STUDY PLAN TRANSLATIONAL ORGANOTYPIC ACUTE HUMAN BRONCHIAL TISSUES STUDY (THS2.2) (S 170900)

Program Name	Systems Toxicology
Project Name	CellSB
PUC Number	J-1002RDA0175/0001/0001
Work Package Name	Translational Framework Smoke Street – Primary Organotypic
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Date	September 2013

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1 Review and Approval

This document has been approved by:

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2 Introduction

2.1 Purpose

The purpose of this study is to expose for 28 min human bronchial organotypic culture to similar doses at the nicotine level of 3R4F whole smoke and of THS2.2 aerosol and to compare the induced effect in both conditions at different post-exposure time points using systems toxicology approaches.

2.2 Background

Organotypic tissue models composed of several cell layers more closely mimic the *in vivo* situation than monolayer cell cultures. The cells to build organotypical airway tissues are arranged in a pseudo-stratified pattern. This epithelial morphology (all cells contacting the basement membrane but only some reaching the luminal surface) is found in the lines of the trachea as well as the upper respiratory tract. Those intact tissues are forming a functional barrier by tight junctions as seen and as are of high importance in the *in vivo* situation. Tight junctions are holding the cells together and maintaining the polarity of cells, thereby defining two distinct compartments, apical (in the *in vitro* situation the air-directed side) and basolateral (in the *in vitro* situation the medium-directed side). By this polarity receptor-mediated endocytosis at the apical surface is distinguished from exocytosis at the basolateral surface to name an example. By ensuring the polarity of the cells and preventing lateral diffusion processes extra-cellularity (i.e. for most materials entering of the cell is required) osmotic balance and transcellular transport processes are maintained. This compartmentalization closely resembles the tissue in situ i.e., airway epithelial cells representing the front line in terms of exposure to toxic stimuli contained in the ambient air but also in cigarette smoke. Furthermore, it is becoming increasingly evident that the interaction of these cells with the extracellular matrix and other cell types resident in lung parenchymal tissue plays an integral role in mechanisms associated with smoking related disease such as inflammation and tissue remodeling and repair.

The existence of the intact air liquid interface (ALI), which represents the apical side of the tissue comprising mucociliary cells covered by a physiological mucus layer, allows the exposure to complex aerosols (as cigarette smoke is) to the target cells in a 'physiologically' relevant environment. Furthermore the organotypic tissues derived from human primary cells are close to the *in vivo* situation, which might allow recapitulation of the transcriptional profile of *in vivo* airway epithelial tissues.

It has been shown that 3 dimensional (3-D) organotypic tissues derived from differentiated bronchial epithelial cells present "*in vivo* like" features at both morphological and gene expression levels (Pezzulo 2011). In this publication the analysis of gene expression profiling showed that global gene expression correlated between 3D organotypic tissues (cultured at the ALI) and bronchial epithelial cells collected directly *in vivo*, but with some differences. ALI cells expressed higher levels of basal cell-related genes, and brushed cells expressed higher levels of cilia-related genes. This correlates well with the higher percentage of basal cells in ALI cultures and higher percentage of ciliated cells in brushed cells (Dvorak 2011).

Organotypic tissues offer broad potential for the detection of toxicity of aerosols and the underlying pathophysiological mechanisms and can be used to analyze smoking related responses linked to disease such as inflammation, tissue remodeling and repair. Moreover, *in vitro* organotypic tissue models (either rat or human) will allow the direct exposure of whole smoke (aerosols) at the ALI (single or repeated exposures) to mimic cessation or switching studies. It has been shown that their differential expression in response to cigarette smoke can be assigned to functional categories and mapped to e.g., signaling pathways like xenobiotic metabolism, oxidant/antioxidant balance, and DNA damage and repair (Maunders 2007).

To conclude human bronchial organotypic cultures are a key in vitro model for the systems toxicology assessment of our new products.

3 Objectives

The objectives of this study are to provide robust gene expression data sets together with biological endpoints from an organotypic bronchial epithelium-like culture (Human) AND directly exposed once to 3R4F smoke or THS2.2 (or also named Zurich) aerosol in order **to assess and identify the biological network perturbations which are:**

1) Only triggered by THS2.2 exposure (single exposure)

2) Similarly observed after both CC and THS2.2 exposure (possibly with a different amplitude – single exposure)

3) Only triggered by whole CC smoke (single exposure)

The following objectives are all exploratory.

Summary of the experimental design

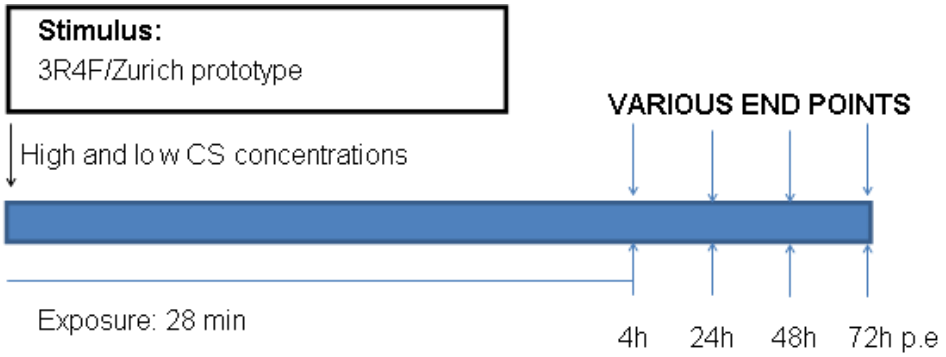


Fig. 1. Various endpoints will be collected after different post-exposure times (see Table 4).

4 Experimental Design

4.1 Test and Reference Items

4.1.1 Conventional cigarette and MRTP

Whole smoke generated from the Reference Cigarette 3R4F will be compared to aerosols generated from THS2.2 platform. All test items (Table 1 and Table 2) will be smoked under Health Canada smoking regimen adapted to meet the special conditions for the Vitrocell system.

Table 1. Test and Reference Items

Product Description	Overall Packs Batch Product Code	Type	Batch number	Short Name
THS2.2/DDA1/C3/ F Reform1 / CAST LEAF – CL /Flavor / Reynaldo	PCK.00067.RD	Test	B-05879	THS2.2
3R4F	3R4F	reference	NA	3R4F_HC

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. The production has been finalized in June 2013 and the expiration date is 10.01.2014. For more information on this production, please see the corresponding batch release report:

PMI Internal Document No.1, 2012

Reference Cigarettes 3R4F (30.000 cigarettes) were purchased from the University of Kentucky, Kentucky Tobacco Research and Development Center (for specifications see 3R4F reference (Kentucky Tobacco Research & Development Center) and Table 2). They were packed 20 cigarettes/soft pack. The packaging capacity was 5000 cigarettes per carton. They were received at PMI R&D Neuchâtel July 2012.

Table 2. 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 (puff/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

Reference: University of Kentucky (Kentucky Tobacco Research & Development Center).

Remarks: The smoke analysis data were generated under 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.

4.1.2 Reagents used for control

Table 3. List of all reagents used as controls for each assay.

Endpoints	Reagents	Duration of the treatment	Site of Application	Dose	Ref.
TEER, LDH	Triton-X100	24hrs	Basal site	1%	Sigma
TEER, LDH	PBS	24hrs	Basal site	1X	Sigma
CYPs assays	TCDD	48hrs	Basal site	30nM	Sigma
Pro-inflammatory markers release (MAP)	Mix of human TNF-α & human IL-1β	24hrs	Basal site	10ng/ml for both	human TNF-alpha, 210-TA-050, R&D systems & human IL-1β, 201-LB-025, R&D systems

The release of the inflammatory markers in the media will be measured using Luminex multiplex based technology as following:

Human MucilAir bronchial tissues: G-CSF, GM-CSF, IP-10, IL-1A, IL-1B, IL-6, IL-8, MCP-1, MIP-1A, IFN-gamma, VEGF, Eotaxin, RANTES (Miliplex MAP Human cytokine/chemokine magnetic bead panel, HCYTOMAG-60K, Millipore), MMP-1 and MMP-9 (Miliplex MAP Human MMP magnetic bead panel 2, HMMP2MAG-55K, Millipore), 6-plex Human Cytokine / TIMP-1 Magnetic Bead Panel EGF, GRO, IL-10, TNF-a, IL-13 and TIMP-1 (Cat#: SPR506) and 3-Plex Human Cytokine / Sepsis Magnetic Bead Panel TSLP, MIP-3a and sICAM-1 (Cat# SPR506).

4.1.3 Media

Cell culture media will be obtained from the tissue culture supplier Mattek.

4.1.4 Exposure

4.1.4.1 Exposure system

To smoke 3R4F reference cigarettes, the smoking machine SM 2000 will be connected to the Vitrocell system. For THS2.2 test item, we will use the smoking machine SM 2000 THS2.2, SM_SDDS_THS2.2.

