for Application and Application: Evaluation:	 October 11, 2011 November 17, 2011	 October 11, 2011 November 17, 2011
Report:	December 29-30, 2011	December 30, 2011

This statement is to confirm that the present report reflects the raw data.

Head of Quality Assurance Unit [redacted]

[redacted]

Date: January 17, 2012

[redacted]

6 COPY OF GLP CERTIFICATE

[redacted]

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

[redacted]

(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
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)
6 Prüfungen zur Bestimmung von Rückständen
8 Analytische Prüfungen an biologischen Materialien

2 Toxicity studies
3 Mutagenicity studies
6 Residues
8 Analytical studies on biological materials

15.08. und 27. – 29.10.2008
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.

[redacted]

(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

[redacted]

(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority

7 CONTENTS

CONTENTS CONTINUED

8 PREFACE

8.1 General

Title:	Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) with Paliogen Violet 5011
Sponsor:	BASF SE [redacted]
Study Monitor:	[redacted]
Test Facility:	[redacted]

8.2 Responsibilities

Study Director:	[redacted]
Deputy Study Director:	[redacted]
Management:	[redacted]
Head of Quality Assurance Unit:	[redacted]

8.3 Schedule

Experimental Starting Date:	October 05, 2011
Experimental Completion Date:	December 09, 2011

8.4 Good Laboratory Practice

The study was performed in compliance with:

"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 [C(97)186/Final].

These regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17), and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

8.5 Guidelines

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

OECD Guidelines for Testing of Chemicals No. 476 "*In vitro* Mammalian Cell Gene Mutation Test" (adopted July 21, 1997)

Commission Regulation (EC) 440/2008; B. 17 "Mutagenicity – *In vitro* Mammalian Cell Gene Mutation Test", dated May 30, 2008

US EPA (TSCA) OPPTS 870.5300 "*In Vitro* Mammalian Cell Gene MutationTest", EPA 712-C-98-221, August 1998

8.6 Archiving

The raw data, specimens (as long as the quality permits evaluation), the study plan, and the final report (or true copies) will be sent by [redacted] to the Sponsor. The Sponsor will archive the raw data, specimens (as long as the quality permits evaluation), the study plan and the final report for at least the period of time specified in the GLP principles at BASF SE ([redacted]).

[redacted] will archive:

Copies of raw data, study plan, report, and specimens (if any) for at least 3 years at the test facility's archive. Thereafter, the material will be transferred to the GLP archive of [redacted] for archiving the remaining time up to a total archiving period of 15 years. No data will be discarded without the sponsor's written consent.

A sample of the test item will be archived two years after the expiration date provided by the sponsor. If no expiration date is given, the archiving period will be the required 15 years. Thereafter the samples will be discarded without further notice.

8.7 Deviations from the Study Plan

There were no deviations from the study plan.

9 SUMMARY

The study was performed to investigate the potential of Paliogen Violet 5011 to induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster.

The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation.

The highest concentration (5500 µg/mL) used in the range finding pre-experiment was chosen with respect to the current OECD guideline 476 and the purity of the test item. The test item was dissolved in deionised water. The concentration range of the main experiments was limited by the occurrence of precipitation of the test item.

The tested concentrations of the main experiments are described in chapter 12.6 (page 18). The evaluated experimental points and the results are summarised in Table 1 (page 13).

No substantial and reproducible dose dependent increase of the mutation frequency was observed up to the maximum concentration with and without metabolic activation.

Appropriate reference mutagens (EMS and DMBA), used as positive controls, induced a distinct increase in mutant colonies and thus, showed the sensitivity of the test system and the activity of the metabolic activation system.

Conclusion

In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, Paliogen Violet 5011 is considered to be non-mutagenic in this HPRT assay.

Table 1: Summary of results

	conc. µg/mL	P	S9 mix	relative cloning efficiency I %	relative cell density %	relative cloning efficiency II %	mutant colonies/ 10^6 cells	induction factor	relative cloning efficiency I %	relative cell density %	relative cloning efficiency II %	mutant colonies/ 10^6 cells	induction factor
Column	1	2	3	4	5	6	7	8	9	10	11	12	13
Experiment I / 4 h treatment				culture I					culture II				
Solvent control water			-	100 0	100 0	100 0	8.6	1.0	100 0	100.0	100 0	23.8	1.0
Positive control (EMS)	150 0		-	90.4	93.1	78.1	171.2	19.8	80 0	106.3	76 5	123.1	5.2
Test item	10 8		-	96 5	139 9	92 0	12.7	1.5	96 0	112.9	89.4	16.0	0.7
Test item	21 5		-	106.4	123.7	89 9	10.2	1.2	97 6	108.1	77 8	20.3	0.9
Test item	43 0		-	98 0	100 3	69 9	13.8	1.6	99.1	68.1	90 5	11.6	0.5
Test item	86 0	P	-	93.1	112 6	68.7	21.0	2.4	101 6	82.9	66 9	11.5	0.5
Test item	172 0	P	-	79 8	82 6	87 5	9.8	1.1	101 6	95.0	68.1	18.7	0.8
Test item	344 0	P	-	77.7	culture was not continued#				91 9	culture was not continued#			
Solvent control water			+	100 0	100 0	100 0	8.5	1.0	100 0	100.0	100 0	9.5	1.0
Positive control (DMBA)	1.1		+	44 0	38.7	69 8	754.4	88.3	46 9	49.6	67 8	856.7	90.4
Test item	5 6		+	91.1	culture was not continued##				98 2	culture was not continued##			
Test item	10 8		+	86 3	80 3	91 8	9.0	1.1	108.4	103.2	94.1	6.1	0.6
Test item	21 5		+	85 6	88 3	119 0	7.4	0.9	102 2	88.3	90 3	4.8	0.5
Test item	43 0		+	94 5	90.4	103 3	7.8	0.9	100.4	85.9	99 5	15.1	1.6
Test item	86 0	P	+	88 3	103.1	112 5	20.9	2.4	102 9	107.2	86 9	10.1	1.1
Test item	172 0	P	+	84 0	81 9	99 9	24.4	2.9	99 2	107.7	92 5	25.6	2.7
Experiment II / 24 h treatment				culture I					culture II				
Solvent control water			-	100 0	100 0	100 0	16.3	1.0	100 0	100.0	100 0	10.8	1.0
Positive control (EMS)	150 0		-	83 6	60 8	107 2	410.7	25.2	98 0	108.7	99 6	307.2	28.5
Test item	10 8		-	97.1	79 5	105.4	16.0	1.0	96 6	119.3	114 5	14.3	1.3
Test item	21 5		-	91.4	83 8	102.7	18.0	1.1	97.4	110.7	110 2	14.1	1.3
Test item	43 0		-	87 5	66.7	101.7	27.5	1.7	95 6	130.8	104 3	13.4	1.2
Test item	86 0	P	-	78 9	80.1	105 0	22.5	1.4	84 3	112.0	104 3	22.9	2.1
Test item	172 0	P	-	71 8	69.1	106 2	18.3	1.1	70 8	109.7	100.4	10.3	1.0
Test item	344 0	P	-	50.4	culture was not continued#				51.1	culture was not continued#			
Experiment II / 4 h treatment													
Solvent control water			+	100 0	100 0	100 0	6.1	1.0	100 0	100.0	100 0	11.0	1.0
Positive control (DMBA)	1.1		+	44 0	57.1	93 0	588.3	96.0	53.7	74.3	91 9	635.3	58.0
Test item	5 6		+	111.7	culture was not continued##				100.4	culture was not continued##			
Test item	10 8		+	100 9	79 5	104 5	9.6	1.6	88 2	98.3	96.4	11.4	1.0
Test item	21 5		+	110 3	105.4	99 0	7.7	1.3	92.7	90.0	92.1	15.4	1.4
Test item	43 0		+	106 8	111 9	102.4	9.2	1.5	93 0	114.8	104.4	13.9	1.3
Test item	86 0	P	+	98 6	79 6	73 5	23.7	3.9	89.7	126.6	98 6	14.8	1.4
Test item	172 0	P	+	109 0	81 2	94.4	8.2	1.3	89 0	101.5	93 8	11.5	1.0

