STUDY PLAN

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)

TABLE OF CONTENTS

1 APPROVAL AND VERIFICATION

Name	Justification	Date / Signature
Vanscheeuwijck, Patrick	Approved by Test Facility Management	17 Aug. 17
Lamboley, Christelle	Approved by Sponsor	17. AUG. 2017 Lamboley
Bosson, Régine	Verified by Quality Assurance	21 Aug. 2017
Smart, Daniel	Approved by Study Director	21 Aug 2017

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
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Supervisor, Statistics	Vuillaume, Grégory

2.2 Study Schedule

Proposed experimental starting week	Calendar Week 34 (2017)
Proposed experimental completion week	Calendar Week 37 (2017)

2.3 Test Guideline

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

2.4 Good Laboratory Practice Compliance

The current study will be performed in compliance with the Swiss Ordinance relating to Good Laboratory Practice, 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].

3 AMENDMENTS AND DEVIATIONS

Any intended (amendment) or unintended (deviation) change to the study plan will be documented according to applicable study management procedures.

4 INTRODUCTION

The *in vitro* micronucleus (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, Chinese hamster ovary (CHO) cells will be exposed either to the aerosol fractions derived from the mainstream aerosol of Tobacco Heating System Regular (THS) tobacco sticks or mainstream smoke fractions from 3R4F research cigarettes, namely the total particulate matter (TPM) fraction and the cell culture medium-soluble portion of the gas/vapour phase (GVP). Cytotoxicity, i.e. relative population doubling (RPD), and genotoxicity, i.e. MN frequency (%MN), parameters will be generated and, if biologically-relevant genotoxicity is induced by both items, comparisons of lowest observed genotoxic effect levels (LOGELs) will be made (see section 6.2.5).

5 OBJECTIVES

The objectives of this study are: 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 is observed.

6 EXPERIMENTAL DESIGN

6.1 Test and Reference Items

TIM will be 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, will be provided by the Sponsor via the batch release certificate. The batch items number will be 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 will be performed according to PMI-RRP-WKI-111777, Preparation of items (RDNEU). All unused test and reference items will be returned to the Sponsor prior to study report finalisation.

6.1.1 Identification and Description

THS tobacco sticks will be regarded as the test item and the 3R4F research cigarettes will be regarded as the reference item (summarised in Table 1).

Table 1. 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

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 6.5.1) will be 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 the 24th September 2015.

6.1.2 Storage and Stability of Test and Reference Items

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

- Test item: at 22 ± 3°C and 60 ± 5% RH until required for the aerosol generation procedure. Test item stability information will be 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.

6.2 Test System

6.2.1 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).

6.2.2 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 29 years of genotoxicity testing at Merck Research Laboratories. 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) to be 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).

6.2.3 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 are 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

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

6.2.4 Acceptability Criteria

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

The following exclusion criteria will be applied in the stated order and, in addition, only remaining data from a previous step will be taken forward into subsequent steps. Furthermore, the Study Director may exclude some samples due to technical or human error and will document the reason of exclusion in the study data. However, if an exclusion applies to a solvent-treated control, statistical tests will consequently become underpowered and, therefore, corresponding test and/or reference item data will be discarded from the statistical analysis.

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

b) The mean RPD of duplicate cultures will be ≥40%:
    i. For test and reference items, if any duplicate cultures do not fulfil this condition, they will be discarded from the analysis.
    ii. This criterion does not apply to positive controls.

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

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

e) The geometric mean %MN of concurrent solvent-treated control replicate cultures must be in the range of its respective historical range. If solvent-treated control replicate cultures do not fulfil this condition, the assay will be discarded from the analysis. The historical range is 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 show a statistically significant ($p \leq 0.05$) positive trend with respect to concurrent solvent-treated control %MN. If both positive control chemicals do not fulfil this condition, the assay will be discarded from the analysis.

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

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

6.2.5 Evaluation Criteria

Refer to section 7 for details of the statistical methods to be applied in this study. Briefly, provided that the acceptability criteria are satisfied, a response to a test and reference item is classified as positive, i.e. genotoxic, and biologically-relevant in the MNvit assay if:

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

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

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

Furthermore, the LOGEL is defined as the lowest concentration of TPM or GVP tested that induces a biologically-relevant genotoxic response in the MNvit assay. Provided that biologically-relevant mutagenic responses are induced by test and reference items on the same test occasion, LOGELs will be compared on a fold-difference basis.

