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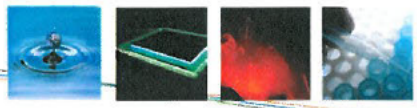

10.2.e

Final Report: GLP study

Study number AME11 005

Feasibility study on an innovative pesticide against viral diseases on tomato crops: research on mutagenic action with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537.

According to OECD Guideline 471 (Ames)

2011/MRG/R/244

October 2011

Test facility:

CARDAM-VITO

Industriezone Vlasmeer 7

B-2400 Mol

Belgium

Sponsor:

De Ceuster n.v.

Fortsesteenweg 30

B-2860 Sint-Katelijne-Waver

Belgium

Sponsor representatives:

[redacted]

CARDAM
Centre for Advanced R&D on Alternative Methods

TEST APPROVAL PAGE

TEST ITEM	SPONSOR CODE	VITO CODE
[redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906	-	2011TOX007/1

10.1.c Wob juncto 63.2.d Vo 1107/2009

TEST FACILITY MANAGER	STUDY DIRECTOR	QA MANAGER
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Date: 18/10/2011 Signatu[redacted]	Date: 18/10/2011 Signat[redacted]	Date: 18-10-2011 Signature: [redacted]

10.1.c wob juncto 63.2ter.d Vo 1107/2009 juncto 39sexies.2 Vo 178/2002

SPONSOR	
De Ceuster n.v. Fortsesteenweg 30 B-2860 Sint-Katelijne Waver Belgium	**Sponsor representative:** [redacted] De Ceuster Meststoffen n.v. Bannerlaan 79 B-2280 Grobbendonk Belgium Tel: [redacted] Mobile: [redacted] Fax: [redacted] E-mail [redacted]@dcm-info.com Date: Signature:

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PERSONNEL
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10.1.c wob juncto 63.2ter.d Vo 1107/2009 juncto 39sexies.2 Vo 178/2002

CONTENTS

STUDY SPECIFIC INFORMATION

TEST ITEM: [redacted] Pepino Mosaic Virus, CH2 strain, isolate 1906	**VITO CODE:** 2011TOX007/1
Experimental data:	**Start:** 23/08/2011 **End:** 29/09/2011
Reports data:	**Draft report:** 03/10/2011 **Final report:** CARDAM promises to provide a final report 10 working days after the study director received all the comments from the sponsor on the draft report.
Test site:	**CARDAM:** Building BIO1, room 0122 and 0234

TEST ITEM INFORMATION

Material Safety Data Sheet: Not available

Certificate of Analyses: Available, received on 28/09/2011. CARDAM makes no GLP-compliance claim for the characterization and verification of the identity of the test item. This is the responsibility of the Sponsor.

SUMMARY

The potential of test item [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' and its metabolites (if relevant) to induce reverse mutations was evaluated in five standard *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 in the absence and in the presence of a rat liver metabolic activation system (S9). Test item [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' was tested in water as solvent. An extra solvent control [redacted] was included to verify if this material does not induce reverse mutations on its own. Also negative (untreated) and positive control plates were included.

Test item [redacted] containing *Pepino Mosaic Virus*, CH2 strain, isolate 1906' and the extra solvent control [redacted] were checked for sterility. No signs of contamination were observed for both.

The concurrent positive controls demonstrated the sensitivity of the assay and the metabolizing activity of the S9-mix. In the second Reverse Mutation Experiment, as well as in the first repeat of this experiment, for strain TA102 in the presence of S9-mix, no 2-fold increase of the positive control (2-aminoanthracene) was obtained compared to solvent control. For that reason, the second Reverse Mutation Experiment in the presence of S9-mix was repeated again for strain TA102 starting from a daughter culture instead of a test culture tube. For the latter experiment, a 2-fold increase of the positive control (2-aminoanthracene) was obtained compared to solvent control.

After a toxicity range-finder experiment, two independent reverse mutation tests were performed. The first was a standard plate incorporation assay and the second involved a pre-incubation stage.

In the toxicity range-finder experiment, no substantial increases in revertant colony numbers over solvent control counts were obtained with strain TA100 following exposure to [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' at 10 selected concentrations up to a concentration of 100 µl/plate in either the presence or absence of S9-mix. The concentration of 100 µl/plate corresponds to a Ct-value of 17.98 measured using TaqMan RT-qPCR. As sufficient concentrations could be scored, these data were used for the actual mutagenicity data of the first Reverse Mutation Experiment. The top concentration for the plate incorporation test with the strains TA98, TA102, TA1535, and TA1537 in the absence and in the presence of S9-mix was the same as for the toxicity range-finder experiment with the strain TA100.

The concentrations for the main study were 1.563, 3.125, 6.25, 12.5, 25, 50, and 100 µl/plate. No evidence of mutagenic activity was seen at any concentration of [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' with any of the five *Salmonella typhimurium* strains in the plate incorporation test as well as in the pre-incubation test in the

absence and in the presence of S9-mix. For strain TA1537 in the absence of S9-mix only, a borderline increased reversion rate (2.92), which was not yet biological significant (IF > 3), was observed for the highest concentration 100 µl/plate, but no dose-related increase in reversion rate was observed for all concentrations tested.

No evidence of mutagenic activity was seen for [redacted] tested at a concentration of 100 µl/plate with any of the five *Salmonella typhimurium* strains in the plate incorporation test as well as in the pre-incubation test in the absence and in the presence of S9-mix.

On basis of these findings, it can be concluded that [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' showed, under the test conditions employed, no evidence of mutagenic activity towards the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 at the tested concentrations.

STATEMENT QA

Study AME11 005:
2011TOX007/1 - Feasibility study on an innovative pesticide against viral diseases on tomato crops: research on mutagenic action with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537.

The data contained in this study report were audited by the Quality Assurance Unit to assure compliance with the protocol, the standard operating procedures and the pertinent Good Laboratory Practice regulations of the OECD and EEC directives.

The audits took place, were reported to study director and the management on the following dates:

Date	Audit	Reported findings to Study Director	Reported findings to Test Facility Management
15/07/11	Study plan	15/07/11	
07/09/11	Inspection 1: preparation and dilution of test item ; usage of negative control (supplied by sponsor); adequate working of biohazard; checking of characteristics of the bacteria; univocal identification of the plates; temperature settings of incubator.	07/09/11	07/09/11
09/09/11	Inspection 2: counting of colonies according SOP TTOES085v03.	09/09/11	09/09/11
29 and 30 /09/11	Draft report : availability of certificate of analysis, batch number and expiry date of test item; usage of SOPs and their versions; control of all the raw data.	03/10/11	
18/10/11	Final report	18/10/11	

We declare that the report completely and accurately describes the used materials and methods and that the results and conclusions accurately reflect the raw data that were obtained during the study.

Date: 18-10-2011

Signatur [redacted]

[redacted]

10.1.c wob juncto 63.2ter.d Vo 1107/2009 juncto 39sexies.2 Vo 178/2002

STATEMENT OF GLP COMPLIANCE BY THE STUDY DIRECTOR

The study described in this report was carried out under my supervision and responsibility and in compliance with the OECD principles of Good Laboratory Practice.

I hereby attest to the authenticity of the study and guarantee that the study was performed according to the procedures described in this report. This study report is a complete and accurate representation of the data obtained.

There were no significant deviations which may have an adverse affect on the quality or integrity of this study.

