STUDY REPORT

DETERMINATION OF THE GENOTOXICITY OF THE MAINSTREAM AEROSOL FRACTIONS GENERATED FROM THE TEST ITEM, TOBACCO HEATING SYSTEM TOBACCO STICKS, AND THE MAINSTREAM SMOKE FRACTIONS GENERATED FROM THE REFERENCE ITEM, 3R4F, IN THE *IN VITRO* MICRONUCLEUS ASSAY

(STUDY NUMBER RLS-ZRH-2017-661)

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1 APPROVAL

1.1 Study Director's GLP Statement of Compliance

The Study Director acknowledges responsibility for the validity of the data and confirms that this study has been performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice (GLP), adopted May 18th, 2005 [RS 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97)186/Final].

Name	**Date / Signature**
Smart, Daniel	13 June 2018

1.2 Quality Assurance Statement

The general facilities and activities are inspected periodically and the results are reported to the responsible person and to the Test Facility Management (TFM).

The verification of the study plan and all study plan amendments, as well as inspections on this study and the reports were performed by the Quality Assurance Unit at Philip Morris Products S.A., Research & Development, PMI Product Testing. All findings of the following inspections were reported to the study director and TFM. The dates are given below.

This statement also confirms that the final study report reflects the raw data.

Inspection Type and Phase Inspected	**Date of Inspection**	**Date Inspection Report Sent to TFM and Study Director**
Study Plan	07-08 Aug. 2017	08 Aug. 2017
Aerosol Collection of Test Item	31 Aug. 2017	31 Aug. 2017
Study Data and Study Report	25-28-29-30-31 May 2018	31 May 2018

The following phases have been inspected in study RLS-ASC-2017-80 and RLS-ASC-2017-83: aerosol collection for the reference item, cell thawing, dilutions and cell exposure, end staining and analysis, nicotine determination, T0 staining and analysis, and washing.

Name	**Date / Signature**
d'Estais, Guy (QA personnel)	13 Jun 2018

2 GENERAL INFORMATION

2.1 Names and Addresses

Sponsor	Lamboley, Christelle Philip Morris Products S.A. Research & Development Pre-clinical Toxicological Evaluation Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Test Facility	Philip Morris Products S.A. Research & Development PMI Product Testing Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Test Facility Management	Vanscheeuwijck, Patrick Jeannet, Cyril
Study Director	Smart, Daniel
Study Director Deputy	McHugh, Damian
Manager, Bio-Analytical Laboratory	McHugh, Damian
Team Leader, Test Item Management (TIM)	Forte, Déborah
Supervisor, Statistics	Vuillaume, Grégory

2.2 Study Schedule

Experimental starting date:	21 August 2017
Experimental completion date:	29 March 2018

2.3 Test Guideline

- OECD Test Guideline (TG) for the Testing of Chemicals 487 (2016), *In Vitro* Mammalian Cell Micronucleus Test.

3 STUDY PLAN DEVIATIONS

During the execution of the study, three study plan deviations were raised (Table 1).

Table 1. Study plan deviations.

Number	Description	Justification of impact on study data and integrity
01	On 29 August 2017, the total particulate matter (TPM) derived from the test item (29.95 mg/stick) was not sufficiently concentrated to permit exposure up to 3500 µg/ml in the 4 h +S9 treatment condition. The following TPM concentration range, as for the 4 h -S9 treatment condition, was therefore implemented: 1125; 1237.5; 1350; 1462.5; 1575; 1687.5; 1800; 2025; 2137.5; 2250 µg/ml.	No impact; data from this experiment were rejected as %MN values from the solvent-treated controls were found to be outside the laboratory's historical range and, therefore, not acceptable.

Number	Description	Justification of impact on study data and integrity
02	Prior to treatment on 29 August 2017, cells seeded in 96-well plates were cultivated for more than 25 h. Specifically, cells were cultured for the following times prior to treatment: 4 h -S9: 25 h 27 minutes; 4 h +S9: 25 h 19 minutes; 24 h -S9: 25 h 20 minutes (plate 1) and 25 h 22 minutes (plate 2).	Population doublings were determined to be ≥1.0 in the solvent-treated controls at the end of the treatment (24 h -S9) and treatment-recovery (4 h ±S9) periods and, therefore the data were considered as acceptable in terms of cell proliferation.
03	On 06 March 2018, the TPM derived from the test item (30.44 mg/stick) was not sufficiently concentrated to permit exposure up to 3181 µg/ml in the 4 h +S9 treatment condition. The following TPM concentration range (using the maximum permitted concentration) was therefore implemented: 1674.2 1826.4; 1978.6; 2130.8; 2283; 2435.2; 2587.4; 2739.6; 2891.8; 3044 µg/ml.	No impact; data from this experiment were rejected as an insufficient range of cytotoxicity was induced by TPM from both items.

4 ABSTRACT

In this *in vitro* micronucleus (MNvit) study, Chinese hamster ovary (CHO) cells were exposed either to the TPM or the cell culture medium-soluble portion of the gas/vapour phase (GVP) derived from the mainstream aerosol of the test item, Tobacco Heating System (THS) tobacco sticks, or the mainstream smoke of the reference item, 3R4F research cigarettes. Cytotoxicity data (relative population doubling; RPD), and genotoxicity data (micronucleus frequency; %MN), were used to determine the *in vitro* genotoxicity of test item-derived fractions under 4 h ±S9 and 24 h -S9 treatment conditions in two independent tests and to compare their lowest observable genotoxic effect levels (LOGELs) with those of counterpart fractions from the reference item. In the 4 h -S9 treatment condition, only TPM from the test item in one of the two tests induced a biologically-relevant level of genotoxicity as TPM from the reference item failed to produce a significant result in the Dunnett's statistical test on the same occasion (the other statistical tests were significant). Biologically-relevant genotoxicity was induced by GVP from both items in the two tests performed; LOGELs for THS tobacco sticks were at least 11.6-fold higher than those for 3R4F. In the 4 h +S9 treatment condition, test item-derived TPM induced biologically-relevant levels of genotoxicity in the two tests, while similar levels of genotoxicity were only induced in the second of the two tests by the reference item as, in the first, it failed to produce a significant result in the Dunnett's statistical test (the other statistical tests were again significant). The LOGEL for THS tobacco sticks-derived TPM was 15.1-fold higher than that for 3R4F-derived TPM. In addition, the level of nicotine in the LOGEL TPM concentration was calculated to be 10.1-fold higher in THS tobacco sticks-derived TPM than 3R4F-derived TPM. For GVP, only the test item-derived fraction induced biologically-relevant levels of genotoxicity, with the reference item-related responses failing to exceed the laboratory's historical range (the other two statistical tests were significant). In the 24 h -S9 treatment condition, TPM from both items was universally non-genotoxic, while for GVP, significant genotoxic results were obtained for the two items in the second of the two tests; the LOGEL was 7.7-fold higher for test item than the reference item. In conclusion, the data from this study indicate that TPM and GVP derived from THS tobacco sticks are genotoxic in the MNvit assay but the LOGELs demonstrate that these fractions possess lower *in vitro* genotoxic potency (between 7.7-15.1-fold) than counterpart fractions from the 3R4F reference item. The data also indicate that the genotoxic potency of THS tobacco sticks-derived TPM is also markedly lower (10.1-fold) than TPM from 3R4F when considered on a nicotine basis.

5 INTRODUCTION

The MNvit assay is used for the detection of micronuclei (MN) which may originate from genotoxin-induced acentric chromosome fragments or whole chromosomes that are unable to migrate to the poles during cell division. In this study, CHO cells were exposed either to the aerosol fractions derived from the mainstream aerosol of THS tobacco sticks or mainstream smoke fractions from 3R4F research cigarettes, namely the TPM fraction and the cell culture medium-soluble portion of the GVP. Cytotoxicity, i.e. RPD, and genotoxicity, i.e. %MN, parameters were generated and, if biologically-relevant genotoxicity was induced by both items, comparisons of LOGELs were made (see section 7.2.5).

6 OBJECTIVES

The objectives of this study were: 1) To determine the genotoxicity of TPM and GVP aerosol fractions derived from the mainstream aerosol of THS tobacco sticks (via heating with the ZRH THD 2.4 tobacco stick holder) following the treatment of CHO cells for 4 hours in presence of S9, and 4 h and 24 h in the absence of S9 (hereafter termed 4 h +S9, 4 h -S9 and 24 h treatment conditions) using the MNvit assay; 2) To compare the LOGEL of aerosol fractions derived from THS tobacco sticks relative to counterpart fractions from 3R4F research cigarettes in the MNvit assay if biologically-relevant genotoxicity was observed.

7 EXPERIMENTAL DESIGN

7.1 Test and Reference Items

TIM was responsible for the reception as well as the identification of the test and reference items according to PMI-RRP-WKI-111735, Reception and storage of items (RDNEU). Test item characteristics, under the conditions of use, were provided by the Sponsor via the batch release certificate. The batch items number was taken as the unique identifier for the test item. Reference item characteristics are made available on the website of the supplier (University of Kentucky, Center for Tobacco Reference Products, www.ctrp.uky.edu). Analysis requests and registration of the samples were performed according to PMI-RRP-WKI-111777, Preparation of items (RDNEU). All unused test and reference items were returned to the Sponsor prior to study report finalisation.

7.1.1 Identification and Description

THS tobacco sticks were regarded as the test item and the 3R4F research cigarettes were regarded as the reference item (summarised in Table 2).

Table 2. Identification and description of the test and reference items.

	Test*	**Reference**
Short name	THS tobacco sticks	3R4F
Description	ZRH Marlboro Dorado II C3.2	Kentucky reference cigarette
Product code (PDIMS)	6AAAAHG.RD / 6AAJB	3R4F
Batch Items (PDIMS)	B-44909	3R4F

*Test item characterisation was carried out by the Sponsor.

A sufficient number of the test item (as well as ZRH THD 2.4 tobacco stick holders used to heat the test item; see section 7.5.1) was received by the Test Facility from the Sponsor in order to execute the study. 3R4F research cigarettes were purchased from the University of Kentucky, Kentucky Tobacco Research and Development Center, Kentucky, USA and received at the Test Facility on 24 September 2015.

7.1.2 Storage and Stability of Test and Reference Items

Once the test and reference items were transferred to the Test Facility, TIM was responsible for their storage according to PMI-RRP-WKI-111735, Reception and storage of items (RDNEU). Items were stored in their original (closed) packaging as follows:

- Test item: at 22 ± 2°C (within the study plan-stipulated specifications of 22 ± 3°C) and 60 ± 5% relative humidity (RH) until required for the aerosol generation procedure. Test item stability information was provided by the Sponsor.
- Reference item: removed from long term storage (4 ± 3°C) and stored in a controlled environment at 22 ± 3°C and 60 ± 5% RH for at least 24 hours (and not more than 3 months) prior to conditioning.

7.2 Test System

7.2.1 Test System Details

- Cell Line Name: CHO-Wolff Bloom Litton (WBL) cells.
- Cell Line Supplier: Merck Research Laboratories, West Point, PA 19486, USA.
- Description: Cells with polygonal morphology originally established as spontaneously-transformed cells from a hamster ovarian biopsy.
- Culture Properties: Adherent growth; cell cycle length and doubling time of approximately 14 h.
- Culture Media: McCoy's 5A + GlutaMAX™ medium supplemented with foetal bovine serum (10% v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml).