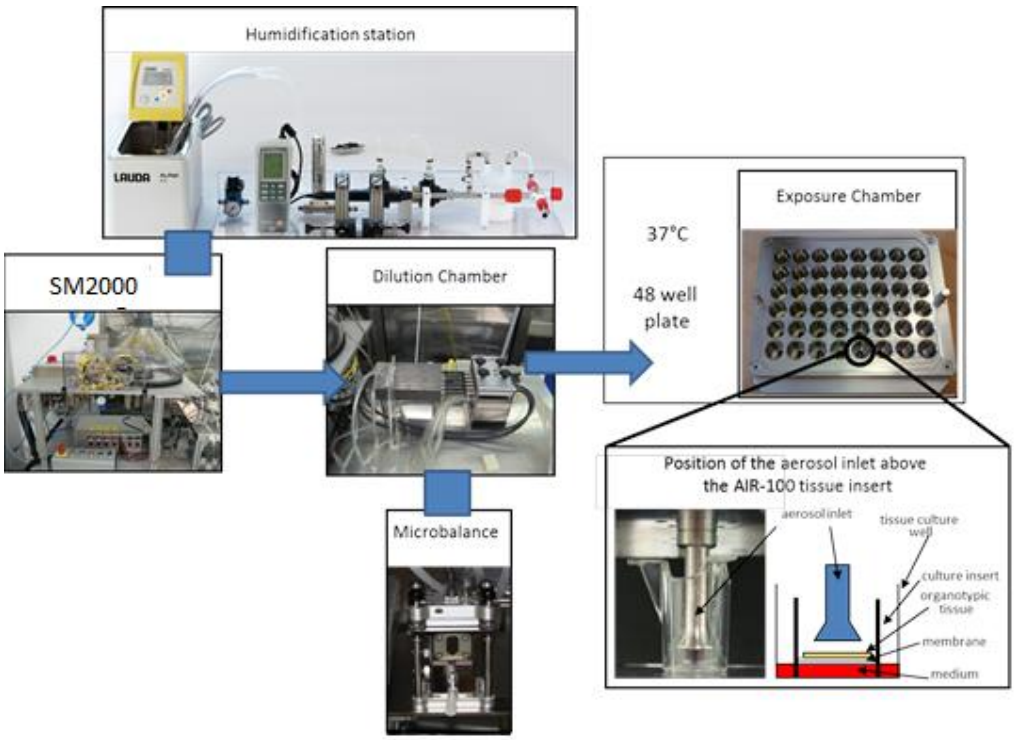


Fig. 2. Schematic representation of the whole CS exposure system and the exposure chamber (Vitrocell® and smoking robot).

To make sure that the aerosol generation meets the requirements the following steps will be implemented:

- 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.

The matching doses of 3R4F smoke and THS2.2 aerosol will be selected based on previous results reported here: PMI Internal Document No. 2, 2013.

And on the data obtained from the testing phase results described in section 4.4.1.

A) TPM deposition:

1. For all smoke runs (with or without tissue exposed), a measurement of the TPM deposition will be obtained using the microbalance for each test items and doses selected.

B) Carbonyls determination:

During the main phase a repetition of the smoke run without tissues cultures will be performed (in the same way than the one done for tissue exposure in the main phase) for both THS2.2 and 3R4F.

18 samples (9= 3X THS2.2 dose 1 + 3X sham + 3X THS2.2 dose 2 AND 9= 3X 3R4F dose 1 + 3X sham + 3X 3R4F dose 2) will be collected and used to determine the carbonyls.

C) Nicotine determination

During the testing phase, 12 samples (12= 3R4F: 3X 8% + 3X 13% and THS2.2: 3X 19% + 3X 12%) will be collected using Extrelute method for nicotine determination.

The results will be compared between 3R4F and THS2.2 to see if the dilution tested allows a good matching at the nicotine level between both test items.

If it is not the case, the dilution for THS2.2 will be change according to the data collected in the following report: PMI Internal Document No. 2, 2013.

During the main phase, 12 samples (12= 3R4F: 3X 8% + 3X 15% and THS2.2: 3X 24% + 3X 12.8%) will be collected using Extrelut method for nicotine determination to confirm that the doses used for 3R4F and for THS2.2 are matching at the level of Nicotine.

Extrelut method:

- Extrelut 3NT columns are prepared by adding 2mL of a 0.5M H_2SO_4 solution and stored at 2-8°C (to be brought to room temperature prior to sample collection)
- After sample collection tubes are sealed at both ends with parafilm
- Triplicate aerosol samples will be prepared for 3R4F and THS2.2 test items for the applied doses

4.1.4.2 Exposure design

Testing phase – doses tested for Nicotine determination:

3R4F 13% = 1.5 L/min ⇔ THS2.2 19% = 0.95L/min

3R4F 8% = 2.5 L/min ⇔ THS2.2 12%= 1.58L/min

Main phase - doses tested for Nicotine determination:

3R4F 15% = L/min ⇔ THS2.2 24% = 0.69L/min

3R4F 8% = 2.5 L/min ⇔ THS2.2 12.8%= 0.82L/min

Correspondence between 3R4F and THS2.2 based on Nicotine measurement (see: PMI Internal Document No. 2, 2013).

4.2 Test System

4.2.1 Human Bronchial tissue culture

Human bronchial organotypic tissue cultures or Epiairway-FT (with fibroblasts) used in this study are purchased at Mattek corporation, USA.

Ref.: AFT-100-PC6.5

For both shipments (18.09 & 25.09), at the delivery date, the tissue will be already cultured at the air-liquid interface for 20 consecutive days and should be already completely differentiated.

Human Bronchial Epithelial cells – Origin:

- Donor characteristics: 23 years old, male, non-disease, non smoker
- Human Fibroblasts – Origin: same donor than the bronchial epithelial cells

Specific features of this organotypic culture:

1. Primary human cells isolated from a healthy donor.
2. Human Bronchial tissues are reconstituted in separable transwells of 6.5 mm of diameter on the polyester membrane with pore size of 0.4 µm (Costar 3470, Corning incorporated).

Rational for choice for this study:

Human bronchial tissue model co-cultured with fibroblasts grow at the air/liquid interface and it is the most appropriate *in vitro* model existing on the market at the moment, which can be used for CS risk assessment. The human bronchial tissue model contains pseudo-stratified epithelium consisting of basal, mucus and ciliated cells. It shows active ion transport, good metabolic activity and secretion of inflammatory markers upon challenge.

Potential source of variation between units of investigation that require stratification during the study to control them:

Potential source of variations in the response of human bronchial tissue cultures to CS could come from different human donors used for the creation of the tissue cultures and also from one batch of tissue culture to the other. The other cause for the variations of the response of the human bronchial tissues to CS can come from the different age of the tissues used for the experiments. Also we can observe some variations between different technical replicates, if there are not exposed to CS at the same time and day, due to aging of the tissues and changes in their functionalities over days in culture.

To be able to compare different experiments using different human bronchial tissue cultures, the best is to perform the exposure of all the replicates at the same time and to include all the appropriate controls in each experiment with the same batch of tissues having similar age.

4.3 Experimental Groups

Two shipments of Epiairway-FT (with fibroblasts) tissues from the same donor will be organized.

The first shipment consists of 96 inserts (18.09.2013) and the second shipment of 327 inserts (25.09.2013).

Table 4. Description of the different endpoints captured for each shipment and inserts allocation per endpoints.

Shipment	Endpoints	Number of inserts	Exposure
18.09.2013	Toxicity tests (MTT, LDH, ATP)	18	Staurosporine dose range
	CYPs assay – pre-test 1	18	TCDD dose range
	CYPs assay – pre-test 2	60	Sham/3R4F
25.09.2013	RNA	144	Sham/3R4F/THS2.2
	CYPs assay	96	
	Histo (+incubator controls)	54	
	AK/TEER (controls)	24	
	MAP (controls)	9	

Table 5. Recapitulation of the collection time for each endpoints for the 2nd shipment of tissues.

Shipment	Endpoints	Post-exposure time			
		4hrs	24hrs	48hrs	72hrs
25.09.2013	Gene Expression (Affymetrix) (n=6)	X	X	X	X
	MicroRNA Expression (Affymetrix) (n=6)	X	X	X	X
	Tissue integrity (TEER measurements)			X	X
	AK assay (n=3)	X	X	X	X
	IHC/Histology (n=3)			X	X
	CYPs assays (n=3)	X	X	X	X
	Release of pro-inflammatory markers release (n=6)	X	X	X	X