The study director makes no GLP-compliance claim for the characterization and verification of the identity of the test item, which is the responsibility of the sponsor.

Date: 18/10/2011

Signatu [redacted]

[redacted]

10.1.c wob juncto 63.2ter.d Vo 1107/2009 juncto 39sexies.2 Vo 178/2002

1. NATURE AND PURPOSE OF THE STUDY

Objective of the study was to evaluate the potential of [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' and its metabolites (if relevant) to induce reverse mutations in five standard *Salmonella typhimurium* strains in the absence and in the presence of a rat liver metabolic activation system (S9).
An extra solvent control [redacted] was included to verify if this material does not induce reverse mutations on its own.

2. TEST SYSTEM

2.1. CHARACTERIZATION

2.1.1. Introduction

This Bacterial Reverse Mutation Test (1,2) uses histidine-requiring strains of *Salmonella typhimurium* to measure the frequency of spontaneous or chemical-induced point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. This Bacterial Reverse Mutation Test detects mutations restoring the functional capability of the bacteria to synthesize histidine. The revertant bacteria are easily detected as they recover the ability to grow in the absence of histidine. Comparing the number of colonies (revertants) growing on histidine poor medium in controls and when exposed to [redacted] Pepino Mosaic Virus, CH2 strain, isolate 1906', allows to evaluate the effect of the test item on the mutation frequency. This test has been shown to detect a wide range of classes of chemical mutagens (3,4).

2.1.2. Scientific background

To detect mutagenic activity, the histidine-requiring *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 both in the absence and in the presence of a metabolic activation system (S9-mix) was tested.

The strains TA98, TA100, TA102, TA1535, and TA1537 were obtained from J&JPRD (Beerse, Belgium). These master stocks were stored, according SOP TBACE001v05, in a -70 °C freezer. The test batches were aliquots of nutrient broth cultures and stored at -70 °C, according to SOP TBACE001v05. Dimethyl sulphoxide was added to the cultures as a cryopreservative. The test batches will be upon usage checked to confirm their histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), ampicillin plus tetracycline resistance (TA102), and UV-sensitivity.

Characteristics of the 5 different strains are given below:

TA strain	Histidine mutation	Type histidine mutation	LPS	Repair	R-factor	
					Ampicillin	Tetracycline
TA98	His D 3052	Frameshift	rfa	Δ UvrB	+R	-
TA100	His G 46	Base pair substitution	rfa	Δ UvrB	+R	-
TA102	His G 428	Base pair substitution	rfa	+	+R	+R
TA1535	His G 46	Base pair substitution	rfa	Δ UvrB	-	-
TA1537	His C 3076	Frameshift	rfa	Δ UvrB	-	-

The rfa mutation causes partial loss of the lipopolysaccharide (LPS) barrier. This mutation enables large test items to penetrate the cell wall more easily. The UvrB mutation causes a reduction in the DNA excision repair activity, resulting in an increased sensitivity to detect mutagens. This mutation is not present in TA102. The R-factor strains contain the plasmid pKM 101, which is an ampicillin resistance marker. This plasmid increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system normally present in *Salmonella typhimurium*. The strain TA102 also contains the plasmid pAQ1 and gives resistance to tetracycline.

For each test, fresh cultures were prepared by inoculating nutrient broth with a thawed aliquot of the stock cultures and incubating the broth overnight for approximately 8 hours at 37 °C while shaking. The inocula were taken from master plates or vials of frozen cultures, which have been checked for strain characteristics (histidine dependence, rfa character, UvrB character, and resistance to ampicillin or ampicillin plus tetracycline).
For each test also the strain characteristics as described above were determined.

2.1.3. Metabolic activation system

Bacteria used in this test do not contain the enzyme systems, which in mammals are known to metabolize pro-mutagens into mutagenic metabolites. To overcome this drawback, an exogenous metabolic activation system, mostly a liver post-mitochondrial fraction (S9) obtained from rats, was added to the bacteria.

S9 is obtained from Molecular Toxicology Incorporated (MolTox™, USA), which is prepared from male Sprague Dawley rats induced with Aroclor 1254. Batches of MolTox™ S9 are stored frozen in aliquots at approximately -70 °C prior to use. Each batch is checked by the manufacturer for sterility, protein content, and ability to metabolize pro-mutagens to bacterial mutagens.

Treatments were carried out both in the absence and presence of a 10 % S9-mix, according to the following table:

Ingredient	Stock concentration (M)	Quantity (µl/plate)	
		10% S9-mix	Buffer solution
Sodium phosphate buffer pH 7.4	0.1	-	500
Sodium phosphate buffer pH 7.4	0.2	250	-
Glucose-6-phosphate	1	2.5	-
NADP	0.1	20	-
KCl/$MgCl_2$	1.65/0.4	10	-
S9		50	-
Water		167.5	-

2.2. JUSTIFICATION OF THE TEST SYSTEM

The bacteria used in this Reverse Mutation Test and recommended by the OECD guideline 471 (1997) are suitable to detect mutagenic effects induced by genotoxic compounds (5).

3. TEST ITEM

3.1. IDENTIFICATION

10.1.c Wob juncto 63.2.d Vo 1107/2009

TEST ITEM	SPONSOR CODE	VITO CODE
[redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906	-	2011TOX007/1

The Certificate of Analysis, received from De Ceuster n.v. on 28 September 2011, revealed:

- Quantification [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906 using TaqMan RT-qPCR resulting in a Ct-value [redacted]
- Batch numbe[redacted]
- Expiring date: [redacted]

10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002

3.2. REGISTRATION

All handling of the test item was registered on forms BTEST001-Frm1v02, BTEST001-Frm3v03, and BTEST001-Frm4v02, which will be kept in room BIO1 – 0321 and afterwards in the archives.

4. CONTROL ITEMS

4.1. POSITIVE AND NEGATIEVE CONTROL ITEMS

Untreated controls were included. Negative controls comprised treatments with the extraction liquid, which in this case was fresh milli-Q-water. The negative controls were included at the same volume per plate (100 µl/plate) as the test item solutions.

The positive controls used for each of the strains in the absence and presence of a metabolizing S9 fraction are listed in the following table:

Chemical	Source	Solvent	Stock conc. (mg/ml)	Final conc. (µg/plate)	Use	
					Strain(s)	S9
Sodium azide (NaN_3)	Sigma	H_2O	5	5	TA100	-
				1	TA1535	-
4-Nitroquinoline oxide (4-NQO)	Sigma	DMSO	2	0.2	TA98	-
				2	TA102	-
9-Aminoacridine (9-AAC)	Sigma	DMSO	5	50	TA1537	-
2-Aminoanthracene (2-AA)	Acros Organics	DMSO	5	2.5	TA98, TA100, TA1535, TA1537	+
				7.5	TA102	+

The final concentrations used for the plate incorporation and the pre-incubation method were the same.

4.2. SOLVENT CONTROL ITEM

An extra solvent control [redacted] was included to verify if this material does not induce reverse mutations on its own.

TEST ITEM	SPONSOR CODE	VITO CODE
[redacted]	-	2011TOX008/1

Material Safety Data Sheet: Not available

The solvent controls were included at the same volume per plate (100 µl/plate) as the test item solutions.

5. TEST DESCRIPTION

5.1. GUIDELINES

This study followed the procedures indicated by the international accepted guidelines and recommendations:

- OECD Guideline 471: Genetic Toxicology: Bacterial Reverse Mutation Test (1997) (5).