P = Precipitation

\# culture was not continued to avoid analysis of too many precipitating concentrations

\## culture was not continued as a minimum of only four analysable concentrations is required

10 OBJECTIVE

11 Aims of the Study

This *in vitro* experiment was performed to assess the potential of the test item to induce gene mutations using the Chinese hamster cell line V79. Two parallel cultures were used throughout the assay. The first experiment was performed with a treatment time of 4 hours with and without metabolic activation. The second experiment was performed with a treatment time of 24 hours without and 4 hours treatment with metabolic activation.

11.1 Relevance of the Test System

In vitro methods are valuable when it is desired to accurately control the concentration and exposure time of cells to the test item under study. However, the limited capacity of metabolic activation of potential mutagens requires an exogenous metabolic activation system.

This *in vitro* test is an assay for the detection of forward gene mutations in mammalian cells. Gene mutations are discussed as an initial step in the carcinogenic process.

The V79 cells are exposed to the test item both with and without exogenous metabolic activation. At a defined time interval after treatment the descendants of the treated original population are monitored for the loss of functional HPRT enzyme.

HPRT (hypoxanthine-guanine phosphoribosyl transferase) catalyzes the conversion of the nontoxic 6-TG (6-thioguanine) to its toxic ribophosphorylated derivative. Therefore, cells deficient in HPRT due to a forward mutation are resistant to 6-TG. These cells are able to proliferate in the presence of 6-TG whereas the non-mutated cells die. However, the mutant phenotype requires a certain period of time before it is completely expressed. The phenotypic expression is achieved by allowing exponential growth of the cells for 7 - 9 days. The expression period is terminated by adding 6-TG to the culture medium.

Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the surviving cells. After a suitable period the colonies are counted. Mutant frequencies are calculated from the number of mutant colonies corrected for cell survival.

In order to establish a concentration response effect of the test item at least four concentration levels are tested. These concentration levels should yield a concentration related toxic effect. The highest concentration level should induce a reduced level of survival.

To demonstrate the sensitivity of the test system reference mutagens are tested in parallel to the test item.

The test item may be formulated in water or appropriate organic solvents (1), the pH should be adjusted to neutral prior to application to the cells (2).

12 MATERIALS AND METHODS

12.1 Test Item

Internal Test Item Number: S 1282511

The test item and the information concerning the test item were provided by the sponsor.

Test Item:	Paliogen Violet 5011
BASF Test Item No.:	11/0223-1
Batch Number:	P 100012
CAS No.:	81-33-4
Purity:	>90% (see analytical report, BASF study code 11L00104); dose calculation adjusted to purity.
Expiration Date:	18 November 2020
Physical state, appearance:	Solid violet
Molecular weight:	Not indicated
Storage conditions:	Room temperature
Stability in Solvent:	Not indicated by the sponsor

On the day of the experiment (immediately before treatment), the test item was dissolved in deionised water. The solvent was chosen to its solubility properties and its relative non-toxicity to the cell cultures. The final concentration of deionised water in the culture medium was 10 % (v/v).

The osmolarity and pH-value were determined in the solvent control and in the highest concentration of the pre-experiment without metabolic activation:

	solvent control	Paliogen Violet 5011 5500 µg/mL
Osmolarity mOsm	278	279
pH-value	7.39	7.35

12.2 Controls

12.2.1 Solvent Controls

Concurrent solvent controls (deionised water (local tap water, deionised at [redacted] were performed.

12.2.2 Positive Control Substances

Without metabolic activation

Name:	EMS; ethylmethane sulfonate
Supplier:	[redacted]
Purity:	≥ 98 %
Lot no.:	A 0297962
Expiry date:	April 2012
Dissolved in:	Nutrient medium
Final concentration:	0.150 mg/mL = 1.2 mM

With metabolic activation

Name:	DMBA; 7,12-dimethylbenz(a)anthracene
Supplier:	[redacted]
Purity:	95 %
Lot no.:	040M1231
Expiry date:	December 2015
Dissolved in	DMSO, Dimethylsulfoxide
Supplier:	[redacted]; final concentration in nutrient medium 0.5 %
Final concentration:	1.1 µg/mL = 4.3 µM

The dilutions of the stock solutions were prepared on the day of the experiment and used immediately.

The stability of both positive control substances in solution was proven by the mutagenic response in the expected range.

12.3 Test System

12.3.1 Reasons for the Choice of the Cell Line V79

The V79 cell line has been used successfully in *in vitro* experiments for many years. Especially the high proliferation rate (doubling time 12 - 16 h in stock cultures) and a good cloning efficiency of untreated cells (as a rule more than 50 %) both necessary for the appropriate performance of the study, recommend the use of this cell line. The cells have a stable karyotype with a modal chromosome number of 22 (3).

12.3.2 Cell Cultures

Large stocks of the V79 cell line (supplied by [redacted]) are stored in liquid nitrogen in the cell bank of [redacted] allowing the repeated use of the same cell culture batch in experiments. Before freezing, the level of spontaneous mutants was depressed by treatment with HAT-medium as described in (4). Each batch is screened for mycoplasm contamination and checked for karyotype stability and spontaneous mutant frequency. Consequently, the parameters of the experiments remain similar because of the reproducible characteristics of the cells.

