STUDY PLAN TRANSLATIONAL ORGANOTYPIC ACUTE HUMAN BUCCAL TISSUES STUDY (THS2.2) (S174300)

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4 Review and Approval

This document has been approved by:

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5 OBJECTIVES

The purpose of this study is to assess the impact of an acute (28 minutes) exposure of aerosols generated from 3R4F or THS2.2 at the comparable doses of nicotine to a human buccal organotypic epithelial tissue culture.

The specific aims of this study are the following:

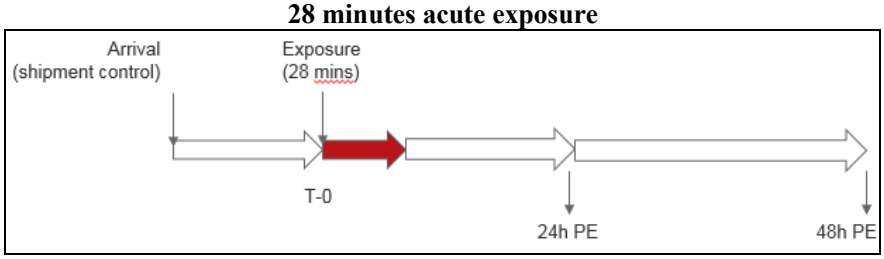
- **Dose Range Finding (DRF) phase**
  - ➢ To determine the dose-dependent and time-dependent responses of the exposure of whole mainstream smoke of conventional CS (3R4F) and THS2.2, in the following context:
  - Primary measures:
  - cytotoxicity (i.e. membrane integrity, cell viability, morphological changes)
  - activity of xenobiotic metabolism enzymes (e.g. cytochrome P450s (CYPs))
  - Secondary measure:
  - Secretion of inflammatory mediators

- **Main Phase**
  - ➢ To determine the systems response profiles upon exposure of 3R4F or THS2.2 at the chosen doses (based on the DRF phase) for assessing the activation (perturbation) of molecular pathways and biological network models
  - ➢ To determine the impact of 3R4F and THS2.2 on the secretion of inflammatory mediators
  - ➢ To determine the impact of 3R4F and THS2.2 on the activity of xenobiotic metabolism enzymes (e.g. CYPs).

Summary of the experimental design:

DRF Phase

28 minutes acute exposure



	Exposure Item		PE Time-point	
Endpoints to measure	**3R4F**	**THS2.2**	**24 h PE**	**24 h PE**
CYP1A1/1B1 activity	5 doses + sham	5 doses + sham	3 inserts	2 inserts
CYP1A1/1B1 activity + protein normalization	5 doses + sham	5 doses + sham	1 insert	2 inserts
CYP3A4 activity	5 doses + sham	5 doses + sham	2 inserts	1 inserts
CYP3A4 activity + protein normalization	5 doses + sham	5 doses + sham	1 insert	-
AK assay	5 doses + sham	5 doses + sham	5 inserts (Max 3 runs)	3 inserts
TEER assay	5 doses + sham	5 doses + sham	-	3 inserts
HE	5 doses + sham	5 doses + sham	-	3 inserts
(MAP)	5 doses + sham	5 doses + sham	-	3 inserts

PE = Postexposure; TEER= Transepithelial electrical resistance; AK= Adenylate kinase; HE = Hematoxylin and Eosin; MAP= Multi Analyte Profile

Main Phase

(The design and details of the Main Phase will be added as an amendment to this Study Plan)

6 BACKGROUND

6.1 The buccal organotypic 3D epithelial tissue model

Organotypic 3D tissues reconstituted from the tissues of the oral cavity, such as 3D oral mucosal tissues (MatTek, SkinEthics), have characteristics resembling the *in vivo* situation. Thus, they are suitable for studying biological process in the oral mucosa, including processes related to innate immunity that frequently occur in gingivitis, candidiasis, oral cancer and inflammatory oral diseases (Andrian 2004, Ceder 2007, Hansson 2001, Klausner 2007, Mostefaoui 2002, Moyes 2010, Walle 2006, Wang 2001). In addition, they are superior as compared with the ordinary 2D oral epithelial cell cultures that are submerged in culture medium, because the 3D tissue cultures are grown on a permeable porous membrane, in an air-liquid interface that allows direct exposure to whole smoke (aerosol) at the apical side (whereas the basolateral side is submerged in culture medium).

In particular, the 3D EpiOral™ tissue model (MatTek Corp.), consists of normal, human-derived epithelial cells. The cells have been cultured to form multilayered, highly differentiated models of the human buccal phenotypes. The tissues, which are cultured on specially prepared cell-culture-inserts using serum free medium, attain signs of differentiation. The EpiOral tissue models exhibiting morphological and growth characteristics similar to the *in vivo* situation. The EpiOral is a multilayerd tissue consisting of an organized basal layer and multiple non-cornified layers analogous to native human buccal tissue (PMI Internal Document No. 1). (For this study, the full-thickness tissue model containing the fibroblast layer will not be used).

The EpiOral™ tissue expresses cytokeratin K13 in all layers except the most apical ones, and weakly expresses cytokeratin K14 in the upper layers (PMI Internal Document No. 1) (Garzon 2009, Hansson 2001, Hansson 2003, Klausner 2007). The EpiOral also produce the naturally occurring antimicrobial peptides (i.e. human beta defensins HBD, such as HBD-1 and HBD-3, but not HBD-2) (PMI Internal Document No. 1). Lipid analysis showed the presence of only ceramide 2 in EpiOral tissue, similar to what is observed in the *in vivo* situation (EpiOral™ Datasheet).The *in vivo*-like morphological and growth characteristics are uniform and highly reproducible (Kimball 2006, Klausner 2007, Mattek 2012).

Moreover, CS can induce similar changes of gene expression in oral epithelia as compared to bronchial and nasal epithelia suggesting a common airway response to smoking-induced injury (Sridhar 2008). The “field of injury” hypothesis indicates common CS-related changes in gene expression that occur throughout the respiratory tract despite of frequent absence of noticeable histological/phenotypical changes (Gower 2011, Spira 2007, Sridhar 2008, Steiling 2008). This could be utilized to potentially develop non-invasive biomarkers of CS expoure in the easily accessible oral epithelia (Boyle 2010, Kupfer 2010, Paszkiewicz 2008, Spivack 2004).

6.2 Cytotoxicity assessment

Cytotoxicity is one of the most common biological parameters measured upon insults because it is easily measured and adheres to the dose-dependence paradigm of Paracelsus (Cho 2008). Cellular membrane integrity has been widely used to assess chemical cytotoxicity. Many insults, such as cigarette smoke (CS), produce physiological actions at lower concentrations, while toxic effects including apoptosis or cell death occur at higher concentrations (Cho 2008).

6.2.1 Membrane integrity

Regardless of the mechanism of cell death, cells undergo a series of morphological changes that lead to the impairment of membrane integrity (Cho 2008). This process is considered irreparable, thus allows a free movement of the previously isolated molecules in the intracellular compartment to leak into the culture medium (Cho 2008). Thus, measurement of intracellular enzymes in the extra-cellular environment is the basis of several cytotoxicity assays for assessing cell membrane integrity (Cho 2008).

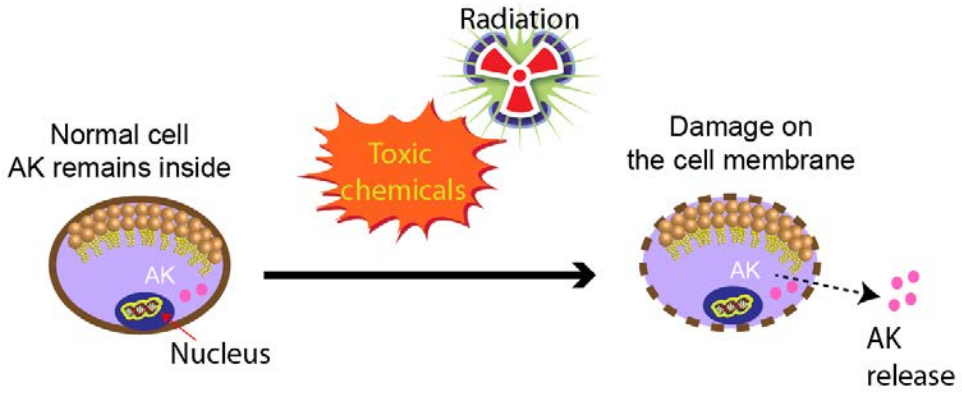


Figure 1. Illustration of adenylate kinase (AK) enzyme released from the intracellular compartment of the cells upon insults.

Several assays are often used to determine cell membrane integrity that rely on the enzymatic "release" assays, including, adenylate kinase (AK), lactate dehydrogenase (LDH) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the culture medium (Cho 2008). These release assays are widely used in *in vitro* toxicology studies. The assay is based on the measurement of the enzymatic activity in the extracellular medium. **Figure 1** shows the illustration of AK being released into the extracellular compartment upon insults. All of the three enzymes are normally compartmentalized within the cell, but their activities are significantly increased in the extracellular environment as a result of cell death (Cho 2008) (which becomes the principle of the assays). Some limitations of these assays include multiple reagent additions, low sensitivity, as well as low-throughput and scalability (Cho 2008).

Membrane permeability is often measured by transepithelial electrical resistance (TEER). Measurement of TEER provides an indirect measure of formation of tight junction and often used as a marker of disruption of the epithelial layer (Blume 2010, Stewart 2012).

6.2.2 Morphological assessment

Morphological changes by histological analyses.

6.3 Systems approach assessment

In this present study, the impact of the complex nature of CS will be assessed not only using classical endpoints of cellular response and toxicity assays described above—but also using transcriptomics, gene-set analysis, and a network-based systems biology approach. The objective of our overall approach is to comprehensively investigate the biological impacts of CS exposure. One of our approaches includes the utilization of the recently published collection of hierarchically structured biological network models to capture biological processes and mechanisms that are specific and relevant to respiratory system: Cell Proliferation (a.k.a. Cell Cycle) network (Westra 2011), Stress network (Schlage 2011), DNA Damage, Autophagy, Cell Death, and Senescence network (Westra 2011), and the Pulmonary Inflammation processes network (Thomson 2013). These biological network models were built based on *a priori* knowledge of cause-and-effect relationships among biological entities derived from published literature within a specific boundary, i.e. mainly within the context of non-diseased mammalian pulmonary tissues and cardiovascular tissues (Hoeng 2012, Thomson 2013). Because the hierarchical network models capturing mechanisms at the levels of biological processes, pathways, and specific molecular entities; the network models would be useful to not only assess the overall impact of exposure but also to investigate the specific molecular mechanisms affected by the exposure. Using the network models and systems biology approach, we will assess the impacts of CS exposure (perturbation of the biological networks) that will be quantitatively computed as described previously (Hoeng 2012, Thomson 2013) from the transcriptomics data that will be generated from the tissue models (exposed and non-exposed, and from the various models).

7 EXPERIMENTAL DESIGN

7.1 Exposure items

All exposures will be conducted according to the Health Canada smoking regimen adapted to meet the special conditions for the Vitrocell system specified in Section 7.3.2.

7.1.1 Reference items

Whole smoke generated from the Reference Cigarette 3R4F will be used as the reference aerosol. They will be purchased from the University of Kentucky, (Kentucky Tobacco Research & Development Center).
Preliminary analysis can be found in:

For specifications see **Table 1**.

Table 1. Specifications of the reference cigarette 3R4F

Parameter	Value
Physical Analysis Data	
butt length (mm)	35
cigarette length (mm)	84
total cigarette weight (g)	1.05
resistance to draw (cm water column)	13.35
circumference (mm)	24.9
paper permeability (Coresta units)	24
paper additive type (citrate) (%)	0.60
FTC Smoke Analysis	
puff count (puffs/cig.)	9.0
TPM (mg/cig.)	11.0
FTC tar (mg/cig.)	9.4
nicotine (mg/cig.)	0.73
CO (mg/cig.)	12.0
Blend Summary	
flue-cured (%)	35.41
Burley (%)	21.62
Maryland (%)	1.35
Oriental (%)	12.07
Reconstituted (Schweitzer Process) (%)	29.55
glycerine (%)	2.67
isosweet (sugar) (%)	6.41

Remarks: The smoke analysis data were generated under Federal Trade Commission (FTC) conditions.

Reference Cigarettes 3R4F and the sensorial media are stored in a cooling chamber at 5±3°C with uncontrolled humidity conditions in the original packaging, before conditioning. Packages of the sensorial media already opened are stored under controlled conditions. All test items will be conditioned between 7 and 21 days under controlled conditions from 22±1ºC and a relative humidity of 60 ± 3% according to ISO guidelines.

Mass-produced test cigarettes are generally homogeneous and remain stable when stored under our standardized storage conditions (according to the results of INBIFO study P 0500/5312, 1998). However, all constituents derived from natural products resulting in a final product which is intrinsically variable. Determination of the test article characteristics including composition will be given in the batch release.
The test aerosols will be generated from THS2.2:

Table 2. Description of the test item (THS2.2)

Product description	ZRH/DDA1/C3/ F Reform1 / CAST LEAF – CL /Flavor / Reynaldo
Overall packs (batch product code)	PCK.00112.RD
Batch number	B-08164
Final production date	10.10.2013
Expiration date	10.10.2014
Batch release certificate	PMI Internal Document No. 2

THS2.2 test item is composed of a device (CH FDP4.2) and a sensorial media with the flavor system Reynaldo. THS2.2 test item will be provided by Philip Morris International, Neuchâtel, Switzerland, with the production of 4025 sensorial media.