7.2.2 Test System Justification

CHO cells are permitted for use in the MNvit assay by OECD TG 487. Specifically, the CHO-WBL cell line has been used during 32 years of genotoxicity testing at Merck Research Laboratories (Lorge *et al.*, 2016). Throughout this time, the karyotype has remained stable and the levels of polyploidy low, and furthermore, there has been no change in growth rate, modal number or background levels of chromosome aberrations and MN.

The CHO-WBL cells (batch: 150204-CHOWBL) used by the laboratory in this study showed no evidence of mycoplasma contamination (Test Facility record). Furthermore, karyotyping analysis (including chromosome number and aberrations) conducted at an external contract laboratory revealed that the karyotype of this batch of cells was consistent with historical data on this cell line (Test Facility record).

7.2.3 Assay Principle

Exposure of CHO cells to a genotoxic agent may result in the production of acentric chromosome fragments or whole chromosomes that are unable to migrate to the poles during cell division. In this event, the acentric chromosome fragments or whole chromosomes appear as MN which can be detected using fluorescence-based methodology. In essence, concentration-dependent induction of MN is proportional to the degree of genotoxicity induced.

Cytotoxicity and genotoxicity parameters in the MNvit assay were calculated as follows:

a) Population Doubling (PD)

$$PD = \frac{LOG(Nuclei\ per\ well\ at\ T_{END}/Average\ nuclei\ per\ well\ at\ T_0)}{LOG(2)}$$

b) RPD

$$RPD = \frac{PD_{\text{test or reference item-treated}}}{PD_{\text{solvent-treated}}} \times 100\%$$

c) %MN

$$\%\text{MN} = \frac{MN\ events}{*\ Nuclei\ events + Hypodiploid\ nuclei\ events}$$

* Nuclei events and hypodiploid events are parameters measured during data acquisition and used in the calculation of %MN (see section 8.2).

7.2.4 Acceptability Criteria

Each MNvit assay carried out, i.e. each treatment condition on each independent test occasion, was evaluated for acceptability. While all the acceptable study data are reported, some data were excluded from the statistical analysis.

The following exclusion criteria were applied in the stated order and, in addition, only remaining data from a previous step were taken forward into subsequent steps. Furthermore, the Study Director excluded some samples due to technical or human error and documented the reason of exclusion in the study data.

a) At least 2000 nuclei per sample were evaluated and:
   - If any of the solvent-treated or positive control samples did not fulfil this condition, the assay was discarded from the analysis.
   - For test and reference items, if one or more samples did not fulfil this condition, they were discarded from the analysis.

b) The mean RPD of duplicate cultures was ≥40%:
   - For test and reference items, if any duplicate cultures did not fulfil this condition, they were discarded from the analysis, except where a non-genotoxic response occurred (confirmation via statistical analysis).
   - This criterion did not apply to positive controls.

c) Cell proliferation in the solvent-treated controls, measured as the number of PDs, was ≥1. If the mean PD of solvent-treated control replicate cultures did not fulfil this condition, the assay was discarded from the analysis.

d) The geometric mean %MN of the solvent-treated control replicates cultures was ≤2%. If solvent-treated control replicate cultures did not fulfil this condition, the assay was discarded from the analysis.

e) The geometric mean %MN of concurrent solvent-treated control replicate cultures must have been within its respective historical range. If solvent-treated control replicate cultures did not fulfil this condition, the assay was discarded from the analysis. The historical range was defined as:

$$e^{\left(\mu_{history}\left(\ln\left(\mu_{plate(\%MN)}\right)\right)\pm 3\sigma_{history}\left(\ln\left(\mu_{plate(\%MN)}\right)\right)\right)}$$

f) Using a linear regression T-Test, the log transformed %MN of the positive controls showed a statistically significant ($p \leq 0.05$) positive trend with respect to concurrent solvent-treated control %MN.

g) The mean RPD of the three lowest concentrations of the test or reference item was ≥40%, otherwise test and/or reference item data were discarded from the analysis.

h) The solvent-treated control %MN data were included in the historical database if criteria b), c), d), e), f) and g) were fulfilled.

7.2.5 Evaluation Criteria

Refer to section 8 for details of the statistical methods applied in this study. Briefly, provided that the acceptability criteria were satisfied, a response to a test and reference item was classified as positive, i.e. genotoxic, and biologically-relevant in the MNvit assay if:

a) At least one of the test concentrations exhibited a statistically significant increase in %MN compared with the concurrent solvent-treated controls.

b) The increase in %MN was concentration-related in at least one experimental condition when evaluated with an appropriate statistical trend test.

c) Any one (or more) of the results was outside the ±2 standard deviation (SD) (95%) controls limits of the laboratory's historical solvent-treated control %MN distribution.

Furthermore, the LOGEL was defined as the lowest concentration of TPM or GVP tested that induced a biologically-relevant genotoxic response in the MNvit assay. Provided that biologically-relevant genotoxic responses were induced by test and reference items on the same test occasion, LOGELs were compared on a fold-difference basis. In addition, the concentration of nicotine (as µg/ml) calculated to be present in the LOGEL concentrations of TPM was compared on a fold-difference basis between test and reference items.

7.3 Preparation of the Reference Item

The reference item was prepared and 100% vent-blocked by taping the ventilation holes in the filter region according to PMI-RRP-WKI-111712, Blocage de la ventilation du papier de bout des cigarettes (RDNEU).

7.4 Conditioning of the Reference Item

The reference item was conditioned in the absence of packaging following the International Organization for Standardization (ISO) standard 3402 (1999), i.e. at least 48 h at target conditions of 22 ± 1°C and 60 ± 3% RH, prior to being used for TPM and GVP generation.

7.5 Aerosol Generation

7.5.1 Test Item-Specific Information

Test item-derived aerosols were generated in combination with tobacco stick holder devices. The tobacco stick holders contain all the required functions to allow one stick to be heated; in particular, the tobacco stick holder includes a battery, controlling electronics, a heating element and a stick extractor. The description and characteristics of the tobacco stick holders used in the study are summarised in Table 3.

Table 3. Identification of the tobacco stick holders.

Device Description	Product Code (PDIMS)	Batch Items (PDIMS)
○ ZRH/THD 2.4/ZRH holder firmware - 1.1.2 (v2.4)/C28/P1 THD V2.4 Holder - White Matte ○ THD version 2.4 ○ Heating Profile C28	DV.000180(5)	B-34548

The batch items number was taken as the unique identifier for the tobacco stick holders.

7.5.2 Aerosol Generation Procedure

Test item- and reference item-derived aerosols were generated via the Health Canada Intense (HCI) smoking regime, applying a bell-shaped puff profile and a defined puff count of 12 puffs for the test item and to a butt length of 35 mm for the reference item (summarised in Table 4). The environmental conditions of the area in which the smoking machine was operational during aerosol generation was maintained at a temperature and RH of 22 ± 2°C and 60 ± 5%, respectively.

Table 4. Smoking machinery and aerosol generation conditions.

Item	Smoking machine	Tobacco stick holder	Puff volume (ml)	Puff duration (s)	Puff frequency (times/min)	Puff count
Test	Burghart RMB20	Yes	55	2	2	Fixed: set to 12
Reference		No				Butt length controlled: set to 35 mm

Test and reference item aerosol generation was performed on a semi-automatic 20-port RMB20 Burghart rotary smoking machine (the settings are described in Table 5), according to PMI-RRP-WKI-111738, Guide d'utilisation pour la machine à fumer rotative semi-automatique RMB20 (RDNEU), PMI-RRP-WKI-111791, Guide d'utilisation pour la mesure de l'air flow (RDNEU), PMI-RRP-WKI-111759, Trappage de l'eau, de la nicotine et de l'acroléine pour les tests biologiques (RDNEU) and PMI-RRP-WKI-111703, Use of Tobacco Heating System with linear and rotary smoking machine (THS) (RDNEU).

Table 5. Settings of the Burghart rotary smoking machine (RMB20) and specific puff parameters.

Item	Smoking volume (ml)	Puff duration (ms)	Gap time (ms)	Intermission (ms)	Activated ports/run
Test	55	1860	140	4000	5
Reference					

Both puff duration and gap time parameters (Table 5) comprise the puff duration parameter that is described in Table 4. In addition, the term smoking volume (Table 5) corresponds to the term puff volume (Table 4).

The used mouth pieces, the filter pad holder and the tubing connecting the glass impinger to the filter pad holder were replaced each time the aerosol generation procedure from an item was completed in order to minimise any potential contamination between test and reference items. Once the aforementioned parts had been replaced, a leak check of the smoking machine was performed prior to execution of the subsequent item aerosol generation procedure.

Aerosol preparations were fractionated into two parts, namely TPM and GVP, during the same aerosol generation. For the test item, TPM was collected on two Cambridge filter pads (44 mm diameter) placed in series in the same filter pad holder and weighed. For the reference item, TPM was collected on one Cambridge filter pad (44 mm diameter) and weighed. Once the TPM had been captured on the filter pad(s), the extraction of the TPM was performed as follows. For the test item, the two filter pads were removed from the filter pad holder and placed into a centrifugal tube device with integrated filtration membrane (0.45 µm) together with dimethylsulfoxide (DMSO). For the reference item, the single filter pad was removed from the filter pad holder and placed into a centrifugal tube device with integrated filtration membrane together with DMSO. The centrifugal tube device containing the filter pad(s) was shaken for 10 minutes prior to centrifugation at 1'600 ×

g for 10 minutes. In contrast, GVP, which is not retained by the filter pad(s), was bubbled into a glass impinger containing ice-cold cell culture medium to capture the cell culture medium-soluble fraction. The number of test and reference item accumulations to produce the fractions, as well as the volume of DMSO and cell culture medium in which TPM and GVP were collected, are shown in Table 6.

Table 6. Conditions for the preparation of TPM and GVP.

		TPM preparation:		**GVP preparation:**	
Item	**Number of accumulations**	**Number of filter pads**	**Volume of DMSO (ml)**	**Number of impingers**	**Volume of medium per impinger (ml)**
Test	40	2	5 or 4	1	24
Reference	5	1	5	1	36

The concentration of TPM (in mg/ml) was calculated from the mass of TPM captured on the filter pad(s) (in mg) and the volume of DMSO (in ml) used to extract the TPM from the filter pad(s). The concentration of GVP (in mg TPM equivalent/ml) was calculated from the mass of TPM captured on the filter pad(s) (in mg) and the total volume of cell culture medium (in ml) that GVP was bubbled through. TPM and GVP fractions were diluted to the required concentrations for genotoxicity evaluation in the MNvit assay (see section 7.6).

7.6 MNvit Assay Procedure

The MNvit assay was performed in accordance with the OECD TG 487. A description that details how the assay was conducted is provided in PMI-RRP-WKI-111805, Micronucleus Assay (RDNEU). For the completion of this study, two independent tests that satisfied assay acceptability criteria (see section 7.2.4) were required for the three treatment conditions.