5.2. EXPERIMENTAL DESIGN

The first and last day of use of pipettes, all the pipettes were controlled for good working. Data were registered on form TTOES083-Frm1v04.

Before use, test item [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' and the extra solvent control [redacted] were checked for sterility.

5.2.1. Toxicity range-finder experiment – plate incorporation method

Selection of an adequate range of doses was based on a toxicity range-finder experiment with the strain TA100 at 10 concentrations. The highest concentration was the highest water-soluble concentration as delivered by the Sponsor (100 µl/plate). The concentration of 100 µl/plate corresponds to a Ct-value of 17.98 measured using TaqMan RT-qPCR. The lower concentrations were separated by approximately half log intervals. In this case for the test item, the 10 concentrations were separated by a factor 2. Untreated, negative, solvent, and positive control plates were included. Suspensions of bacterial cells were exposed to [redacted] *epino Mosaic Virus*, CH2 strain, isolate 1906' in the presence and in the absence of S9. The plate incorporation method was used as described by Ames *et al.* (1) and updated by Maron and Ames (2). The test solution was mixed in triplicate with the strain TA100, the sterile buffer (-S9) or the metabolic activation system (+S9) and with the overlay (histidine-containing) agar containing a trace L-histidine and biotine (0.5 mM) to allow a few cell divisions. Plating was achieved by the following sequence of additions to 2 ml of molten agar at 45 +/- 2 °C:

- 0.1 ml of TA100 bacterial culture containing approximately 10^8 viable bacteria
- 0.1 ml o[redacted] *epino Mosaic Virus*, CH2 strain, isolate 1906' or control
- 0.5 ml of 10 % S9-mix (+S9) or buffer solution (-S9)

The mixture was rapidly mixed and poured on to Vogel-Bonner E agar plates. When set, the plates were inverted and incubated at 37 +/- 1 °C in the dark for 48 hours +/- 4 hours. Each petri dish was individually labelled with a unique code corresponding to form TBACE001v05-Frm09v01, identifying the content of the dish.

If at least five concentrations were scored, the countings on the number of revertant colonies were used for the actual mutagenicity data of the first Reverse Mutation Experiment.

5.2.2. Reverse Mutation Experiment 1 – plate incorporation method

As sufficient scorable concentrations were obtained in the range finding study with the strain TA100, this strain was not retested in Reverse Mutation Experiment 1.
The test item was tested for reverse mutations in the *Salmonella typhimurium* strains TA98, TA102, TA1535, and TA1537.
Seven concentrations, separated by a factor 2 were tested in triplicate in the absence and in the presence of rat liver S9-mix. Concentrations applied were 1.563, 3.125, 6.25, 12.5, 25, 50, and 100 µl/plate. Untreated, negative, solvent, and positive controls were included.

The highest dose level was selected on basis of data generated in the preliminary toxicity range-finder study.

Suspensions of bacterial cells were exposed to [redacted] *epino Mosaic Virus*, CH2 strain, isolate 1906' in the presence and in the absence of S9. The plate incorporation method was used as described by Ames *et al.* (1) and updated by Maron and

Ames (2). The test solution was mixed in triplicate with the strains, the sterile buffer (-S9) or the metabolic activation system (+S9) and with the overlay (histidine-containing) agar containing a trace L-histidine and biotine (0.5 mM) to allow a few cell divisions. Plating were achieved by the following sequence of additions to 2 ml of molten agar at 45 +/- 2 °C:

- 0.1 ml of bacterial culture containing approximately 10^8 viable bacteria
- 0.1 ml of [redacted] *epino Mosaic Virus*, CH2 strain, isolate 1906' or control
- 0.5 ml of 10 % S9 mix (+S9) or buffer solution (-S9)

The mixture was rapidly mixed and poured on to Vogel-Bonner E agar plates. When set, the plates were inverted and incubated at 37 +/- 1 °C in the dark for 48 +/- 4 hours. Each petri dish was individually labelled with a unique code corresponding to form TBACE001v05-Frm09v01, identifying the content of the dish.

5.2.3. Reverse Mutation Experiment 2 – pre-incubation method

As the first Reverse Mutation Test gave clearly negative results, the second Mutation Experiment included a pre-incubation step. Volumes of 0.3 ml of bacterial culture, 0.3 ml of [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' or control, and 1.5 ml of 10 % S9-mix (+S9) or buffer solution (-S9) were mixed together for 30 minutes at 37 +/- 1 °C, where after 6 ml of molten top agar (+/- 45 °C) was added. This was spread among three Vogel-Bonner E agar plates.

5.2.4. Colony scoring

Colonies were scored automatically by using the Sorcerer Image Analysis/Colony counting system (Perspective Instruments, UK) as described in SOP TTOES085v03.

The plates were examined for their background layer (signs of toxicity) and revertant colonies counted.

5.2.5. Data recording

Data are presented as the number of revertant colonies per plate. Individual plate counts, the mean number of revertant colonies per plate, and the standard deviation are presented for [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' and for the positive, untreated, negative, and solvent control. Counts were compared with the accepted normal ranges for our laboratory. To facilitate the follow up of the reversion induction, the induction factor (IF) was calculated and reported. The IF was obtained by dividing the mean number of revertant colonies obtained with the [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906'by the mean number of revertant colonies obtained with the negative control.

5.3. STANDARD OPERATING PROCEDURES CONCERNING THE STUDY

- SOP TBACE001v05: Ames-test
- SOP TTOES085v03: Sorcerer Colony Counter

- SOP TTOES091v03: Operation and maintenance of analytical scales.
- SOP TTOES062v02: Use and maintenance of pH meter.
- SOP TTOES083v03: Use of micropipettes (1 or more channels and repetition).
- SOP BTOES075v03: Calibration of Thermometers.
- SOP BTOES031v07: Use and maintenance of fridge or freezer.
- SOP TTOES027v04: Use and maintenance of Biohazards.
- SOP's concerning safety aspects: SOP BVEIL001v02, SOP BVEIL004v02; SOP BVEIL005v02; SOP BVEIL006v02, SOP BVEIL007v02; SOP BVEIL009v02; VITO-PRO-M004v02; VITO-WER-M004-1v08; VITO-PRO-M005v02; VITO-WER-M005-01v06

5.4. ACCEPTANCE CRITERIA OF THE STUDY

The experiment was considered valid if the following criteria were met:

- the mean number of spontaneous revertants and solvent control revertants fall reasonable within the laboratory historical control range for each strain. (See 11: Annex 1)
- the positive control chemicals induced a biological significant increase in the number of revertant colonies.
- the top concentration was tested at the highest water-soluble concentration as delivered by the Sponsor or should induce toxic effects or should show limited solubility as demonstrated in the preliminary toxicity range-finder study.

5.5. EVALUATION CRITERIA OF RESULTS

[redacted]epino Mosaic Virus, CH2 strain, isolate 1906 was considered to be mutagenic towards bacteria in the study if:

- the study is considered valid (acceptance criteria are met)
- a concentration related increase in the number of mean revertant colonies was observed with at least a 2-fold increase with the strains TA98, TA100, and TA102, and at least a 3-fold increase with the strains TA1535 and TA1537 in the absence and/or in the presence of rat liver S9-mix
- the results were repeated in an independent experiment

5.6. AMENDMENTS AND DEVIATIONS TO THE STUDY PLAN

During the process of the study, decisions and actions had to be taken which are detailed by amendments. Amendments and deviations are listed in function of time.