7.2 Exposure dose

7.2.1 Concentrations of aerosols for the DRF phase

Table 3. Concentrations of aerosols applied to the DRF phase

	3R4F					THS2.2				
Concentration	1	2	3	4	5	1	2	3	4	5
Percent smoke (%)	10	20	30	40	50	15	30	45	60	80
Flow rate (L/min)	1.9	0.9	0.53	0.34	0.22	1.28	0.55	0.30	0.15	0.055

7.2.2 Concentrations of aerosols for the main phase experiment

The concentrations will be specified as an amendment to this study plan when the data from the DRF phase is available.

7.3 Smoking machine

The whole smoke exposure run will be conducted for 28 min according to the Health Canada regimen.

7.3.1 Overview of the smoking system

The following smoking machines will be used to generate the aerosol:
3R4F : SM 2000
THS2.2: SM 2000 ZRH, SM_SDDS_ZRH

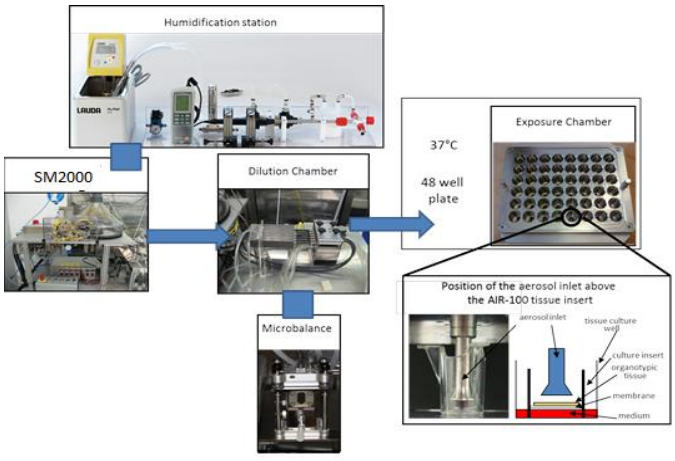


Figure 2. Schematic representation of the whole smoke exposure system.
The exposure chamber (VITROCELL® and smoking robot) are illustrated.

7.3.2 QC for the smoke run

The quality control (QC) will generate 3 TPM filters per day and 3 smoke bubbled PBS (sbPBS) for 3 consecutive days. The following actions will be taken for the QC of the aerosol generation, approximately starting 2 weeks before the actual exposure experiment:

- Installation and qualification of the aerosol generators.
- Determination of the chemical characterization of the undiluted aerosol generated under the adapted HC protocol.
- Any leakage of the exposure chamber will be noticed via checking valves and Borgwaldt leakage tester.
- The results of these QC will be collected and reported (approximately 1 week before the actual exposure experiment starts.

7.3.3 Characterization of the smoke

Characterization of the smoke exposure can be done for the measurement of total particulate matter (TPM), nicotine, and carbonyls. Detailed description and protocol are specified in Section 9.1.11.

7.3.3.1 DRF Phase-smoke characterization

Characterization of the smoke during the DRF phase will be TPM measurement for all of the doses during the smoking run. Nicotine determination using Extrelute will be conducted if deemed necessary following the exposure DRF study (which would likely to be specific only for the chosen doses of smoke that would be used for the Main Phase).

7.3.3.2 Main Phase-smoke characterization

Characterization of the smoke exposure for the main phase will include: TPM measurement, nicotine, and carbonyls of all of the doses used during the Main Phase study.

7.4 Test system

7.4.1 Tissue culture information

The 3D EpiOral™ tissue model (MatTek Corp.), consists of normal, human-derived epithelial cells (PMI Internal Document No. 1) and will be utilized as the test system for this study.
Table 4 summarizes the specific details on the EpiOral™ tissue culture that will be used for this study:

Table 4. Information of the EpiOral tissue culture used in this study

	DRF Phase		Main Phase1	Main Phase 2
	Buccal (without fibroblast)			
	Donor 1	Donor 2	Donor selected based on DRF	Donor selected based on DRF
Donor ID	G-26	G-28		
Gender	Male	Male		
Age	46	40		
Diameter of transwell	6.5 mm	6.5 mm		

More information (e.g. smoking status and health condition) are stated in PMI Internal Document No. 3.
Tissues generated from the same donor(s) will be ordered and used for the Main Phase study. Based on the results of the DRF one of the two donors will be chosen for the entire duration of the study and will be indicated, as well as any other deviation, in an amendment to the study plan.

7.4.2 Cell culture media

The culture media will be obtained from the tissue culture supplier (MatTek Corp): ORAL-200-MM medium.

7.4.3 QC for the test system

The QC of the test system will be conducted upon the tissue arrival as described in Section 9.1.1.

7.4.4 Variability of the test system

The following factors may contribute to the variability of the test systems:

Table 5. List of variability of the test system

	Potential variability	Potential solution to reduce variability
1	**Different batches**	Ensuring similar batch and age of tissues. Identical batch is not feasible to obtain for both the DRF and Main Phase studies. Thus, batch variability would occur between these two phases. However, appropriate control (e.g. air-exposed) would be tested as described in row no. 4
2	**Different age of tissues**	Ensuring similar batch and age of tissues
3	**Different replicates**	Ensuring similar batch and age of tissues
4	**Different time of exposure/smoke run**	Ensuring appropriate control (e.g. air-exposed) for each run

7.5 Endpoints to measure

7.5.1 List of endpoints for the dose range finding (DRF) phase

The list of the endpoints and relative post exposure timepoints are reported in Table 6. Please note: There will not be GEX analysis for the DRF phase. Samples collected for MAP analysis will only be used for back-up samples only.

PMI Internal Document No. 4

PMI Internal Document No. 5

Table 6. Endpoints for the DRF

Tissue	Exposure	Dose of Exposure	Shared endpoints from the same insert		Shared endpoints from the same insert				Terminal Collection PE Time-point	Longitudinal Collection PE Time-point		Terminal Collection PE Time-point	No Replicate	No Donor	No Insert	Run	Day
			1	2	3	4	5	6	CYP(p)	AK	CYP	TEER/AK/ MAP/H&E					
BU	Air-for 3R4F	NA	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run A/C	Mon
BU	3R4F	10%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run A/C	Mon
BU	3R4F	20%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run A/C	Mon
BU	3R4F	30%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run A/C	Mon
BU	3R4F	40%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run A/C	Mon
BU	3R4F	50%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run A/C	Mon
BU	Air-for 3R4F	NA	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run A	Mon
BU	3R4F	10%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run A	Mon
BU	3R4F	20%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run A	Mon
BU	3R4F	30%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run A	Mon
BU	3R4F	40%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run A	Mon
BU	3R4F	50%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run A	Mon
BU	Air-for 3R4F	NA			TEER	H&E	AK	(MAP)				48	2	2	4	Run A/C	Mon
BU	3R4F	10%			TEER	H&E	AK	(MAP)				48	2	2	4	Run A/C	Mon
BU	3R4F	20%			TEER	H&E	AK	(MAP)				48	2	2	4	Run A/C	Mon
BU	3R4F	30%			TEER	H&E	AK	(MAP)				48	2	2	4	Run A/C	Mon
BU	3R4F	40%			TEER	H&E	AK	(MAP)				48	2	2	4	Run A/C	Mon
BU	3R4F	50%			TEER	H&E	AK	(MAP)				48	2	2	4	Run A/C	Mon

(table continues)

Table 6. Endpoints for the DRF

Tissue	Exposure	Dose of Exposure	Shared endpoints from the same insert		Shared endpoints from the same insert				Terminal Collection PE Time-point	Longitudinal Collection PE Time-point		Terminal Collection PE Time-point	No Replicate	No Donor	No Insert	Run	Day
			1	2	3	4	5	6	CYP(p)	AK	CYP	TEER/AK/MAP/H&E					
BU	Air-for 3R4F	NA	AK	CYP1A1/1B1					24				1	2	2	Run E	Tues
BU	3R4F	10%	AK	CYP1A1/1B1					24				1	2	2	Run E	Tues
BU	3R4F	20%	AK	CYP1A1/1B1					24				1	2	2	Run E	Tues
BU	3R4F	30%	AK	CYP1A1/1B1					24				1	2	2	Run E	Tues
BU	3R4F	40%	AK	CYP1A1/1B1					24				1	2	2	Run E	Tues
BU	3R4F	50%	AK	CYP1A1/1B1					24				1	2	2	Run E	Tues
BU	Air-for 3R4F	NA	AK	CYP3A4					24				1	2	2	Run E	Tues
BU	3R4F	10%	AK	CYP3A4					24				1	2	2	Run E	Tues
BU	3R4F	20%	AK	CYP3A4					24				1	2	2	Run E	Tues
BU	3R4F	30%	AK	CYP3A4					24				1	2	2	Run E	Tues
BU	3R4F	40%	AK	CYP3A4					24				1	2	2	Run E	Tues
BU	3R4F	50%	AK	CYP3A4					24				1	2	2	Run E	Tues
BU	Air-for 3R4F	NA			TEER	H&E	AK	(MAP)				48	1	2	2	Run G	Wed
BU	3R4F	10%			TEER	H&E	AK	(MAP)				48	1	2	2	Run G	Wed
BU	3R4F	20%			TEER	H&E	AK	(MAP)				48	1	2	2	Run G	Wed
BU	3R4F	30%			TEER	H&E	AK	(MAP)				48	1	2	2	Run G	Wed
BU	3R4F	40%			TEER	H&E	AK	(MAP)				48	1	2	2	Run G	Wed
BU	3R4F	50%			TEER	H&E	AK	(MAP)				48	1	2	2	Run G	Wed

(table continues)

Table 6. Endpoints for the DRF

Tissue	Exposure	Dose of Exposure	Shared endpoints from the same insert		Shared endpoints from the same insert				Terminal Collection PE Time-point	Longitudinal Collection PE Time-point		Terminal Collection PE Time-point	No Replicate	No Donor	No Insert	Run	Day
			1	2	3	4	5	6	CYP(p)	AK	CYP	TEER/AK/ MAP/H&E					
BU	Air-for ZRH	NA	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run B/D	Mon
BU	ZRH	15%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run B/D	Mon
BU	ZRH	30%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run B/D	Mon
BU	ZRH	45%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run B/D	Mon
BU	ZRH	60%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run B/D	Mon
BU	ZRH	80%	AK	CYP1A1/1B1					48	24, 48	24, 48		2	2	4	Run B/D	Mon
BU	Air-for ZRH	NA	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run B	Mon
BU	ZRH	15%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run B	Mon
BU	ZRH	30%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run B	Mon
BU	ZRH	45%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run B	Mon
BU	ZRH	60%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run B	Mon
BU	ZRH	80%	AK	CYP3A4					48	24, 48	24, 48		1	2	2	Run B	Mon
BU	Air-for ZRH	NA			TEER	H&E	AK	(MAP)				48	2	2	4	Run B/D	Mon
BU	ZRH	15%			TEER	H&E	AK	(MAP)				48	2	2	4	Run B/D	Mon
BU	ZRH	30%			TEER	H&E	AK	(MAP)				48	2	2	4	Run B/D	Mon
BU	ZRH	45%			TEER	H&E	AK	(MAP)				48	2	2	4	Run B/D	Mon
BU	ZRH	60%			TEER	H&E	AK	(MAP)				48	2	2	4	Run B/D	Mon
BU	ZRH	80%			TEER	H&E	AK	(MAP)				48	2	2	4	Run B/D	Mon

(table continues)

Table 6. Endpoints for the DRF

Tissue	Exposure	Dose of Exposure	Shared endpoints from the same insert		Shared endpoints from the same insert				Terminal Collection PE Time-point	Longitudinal Collection PE Time-point		Terminal Collection PE Time-point	No Replicate	No Donor	No Insert	Run	Day
			1	2	3	4	5	6	CYP(p)	AK	CYP	TEER/AK/ MAP/H&E					
BU	Air-for ZRH	NA	AK	CYP1A1/1B1					24				1	2	2	Run F	Tues
BU	ZRH	15%	AK	CYP1A1/1B1					24				1	2	2	Run F	Tues
BU	ZRH	30%	AK	CYP1A1/1B1					24				1	2	2	Run F	Tues
BU	ZRH	45%	AK	CYP1A1/1B1					24				1	2	2	Run F	Tues
BU	ZRH	60%	AK	CYP1A1/1B1					24				1	2	2	Run F	Tues
BU	ZRH	80%	AK	CYP1A1/1B1					24				1	2	2	Run F	Tues
BU	Air-for ZRH	NA	AK	CYP3A4					24				1	2	2	Run F	Tues
BU	ZRH	15%	AK	CYP3A4					24				1	2	2	Run F	Tues
BU	ZRH	30%	AK	CYP3A4					24				1	2	2	Run F	Tues
BU	ZRH	45%	AK	CYP3A4					24				1	2	2	Run F	Tues
BU	ZRH	60%	AK	CYP3A4					24				1	2	2	Run F	Tues
BU	ZRH	80%	AK	CYP3A4					24				1	2	2	Run F	Tues
BU	Air-for ZRH	NA			TEER	H&E	AK	(MAP)				48	1	2	2	Run H	Wed
BU	ZRH	15%			TEER	H&E	AK	(MAP)				48	1	2	2	Run H	Wed
BU	ZRH	30%			TEER	H&E	AK	(MAP)				48	1	2	2	Run H	Wed
BU	ZRH	45%			TEER	H&E	AK	(MAP)				48	1	2	2	Run H	Wed
BU	ZRH	60%			TEER	H&E	AK	(MAP)				48	1	2	2	Run H	Wed
BU	ZRH	80%			TEER	H&E	AK	(MAP)				48	1	2	2	Run H	Wed