Although the aneugenicity-related endpoint, namely hypodiploid nuclei, is not formally part of the evaluation criteria, it may be used to support the conclusion drawn on the genotoxicity of a test substance. For example, any signs of aneugenicity observed in a MNvit assay would instigate further investigations with the view of understanding better the test substance's genotoxic mode of action.

6.3 Preparation of the Reference Item

The reference item will be 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).

6.4 Conditioning of the Reference Item

The reference item will be 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.

6.5 Aerosol Generation

6.5.1 Test Item-Specific Information

Test item-derived aerosols will be 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 to be used in the study are summarised in Table 2.

Table 2. 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 will be taken as the unique identifier for the tobacco stick holders.

6.5.2 Aerosol Generation Procedure

Test item- and reference item-derived aerosols will be 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 3). The environmental conditions of the area in which the smoking machine will be operational during aerosol generation will be maintained at a temperature and RH of 22 ± 2°C and 60 ± 5%, respectively.

Table 3. 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 will be performed on a semi-automatic 20-port RMB20 Burghart rotary smoking machine (the settings are described in Table 4), 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 4. 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	1000	10
Reference				4000	5

Both puff duration and gap time parameters (Table 4) comprise the puff duration parameter that is described in Table 3. In addition, the term smoking volume (Table 4) corresponds to the term puff volume (Table 3).

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

Aerosol preparations will be fractionated into two parts, namely TPM and GVP, during the same aerosol generation. For the test item, TPM will be 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 will be collected on one Cambridge filter pad (44 mm diameter) and weighed. Once the TPM has been captured on the filter pad(s), the extraction of the TPM will be performed as follows. For the test item, the two filter pads will be 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 will be 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) will be 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), will be 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 will be collected, are shown in Table 5.

Table 5. 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	1	24
Reference	5	1	5	1	36

The concentration of TPM (in mg/ml) will be 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) will be 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 will be diluted to the required concentrations for genotoxicity evaluation in the MNvit assay (see section 6.6.1).

6.6 Testing Procedure

6.6.1 MNvit Assay Procedure

The MNvit assay will be performed in accordance with the OECD TG 487. A description that details how the assay will be conducted is provided in PMI-RRP-WKI-111805, Micronucleus Assay (RRPCE). For the completion of this study, two independent tests that satisfy assay acceptability criteria (see section 6.2.4) are required for the three treatment conditions.

Briefly, liquid nitrogen-stored CHO-WBL cells will be thawed and sub-cultured for at least two passages prior to treatment. CHO-WBL cells will be seeded (4500 cells/well) into 96-well plates and

cultivated for 24 ± 1 h. Cells will then be exposed to the various concentrations of test and reference items under 4 h ±S9 and 24 h -S9 treatment conditions (Table 6). Test and reference item-related concentration ranges are to be defined based upon the results of preliminary dose range finding studies. Following 4 h ±S9 treatment, cells will be 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 is 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 will be 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 LSRII flow cytometer and/or a BD FACSCanto II will be used (interchangeably) to analyse the samples according to PMI-RRP-WKI-111803, LSR II Flow Cytometer (RDNEU) and PMI-RRP-WKI-111861, FACSCanto II Flow Cytometer (RDNEU), respectively.

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}) will be 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 6. Concentrations to be tested.

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9 4 h +S9 24 h	Concentrations to be added via a study plan amendment			

In addition, appropriate reference chemical genotoxins (clastogens and an aneugen) will also be included as positive controls in each treatment condition (Table 7).

Table 7. The positive controls to be 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	Colchicine (COL)	0.8; 1.0
4 h +S9	Cyclophosph-amide (CPA)	1; 2	COL	0.6; 0.8
24 h -S9	MMS	15; 20	COL	0.15; 0.20

6.6.2 Analytical Procedures (TPM and Nicotine Determination)

The mass of TPM collected on the filter pad(s) will be 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 will correspond 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 will be 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 will be measured using a gas chromatography with a flame ionisation detector; nicotine will be quantified using the ratio of the nicotine peak area to the peak area of isoquinoline. DMSO will serve as the blank control.