- **Amendment 1**: On request of the Sponsor, the name of the test item '*Pepino mosaic virus*, CH2 strain, isolate 1906' will be changed into [redacted]*Pepino Mosaic Virus*, CH2 strain, isolate 1906' and the name of the control [redacted] will be changed into [redacted] These new names will be used in further communication, results, and reports. 17 August 2011.
- **Amendment 2**: Following OECD 471, all plates in a given assay should be incubated at 37 °C for 48-72 hours. At CARDAM, all plates are incubated at 37 °C for 48 +/- 4 hours. This is not the same as described in OECD 471, but historical data from the database of CARDAM has proven that an incubation of 48 +/- 4 hours has no influence on the obtained results. 17 August 2011.

- **Deviation 1**: Strain TA100 was tested for crystalline violet sensitivity, however, the crystalline violet solution was expired (exp. from 13/04/2011). Strain TA100 was clearly crystal violet sensitive. Therefore, we can conclude that strain TA100 still had its required rfa mutation. This notification has no impact on the obtained results. 7 September 2011.
- **Deviation 2**: The overnight culture strain TA100 was intended to provide $>10^8$ cells/ml. This was measured by spreading aliquots (0.1 ml) of a 10-6 dilution of the overnight culture TA100 with top-agar containing saturated histidine solution and phosphate buffer on the surface of the plates. An expired saturated histidine solution (exp. 12/04/2011 and stored at -20°C) was used, but the Colony Forming Units were good (1.69×10^9 cells/ml). Therefore, we can conclude that the overnight culture strain TA100 was sufficiently grown. This notification has no impact on the obtained results. 7 September 2011.
- **Deviation 3**: The solvent control reversion rate (123.3 ± 11.2) with the strain TA102 in the presence of S9-mix was below the historical control range (132-514). But as the spontaneous reversion rate (192.7 ± 15.5) was within the historical control range (91-464), the revertant counts with the strain TA102 were accepted. 12 September 2011.
- **Deviation 4**: The solvent control reversion rate (91.0 ± 12.3) with the strain TA100 in the presence of S9-mix in the second Reverse Mutation Experiment was below the historical control range (136-218). But as the spontaneous reversion rate (108.0 ± 10.5) was within the historical control range (100-180), the revertant counts with the strain TA100 were accepted. 16 September 2011.
- **Deviation 5**: In the second Reverse Mutation Experiment (pre-incubation), for strain TA102 in the presence of rat liver S9-mix, no 2-fold increase of the positive control (2-aminoanthracene) compared to solvent control was obtained. For this reason, the second Reverse Mutation Experiment in the presence of S9-mix will be repeated for strain TA102 in week 38. 16 September 2011.
- **Deviation 6**: In the second Reverse Mutation Experiment (pre-incubation) repeat experiment (week 38), for strain TA102 in the presence of rat liver S9-mix, no 2-fold increase of the positive control (2-aminoanthracene) was obtained compared to solvent control. For this reason, the second Reverse Mutation Experiment in the presence of S9-mix will be repeated for strain TA102 in week 39. This time a daughter culture tube will be used instead of a test culture tube of strain TA102. 22 September 2011.

6. RESULTS

Before use, test item [redacted] *epino Mosaic Virus*, CH2 strain, isolate 1906' and the extra solvent control [redacted] vere checked for sterility. No signs of contamination were observed for both and no filtration was therefore needed.

The results obtained with [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' and positive control compounds are presented in tables 10.1.1 to 10.3.7.

6.1. TOXICITY RANGE-FINDER EXPERIMENT - PLATE INCORPORATION METHOD

The top concentration for the toxicity range-finder experiment was 100 µl/plate, which was the highest water-soluble concentration as delivered by the Sponsor. The concentration of 100 µl/plate corresponds to a Ct-value of 17.98 measured using TaqMan RT-qPCR. Concentrations to test were: 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50, and 100 µl/plate.

For strain TA100, the characteristics (histidine-dependence, rfa character, UvrB character, and resistance to ampicillin) were determined and the CFU were counted. Despite solutions crystalline violet and histidine were expired (deviations 1 and 2), strain TA100 was found to be genetically found.

The spontaneous reversion rate was within the historical control range of the laboratory (range 100 - 180) with a value of 120.3. The mean revertant colony counts for the solvent control (water) were within the current historical control range of the laboratory (table Annex 1) for *Salmonella typhimurium* strain TA100 with a value of 131.3 in the absence of rat liver S9 and 147.7 in the presence of S9.

Appropriate positive control chemicals induced a substantial increase in the revertant colony number with TA100, confirming sensitivity of the culture and activity of S9-mix. In the study plan was included an extra acceptance criteria that revertants of positive controls should be in the historical positive control range of the laboratory. This criteria was not for every strain fulfilled, but the extra criteria is not required according to SOP TBACE001v05, nor according to OECD 471, so these results are accepted and in line with the official guidelines.

On basis of these findings, the toxicity range-finder experiment generated with [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' for strain TA100 was accepted.

In the preliminary range finding plate incorporation test, [redacted] was found to be not mutagenic with the strain TA100 in the absence and in the presence of rat liver S9-mix.

No substantial increases in revertant colony numbers over solvent control counts were obtained with strain TA100 following exposure to [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' at 10 selected concentrations in either the presence or absence of S9-mix (table 10.1.1). As sufficient scorable concentrations were obtained in the range finding study with the strain TA100, this strain was not retested in Reverse Mutation Experiment 1.

6.2. REVERSE MUTATION EXPERIMENT 1 - PLATE INCORPORATION METHOD

The top concentration for the main test with the strains TA98, TA102, TA1535, and TA1537 in the absence and in the presence of S9-mix was the same as for the toxicity range-finder

experiment with the strain TA100. The concentrations for the main study were 1.563, 3.125, 6.25, 12.5, 25, 50, and 100 µl/plate.

For every strain, the characteristics (histidine-dependence, rfa character, UvrB character, and resistance to ampicillin or ampicillin plus tetracycline) were determined and the CFU were counted. All strains were found to be genetically fine.

The spontaneous reversion rate for the strains TA98, TA102, TA1535, and TA1537 was within the historical control range of the laboratory (table Annex 1). The mean revertant colony counts for the solvent control (water) were within the current historical control range of the laboratory (table Annex 1) for the *Salmonella typhimurium* strains TA98, TA1535, and TA1537 in the absence and in the presence of rat liver S9, respectively. The solvent control reversion rate with the strain TA102 in the absence of S9-mix was within the current historical control range of the laboratory (table Annex 1). The solvent control reversion rate (123.3 ± 11.2) with the strain TA102 in the presence of S9-mix was below the historical control range (132-514). But as the spontaneous reversion rate was within the historical control range, the revertant counts with the strain TA102 were accepted (deviation 3).

Appropriate positive control chemicals induced substantial increase in revertant colony number with all strains, confirming sensitivity of the cultures and activity of S9-mix. In the study plan was included an extra acceptance criteria that revertants of positive controls should be in the historical positive control range of the laboratory. This criteria was not for every strain fulfilled, but the extra criteria is not required according to SOP TBACE001v05, nor according to OECD 471, so these results are accepted and in line with the official guidelines.

On basis of these findings, all strains were considered to be of good quality to assess the mutagenic potential of [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906'.