Briefly, liquid nitrogen-stored CHO-WBL cells were thawed and sub-cultured for at least two passages prior to treatment. CHO-WBL cells were seeded (4500 cells/well) into 96-well plates and cultivated for 24 ± 1 h. Cells were then exposed to the various concentrations of test and reference items under 4 h ±S9 and 24 h -S9 treatment conditions (Table 7, Table 8 and Table 9). Test and reference item-related concentration ranges were defined based upon the results of preliminary dose range finding studies as well as data generated in this study. Following 4 h ±S9 treatment, cells were sub-cultured for a further 24 ± 1 h (approximately 1.5-2.0 cell cycle lengths from the start of treatment) to allow any potential chromosome damage to lead to the formation of MN. For the 24 h -S9 treatment condition, no recovery time was required as this extended treatment period is sufficient to permit the formation of MN. After these times (and also immediately prior to treatment; see below for the explanation of this step), nuclei and micronuclei were harvested using the *in vitro* MicroFlow® kit (Litron Laboratories, USA). The *in vitro* MicroFlow® kit is composed of several proprietary reagents that liberate nuclei and micronuclei from intact cells and render them amenable to flow cytometric analysis (Bryce *et al.*, 2007). A BD FACSCanto II was used to analyse the samples according to PMI-RRP-WKI-111861, FACSCanto II Flow Cytometer (RDNEU).

Since this is the non-cytokinesis-block version of the assay, absolute nuclei counts measured immediately prior to treatment (T_0) and at the end of recovery (4 h ±S9 treatment conditions) or treatment (24 h -S9 condition) (T_{END}) were used to calculate the index of cytotoxicity applied in this approach, namely RPD. RPD is one of the two indices recommended for use in the MNvit assay by OECD TG 487 and it estimates both the cytotoxic and cytostatic activity of a test substance (Lorge *et al.*, 2008).

Table 7. The concentrations tested in the 4 h -S9 treatment condition.

4 h -S9 Treatment Condition	Test item		Reference item	
	TPM (µg/ml)	GVP (µg TPM equivalent/ml)	TPM (µg/ml)	GVP (µg TPM equivalent/ml)
Test 1	1000; 1200; 1400; 1600; 1800; 2000; 2200; 2400; 2600; 2800	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	70; 90; 100; 110; 120; 130; 140; 150; 160; 180	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
Test 2	1000; 1200; 1400; 1600; 1800; 1900; 2000; 2100; 2200; 2400; 2600; 2800		40; 50; 60; 70; 75; 80; 85; 90; 95; 100; 110; 130	

Table 8. The concentrations tested in the 4 h +S9 treatment condition.

4 h +S9 Treatment Condition	Test item		Reference item	
	TPM (µg/ml)	GVP (µg TPM equivalent/ml)	TPM (µg/ml)	GVP (µg TPM equivalent/ml)
Test 1	1749.1; 1908.2; 2067.3; 2226.4; 2385.5; 2544.6; 2703.7; 2862.8; 3021.9; 3181	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 125; 150; 175; 200; 225; 250; 265; 275; 300	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
Test 2			100; 125; 150; 175; 187.5; 200; 212.5; 225; 237.5; 250; 255; 265; 275; 300	

Table 9. The concentrations tested in the 24 h -S9 treatment condition.

24 h -S9 Treatment Condition	Test item		Reference item	
	TPM (µg/ml)	GVP (µg TPM equivalent/ml)	TPM (µg/ml)	GVP (µg TPM equivalent/ml)
Test 1	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600	1000; 1500; 2000; 2250; 2500; 2750; 3000; 3250; 3500; 4000	50; 60; 70; 80; 90; 100; 110; 120; 130; 140	100; 150; 200; 225; 250; 300; 325; 350; 375; 400
Test 2	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600; 1800; 2000	1000; 1500; 2000; 2250; 2500; 2625; 2750; 3000; 3250; 3500; 4000	50; 60; 70; 80; 90; 95; 100; 105; 110; 120; 130; 140	100; 150; 200; 225; 250; 300; 325; 350; 375; 400; 425; 450

In addition, appropriate reference chemical genotoxins (clastogens and an aneugen) were also included as positive controls in each treatment condition (Table 10).

Table 10. The positive controls used in the MNvit assay.

	Clastogen Controls		Aneugen Control	
Treatment condition	**Chemical**	**Concentra-tions (µg/ml)**	**Chemical**	**Concentra-tions (µg/ml)**
4 h -S9	Methyl methanesulfonate (MMS)	15; 20 or 30; 35	Colchicine (COL)	0.8; 1.0
4 h +S9	Cyclophosph-amide (CPA)	1; 2 or 3; 4	COL	0.6; 0.8
24 h -S9	MMS	15; 20 or 30; 35	COL	0.15; 0.20

7.7 Analytical Procedures (TPM and Nicotine Determination)

The mass of TPM collected on the filter pad(s) was determined by weighing the whole filter pad holder containing the filter pad(s) on a balance instrument before and after aerosol generation procedures, according to PMI-RRP-WKI-111759, Trappage de l'eau, de la nicotine et de l'acroléine pour les tests biologiques (RDNEU). The difference between the two masses corresponded to the mass of TPM collected on the filter pad(s).

Determination of nicotine content in the DMSO-solubilised TPM fractions derived from the test and reference items was conducted according to PMI-RRP-WKI-111817, Determination of nicotine in smoke or aerosol condensates for *in vitro* tests (RRPCE) and PMI-RRP-WKI-111836, Performing and recording analytical activities using the GC with Total Chrom software (RDNEU). The nicotine concentration was measured using a gas chromatography with a flame ionisation detector; nicotine was quantified using the ratio of the nicotine peak area to the peak area of isoquinoline. DMSO served as the blank control.

8 STATISTICAL METHODS

8.1 Data Analysis

8.1.1 General

Statistical analysis was performed according to PMI-RRP-WKI-111852, Statistical Analysis with Double Programming (RDNEU), using SAS Enterprise guide 6.1 with SAS 9.2.

8.1.2 Missing Data and Extreme Data

See section 7.2.4.

8.1.3 Confidence/Significance Level

This study was exploratory from a statistical perspective and, consequently, no formal hypothesis testing was performed and nor was there any pre-specification or adjustment for any overall or local alpha level. However, a raw p-value or a p-value adjusted, e.g. for false discovery rate, has been descriptively reported as a noteworthy finding when below the usual threshold (P<=0.05, 0.01, or 0.001). These usual thresholds were also used to build confidence intervals.

8.2 Parameters for Data Analysis

8.2.1 Measured Parameters

- C_{Nuc}^{T0} = absolute nuclei count per sample immediately prior to treatment.
- C_{Nuc}^{END} = absolute nuclei count per sample at the end of treatment/recovery.
- C_{B+T}^{T0} = bead count and time events immediately prior to treatment.
- C_{B+T}^{END} = bead and time events count at the end of treatment/recovery.
- C_{HD} = hypodiploid events count.
- C_{MN} = MN events count.

8.2.2 Derived Parameters

- $N_{Nuc}^{END} = (C_{Nuc}^{END} + C_{HD}) \div C_{B+T}^{END} \times Beads\ Concentration \times Beads\ Volume$ = the number of nuclei per sample at the end treatment/recovery.

- $N_{Nuc}^{T0} = C_{Nuc}^{T0} \div C_{B+T}^{T0} \times Beads\ Concentration \times Beads\ Volume$ = the number of nuclei per sample immediately prior to treatment.
- $PD = \log\left(N_{Nuc}^{END} \div \overline{N_{Nuc}^{T0}}\right) \div log(2)$
- $\%RPD = 100 \times PD_{item} \div \overline{PD_{solvent}}$

8.2.3 Endpoints

- $\%MN = C_{MN} \div (C_{Nuc}^{END} + C_{HD})$
- $\%HD = C_{HD} \div (C_{Nuc}^{END} + C_{HD})$

8.3 Acceptability Criteria

See to section 7.2.4.

8.4 Descriptive Statistics

8.4.1 Historical Solvent-Treated Control %MN Data

The following data were reported by treatment condition and solvent-treated control: the sample size, the geometric mean and bounds at ±2 and ±3 geometric SD using the formula:

$$e^{\left(\mu_{history}\left(\ln\left(\mu_{plate}(\%MN)\right)\right)\pm X\sigma_{history}\left(\ln\left(\mu_{plate}(\%MN)\right)\right)\right)}$$

The PBS-treated and non-treated historical %MN ranges have been merged for comparison purposes as they are extensive, i.e. contain data from at least 30 experiments and encompass a time period of more than one year, and, therefore, represent a reasonable reflection of the CHO-WBL cell line's background %MN variability.

8.4.2 Study Data

The measured and derived parameters for the different endpoints were reported for each replicate culture and concentration in the validated spreadsheet PMI_RD_FOR_000726, Micronucleus assay (V2.0).

8.5 Statistical Design and Power

Given this study was exploratory from a statistical perspective as a result of a lack of historical data for the test item, no power calculations were performed.

The concentrations of test and reference items tested in each treatment condition were based on data from separate dose-range finding experiments or from data generated during this study. The number of concentrations and their nominal value varied between assays. Two assays that satisfied acceptability criteria were required for test and reference items in each treatment condition (see section 7.2.4).

8.6 Exploratory Hypothesis

In order to determine whether biologically-relevant genotoxicity had been induced in a particular assay, the following statistical questions were addressed:

a) Whether the %MN induced by at least one concentration of test or reference item was higher than the concurrent solvent-treated control %MN using a one-sided Dunnett's test ($P \leq 0.05$ indicated statistical relevance). Only the lowest relevant concentration was reported.

b) Whether test or reference item-induced %MN showed a positive trend using the Kendall correlation (a positive correlation and $P \leq 0.05$ indicated statistical relevance). Initially, only the three lowest concentrations were used, then iteratively the next lowest concentration was included in the statistical test until statistical relevance or the highest acceptable concentration was reached. Only the lowest relevant concentration was reported.

c) Whether at least one of the biological replicate geometric mean of the induced %MN was above the upper limit (geometric mean × 2 × geometric SD) of the laboratory's historical solvent-treated control %MN distribution.

9 RESULTS

9.1 Assay Acceptability

All assays met the acceptability criteria stated in sections 7.2.4 a) c) d) e) f) g) h) (Table 11, Table 14, Table 17, Table 20, Table 23, Table 26, Table 29, Table 32, Table 35, Table 38, Table 41 and Table 44). On several occasions a number of test and reference item concentrations induced RPDs <40% and, therefore, did not meet the acceptability criterion stated in section 7.2.4 b) (Table 13, Table 15, Table 18, Table 19, Table 21, Table 22, Table 25, Table 28, Table 30, Table 31, Table 33, Table 36, Table 37, Table 39, Table 40, Table 42, Table 45 and Table 46); these concentrations were excluded from downstream statistical analysis. On the occasions where only one of the two replicate cultures met the criterion stated in section 7.2.4 b), their data were pooled and, if their averaged RPD (%) was in accordance with the acceptability criterion stated in section 7.2.4 b), then the data were accepted and included in downstream statistical analysis (Table 13, Table 15, Table 16, Table 19, Table 21, Table 36, Table 37, Table 40, Table 43, Table 45 and Table 46).