7 STATISTICAL METHODS

7.1 Data Analysis

7.1.1 General

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

7.1.2 Missing Data and Extreme Data

See section 6.2.4.

7.1.3 Confidence/Significance Level

This study is exploratory from a statistical perspective and, consequently, no formal hypothesis testing will be performed and nor will there be 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, may be descriptively reported as a noteworthy finding when below the usual threshold (P<=0.05, 0.01, or 0.001). These usual thresholds may also be used to build confidence intervals.

7.2 Parameters for Data Analysis

7.2.1 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.

7.2.2 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.
- $PD = \log\left(N_{Nuc}^{END} \div \overline{N_{Nuc}^{T0}}\right) \div log(2)$
- $\%RPD = 100 \times PD_{item} \div \overline{PD_{solvent}}$

7.2.3 Endpoints

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

7.3 Acceptability Criteria

See to section 6.2.4.

7.4 Descriptive Statistics

7.4.1 Historical Solvent-Treated Control %MN Data

The following data will be 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:

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

Certain historical %MN ranges, e.g. phosphate-buffered saline-treated and untreated, may be 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.

7.4.2 Study Data

The measured and derived parameters for the different endpoints will be reported for each replicate culture and concentration in the validated spreadsheet PMI-RRP-FOR-116343, Micronucleus assay (V2.0).

7.5 Statistical Design and Power

Given this study is exploratory from a statistical perspective as a result of lack of historical data for the test item THS tobacco sticks, no power calculations will be performed.

The concentrations of test and reference items tested in each treatment condition will be based on data from separate dose-range finding experiments or from data generated during this study. The number of concentrations and their nominal value may vary between assays. Two assays that satisfy acceptability criteria are required for test and reference items in each treatment condition (see section 6.2.4).

7.6 Exploratory Hypothesis

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

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

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

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

8 ARCHIVING

After the creation of the sub-stock, the test and reference item retention samples will be 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 need 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 and materials 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 PMI Product Testing e-archivist on the central archiving server at Philip Morris S.A., Lausanne, Switzerland.

9 REFERENCES

Bryce SM, Bemis JC, Avlasevich SL, Dertinger SD. *In vitro* micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. *Mutat. Res.* 2007 630: 78-91.

Lorge E, Hayashi M, Albertini S, Kirkland D. Comparison of different methods for an accurate assessment of cytotoxicity in the *in vitro* micronucleus test. I. Theoretical aspects. *Mutat. Res.* 2008 655: 1-3.

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)).

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

The procedures and instructions to be followed in order to carry out the study are listed below:

- PMI-RRP-SOP-111686, Perform Analysis (RDNEU).
- PMI-RRP-SOP-111687, Manage Biological Test Systems (RDNEU).
- PMI-RRP-SOP-111691, Manage Sample (RDNEU).
- PMI-RRP-SOP-111696, Role of Statistician in GLP study (RDNEU).
- PMI-RRP-WKI-111701, Sélection du type de lèvres à utiliser lors du fumage (RDNEU).
- PMI-RRP-WKI-111702, Utilisation et gestion des armoires climatiques (RDNEU).
- PMI-RRP-WKI-111703, Use of Tobacco heating system with linear and rotary smoking machine (THS) (RDNEU).
- PMI-RRP-WKI-111711, Gestion des conditions environnementales du laboratoire de collection d'aérosol et de la chambre de conditionnement (RDNEU).
- PMI-RRP-WKI-111712, Blocage de la ventilation du papier de bout des cigarettes (RDNEU).