(table continues)

Table 6. Endpoints for the DRF

Tissue	Exposure	Dose of Exposure	Shared endpoints from the same insert		Shared endpoints from the same insert				Terminal Collection PE Time-point	Longitudinal Collection PE Time-point		Terminal Collection PE Time-point	No Replicate	No Donor	No Insert	Run	Day
			1	2	3	4	5	6	CYP(p)	AK	CYP	TEER/AK/ MAP/H&E					
BU	Triton-X	1 or 10% (24 or 2 h)			TEER (+)		AK(+)		freeze for all				3	2	6	NA	NA
BU	TCDD	30 nM (48 h)		CYP1A/B1(+)					freeze for all				3	2	6	NA	NA
BU	Unexposed				TEER(-)	H&E(-) 24h fix			arrival/shipment				3	2	6	NA	NA
BU	Unexposed				TEER(-)	H&E(-) 1h fix			arrival/shipment				3	2	6	NA	NA
BU	Unexposed				TEER(-)	H&E(-)	AK(-)		t0; freeze for all				3	2	6	NA	NA
BU	Unexposed			CYP1A/B1(-)					freeze for all				2	2	4	NA	NA
BU	TNFa/IL1b	10 ng/ml (24 h)				H&E(-)		(MAP(+))	freeze for all				3	2	6	NA	NA
BU	PBS					H&E(-)		(MAP(-))	freeze for all				3	2	6	NA	NA
BU	Rifampicin	25 uM (48 h)		CYP3A4(+)					freeze for all				3	2	6	NA	NA
BU	Unexposed			CYP3A4(-)	TEER(-)		AK(-)		freeze for all				2	2	4	NA	NA
BU	Testing CYP3A4												2	2	4	NA	NA

(end of the table)

t0, t-zero = Monday before exposure; arrival = shipment control; CYP(p) = Measurement of the CYP activity along with its protein levels; (MAP) = sample collection only for backup analyses; MAP savings; ZRH = alternative name for THS2.2; BU = Human BUCCAL w/o fibroblast, other name for EpiOral™ cultures

7.5.2 List of endpoints for the main phase

The Endpoints for the Main Phase experiment will be described in an amendment to this Study Plan.

7.5.3 Negative and positive controls

Table 7. Negative and positive controls use in the study

Endpoints	Negative Controls			Positive Controls		
	Reagent	**Dose**	**Company**	**Reagent**	**Dose**	**Company**
TEER and AK	Untreated	NA	NA	Triton-X100	1% (24 h at the basal medium) or 10% (2 h at the apical side)	Sigma
CYP1A1/1B1 assays	Untreated	NA	NA	TCDD	30 nM	Sigma
CYP3A4	Untreated	NA	NA	Rifampicin	25 µM	Sigma
Multi Analyte Profiling (MAP)	PBS	Equal volume as compared to the TNFα& IL-1 β	MatTek	Mix of human TNFα& human IL-1 β	10 ng/ml (for each)	TNFα, 210-TA-050, R&D systems & human IL-1β, 201-LB-025, R&D systems

NA: not applicable

8 SCHEDULE OF THE EXPERIMENT

8.1 Schedule-DRF phase

The DRF phase will be started on the week of April 28th, 2014.

8.1.1 Exposure plan

The exposure plan can be found at the following link:

PMI Internal Document No.4

and is illustrated in **Table 8**

Table 8. Exposure Plan

Donor 1								
Donor 2								
Run A	3R4F							SHAM
DRF	dose 1	dose 2	dose 3	dose 4	dose 5	dose 6	dose 7	
	50%	40%	30%	20%	10%			Air
A	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)			BU TEER/HE/AK/(MAP) (48h)
B	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)			BU TEER/HE/AK/(MAP) (48h)
C	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)			BU AK/CYP1A-B1 (24 longtd, 48h)
D	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)			BU AK/CYP1A-B1 (24 longtd, 48h)
E	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)			BU AK/CYP3A4 (24 longtd, 48h)
F	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)			BU AK/CYP3A4 (24 longtd, 48h)
Run B	ZRH							SHAM
DRF	dose 1	dose 2	dose 3	dose 4	dose 5	dose 6	dose 7	
	80%	60%	45%	30%	15%			Air
A	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)			BU TEER/HE/AK/(MAP)
B	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)			BU TEER/HE/AK/(MAP)
C	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)			BU AK/CYP1A-B1 (24 longtd, 48h)
D	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)			BU AK/CYP1A-B1 (24 longtd, 48h)
E	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)			BU AK/CYP3A4 (24 longtd, 48h)
F	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)	BU AK/CYP3A4 (24 longtd, 48h)			BU AK/CYP3A4 (24 longtd, 48h)

(table continues)

Table 8. Exposure Plan

Run C	3R4F							SHAM
DRF	dose 1	dose 2	dose 3	dose 4	dose 5	dose 6	dose 7	
	50%	40%	30%	20%	10%			Air
A	BU TEER/AK/MAP/HE (48h)	BU TEER/AK/MAP/HE (48h)	BU TEER/AK/MAP/HE (48h)	BU TEER/AK/MAP/HE (48h)	BU TEER/AK/MAP/HE (48h)			BU TEER/AK/MAP/HE (48h)
B	BU TEER/AK/MAP/HE (48h)	BU TEER/AK/MAP/HE (48h)	BU TEER/AK/MAP/HE (48h)	BU TEER/AK/MAP/HE (48h)	BU TEER/AK/MAP/HE (48h)			BU TEER/AK/MAP/HE (48h)
C	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)			BU AK/CYP1A-B1 (24 longtd, 48h)
D	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)			BU AK/CYP1A-B1 (24 longtd, 48h)
E								
F								

Run D	ZRH							SHAM
DRF	dose 1	dose 2	dose 3	dose 4	dose 5	dose 6	dose 7	
	80%	60%	45%	30%	15%			Air
A	BU TEER/AK/MAP/HE	BU TEER/AK/MAP/HE	BU TEER/AK/MAP/HE	BU TEER/AK/MAP/HE	BU TEER/AK/MAP/HE			BU TEER/AK/MAP/HE
B	BU TEER/AK/MAP/HE	BU TEER/AK/MAP/HE	BU TEER/AK/MAP/HE	BU TEER/AK/MAP/HE	BU TEER/AK/MAP/HE			BU TEER/AK/MAP/HE
C	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)			BU AK/CYP1A-B1 (24 longtd, 48h)
D	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)	BU AK/CYP1A-B1 (24 longtd, 48h)			BU AK/CYP1A-B1 (24 longtd, 48h)
E								
F								

(table continues)

Table 8. Exposure Plan

Run E	3R4F							SHAM
DRF	dose 1	dose 2	dose 3	dose 4	dose 5	dose 6	dose 7	
	50%	40%	30%	20%	10%			Air
A	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)			BU AK/CYP1A-B1 (24h)
B	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)			BU AK/CYP1A-B1 (24h)
C	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)			BU AK/CYP3A4 (24h)
D	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)			BU AK/CYP3A4 (24h)
E								
F								

Run F	ZRH							SHAM
DRF	dose 1	dose 2	dose 3	dose 4	dose 5	dose 6	dose 7	
	80%	60%	45%	30%	15%			Air
A	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)			BU AK/CYP1A-B1 (24h)
B	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)	BU AK/CYP1A-B1 (24h)			BU AK/CYP1A-B1 (24h)
C	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)			BU AK/CYP3A4 (24h)
D	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)	BU AK/CYP3A4 (24h)			BU AK/CYP3A4 (24h)
E								
F								

(table continues)

Table 8. Exposure Plan

Run G	3R4F							SHAM
DRF	dose 1	dose 2	dose 3	dose 4	dose 5	dose 6	dose 7	
	50%	40%	30%	20%	10%			Air
A	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)			BU TEER/HE/AK/(MAP) (48h)
B	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)	BU TEER/HE/AK/(MAP) (48h)			BU TEER/HE/AK/(MAP) (48h)
C								
D								
E								
F								

Run H	ZRH							SHAM
DRF	dose 1	dose 2	dose 3	dose 4	dose 5	dose 6	dose 7	
	80%	60%	45%	30%	15%			Air
A	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)			BU TEER/HE/AK/(MAP)
B	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)	BU TEER/HE/AK/(MAP)			BU TEER/HE/AK/(MAP)
C								
D								
E								
F								

(end of the table)

Longtd = Longitudinal measurement only (without tissue harvesting).

8.1.2 Overview day-to-day schedule

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY/ SUNDAY
21 APRIL 2014	22	23	24	25	26/27
		ACUTE BUCCAL TISSUE DELIVERY DRF **AK/HE on arrival (HE 24h fix and 1h fix, for method establishment)**			
28	29	30	**1 MAY 2014**	2	3/4
ACUTE BUCAL DRF **AK/HE T-zero (HE 1h or 48h fixation)** **Run A, B, C, D**	**24 h PE** **AK/Two CYPs** **Longitudinal collection** **Run E, F**	**48 h PE** **AK/Two CYP(p)s** **TEER/HE/AK/(MAP)** **(HE 1h or 48h fixation)** ***MAP = sample collection only (back up samples)*** **24 h PE** **AK/Two CYP(p)s** **Run G, H**	HOLIDAY	**48 h PE** **TEER/HE/AK/(MAP)** **(HE 1h or 72h fixation)** ***MAP = sample collection only (back up samples)***	
5	6	7	8	9	10/11
			ACUTE BUCAL ORDERING		
12	13	14	15	16	17/18
19	20	21	22	23	24/25
26	27	28	29	30	**31 MAY / 1 JUNE 2014**
		ACUTE BUCCAL TISSUE DELIVERY **HE on arrival**	HOLIDAY		
2	3	4	5	6	7/8
MAIN PHASE ACUTE BUCCAL					
9	10	11	12	13	14/15
HOLIDAY					

Repetition of the Main Phase study is planned for 30th of July, 2014.

8.2 Schedule-Main phase

Will be added as an amendment to this Study Plan.

9 PROCEDURES

9.1.1 Tissues handling upon arrival

Upon arrival, the human organotypic oral tissue will be handled under sterile conditions as follows:

- The oral inserts arrive from MatTek in sealed 24-well plates containing agarose. Put them in the fridge until you begin the culture.
- Pre-warm the ORL-200-MM medium to 37ºC. Using sterile technique pipet 0.5ml media in each well of new sterile 24-well plates. Transfer the inserts in the new plate containing the medium.
- Take out the plates from the fridge, 1 hour before putting them in culture. Under sterile conditions open the package and using sterile forceps remove the inserts from the agarose in the 24-well plates while there are cold.
- Incubate the inserts in the 0.5 ml medium for 1h at 37ºC, 5% CO2, which allows the removal of the agarose from the inserts and the recovery of the tissues from the shipment.
- After 1h equilibration, the cultures can be airlifted (expose the apical part of the tissues to the air) in 12-well plates.
- Under sterile conditions open the lid of 12-well plates and stack 2 modified 12-well tops provided by MatTek (HNG-TOP-2X) on top.
- Add 5.5 ml warm medium in each well of the 12-well plates and using sterile technique remove the inserts from the 24-well plates and hang the inserts atop of the stack of 2 modified 12-well tops. The medium must be in contact with the bottom of the inserts in the 12-well plates.
- Cover the inserts with the original cover and place the plates in the incubator at 37ºC, 5% CO2.

9.1.2 QC of the test system upon arrival

9.1.2.1 Phase 1 QC of test system

- Assessment of tissue (e.g. to identify tissue damage, contamination) will be conducted using a light microscope.
- Scenario of the assessment:

Tissue damage	**Contamination**	**Solution**
Yes	-	Stop*
-	Yes	Stop*
-	-	Stop*
Yes	Yes	Stop*
-	Yes	Stop*
Yes	-	Stop*

* When Phase 1 OQ fails and experiment needs to be terminated, the study will be postponed, and a new tissue batch will be requested from the supplier.

9.1.2.2 Phase 2 QC of the test system

Additional number of insert (arrival time-point) will be subjected to histology assessment for QC, e.g. standard H&E to determine the normal structure of the tissue. Apical washing will not be performed because the oral tissues do not contain mucus secreting cells, thus TEER measurement will not be performed. This is to ensure that all inserts will be treated equally.