Exposure Plan: PMI Internal Document No. 3

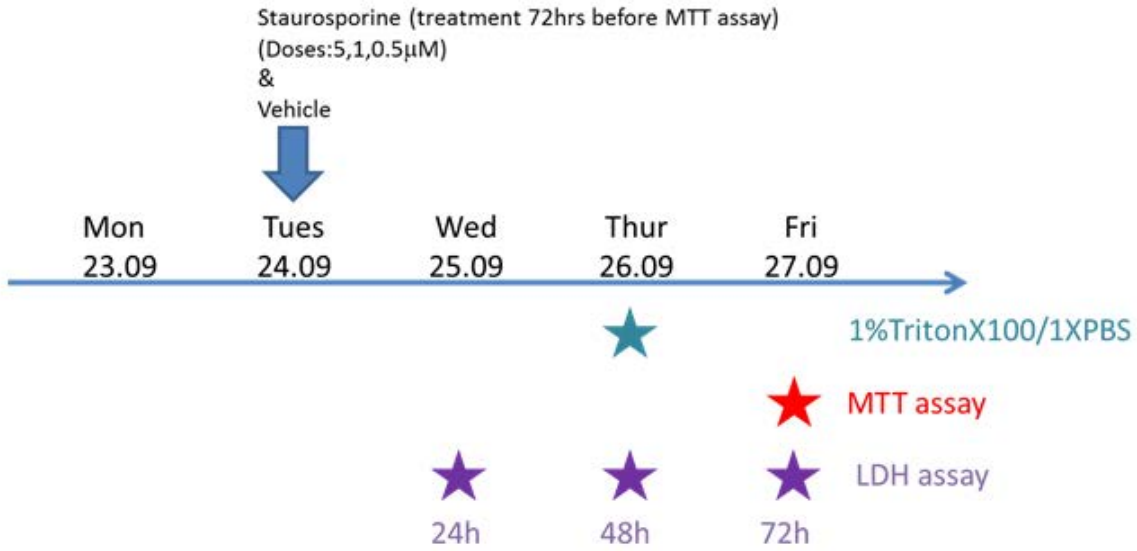
Metadata Sheet: PMI Internal Document No. 4, 2013

4.4 Sample Generation

4.4.1 Testing Phase – PART 1

The aim of the testing phase is to further assess different toxicity assays (LDH assay, MTT assay, TEER, AK assay) on bronchial organotypic cultures exposed to a dose range of Staurosporine (toxic compounds) in order to compare the results obtained between each assay and to assess their sensitivity in detecting the toxic impact of the applied compound. For the last time point, a positive control with Triton-X100 1% treatment will be added.

TOTAL NUMBER OF INSERTS USED: **18BR**



Number of samples per tasks per day

TASKS	18.09	20.09	23.09	24.09	25.09	26.09	27.09
Tissue delivery	96BR						
TEER		96BR					18BR
Medium change	96BR	96BR	96BR				
Staurosporine				9BR			
Vehicle				3BR			
Triton X100						3BR	
PBS						3BR	
LDH assay (supernatants)					12BR	12BR	18BR
MTT assay (inserts)							18BR
AK assay (supernatants)					12BR	12BR	18BR

For LDH assay and AK assay: Collect only 50µl of basal medium from inserts treated (done on 24.09) with different doses of Staurosporine and vehicle on the following days: 25.09 and 26.09.

On the 27.09, basal medium from tissues treated with PBS1X (N=3) and 1% Triton X100 (N=3) will also be collected in plus of the basal medium from tissues treated with vehicle (N=3) and Staurosporine (N=12).

LDH assay and AK assay will be performed on the day of samples collection. (see paragraph 4.5.5 for both LDH assay and AK assay protocol)

Before starting the MTT assay (27.09), all tissues (treated with Staurosporine and vehicle as well as PBS and Triton X100) will be used to measure TEER (N=18). (see paragraph 4.5. for TEER measurement protocol)

For MTT assay (N=18), inserts treated during 72hrs with different doses of Staurosporine and vehicle will be used as well as the inserts treated for 24hrs with PBS1x or with Triton X100 1%. (see paragraph 4.5.7 for MTT assay protocol).

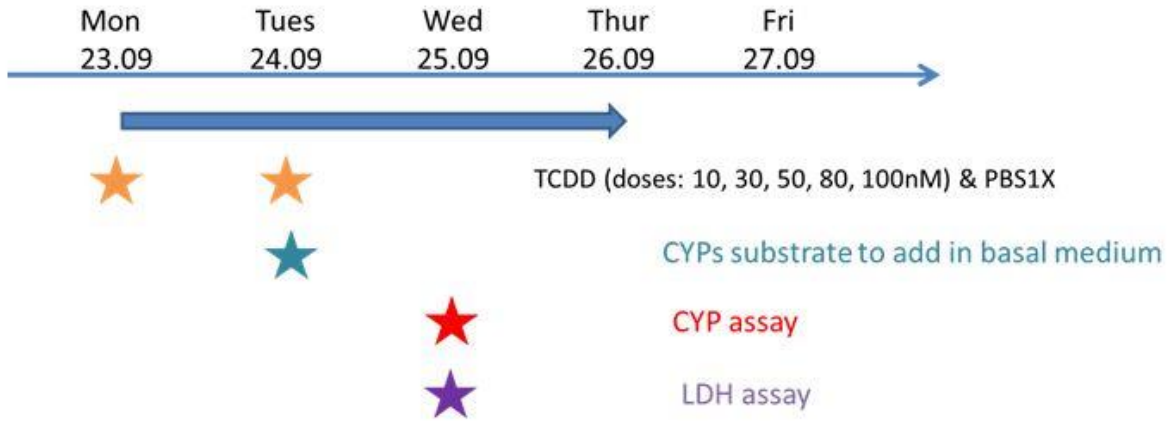
4.4.2 Testing Phase – PART 2

The aim of the testing phase – PART 2 is to further establish CYPs activity assay by measuring in parallel in tissue inserts (human bronchial epithelial cells) treated daily for 48hrs with a dose range of TCDD or PBS the following endpoints:

- CYP1A1 and CYP1B1 activity in the basal medium of tissue inserts (see paragraph 4.5.10–

- CYP1A1 and CYP1B1 proteins in the tissue insert (see paragraph 4.5.10–PMI Internal Document No. 5)
- Total protein amount in the tissue insert (see paragraph 4.5.10- PMI Internal Document No. 11)
- LDH assay (high dose of TCDD can be toxic)

TOTAL NUMBER OF INSERTS USED: **18BR**



Number of samples per tasks per day

TASKS	18.09	20.09	23.09	24.09	25.09
Tissue delivery	96BR				
TEER		96BR			
Medium change	96BR	96BR	96BR		
TCDD			15BR	15BR	
PBS			3BR	3BR	
LDH assay (supernatants)					18BR
CYPs proteins (inserts)					18BR
CYPs activity (supernatants)					18BR
Add Substrate for CYP activity				18BR	

A modification of the previous protocol is planned here:

- Substrates to measure CYP1A1 or CYP1B1 activity will only be added on the basolateral side to avoid cell stress when applied on the apical side.

- Incubation with substrates to measure CYP1A1 or CYP1B1 activity will be performed overnight and not during 3hrs only.
- 48hrs TCDD treatment is sufficient to induce CYPs activity. (Cf. Experiment done on CW37 – 2013)

4.4.3 Testing Phase – PART 3

The aim of the testing phase – PART 3 is to repeat CYPs activity assay by measuring in parallel in tissue inserts exposed to 3R4F whole smoke/SHAM (for 28min) the following endpoints:

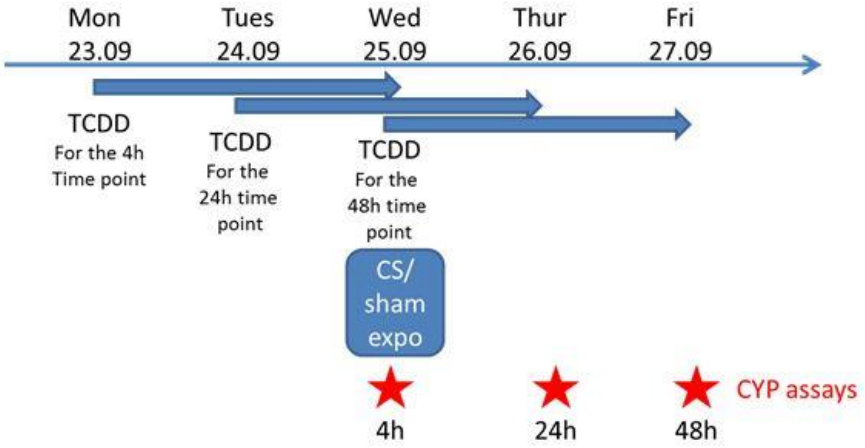
- CYP1A1 and CYP1B1 activity in the basal medium of tissue inserts (see paragraph 4.5.10–
- CYP1A1 and CYP1B1 proteins in the tissue insert (see paragraph 4.5.10– PMI Internal Document No. 5)
- Total protein amount in the tissue insert (see paragraph 4.5.10– PMI Internal Document No. 11)
- LDH assay (high dose of TCDD can be toxic)

TOTAL NUMBER OF INSERTS USED: **60BR**

N=33: CS/Sham exposure + N=9: TCDD treatment + N=9: PBS treatment + N=9: Triton x100

In addition to the tissue inserts exposed to CS/Sham, controls will also be included (30nM TCDD/PBS 1X treatment for 48hrs).