In the plate incorporation test, [redacted] was found to be not mutagenic with any of the strains in the absence and in the presence of rat liver S9-mix.

No biological significant increase in the reversion rate was observed with any of the tester strains following exposure to 'Tomato watery leaf extract containing *Pepino Mosaic Virus*, CH2 strain, isolate 1906' at any concentration in either the absence or presence of a rat liver metabolic activation system (tables 10.2.1 to 10.2.4).

6.3. REVERSE MUTATION EXPERIMENT 2 – PRE-INCUBATION METHOD

As the plate incorporation test gave clearly negative results, a second Reverse Mutation Experiment including a pre-incubation step was performed with the strains TA98, TA100, TA102, TA1535, and TA1537.

For every strain, the characteristics (histidine-dependence, rfa character, UvrB character, and resistance to ampicillin or ampicillin plus tetracycline) were determined and the CFU were counted. All strains were found to be genetically fine.

The spontaneous reversion rate for all the strains was within the historical control range of the laboratory (table Annex 1). The mean revertant colony counts for the solvent control (water) were within the current historical control range of the laboratory (table Annex 1) for the *Salmonella typhimurium* strains TA98, TA102, TA1535, and TA1537 in the absence and in the presence of rat liver S9, respectively.

The solvent control reversion rate with the strain TA100 in the absence of S9-mix was within the current historical control range of the laboratory (table Annex 1). The solvent control reversion rate (91.0 ± 12.3) with the strain TA100 in the presence of S9-mix was below the historical control range (136-218). But as the spontaneous reversion rate was within the historical control range, the revertant counts with the strain TA100 were accepted (deviation 4).

Appropriate positive control chemicals induced substantial increase in revertant colony number with the strains TA98, TA100, TA1535, and TA1537 confirming sensitivity of the cultures and activity of S9-mix. The same was true for strain TA102 in the absence of S9-mix. In the second Reverse Mutation Experiment (table 10.3.3), as well as in the first repeat of this experiment (table 10.3.6), for strain TA102 in the presence of S9-mix, no 2-fold increase of the positive control (2-aminoanthracene) was obtained compared to solvent control. For that reason, the second Reverse Mutation Experiment in the presence of S9-mix was repeated again for strain TA102 starting from a daughter culture instead of a test culture tube (table 10.3.7). For the latter experiment, a 2-fold increase of the positive control (2-aminoanthracene) was obtained compared to solvent control. In the study plan was included an extra acceptance criteria that revertants of positive controls should be in the historical positive control range of the laboratory. This criteria was not for every strain fulfilled, but the extra criteria is not required according to SOP TBACE001v05, nor according to OECD 471, so these results are accepted and in line with the official guidelines.

On basis of these findings, the data generated with [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' were accepted.

In the pre-incubation test, tomato watery leaf extract was found to be not mutagenic with any of the strains in the absence and in the presence of rat liver S9-mix.

No biological significant increase in the reversion rate was observed with any of the tester strains following exposure to [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' at any concentration in either the absence or presence of a rat liver metabolic activation system (tables 10.3.1 to 10.3.7). For strain TA1537 in the absence of S9-mix only, a borderline increased reversion rate (2.92), which was not yet biological significant

(IF > 3), was observed for the highest concentration 100 µl/plate. As no dose-related increase in reversion rate was observed for all concentrations tested, this finding is considered to be a fortuitous finding of no importance.

6.4. CONCLUSION

On basis of these findings, it can be concluded that [redacted] *Pepino Mosaic Virus*, CH2 strain, isolate 1906' showed, under the test conditions employed, no evidence of mutagenic activity towards the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 at the tested concentrations.

7. ARCHIVES

The study dossier (original study plan, amendments, deviations, draft report, certified copies of the study report and attached documents, the original raw data, the product registration forms) and for identification purpose a small amount of the test item will be retained in archives (BIOL, room 0110 and BIO2 room 0068) of VITO during 5 years after issue of the final report. After this time, the sponsor will be contacted and his advice sought to return the study file and materials to the sponsor, retain at VITO or store at a location designated by the sponsor. If requested, VITO will continue to retain the study file and materials or help the sponsor to find another location for archiving. The sponsor will be notified of the financial implications of each of these options at that time.

8. QUALITY ASSURANCE

The following will be inspected or audited in relation to this study:

- study plan and its specific pages
- the critical phases (at least one of the aspects for each of these critical phases will be controlled by the quality manager)
- the report and study data will be audited before issue of the draft report to the Sponsor for review and comments

The study will be conducted in compliance with the Good Laboratory Practice Standards as set forth in OECD series on Principles of Good Laboratory Practice and Compliance Monitoring: Number 14: The Application of the Principles of GLP to *in vitro* Studies (ENV/JM/MONO(2004)26) + Number 1: OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98)17).

9. REFERENCES

1. Ames BN, McCann J and Yamasaki E (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31: 347-364.

2. Maron DM and Ames BN (1983). Revised methods for the Salmonella mutagenicity test. Mutation Res. 113:173-215.
3. McCann J and Ames BN (1976). Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA. 73:950-954.
4. McCann J, Choi E, Yamasaki E and Ames BN (1975). Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA. 72: 5135-5139.
5. OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test, 1997).

10. TABLE OF RESULTS

10.1. TOXICITY RANGE-FINDER EXPERIMENT - PLATE INCORPORATION METHOD

10.1.1. Strain *Salmonella typhimurium* TA100

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	95								
	-	-	124	120.3	23.7	-					
	-	-	142								
Solvent: water	-	Nt	142				Nt	151			
	-	-	127	131.3	9.3	**1.00**	-	145	147.7	3.1	**1.00**
	-	-	125				-	147			
[redacted]	100.00	Nt	132				Nt	125			
		-	111	125.0	12.1	**0.95**	-	115	132.0	21.4	**0.89**
		-	132				-	156			
Pepino Mosaic Virus, CH2 strain, isolate 1906 [redacted]	0.20	Nt	121				Nt	130			
		-	129	129.0	8.0	**0.98**	-	139	129.3	10.0	**0.88**
		-	137				-	119			
	0.39	Nt	117				Nt	140			
		-	95	116.7	21.5	**0.89**	-	129	139.7	10.5	**0.95**
		-	138				-	150			
	0.78	Nt	113				Nt	150			
		-	125	121.3	7.2	**0.92**	-	143	145.0	4.4	**0.98**
		-	126				-	142			
	1.56	Nt	130				Nt	121			
		-	112	118.7	9.9	**0.90**	-	124	125.0	4.6	**0.85**
		-	114				-	130			
	3.13	Nt	C				Nt	154			
		-	191	154.0	52.3	**1.17**	-	127	132.0	20.0	**0.89**
		-	117				-	115			
	6.25	Nt	126				Nt	149			
		-	116	121.7	5.1	**0.93**	-	159	160.3	12.1	**1.09**
		-	123				-	173			
	12.50	Nt	C				Nt	146			
		-	156	155.0	1.4	**1.18**	-	130	137.0	8.2	**0.93**
		-	154				-	135			
	25.00	Nt	124				Nt	151			
		-	122	126.0	5.3	**0.96**	-	86	126.7	35.4	**0.86**
		-	132				-	143			
	50.00	Nt	138				Nt	143			
		-	140	128.7	17.9	**0.98**	-	207	168.0	34.2	**1.14**
		-	108				-	154			
	100.00	Nt	137				Nt	158			
		-	135	132.7	5.9	**1.01**	-	155	153.0	6.2	**1.04**
			126					146			
SA	5.00	Nt	823								
		-	687	699.7	117.5	**5.33**					
		-	589								
2-AA	2.50						Nt	620			
							-	631	622.3	7.8	**4.21**
							-	616			