9.2 4 h -S9 Treatment Condition

TPM and GVP fractions derived from test and reference items under the 4 h -S9 treatment condition generally induced concentration-dependent increases in cytotoxicity when compared with solvent-treated controls, as determined by a reduction in the RPD parameter (Table 11, Table 12, Table 13, Table 14, Table 15, Table 16, Table 17, Table 18, Table 19, Table 20, Table 21 and Table 22). Concentration-dependent increases in %MN were also observed for TPM derived from both items in the first independent test, however, significant genotoxicity was only detected for the test item (Table 12 and Table 13). The second independent test yielded non-significant genotoxic findings for both items (Table 15 and Table 16). In contrast, the GVP from both items was determined to induce significant levels of genotoxicity in the two independent tests carried out (Table 18, Table 19, Table 21 and Table 22). However, the lowest concentration at which significant genotoxicity was induced, i.e. the LOGEL, was markedly higher (at least 11.6-fold) for test item-derived GVP than the counterpart fraction from the reference item (Table 48).

9.3 4 h +S9 Treatment Condition

TPM and GVP fractions derived from test and reference items under the 4 h +S9 treatment condition generally induced concentration-dependent increases in cytotoxicity and genotoxicity when compared with solvent-treated controls, as determined by a reduction in the RPD parameter and an increase in the %MN parameter (Table 23, Table 24, Table 25, Table 26, Table 27, Table 28, Table 29, Table 30, Table 31, Table 32, Table 33 and Table 34). Concentration-dependent increases in %MN were also observed for TPM derived from both items in the first independent test, however, significant genotoxicity was only detected for the test item (Table 24 and Table 25). The second independent test on TPM yielded significant genotoxic findings for both items but the LOGELs were markedly different (15.1-fold) (Table 27, Table 28 and Table 49). Furthermore, this marked difference in genotoxic potencies between test and reference item-derived TPM on this test occasion was also apparent when LOGELs were considered on a nicotine basis (10.1-fold) (Table 49). Concentration-dependent increases in %MN were observed for GVP derived from both items in the two independent tests, however, significant genotoxicity was only detected for the test item on both occasions (Table 30, Table 31, Table 33 and Table 34).

9.4 24 h -S9 Treatment Condition

TPM and GVP fractions derived from test and reference items under the 24 h -S9 treatment condition generally induced concentration-dependent increases in cytotoxicity when compared with solvent-treated controls, as determined by a reduction in the RPD parameter (Table 35, Table 36, Table 37, Table 38, Table 39, Table 40, Table 41, Table 42, Table 43, Table 44, Table 45 and Table 46). However, no significant increases in %MN were observed for test and reference item-derived TPM in both independent tests (Table 36, Table 37, Table 39 and Table 40). For GVP, no significant increases in %MN were observed for both items in the first independent test but, in contrast, significant genotoxic results were obtained for the two items in the second independent test (Table 42, Table 43, Table 45 and Table 46). In this case, the LOGEL was markedly higher (7.7-fold) for test item-derived GVP than the counterpart fraction from the reference item (Table 52).

9.5 Chemical Analysis of TPM

Both analytical characteristics of TPM, i.e. mass generated and level of nicotine, evaluated were broadly consistent for both test and reference items through the six rounds of aerosol fraction generation that are reported in this study (Table 59).

10 DISCUSSION AND CONCLUSION

In this study, the MNvit assay was used to evaluate the genotoxicity of TPM and GVP aerosol fractions derived from the mainstream aerosol of THS tobacco sticks (via heating with the ZRH THD 2.4 tobacco stick holder) following the treatment of CHO cells in 4 h +S9, 4 h -S9 and 24 h treatment conditions as well as compare the LOGEL of aerosol fractions derived from THS tobacco sticks relative to counterpart fractions from 3R4F research cigarettes in the MNvit assay where biologically-relevant genotoxicity was observed.

In both independent tests carried out using the 4 h -S9 treatment condition, TPM aerosol fractions derived from test and reference items were found to induce concentration-dependent MN effects. However, only TPM from the test item in the first of the two independent tests induced a biologically-relevant level of genotoxicity (although reference item-derived TPM only failed to produce a statistically significant result in the Dunnett's test on this test occasion; the other statistical tests were significant). Biologically-relevant genotoxicity was also induced by both test and reference item-derived GVP in the two tests carried out; the LOGELs determined for THS tobacco sticks was at least 11.6-fold higher than the LOGELs determined for 3R4F on these occasions.

In the 4 h +S9 treatment condition, TPM derived from test item was found to induce concentration-dependent MN effects up to a biologically-relevant level of genotoxicity in the two independent tests. Although concentration-dependent MN effects were also observed for reference item-derived TPM, a biologically-relevant level of genotoxicity was only reached in the second of the two test occasions as, on the first occasion, it failed to produce a statistically significant result in the Dunnett's statistical test (the other statistical tests were significant). The LOGEL determined for THS tobacco sticks-derived TPM was 15.1-fold higher than the LOGEL determined for 3R4F-derived TPM. At these LOGELs, the concentration of nicotine was 10.1-fold higher in test item-derived TPM than reference item-derived TPM. For GVP, concentration-dependent MN effects were observed for both items but only GVP derived from the test item reached biologically-relevant levels of genotoxicity, with the reference item-related responses failing to exceed the laboratory's historical range for solvent-treated/non-treated controls (the other statistical tests were significant).

In the 24 h -S9 treatment condition, TPM from both items was universally non-genotoxic in the assay. While for GVP, no significant increases in MN were observed for both items in the first independent test but, in contrast, significant genotoxic results were obtained for the two items in the second independent test; however, the LOGEL was 7.7-fold higher for test item-derived GVP than the reference item-derived counterpart fraction.

In conclusion, the data generated in this study indicate that both TPM and GVP aerosol fractions derived from THS tobacco sticks are genotoxic in the MNvit assay. However, the LOGELs demonstrate that THS tobacco sticks-derived aerosol fractions possess lower *in vitro* genotoxic potency (between 7.7-15.1-fold) than counterpart fractions derived from the 3R4F reference item in this assay. Furthermore, this marked difference in genotoxic potencies between THS tobacco sticks- and 3R4F-derived TPM is also apparent when they are considered on a nicotine basis (10.1-fold).

11 ARCHIVING

After the creation of the sub-stock, the test and reference item retention samples were archived. In addition, after completion of the study, the study plan with any amendment, all raw data, the report with any amendment and all further study-related records needed to reconstruct the study will be archived. They will be retained for at least 10 years in compliance with the Swiss Ordinance on Good Laboratory Practice, adopted May 18th, 2005 [RS 813.112.1] and as reflected in the Test Facility's applicable archiving procedures. Test and reference items can be discarded after their deterioration as, in this case, they would be no longer amenable to further evaluation. If the storage period for paper and electronic records needs to be further extended in order to satisfy additional legal or company requirements, the storage location will be specified in a dedicated Test Facility Management Statement. Paper records will be archived in the archive at Philip Morris Products S.A., Research & Development, PMI Product Testing, Neuchâtel, Switzerland and electronic records will be managed by the PMI Product Testing e-archivist on the central archiving server at Philip Morris S.A., Lausanne, Switzerland.

12 REFERENCES

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OECD series on Principles on GLP and Compliance Monitoring (number 1), OECD Principles on GLP (as revised in 1997), Environment Directorate Chemicals Group and Management Committee (ENV/MC/CHEM(98(17)).

OECD TG for the Testing of Chemicals 487 (2016), *In Vitro* Mammalian Cell Micronucleus Test.

13 LIST OF STANDARD OPERATING PROCEDURES (SOP) AND WORK INSTRUCTIONS (WKI)

The procedures and instructions that were followed to perform the study are listed below:

- PMI-RRP-SOP-111686 V8.0.0, Perform Analysis (RDNEU).
- PMI-RRP-SOP-111687 V3.0.0, Manage Biological Test Systems (RDNEU).
- PMI-RRP-SOP-111691 V6.0.0, Manage Sample (RDNEU).
- PMI-RRP-SOP-111696 V4.0.0 and V5.0.0, Role of Statistician in GLP study (RDNEU).
- PMI-RRP-WKI-111701 V6.0.0, Sélection du type de lèvres à utiliser lors du fumage (RDNEU).
- PMI-RRP-WKI-111702 V4.0.0, Utilisation et gestion des armoires climatiques (RDNEU).
- PMI-RRP-WKI-111703 V7.0.0, Use of Tobacco heating system with linear and rotary smoking machine (THS) (RDNEU).
- PMI-RRP-WKI-111711 V17.0.0, Gestion des conditions environnementales du laboratoire de collection d'aérosol et de la chambre de conditionnement (RDNEU).
- PMI-RRP-WKI-111712 V5.0.0, Blocage de la ventilation du papier de bout des cigarettes (RDNEU).
- PMI-RRP-WKI-111734 V6.0.0 and V7.0.0, Performing and recording analytical activities using the LC-MS/MS Thermo with Xcalibur and LC Quan software (RDNEU).
- PMI-RRP-WKI-111735 V10.0.0, Reception and storage of items (RDNEU).
- PMI-RRP-WKI-111737 V6.0.0, Aerosol Data Management (RDNEU).
- PMI-RRP-WKI-111738 V5.0.0, Guide d'utilisation pour la machine à fumer rotative semi-automatique RMB20 (RDNEU).

- PMI-RRP-WKI-111759 V9.0.0, Trappage de l'eau, de la nicotine et de l'acroléine pour les tests biologiques (RDNEU).
- PMI-RRP-WKI-111777 V8.0.0 and V9.0.0, Preparation of items (RDNEU).
- PMI-RRP-WKI-111791 V6.0.0, Guide d'utilisation pour la mesure de l'air flow (RDNEU).
- PMI-RRP-WKI-111794 V2.0.0, V3.0.0 and V4.0.0, User guide of RDLims for Logistics activities (RDNEU).
- PMI-RRP-WKI-111802 V3.0.0 and V4.0.0, Cultivation of CHO cell lines (RDNEU).
- PMI-RRP-WKI-111805 V2.0.0 and V3.0.0, Micronucleus Assay (RRPCE).
- PMI-RRP-WKI-111817 V10.0.0, Determination of nicotine in smoke or aerosol condensates for *in vitro* tests (RRPCE).
- PMI-RRP-WKI-111826 V7.0.0, Freezing and thawing of mammalian cells (RDNEU).
- PMI-RRP-WKI-111834 V5.0.0 and V6.0.0, GLP archiving (RDNEU).
- PMI-RRP-WKI-111836 V7.0.0 and V8.0.0, Performing and recording analytical activities using the GC with Total Chrom software (RDNEU).
- PMI-RRP-WKI-111843 V5.0.0, E-archiving of GLP Data (RDNEU).
- PMI-RRP-WKI-111848 V5.0.0 and V6.0.0, Management of the Multisizer 4 - Particle Analyzer (RDNEU).
- PMI-RRP-WKI-111852 V2.0.0, Statistical Analysis with Double Programming (RDNEU).
- PMI-RRP-WKI-111861 V1.0.0 and V2.0.0, FACSCanto II Flow Cytometer (RDNEU).