Experimental design:



Exposure plan:

run 1	3R4F							
xposure pl	dose 1	dose 1	dose 2	dose 2			SHAM	SHAM
	1	2	3	4			7	8
A	BR	BR	BR	BR			BR	BR
B	BR	BR	BR	BR			BR	BR
C	BR	BR	BR	BR			BR	BR
D	BR		BR	BR			BR	BR
E	BR		BR	BR			BR	BR
F	BR		BR	BR			BR	BR

run 1	3R4F							
ost-exposu	dose 1	dose 1	dose 2	dose 2			SHAM	SHAM
		2	3	4			7	8
A	4hrs	4hrs	4hrs	4hrs			4hrs	4hrs
B	24 hrs	24 hrs	24 hrs	24 hrs			24 hrs	24 hrs
C	48 hrs	48 hrs	48 hrs	48 hrs			48 hrs	48 hrs
D	4hrs		4hrs	histo			4hrs	histo
E	24 hrs		24 hrs	histo			24 hrs	histo
F	48 hrs		48 hrs	histo			48 hrs	histo

DOSES SELECTED:

- 3R4F: DOSE 1 = 8% and DOSE 2= 13%

Number of samples per tasks per day:

TASKS	18.09	20.09	23.09	24.09	25.09	26.09	27.09
Tissue delivery	96BR						
TEER		96BR					
Medium change	96BR	96BR					
CS exposure					33BR		
Tissue Fixation							3xSham + 3x13%CS
TCDD			3BR	6BR	9BR	6BR	3BR
Staurosporine/ TritonX100?					3BR	3BR	3BR
PBS					3BR	3BR	3BR
LDH assay					18BR	18BR	18BR
CYP proteins (inserts)					12BR	12BR	12BR
CYP activity (supernatants)					12BR	12BR	12BR
Add Substrate for CYP activity				12BR	12BR	12BR	

4.4.4 Main Phase

The aim of the main phase is to generate valuable systems biology data representing the molecular and cellular changes (transcriptomics and microRNA expression, release of pro-inflammatory markers, histology/immunohistochemistry, CYPs assay, Toxicity assay, TEER) triggered by a single exposure (28min) with two doses of THS2.2 aerosol and two doses of 3R4F smoke in human organotypic bronchial tissue cultures at different post-exposure time points.

Experimental design: see Table 5 **– paragraph 4.3**

Exposure:

THS2.2 aerosol and 3R4F whole smoke will be generated during 28 min according to Health Canada regimen.

DOSES SELECTED:

3R4F 15% = 1.25 L/min ⇔ THS2.2 24% = 0.69L/min

3R4F 8% = 2.5 L/min ⇔ THS2.2 12.8%= 0.82L/min

Exposure Plan

PMI Internal Document No. 3

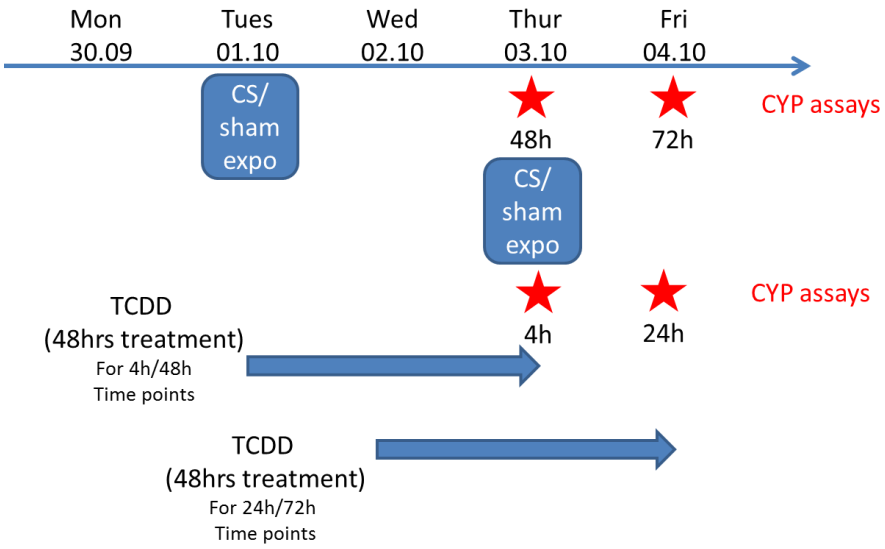
Transcriptomics

Date of exposure	Time of Exposure	Smoke run	Post exposure time	Endpoints	Number of samples	Date of samples delivery	Time of samples delivery
30.09.2013	Morning	A1/A2	4h	RNA	18	30.09.2013	Afternoon
30.09.2013	Morning	A1/A2	24h	RNA	18	01.10.2013	Morning
30.09.2013	Morning	A1/A2	48h	RNA	18	02.10.2013	Morning
30.09.2013	Morning	A1/A2	72h	RNA	18	03.10.2013	Morning
01.10.2013	Morning	B1/B2	4h	RNA	18	01.10.2013	Afternoon
01.10.2013	Morning	B1/B2	48h	RNA	18	03.10.2013	Morning
01.10.2013	Afternoon	C1/C2	72h	RNA	18	04.10.2013	Afternoon
03.10.2013	Morning	D1/D2	24h	RNA	18	04.10.2013	Morning

Randomization Plan: PMI Internal Document No. 6, 2013

CYPs ASSAYs

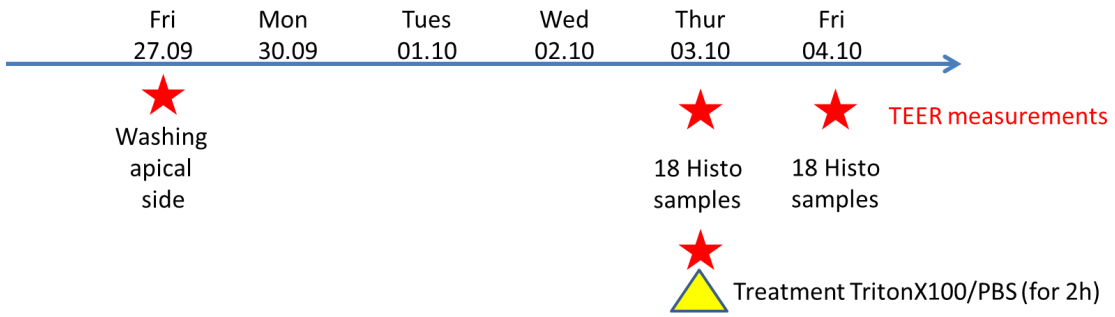
Date of exposure	Time of Exposure	Smoke run	Post exposure time	Endpoints	Number of samples	Date of samples delivery	Time of samples delivery
01.10.2013	Morning	B1/B2	48h	CYP	18 + 6 CTRLs (TCDD/PBS)	03.10.2013	Morning
01.10.2013	Morning	B1/B2	72h	CYP	18 + 6 CTRLs (TCDD/PBS)	04.10.2013	Morning
03.10.2013	Morning	D1/D2	4h	CYP	18 + 6 CTRLs (TCDD/PBS)	03.10.2013	Afternoon
03.10.2013	Morning	D1/D2	24h	CYP	18 + 6 CTRLs (TCDD/PBS)	04.10.2013	Morning



HISTOLOGY/IHC

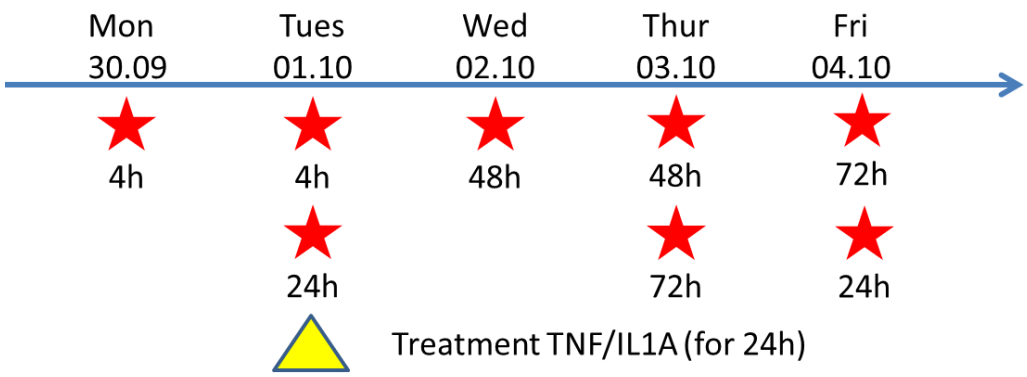
Date of exposure	Time of Exposure	Smoke run	Post exposure time	Endpoints	Number of samples	Date of samples delivery	Time of samples delivery
NA	NA	NA	NA	Histo	3 (incubator ctrls)	25.09.2013	Afternoon
NA	NA	NA	NA	Histo	3 (incubator ctrls)	30.09.2013	Afternoon
NA	NA	NA	NA	Histo	6 (incubator ctrls treated PBS/TNF, IL1A)	02.10.2013	Morning
NA	NA	NA	NA	Histo	3 (incubator ctrls)	03.10.2013	Afternoon
NA	NA	NA	NA	Histo	3 (incubator ctrls)	04.10.2013	Afternoon
01.10.2013	Afternoon	C1/C2	48h	Histo	18	03.10.2013	Afternoon
01.10.2013	Afternoon	C1/C2	72h	Histo	18	04.10.2013	Afternoon