SR: spontaneous reversion
IF: induction factor
SA: sodium azide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.2. REVERSE MUTATION EXPERIMENT 1 – PLATE INCORPORATION RESULTS

10.2.1. Strain *Salmonella typhimurium* TA98

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	28								
	-	-	21	23.0	4.4	-					
	-	-	20								
Solvent: water	-	Nt	20				Nt	31			
	-	-	32	23.3	7.6	**1.00**	-	40	34.0	5.2	**1.00**
	-	-	18					31			
[redacted]	100.00	Nt	21				Nt	38			
		-	30	28.0	6.2	**1.20**	-	31	33.3	4.0	**0.98**
		-	33					31			
Pepino Mosaic Virus, CH2 strain, isolate 1906 [redacted]	1.56	Nt	19				Nt	32			
		-	18	22.0	6.1	**0.94**	-	31	30.0	2.6	**0.88**
		-	29				-	27			
	3.13	Nt	23				Nt	30			
		-	20	20.7	2.1	**0.89**	-	28	30.0	2.0	**0.88**
		-	19				-	32			
	6.25	Nt	18				Nt	39			
		-	18	20.3	4.0	**0.87**	-	29	33.3	5.1	**0.98**
		-	25				-	32			
	12.50	Nt	30				Nt	34			
		-	29	28.7	1.5	**1.23**	-	33	32.7	1.5	**0.96**
		-	27				-	31			
	25.00	Nt	20				Nt	34			
		-	29	26.3	5.5	**1.13**	-	28	30.7	3.1	**0.90**
		-	30				-	30			
	50.00	Nt	32				Nt	45			
		-	27	27.3	4.5	**1.17**	-	32	36.3	7.5	**1.07**
		-	23				-	32			
	100.00	Nt	20				Nt	25			
		-	32	29.0	7.9	**1.24**	-	34	31.0	5.2	**0.91**
		-	35				-	34			
4-NQO	0.20	Nt	98								
		-	111	103.3	6.8	**4.43**					
		-	101								
2-AA	2.50						Nt	1861			
							-	1474	1609.0	218.4	**47.32**
							-	1492			

SR: spontaneous reversion
IF: induction factor
4-NQO: 4-nitroquinoline N-oxide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.2.2. Strain *Salmonella typhimurium* TA102

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	210								
	-	-	188	192.7	15.5	-					
	-	-	180								
Solvent: water	-	Nt	179				Nt	115			
	-	-	169	173.7	5.0	**1.00**	-	119	123.3	11.2	**1.00**
	-	-	173				-	136			
[redacted]	100.00	Nt	217				Nt	190			
		-	231	222.7	7.4	**1.28**	-	173	187.0	12.8	**1.52**
		-	220				-	198			
[redacted] Pepino Mosaic Virus, CH2 strain, isolate 1906	1.56	Nt	121				Nt	127			
		-	156	149.0	25.2	**0.86**	-	128	127.3	0.6	**1.03**
		-	170				-	127			
	3.13	Nt	171				Nt	122			
		-	191	167.0	26.2	**0.96**	-	140	126.3	12.1	**1.02**
		-	139				-	117			
	6.25	Nt	143				Nt	309			
		-	174	158.3	15.5	**0.91**	-	145	182.7	112.3	**1.48**
		-	158				-	94			
	12.50	Nt	208				Nt	107			
		-	190	194.3	12.1	**1.12**	-	136	123.3	14.8	**1.00**
		-	185				-	127			
	25.00	Nt	189				Nt	139			
		-	177	189.3	12.5	**1.09**	-	144	126.7	25.8	**1.03**
		-	202				-	97			
	50.00	Nt	166				Nt	215			
		-	249	204.7	41.8	**1.18**	-	171	176.7	35.8	**1.43**
		-	199				-	144			
	100.00	Nt	212				Nt	161			
		-	240	225.3	14.0	**1.30**	-	174	152.7	26.5	**1.24**
		-	224				-	123			
4-NQO	2.00	Nt	1514								
		-	1451	1403.3	140.7	**8.08**					
		-	1245								
2-AA	7.50						Nt	462			
							-	415	391.0	85.6	**3.17**
							-	296			

SR: spontaneous reversion
IF: induction factor
4-NQO: 4-nitroquinoline N-oxide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.2.3. Strain *Salmonella typhimurium* TA1535

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	36								
	-	-	34	35.3	1.2	-					
	-	-	36								
Solvent: water	-	Nt	42				Nt	8			
	-	-	38	42.0	4.0	**1.00**	-	10	9.0	1.0	**1.00**
	-	-	46				-	9			
[redacted]		Nt	25				Nt	9			
	100.00	-	42	34.3	8.6	**0.82**	-	9	11.0	3.5	**1.22**
		-	36				-	15			
[redacted] Pepino Mosaic Virus, CH2 strain, isolate 1906		Nt	36				Nt	13			
	1.56	-	21	29.7	7.8	**0.71**	-	9	10.3	2.3	**1.15**
		-	32				-	9			
		Nt	30				Nt	7			
	3.13	-	23	29.3	6.0	**0.70**	-	6	7.3	1.5	**0.81**
		-	35				-	9			
		Nt	30				Nt	12			
	6.25	-	45	35.7	8.1	**0.85**	-	4	7.7	4.0	**0.85**
		-	32				-	7			
		Nt	48				Nt	11			
	12.50	-	28	39.3	10.3	**0.94**	-	7	9.7	2.3	**1.07**
		-	42				-	11			
		Nt	32				Nt	14			
	25.00	-	29	30.5	2.1	**0.73**	-	13	10.3	5.5	**1.15**
		-	C				-	4			
		Nt	C				Nt	24			
	50.00	-	43	38.0	7.1	**0.90**	-	7	15.3	8.5	**1.70**
		-	33				-	15			
		Nt	32				Nt	9			
	100.00	-	45	39.0	6.6	**0.93**	-	9	11.0	3.5	**1.22**
		-	40				-	15			
SA		Nt	524								
	1.00	-	614	587.3	55.1	**13.98**					
		-	624								
2-AA							Nt	148			
	2.50						-	132	131.0	17.5	**14.56**
							-	113			

SR: spontaneous reversion
IF: induction factor
SA: sodium azide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.2.4. Strain *Salmonella typhimurium* TA1537