9.1.3 Apical side washing before exposure

Apical washing will not be performed because the oral tissues do not contain mucus secreting cells. The apical side washing usually is conducted prior to the exposure day (T-zero), to minimize the risk of interference from the mucous layer at the apical side, which could affect the impact of exposure.

9.1.4 Cell culture maintenance

The organotypic oral tissue will be delivered fully differentiated. They will be cultured in the culture medium (ORAL-300-FT-MM, MatTek) at their basolateral side and maintain in the incubator (at 37 °C). The culture medium will be changed every 3 days. According to the manufacturer's recommendation, the tissues will be kept with 5 mL medium over the weekend(s).

During the medium change, the tissues will further be examined under the light microscope to monitor their growth and to ensure that the tissues are healthy.

9.1.5 Tissue fixation & histology analysis

9.1.5.1 Protocol of tissue fixation and processing

There are two different protocols available for the tissue fixation and processing (see below). During the DRF phase, the most suitable protocol of fixation and processing will be implemented based on the collection time (Monday-Thursday or Friday, for 24 h fixation vs. 1h fixation, respectively). We will also determine whether the shorter fixation and processing could be adapted for this specific type of tissue (i.e. buccal organotypic tissue cultures without fibroblast layer purchased from MatTek). The most effective and efficient protocol will then be implemented in the Main Phase study.

Protocol based on Epithelix's recommendation (total duration: ~ 5 hours)

Fixation protocol

The fixation of human bronchial tissues with and without fibroblast will be performed under the hood as follows:

- Inserts from each tissue will be washed 3 times with 1x PBS at RT in both basal and apical side.
- Aspirate PBS buffer.
- Add 3ml of 4% paraformaldehyde (PFA) (see below the preparation of 4% PAF) diluted in 1x PBS containing calcium/magnesium to cover all the tissue.

Preparation of the 4% PFA:

Preparation **1 L of 4% paraformaldehyde (PFA, P-6148, Sigma-Aldrich)**
PFA fixatives should be prepared on the day of use. PFA is not stable in solution. Composition: 4% PFA in 0.15 M Na-phosphate buffer
Procedure 1. Dissolve 40g PFA in 625ml dH_2O; warm up to max. 60°C while stirring continuously until entirely dissolved (let the solution clear) 2. **If the solution is not clear**, add drop by drop of NaOH (10 M) while stirring continuously until entirely cleared 3. Add 375 ml 0.4 M Na-phosphate buffer 4. Cool the solution to room temperature, adjust the pH to 7.4 5. Filter the solution and label the bottle: solution name, pH, date, and owner 6. Put the solution on ice or at 4°C until use
Buffer Solutions

Na-phosphate buffer stock (0.4M). For 1000 ml:	
Na_2HPO_4	46.0 g
NaH_2PO_4 H_2O	10.5 g
dH_2O	1000 ml
Mix well by stirring and store at 4°C. Before use: warm to room temperature and stir to dissolve crystals.	
Phosphate buffered saline (10 mM). - Ready to use - Fisher Scientific – BP399-4	
Mix well by stirring, adjust the pH to 7.4 and store at 4°C or room temperature	

- Incubate in PFA for 1 hour at room temperature (RT).
- Aspirate PAF under the hood.
- Wash 3 times for 5 min with 1xPBS in both basal and apical side at RT.

Processing

In-house histology quality check will be performed only on incubator control inserts. The inserts will be fixed as above mentioned procedure. After fixation and PBS washing, the 3D cell culture will be separated from the inserts by detachment of the membrane from the plastic with a forceps. The inserts will be processed using Leica ASP300S and embedded into paraffin wax.

Insert processing protocol:

Steps	**Reagent**	**Incubation time**
1	70% Alcohol	30 minutes
2	95% Alcohol	15 minutes
3	95% Alcohol	15 minutes
4	100% Alcohol	15 minutes
5	100% Alcohol	15 minutes
6	100% Alcohol	15 minutes
7	Xylene	15 minutes
8	Xylene	15 minutes
9	Paraffin-wax	20 minutes
10	Paraffin-wax	20 minutes

The paraffin blocks will be sliced at 3-4 µm using Microtome. The produced slide will be processed for Hematoxyline & Eosin (H&E).

Protocol based on MatTek's recommendation (total duration: ~ 24 hours)

Fixation protocol

The fixation of human buccal tissues will be performed under the hood as follows:

- Prior to fixation, culture inserts are washed 3 times with 1x PBS at RT in both basal and apical side to remove cellular debris
- Following the aspiration of the last wash, 1.5mL of ready to use 10% neutral buffered formalin(NBF) (Sigma-Aldrich HT501128-4L) fixative solution are added to each insert and the cultures are fixed at room temperature for 24 hours
- After completion of fixation, the fixative is aspirated and the fixed organotypic cultures are washed at room temperature both apically and basally 3 times with 1 x PBS for 5 minutes each
- Following the wash, the fixed cultures are separated from the inserts by detaching the membrane from the plastic with a forceps and bisected through the middle prior to processing using the Leica ASP300S tissue processor with the following program

Processing (aligned with MatTek's Recommendation)

Steps	Reagent	Incubation time
1	Formalin	60 minutes
2	70% Alcohol	45 minutes
3	90% Alcohol	45 minutes
4	90% Alcohol	45 minutes
5	100% Alcohol	45 minutes
6	100% Alcohol	45 minutes
7	100% Alcohol	45 minutes
8	Xylene	45 minutes
9	Xylene	45 minutes
10	Xylene	45 minutes
11	Paraffin-wax	30 minutes
12	Paraffin-wax	30 minutes

After processing, both part of each insert will be embedded flat side down using paraffin wax. Microscopy sections of 3-4 µm thickness are obtained using a microtome and mounted on glass slides which are then transferred to the automated slide stainer Medite TST44 for staining with hematoxylin and eosin according to the following sequence

9.1.5.2 Hematoxylin and Eosin staining protocol

Procedure	Reagent	Station number	Timing
De-waxing and rehydration	Xylene	16	5min
	Xylene	17	5min
	Xylene	18	5min
	Ethanol Absolut	19	5min
	Ethanol Absolut	20	5min
	Ethanol 90	21	5min
	Ethanol 90	22	5min
	Ethanol 70	23	5min
	Distilled water	24	5min
Staining	Hematoxylin	27	3min 30sec
	Tap water bath	14	3min
	Acid Rinse	29	15sec
	Scott's tap Water	30	1min
	Eosin	28	1min 30sec
	Distilled Water	29	1min
	Ethanol 70	39	3min
	Ethanol 90	40	3min
Dehydration	Ethanol 90	41	3min
	Ethanol absolute	42	3min
	Ethanol Absolute	43	3min
	Xylene	44	3min
	Xylene	45	3min

9.1.5.3 Result output for the histology analysis

Each section is carefully reviewed under the microscope at a minimum of 2 different magnifications (x10 and x40). Digital images of each slide are generated using the Hamamatsu NanoZoomer slide scanner and saved in the internal server PMI Internal Document No.6.

9.1.6 TEER protocol

9.1.6.1 Protocol for TEER

Trans Epithelium Electrical Resistance (TEER) will be measured under the hood in sterile conditions using Chopstick Electrode (STX-2) connected to the EVOMTM Epithelial Voltohmmeter (World Precision Instruments) as follows:

- Add 200 µl of culture media on the apical surface of the human buccal tissues
- Turn on the EVOMX machine
- Wash the electrode with 70 % ethanol, subsequently the screen of EVOMX will indicate a reading of -1
- Wash the electrode with the culture medium, subsequently the screen of EVOMX will indicate a reading of 0.00
- Measure the resistance (Ω) with the EVOMX, the screen will indicate the value of the resistance
- After the measurements, gently remove (by aspirating) the medium from the apical surface of the tissues
- Place the tissues back in the incubator at 37°C
- Repeat step #5 for 5 times

9.1.6.2 Positive/Negative controls for TEER

Positive control will be generated from Triton-X treated tissues:

- For the positive control of the 4 h postexposure samples: tissues will be treated with 10% Trition-X 100 in the basal medium for 2 h.
- For the positive control of the 24 h postexposure samples: tissues will be treated with 1% Triton-X100 in the basolateral medium for 24 h.

Negative control will be generated from PBS treated tissues.
The controls are summarized in Section 7.5.3.

9.1.6.3 Result output for TEER

The result of the TEER assay will be calculated as follows:

$$TEER\ (\Omega.cm^2) = (R_{readout} - R_m) \times A_{epithelium\ surface}$$

Note:

$R_{readout}$ = Resistance value from the readout (Ω)
R_m = Resistance of the membrane (constant value of 100 Ω)
$A_{epithelium\ surface}$ = Area of the epithelium's surface (constant value of 0.33 cm^2)

9.1.7 AK assay

9.1.7.1 Protocol for AK assay

The activity of adenylate kinase (AK) that will be released in the culture medium upon plasma membrane damage will be measured using the kit indicated in PMI Internal Document No.7.

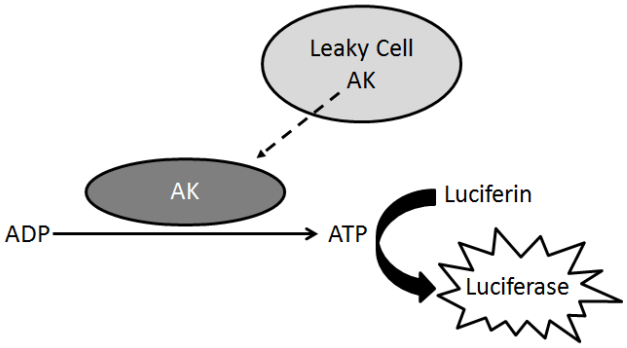


Figure 3. Illustration of the AK assay principal

Briefly, the protocol of the AK assay is the following:

- Collect basal medium from each insert (50 µL)
- Place the medium samples in a 96 well plate for the AK assay
- Add 50 µL AK Reagent Working Solution to each well. Incubate 5 minutes
- Read the microplate Luminometer. Samples should be read within 30 minutes following the addition of the AK Reagent Working Solution. The reaction time should be kept consistent for all samples.

9.1.7.2 Positive/Negative controls for AK

Positive control will be generated from Triton-X treated tissues:

- For the positive control of the 4 h postexposure samples, treatment of 10% Trition-X 100 in the basal medium for 2 h
- For the positive control of the 24 h postexposure samples, treatment of 1% of Triton-X100 in the basolateral medium for 24 h.

Negative control will be generated from PBS treated tissues.
Another blank control will be generated from culture medium only for the calculation of AK result.
The controls are summarized in Section 7.5.3.

9.1.7.3 Result output for AK assay

A blank control (i.e. culture medium only) will be obtained. The result of the AK assay will be reported after subtracting the measured value to the blank control.

9.1.8 CYPs assay

9.1.8.1 Protocol for CYP1A1 and CYP1B1, as well as CYP3A4 activity measurement

The activity of CYP1A1 and CYP1B1, as well as CYP3A will be measured from the medium at the basolateral side of the tissues after the incubation of the medium with the respective enzyme substrate according to the manufacturer's recommendation (PMI Internal Document No.8).

The assay measures the activities of CYP1A1/CYP1B1 enzymes from recombinant and native sources to measure CYP activities. The P450-Glo™ Substrates are CYP enzyme substrates that are derivatives of beetle luciferin [(4S)-4,5-dihydro-2-(6′-hydroxy-2′-benzothiazolyl)-4-thiazolecarboxylic acid]. The derivatives are converted by CYP enzymes to luciferin products. D-luciferin is formed and detected in a second reaction with the Luciferin Detection Reagent. The amount of light produced in the second reaction is proportional to CYP activity.
Specific substrate for CYP1A1/CYP1B1 is: Luciferin-CEE
Specific substrate for CYP3A4 is: Luciferin-IPA

For measurement of the basal CYP activity:

- Incubate tissue inserts overnight with the Luciferin-CEE substrate in the basal culture medium (the Luciferen-CEE substrate is diluted at 1:50 in culture medium; the Luciferin-IPA substrate is diluted at 1:1000 in culture medium).
- Prepare the Luciferin Detection Reagent by transferring the entire contents of the bottle of the appropriate reconstitution buffer to the amber bottle containing the Luciferin Detection Reagent (see manual instructions in PMI Internal Document No.8). Prepare this mixture before the start of the study (usually this will be prepared 1 week before the exposure study will be conducted). Store aliquots of 2 ml at -20°C for maximum 1 month.
- Transfer 50 µl of culture medium from each tissue inserts to a 96-well opaque white luminometer plate at room temperature.
- Add 50 µl of Luciferin Detection Reagent to each well to initiate a luminescent reaction.
- Incubate the plate at room temperature for 20 minutes.
- Read the luminescence using a luminometer.
- NB: Use for the luminometer an integration time of 0.25–1 second per well as a guideline.

9.1.8.2 Positive/Negative controls for CYP activity measurement

Positive control for CYP1A1/1B1: Tissue inserts treated with 30 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) diluted in the basal medium for 72 h at 37°C, 5%CO_2. The treatment should be changed every 24 h within the 24 h period.

Positive control for CYP3A4: Tissue inserts treated with rifampicin diluted in the basal medium for 48 h at 37°C, 5%CO_2. The treatment should be changed every 24 h within the 48 h period.