- PMI-RRP-WKI-111734, Performing and recording analytical activities using the LC-MS/MS Thermo with Xcalibur and LC Quan software (RDNEU).
- PMI-RRP-WKI-111735, Reception and storage of items (RDNEU).
- PMI-RRP-WKI-111737, Aerosol Data Management (RDNEU).
- PMI-RRP-WKI-111738, Guide d'utilisation pour la machine à fumer rotative semi-automatique RMB20 (RDNEU).
- PMI-RRP-WKI-111759, Trappage de l'eau, de la nicotine et de l'acroléine pour les tests biologiques (RDNEU).
- PMI-RRP-WKI-111775, Testing mammalian cell cultures for Mycoplasma (RDNEU).
- PMI-RRP-WKI-111777, Preparation of items (RDNEU).
- PMI-RRP-WKI-111791, Guide d'utilisation pour la mesure de l'air flow (RDNEU).
- PMI-RRP-WKI-111794, User guide of RDLims for Logistics activities (RDNEU).
- PMI-RRP-WKI-111802, Cultivation of CHO cell lines (RDNEU).
- PMI-RRP-WKI-111803, LSR II Flow Cytometer (RDNEU).
- PMI-RRP-WKI-111805, Micronucleus Assay (RRPCE).
- PMI-RRP-WKI-111817, Determination of nicotine in smoke or aerosol condensates for *in vitro* tests (RRPCE).
- PMI-RRP-WKI-111826, Freezing and thawing of mammalian cells (RDNEU).
- PMI-RRP-WKI-111834, GLP archiving (RDNEU).
- PMI-RRP-WKI-111836, Performing and recording analytical activities using the GC with Total Chrom software (RDNEU).
- PMI-RRP-WKI-111843, E-archiving of GLP Data (RDNEU).
- PMI-RRP-WKI-111848, Management of the Multisizer 4 - Particle Analyzer (RDNEU).
- PMI-RRP-WKI-111852, Statistical Analysis with Double Programming (RDNEU).
- PMI-RRP-WKI-111861, FACSCanto II Flow Cytometer (RDNEU).

11 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 Observed 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
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

Abbreviation/Term	
SD	Standard Deviation
SOP	Standard Operating Procedure
TG	Test Guideline
THD	Tobacco Heating Device
THS	Tobacco Heating System
TIM	Test Item Management
TPM	Total Particulate Matter
WKI	Work Instruction
ZRH	Zürich

Study Number RLS-ZRH-2017-661 Plan Amendment Number 01

Present Text Version

Concerning Page

1.

Item	Smoking volume (ml)	Puff duration (ms)	Gap time (ms)	Intermission (ms)	Activated ports/run
Test	55	1860	140	1000	10
Reference				4000	5

2. Following 4 h ±S9 treatment, cells will be 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.

3.

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	Test item	Reference item	Test item	Reference item
4 h -S9 4 h +S9 24 h	Concentrations to be added via a study plan amendment			

Study Number RLS-ZRH-2017-661 Plan Amendment Number 01

New Text Version

1.

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

2. Following 4 h ±S9 treatment, cell culture medium will be removed from the cells and replaced with fresh cell culture medium. They will then be 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.

3.

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	Test item	Reference item	Test item	Reference item
4 h -S9	1125; 1237.5; 1350; 1462.5; 1575; 1687.5; 1800; 2025; 2137.5; 2250	70; 90; 100; 110; 120; 130; 140; 150; 160; 180	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	1250; 1500; 1750; 2000; 2250; 2500; 2750; 3000; 3250; 3500	100; 125; 150; 162.5; 175; 187.5; 200; 212.5 225; 250	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600	50; 60; 70; 80; 90; 100; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400

Study Number RLS-ZRH-2017-661 Plan Amendment Number 01

Reason for Change

1. Inaccuracy in the original table 4 concerning the intermission time and number of ports activated/run.
2. Refinement of the assay procedure to clarify the absence of the washing step.
3. Update of the study plan with the concentrations of test and reference item-derived TPM and GVP to be tested.

Approval	Name	Date and Signature
Study Director	Smart, Daniel	28 Aug 2017
Sponsor	Lamboley, Christelle	28. AUG. 2017
Verification	Name	Date and Signature
Quality Assurance	Bosson, Régine	28 Aug. 2017
Acknowledgement in Case of Deputy Study Director[1] Approval		
Study Director	N/A	N/A
In Case of a Multi Site Study and if concerned		
Principal Investigator	N/A	N/A
Management of Test Site	N/A	N/A
In Case of Change of Study Director		
Management of Test Facility	N/A	N/A

Distribution	
Original	Study Director
Copies	QA, Sponsor, TIM, AC, Statistics, GC, Bio labs

[1] Defined by name and address in the study plan

This form is related to PMI_WKI_000447

Study Number RLS-ZRH-2017-661 Plan Amendment Number 02

Present Text Version

Concerning

1. Table 5. Conditions for the preparation of TPM and GVP. Page 17
2. Table 6. Concentrations to be tested ~~(Study plan deviation 01 [test item] and~~ study plan amendment 01 ~~[reference item])~~. * EE. DSM 01 Sept. 2017 19

1.