14 ABBREVIATIONS

Abbreviation/Term	
%MN	Micronuclei Frequency
3R4F	3R4F Reference Cigarette
CHO-WBL	Chinese Hamster Ovary-Wolff Bloom Litton
COL	Colchicine
CPA	Cyclophosphamide
DMSO	Dimethylsulfoxide
GLP	Good Laboratory Practice
GVP	Gas-Vapour Phase
HCI	Health Canada Intensive
ISO	International Organization for Standardization
LOGEL	Lowest Observable Genotoxic Effect Level
MMS	Methyl Methanesulfonate
MN	Micronucleus or Micronuclei
MNvit	*In Vitro* Micronucleus
N/A	Not Applicable
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate-Buffered Saline
PD	Population Doubling
PDIMS	Product Development Information Management System
PMI	Philip Morris International

RH	Relative Humidity
RPD	Relative Population Doubling
S9	Supernatant fraction obtained from Aroclor 1254-induced rat liver homogenate via centrifugation at 9’000 × g
SAS	Statistical Analysis Software
SD	Standard Deviation
SOP	Standard Operating Procedure
TFM	Test Facility Management
TG	Test Guideline
THD	Tobacco Heating Device
THS	Tobacco Heating System
TIM	Test Item Management
TPM	Total Particulate Matter
WKI	Work Instruction
ZRH	Zürich

15 TABLES

It should be noted that the values presented here may differ slightly from the raw data due to the use of rounding procedures.

15.1 Tables

Table 11. 4 h -S9 Test 1: Response to DMSO, MMS and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
14-Sept-2017	Solvent	0 (1% v/v DMSO)	1	3.4	100	35	5000	0.70	N/A
			2	3.3		46		0.92	
			3	3.5		42		0.84	
	MMS	20	1	N/A	88.8	91	5000	1.81	Yes
		15	1		93.4	60		1.20	
	COL	1.0	1		75.0	277	5000	5.20	Yes
		0.8	1		84.7	261		4.93	

Table 12. 4 h -S9 Test 1: Response to test item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
14-Sept-2017	Test; 1706509	2800	1	40.7	170	4251	3.95	-
			2	47.0	115	4896	2.33	
		2600	1	47.8	183	5000	3.61	-
			2	54.5	95	5000	1.87	
		2400	1	62.2	148	5000	2.91	Yes
			2	61.0	97	5000	1.91	
		2200	1	68.7	119	5000	2.33	-
			2	71.3	69	5000	1.37	
		2000	1	70.6	158	5000	3.11	-
			2	68.4	85	5000	1.68	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
14-Sept-2017	Test; 1706509	1800	1	76.0	159	5000	3.14	-
			2	85.4	61	5000	1.21	
		1600	1	83.4	86	5000	1.70	-
			2	87.4	43	5000	0.85	
		1400	1	94.4	82	5000	1.63	-
			2	91.2	45	5000	0.90	
		1200	1	87.3	58	5000	1.16	-
			2	105.1	32	5000	0.64	
		1000	1	104.3	52	5000	1.04	-
			2	98.7	36	5000	0.72	

(Table continued from previous page)

Table 13. 4 h -S9 Test 1: Response to reference item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
14-Sept-2017	Reference; 1706512	180	1	-4.9	102	1542	6.51	-
			2	-18.2	113	1139	9.80	
		160	1	14.3	216	2490	8.56	-
			2	-5.2	162	1508	10.62	
		150	1	20.7	179	2793	6.31	-
			2	26.8	422	3067	13.51	
		140	1	21.9	189	2866	6.47	-
			2	24.5	293	3036	9.48	
		130	1	35.4	196	4311	4.48	-
			2	20.7	378	2986	12.39	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
14-Sept-2017	Reference; 1706512	120	1	40.1	109	4954	2.18	-
			2	26.0	452	3035	14.60	
		110	1	67.6	57	5000	1.13	-
			2	37.4	377	4267	8.69	
		100	1	83.2	57	5000	1.13	-
			2	59.8	101	5000	1.99	
		90	1	87.4	62	5000	1.23	-
			2	74.6	79	5000	1.57	
		70	1	95.3	69	5000	1.38	-
			2	98.3	42	5000	0.84	

(Table continued from previous page)

Table 14. 4 h -S9 Test 2: Response to DMSO, MMS and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
20-Mar-2018	Solvent	0 (1% v/v DMSO)	1	2.0	100	11	5000	0.22	N/A
			2	1.9		50		1.00	
			3	2.0		15		0.30	
	MMS	35	1	N/A	79.5	88	5000	1.75	Yes
		30	1		77.7	85		1.69	
	COL	1.0	1		69.2	249	5000	4.59	Yes
		0.8	1		70.4	185		3.45	

Table 15. 4 h -S9 Test 2: Response to test item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
20-Mar-2018	Test; 1779879	2800	1	28.8	69	5000	1.37	-
			2	20.1	52	5000	1.03	
		2600	1	37.6	41	5000	0.81	-
			2	32.6	43	5000	0.85	
		2400	1	40.0	40	5000	0.79	-
			2	38.1	32	5000	0.63	
		2200	1	53.1	38	5000	0.75	-
			2	35.4	35	5000	0.69	
		2100	1	63.9	29	5000	0.58	-
			2	28.9	30	5000	0.60	
		2000	1	59.3	28	5000	0.56	-
			2	61.2	30	5000	0.60	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
20-Mar-2018	Test; 1779879	1900	1	70.0	35	5000	0.70	-
			2	73.3	36	5000	0.72	
		1800	1	69.3	35	5000	0.70	-
			2	54.0	20	5000	0.40	
		1600	1	74.3	14	5000	0.28	-
			2	61.3	20	5000	0.40	
		1400	1	75.6	33	5000	0.66	-
			2	86.5	30	5000	0.60	
		1200	1	72.0	15	5000	0.30	-
			2	71.3	19	5000	0.38	
		1000	1	86.8	23	5000	0.46	-
			2	87.1	23	5000	0.46	

(Table continued from previous page)

Table 16. 4 h -S9 Test 2: Response to reference item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
20-Mar-2018	Reference; 1779921	130	1	63.0	44	5000	0.87	-
			2	35.9	41	5000	0.81	
		110	1	79.1	36	5000	0.72	-
			2	51.4	42	5000	0.83	
		100	1	71.6	37	5000	0.74	-
			2	72.3	31	5000	0.62	
		95	1	79.9	29	5000	0.58	-
			2	75.3	28	5000	0.56	
		90	1	85.3	27	5000	0.54	-
			2	72.3	33	5000	0.66	
		85	1	89.6	32	5000	0.64	-
			2	87.8	27	5000	0.54	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
20-Mar-2018	Reference; 1779921	80	1	84.4	25	5000	0.50	-
			2	91.8	35	5000	0.70	
		75	1	86.7	27	5000	0.54	-
			2	86.3	31	5000	0.62	
		70	1	95.4	27	5000	0.54	-
			2	85.3	27	5000	0.54	
		60	1	89.9	30	5000	0.60	-
			2	93.1	22	5000	0.44	
		50	1	85.2	38	5000	0.76	-
			2	69.4	6	5000	0.12	
		40	1	76.5	21	5000	0.42	-
			2	75.6	13	5000	0.26	

(Table continued from previous page)

Table 17. 4 h -S9 Test 1: Response to medium, MMS and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
29-Aug-2017	Non-treated	0 (100% v/v medium)	1	1.7	100	21	5000	0.42	N/A
			2	1.8		31		0.62	
			3	1.8		13		0.26	
	MMS	20	1	N/A	77.0	92	5000	1.83	Yes
		15	1		85.5	100		1.99	
	COL	1.0	1		33.8	278	5000	5.12	Yes
		0.8	1		47.8	243		4.54	

Table 18. 4 h -S9 Test 1: Response to test item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Test; 1702178	3250	1	-4.0	219	5000	4.24	-
			2	-1.2	237	5000	4.58	
		2925	1	10.3	181	5000	3.52	-
			2	16.2	188	5000	3.62	
		2762.5	1	33.0	173	5000	3.36	-
			2	39.9	183	5000	3.53	
		2600	1	43.3	160	5000	3.11	-
			2	42.1	180	5000	3.48	
		2437.5	1	47.1	151	5000	2.93	-
			2	62.6	177	5000	3.45	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Test; 1702178	2275	1	58.0	146	5000	2.86	-
			2	65.0	139	5000	2.71	
		2112.5	1	67.2	112	5000	2.21	-
			2	68.3	128	5000	2.51	
		1950	1	78.5	99	5000	1.96	Yes
			2	71.9	90	5000	1.78	
		1625	1	86.0	70	5000	1.39	-
			2	84.9	79	5000	1.57	
		1462.5	1	87.7	46	5000	0.92	-
			2	80.2	68	5000	1.35	

(Table continued from previous page)

Table 19. 4 h -S9 Test 1: Response to reference item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Reference; 1702180	225	1	-10.9	105	4639	2.18	-
			2	-13.7	166	4447	3.60	
		202.5	1	17.8	144	5000	2.78	-
			2	3.8	213	5000	4.05	
		191.25	1	32.9	163	5000	3.12	-
			2	30.5	125	5000	2.40	
		180	1	46.7	105	5000	2.03	-
			2	36.5	121	5000	2.34	
		168.75	1	51.8	113	5000	2.19	Yes
			2	56.8	102	5000	1.98	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Reference; 1702180	157.5	1	67.8	76	5000	1.49	-
			2	63.1	90	5000	1.76	
		146.25	1	67.5	80	5000	1.57	-
			2	78.0	68	5000	1.34	
		135	1	68.7	69	5000	1.37	-
			2	73.2	68	5000	1.34	
		123.75	1	83.6	46	5000	0.91	-
			2	81.8	42	5000	0.83	
		112.5	1	83.3	108	5000	2.14	-
			2	76.7	54	5000	1.07	

(Table continued from previous page)

Table 20. 4 h -S9 Test 2: Response to medium, MMS and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
31-Aug-2017	Non-treated	0 (100% v/v medium)	1	1.4	100	28	5000	0.56	N/A
			2	1.4		27		0.54	
			3	1.6		15		0.30	
	MMS	20	1	N/A	67.5	96	5000	1.91	Yes
		15	1		71.5	79		1.58	
	COL	1.0	1		38.3	317	5000	5.85	Yes
		0.8	1		34.2	252		4.76	

Table 21. 4 h -S9 Test 2: Response to test item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
31-Aug-2017	Test; 1702187	3250	1	-25.9	180	4205	4.13	-
			2	-11.5	226	5000	4.40	
		2925	1	-7.6	235	5000	4.52	-
			2	14.9	198	5000	3.81	
		2762.5	1	0.2	186	5000	3.58	-
			2	38.7	194	5000	3.74	
		2600	1	20.7	190	5000	3.67	-
			2	53.6	191	5000	3.70	
		2437.5	1	36.8	168	5000	3.25	-
			2	55.1	178	5000	3.46	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
31-Aug-2017	Test; 1702187	2275	1	37.5	176	5000	3.43	-
			2	64.1	146	5000	2.85	
		2112.5	1	52.8	143	5000	2.80	-
			2	67.1	121	5000	2.37	
		1950	1	54.3	106	5000	2.10	Yes
			2	82.1	94	5000	1.86	
		1625	1	65.6	77	5000	1.53	-
			2	99.4	66	5000	1.31	
		1462.5	1	92.9	64	5000	1.27	-
			2	108.3	85	5000	1.69	

(Table continued from previous page)