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	7	5.0	1.7	-					
	-	-	4								
	-	-	4								
Solvent: water	-	Nt	4	4.0	0.0	**1.00**	Nt	2	5.3	2.9	**1.00**
	-	-	4				-	7			
	-	-	4				-	7			
[redacted]	100.00	Nt	9	8.7	0.6	**2.17**	Nt	3	8.7	5.1	**1.63**
		-	9				-	13			
		-	8				-	10			
Pepino Mosaic Virus, CH2 strain, isolate 1906 [redacted]	1.56	Nt	7	6.3	0.6	**1.58**	Nt	12	8.7	2.9	**1.63**
		-	6				-	7			
		-	6				-	7			
	3.13	Nt	4	8.3	5.9	**2.08**	Nt	4	4.3	1.5	**0.81**
		-	6				-	6			
		-	15				-	3			
	6.25	Nt	10	6.7	3.1	**1.67**	Nt	7	6.0	1.7	**1.13**
		-	6				-	7			
		-	4				-	4			
	12.50	Nt	11	9.7	1.5	**2.42**	Nt	7	5.7	1.5	**1.06**
		-	10				-	6			
		-	8				-	4			
	25.00	Nt	9	11.3	5.9	**2.83**	Nt	3	6.7	4.0	**1.25**
		-	18				-	11			
		-	7				-	6			
	50.00	Nt	8	8.0	0.0	**2.00**	Nt	4	11.0	6.6	**2.06**
		-	8				-	12			
		-	8				-	17			
	100.00	Nt	7	8.0	2.6	**2.00**	Nt	1	6.3	5.0	**1.19**
		-	11				-	7			
		-	6				-	11			
9-AAC	50.00	Nt	109	156.0	58.1	**39.00**					
		-	221								
		-	138								
2-AA	2.50						Nt	129	115.3	14.6	**21.63**
							-	117			
							-	100			

SR: spontaneous reversion
IF: induction factor
9-AAC: 9-aminoacridine
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.3. REVERSE MUTATION EXPERIMENT 2 – PRE-INCUBATION RESULTS

10.3.1. Strain *Salmonella typhimurium* TA98

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	24								
	-	-	18	21.3	3.1	-					
	-	-	22								
Solvent: water	-	Nt	25				Nt	23			
	-	-	32	26.0	5.6	1.00	-	33	27.7	5.0	1.00
	-	-	21					27			
[redacted]	100.00	Nt	20				Nt	27			
		-	22	21.0	1.0	0.81	-	24	25.3	1.5	0.92
		-	21					25			
Pepino Mosaic Virus, CH2 strain, isolate 1906 [redacted]	1.56	Nt	18				Nt	18			
		-	23	22.0	3.6	0.85	-	23	20.7	2.5	0.75
		-	25				-	21			
	3.13	Nt	18				Nt	24			
		-	29	19.7	8.6	0.76	-	20	22.7	2.3	0.82
		-	12				-	24			
	6.25	Nt	23				Nt	25			
		-	23	20.0	5.2	0.77	-	27	27.0	2.0	0.98
		-	14				-	29			
	12.50	Nt	23				Nt	23			
		-	28	24.0	3.6	0.92	-	32	25.7	5.5	0.93
		-	21				-	22			
	25.00	Nt	20				Nt	32			
		-	29	24.3	4.5	0.94	-	43	36.3	5.9	1.31
		-	24				-	34			
	50.00	Nt	29				Nt	34			
		-	14	24.0	8.7	0.92	-	40	32.0	9.2	1.16
		-	29				-	22			
	100.00	Nt	30				Nt	31			
		-	23	23.7	6.0	0.91	-	33	31.7	1.2	1.14
		-	18				-	31			
4-NQO	0.20	Nt	192								
		-	173	194.0	22.1	7.46					
		-	217								
2-AA	2.50						Nt	924			
							-	874	927.7	55.6	33.53
							-	985			

SR: spontaneous reversion
IF: induction factor
4-NQO: 4-nitroquinoline N-oxide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.3.2. Strain *Salmonella typhimurium* TA100

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	97	108.0	10.5	-					
	-	-	109								
	-	-	118								
Solvent: water	-	Nt	95	103.3	15.3	1.00	Nt	77	91.0	12.3	1.00
	-	-	121				-	96			
	-	-	94				-	100			
[redacted]	100.00	Nt	140	142.0	5.3	1.37	Nt	104	106.7	7.4	1.17
		-	138				-	115			
		-	148				-	101			
Pepino Mosaic Virus, CH2 strain, isolate 1906 [redacted]	1.56	Nt	105	109.0	10.6	1.05	Nt	93	110.7	15.9	1.22
		-	101				-	115			
		-	121				-	124			
	3.13	Nt	142	123.7	17.6	1.20	Nt	109	107.0	9.2	1.18
		-	122				-	97			
		-	107				-	115			
	6.25	Nt	119	116.0	4.4	1.12	Nt	116	107.0	10.1	1.18
		-	111				-	109			
		-	118				-	96			
	12.50	Nt	115	114.7	8.5	1.11	Nt	97	116.0	18.1	1.27
		-	106				-	118			
		-	123				-	133			
	25.00	Nt	171	150.0	27.4	1.45	Nt	125	140.7	17.8	1.55
		-	119				-	160			
		-	160				-	137			
	50.00	Nt	176	146.0	26.9	1.41	Nt	145	135.0	11.1	1.48
		-	124				-	137			
		-	138				-	123			
	100.00	Nt	132	125.3	6.5	1.21	Nt	132	130.7	19.0	1.44
		-	125				-	149			
			119					111			
SA	5.00	Nt	805	812.3	6.4	7.86					
		-	817								
		-	815								
2-AA	2.50						Nt	1072	1089.0	37.5	11.97
							-	1063			
							-	1132			

SR: spontaneous reversion
IF: induction factor
SA: sodium azide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.3.3. Strain *Salmonella typhimurium* TA102 (invalidated results in the presence of S9)

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	145								
	-	-	136	149.3	15.9	-					
	-	-	167								
Solvent: water	-	Nt	145				Nt	127			
	-	-	151	153.7	10.3	**1.00**	-	132	133.7	7.6	**1.00**
	-	-	165				-	142			
[redacted]	100.00	Nt	275				Nt	180			
		-	285	280.3	5.0	**1.82**	-	163	156.0	28.2	**1.17**
		-	281				-	125			
Pepino [redacted] Mosaic Virus, CH2 strain, isolate 1906	1.56	Nt	126				Nt	113			
		-	156	154.0	27.1	**1.00**	-	98	113.7	16.0	**0.85**
		-	180				-	130			
	3.13	Nt	180				Nt	139			
		-	177	175.0	6.2	**1.14**	-	80	115.7	31.4	**0.87**
		-	168				-	128			
	6.25	Nt	255				Nt	74			
		-	205	212.0	40.0	**1.38**	-	153	123.7	43.2	**0.93**
		-	176				-	144			
	12.50	Nt	108				Nt	130			
		-	168	146.7	33.5	**0.95**	-	133	125.0	11.4	**0.94**
		-	164				-	112			
	25.00	Nt	205				Nt	103			
		-	227	219.7	12.7	**1.43**	-	113	95.7	21.9	**0.72**
		-	227				-	71			
	50.00	Nt	274				Nt	117			
		-	199	226.0	41.7	**1.47**	-	85	106.7	18.8	**0.80**
		-	205				-	118			
	100.00	Nt	280				Nt	144			
		-	218	229.3	46.1	**1.49**	-	90	112.0	28.4	**0.84**
		-	190				-	102			
4-NQO	2.00	Nt	527								
		-	634	623.0	91.0	**4.05**					
		-	708								
2-AA	7.50						Nt	135			
							-	174	164.3	25.9	**1.23**
							-	184			

SR: spontaneous reversion
IF: induction factor
4-NQO: 4-nitroquinoline N-oxide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.3.4. Strain *Salmonella typhimurium* TA1535