		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	1	24
Reference	5	1	5	1	36

2.

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9	1125; 1237.5; 1350; 1462.5; 1575; 1687.5; 1800; 2025; 2137.5; 2250	70; 90; 100; 110; 120; 130; 140; 150; 160; 180	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	* ~~1125; 1237.5; 1350; 1462.5; 1575; 1687.5; 1800; 2025; 2137.5; 2250~~	100; 125; 150; 162.5; 175; 187.5; 200; 212.5 225; 250	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600	50; 60; 70; 80; 90; 100; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400

* EE. 1250; 1500; 1750; 2000; 2250; 2500; 2750; 3000; 3250; 3500. DSM 31 Aug 2017

Study Number RLS-ZRH-2017-661 Plan Amendment Number 02

New Text Version

1.

		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	4	1	24
Reference	5	1	5	1	36

2.

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9	1125; 1237.5; 1350; 1462.5; 1575; 1687.5; 1800; 2025; 2137.5; 2250	70; 90; 100; 110; 120; 130; 140; 150; 160; 180	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	1125; 1350; 1575; 1800; 2025; 2250; 2400; 2500; 2600; 2800	100; 125; 150; 175; 200; 225; 250; 265; 275; 300	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600	50; 60; 70; 80; 90; 100; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400

Study Number RLS-ZRH-2017-661 Plan Amendment Number 02

Reason for Change

1. Refinement in the conditions for the preparation of TPM from the test item in order to increase its concentration.
2. Refinement of the concentrations of test and reference item-derived TPM to be tested in the 4 h +S9 treatment condition.

Approval	Name	Date and Signature
Study Director	Smart, Daniel	30 Aug 2017
Sponsor	Lamboley, Christelle	04. SEP. 2017
Verification	Name	Date and Signature
Quality Assurance	Bosson, Régine	31 Aug. 2017
Acknowledgement in Case of Deputy Study Director[1] Approval		
Study Director	N/A	N/A
In Case of a Multi Site Study and if concerned		
Principal Investigator	N/A	N/A
Management of Test Site	N/A	N/A
In Case of Change of Study Director		
Management of Test Facility	N/A	N/A

Distribution	
Original	Study Director
Copies	QA, Sponsor, TIM, AC, Statistics, GC, Bio labs

[1] Defined by name and address in the study plan

This form is related to PMI_WKI_000447

Study Number RLS-ZRH-2017-661 Plan Amendment Number 03

Present Text Version

Concerning Table 6. Concentrations to be tested (Study plan amendment 02). Page 19

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9	1125; 1237.5; 1350; 1462.5; 1575; 1687.5; 1800; 2025; 2137.5; 2250	70; 90; 100; 110; 120; 130; 140; 150; 160; 180	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	1125; 1350; 1575; 1800; 2025; 2250; 2400; 2500; 2600; 2800	100; 125; 150; 175; 200; 225; 250; 265; 275; 300	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600	50; 60; 70; 80; 90; 100; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400

Study Number RLS-ZRH-2017-661 Plan Amendment Number 03

New Text Version

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9	1000; 1200; 1400; 1600; 1800; 2000; 2200; 2400; 2600; 2800	70; 90; 100; 110; 120; 130; 140; 150; 160; 180	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	1749.1; 1908.2; 2067.3; 2226.4; 2385.5; 2544.6; 2703.7; 2862.8; 3021.9; 3181	100; 125; 150; 175; 200; 225; 250; 265; 275; 300	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600	50; 60; 70; 80; 90; 100; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400

Study Number RLS-ZRH-2017-661 Plan Amendment Number 03

Reason for Change

Refinement of the concentrations of test item-derived TPM to be tested in the 4 h ±S9 treatment conditions.