Thawed stock cultures are propagated at 37 °C in 80 cm^2 plastic flasks. About 5· 10^5 cells were seeded into each flask with 15 mL of MEM (minimal essential medium) containing Hank's salts supplemented with 10 % foetal bovine serum (FBS), neomycin (5 µg/mL) and amphotericin B (1 %). The cells were sub-cultured twice weekly. The cell cultures were incubated at 37 °C in a 1.5 % carbon dioxide atmosphere (98.5 % air).

12.4 Mammalian Microsomal Fraction S9 Mix

Lacking metabolic activities of cells under *in vitro* conditions are a disadvantage of assays with cell cultures as many chemicals only develop a mutagenic potential when they are metabolized by the mammalian organism. However, metabolic activation of chemicals can be achieved at least partially by supplementing the cell cultures with mammalian liver microsome preparations (S9 mix).

12.4.1 S9 (Preparation by [redacted]

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 were prepared from 8 – 12 weeks old male Wistar rats (Hsd Cpb: WU, weight approx. 220 – 320 g, [redacted]) induced by intraperitoneal applications of 80 mg/kg b.w. phenobarbital (Desitin, [redacted]) and by peroral administrations of 80 mg/kg b.w. β-naphthoflavone (Sigma-Aldrich Chemie GmbH, [redacted]) 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 were kept at –20 °C for up to one week. Each batch of S9 mix was routinely tested with 2-aminoanthracene as well as benzo(a)pyrene.

The protein concentration of the S9 preparation was 29.4 mg/mL (Lot. No.: 150711) in the pre-experiment and 32.2 mg/mL (Lot. No.: 300911) in experiments I and II.

12.4.2 S9 Mix

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. Cofactors were added to the S9 supernatant to reach following concentrations in the S9 mix:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP

in 100 mM sodium-phosphate-buffer, pH 7.4.

During the experiment, the S9 mix was stored in an ice bath.

12.5 Pre-Test on Toxicity

A pre-test was performed in order to determine the concentration range for the mutagenicity experiments. The general culture conditions and experimental conditions in this pre-test were the same as described for the mutagenicity experiment below. In this pre-test the colony forming ability of approximately 500 single cells (duplicate cultures per concentration level) after treatment with the test item was observed and compared to the controls. Toxicity of the test item is indicated by a reduction of the cloning efficiency (CE).

12.6 Dose Selection

According to the current OECD Guideline for Cell Gene Mutation Tests at least four analysable concentrations should be used in two parallel cultures. For freely-soluble and non-cytotoxic test items the maximum concentration should be 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest. For cytotoxic test items the maximum concentration should result in approximately 10 to 20 % relative survival or cell density at subcultivation and the analysed concentrations should cover a range from the maximum to little or no cytotoxicity. Relatively insoluble test items should be tested up to the highest concentration that can be formulated in an appropriate solvent as solution or homogenous suspension. These test items should be tested up or beyond their limit of solubility. Precipitation should be evaluated at the end of treatment by the unaided eye.

The maximum concentration (5500 µg/mL) used in the range finding pre-experiment was chosen with respect to the current OECD guideline 476 and the purity of the test item. Test item concentrations between 43.0 and 5500 µg/mL were used to evaluate cytotoxicity in the presence (4 hours treatment) and absence (4 hours and 24 hours treatment) of metabolic activation. Relevant toxic effects occurred at the maximum concentration of 5500 µg/mL without metabolic activation following 4 hours treatment. In the presence of metabolic activation (4 hours treatment) no relevant cytotoxicity were observed up to the maximum concentration. Following 24 hours treatment without metabolic activation, increasing cytotoxicity was observed at 343.8 µg/mL and above.

The test medium was checked for precipitation or phase separation at the end of each treatment period (4 or 24 hours) prior to removal to the test item. Precipitation occurred at 171.9 µg/mL and above without metabolic activation following 4 and 24 hours treatment and at 85.9 µg/mL and above with metabolic activation (4 hours treatment).

There was no relevant shift of pH and osmolarity of the medium even at the maximum concentration of the test item.

Based on the occurrence of precipitation in the pre-experiment, the individual concentrations of the main experiments were selected. The individual concentrations were spaced by a factor of 2.

Doses applied in the gene mutation assay with Paliogen Violet 5011 (concentrations given in bold letters were chosen for the mutation rate analysis):

exposure period	S9 mix	concentrations in µg/mL					
		Experiment I					
4 hours	-	**10.8**	**21.5**	**43.0**	**86.0** [P]	**172.0** [P]	344.0 [P]
4 hours	+	5.6	**10.8**	**21.5**	**43.0**	**86.0** [P]	**172.0** [P]
		Experiment II					
24 hours	-	**10.8**	**21.5**	**43.0**	**86.0** [P]	**172.0** [P]	344.0 [P]
4 hours	+	5.6	**10.8**	**21.5**	**43.0**	**86.0** [P]	**172.0** [P]

P = Precipitation

The cultures at the lowest concentrations with metabolic activation were not continued, since a minimum of only four analysable concentrations is required by the guidelines. The cultures at the maximum concentration without metabolic activation were not continued to avoid evaluation of too many precipitating concentrations.

12.7 Experimental Performance

12.7.1 Culture Medium

For seeding and treatment of the cell cultures the complete culture medium was MEM (minimal essential medium) containing Hank's salts, neomycin (5 µg/mL) and amphotericin B (1 %). For the selection of mutant cells the complete medium was supplemented with 11 µg/mL 6-thioguanine. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

12.7.2 Seeding

Two to three days after sub-cultivation stock cultures were trypsinized at 37 °C for 5 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10 % FBS and a single cell suspension was prepared. The trypsin concentration for all sub-culturing steps was 0.2 % in PBS.

The PBS is composed as follows (per litre):

NaCl	8000 mg
KCl	200 mg
KH_2PO_4	200 mg
Na_2HPO_4	150 mg

Prior to the trypsin treatment the cells were rinsed with Ca-Mg-free salt solution containing 200 mg/L EDTA (ethylene diamine tetraacetic acid). Approximately $1.5 \cdot 10^6$ (single culture) and $5 \cdot 10^2$ cells (in duplicate) were seeded in plastic culture flasks. The cells were grown for 24 hours prior to treatment.

12.7.3 Treatment

After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µL/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G". In the second experiment the cells were exposed to the test item for 24 hours in complete medium, supplemented with 10 % FBS, in the absence of metabolic activation.