Negative control: Tissue inserts treated with PBS (basal side) for 48 h.

The controls are summarized in Section (missing).

9.1.8.3 Protocol for CYP protein levels

Normalization of the CYP activity to their protein levels will be conducted for some of the samples, only when tissues will be harvested (thus, longitudinal medium sample collection would not be normalized to the CYP protein levels). This will allow us to determine whether normalization to the protein levels could be implemented for future studies. The protein levels of CYP will be measured using the ELISA kits using 50 µL of samples reported in:

- (CYP1A1 ELISAkit)
- (CYP1B1 ELISA kit)
- (CYP3A4 ELISA kit)

Protocol for total protein amount determination per tissue insert using a BCA assay (BCA Protein Assay Kit)

9.1.8.4 Result output for CYP assay

The calculation of the net signals will be performed by subtracting background luminescence values (no-cell control) from the signal obtained from the cell incubated with the substrate. Absolute value (relative light unit, RLU) of the luminescence indicating the CYP activity will be reported.

9.1.9 Multi analyte profiling (MAP) analysis

9.1.9.1 Protocol for MAP analysis

The following cytokines/chemokines/MMPs (secretion of pro-inflammatory markers) in culture basolateral medium samples would be measured using Luminex-based technology. The final list of the analytes will be further clarified in the amendment of the Study Plan (that is specific to the Main Phase study, where MAP will be analyzed).

The media will be collected during the study according to the study plan (see section 7.5) and stored at -80°C until the measurement will be conducted.

Randomization of the samples (for the plate set-up for MAP analysis) will be generated by the Statistician to avoid any biases in the analysis.

Table 9. List of the proposed inflammatory markers analyzed using Luminex

Marker	Miliplex panel
G-CSF **GM-CSF** **IP-10** **IL-1A** **IL-1B** **IL-6** **IL-8** **VEGF**	Miliplex MAP Human cytokine/chemokine magnetic bead panel; **Millipore** (PMI Internal Document No.9)
MMP-1	Miliplex MAP Human MMP magnetic bead panel; **Millipore** (PMI Internal Document No.10)

Luminex instructions:

Prepare all reagents, standards, and samples as described in the manufactures instructions ((Miliplex MAP Human cytokine/chemokine magnetic bead panel (PMI Internal Document No.9, PMI Internal Document No.10)).

1. Add 200 µL of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25ºC).
2. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells (The Assay Buffer should be used to establish the Background reading (0 pg/mL).
4. Add 25 µL of Assay Buffer to the background and sample wells.
5. Add 25 µL of appropriate matrix to the background, standards, and control wells.
6. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution (this can be done only if the medium was tested before negative for the analytes)
7. Add 25 µL of sample into the appropriate wells.
8. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
9. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker 2 h at room temperature (20-25ºC).
10. Gently remove well contents and wash plate 2 times as following: For hand-held magnet, rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 uL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash step.
11. Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
12. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). DO NOT ASPIRATE AFTER INCUBATION.
13. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
14. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
15. Gently remove well contents and wash plate 2 times as outlined above
16. Add 100 µL or 150 µL of Sheath Fluid to all wells for MMP or cytokine measurement respectively. Resuspend the beads on a plate shaker for 5 minutes.
17. Run plate on Luminex, 200™, HTS with xPONENT software.
18. Save and analyze the Median Fluorescent Intensity (MFI) data.

9.1.9.2 Positive/Negative controls for MAP analysis

Positive control will be obtained from tissues treated with a mixture of human TNF-α/IL-1β (10 ng/ml each) diluted in the basolateral medium for a 24 h incubation.

Negative controls would be PBS-treated tissues.

The controls are summarized in Section 7.5.3.

9.1.9.3 Result output for MAP

The Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating the Cytokine or MMP concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor).

9.1.10 RNA analysis

9.1.10.1 Tissues harvesting and storage prior to RNA/miRNA extraction

For collection of the tissues (buccal without fibroblast):

1. Washed the tissues with 1x PBS (pre cooled at 4°C) in both basal and apical side under the hood.
2. Aspirate the PBS buffer carefully.
3. Repeat steps 1 and 2 two times.
4. Place the tissues on the membranes in Magna Lyser tubes (Roche, Cat. 03358941001) containing 700 uL QIAzol lysis buffer (miRNeasy Mini Kit Qiagen kit (cat # 217004)) and store the tissues on dry ice and for a long term storage in -80°C until the extraction of RNA/microRNA.

9.1.10.2 RNA extraction

RNA/microRNA extraction in Qiazol

1. Extract the RNA/microRNA extraction protocol according to the recommendations from the manual provided along the miRNeasy Mini Kit Qiagen (cat # 217004) (PMI Internal Document No.11)
2. The Macherey Nagel Nucleospin miRNA (Cat.740971.50) may be also used at this step.
3. Elute the RNA in 30 µl RNAse-free water.
4. The miRNeasy and Nucleospin kits are designed for purification of total RNA, including miRNA and other small RNA molecules, from small amounts of cultured cells and various animal and human tissues. The QIAcube robot can process 12 samples simultaneously and the procedure will be adjusted for isolation of mRNA as well as miRNA for possible future analyses.

9.1.10.3 Quality test and RNA yield determination

1. Determine the quantity of the isolated RNA with NanoDrop 1000 or 8000 spectrophotometer (Thermo Scientific) or Qubit (Thermo Scientific).
2. For the subsequent RNA amplification protocol HT 3'IVT an amount of 100 ng RNA is required. The concentration of RNA should be at least 33.3 ng/µl. The RNA will be dissolved in RNAse free water in an appropriate volume.
3. RNA quality will be measured using Agilent 2100 Bioanalyzer.
4. NB: The minimum RNA concentration required for Human DNA Affymetrix array (Human genome U133 plus 2 array) is around 34 ng/µl of RNA with a minimum RIN number of 6.0.
5. The minimal amount of miRNA necessary for GeneChip miRNA 3.0 array is 130 ng.

9.1.10.4 Affymetrix gene chips

Depending on the extraction protocol, the yields and the quality of RNA, 2 main protocols may be used to perform the Gene Expression Profiling of these samples:

Case 1:

If concentrations are below 33.3 ng/uL, the Ovation RNA Amplification System V2 may be used (Nugen, The Netherland). The Ovation® RNA Amplification System V2 provides a sensitive method for preparing microgram quantities of amplified cDNA from Total RNA for gene expression analysis and it is powered by Ribo-SPIA® technology. The Encore® Biotin Module allows doing the fragmentation and the labeling process by combining enzymatic and chemical processes for the preparation of labeled cDNA to generate labeled targets suitable for hybridization to Affymetrix GeneChip® arrays.

All RNA samples will be analyzed with GeneChip Human Genome U133 Plus 2.0 Array (Ref. number 900467, Affymetrix). A randomization of the samples will be determined for Affymetrix hybridization. Also only one chip lot will be used (lot number will be provided in the final report). Afterwards, the analysis of this raw data will be performed by the Computational Sciences and Bioinformatics team within the Systems Biology Development, Application & Verification program.

Ovation® RNA Amplification System V2 and Encore® Biotin Module

Affymetrix 3' expression arrays have probe sets targeting the 3' end of the transcript. Targets for these arrays are prepared by using amplification and labeling methodologies that generated labeled targets initially primed from the Poly-A tail of the transcript. The procedure will be carried out as described (Encore® Biotin Module User Guide NuGen® , Ovation® RNA Amplification System V2 User Guide.) (NuGen®). The Ovation® RNA Amplification System V2 provides a fast, simple and sensitive method for preparing microgram quantities of amplified cDNA from Total RNA for gene expression analysis and it is powered by Ribo-SPIA® technology. The Encore® Biotin Module allows doing the fragmentation and the labeling process by combining enzymatic and chemical processes for the preparation of labeled cDNA to generate labeled targets suitable for hybridization to Affymetrix GeneChip® arrays. As starting material 50 ng total RNA gained from the different samples will be used. All samples will be processed in one series after all RNA preparations have been completed **(Figure 4)**.

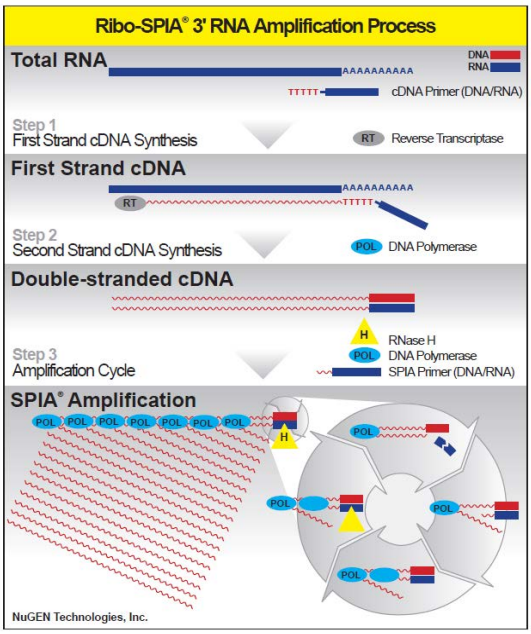


Figure 4. The Ribo-SPIA Amplification Process used in the Ovation RNA Amplification System V2.

First strand cDNA is prepared from total RNA using a unique first strand DNA/RNA chimeric primer and a reverse transcriptase. The primer has a DNA portion that hybridizes to the 5' portion of the poly-A sequence. The resulting cDNA/mRNA hybrid molecule contains a unique RNA sequence at the 5' end of the cDNA strand.

Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthetize a second strand. The result is a doubled stranded cDNA with a unique DNA/RNA heteroduplex at one end.

SPIA amplification is a linear isothermal DNA Amplification using a SPIA DNA/RNA chimeric primer, DNA polymerase and RNAse H.

The cDNA is then purified to remove unincorporated dNTPs, salts, enzyme and inorganic phosphate. Then the cDNA is fragmented with a combined chemical and enzymatic reaction. The fragmented product is labeled via enzymatic attachment of a biotin-labeled nucleotide to the 3-hydroxyl end of the fragmented cDNA. The Fragmentation and labeling process prepare the samples for the hybridization onto GeneChip® arrays.

After the purification step, the quantity of the cDNA will be measured with the Nanodrop 1000 or 8000 spectrophotometer (Thermo Scientific). The accurate calculation of the yield is necessary so that the correct amount of cDNA will be added to the fragmentation reaction. The quality of the cDNA will be determined by assessing the size of the un-fragmented cDNA by using the Agilent 2100 Bioanalyzer. The expected SPIA cDNA profile is a distribution between 200 bases and 2 Kb in length (see **Figure 5**). A shift in size to a much smaller size may be indicative of significantly

degraded input RNA or failure of amplification. The shape of the Bioanalyzer curve will vary depending on the tissue origin of the RNA sample. The size distribution of the final fragmented and biotinylated product will also be monitored using electropherograms. For good results on the GeneChip® arrays, the fragmented cDNA product should be smaller than 200 bases in length.

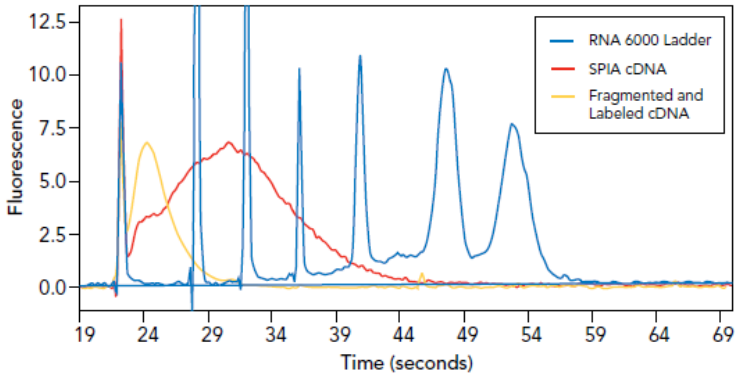


Figure 5. Bionalyzer trace of Amplified, Un-fragmented and Fragmented cDNA.

The un-fragmented and fragmented cDNA can also be monitored by electrophoresis in agarose gel with a ladder. Then use a flatbed scanner or an UV transilluminator equipped with a digital camera to capture an image.

Case 2:

If concentrations are greater than 33.3 ng/uL: High Throughput 3' In Vitro Transcription (HT 3' IVT)
Affymetrix 3’ expression arrays have probe sets targeting the 3’ end of the transcript. Targets for these arrays are prepared by using amplification and labeling methodologies that generate labeled targets initially primed from the poly-A tail of the transcript. The procedure will be carried out as described in the GeneChip® HT 3' IVT Express Kit User Manual (Affymetrix). The GeneChip® HT 3’ IVT Express Kit represents the newest high-throughput target generation assay for 3’-based arrays. The configuration of the HT 3’ IVT Express Kit is optimized for use with liquid-handling robotics and generates a high-quality, array-ready target and requires minimal manual intervention. As starting material 100ng total RNA gained from the different samples will be used. All samples will be processed in one series after all RNA preparations have been completed. A positive and negative control will be added to the sample preparation in order to have a positive control and check potential cross contamination issues. This whole reaction will be pipetted automatically by using the robotic technology of the Beckman Biomek® FXp Target Prep Express (TPE) System. After the amplification step, the quantity of the aRNA will be measured as A260 determined with the Spectramax. The accurate calculation of the yield is necessary so that the correct amount of aRNA will be added to the fragmentation reaction. If necessary, aRNA will be concentrated by vacuum centrifugation (Eppendorf speedvac). The quality of the aRNA will be determined by assessing the size of the unfragmentated aRNA by using the Agilent Bioanalyzer electropherogram. The expected aRNA profile is a distribution of sizes from 250–5500 nt with most of the aRNA

between 500–1500 nt. Average aRNA size may vary slightly depending on RNA quality and total RNA input amount. Average sizes below 500 nt could hint for partially degraded input RNA.