Table 22. 4 h -S9 Test 2: Response to reference item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
31-Aug-2017	Reference; 1702189	225	1	36.3	141	5000	2.71	-
			2	22.8	192	5000	3.65	
		202.5	1	32.6	145	5000	2.79	-
			2	32.8	146	5000	2.81	
		191.25	1	47.6	94	5000	1.83	-
			2	40.1	113	5000	2.19	
		180	1	61.0	115	5000	2.26	-
			2	62.8	94	5000	1.84	
		168.75	1	67.9	104	5000	2.04	-
			2	73.8	74	5000	1.45	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
31-Aug-2017	Reference; 1702189	157.5	1	86.7	77	5000	1.52	-
			2	70.1	80	5000	1.58	
		146.25	1	85.0	73	5000	1.45	Yes
			2	68.6	54	5000	1.07	
		135	1	77.2	49	5000	0.97	-
			2	82.8	47	5000	0.93	
		123.75	1	94.7	44	5000	0.88	-
			2	100.2	34	5000	0.68	
		112.5	1	111.1	212	5000	4.21	-
			2	118.6	47	5000	0.94	

(Table continued from previous page)

Table 23. 4 h +S9 Test 1: Response to DMSO, CPA and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
14-Sept-2017	Solvent	0 (1% v/v DMSO)	1	3.2	100	74	5000	1.48	N/A
			2	3.0		31		0.62	
			3	3.2		43		0.86	
	CPA	2	1	N/A	99.0	75	5000	1.50	No
		1	1		96.1	87		1.74	
	COL	0.8	1		74.3	444	5000	8.12	Yes
		0.6	1		89.9	194		3.73	

Table 24. 4 h +S9 Test 1: Response to test item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
14-Sept-2017	Test; 1706509	3181	1	82.8	122	5000	2.41	Yes
			2	80.9	115	5000	2.28	
		3021.9	1	88.3	81	5000	1.61	-
			2	84.2	65	5000	1.29	
		2862.8	1	78.4	65	5000	1.29	-
			2	92.4	81	5000	1.61	
		2703.7	1	90.1	68	5000	1.36	-
			2	92.6	75	5000	1.49	
		2544.6	1	89.5	45	5000	0.90	-
			2	91.4	80	5000	1.59	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
14-Sept-2017	Test; 1706509	2385.5	1	103.7	60	5000	1.19	-
			2	94.0	58	5000	1.15	
		2226.4	1	81.9	53	5000	1.06	-
			2	94.7	65	5000	1.29	
		2067.3	1	93.8	37	5000	0.74	-
			2	102.1	58	5000	1.16	
		1908.2	1	99.3	63	5000	1.25	-
			2	104.5	55	5000	1.10	
		1749.1	1	96.7	62	5000	1.24	-
			2	99.1	75	5000	1.49	

(Table continued from previous page)

Table 25. 4 h +S9 Test 1: Response to reference item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
14-Sept-2017	Reference; 1706512	300	1	4.4	352	1924	17.77	-
			2	71.0	156	5000	3.06	
		275	1	-1.8	385	1631	22.90	-
			2	69.2	222	5000	4.35	
		265	1	32.2	412	3393	11.87	-
			2	89.9	101	5000	1.99	
		250	1	40.9	371	4229	8.60	-
			2	87.7	84	5000	1.66	
		225	1	75.4	105	5000	2.07	-
			2	71.3	58	5000	1.15	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
14-Sept-2017	Reference; 1706512	200	1	85.4	123	5000	2.43	-
			2	84.0	50	5000	1.00	
		175	1	91.9	61	5000	1.21	-
			2	91.5	48	5000	0.96	
		150	1	92.3	45	5000	0.89	-
			2	108.2	43	5000	0.86	
		125	1	97.2	45	5000	0.90	-
			2	95.4	39	5000	0.78	
		100	1	100.3	62	5000	1.24	-
			2	104.4	67	5000	1.34	

(Table continued from previous page)

Table 26. 4 h +S9 Test 2: Response to DMSO, CPA and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
08-Mar-2018	Solvent	0 (1% v/v DMSO)	1	2.0	100	39	5000	0.78	N/A
			2	1.7		23		0.46	
			3	1.8		23		0.46	
	CPA	4	1	N/A	85.6	194	5000	3.87	Yes
		3	1		93.0	70		1.40	
	COL	0.8	1		56.2	427	5000	7.72	Yes
		0.6	1		86.8	112		2.19	

Table 27. 4 h +S9 Test 2: Response to test item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
08-Mar-2018	Test; 1767512	3181	1	69.9	129	5000	2.52	-
			2	61.3	114	5000	2.24	
		3021.9	1	61.4	100	5000	1.98	Yes
			2	63.2	81	5000	1.60	
		2862.8	1	50.9	85	5000	1.68	-
			2	71.0	70	5000	1.39	
		2703.7	1	73.6	66	5000	1.31	-
			2	70.7	100	5000	1.04	
		2544.6	1	61.1	147	4242	1.17	-
			2	81.0	62	5000	1.23	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
08-Mar-2018	Test; 1767512	2385.5	1	74.2	51	5000	1.01	-
			2	92.9	83	5000	1.26	
		2226.4	1	80.8	47	5000	0.94	-
			2	81.8	108	5000	0.99	
		2067.3	1	83.5	39	5000	0.78	-
			2	98.8	41	5000	0.81	
		1908.2	1	92.6	40	5000	0.80	-
			2	92.5	58	5000	1.16	
		1749.1	1	99.1	25	5000	0.50	-
			2	96.5	48	5000	0.96	

(Table continued from previous page)

Table 28. 4 h +S9 Test 2: Response to reference item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
08-Mar-2018	Reference; 1767554	300	1	-37.2	134	3243	4.09	-
			2	-30.4	153	3617	4.19	
		275	1	-53.7	369	2713	13.36	-
			2	-31.0	248	3812	6.44	
		265	1	25.6	156	5000	3.07	-
			2	5.3	203	5000	4.00	
		255	1	-23.4	171	5000	4.49	-
			2	3.1	194	5000	3.83	
		250	1	32.8	173	5000	3.41	-
			2	34.6	146	5000	2.88	
		237.5	1	28.1	162	5000	3.18	-
			2	28.7	121	5000	2.38	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
08-Mar-2018	Reference; 1767554	225	1	21.4	114	5000	2.25	-
			2	31.4	143	5000	2.81	
		212.5	1	44.9	105	5000	2.07	-
			2	47.8	111	5000	2.19	
		200	1	40.5	121	5000	2.37	Yes
			2	64.3	75	5000	1.48	
		187.5	1	73.8	88	5000	1.74	-
			2	81.3	58	5000	1.15	
		175	1	83.9	49	5000	0.97	-
			2	92.9	54	5000	1.07	
		150	1	86.4	49	5000	0.97	-
			2	94.2	43	5000	0.86	
		125	1	84.1	37	5000	0.73	-
			2	96.7	47	5000	0.93	
		100	1	95.0	35	5000	0.70	-
			2	99.9	46	5000	0.92	

(Table continued from previous page)

Table 29. 4 h +S9 Test 1: Response to medium, CPA and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
29-Aug-2017	Non-treated	0 (100% v/v medium)	1	1.5	100	35	5000	0.70	N/A
			2	1.5		22		0.44	
			3	1.6		22		0.44	
	CPA	2	1	N/A	85.5	134	5000	2.67	Yes
		1	1		105.5	56		1.12	
	COL	0.8	1		57.6	258	5000	4.81	Yes
		0.6	1		63.8	158		3.04	

Table 30. 4 h +S9 Test 1: Response to test item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Test; 1702178	5000	1	-69.1	83	2649	3.10	-
			2	-83.1	63	2203	2.83	
		4750	1	-74.5	97	2365	4.07	-
			2	-62.7	97	2670	3.58	
		4500	1	-42.9	130	3448	3.71	-
			2	-33.4	182	3844	4.66	
		4250	1	-21.8	189	4364	4.27	-
			2	-10.8	191	4852	3.88	
		4000	1	10.7	198	5000	3.89	-
			2	14.5	180	5000	3.53	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Test; 1702178	3750	1	28.0	167	5000	3.28	-
			2	48.0	160	5000	3.14	
		3500	1	43.9	115	5000	2.26	Yes
			2	53.4	140	5000	2.77	
		3000	1	80.0	61	5000	1.21	-
			2	83.5	67	5000	1.33	
		2000	1	95.4	45	5000	0.90	-
			2	95.4	46	5000	0.92	
		1000	1	118.8	26	5000	0.52	-
			2	108.8	23	5000	0.46	

(Table continued from previous page)

Table 31. 4 h +S9 Test 1: Response to reference item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Reference; 1702180	550	1	-72.0	88	2459	3.49	-
			2	-85.9	53	2044	2.53	
		525	1	-42.5	92	3370	2.65	-
			2	-57.3	76	2705	2.74	
		500	1	-18.2	141	4339	3.15	-
			2	-21.2	139	4172	3.23	
		475	1	9.4	142	5000	2.76	-
			2	-4.4	154	4943	3.03	
		450	1	39.0	105	5000	2.05	-
			2	28.4	144	5000	2.80	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Reference; 1702180	425	1	58.7	68	5000	1.34	-
			2	54.5	87	5000	1.70	
		400	1	70.4	47	5000	0.93	-
			2	65.9	68	5000	1.34	
		300	1	97.4	30	5000	0.60	-
			2	81.7	36	5000	0.72	
		200	1	94.3	18	5000	0.36	-
			2	79.1	46	5000	0.92	
		100	1	108.9	29	5000	0.58	-
			2	110.0	18	5000	0.36	

(Table continued from previous page)

Table 32. 4 h +S9 Test 2: Response to medium, CPA and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
31-Aug-2017	Non-treated	0 (100% v/v medium)	1	1.3	100	38	5000	0.76	N/A
			2	1.3		38		0.76	
			3	1.2		26		0.52	
	CPA	2	1	N/A	70.5	212	5000	4.21	Yes
		1	1		96.8	80		1.54	
	COL	0.8	1		34.6	423	5000	7.75	Yes
		0.6	1		51.7	202		3.89	

Table 33. 4 h +S9 Test 2: Response to test item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
31-Aug-2017	Test; 1702187	5000	1	-67.3	134	3164	4.19	-
			2	-60.6	138	3363	4.05	
		4750	1	-60.5	114	3332	3.36	-
			2	-40.2	151	3903	3.79	
		4500	1	-28.9	179	4372	4.04	-
			2	-37.2	186	4276	4.29	
		4250	1	8.2	197	5000	3.88	-
			2	11.9	196	5000	3.85	
		4000	1	6.4	202	5000	3.97	-
			2	27.3	191	5000	3.75	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
31-Aug-2017	Test; 1702187	3750	1	25.8	183	5000	3.59	-
			2	50.4	201	5000	3.95	
		3500	1	45.9	121	5000	2.39	Yes
			2	52.0	168	5000	3.31	
		3000	1	61.8	75	5000	1.49	-
			2	73.7	113	5000	2.24	
		2000	1	77.9	55	5000	1.10	-
			2	95.3	55	5000	1.10	
		1000	1	104.7	42	5000	0.83	-
			2	116.2	50	5000	0.76	

(Table continued from previous page)

Table 34. 4 h +S9 Test 2: Response to reference item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
31-Aug-2017	Reference; 1702189	550	1	49.8	80	5000	1.57	-
			2	43.6	104	5000	2.05	
		525	1	42.5	83	5000	1.64	-
			2	40.5	76	5000	1.50	
		500	1	60.3	59	5000	1.17	-
			2	51.7	67	5000	1.32	
		475	1	65.7	57	5000	1.13	-
			2	54.3	61	5000	1.21	
		450	1	69.0	44	5000	0.88	-
			2	54.7	49	5000	0.97	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
31-Aug-2017	Reference; 1702189	425	1	62.6	41	5000	0.82	-
			2	68.8	56	5000	1.11	
		400	1	81.3	36	5000	0.72	-
			2	64.0	55	5000	1.09	
		300	1	81.5	29	5000	0.58	-
			2	72.9	31	5000	0.62	
		200	1	80.4	32	5000	0.64	-
			2	86.9	31	5000	0.62	
		100	1	95.1	31	5000	0.61	-
			2	108.1	33	5000	0.66	