TEER MEASUREMENTS



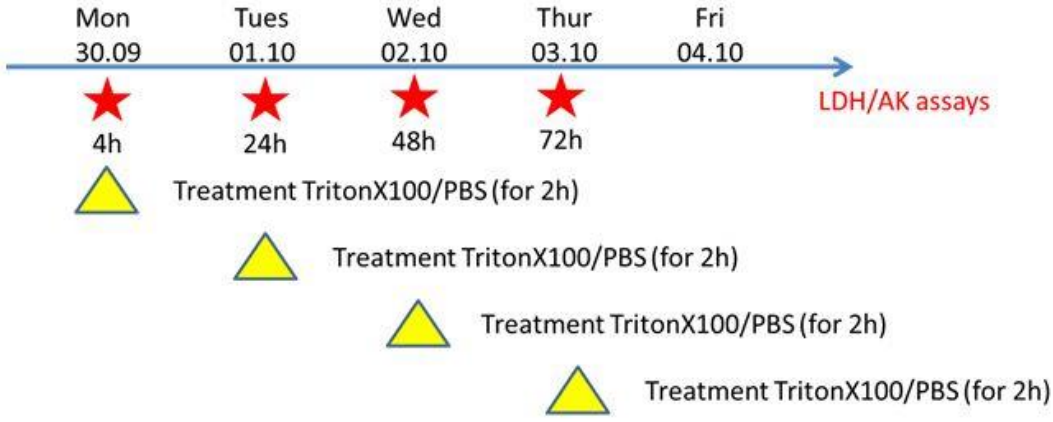
Pro-Inflammatory markers measurement (MAP)

Date of exposure	Time of Exposure	Smoke run	Post exposure time	Endpoints	Number of samples	Date of samples delivery	Time of samples delivery
30.09.2013	Morning	A1/A2	4h	MAP	18 + 3 CTRLs (IncubatorT0)	30.09.2013	Afternoon
30.09.2013	Morning	A1/A2	24h	MAP	18	01.10.2013	Morning
30.09.2013	Morning	A1/A2	48h	MAP	18 + 6 ctrls (PBS/TNF,IL1A)	02.10.2013	Morning
30.09.2013	Morning	A1/A2	72h	MAP	18	03.10.2013	Morning
01.10.2013	Morning	B1/B2	4h	MAP	18	01.10.2013	Afternoon
01.10.2013	Morning	B1/B2	48h	MAP	18	03.10.2013	Morning
01.10.2013	Afternoon	C1/C2	72h	MAP	18	04.10.2013	Afternoon
03.10.2013	Morning	D1/D2	24h	MAP	18	04.10.2013	Morning



LDH/AK ASSAYS

Date of exposure	Time of Exposure	Smoke run	Post exposure time	Endpoints	Number of samples	Date of samples delivery	Time of samples delivery
30.09.2013	Morning	A1/A2	4h	LDH	18 + 6 (PBS/TritonX100)	30.09.2013	Afternoon
30.09.2013	Morning	A1/A2	24h	LDH	18 + 6 (PBS/TritonX100)	01.10.2013	Morning
30.09.2013	Morning	A1/A2	48h	LDH	18 + 6 (PBS/TritonX100)	02.10.2013	Morning
30.09.2013	Morning	A1/A2	72h	LDH	18 + 6 (PBS/TritonX100)	03.10.2013	Morning



4.5 Procedures

4.5.1 Tissues handling upon reception

Upon arrival, human bronchial tissues will be handled under sterile conditions and as follow:

1) Under the hood, fill a new sterile 24-well plate with 0.5ml of pre-warmed assay medium

2) Remove the packaging from the plate under the hood and transfer the inserts to the prepared 24-well plate

3) Incubate the tissues at 37°C (5% CO_2, 90% humidity) for 1h for the tissues to recover from the shipment

4) In the meantime, prepare the 12-well plates with modified lids (MatTek) by adding 5.5ml of pre-warmed maintenance medium to each well

5) After the incubation, transfer the inserts to the 12-well plates, placing them on the modified lids

6) Place the inserts back into the incubator for normal culturing

Tissue quality will also be checked:

1. under a light microscope to visualize cilia beating as well as to control for unwanted tissue damage and contaminations. If the tissue batch shows any defect, the study will be postponed and a new tissue batch will be requested to our supplier.
2. with TEER measurement done during the washing step (paragraph 4.4.2) three days before the beginning of the experiments.

4.5.2 Apical side washing

Three days before exposure, the apical side of human bronchial tissue cultures will be very gently washed for not more than 15 min with 200µl culture medium. This washing step allows removing accumulated mucus and therefore minimizes the risks of interference with the tests.

4.5.3 Culture medium change

Before exposure, human bronchial tissues will be cultured with culture medium.

Volume = 5500 µl per well over the week-end and 700 µl per well during experiment (like previously done) – except for the MTT assay, where the culture will be kept with 1ml media.

4.5.4 TEER Measurement

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

1) Add 200 µl of culture media (control) on the apical surface of the human bronchial EpiAirway tissues.
2) Turn on the EVOMX machine.
3) Wash the electrode with 70 % ethanol and then in the EVOMX screen will appear: -1
4) Wash the electrode with the culture medium and then in the EVOMX screen will appear: 0.00
5) Measure the resistance (Ω) with the EVOMX
6) Gently remove (aspirate) the medium from the apical surface of the tissues
7) Place the tissues in the incubator at 37 °C.
8) For each tissue the TEER value will be taken 5 times.

4.5.5 Cellular toxicity determination

4.5.5.1 LDH assay

For the detection of cell viability and cytotoxicity, Lactate Deshydrogenase (LDH) release will be measured in the media following the working manual PMI Internal Document No.7.

- Collect in a 96 well plate, 50µl of basal medium from each samples where toxicity should be assessed.
- Equilibrate the medium to 22°C for 20-30 min.
- Add to each media sample in each well of 96 well plate 50µl of CytoTox-ONE™ Reagent and mix or shake for 30 seconds
- Incubate at 22°C for 10 minutes.
- Add to each sample 25µl of stop solution (kit component)
- Shake plate for 10 seconds and record fluorescence with an excitation wavelength of 560nm and an emission wavelength of 590nm using the FluoStar OPTIMA (BMG Labtech).
- Record the data within one hour to avoid increased background fluorescence.

4.5.5.2 AK assay

Activity of Adenylate kinase is measured in basal culture medium samples.
Protocol for the AK assay:
PMI Internal Document No.8

4.5.5.3 MTT assay

Suggested Protocol for the MTT assay (the final protocol will be given in the report):

4.5.6 Tissues Fixation & IHC/Histology analysis

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

- Inserts from each tissue will be washed 3 times with 1xPBS at RT in both basal and apical side.
- Aspirate PBS buffer
- Add 3ml of 4% paraformaldehyde (PAF) (see below the preparation of 4% PAF, P-6148, Sigma) diluted in 1xPBS containing calcium/magnesium to cover all the tissue.

- Incubate 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.
- Transfer the inserts in a labelled with the identification number from the metadata sheet 50mL falcon tube filled with PBS1X for shipment to Mattek, USA. (Project Agreement: PMI Internal Document No. 9, 2013)

Preparation of 4% PAF:

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

For 1 liter:

1. Dissolve 40 g PFA in 625 ml dH_2O; warm up to max. 60°C while stirring continuously until entirely dissolved (let the solution clear).
2. **If not clear**, add drop by drop of NaOH (10M) 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)

For 1 liter:

Na_2HPO_4 $2H_2O$	14.4 g
KCl	2 g
NaCl	80 g
KH_2PO_4	2 g
dH_2O	1000 ml

Mix well by stirring, adjust the pH to 7.4 and store at 4°C or room temperature.

4.5.7 Pro-inflammatory markers release measurement

We will measure the following cytokines/chemokines/MMPs in culture medium samples collected during the study and stored at -80°C until measurement:

Human MucilAir bronchial tissues: G-CSF, GM-CSF, IP-10, IL-1A, IL-1B, IL-6, IL-8, MCP-1, MIP-1A, IFN-gamma, VEGF, Eotaxin, RANTES (Miliplex MAP Human cytokine/chemokine magnetic bead panel, HCYTOMAG-60K, Millipore), MMP-1 and MMP-9 (Miliplex MAP Human MMP magnetic bead panel 2, HMMP2MAG-55K, Millipore) using the Luminex based technology.

Luminex instructions:

- Prepare all reagents, standards, and samples as described in the manufactures instructions (Put the link to Milipore manuals).
- 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).
- Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
- Add 25 µL of each Standard or Control into the appropriate wells (Assay Buffer should be used for 0 pg/mL standard (Background))
- Add 25 µL of Assay Buffer to the background and sample wells.
- Add 25 µL of appropriate matrix to the background, standards, and control wells.
- 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)
- Add 25 µL of Sample into the appropriate wells.
- 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.)
- Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker 2h at room temperature (20-25ºC). (For the cytokine measurement, overnight incubation at 4°C may increase signal intensity)
- 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.
- Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
- 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.
- Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
- Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).

- Gently remove well contents and wash plate 2 times as outlined above
- Add 100 µL or 150 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells for MMP or cytokine measurement respectively. Resuspend the beads on a plate shaker for 5 minutes.
- Run plate on Luminex, 200™, HTS with xPONENT software.
- Save and analyze 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.)