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	41	39.7	9.1	-					
	-	-	48								
	-	-	30								
Solvent: water	-	Nt	48	42.0	6.0	**1.00**	Nt	14	11.7	2.5	**1.00**
	-	-	42				-	9			
	-	-	36				-	12			
[redacted]	100.00	Nt	39	51.0	10.6	**1.21**	Nt	9	10.7	1.5	**0.91**
		-	55				-	12			
		-	59				-	11			
Pepino [redacted] Mosaic Virus, CH2 strain, isolate 1906	1.56	Nt	35	43.3	9.1	**1.03**	Nt	10	13.7	4.0	**1.17**
		-	53				-	18			
		-	42				-	13			
	3.13	Nt	40	46.7	13.3	**1.11**	Nt	9	8.7	1.5	**0.74**
		-	62				-	10			
		-	38				-	7			
	6.25	Nt	46	44.0	2.6	**1.05**	Nt	14	13.7	0.6	**1.17**
		-	45				-	14			
		-	41				-	13			
	12.50	Nt	55	45.3	8.5	**1.08**	Nt	10	12.0	4.4	**1.03**
		-	39				-	9			
		-	42				-	17			
	25.00	Nt	52	43.0	18.2	**1.02**	Nt	12	11.7	0.6	**1.00**
		-	55				-	11			
		-	22				-	12			
	50.00	Nt	29	23.7	5.5	**0.56**	Nt	4	8.3	5.1	**0.71**
		-	24				-	7			
		-	18				-	14			
	100.00	Nt	28	28.0	3.0	**0.67**	Nt	11	18.0	6.1	**1.54**
		-	25				-	22			
		-	31				-	21			
SA	1.00	Nt	485	567.0	105.5	**13.50**					
		-	530								
		-	686								
2-AA	2.50						Nt	64	84.7	20.5	**7.26**
							-	85			
							-	105			

SR: spontaneous reversion
IF: induction factor
SA: sodium azide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.3.5. Strain *Salmonella typhimurium* TA1537

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	6	3.7	2.5	-					
	-	-	1								
	-	-	4								
Solvent: water	-	Nt	2	4.3	2.5	**1.00**	Nt	3	4.0	2.6	**1.00**
	-	-	7				-	7			
	-	-	4				-	2			
[redacted]	100.00	Nt	2	6.0	5.3	**1.38**	Nt	4	3.0	1.7	**0.75**
		-	4				-	1			
		-	12				-	4			
Pepino Mosaic Virus, CH2 strain, isolate P06 [redacted]	1.56	Nt	9	6.3	2.5	**1.46**	Nt	2	3.0	1.0	**0.75**
		-	4				-	3			
		-	6				-	4			
	3.13	Nt	9	7.3	1.5	**1.69**	Nt	7	4.0	2.6	**1.00**
		-	6				-	2			
		-	7				-	3			
	6.25	Nt	11	9.7	2.3	**2.23**	Nt	1	2.7	1.5	**0.67**
		-	11				-	4			
		-	7				-	3			
	12.50	Nt	8	7.0	3.6	**1.62**	Nt	3	3.7	0.6	**0.92**
		-	10				-	4			
		-	3				-	4			
	25.00	Nt	8	9.7	2.1	**2.23**	Nt	11	5.7	4.6	**1.42**
		-	9				-	3			
		-	12				-	3			
	50.00	Nt	7	7.7	0.6	**1.77**	Nt	4	3.0	1.0	**0.75**
		-	8				-	2			
		-	8				-	3			
	100.00	Nt	14	12.7	3.2	**2.92**	Nt	6	4.0	1.7	**1.00**
		-	15				-	3			
		-	9				-	3			
9-AAC	50.00	Nt	101	99.3	3.8	**22.92**					
		-	102								
		-	95								
2-AA	2.50						Nt	220	367.3	137.8	**91.83**
							-	389			
							-	493			

SR: spontaneous reversion
IF: induction factor
9-AAC: 9-aminoacridine
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.3.6. Strain *Salmonella typhimurium* TA102 – repeat experiment: invalidated results

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	-	-	178	171.3	18.9	-					
	-	-	150								
	-	-	186								
Solvent: water	-						Nt	250			
	-						-	236	242.7	7.0	**1.00**
	-						-	242			
[redacted]	100.00						Nt	289			
							-	312	260.0	71.1	**1.07**
							-	179			
Pepino Mosaic virus, CH2 strain, isolate 1906	1.56						Nt	184			
							-	249	218.3	32.7	**0.90**
							-	222			
	3.13						Nt	236			
							-	266	253.0	15.4	**1.04**
							-	257			
	6.25						Nt	260			
							-	260	261.0	1.7	**1.08**
							-	263			
	12.50						Nt	187			
							-	248	225.3	33.4	**0.93**
							-	241			
	25.00						Nt	187			
							-	190	170.7	30.9	**0.70**
							-	135			
	50.00						Nt	260			
							-	292	258.0	35.0	**1.06**
							-	222			
	100.00						Nt	217			
							-	146	204.0	52.7	**0.84**
							-	249			
4-NQO	2.00										
2-AA	7.50						t	374			
							t	375	378.0	6.1	**1.56**
							t	385			

SR: spontaneous reversion
IF: induction factor
4-NQO: 4-nitroquinoline N-oxide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

10.3.7. Strain *Salmonella typhimurium* TA102 – repeat experiment 2

Test item	Conc. (µl/plate)	In the absence of S9					In the presence of S9				
		Signs of toxicity	Revertant countings			IF	Signs of toxicity	Revertant countings			IF
			Per plate	Mean	SD			Per plate	Mean	SD	
SR	- - -	- - -	217 211 215	214.3	3.1	-					
Solvent: water	- - -						Nt - -	134 104 177	138.3	36.7	**1.00**
[redacted]	100.00						Nt - -	231 137 282	216.7	73.6	**1.57**
[redacted] Pepino Mosaic Virus, CH2 strain, isolate P706	1.56						Nt - -	133 124 142	133.0	9.0	**0.96**
	3.13						Nt - -	147 147 128	140.7	11.0	**1.02**
	6.25						Nt - -	139 143 165	149.0	14.0	**1.08**
	12.50						Nt - -	126 165 123	138.0	23.4	**1.00**
	25.00						Nt - -	123 147 104	124.7	21.5	**0.90**
	50.00						Nt - -	237 207 115	186.3	63.6	**1.35**
	100.00						Nt - -	122 208 180	170.0	43.9	**1.23**
4-NQO	2.00										
2-AA	7.50						Nt Nt Nt	1162 1073 1380	1205.0	158.0	**8.71**

SR: spontaneous reversion
IF: induction factor
4-NQO: 4-nitroquinoline N-oxide
2-AA: 2-aminoanthracene
Nt: not toxic; -: not determined; t: partly no background; T: no background layer; pp: pinpoints; p: precipitation; C: contamination

11. ANNEX 1: HISTORICAL CONTROL DATA

11.1. SPONTANEOUS REVERSION RATE

Strain	Minimum	Maximum
TA98	13	49
TA100	100	180
TA102	91	464
TA1535	6	47
TA1537	3	13

The values for the strains TA98, TA100, TA102, TA1535 and TA1537 represent our laboratory historical control data since April 2008 to February 2011.

11.2. MEAN VALUE OF REVERTANTS/PLATE FOR THE SOLVENT CONTROL

Strain	Without metabolic activation	With metabolic activation
TA98	9-45	15-60
TA100	99-205	136-218
TA102	104-491	132-514
TA1535	9-55	3-20
TA1537	2-14	2-17

The values for the strains TA98, TA100, TA102, TA1535 and TA1537 represent our laboratory historical control data since April 2008 to February 2011.