(Table continued from previous page)

Table 35. 24 h -S9 Test 1: Response to DMSO, MMS and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
29-Aug-2017	Solvent	0 (1% v/v DMSO)	1	1.8	100	26	5000	0.52	N/A
			2	1.8		30		0.60	
			3	2.0		27		0.54	
	MMS	20	1	N/A	61.8	136	5000	2.68	Yes
		15	1		78.7	89		1.77	
	COL	0.2	1		53.8	250	5000	4.51	Yes
		0.15	1		74.4	96		1.81	

Table 36. 24 h -S9 Test 1: Response to test item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Test; 1702178	1600	1	42.0	32	5000	0.64	-
			2	29.6	35	5000	0.70	
		1500	1	49.1	26	5000	0.52	-
			2	38.6	31	5000	0.62	
		1400	1	54.4	46	5000	0.92	-
			2	43.6	34	5000	0.68	
		1300	1	61.1	23	5000	0.46	-
			2	55.2	34	5000	0.68	
		1200	1	48.4	36	5000	0.72	-
			2	58.3	36	5000	0.72	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Test; 1702178	1100	1	65.9	27	5000	0.54	-
			2	66.2	19	5000	0.38	
		1000	1	49.4	2	134	1.48	-
			2	78.7	19	5000	0.38	
		900	1	72.6	23	5000	0.46	-
			2	69.6	16	5000	0.32	
		700	1	81.2	18	5000	0.36	-
			2	87.1	17	5000	0.34	
		500	1	87.9	24	5000	0.48	-
			2	88.1	26	5000	0.52	

(Table continued from previous page)

Table 37. 24 h -S9 Test 1: Response to reference item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Reference; 1702180	140	1	-5.8	49	4400	1.10	-
			2	8.1	70	5000	1.37	
		130	1	32.5	55	5000	1.08	-
			2	15.9	76	5000	1.48	
		120	1	43.5	33	5000	0.65	-
			2	29.6	62	5000	1.21	
		110	1	44.9	34	5000	0.67	-
			2	33.9	43	5000	0.85	
		100	1	52.1	47	5000	0.94	-
			2	38.9	46	5000	0.91	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Reference; 1702180	90	1	58.9	17	5000	0.34	-
			2	54.9	36	5000	0.71	
		80	1	63.4	32	5000	0.64	-
			2	55.2	27	5000	0.54	
		70	1	75.3	26	5000	0.52	-
			2	65.2	26	5000	0.52	
		60	1	78.1	16	5000	0.32	-
			2	74.4	29	5000	0.58	
		50	1	83.5	17	5000	0.34	-
			2	93.3	28	5000	0.56	

(Table continued from previous page)

Table 38. 24 h -S9 Test 2: Response to DMSO, MMS and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
08-Mar-2018	Solvent	0 (1% v/v DMSO)	1	1.8	100	24	5000	0.48	N/A
			2	1.8		25		0.50	
			3	1.7		41		0.82	
	MMS	35	1	N/A	60.0	279	5000	5.43	Yes
		30	1		56.9	165		3.22	
	COL	0.2	1		23.1	930	5000	15.71	Yes
		0.15	1		50.7	373		6.54	

Table 39. 24 h -S9 Test 2: Response to test item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
08-Mar-2018	Test; 1767512	2000	1	24.4	100	5000	1.97	-
			2	16.5	82	5000	1.61	
		1800	1	25.5	109	5000	2.15	-
			2	33.6	118	5000	2.33	
		1600	1	48.4	54	5000	1.07	-
			2	54.7	54	5000	1.07	
		1500	1	43.8	79	5000	1.57	-
			2	52.0	63	5000	1.25	
		1400	1	56.2	47	5000	0.93	-
			2	60.7	70	5000	1.39	
		1300	1	57.7	47	5000	0.94	-
			2	64.0	42	5000	0.84	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
08-Mar-2018	Test; 1767512	1200	1	66.2	41	5000	0.82	-
			2	71.8	35	5000	0.70	
		1100	1	70.9	39	5000	0.78	-
			2	72.2	35	5000	0.70	
		1000	1	80.8	35	5000	0.70	-
			2	95.2	35	5000	0.70	
		900	1	92.0	30	5000	0.60	-
			2	89.9	27	5000	0.54	
		700	1	97.8	30	5000	0.60	-
			2	94.2	28	5000	0.56	
		500	1	93.1	23	5000	0.46	-
			2	87.6	28	5000	0.56	

(Table continued from previous page)

Table 40. 24 h -S9 Test 2: Response to reference item-derived TPM.

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
08-Mar-2018	Reference; 1767554	140	1	35.1	57	5000	1.13	-
			2	32.0	32	5000	0.63	
		130	1	24.7	54	5000	1.07	-
			2	33.2	32	5000	0.63	
		120	1	38.8	35	5000	0.69	-
			2	43.0	40	5000	0.79	
		110	1	48.8	33	5000	0.66	-
			2	44.7	36	5000	0.72	
		105	1	52.4	45	5000	0.89	-
			2	51.6	48	5000	0.96	
		100	1	45.8	24	5000	0.48	-
			2	64.9	50	5000	0.99	

Treatment Date	Item; Sample ID	Concen-tration (µg/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
08-Mar-2018	Reference; 1767554	95	1	53.1	32	5000	0.64	-
			2	59.8	52	5000	1.04	
		90	1	69.8	31	5000	0.62	-
			2	68.1	58	5000	1.15	
		80	1	66.5	32	5000	0.64	-
			2	75.0	39	5000	0.78	
		70	1	78.3	35	5000	0.70	-
			2	90.6	48	5000	0.96	
		60	1	89.5	29	5000	0.58	-
			2	95.5	22	5000	0.44	
		50	1	105.2	24	5000	0.48	-
			2	97.0	25	5000	0.50	

(Table continued from previous page)

Table 41. 24 h -S9 Test 1: Response to medium, MMS and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
29-Aug-2017	Non-treated	0 (100% v/v medium)	1	1.7	100	21	5000	0.42	N/A
			2	1.7		13		0.26	
			3	1.6		18		0.36	
	MMS	20	1	N/A	65.7	108	5000	2.13	Yes
		15	1		79.2	81		1.60	
	COL	0.2	1		38.7	230	5000	4.18	Yes
		0.15	1		72.4	77		1.46	

Table 42. 24 h -S9 Test 1: Response to test item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Test; 1702178	4000	1	18.3	95	5000	1.86	-
			2	22.8	123	5000	2.41	
		3500	1	40.6	47	5000	0.93	-
			2	32.7	75	5000	1.47	
		3250	1	53.7	46	5000	0.91	-
			2	48.5	54	5000	1.07	
		3000	1	63.4	31	5000	0.62	-
			2	55.1	46	5000	0.91	
		2750	1	72.1	31	5000	0.62	-
			2	76.3	24	5000	0.48	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Test; 1702178	2500	1	72.0	29	5000	0.58	-
			2	87.0	37	5000	0.74	
		2250	1	90.3	21	5000	0.42	-
			2	87.4	25	5000	0.50	
		2000	1	85.8	22	5000	0.44	-
			2	91.2	29	5000	0.58	
		1500	1	99.8	13	5000	0.26	-
			2	111.1	15	5000	0.30	
		1000	1	108.6	13	5000	0.26	-
			2	107.2	20	5000	0.40	

(Table continued from previous page)

Table 43. 24 h -S9 Test 1: Response to reference item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Reference; 1702180	400	1	34.0	54	5000	1.06	-
			2	46.2	33	5000	0.65	
		375	1	41.1	84	5000	1.64	-
			2	41.2	41	5000	0.81	
		350	1	79.0	31	5000	0.62	-
			2	62.9	27	5000	0.54	
		325	1	87.8	18	5000	0.36	-
			2	92.9	26	5000	0.52	
		300	1	84.5	26	5000	0.52	-
			2	88.3	27	5000	0.54	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equiva-lent/ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
29-Aug-2017	Reference; 1702180	250	1	104.0	13	5000	0.26	-
			2	98.8	8	5000	0.16	
		225	1	106.5	14	5000	0.28	-
			2	107.3	18	5000	0.36	
		200	1	97.8	14	5000	0.28	-
			2	109.3	14	5000	0.28	
		150	1	100.5	11	5000	0.22	-
			2	105.7	11	5000	0.22	
		100	1	107.4	13	5000	0.26	-
			2	103.4	13	5000	0.26	

(Table continued from previous page)

Table 44. 24 h -S9 Test 2: Response to medium, MMS and COL treatment.

Treatment Date	Control	Concen-tration (µg/ml)	Replicate Culture	No. PD	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	Signifi-cantly Different From Solvent
13-Mar-2018	Non-treated	0 (100% v/v medium)	1	2.0	100	27	5000	0.54	N/A
			2	1.9		26		0.52	
			3	1.9		50		1.00	
	MMS	35	1	N/A	39.6	210	5000	4.08	Yes
		30	1		40.1	223		4.34	
	COL	0.2	1		54.5	696	5000	11.72	Yes
		0.15	1		55.6	319		5.63	

Table 45. 24 h -S9 Test 2: Response to test item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
13-Mar-2018	Test; 1777587	4000	1	-16.7	165	4692	3.43	-
			2	-22.3	163	4481	3.57	
		3500	1	-4.7	233	5000	4.55	-
			2	-20.7	139	4391	3.10	
		3250	1	11.6	292	5000	5.59	-
			2	-19.3	256	4455	5.57	
		3000	1	10.1	325	5000	6.25	-
			2	-6.3	330	4996	6.36	
		2750	1	28.4	240	5000	4.60	-
			2	-5.3	240	4697	4.88	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
13-Mar-2018	Test; 1777587	2625	1	41.7	204	5000	3.93	-
			2	-1.3	235	5000	4.52	
		2500	1	50.8	162	5000	3.13	Yes
			2	33.3	187	5000	3.64	
		2250	1	60.1	75	5000	1.48	-
			2	30.4	96	5000	1.88	
		2000	1	71.0	83	5000	1.64	-
			2	46.0	59	5000	1.17	
		1500	1	85.3	49	5000	0.97	-
			2	58.7	52	5000	1.03	
		1000	1	60.9	31	5000	0.62	-
			2	76.4	30	5000	0.60	

(Table continued from previous page)

Table 46. 24 h -S9 Test 2: Response to reference item-derived GVP.