The "saline G" solution had the following constituents (per litre):

NaCl	8000 mg
KCl	400 mg
Glucose	1100 mg
$Na_2HPO_4 \cdot 2H_2O$	192 mg
KH_2PO_4	150 mg

The pH was adjusted to 7.2

The colonies used to determine the cloning efficiency (survival) were fixed and stained approx. 7 days after treatment as described below.

Three or four days after treatment $1.5 \cdot 10^6$ cells per experimental point were sub-cultivated in 175 cm² flasks containing 30 mL medium. Following the expression time of 7 days five 80 cm² cell culture flasks were seeded with about $3 - 5 \cdot 10^5$ cells each in medium containing 6-TG. Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability.

The cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 for about 8 days. The colonies were stained with 10 % methylene blue in 0.01 % KOH solution.

The stained colonies with more than 50 cells were counted. In doubt the colony size was checked with a preparation microscope.

12.8 Data Recording

The data generated were recorded in the raw data. The results are presented in tabular form, including experimental groups with the test item, solvent and positive controls.

12.9 Acceptability of the Assay

The gene mutation assay is considered acceptable if it meets the following criteria:

The numbers of mutant colonies per 10^6 cells found in the solvent controls falls within the laboratory historical control data (see Annex II).

The positive control substances should produce a significant increase in mutant colony frequencies (see Annex II).

The cloning efficiency II (absolute value) of the solvent controls should exceed 50 %.

The data of this study comply with the above mentioned criteria [see Annex I (tables of results, mutation rate and factor calculated referring to the cloning efficiency of the untreated cultures) and Annex II (Historical data)].

12.10 Evaluation of Results

A test item is classified as positive if it induces either a concentration-related increase of the mutant frequency or a reproducible and positive response at one of the test points.

A test item producing neither a concentration-related increase of the mutant frequency nor a reproducible positive response at any of the test points is considered non-mutagenic in this system.

A positive response is described as follows:

A test item is classified as mutagenic if it reproducibly induces a mutation frequency that is three times above the spontaneous mutation frequency at least at one of the concentrations in the experiment.

The test item is classified as mutagenic if there is a reproducible concentration-related increase of the mutation frequency. Such evaluation may be considered also in the case that a threefold increase of the mutant frequency is not observed.

However, in a case by case evaluation this decision depends on the level of the corresponding solvent control data. If there is by chance a low spontaneous mutation rate within the laboratory´s historical control data range, a concentration-related increase of the mutations within this range has to be discussed. The variability of the mutation rates of solvent controls within all experiments of this study was also taken into consideration.

12.11 Statistical Analysis

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. The number of mutant colonies obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05. However, both, biological and statistical significance was considered together.

experimental group	p-value
experiment I, culture I without S9 mix	0.777
experiment I, culture II without S9 mix	0.674
experiment I, culture I with S9 mix	0.010*
experiment I, culture II with S9 mix	0.026*
experiment II, culture I without S9 mix	0.754
experiment II, culture II without S9 mix	0.995
experiment II, culture I with S9 mix	0.628
experiment II, culture II with S9 mix	0.928

* inverse trend without biological relevance

13 CALCULATION OF THE DATA

The data listed in the tables (Annex I) are calculated and processed as described below.

Pre-test

cloning efficiency, absolute	mean number of colonies per flask divided by the number of cells seeded · 100
cloning efficiency, relative	(mean number of colonies per flask divided by the mean number of colonies per flask of the corresponding control) · 100

Main test

cloning efficiency I (survival)	cloning efficiency determined immediately after treatment to measure toxicity.
cloning efficiency II (viability)	cloning efficiency determined after the expression period to measure viability of the cells without selective agent.
cloning efficiency I (survival, absolute)	mean number of colonies per flask divided by the number of cells seeded
cloning efficiency I (survival, relative)	(mean number of colonies per flask divided by the mean number of colonies per flask of the corresponding control) · 100
cell density % of control	(cell density at 1st subcultivation divided by the cell density at 1st subcultivation of the corresponding control) · 100
cloning efficiency II (viability, absolute)	mean number of colonies per flask divided by the number of cells seeded
cloning efficiency II (viability, relative)	cloning efficiency II absolute divided by the cloning efficiency II absolute of the corresponding control · 100
cells survived (after plating in TG containing medium)	number of cells seeded · cloning efficiency II absolute
mutant colonies / 10^6 cells	mean number of mutant colonies per flask found after plating in TG medium · 10^6 divided by the number of cells survived
induction factor	mutant colonies per 10^6 cells / mutant colonies per 10^6 cells of the corresponding solvent control

14 RESULTS AND DISCUSSION

The test item Paliogen Violet 5011 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster.

The study was performed in two independent experiments, using identical experimental procedures. In the first experiment the treatment period was 4 hours with and without metabolic activation. The second experiment was performed with a treatment time of 4 hours with and 24 hours without metabolic activation.

In experiment I and II the cell cultures were evaluated at the following concentrations:

exposure period	S9 mix	concentrations in µg/mL				
		Experiment I				
4 hours	-	**10.8**	**21.5**	**43.0**	**86.0** [P]	**172.0** [P]
4 hours	+	**10.8**	**21.5**	**43.0**	**86.0** [P]	**172.0** [P]
		Experiment II				
24 hours	-	**10.8**	**21.5**	**43.0**	**86.0** [P]	**172.0** [P]
4 hours	+	**10.8**	**21.5**	**43.0**	**86.0** [P]	**172.0** [P]

P = Precipitation

In both main experiments precipitation was observed at 86.0 µg/mL and above with metabolic activation (4 hours treatment) and without metabolic activation (4 and 24 hours treatment).

No relevant toxic effects occurred up to the maximum concentration with and without metabolic activation following 4 and 24 hours treatment.

No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration. All mutant frequencies remained well within the historical range of solvent controls.

An increase of the induction factor exceeding the threshold of three times the mutation frequency of the corresponding solvent control was observed in the first culture of the second experiment with metabolic activation at 86.0 µg/mL. However, the increase was based on a rather low mutation frequency of the solvent control of just 6.1 colonies per 10^6 cells. Furthermore, the effect was not reproduced in the parallel culture under identical experimental conditions. Therefore, the increase of the induction factor was judged as biologically irrelevant fluctuation.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. A p-value below 0.05 was detected in both cultures of the first experiment with metabolic activation. This trend however, was judged as biologically irrelevant since it actually was reciprocal, going down versus increasing concentrations.

In both experiments of this study (with and without S9 mix) the range of the solvent controls was from 6.1 up to 23.8 mutants per 10^6 cells; the range of the groups treated with the test item was from 4.8 up to 27.5 mutants per 10^6 cells.

EMS (150 µg/mL) and DMBA (1.1 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies.

Conclusion

In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, Paliogen Violet 5011 is considered to be non-mutagenic in this HPRT assay.