After the aRNA yield calculation, the Biomek FXp TPE System will automatically proceed through Fragmentation setup. The instrument will place the plate in the on-deck thermal cycler. Fragmentation of aRNA target before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay sensitivity. The fragmentation process will also be monitored using electropherograms. The reaction should produce a distribution of 35–200 nt aRNA fragments with a peak.

The unfragmented and fragmented aRNA can also be monitored by electrophoresis in agarose gel with a ladder. Then use a flatbed scanner or an UV transilluminator equipped with a digital camera to capture an image.

9.1.10.5 Hybridization, Wash, Stain, and Scan using the Fluidic Station FS450

The Biomek FXp TPE System will proceed to make the hybridization ready sample by mixing appropriate amounts of fragmented aRNA and hybridization cocktail master mix according to the manufacturer's manual. The final array format will be selected and this option will produce approximately 230µl of hybridization cocktail per well including the fragmented aRNA and the controls (processed poly-A RNA controls, hybridization controls, control oligonucleotide B2). Each sample is hybridized to an Affymetrix Human genome U133 plus 2 array Uni Gene Database. All the arrays are moved to the hybridization oven for 16 hours at 45°C with 60 rpm. After hybridization arrays will be washed several times and then stained with Fluidic Stations FS450 using AGCC Software with the protocol FS450_0001 for the Affymetrix IVT protocol and FS450_0004 for the Nugen Protocol. In the last step, the arrays will be moved to GeneChip Scanner 3000 7G and each array will be scanned.

9.1.10.6 Monitoring the Quality Check of 3' IVT Expression Arrays

By default, the AGCC Software automatically grids the DAT file and create the CEL data file (probe cell intensity data). Thereby the information from each probe on the chip is extracted from the image data and the information is stored in the CEL file. CEL files are then further processed in the AGCC Software using robust multi-array analysis (RMA) for probe set summarization. The array is further analyzed using the AGCC software application. Quantile normalization and probe set signal summarization will be computed according to the RMA procedure.

The results are reported in tabular and graphical formats (Expression Report (.rpt) file).
In addition, pseudo images will be calculated using R packages. These pseudo images provide a better means to monitor artifacts that are not visible on the original image like non-uniform distribution of signals.

For each transcriptomics experiment the RIN, the electrospherograms of the Bioanalyzer, the amount of amplified RNA and the Affymetrix report will be delivered. A metadata sheet describing the sample name, exposure conditions, CEL file name will be provide along with the CEL files.

9.1.11 Procedure on the smoke characterization

9.1.11.1 TPM deposition measurement

During a whole-smoke exposure run, a microbalance is used to determine the real-time deposition of particles on a quartz crystal. The microbalance will be placed during each smoke run and it will measure the amount of particles deposited during the smoke exposure (generated from the 3R4F) to organotypic tissues. The method allows a measurement of the deposition with a resolution of 10 $ng.cm^{-2}$ s^{-1}. The lowest detection limit is approximately 20 $ng.cm^{-2}$ h^{-1}. The measurement will indicate the amount of delivered particles for a given dose. Because the measurement on the microbalance indicate the deposition at cm^{-2} and the culture area of each tissue culture insert is $0.33cm^2$; therefore, this discrepancy will be considered when calculating the deposited amount on each tissue culture insert (i.e. only 33% will deposited on the insert).

Protocol for the measurement

1. Connect quartz crystal with the microbalance.
2. Start the computer; open Microbalance Software.
3. Microbalance will be connected with all relevant rows of smoking chamber where the tissues were exposed.
4. Set the scale (of the output displayed on the computer) to zero and wait a couple of minutes until baseline is set.
5. Start whole smoke exposure according to this study plan (see Section 8.1.1).
6. After the end of each smoke run, set the scale again back to zero for the next measurement.
7. After completing all smoke runs save the files. Microbalance software will generate the raw data file including a graphical display of particle deposition.

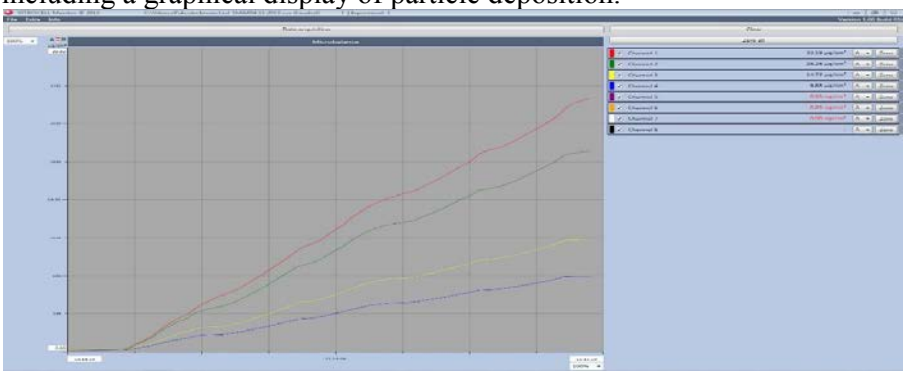


Figure 6. Example Screenshot of the Software representing tissue exposed with different smoke concentration during each smoke run.

9.1.11.2 Nicotine determination

The amount of nicotine in the eluate will be determined by gas chromatography-flame ionization detection (GC-FID) after trapping the aerosol by the Extrelut 3NT column (Merck, cat # 15095) to the individual exhaust of the Dilution/Distribution system of the Vitrocell®. Diluted aerosol is trapped on an Extrelut 3NT column that will be infused with sulfuric acid. After the collection of the aerosol (for each of the target dilution of the smoke concentration), an internal standard (i.e. isoquinoline) will be added to the column. The trapped components on the column are subsequently eluted with a 5% v/v of Triethylamine in N-butylacetate.

Protocol of the measurement:

1. Extrelut 3NT columns will be prepared by adding 2 mL of a 0.5M H_2SO_4 solution and stored at 2-8°C (measurements will be conducted at room temperature).
2. After sample collection, the Extrelut 3NT columns will be labeled accordingly and sealed at both ends with caps or parafilm. Note: the columns can be stored at 2-8°C for a maximum of 4 weeks.
3. An internal standard (250 µL of isoquinoline) will be added prior to the elution.
4. 5 mL of 5% v/v Triethylamine in N-butylacetate will be added in a total of three cycles (total volume = 15 mL) to elute the constituents; the eluate will be collected in 20 mL pyrex glass tubes.
5. 1 mL of the sample is transferred to an amber glass vial for GC-FID analysis
6. For each GC-FID analysis, a series of control samples will be run: i) A blank sample, ii) an internal standard sample (i.e. isoquinoline), iii) a low concentration (of the working range) of the quality control (QC) sample, and iv) a high concentration of the working range of the QC sample will be included at the start and end of each sequence and after every 10 runs to ensure the quality of the results.
7. Peak detection, integration, and quantification (according to internal standard calibration curve) will be automatically performed using the Agilent ChemStation software.

9.1.11.3 Carbonyls determination

Carbonyls determination will be performed as follows:

1) Sample fraction generation:
a. Before starting the exposure, 18.5 mL of PBS will be loaded in each row of the exposure plate
b. Following the completion of the smoke run, an aliquot of 1.2 mL from each row will be taken
c. Add 1.8 mL of DNPH solution (15 mM) to each aliquot.
d. After 30 minutes, 150 µL pyridine solution will subsequently be added to the aliquot to stabilize the carbonyls derivatives.

2) Quantification of acetaldehyde, acetone, acrolein, methyl ethyl ketone, formaldehyde, crotonaldehyde, butyraldehyde, and propionaldehyde is determined by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) using isotope dilution technique :

   a. An aliquot of 500 µL of the above solutions (stabilized smoke bubbled PBS (sbPBS) will be placed into a LC-MS amber glass vial.
   b. Add 485 µL acetonitrile solution.
   c. Add 15 µL of the internal standard mixture (24 µg/mL d6-acetone and 21 µg/mL d5-methyl ethyl ketone).
   d. In parallel, prepare and test the 4 QC samples (i.e. i) A blank sample, ii) an internal standard sample (i.e. isoquinoline), iii) a low concentration (of the working range) of the quality control (QC) sample, and iv) a high concentration of the working range of the QC sample) to confirm the expected concentration (with an accuracy between 85-115%) before acceptance of the results.

e. A 5 µL injection volume will be analyzed using LC-MS/MS.

10 STATISTICAL METHODS

10.1 General Considerations for Data Analysis

Analysis will be performed using SAS Enterprise Guide 4.3.

10.1.1 Missing values

No imputation for missing values.

10.1.2 Values below limit of quantification/detection

If the value is available, it will be used as it is, if not the value will be imputed to either LOQ/2 or LOD/2.

10.1.3 Outliers

If an extreme value is observed, it will be checked for mis-typing/mis-recording. Subsequently, the checked value will be used in the analysis.

10.1.4 Significance Level

Since the study is exploratory, the notion of significance level and alpha level do not apply.

P-value will not be adjusted for multiplicity, and raw p-values be reported. P-values could be descriptively reported using for instance:

- a lower case letter* when p-value is less or equal to 0.05
- an upper case letter* when p-value is less or equal to 0.01

*The letter indicating the comparison tested.

10.1.5 Randomization

To generate MAP data, a randomization plan will be provided to the lab by the Statistician to avoid any biases in the data.

To generate transcriptomics data, a randomization plan will be provided to the lab for the processing and hybridization of mRNA samples. This step is **mandatory** to avoid the introduction of biases and confounding effects in the data.

10.2 Exploratory Objectives

Given the different endpoints, the exploratory objectives of this study are to show differences between:

- Exposures (sham, doses of 3R4F, doses of THS2.2)
- Positive controls and negative controls

In addition, same comparisons could be performed using normalized data. It is foreseen that data will be normalized to the level of positive controls or total protein levels (not exhaustive).

10.3 Study design

10.3.1 Sample Size

No sample size calculation can be done, given the fact that very few knowledge is available.

10.3.2 Source of variation

The design takes into account the variability between tissue inserts and smoking runs.

10.4 Descriptive and Inferential Statistics

10.4.1 Descriptive statistics

Descriptive statistics will be provided for each measurement, substance, exposure time, post-exposure time, dose: the mean, the standard deviation, the sample size and the number of value under limit of quantification.

10.4.2 Inferential statistics

Because very little knowledge is available from this type of experiment, few precision can be given on the concrete way the data will be analyzed, however, it is foreseen that:

- Linear Models will be used to compare substance,
- Dose will be tested and included in models if statistically relevant.
- A correction for non-equal variance, and a heterogenous variance structure will be used when applicable.

11 SUMMARY AND SPECIFIC CONSIDERATION FOR EACH ENDPOINTS

11.1 DRF Phase

11.1.1 H&E analysis

H&E analysis will be conducted to assess the morphological changes upon the various doses of 3R4F or THS2.2.
Note: Thursday will be May 1, 2014 (holiday)

Tissue	Item	Conc	Endpoint	PE	Replicat	Dor	Total Inse	Run	Day Run	Date Ru
BU	Air-for 3R4F	n/a	H&E	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	10%	H&E	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	20%	H&E	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	30%	H&E	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	40%	H&E	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	50%	H&E	48	2	2	4	Run A/C	Mon	28.04.2014
BU	Air-for 3R4F	n/a	H&E	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	10%	H&E	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	20%	H&E	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	30%	H&E	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	40%	H&E	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	50%	H&E	48	1	2	2	Run G	Wed	30.04.2014
BU	Air-for ZRH	n/a	H&E	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	15%	H&E	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	30%	H&E	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	45%	H&E	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	60%	H&E	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	80%	H&E	48	2	2	4	Run B/D	Mon	28.04.2014
BU	Air-for ZRH	n/a	H&E	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	15%	H&E	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	30%	H&E	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	45%	H&E	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	60%	H&E	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	80%	H&E	48	1	2	2	Run H	Wed	30.04.2014
BU	Unexposed		H&E(-) 24h fix	arrival/shipment	3	2	6	NA	NA	
BU	Unexposed		H&E(-) 1h fix	arrival/shipment	3	2	6	NA	NA	
BU	Unexposed		H&E(-)	t0; freeze for all	3	2	6	NA	NA	
BU	TNFa/IL1b	10 ng/ml (24 h)	H&E(-)	freeze for all	3	2	6	NA	NA	
BU	PBS		H&E(-)	freeze for all	3	2	6	NA	NA	
						Total	102			