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
13-Mar-2018	Reference; 1777590	450	1	-15.0	170	4787	3.40	-
			2	-11.3	118	4905	2.33	
		425	1	20.7	125	5000	2.42	-
			2	5.4	165	5000	3.18	
		400	1	30.2	140	5000	2.72	-
			2	20.2	152	5000	2.91	
		375	1	21.6	195	5000	3.67	-
			2	29.4	149	5000	2.88	
		350	1	40.6	119	5000	2.32	-
			2	56.3	139	5000	2.70	
		325	1	49.4	111	5000	2.15	Yes
			2	35.3	103	5000	1.98	

Treatment Date	Item; Sample ID	Concen-tration (µg TPM equivalent/ ml)	Replicate Culture	RPD (%)	No. MN	No. Nuclei Evaluated	%MN	LOGEL
13-Mar-2018	Reference; 1777590	300	1	82.6	59	5000	1.16	-
			2	83.2	65	5000	1.28	
		250	1	70.1	54	5000	1.07	-
			2	103.1	38	5000	0.76	
		225	1	93.3	44	5000	0.87	-
			2	83.0	50	5000	0.99	
		200	1	91.0	50	5000	0.99	-
			2	89.8	44	5000	0.88	
		150	1	72.9	29	5000	0.58	-
			2	70.5	21	5000	0.42	
		100	1	87.9	20	5000	0.40	-
			2	75.3	26	5000	0.52	

(Table continued from previous page)

Table 47. 4 h -S9 TPM-induced LOGELs.

Test Number	Fraction	Item	LOGEL (µg/ml)	LOGEL Ratio	Nicotine at LOGEL (µg/ml)	Nicotine at LOGEL Ratio
1	TPM	Test	2400	N/A	66.9235	N/A
		Reference	N/A	N/A	N/A	N/A
2	TPM	Test	N/A	N/A	N/A	N/A
		Reference	N/A	N/A	N/A	N/A

Table 48. 4 h -S9 GVP-induced LOGELs.

Test Number	Fraction	Item	LOGEL (µg TPM equivalent/ml)	LOGEL Ratio
1	GVP	Test	1950	11.6
		Reference	168.75	1
2	GVP	Test	1950	13.3
		Reference	146.25	1

Table 49. 4 h +S9 TPM-induced LOGELs.

Test Number	Fraction	Item	LOGEL (µg/ml)	LOGEL Ratio	Nicotine at LOGEL (µg/ml)	Nicotine at LOGEL Ratio
1	TPM	Test	3181	N/A	88.7015	N/A
		Reference	N/A	N/A	N/A	N/A
2	TPM	Test	3021.9	15.1	88.8106	10.1
		Reference	200	1	8.8008	1

Table 50. 4 h +S9 GVP-induced LOGELs.

Test Number	Fraction	Item	LOGEL (µg TPM equivalent/ml)	LOGEL Ratio
1	GVP	Test	3500	N/A
		Reference	N/A	N/A
2	GVP	Test	3500	N/A
		Reference	N/A	N/A

Table 51. 24 h TPM-induced LOGELs.

Test Number	Fraction	Item	LOGEL (µg/ml)	LOGEL Ratio	Nicotine at LOGEL (µg/ml)	Nicotine at LOGEL Ratio
1	TPM	Test	N/A	N/A	N/A	N/A
		Reference	N/A	N/A	N/A	N/A
2	TPM	Test	N/A	N/A	N/A	N/A
		Reference	N/A	N/A	N/A	N/A

Table 52. 24 h GVP-induced LOGELs.

Test Number	Fraction	Item	LOGEL (µg TPM equivalent/ml)	LOGEL Ratio
1	GVP	Test	N/A	N/A
		Reference	N/A	N/A
2	GVP	Test	2500	7.7
		Reference	325	1

Table 53. The laboratory's historical %MN database derived from 1% DMSO- and 2-10% PBS-treated/non-treated cells in the 4 h -S9 treatment condition.

%MN	**1% v/v DMSO**	**2-10% v/v PBS/ non-treated**
Mean=	0.950	0.970
N=	57	81
Mean-2SD=	0.516	0.515
Mean+2SD=	1.749	1.827
Mean-3SD=	0.380	0.375
Mean+3SD=	2.374	2.508

Table 54. The laboratory's historical %MN database derived from 1% DMSO- and 2-10% PBS-treated/non-treated cells in the 4 h +S9 treatment condition.

%MN	**1% v/v DMSO**	**2-10% v/v PBS/ non-treated**
Mean=	1.005	1.055
N=	38	75
Mean-2SD=	0.583	0.561
Mean+2SD=	1.732	1.985
Mean-3SD=	0.444	0.409
Mean+3SD=	2.274	2.722

Table 55. The laboratory's historical %MN database derived from 1% DMSO- and 2-10% PBS-treated/non-treated cells in the 24 h -S9 treatment condition.

%MN	1% v/v DMSO	2-10% v/v PBS/ non-treated
Mean=	1.062	0.968
N=	45	69
Mean-2SD=	0.549	0.479
Mean+2SD=	2.055	1.954
Mean-3SD=	0.395	0.337
Mean+3SD=	2.858	2.776

Table 56. The laboratory's historical %MN database derived from 15 and 20 µg/ml MMS-treated cells in the 4 h -S9 treatment condition.

%MN	MMS 15 µg/ml	MMS 20 µg/ml
Mean=	3.132	3.889
N=	65	67
Mean-2SD=	1.275	1.870
Mean+2SD=	7.695	8.089
Mean-3SD=	0.814	1.297
Mean+3SD=	12.061	11.665

Note: there is currently no laboratory historical %MN database for cells treated with 30 and 35 µg/ml MMS as at least ten experiments have not yet been carried out.

Table 57. The laboratory's historical %MN database derived from CPA-treated cells in the 4 h +S9 treatment condition.

%MN	CPA 1 µg/ml	CPA 2 µg/ml
Mean=	2.864	6.330
N=	52	56
Mean-2SD=	1.463	2.483
Mean+2SD=	5.607	16.137
Mean-3SD=	1.046	1.555
Mean+3SD=	7.844	25.765

Note: there is currently no laboratory historical %MN database for cells treated with 3 and 4 µg/ml CPA as at least ten experiments have not yet been carried out.

Table 58. The laboratory's historical %MN database derived from 15 and 20 µg/ml MMS-treated cells in the 24 h treatment condition.

%MN	MMS 15 µg/ml	MMS 20 µg/ml
Mean=	4.131	6.417
N=	59	64
Mean-2SD=	2.168	3.380
Mean+2SD=	7.871	12.182
Mean-3SD=	1.571	2.453
Mean+3SD=	10.864	16.785

Note: there is currently no laboratory historical %MN database for cells treated with 30 and 35 µg/ml MMS as at least ten experiments have not yet been carried out.

Table 59. Chemical analysis of test and reference item-derived TPM.

Preparation Date	Item; Sample ID	Yield (mg/cigarette)		Yield (mg/ml)	
		TPM	Nicotine	TPM	Nicotine
29-Aug-2017	Test; 1702178	29.95	0.967	239.60	7.7356
	Reference; 1702180	40.38	1.812	40.38	1.8118
31-Aug-2017	Test; 1702187	33.91	0.950	339.10	9.5024
	Reference; 1702189	41.06	1.795	41.06	1.7946
14-Sept-2017	Test; 1706509	32.98	0.920	329.80	9.1964
	Reference; 1706512	40.94	1.849	40.94	1.8494

Preparation Date	Item; Sample ID	Yield (mg/cigarette)		Yield (mg/ml)	
		TPM	Nicotine	TPM	Nicotine
08-Mar-2018	Test; 1767512	32.11	0.944	321.10	9.4368
	Reference; 1767554	43.06	1.895	43.06	1.8948
13-Mar-2018	Test; 1777587	32.72	0.948	327.20	9.4788
	Reference; 1777590	45.28	1.904	45.28	1.9036
20-Mar-2018	Test; 1779879	32.58	0.937	325.80	9.3676
	Reference; 1779921	40.16	1.797	40.16	1.7972

(Table continued from previous page)

16 APPENDIX A: POST MITOCHONDRIAL SUPERNATANT (S9) QUALITY CONTROL & PRODUCTION CERTIFICATE

TRINOVA Biochem GmbH

MOLTOX®
Molecular Toxicology, Inc.

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information	Part Number Information	
SPECIES: Rat	**LOT NO.:** 1546	**PREP:** October 28, 2015
STRAIN: Sprague Dawley	**PART NO.:** 11-101	**EXPIRY:** October 28, 2017
SEX: Male	**VOLUME:** 1 & 2 mL	**INDUCING AGENT:** Aroclor 1254, (Monsanto KL615), 500 mg/kg i.p.
AGE: 5 – 6 weeks	**BUFFER:** 0.15 M KCl	
WEIGHT: 175 – 199 g	**STORAGE:** At or below -70°C	
TISSUE: Liver		

REFERENCE: Maron, D & Ames, B., *Mutat Res*, **113**: 173, 1983. **For Research Purposes Only**

BIOCHEMISTRY:
- PROTEIN: 38.6 mg/ml — Assayed according to the method of Lowry et al., *JBC* **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-O-DEALKYLASE ACTIVITIES

Activity	P450	Fold-Induction	
BROD	2B1, 2B2	61.5	Assays for ethoxyresorufin-O-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-O-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 67.4, 21.9, 4.3, & 17.4 for BROD, EROD, MROD and PROD, respectively.
EROD	1A1, 1A2	262.8	
MROD	1A1, 1A2	265.7	
PROD	2B1, 2B2	33.6	

BIOASSAY:
- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS
Samples of S-9 were assayed for the presence of contaminating microorganisms by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Duplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

PROMUTAGEN ACTIVATION — The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**: 299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

No. His+ Revertants	
TA98	TA1535
91.6	752

Dilutions of the sample S9, ranging from 0.2 – 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to metabolites mutagenic to TA100. Assays were conducted as described by Maron & Ames, (*Mutat Res* **113**: 173, 1983).

	µl S9 per plate/number his+ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	79	134	484	665	805	1124
2-AA (2.5 µg)	87	429	1756	2053	1934	1108

Approved: 11/02/15

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17 APPENDIX B: CELL LINE PROVENANCE

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Dr Daniel Smart
PMI R&D
Quai Jeanrenaud 5
2000 Neuchâtel
Switzerland

Dear Dr Smart,

The CHO cells were originally established from a Chinese hamster ovarian biopsy by T. Puck and I obtained them many years later from the laboratory of Dr Sheldon Wolff, at the lab of Radiobiology, University of California, San Francisco. The cells were cloned at the laboratory of Dr Arthur Bloom at Columbia University (CHO-W-B1) in about 1980 and used extensively in testing for the National Toxicology Program (NTP) by that laboratory and by my laboratory at Litton Bionetics (see publications such as Galloway et al, Environmental Mutagenesis 7, 1 – 51, 1985; and Galloway et al, Environmental and Molecular Mutagenesis, 10, suppl10, 1 – 175, 1987). The cells were re-cloned at Litton Bionetics in about 1982 (CHO-WBL) and used in further NTP studies (e.g., Ivett et al, Environmental and Molecular Mutagenesis, 14, 165- 187, 1989 and Anderson et al, Environmental and Molecular Mutagenesis, 16, suppl 18, 55-137, 1990). We have continued to use these "WBL" (Wolff, Bloom, Litton) cells during 30 years of testing here at Merck.

As part of an ILSI initiative to arrange for standard sources of cells for genotoxicity testing, the derivation and handling procedures for CHO-WBL cells are described in detail in a draft manusctript which was provided to you. This includes for reference expected growth rate, and background levels of metaphase chromosome aberrations, micronuclei and polyploidy.

The cells we sent you were frozen on 19-Aug-2010. They were at passage 8.

Yours faithfully,

Sheila M Galloway

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