15 REFERENCES

1. Easterbrook, J., Lu, C., Sakai, Y. and Li, A.P. (2001)
Effects of organic solvents on the activities of cytochrome P450 isoforms, UDP-dependent glucuronyl transferase, and phenol sulfotransferase in human hepatocytes
Drug Metabolism and Disposition, 29, 141-144

2. Mitchell, A.D., Auletta, A.E., Clive, D., Kirby, P.E., Moore, M.M. and Myhr, B.C. (1997)
The L5178Y/tk+/- mouse lymphoma specific gene and chromosomal mutation assay - A phase III report of the U.S. Environmental Protection Agency Gene-Tox Program
Mutation Research, 394, 177-303

3. P. Howard-Flanders
Mutagenesis in mammalian cells
Mutation Research 86, 307-327, 1981

4. M.O. Bradley, B. Bhuyan, M.C. Francis, R. Langenbach, A. Peterson, and E. Huberman
Mutagenesis by chemical agents in V79 Chinese hamster cells: a review and analysis of the literature. A report of the gene-tox program
Mutation Research 87, 81-142, 1981

16 DISTRIBUTION OF THE REPORT

Sponsor	2x (1x original, 1x electronic copy as PDF-file)
Study Director	1x (copy)

17 ANNEX I: TABLES OF RESULTS

17.1 Pre-test

Table 2: Toxicity data

	conc.	S9	Duration of	cells	number of colonies per flask			CE%	CE%	
	µg/mL	mix	treatment	seeded	found			absolute	relative	precipitation
Test group			(h)	I/II	I	II	mean			
Column	1	2	3	4	5	6	7	8	9	10
Solvent control with water		-	4	506	330	326	328	64.8	100.0	
Test item	43.0	-	4	506	305	312	309	61.0	94.1	
Test item	85.9	-	4	506	310	285	298	58.8	90.7	
Test item	171.9	-	4	506	261	275	268	53.0	81.7	precipitation
Test item	343.8	-	4	506	241	226	234	46.1	71.2	precipitation
Test item	687.5	-	4	506	214	248	231	45.7	70.4	precipitation
Test item	1375.0	-	4	506	213	208	211	41.6	64.2	precipitation
Test item	2750.0	-	4	506	163	171	167	33.0	50.9	precipitation
Test item	5500.0	-	4	506	119	131	125	24.7	38.1	precipitation
Solvent control with water		+	4	506	371	382	377	74.4	100.0	
Test item	43.0	+	4	506	386	364	375	74.1	99.6	
Test item	85.9	+	4	506	358	376	367	72.5	97.5	precipitation
Test item	171.9	+	4	506	382	365	374	73.8	99.2	precipitation
Test item	343.8	+	4	506	349	372	361	71.2	95.8	precipitation
Test item	687.5	+	4	506	350	363	357	70.5	94.7	precipitation
Test item	1375.0	+	4	506	347	341	344	68.0	91.4	precipitation
Test item	2750.0	+	4	506	338	342	340	67.2	90.3	precipitation
Test item	5500.0	+	4	506	351	366	359	70.8	95.2	precipitation
Solvent control with water		-	24	506	394	371	383	75.6	100.0	
Test item	43.0	-	24	506	378	358	368	72.7	96.2	
Test item	85.9	-	24	506	324	282	303	59.9	79.2	
Test item	171.9	-	24	506	256	243	250	49.3	65.2	precipitation
Test item	343.8	-	24	506	156	116	136	26.9	35.6	precipitation
Test item	687.5	-	24	506	87	97	92	18.2	24.1	precipitation
Test item	1375.0	-	24	506	76	61	69	13.5	17.9	precipitation
Test item	2750.0	-	24	506	92	93	93	18.3	24.2	precipitation
Test item	5500.0	-	24	506	59	65	62	12.3	16.2	precipitation

17.2 Main Experiment

17.2.1 Experiment I Culture I

Table 3: Cloning Efficiency I (Survival)

	conc.	P	S9	cells	number of colonies per flask			CE I	CE I	cells/mL	cell density
Test group	µg/mL		mix	seeded	found			absolute	relative	at 1st	%
				I/II	I	II	mean		%	subcultivation	of control
Column	1	2	3	4	5	6	7	8	9	10	11
Solvent control with water			-	562	372	365	368.5	0.7	100.0	1382000	100.0
Positive control with EMS	150.0		-	562	317	349	333.0	0.6	90.4	1286000	93.1
Test item	10.8		-	562	349	362	355.5	0.6	96.5	1934000	139.9
Test item	21.5		-	562	402	382	392.0	0.7	106.4	1710000	123.7
Test item	43.0		-	562	363	359	361.0	0.6	98.0	1386000	100.3
Test item	86.0	P	-	562	335	351	343.0	0.6	93.1	1556000	112.6
Test item	172.0	P	-	562	305	283	294.0	0.5	79.8	1142000	82.6
Test item	344.0	P	-	562	285	288	286.5	0.5	77.7	culture was not continued[#]	
Solvent control with water			+	562	514	488	501.0	0.9	100.0	2575000	100.0
Positive control with DMBA	1.1		+	562	206	235	220.5	0.4	44.0	997500	38.7
Test item	5.6		+	562	461	452	456.5	0.8	91.1	culture was not continued[##]	
Test item	10.8		+	562	447	418	432.5	0.8	86.3	2067000	80.3
Test item	21.5		+	562	425	433	429.0	0.8	85.6	2275000	88.3
Test item	43.0		+	562	481	466	473.5	0.8	94.5	2328000	90.4
Test item	86.0	P	+	562	452	433	442.5	0.8	88.3	2655000	103.1
Test item	172.0	P	+	562	427	415	421.0	0.7	84.0	2110000	81.9

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

Table 4: Cloning Efficiency II (Viability), experiment I, culture I

	conc.	P	S9	cells	number of colonies per flask			CE II	CE II	cells	cells
Test group	µg/mL		mix	seeded	found			absolute	relative	seeded	survived
				I/II	I	II	mean		%		
Column	1	2	3	4	5	6	7	8	9	10	11
Solvent control with water			-	512	422	387	404.5	0.8	100.0	498000	393439.5
Positive control with EMS	150.0		-	501	302	316	309.0	0.6	78.1	403500	248865.3
Test item	10.8		-	503	372	359	365.5	0.7	92.0	476700	346389.4
Test item	21.5		-	599	415	436	425.5	0.7	89.9	498300	353967.7
Test item	43.0		-	609	325	348	336.5	0.6	69.9	394800	218144.8
Test item	86.0	P	-	620	341	332	336.5	0.5	68.7	474900	257748.1
Test item	172.0	P	-	500	356	335	345.5	0.7	87.5	499950	345465.5
Test item	344.0	P	-	culture was not continued#							
Solvent control with water			+	600	486	495	490.5	0.8	100.0	400800	327654.0
Positive control with DMBA	1.1		+	628	376	341	358.5	0.6	69.8	353400	201741.9
Test item	5.6		+	culture was not continued##							
Test item	10.8		+	583	448	427	437.5	0.8	91.8	355500	266777.4
Test item	21.5		+	501	503	472	487.5	1.0	119.0	390900	380366.8
Test item	43.0		+	570	477	486	481.5	0.8	103.3	425700	359604.5
Test item	86.0	P	+	504	501	426	463.5	0.9	112.5	343500	315897.3
Test item	172.0	P	+	628	534	492	513.0	0.8	99.9	361500	295301.8