4.5.8 RNA/microRNA extraction

Step 1: Tissues will be harvest/store prior RNA/microRNA extraction

- Tissue inserts will be washed 3 times with 1xPBS at RT in both basal and apical side under the hood.
- Aspirate PBS buffer
- Place the tissues on the membranes in Eppendorfs containing QIAzol lysis buffer (miRNeasy Mini Kit Qiagen kit (cat # 217004)) and stored the tissues directly in -80°C until RNA/microRNA extraction

Step 2: RNA/microRNA extraction

- RNA/microRNA extraction protocol is based on the recommendations from the miRNeasy Mini Kit Qiagen kit (cat # 217004) (PMI Internal Document No. 10)
- RNA will be eluted in 30µl RNAse-free water.
- The miRNeasy 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.

Step 3: Quality test and RNA yield determination

The quantity of the isolated RNA will be determined with the NanoDrop 1000 or 8000 spectrophotometer (Thermo Scientific) or Spectramax Plus 384 (Molecular device).

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.

- RNA quality will be measured using Agilent 2100 Bioanalyzer.

NB: The minimum RNA concentration required for Human DNA Affymetrix array (Human genome U133 plus 2 array) is around 34ng/µl of RNA with a minimum RIN number of 6.0.

The minimal amount of miRNA necessary for GeneChip miRNA 3.0 array is 130ng.

Step 4: Affymetrix microarray

This description includes the processing of samples, isolation of mRNA and performing microarray hybridization using Affymetrix cartridges. Afterwards, the analysis of this raw data will be performed by the HPSCA project within the Systems Biology application & Verification program.

Step 5: 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. 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.

Step 6: 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 250µl 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. In the last step, the arrays will be moved to GeneChip Scanner 3000 7G and each array will be scanned.

Step 7: Monitoring the Quality Check of 3' IVT Expression Arrays

The quality of the arrays can be assessed after scanning. The raw image data from the scanner will be saved in a DAT file. The DAT file contains pixel intensity values collected from the scanner. The DAT file will be monitored by using the Affymetrix® GeneChip Command Console™ software (AGCC) application where an image of the DAT file is created.

This image will be monitored for

- artefacts
- overall intensity and intensity distribution
- checkerboards at the corners
- readability of the array name
- central cross

It will be further checked that the automatic grid alignment, which uses the B2 oligos, works.

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 Bioanalyser, the amount of amplified RNA and the Affymetrix report will be delivered. A meta-data sheet describing the sample name, exposure conditions, CEL file name will be provide along with the CEL files.

Randomization Plan: PMI Internal Document No. 6, 2013

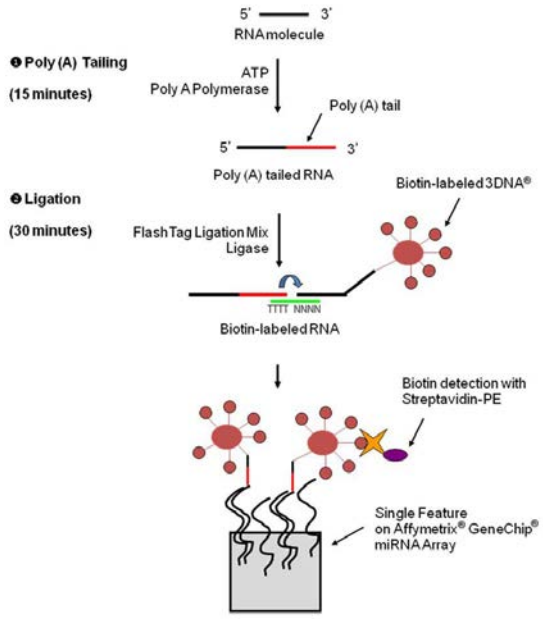
Measurement of the miRNA expression using Affymetrix microarrays

The FlashTag™ Biotin HSR Labeling Kit will label any RNA sample, including total RNA, severely degraded RNA, plant RNA, and low molecular weight RNA. This protocol describes labeling total RNA or low molecular weight (LMW) RNA for analysis by Affymetrix® GeneChip® miRNA Arrays.

LMW RNA molecules (snRNA, hnRNA, piRNA, miRNA, etc.) have recently been shown to be involved in important biological processes such as mRNA degradation, transcriptional gene silencing (TGS) and translational repression. As a result, these newly discovered biomolecules are gaining the interest of the scientific community as possible new drug targets and for use in diagnostics. FlashTag Biotin HSR provides the necessary tools to identify such targets.

FlashTag Biotin HSR labeling is fast, simple, accurate, highly sensitive and reproducible. Starting with total RNA, the process begins with a brief tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample.

The labeling process is complete in less than two hour.



FlashTag™ HSR: Procedure Overview

Step 1: Materials Required

FlashTag™ Biotin HSR RNA Labeling Kit

FlashTag Biotin HSR RNA Labeling Kit, 10 rxn (P/N 901910) or 30 rxn (P/N 901911).

Table 6. Required materials and reagents

Vial	Component	Storage	Handling
1	10X Reaction Buffer	-20°C	Thaw at room temperature, vortex, and briefly microfuge.
2	25mM $MnCl_2$	-20°C	Thaw at room temperature, vortex, and briefly microfuge.
3	ATP Mix	-20°C	Thaw on ice, microfuge if necessary, and keep on ice at all times.
4	PAP Enzyme	-20°C	Remove from freezer just prior to use, and briefly microfuge. Keep on ice at all times. Do not vortex.
5	5X FlashTag Biotin HSR Ligation Mix	-20°C	Thaw at room temperature, vortex, and briefly microfuge.
6	T4 DNA Ligase	-20°C	Remove from freezer just prior to use, and briefly microfuge. Keep on ice at all times. Do not vortex.
7	HSR Stop Solution	-20°C	Thaw at room temperature, vortex, and briefly microfuge.
8	RNA Spike Control Oligos	-20°C	Thaw on ice, microfuge if necessary, and keep on ice at all times.

Step 2: RNA Sample and Quantitation

Either Total RNA or LMW (Low Molecular Weight) RNA can be labeled with FlashTag Biotin HSR.

Using total RNA can save time and money, and prevent sample loss.

To accurately determine the concentration of the RNA sample, Affymetrix recommends the use of the Quant-iT™ RiboGreen RNA Assay Kit (Invitrogen P/N R11490) or the NanoDrop® ND-1000 or 8000 Spectrophotometer (NanoDrop Technologies).

Table 7. RNA input for Flash Tag Biotin HSR labeling

RNA Sample	Input for FlashTag Biotin HSR Labeling for miRNA 400/169 Format Arrays (miRNA 1.0 and 2.0 Arrays)	Input for FlashTag Biotin HSR Labeling for miRNA 100 Format Arrays (miRNA 3.0 Arrays)
Total RNA containing LMW RNAT	100 – 1000 ng total RNA	130 – 1000 ng total RNA
Enriched LMW RNA, quantitated	100 – 400 ng LMW RNA	130 – 400 ng LMW RNA
Enriched LMW RNA, not quantitated	Enriched from 100 – 1000 ng total RNA`	Enriched from 130 – 1000 ng total RNA

4.5.9 FlashTag™ Biotin HSR RNA Labeling Procedure

Important Note:

An automated script is being developed and validated internally allowing a throughput of 96 samples / day.

Both techniques (manual and automated) may be used for this study depending on needs.

Step 3: Poly (A) Tailing

1. Adjust the volume of RNA to 8 µL with Nuclease-Free Water (Vial 11).

2. Transfer the 8 µL RNA to ice. Add 2 µL RNA Spike Control Oligos (Vial 8) and return to ice.

3. Dilute the ATP mix (Vial 3) in 1 mM Tris as follows:

> For total RNA samples, dilute the ATP Mix 1:500.

> For enriched, quantitated samples, calculate the dilution factor according to the following formula:

5000 ÷ ng input LMW RNA

Example: If using 100 ng of enriched LMW RNA, the dilution factor is 5000 ÷ 100 = 50.

Dilute the ATP Mix 1:50.

> For enriched samples that are not quantitated, calculate the dilution factor according to the following formula:

1000 ÷ µg input total RNA

Example: If the sample was enriched from 500 ng total RNA, the dilution factor is 1000 ÷ 0.5 = 2000. Dilute the ATP Mix 1:2000.

4. Assemble a Poly A Tailing Master Mix in a nuclease-free tube in the order listed in Table 8 . Include 10% overage to cover pipetting errors.

Table 8. Poly A Tailing Master Mix (for a single reaction)

Component	Amount
10X Reaction Buffer (Vial 1)	1.5 µL
25 mM MnCl2 (Vial 2)	1.5 µL
Diluted ATP Mix (Vial 3 dilution from Step 3)	1.0 µL
PAP Enzyme (Vial 4)	1.0 µL

5. Add 5 µL of Master Mix to the 10 µL RNA/Spike Control Oligos (Step 2 above), for a volume of 15 µL.

6. Mix gently (do not vortex) and microfuge.

7. Incubate in a 37°C heat block for 15 minutes. Discard any unused, diluted ATP mix from Step 3.

Step 4: FlashTag™ Biotin HSR Ligation

1. Briefly microfuge the 15 µL of tailed RNA and place on ice.

2. Add 4 µL 5X FlashTag Biotin HSR Ligation Mix (Vial 5) to each sample.

3. After the Ligation Mix has been added, add 2 µL of T4 DNA Ligase (Vial 6) to each sample. Do not make a master mix at this step, as auto-ligation can occur.