Approval	Name	Date and Signature
Study Director	Smart, Daniel	07 Sept. 2017
Sponsor	Lamboley, Christelle	03 OCT. 2017
Verification	Name	Date and Signature
Quality Assurance	Bosson, Régine	11 Sept 2017
Acknowledgement in Case of Deputy Study Director[1] Approval		
Study Director	N/A	N/A
In Case of a Multi Site Study and if concerned		
Principal Investigator	N/A	N/A
Management of Test Site	N/A	N/A
In Case of Change of Study Director		
Management of Test Facility	N/A	N/A

Distribution	
Original	Study Director
Copies	QA, Sponsor, TIM, AC, Statistics, GC, Bio labs

[1] Defined by name and address in the study plan

This form is related to PMI_WKI_000447

Study Number	RLS-ZRH-2017-661	Plan Amendment Number	04

Present Text Version

Concerning		Page	
	a) Table 6. Concentrations to be tested (study plan amendment 03).		19
	b) Table 7. The positive controls to be used in the MNvit assay.		20

a)

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9	1000; 1200; 1400; 1600; 1800; 2000; 2200; 2400; 2600; 2800	70; 90; 100; 110; 120; 130; 140; 150; 160; 180	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	1749.1; 1908.2; 2067.3; 2226.4; 2385.5; 2544.6; 2703.7; 2862.8; 3021.9; 3181	100; 125; 150; 175; 200; 225; 250; 265; 275; 300	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600	50; 60; 70; 80; 90; 100; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400

b)

	Clastogen Controls		Aneugen Control	
Treatment condition	**Chemical**	**Concentrations (µg/ml)**	**Chemical**	**Concentrations (µg/ml)**
4 h -S9	Methyl methanesulfonate (MMS)	15; 20	Colchicine (COL)	0.8; 1.0
4 h +S9	Cyclophosphamide (CPA)	1; 2	COL	0.6; 0.8
24 h -S9	MMS	15; 20	COL	0.15; 0.20

Study Number	RLS-ZRH-2017-661	Plan Amendment Number	04

New Text Version

a)

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9	1000; 1200; 1400; 1600; 1800; 1900; 2000; 2100; 2200; 2400; 2600; 2800	40; 50; 65; 70; 75; 80; 85; 90; 95; 100; 110; 130	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	1749.1; 1908.2; 2067.3; 2226.4; 2385.5; 2544.6; 2703.7; 2862.8; 3021.9; 3181	100; 125; 150; 175; 187.5; 200; 212.5; 225; 237.5; 250; 255; 265; 275; 300	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600; 1800; 2000	50; 60; 70; 80; 90; 95; 100; 105; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2625; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400; 425; 450

b)

	Clastogen Controls		Aneugen Control	
Treatment condition	**Chemical**	**Concentrations (µg/ml)**	**Chemical**	**Concentrations (µg/ml)**
4 h -S9	Methyl methanesulfonate (MMS)	30; 35	Colchicine (COL)	0.8; 1.0
4 h +S9	Cyclophosphamide (CPA)	3; 4	COL	0.6; 0.8
24 h -S9	MMS	30; 35	COL	0.15; 0.20

Study Number	RLS-ZRH-2017-661	Plan Amendment Number	04

Reason for Change
a) Refinement of the concentrations of test and reference item-derived TPM and GVP to be tested in the 4 h ±S9 and 24 h treatment conditions. b) Concentrations of the clastogen controls are increased in order to enhance their effectiveness to induce unequivocal genotoxicity in the assay.

Approval	Name	Date and Signature
Study Director	Smart, Daniel	08 Mar. 2018
Sponsor	Lamboley, Christelle	14. MAR. 2018
Verification	Name	Date and Signature
Quality Assurance	Bosson, Régine	15 March 2018
Acknowledgement in Case of Deputy Study Director[1] Approval		
Study Director	N/A	N/A
In Case of a Multi Site Study and if concerned		
Principal Investigator	N/A	N/A
Management of Test Site	N/A	N/A
In Case of Change of Study Director		
Management of Test Facility	N/A	N/A

Distribution	
Original	Study Director
Copies	QA, Sponsor, TIM, AC, Statistics, GC, Bio labs

[1] Defined by name and address in the study plan

This form is related to PMI_WKI_000447

Study Number	RLS-ZRH-2017-661	Plan Amendment Number	05

Present Text Version

Concerning a) Table 6. Concentrations to be tested (study plan amendment 04). Page 19

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9	1000; 1200; 1400; 1600; 1800; 1900; 2000; 2100; 2200; 2400; 2600; 2800	40; 50; 65; 70; 75; 80; 85; 90; 95; 100; 110; 130	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	1749.1; 1908.2; 2067.3; 2226.4; 2385.5; 2544.6; 2703.7; 2862.8; 3021.9; 3181	100; 125; 150; 175; 187.5; 200; 212.5; 225; 237.5; 250; 255; 265; 275; 300	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600; 1800; 2000	50; 60; 70; 80; 90; 95; 100; 105; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2625; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400; 425; 450