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

Table 5: Mutagenicity data (Mutation rates), experiment I, culture I

Test group	conc. µg/mL	P	S9 mix	number of mutant colonies per flask found after plating in TG medium						standard deviation	mutant colonies per 10^6 cells	induction factor
				I	II	III	IV	V	mean			
Column	1	2	3	4	5	6	7	8	9	10	11	12
Solvent control with water			-	2	4	5	5	1	3.4	1.8	8.6	1.0
Positive control with EMS	150.0		-	42	47	39	44	41	42.6	3.0	171.2	19.8
Test item	10.8		-	4	4	5	3	6	4.4	1.1	12.7	1.5
Test item	21.5		-	1	3	4	3	7	3.6	2.2	10.2	1.2
Test item	43.0		-	2	4	3	5	1	3.0	1.6	13.8	1.6
Test item	86.0	P	-	6	2	6	8	5	5.4	2.2	21.0	2.4
Test item	172.0	P	-	3	6	3	3	2	3.4	1.5	9.8	1.1
Test item	344.0	P	-	culture was not continued#								
Solvent control with water			+	3	4	3	3	1	2.8	1.1	8.5	1.0
Positive control with DMBA	1.1		+	146	153	141	162	159	152.2	8.8	754.4	88.3
Test item	5.6		+	culture was not continued##								
Test item	10.8		+	2	5	1	3	1	2.4	1.7	9.0	1.1
Test item	21.5		+	4	4	3	1	2	2.8	1.3	7.4	0.9
Test item	43.0		+	3	4	1	3	3	2.8	1.1	7.8	0.9
Test item	86.0	P	+	4	7	7	11	4	6.6	2.9	20.9	2.4
Test item	172.0	P	+	2	11	10	7	6	7.2	3.6	24.4	2.9

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

17.2.2 Experiment I Culture II

Table 6: Cloning Efficiency I (Survival), culture II

	conc.	P	S9	cells	number of colonies per flask			CE I	CE I	cells/mL	cell density
Test group	µg/mL		mix	seeded	found			absolute	relative	at 1st	%
				I/II	I	II	mean		%	subcultivation	of control
Column	1	2	3	4	5	6	7	8	9	10	11
Solvent control with water			-	562	385	371	378.0	0.7	100.0	1942000	100.0
Positive control with EMS	150.0		-	562	292	313	302.5	0.5	80.0	2064000	106.3
Test item	10.8		-	562	357	369	363.0	0.6	96.0	2192000	112.9
Test item	21.5		-	562	383	355	369.0	0.7	97.6	2100000	108.1
Test item	43.0		-	562	369	380	374.5	0.7	99.1	1322000	68.1
Test item	86.0	P	-	562	392	376	384.0	0.7	101.6	1610000	82.9
Test item	172.0	P	-	562	382	386	384.0	0.7	101.6	1844000	95.0
Test item	344.0	P	-	562	361	334	347.5	0.6	91.9	culture was not continued#	
Solvent control with water			+	562	422	431	426.5	0.8	100.0	2303000	100.0
Positive control with DMBA	1.1		+	562	188	212	200.0	0.4	46.9	1142000	49.6
Test item	5.6		+	562	410	428	419.0	0.7	98.2	culture was not continued##	
Test item	10.8		+	562	468	457	462.5	0.8	108.4	2377000	103.2
Test item	21.5		+	562	442	430	436.0	0.8	102.2	2034000	88.3
Test item	43.0		+	562	423	433	428.0	0.8	100.4	1979000	85.9
Test item	86.0	P	+	562	450	428	439.0	0.8	102.9	2469000	107.2
Test item	172.0	P	+	562	434	412	423.0	0.8	99.2	2481000	107.7

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

Table 7: Cloning Efficiency II (Viability), experiment I, culture II

	conc.	P	S9	cells	number of colonies per flask			CE II	CE II	cells	cells
Test group	µg/mL		mix	seeded	found			absolute	relative	seeded	survived
				I/II	I	II	mean		%		
Column	1	2	3	4	5	6	7	8	9	10	11
Solvent control with water			-	502	437	446	441.5	0.9	100.0	352800	310281.3
Positive control with EMS	150.0		-	535	364	356	360.0	0.7	76.5	478200	321779.4
Test item	10.8		-	506	394	402	398.0	0.8	89.4	413400	325164.4
Test item	21.5		-	628	437	422	429.5	0.7	77.8	446400	305300.6
Test item	43.0		-	500	405	391	398.0	0.8	90.5	411600	327633.6
Test item	86.0	P	-	554	334	318	326.0	0.6	66.9	472800	278218.1
Test item	172.0	P	-	570	322	361	341.5	0.6	68.1	428700	256843.9
Test item	344.0	P	-	culture was not continued#							
Solvent control with water			+	500	434	438	436.0	0.9	100.0	363150	316666.8
Positive control with DMBA	1.1		+	534	321	310	315.5	0.6	67.8	406200	239992.7
Test item	5.6		+	culture was not continued##							
Test item	10.8		+	504	401	426	413.5	0.8	94.1	438000	359351.2
Test item	21.5		+	503	385	407	396.0	0.8	90.3	422400	332545.5
Test item	43.0		+	605	527	523	525.0	0.9	99.5	396000	343636.4
Test item	86.0	P	+	593	458	441	449.5	0.8	86.9	444000	336556.5
Test item	172.0	P	+	625	506	502	504.0	0.8	92.5	427000	344332.8