11.1.2 TEER measurement

Tissue	Item	Conc	Endpoin	PE	Replicat	Dor	Total Inse	Run	Day Run	Date Ru
BU	Air-for 3R4F	n/a	TEER	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	10%	TEER	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	20%	TEER	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	30%	TEER	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	40%	TEER	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	50%	TEER	48	2	2	4	Run A/C	Mon	28.04.2014
BU	Air-for 3R4F	n/a	TEER	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	10%	TEER	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	20%	TEER	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	30%	TEER	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	40%	TEER	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	50%	TEER	48	1	2	2	Run G	Wed	30.04.2014
BU	Air-for ZRH	n/a	TEER	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	15%	TEER	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	30%	TEER	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	45%	TEER	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	60%	TEER	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	80%	TEER	48	2	2	4	Run B/D	Mon	28.04.2014
BU	Air-for ZRH	n/a	TEER	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	15%	TEER	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	30%	TEER	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	45%	TEER	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	60%	TEER	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	80%	TEER	48	1	2	2	Run H	Wed	30.04.2014
BU	Triton-X	1 or 10% (24 or 2 h)	TEER (+)	freeze for all	3	2	6	NA	NA	
BU	Unexposed		TEER(-)	arrival/shipment	3	2	6	NA	NA	
BU	Unexposed		TEER(-)	arrival/shipment	3	2	6	NA	NA	
BU	Unexposed		TEER(-)	t0; freeze for all	3	2	6	NA	NA	
BU	Unexposed		TEER(-)	freeze for all	2	2	4	NA	NA	
						Total	100			

11.1.3 AK assay

Tissue	Item	Conc	Endpoin	PE	Replicat	Dor	Total Inse	Run	Day Run	Date Ru
BU	Air-for 3R4F	n/a	AK	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	10%	AK	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	20%	AK	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	30%	AK	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	40%	AK	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	50%	AK	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	Air-for 3R4F	n/a	AK	24, 48	1	2	2	Run A/C	Mon	28.04.2014
BU	3R4F	10%	AK	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	3R4F	20%	AK	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	3R4F	30%	AK	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	3R4F	40%	AK	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	3R4F	50%	AK	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	Air-for 3R4F	n/a	AK	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	10%	AK	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	20%	AK	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	30%	AK	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	40%	AK	48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	50%	AK	48	2	2	4	Run A/C	Mon	28.04.2014
BU	Air-for 3R4F	n/a	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	10%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	20%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	30%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	40%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	50%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	Air-for 3R4F	n/a	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	10%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	20%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	30%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	40%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	50%	AK	24	1	2	2	Run E	Tues	29.04.2014
BU	Air-for 3R4F	n/a	AK	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	10%	AK	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	20%	AK	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	30%	AK	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	40%	AK	48	1	2	2	Run G	Wed	30.04.2014
BU	3R4F	50%	AK	48	1	2	2	Run G	Wed	30.04.2014

(table continues)

11.1.3 AK assay

Tissue	Item	Conc	Endpoin	PE	Replicat	Dor	Total Inse	Run	Day Run	Date Ru
BU	Air-for ZRH	n/a	AK	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	15%	AK	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	30%	AK	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	45%	AK	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	60%	AK	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	80%	AK	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	Air-for ZRH	n/a	AK	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	15%	AK	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	30%	AK	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	45%	AK	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	60%	AK	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	80%	AK	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	Air-for ZRH	n/a	AK	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	15%	AK	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	30%	AK	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	45%	AK	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	60%	AK	48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	80%	AK	48	2	2	4	Run B/D	Mon	28.04.2014
BU	Air-for ZRH	n/a	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	15%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	30%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	45%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	60%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	80%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	Air-for ZRH	n/a	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	15%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	30%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	45%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	60%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	80%	AK	24	1	2	2	Run F	Tues	29.04.2014
BU	Air-for ZRH	n/a	AK	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	15%	AK	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	30%	AK	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	45%	AK	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	60%	AK	48	1	2	2	Run H	Wed	30.04.2014
BU	ZRH	80%	AK	48	1	2	2	Run H	Wed	30.04.2014
BU	Triton-X	1 or 10% (24 or 2 h)	AK(+)	freeze for all	3	2	6	NA	NA	
BU	Unexposed		AK(-)	t0; freeze for all	3	2	6	NA	NA	
BU	Unexposed		AK(-)	freeze for all	2	2	4	NA	NA	
						Total	208			

(end of the table)

11.1.4 CYPs activity

11.1.4.1 CYP1A1/CYP1B1

Tissue	Item	Conc	Endpoint	PE	Replicat	Dor	Total Inse	Run	Day Run	Date Ru
BU	Air-for 3R4F	n/a	CYP1A1/1B1	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	10%	CYP1A1/1B1	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	20%	CYP1A1/1B1	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	30%	CYP1A1/1B1	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	40%	CYP1A1/1B1	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	3R4F	50%	CYP1A1/1B1	24, 48	2	2	4	Run A/C	Mon	28.04.2014
BU	Air-for 3R4F	n/a	CYP1A1/1B1	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	10%	CYP1A1/1B1	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	20%	CYP1A1/1B1	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	30%	CYP1A1/1B1	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	40%	CYP1A1/1B1	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	50%	CYP1A1/1B1	24	1	2	2	Run E	Tues	29.04.2014
BU	Air-for ZRH	n/a	CYP1A1/1B1	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	15%	CYP1A1/1B1	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	30%	CYP1A1/1B1	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	45%	CYP1A1/1B1	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	60%	CYP1A1/1B1	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	ZRH	80%	CYP1A1/1B1	24, 48	2	2	4	Run B/D	Mon	28.04.2014
BU	Air-for ZRH	n/a	CYP1A1/1B1	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	15%	CYP1A1/1B1	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	30%	CYP1A1/1B1	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	45%	CYP1A1/1B1	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	60%	CYP1A1/1B1	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	80%	CYP1A1/1B1	24	1	2	2	Run F	Tues	29.04.2014
BU	TCDD	30 nM (48 h)	CYP1A/B1(+)	freeze for all	3	2	6	NA	NA	
BU	Unexposed		CYP1A/B1(-)	freeze for all	2	2	4	NA	NA	
						Total	82			

11.1.4.2 CYP3A4

Tissue	Item	Conc	Endpoint	PE	Replicat	Dor	Total Inse	Run	Day Run	Date Ru
BU	Air-for 3R4F	n/a	CYP3A4	24, 48	1	2	2	Run A/C	Mon	28.04.2014
BU	3R4F	10%	CYP3A4	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	3R4F	20%	CYP3A4	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	3R4F	30%	CYP3A4	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	3R4F	40%	CYP3A4	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	3R4F	50%	CYP3A4	24, 48	1	2	2	Run A	Mon	28.04.2014
BU	Air-for 3R4F	n/a	CYP3A4	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	10%	CYP3A4	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	20%	CYP3A4	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	30%	CYP3A4	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	40%	CYP3A4	24	1	2	2	Run E	Tues	29.04.2014
BU	3R4F	50%	CYP3A4	24	1	2	2	Run E	Tues	29.04.2014
BU	Air-for ZRH	n/a	CYP3A4	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	15%	CYP3A4	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	30%	CYP3A4	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	45%	CYP3A4	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	60%	CYP3A4	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	ZRH	80%	CYP3A4	24, 48	1	2	2	Run B	Mon	28.04.2014
BU	Air-for ZRH	n/a	CYP3A4	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	15%	CYP3A4	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	30%	CYP3A4	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	45%	CYP3A4	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	60%	CYP3A4	24	1	2	2	Run F	Tues	29.04.2014
BU	ZRH	80%	CYP3A4	24	1	2	2	Run F	Tues	29.04.2014
BU	Rifampicin	25 uM (48 h)	CYP3A4(+)	freeze for all	3	2	6	NA	NA	
BU	Unexposed		CYP3A4(-)	freeze for all	2	2	4	NA	NA	
						Total	58			

Two additional inserts per donor will be used for method development for testing CYP3A4 activity and measurement of protein levels. This step is taken because this is a new implemented test.

11.1.5 MAP for inflammatory markers

Collection of samples for Map during the DRF phase would only be used for back-up samples. During DRF phase study, the samples for MAP will be collected for backup. Moreover, the doses selected for the Main Phase could be analyzed later to add additional statistical power for the analysis and/or to investigate the batch to batch variability of the signals.

11.1.6 Gene expression (GEX) analysis

GEX will only be performed for the Main Phase. Detailed consideration of the analysis will be added as an amendment to this Study Plan.

12 ADMINISTRATIVE ASPECTS

12.1 Study Timelines

Proposed experimental starting date:	DRF— Main Phase— Repetition of Main Phase—	28 April, 2014 16 June, 2014 30 July, 2014
Proposed experimental completion date:	DRF— Main Phase— Repetition of Main Phase—	2 May, 2014 20 June, 2014 8 August, 2014
Delivery of the raw data		
DRF phase		
CYP1A/B1 activity	7 May 2014	
CYP3A4 activity	7 May 2014	
AK assay	7 May 2014	
TEER assay	7 May 2014	
HE	31 May 2014	
Main phase	TBD	
Proposed date for draft study report:	30 September, 2014	

12.2 Names and Addresses

Study Director	Anita Iskandar Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Deputy Study Director	Radina Kostadinova Quai Jeanrenaud 5 2000 Neuchâtel Switzerland

12.3 Quality Assurance

This study will not be conducted in accordance with Good Laboratory Practice (GLP).

13 ARCHIVING

After finalization of the study the study plan, amendments, raw data, test and reference items, retention samples, specimens collected, the report, and all study-related records will be stored for a maximal period of one year at the archive at Philip Morris Products S.A, Research & Development. Thereafter, all records and materials will be transferred to the external GLP-certified service archive Iron Mountain Deutschland GmbH, Harpener Hellweg, 44805 Bochum, Germany.

Electronic records will be archived on the central archiving server at Philip Morris Products S.A, Research & Development, which is managed by the local IT specialists.

Study folder reported in: PMI Internal Document No.12

Electronic and paper records will be archived for at least 10 years after finalization of the final report according to the specific archiving SOPs.

14 ABBREVIATIONS

Abbreviation	
AK	Adenylate Kinase
ALI	Air Liquid Interface
CC	Conventional Cigarettes
CS	Cigarette Smoke
CW	Calendar Week
EDMS	Electronic Document Management System
GC-FID	Gas Chromatography-Flame Ionization Detection
IHC	Immunohistochemistry
IL	Interleukin
LDH	Lactate Dehydrogenase
MAP	Multi Analyte Profile
MMP	Matrix Metalloproteinase
RIN	RNA Integrity Number
RNA	RiboNucleic Acid
SDMS	Scientific Data Management System
SP	Study Plan
TEER	Trans Epithelial Electrical Resistance
TGF	Transforming Growth Factor
THS	Tobacco Heating System

For complete definition, refer to PMI Internal Document No.13 and PMI Internal Document No.14.

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16 APPENDIX

Study Plan Amendment 1

Study N°.	S174300	Study Plan Amendment N°1	

Short Title	**Main Phase Part 1 and Part 2-Acute Human Buccal Tissues Study (THS2.2)**

Study Director	Anita Iskandar	Date issued	11 June 2014

PREVIOUS TEXT VERSION

No previous version of the amendment exists.
We are adding additional information to the original study plan:
1743_Organotypic_Buccal_SP
This amendment describes the designs of the Main Phase Part 1 and Part 2 study for the Acute Buccal Tissue Study (THS2.2) to be conducted in the CW25 and CW 27, respectively.
Part 2 is the repetition study of Part 1.
The design and endpoints for the Main Phase Part 2 in identical to the Part 1.

CURRENT TEXT VERSION

Test System

Human Buccal Epithelial tissue model without fibroblast from MatTek Donor 1, with the following characteristics: ORL-200-PC6.5; G26; 46 years; Male; Non-smokers; with 12 days of age of reconstructed tissue at time of shipment. Based on the dose range finding (DRF) experiment in CW18, Donor 1 was chosen as the test system in this Main Phase Part 1. The DRF phase showed that the tissue histology of the inserts constructed from Donor 1 was good throughout the experimental week (incubator controls and sham-exposed tissues) but not that from Donor 2. Summary of the HE staining from the DRF phase can be found in:
PMI Internal Document No.1

Test Items

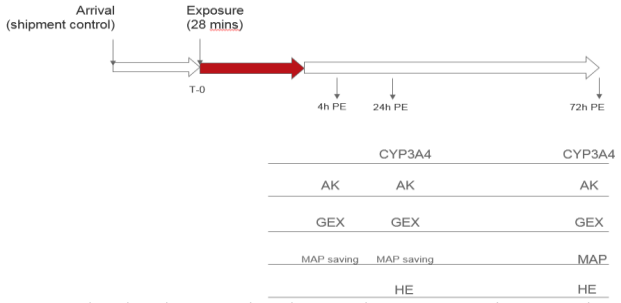
- 3R4F → Doses of 20% and 30%
- THS2.2 batch B-08164 (identical to those used in the DRF phase, see the original study plan) → Doses of 30%; 45%; and 80%

The doses were chosen based on the results of the DRF conducted in CW18; in which tissues exposed to 40% 3R4F already displayed histological changes (PMI Internal Document No.1). Therefore, the 30% smoke is chosen as the highest dose of 3R4F exposure and a 20% smoke as the lower dose of 3R4F exposure.
The equivalent doses for THS2.2 based on the nicotine concentrations were 45% and 30%. Additionally, a highest dose of THS2.2 (i.e. 80%) will be tested which was the highest dose tested during the DRF phase.