Study Number	RLS-ZRH-2017-661	Plan Amendment Number	05

New Text Version

	TPM (µg/ml) derived from:		GVP (µg TPM equivalent/ml) derived from:	
Treatment condition	**Test item**	**Reference item**	**Test item**	**Reference item**
4 h -S9	1000; 1200; 1400; 1600; 1800; 1900; 2000; 2100; 2200; 2400; 2600; 2800	40; 50; 65; 70; 75; 80; 85; 90; 95; 100; 110; 130	1462.5; 1625; 1950; 2112.5; 2275; 2437.5; 2600; 2762.5; 2925; 3250	112.5; 123.75; 135; 146.25; 157.5; 168.75; 180; 191.25; 202.5; 225
4 h +S9	2544.6; 2703.7; 2862.8; 3021.9; 3181	130; 145; 160; 175; 190; 205; 220; 235; 250; 265; 280; 295; 310; 325; 340; 355; 370; 385; 400	1000; 2000; 3000; 3500; 3750; 4000; 4250; 4500; 4750; 5000	100; 200; 300; 400; 425; 450; 475; 500; 525; 550
24 h	500; 700; 900; 1000; 1100; 1200; 1300; 1400; 1500; 1600; 1800; 2000	50; 60; 70; 80; 90; 95; 100; 105; 110; 120; 130; 140	1000; 1500; 2000; 2250; 2500; 2625; 2750; 3000; 3250; 3500; 4000	100; 150; 200; 225; 250; 300; 325; 350; 375; 400; 425; 450

Study Number	RLS-ZRH-2017-661	Plan Amendment Number	05

Reason for Change
a) Refinement of the concentrations of test and reference item-derived TPM to be tested in the 4 h +S9 treatment condition.

Approval	Name	Date and Signature
Study Director	Smart, Daniel	22 Mar. 2018
Sponsor	Lamboley, Christelle	17. APR 2018
Verification	Name	Date and Signature
Quality Assurance	Bosson, Régine	29 March 2018
Acknowledgement in Case of Deputy Study Director[1] Approval		
Study Director	N/A	N/A
In Case of a Multi Site Study and if concerned		
Principal Investigator	N/A	N/A
Management of Test Site	N/A	N/A
In Case of Change of Study Director		
Management of Test Facility	N/A	N/A

Distribution	
Original	Study Director
Copies	QA, Sponsor, TIM, AC, Statistics, GC, Bio labs

[1] Defined by name and address in the study plan

This form is related to PMI_WKI_000447

Study Number RLS-ZRH-2017-661

Study Plan Amendment Number 06

Present Text Version

Concerning 6.2.5 Evaluation Criteria Page 13

Furthermore, the LOGEL is defined as the lowest concentration of TPM or GVP tested that induces a biologically-relevant genotoxic response in the MNvit assay. Provided that biologically-relevant mutagenic responses are induced by test and reference items on the same test occasion, LOGELs will be compared on a fold-difference basis.

New Text Version

Furthermore, the LOGEL is defined as the lowest concentration of TPM or GVP tested that induces a biologically-relevant genotoxic response in the MNvit assay. Provided that biologically-relevant mutagenic responses are induced by test and reference items on the same test occasion, LOGELs will be 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 will be illustrated and compared on a fold-difference basis between test and reference items.

Reason for change

LOGELs must also be compared on a nicotine basis in addition to the TPM basis.

Study Number RLS-ZRH-2017-661

Study Plan Amendment Number 06

Approval	**Name**	**Date and Signature**
Study Director	Smart, Daniel	31 May 2018
Sponsor	Lamboley, Christelle	31.MAY.2018
Verification	**Name**	**Date and Signature**
Quality Assurance	d'Estais, Guy	31 MAY 2018
Acknowledgement in Case of Deputy Study Director[1] Approval		
Study Director	N/A	N/A
In Case of Study Director Change		
Management of Test Facility	N/A	N/A

Distribution

Original	Study Director
Copies	QA, Sponsor, TIM, AC, Statistics, GC, Bio labs

[1] Defined by name and address in the study plan