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

Table 8: Mutagenicity data (Mutation rates), experiment I, culture II

	conc.	P	S9	number of mutant colonies per flask							mutant	
Test group	µg/mL		mix	found after plating in TG medium						standard	colonies	induction
				I	II	III	IV	V	mean	deviation	per 10^6 cells	factor
Column	1	2	3	4	5	6	7	8	9	10	11	12
Solvent control with water			-	8	6	7	7	9	7.4	1.1	23.8	1.0
Positive control with EMS	150.0		-	31	44	38	46	39	39.6	5.9	123.1	5.2
Test item	10.8		-	5	6	6	3	6	5.2	1.3	16.0	0.7
Test item	21.5		-	4	6	7	6	8	6.2	1.5	20.3	0.9
Test item	43.0		-	3	7	5	2	2	3.8	2.2	11.6	0.5
Test item	86.0	P	-	2	5	3	2	4	3.2	1.3	11.5	0.5
Test item	172.0	P	-	4	1	8	7	4	4.8	2.8	18.7	0.8
Test item	344.0	P	-	culture was not continued#								
Solvent control with water			+	6	4	4	1	0	3.0	2.4	9.5	1.0
Positive control with DMBA	1.1		+	198	211	223	195	201	205.6	11.4	856.7	90.4
Test item	5.6		+	culture was not continued##								
Test item	10.8		+	4	1	3	2	1	2.2	1.3	6.1	0.6
Test item	21.5		+	1	1	2	1	3	1.6	0.9	4.8	0.5
Test item	43.0		+	3	6	5	8	4	5.2	1.9	15.1	1.6
Test item	86.0	P	+	4	4	5	1	3	3.4	1.5	10.1	1.1
Test item	172.0	P	+	14	7	9	9	5	8.8	3.3	25.6	2.7

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

17.2.3 Experiment II, Culture I

Table 9: Cloning Efficiency I (Survival)

	conc.	P	S9	cells	number of colonies per flask			CE I	CE I	cells/mL	cell density
Test group	µg/mL		mix	seeded	found			absolute	relative	at 1st	%
				I/II	I	II	mean		%	subcultivation	of control
Column	1	2	3	4	5	6	7	8	9	10	11
Solvent control with water			-	545	515	507	511.0	0.9	100.0	3134000	100.0
Positive control with EMS	150.0		-	545	433	421	427.0	0.8	83.6	1905000	60.8
Test item	10.8		-	545	489	503	496.0	0.9	97.1	2493000	79.5
Test item	21.5		-	545	460	474	467.0	0.9	91.4	2627000	83.8
Test item	43.0		-	545	462	432	447.0	0.8	87.5	2090000	66.7
Test item	86.0	P	-	545	398	408	403.0	0.7	78.9	2509000	80.1
Test item	172.0	P	-	545	363	371	367.0	0.7	71.8	2167000	69.1
Test item	344.0	P	-	545	210	305	257.5	0.5	50.4	culture was not continued#	
Solvent control with water			+	545	498	358	428.0	0.8	100.0	1996000	100.0
Positive control with DMBA	1.1		+	545	175	202	188.5	0.3	44.0	1139000	57.1
Test item	5.6		+	545	497	459	478.0	0.9	111.7	culture was not continued##	
Test item	10.8		+	545	484	380	432.0	0.8	100.9	1587000	79.5
Test item	21.5		+	545	466	478	472.0	0.9	110.3	2104000	105.4
Test item	43.0		+	545	485	429	457.0	0.8	106.8	2234000	111.9
Test item	86.0	P	+	545	370	474	422.0	0.8	98.6	1588000	79.6
Test item	172.0	P	+	545	465	468	466.5	0.9	109.0	1621000	81.2

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

Table 10: Cloning Efficiency II (Viability), experiment II, culture I

	conc.	P	S9	cells	number of colonies per flask			CE II	CE II	cells	cells
Test group	µg/mL		mix	seeded	found			absolute	relative	seeded	survived
				I/II	I	II	mean		%		
Column	1	2	3	4	5	6	7	8	9	10	11
Solvent control with water			-	531	287	303	295.0	0.6	100.0	486300	270166.7
Positive control with EMS	150.0		-	524	328	296	312.0	0.6	107.2	394200	234714.5
Test item	10.8		-	542	323	312	317.5	0.6	105.4	533400	312462.2
Test item	21.5		-	525	305	294	299.5	0.6	102.7	526200	300184.6
Test item	43.0		-	569	328	315	321.5	0.6	101.7	398700	225276.0
Test item	86.0	P	-	540	309	321	315.0	0.6	105.0	396600	231350.0
Test item	172.0	P	-	546	328	316	322.0	0.6	106.2	407400	240261.5
Test item	344.0	P	-	culture was not continued#							
Solvent control with water			+	507	367	351	359.0	0.7	100.0	368850	261177.8
Positive control with DMBA	1.1		+	501	322	338	330.0	0.7	93.0	396900	261431.1
Test item	5.6		+	culture was not continued##							
Test item	10.8		+	596	429	453	441.0	0.7	104.5	421200	311659.7
Test item	21.5		+	500	355	346	350.5	0.7	99.0	446400	312926.4
Test item	43.0		+	500	370	355	362.5	0.7	102.4	452100	327772.5
Test item	86.0	P	+	618	328	315	321.5	0.5	73.5	406200	211316.0
Test item	172.0	P	+	502	342	329	335.5	0.7	94.4	401400	268266.3

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

Table 11: Mutagenicity data (Mutation rates), experiment II, culture I

	conc.	P	S9	number of mutant colonies per flask							mutant	
Test group	µg/mL		mix	found after plating in TG medium						standard	colonies	induction
				I	II	III	IV	V	mean	deviation	per 10^6 cells	factor
Column	1	2	3	4	5	6	7	8	9	10	11	12
Solvent control with water			-	2	3	4	8	5	4.4	2.3	16.3	1.0
Positive control with EMS	150.0		-	91	97	101	94	99	96.4	4.0	410.7	25.2
Test item	10.8		-	7	6	2	5	5	5.0	1.9	16.0	1.0
Test item	21.5		-	7	2	8	6	4	5.4	2.4	18.0	1.1
Test item	43.0		-	5	9	6	4	7	6.2	1.9	27.5	1.7
Test item	86.0	P	-	9	4	4	7	2	5.2	2.8	22.5	1.4
Test item	172.0	P	-	6	4	4	6	2	4.4	1.7	18.3	1.1
Test item	344.0	P	-	culture was not continued#								
Solvent control with water			+	2	2	1	1	2	1.6	0.5	6.1	1.0
Positive control with DMBA	1.1		+	140	158	147	160	164	153.8	10.0	588.3	96.0
Test item	5.6		+	culture was not continued##								
Test item	10.8		+	2	2	5	4	2	3.0	1.4	9.6	1.6
Test item	21.5		+	2	3	0	4	3	2.4	1.5	7.7	1.3
Test item	43.0		+	2	4	2	1	6	3.0	2.0	9.2	1.5
Test item	86.0	P	+	6	4	2	5	8	5.0	2.2	23.7	3.9
Test item	172.0	P	+	3	1	3	2	2	2.2	0.8	8.2	1.3