4. Mix gently (do not vortex) and microfuge.

5. Incubate at 25°C (room temperature) for 30 minutes.

6. Stop the reaction by adding 2.5 µL HSR Stop Solution (Vial 7).

Mix and microfuge the 23.5 µL of ligated sample.

7. If ELOSA QC is performed, remove 2 µL of the biotin-labeled sample for use with the ELOSA QC Assay. It is acceptable to store the 2 µL of biotin-labeled sample on ice for up to 6 hours or at –20°C for up to 2 weeks. If the ELOSA QC Assay is not performed, it is recommended that 2 uL of biotin-labeled sample be saved until the array QC is complete. Retaining this sample will enable one the ability to troubleshoot possible target preparation issues, if needed.

8. The remaining 21.5 µL biotin-labeled sample may be stored on ice for up to 6 hours, or at –20°C for up to 2 weeks, prior to hybridization on Affymetrix GeneChip miRNA Arrays.

Hybridization

1. Bring the reagents listed in Step 3, below, to room temperature.

2. Completely thaw and then heat the 20X Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre from GeneChip® Eukaryotic Hybridization Control Kit) for 5 minutes at 65°C.

3. Add the following components in Table 9 to the 21.5 µL biotin-labeled sample in the order listed, to prepare the array hybridization cocktail:

Table 9. Hybridization Cocktail (for a single reaction)

Component	Volume for a 400/169 Format Array (miRNA 1.0 and 2.0 Arrays)	Volume for a 100 Format Array (miRNA 3.0 Arrays)	Final Concentration
2X Hybridization Mix	50 µL	66 µL	1X
27.5% Formamide (Vial 12)	15 µL	19.2 µL	4%
DMSO	10 µL	12.8 µL	9.7%
20X Hybridization Controls	5 µL	6.6 µL	1X
Control Oligo B2, 3nM	1.7 µL	2.2 µL	50 pM
Nuclease-free Water	N/A	3.7 µL	
Total Volume	81.7 µL	110.5 µL	

NOTE: If preparing multiple samples, a master mix of the hybridization cocktail components in Table above may be prepared by multiplying the volumes by the number of arrays plus 10% overage to cover pipetting error.

Table 10. Hybridization Cocktail using a Hybridization Master Mix

Component	400/169 Format Array (miRNA 1.0 and 2.0 Arrays)	100 Format Array (miRNA 3.0 Array)
Biotin-labeled Sample	21.5 µL	21.5 µL
Hybridization Master Mix	81.7 µL	110.5 µL

4. Incubate at 99°C for 5 minutes, then 45°C for 5 minutes.

5. Aspirate 100 µL (400/169 format array) or 130 µL (100 format array) and inject into an array.

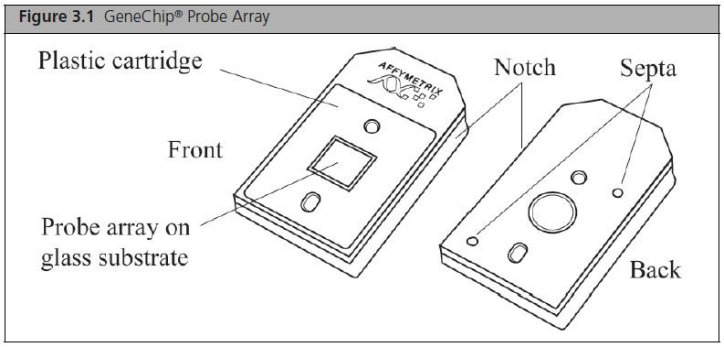


Fig. 3. GeneChip® Probe Array

6. Remove the pipet tip from the upper right septum of the array.
7. Cover both septa with 1/2" Tough-Spots® to minimize evaporation and/or prevent leaks.
8. Place the arrays into hybridization oven trays.
9. Load the trays into the hybridization oven.
10. Incubate the arrays at 48°C and 60 rpm for 16 to 18 hours.

Step 5: Washing and Staining

For additional information about washing, staining, and scanning, refer to the Genechip® Expression Wash, Stain, and Scan User Manual for Cartridge Arrays (PN 702731) and the Affymetrix® Command Console® User Manual (P/N 702569).

1. After 16 to 18 hours of hybridization, remove the arrays from the oven. Remove the Tough-Spots from the arrays.
2. Extract the hybridization cocktail from each array and transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail. Store on ice during the procedure, or at –80°C for long-term storage. Refer to Appendix B, Array Rehybridization Procedure on page 20, if necessary.
3. Fill each array completely with Array Holding Buffer.
4. Allow the arrays to equilibrate to room temperature before washing and staining.

NOTE: Arrays can be stored in the Array Holding Buffer at 4°C for up to 3 hours before proceeding with washing and staining. Equilibrate arrays to room temperature before washing and staining.

5. Place vials into sample holders on the fluidics station:

A. Place one (amber) vial containing 600 µL Stain Cocktail 1 in sample holder 1.
B. Place one (clear) vial containing 600 µL Stain Cocktail 2 in sample holder 2.
C. Place one (clear) vial containing 800 µL Array Holding Buffer in sample holder 3.

7. Wash and stain with Fluidics Station 450 using the appropriate fluidics script : FS450_0002

Table 11. Fluidics Protocols

	Fluidics Station 450 Protocol FD450_0002 (100 format array)	Fluidics Station 450 Protocol FS450_0003 (400/169 format array)
Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C	8 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
1st Stain	Stain the probe array for 5 minutes with Stain Cocktail 2 (Vial Position 2) at 35°C	Stain the probe array for 10 minutes with Stain Cocktail 1 (Vial Position 1) at 25°C
Post Stain Wash	Wash 10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C	Wash 10 cycles of mixes/cycle with Wash Buffer A at 30°C
2nd Stain	Stain the probe array for 5 minutes with Stain Cocktail 2 (Vial Position 2) at 35°C	Stain the probe array for 10 minutes with Stain Cocktail 2 (Vial Position 2) at 25°C
3rd Stain	Stain the probe array for 5 minutes with Stain Cocktail 1 (Vial Position 1) at 35°C	Stain the probe array for 10 minutes with Stain Cocktail 1 (Vial Position 1) at 25°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C
Array Holding Buffer	Fill the probe array with Array Holding Buffer (Vial Position 3)	Fill the probe array with Array Holding Buffer (Vial Position 3)

8. Check for air bubbles. If there are air bubbles, manually fill the array with Array Holding Buffer. If there are no air bubbles, cover both septa with 3/8" Tough-Spots. Inspect the array glass surface for dust and/or other particulates and, if necessary, carefully wipe the surface with a clean lab wipe before scanning.

Step 8: Scanning

The instructions for using the scanner and scanning arrays can be found in the Affymetrix® Command Console® User Manual (P/N 702569).

Table 12. Fluidics Protocols Array Type

Array Type	Software
GeneChip® miRNA 1.0 Array	miRNA QC Tool Version 1.0.33.0
GeneChip® miRNA 2.0 Array	miRNA QC Tool Version 1.1.1.0/Expression Console 1.2 or higher
GeneChip® miRNA 3.0 Array	Expression Console 1.2 or higher

Step 9: Data normalization and quality control

Use Expression Console software for data summarization, normalization, and quality control (v. 1.2 and higher). Please refer to the website for Expression Console for instructions on miRNA Array analysis. Affymetrix recommends using RMA + DABG for analysis. To evaluate the success of the labeling protocol and array processing, open line graphs for the spike-in labeling control probe sets in Expression Console. Once the RMA+DABG analysis is complete, click on the icon for line graphs or select Graph > Line Graph - Report Metrics and select the check boxes for desired probe sets.

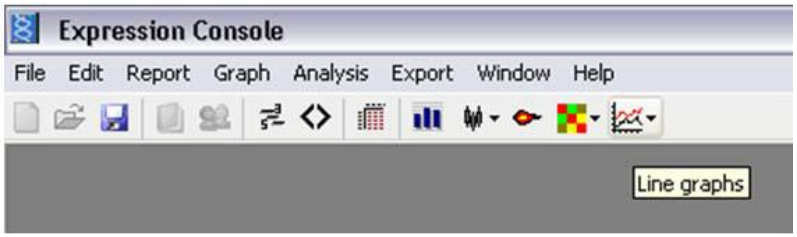


Fig. 4. Expression console window

miRNAs present in vial 8 should have signal greater than or equal to 1000 (or 9.96 for log2 signal):

> spike_in-control-2_st

> spike_in-control-23_st

> spike_in-control-29_st

> spike_in-control-31_st

> spike_in-control-36_st.

Oligos 2, 23 and 29 are RNA, and confirm the poly(A) tailing and ligation.
Oligo 31 is poly(A) RNA and confirms ligation.
Oligo 36 is poly(dA) DNA and confirms ligation and the lack of RNases in the RNA sample.