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Study N°.	S174300	Study Plan Amendment N°1	

Endpoints for Part 1 and Part 2



Human Acute BUCCAL w/o fibroblasts (1 donor, 2 dose 3R4F, 3 dose ZRH)
1 Replicate = 1 Run

			Shared endpoints from the							Number of			
Tissue	Exposure	Dose	1	2	3	4	5	Collection Time Point	Note	Replicate	Donor	Insert	Run
BU	Air_3R4F	air	AK	GEX	MAP saving			4h PE		3	1	3	A1, B1, C1
BU	3R4F	20%	AK	GEX	MAP saving			4h PE		3	1	3	
BU	3R4F	30%	AK	GEX	MAP saving			4h PE		3	1	3	
BU	Air_ZRH	air	AK	GEX	MAP saving			4h PE		3	1	3	A2, B2, C2
BU	ZRH	30%	AK	GEX	MAP saving			4h PE		3	1	3	
BU	ZRH	45%	AK	GEX	MAP saving			4h PE		3	1	3	
BU	ZRH	80%	AK	GEX	MAP saving			4h PE		3	1	3	
BU	Air_3R4F	air	AK	GEX	MAP saving			24h PE		3	1	3	A1, B1, C1
BU	3R4F	20%	AK	GEX	MAP saving			24h PE		3	1	3	
BU	3R4F	30%	AK	GEX	MAP saving			24h PE		3	1	3	
BU	Air_ZRH	air	AK	GEX	MAP saving			24h PE		3	1	3	A2, B2, C2
BU	ZRH	30%	AK	GEX	MAP saving			24h PE		3	1	3	
BU	ZRH	45%	AK	GEX	MAP saving			24h PE		3	1	3	
BU	ZRH	80%	AK	GEX	MAP saving			24h PE		3	1	3	
BU	Air_3R4F	air	AK	GEX	MAP			72h PE		3	1	3	A1, B1, C1
BU	3R4F	20%	AK	GEX	MAP			72h PE		3	1	3	
BU	3R4F	30%	AK	GEX	MAP			72h PE		3	1	3	
BU	Air_ZRH	air	AK	GEX	MAP			72h PE		3	1	3	A2, B2, C2
BU	ZRH	30%	AK	GEX	MAP			72h PE		3	1	3	
BU	ZRH	45%	AK	GEX	MAP			72h PE		3	1	3	
BU	ZRH	80%	AK	GEX	MAP			72h PE		3	1	3	
BU	Air_3R4F	air	AK			H&E	CYP3A4	24h PE		3	1	3	A1, B1, C1
BU	3R4F	20%	AK			H&E	CYP3A4	24h PE		3	1	3	
BU	3R4F	30%	AK			H&E	CYP3A4	24h PE		3	1	3	
BU	Air_ZRH	air	AK			H&E	CYP3A4	24h PE		3	1	3	A2, B2, C2
BU	ZRH	30%	AK			H&E	CYP3A4	24h PE		3	1	3	
BU	ZRH	45%	AK			H&E	CYP3A4	24h PE		3	1	3	
BU	ZRH	80%	AK			H&E	CYP3A4	24h PE		3	1	3	
BU	Air_3R4F	air	AK			H&E	CYP3A4	72h PE		3	1	3	A1, B1, C1
BU	3R4F	20%	AK			H&E	CYP3A4	72h PE		3	1	3	
BU	3R4F	30%	AK			H&E	CYP3A4	72h PE		3	1	3	
BU	Air_ZRH	air	AK			H&E	CYP3A4	72h PE		3	1	3	A2, B2, C2
BU	ZRH	30%	AK			H&E	CYP3A4	72h PE		3	1	3	
BU	ZRH	45%	AK			H&E	CYP3A4	72h PE		3	1	3	
BU	ZRH	80%	AK			H&E	CYP3A4	72h PE		3	1	3	
										Total number of inserts		105	

Figure 7. Inserts and endpoints

Note:

- 1 Replicate (= 1 tissue insert) will be tested per 1 smoke run as illustrated in no. 4 below.
- For Main Phase Part 2, additional inserts will be exposed for metabolite determination in the tissues.

The original excel sheet for the above figure along in a greater detail (including the positive and negative control samples, as well as incubator controls) can be found in:
PMI Internal Document No.2

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Study N°.	S174300	Study Plan Amendment N°1	

Exposure Plan

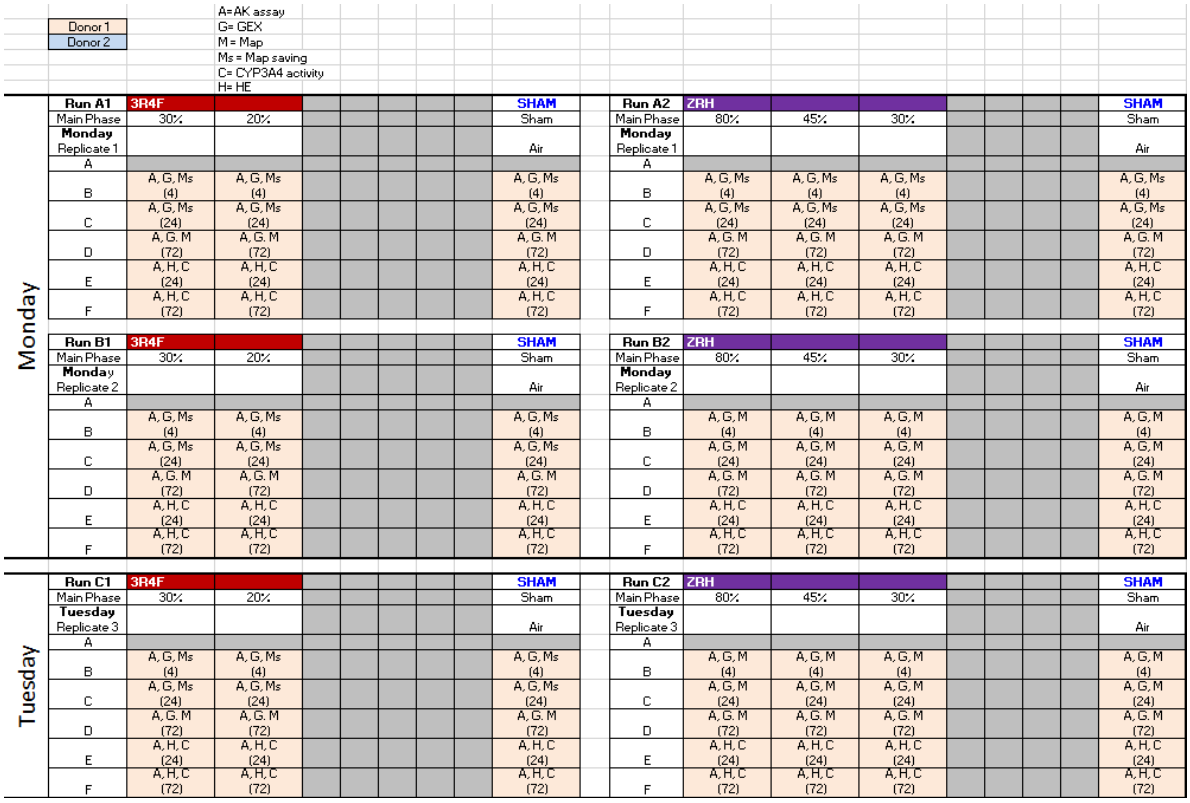


Figure 8. Exposure plan.

Main Phase Part 1: Monday exposure will be on 16 June 2014.
Main Phase Part 2: Monday exposure will be on 30 June 2014
Detailed exposure plans can be found in:
PMI Internal Document No.2

Histology Analysis

- The histology results of the DRF phase shown that a shorter fixation time (i.e. 1 hour) was sufficient and good for the buccal tissue processing. Therefore, in this Main Phase Part 1 and Part 2, we will implement the 1 hour fixation processing time; see summary DRF results here: PMI Internal Document No.3

  Histological analysis (i.e. HE staining) will be performed to examine the effect of tissue aging (i.e. incubator controls from day of shipment and throughout the week of CW25 and CW27, for Part 1 and Part 2, respectively).

- We will determine whether the tissue morphology is good throughout the exposure week of CW 25 (i.e., absence of tissue damage until 10 days following the tissue delivery with or without

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exposure) by reviewing the preliminary histology results during the week of CW 26. These preliminary results will indicate whether the experimental design of the Main Phase Part 2 in CW 27 would need to be revised.

Gene Expression analysis

- Tissues will be processed for gene expression analysis using the protocol described in the original study plan after the histology results confirmed that the tissues were not damaged.
- **For Part 1: These histology results will be reviewed in CW26 as described above in no. 5**
- **For Part 2: We will leverage the learning from Part 1**

Randomization plan will be conducted to avoid bias in the analysis (see PMI Internal Document No.7)

MAP Analysis

- Tissues will be processed for MAP analysis using the protocol described in the original study plan after histology results confirmed that the tissues were not damaged.
- **For Part 1: These histology results will be reviewed in CW26 as described above in no. 5**
- **For Part 2: We will leverage the learning from Part 1**
- Specific analytes, including cytokines that were released by the tissues in the basolateral medium will be measured using a luminex-based analysis as described in the original study plan. Only the following analytes will be measured in this Main Phase Part 1 because they were detected and shown to be increased upon 3R4F exposure in the previous buccal study (S172000): MMP-1, IL-1β; IL-1α; VEGF; IP-10; IL-6; G-CSF; GM-CSF; and TGF-α
- Summary luminex and corresponding gene expression data from the S172000 can be found here:
  PMI Internal Document No.4
  PMI Internal Document No.5

  Basolateral media for the 4h PE and 24 h PE MAP samples will be collected and stored as back-up samples (specified as "MAP saving" in **Figure 7**). Only the 72h PE samples will be analyzed (priority) because we expect to detect the highest concentration of MAP in the media. If needed, to increase statistical power, the MAP samples collected during the DRF phase (for the same doses of exposures) will be analyzed.

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Study N°.	S174300	Study Plan Amendment N°1	

CYP Activity

- Based on the DRF results, the activity of CYP3A4 increased following 3R4F exposures but not the CYP1A/B1 activity. Thus, we will only conduct CYP3A4 activity assay in this Main Phase Part 1 and Part 2 studies. Please refer to the summary of results from the DRF phase reported in PMI Internal Document No.3.
- For Part 1: Positive controls for CYP3A4 activity will be tested as follow:
- Inducer treatment for 72 h (changed every day with new medium) with 24h Luciferin-IPA substrate incubation prior to the activity measurement.
  Day 1 ----------> Day 2 ----------Day 3 ----------Day 4
  + Inducer + Inducer + Inducer
  + Luciferin-IPA collection of sample for CYP3A4 activity
- Three different condition of inducers: 75 µM water soluble rifampicin (R3501, Sigma); 125 µM water soluble rifampicin; or 125 µM water soluble dexamethasone (D2915).
- Negative control will be Luciferin-IPA-treated inserts.
- For Part 2: We will leverage the learning from Part 1

ADDITIONAL LINKS

- Metadatasheet:

  PMI Internal Document No.6
- Randomization sheet for gene expression analysis:

  PMI Internal Document No.7

Time estimation

Detail	Start	Finish
Exposure experiment Part 1	16 June 2014	20 June 2014
Exposure experiment Part 1	30 June 2014	4 July 2014
Raw data delivery	Data available	
Main Phase Part 1		
AK and CYP activity results	23 June 2014	30 June 2014
HE staining	23 June 2014	27 June 2014
MAP		TBD
Gene expression data	23 June 2014	TBD
Main Phase Part 2		
AK and CYP activity results	7 July 2014	18 July 2014
HE staining	30 June 2014	25 July 2014
MAP		TBD
Gene expression data		TBD
Proposed data of study report		30 Sep 2014 as in SP

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Study N°.	S174300	Study Plan Amendment N°1	

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Study N°. S174300 Study Plan Amendment N°1

Reason for Change

This amendment describes the design and revised protocols for the Main Phase Part 1 of the S174300 Acute Buccal Tissue Study (ZRH4.2).

Actions Required

- The preliminary histological analysis will be reviewed during CW26 to determine if the tissue histology from the Main Phase Part 1 were good, thus gene expression and MAP analyses will be conducted. The study director will communicate the results and decisions to the whole team.

Approval

Approval	Name	Date and Signature
Mandatory		
WP and Project Leader	Carole Mathis	10/06/2014
Study Director	Anita Iskandar	11 June 2014
Deputy Study Director	Radina Kostadinova	11 June 2014
Cellular Research Lab Manager	Stefan Frentzel	19 June 2014
Manager Proteomics	Ashraf Elamin	11.06.2014
Supervisor Tissue Research Laboratory	Keyur Trivedi	11/06/2014
Supervisor Gene Expression	Emmanuel Guedj	11/06/2014
Statistician	Patrice Leroy	11/06/2014
Computational Scientist	Alain Sewer	11 JUIN 2014
Program Leader	Julia Hoeng	16.6.2014

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