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

17.2.4 Experiment II, Culture II

Table 12: Cloning Efficiency I (Survival)

Test group	conc. µg/mL	P	S9 mix	cells seeded I/II	number of colonies per flask found I	II	mean	CE I absolute	CE I relative %	cells/mL at 1st subcultivation	cell density % of control
Column	1	2	3	4	5	6	7	8	9	10	11
Solvent control with water			-	545	393	407	400.0	0.7	100.0	2419000	100.0
Positive control with EMS	150.0		-	545	386	398	392.0	0.7	98.0	2630000	108.7
Test item	10.8		-	545	401	372	386.5	0.7	96.6	2885000	119.3
Test item	21.5		-	545	386	393	389.5	0.7	97.4	2678000	110.7
Test item	43.0		-	545	369	396	382.5	0.7	95.6	3165000	130.8
Test item	86.0	P	-	545	316	358	337.0	0.6	84.3	2709000	112.0
Test item	172.0	P	-	545	276	290	283.0	0.5	70.8	2653000	109.7
Test item	344.0	P	-	545	208	201	204.5	0.4	51.1	culture was not continued#	
Solvent control with water			+	545	344	412	378.0	0.7	100.0	1871000	100.0
Positive control with DMBA	1.1		+	545	199	207	203.0	0.4	53.7	1391000	74.3
Test item	5.6		+	545	389	370	379.5	0.7	100.4	culture was not continued##	
Test item	10.8		+	545	326	341	333.5	0.6	88.2	1839000	98.3
Test item	21.5		+	545	361	340	350.5	0.6	92.7	1684000	90.0
Test item	43.0		+	545	333	370	351.5	0.6	93.0	2148000	114.8
Test item	86.0	P	+	545	328	350	339.0	0.6	89.7	2369000	126.6
Test item	172.0	P	+	545	347	326	336.5	0.6	89.0	1899000	101.5

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

Table 13: Cloning Efficiency II (Viability), experiment II, culture II

Test group	conc. µg/mL	P	S9 mix	cells seeded I/II	number of colonies per flask found I	II	mean	CE II absolute	CE II relative %	cells seeded	cells survived
Column	1	2	3	4	5	6	7	8	9	10	11
Solvent control with water			-	502	361	387	374.0	0.7	100.0	398400	296815.9
Positive control with EMS	150.0		-	524	395	383	389.0	0.7	99.6	412200	306003.4
Test item	10.8		-	610	536	505	520.5	0.9	114.5	345000	294381.1
Test item	21.5		-	578	487	462	474.5	0.8	110.2	414000	339866.8
Test item	43.0		-	524	411	403	407.0	0.8	104.3	345000	267967.6
Test item	86.0	P	-	569	435	449	442.0	0.8	104.3	393600	305749.0
Test item	172.0	P	-	563	418	424	421.0	0.7	100.4	339000	253497.3
Test item	344.0	P	-	culture was not continued#							
Solvent control with water			+	528	412	428	420.0	0.8	100.0	413100	328602.3
Positive control with DMBA	1.1		+	587	407	451	429.0	0.7	91.9	426000	311335.6
Test item	5.6		+	culture was not continued##							
Test item	10.8		+	554	431	419	425.0	0.8	96.4	432900	332098.4
Test item	21.5		+	546	403	397	400.0	0.7	92.1	495000	362637.4
Test item	43.0		+	626	512	528	520.0	0.8	104.4	381600	316984.0
Test item	86.0	P	+	505	405	387	396.0	0.8	98.6	378300	296647.1
Test item	172.0	P	+	502	386	363	374.5	0.7	93.8	419700	313102.9

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

Table 14: Mutagenicity data (Mutation rates), experiment II, culture II

Test group	conc. µg/mL	P	S9 mix	number of mutant colonies per flask found after plating in TG medium						standard deviation	mutant colonies per 10^6 cells	induction factor
				I	II	III	IV	V	mean			
Column	1	2	3	4	5	6	7	8	9	10	11	12
Solvent control with water			-	1	4	3	4	4	3.2	1.3	10.8	1.0
Positive control with EMS	150.0		-	83	95	115	92	85	94.0	12.7	307.2	28.5
Test item	10.8		-	3	2	4	8	4	4.2	2.3	14.3	1.3
Test item	21.5		-	4	8	4	4	4	4.8	1.8	14.1	1.3
Test item	43.0		-	2	7	5	3	1	3.6	2.4	13.4	1.2
Test item	86.0	P	-	6	8	11	4	6	7.0	2.6	22.9	2.1
Test item	172.0	P	-	2	1	5	1	4	2.6	1.8	10.3	1.0
Test item	344.0	P	-	culture was not continued#								
Solvent control with water			+	2	6	4	5	1	3.6	2.1	11.0	1.0
Positive control with DMBA	1.1		+	189	192	197	203	208	197.8	7.8	635.3	58.0
Test item	5.6		+	culture was not continued##								
Test item	10.8		+	2	3	6	2	6	3.8	2.0	11.4	1.0
Test item	21.5		+	4	9	8	2	5	5.6	2.9	15.4	1.4
Test item	43.0		+	6	5	4	3	4	4.4	1.1	13.9	1.3
Test item	86.0	P	+	7	7	4	2	2	4.4	2.5	14.8	1.4
Test item	172.0	P	+	5	4	2	3	4	3.6	1.1	11.5	1.0

culture was not continued to avoid analysis of too many precipitating concentrations

culture was not continued as a minimum of only four analysable concentrations is requred

P Precipitation

18 ANNEX II: Historical Data

These values represent the historical control data from 2009 – 2010

Number of mutant colonies per 10^6 cells		
without metabolic activation (4 hours treatment time)		
	Positive control EMS 150 µg/mL	Solvent control (medium, water, acetone DMSO, ethanol, THF)
Range:	65.0 – 1386.4	4.4 – 42.9
Mean value:	181.8	16.7
Standard deviation:	170.5	8.2
Number of studies:	59	60
with metabolic activation (4 hours treatment time)		
	Positive control DMBA 1.1 - 2.0 µg/mL	Solvent control (medium, water, acetone DMSO, ethanol, THF)
Range:	64.3 – 2634.0	3.3 – 45.3
Mean value:	939.4	17.1
Standard deviation:	406.1	7.6
Number of studies:	59	59
without metabolic activation (24 hours treatment time)		
	Positive control EMS 75 – 300 µg/mL	Solvent control (medium, water, acetone DMSO, ethanol, THF)
Range:	51.6 – 1925.5	2.6 – 40.3
Mean value:	358.2	18.0
Standard deviation:	246.6	8.2
Number of studies:	48	48