If the array hybridization, wash, stain and scan procedure was successful, the arrays should be gridded successfully and cel and chp files should be generated. The hybridization control probe sets should have signal commensurate with concentration:
AFFX-r2-Ec-c1-BioB-3_at < AFFX-r2-Ec-c1-BioC-3_at < AFFX-r2-Ec-c1-BioD-3_at < AFFX-r2-Ec-c1-cre-3_at Export the data into third party software for further analysis.

4.5.10 CYPs assay

Protocol for total protein amount determination per tissue insert:

PMI Internal Document No. 11

Protocol for CYPs protein amount determination per tissue insert:

PMI Internal Document No. 11

References for the two ELISA kits used to detect CYP1A1 and CYP1B1 proteins:

(CYP1A1.)

(CYP1B1.)

Protocol for CYP1A1 and CYP1B1 activity measurement:

PMI Internal Document No.12

The assay measures the activities of CYP enzymes from recombinant and native sources and tests the effects of drugs and new chemical entities on 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.

For measurement of the basal CYP activity:

- Incubate tissue inserts overnight with the Luciferin-CEE substrate diluted 1:50 in the basal culture medium.
- Luciferin Detection Reagent is prepared by transferring the entire contents of the bottle of the appropriate reconstitution buffer to the amber bottle containing Luciferin Detection Reagent (see the manual instructions from Promega). This mixture is prepared before the start of the study and aliquots of 2 mL are then stored at -20°C for 1 month max.
- Transfer 50µl of culture medium from each tissue inserts to a 96-well opaque white luminometer plate at room temperature
- Add 50µl 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.

Metabolic cytochrome P450 activity such as CYP1A1 and CYP1B1 will be measured in the basolateral side of the tissues after the incubation of the medium with the respective enzyme substrate.

Positive control:

Tissue inserts treated with 30nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) diluted in the basal medium for 48h at 37°C, 5%CO_2. The treatment should be changed daily within 48h.

Negative control:

Tissue inserts treated with PBS (basal side) for 72h.

4.5.11 TPM deposition measurement

The microbalance is used to determine real time deposition of particles on a quartz crystal during a whole smoke exposure run. The method allows the determination of the deposition with a resolution of 10 ng/cm^{2}s. The lowest detection limit is approx. 20 ng/cm^{2}h. In the present study, the microbalance will be applied during each smoke run and it will measure the amount of particles deposited during CS exposure of organotypical tissues with both 3R4F smoke and THS2.2 aerosol. The measurement will give an indication on the amount of delivered particles for a given dose.

Protocol for the measurement

1) Connect quartz crystal with the microbalance.
2) Open Microbalance Software.
3) Microbalance will be connected with measurement channel 1.
4) Set the scale to zero and wait a couple of minutes until baseline is set
5) Start whole smoke exposure according to study plan.
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.

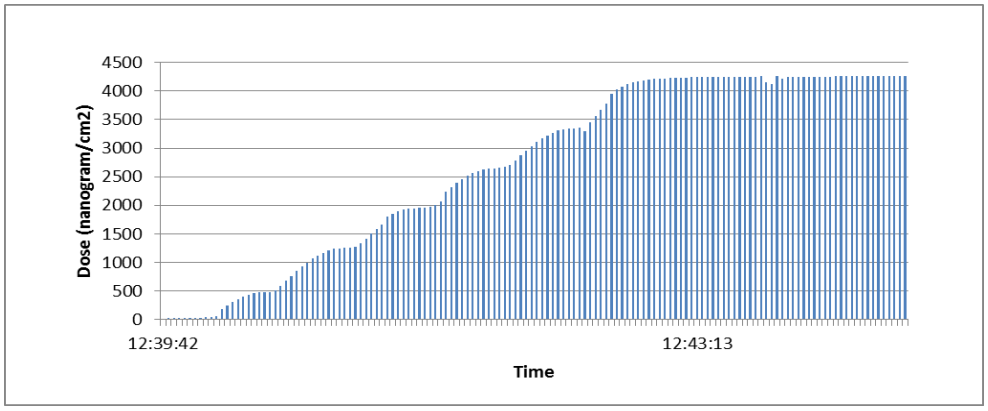


Fig. 5 Example graph representing each puff generated by the cigarette as a step in each smoke run.

For each smoke run with the help of the raw data file similar graph will be generated and in the graph each step represents the puff generated from the cigarette.

4.5.12 Nicotine determination

Diluted aerosol is trapped on an Extrelut 3NT column impregnated with sulfuric acid. After sample collection the internal standard isoquinoline is added to the column. The trapped components on the column are eluted with a 5% v/v of Triethylamine in N-butylacetate. The nicotine in the eluate is determined by gas chromatography – flame ionization detection (GC-FID).

Individual steps:

- Extrelut 3NT columns are prepared by adding 2mL of a 0.5M H_2SO_4 solution and stored at 2-8°C (to be brought to room temperature before use)

- After sample collection the Extrelut 3NT columns must be labeled accordingly and sealed at both ends with caps or parafilm
- The columns can be stored at 2-8°C for a maximum of 4 weeks
- Prior to elution 250µL of isoquinoline is added as an internal standard
- 5mL of 5% v/v Triethylamine in N-butylacetate is added in a total of three cycles (total volume = 15mL) to elute the constituents; the eluate is collected in 20mL pyrex glass tubes
- 1mL of the dilution is transferred to an amber glass vial for GC-FID analysis
- A blank sample, an internal standard sample and two quality control (QC) samples (a high and a low level of the working range) are included at the start and end of each sequence, and after every 10 runs to ensure the quality of the results
- Peak detection, integration, and quantification (according to internal standard calibration curve) is automatically performed by the Agilent ChemStation software

4.5.13 Carbonyls determination

Carbonyls determination will be performed as follows:

1) Sample fraction generation:

- Before starting the exposure, 18.5 mL of PBS will be loaded in each row of the exposure plate
- Once the smoke run is completed, an aliquot of 1.2 mL from each row is taken
- Add 1.8 mL of DNPH solution (15 mM) to each aliquot
- Then add 150 µL pyridine after 30 min 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 :

- An aliquot of 500 µL of the sbPBS aerosol sample is introduced into a LC-MS amber glass vial.
- Add 485 µL acetonitrile.
- Add 15 µL internal standard mixture (24 µg/mL d_6-acetone and 21 µg/mL d_5-methyl ethyl ketone).
- In parallel, 4 QC samples are prepared in order to confirm the expected concentration (with an accuracy between 85-115%) before acceptance of the results.

- A 5 µL injection volume is analyzed by LC-MS/MS.

4.5.14 Data Processing

Not Applicable

5 Statistical Methods

5.1 General Considerations for Data Analysis

Analysis will be performed using SAS Enterprise Guide 4.3.

5.1.1 Missing Values

No imputation for missing values.

5.1.2 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.

5.1.3 Outliers

If an extreme value is observed:

- It will be checked for mistyping/misrecording,
- Then the checked value will be used in the analysis.

5.1.4 Significance Level

This study is exploratory, consequently:

- No effect can be reported as statistically significant.
- No significance level will be defined.
- Raw p-values will be reported as they are. However p-values, for instance less than 5%, may be marked with a “+” sign for reading ease in the final report.

5.1.5 Randomization

Given the sample size per group (N=3) and the limitation of the Vitrocell system (1 exposition per columns and monotonic evolution of the smoke concentration across the columns) a randomization would not be able to control the position bias efficiently (row+column).

So we will prefer a design allowing only the estimation of the column bias within the same dose. Then effect size of results will be compared to the column bias, and carefully interpreted.

5.1.6 Definition and Calculation of derived variables

- LDH assay Cytotoxicity

Calculation:

1. Subtract the average fluorescence values of the culture medium background from all fluorescence values of experimental wells.
2. Use the average fluorescence values from experimental, maximum LDH release, and culture medium background to calculate the percent cytotoxicity for a given experimental treatment.

$$\text{Percent cytotoxicity} = \frac{100 \times (\text{Experimental} - \text{Culture Medium Background})}{(\text{Maximum LDH Release} - \text{Culture Medium Background})}$$

- TEER measurement

Calculation:

Convert the resistance (Ω) to TEER (Ω.cm^2) using the following formula:

TEER (Ώ.cm^2) = (resistance (Ώ) -100(Ώ) x 0.33(cm^2)

100 Ώ = resistance of the membrane

0.33 cm^2 = epithelium's surface

- CYPs activity

Calculation:

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. Calculate percent change by dividing net treated values by net untreated values and multiplying by 100.

5.2 Statistical Design

5.2.1 Part 1

- Design (BR=18):

			Time points*		
Substance	Dose	N	LDH	MTT	AK
Vehicle	-	3	24h	72h	24h
Triton-X100	1%	3	48h		48h
PBS	1X	3	72h		72h
Staurosporine	0.5 uM	3			
	1.0 uM	3			
	5.0 uM	3			

*same replicate measured at different time points

- Objective: find the most sensitive cytotoxicity assay between LDH, MTT, AK.

5.2.2 Part 2

- Design (BR=18):

Substance	Dose	N	Measurements
PBS	-	3	CYP1A1 activity
TCDD	10	3	CYP1B1 activity
	30	3	CYP1A1 + CYP1B1 protein
	50	3	Total protein
	80	3	LDH assay
	100	3	

- Objective: find the dose for which the ratio of CYPs active over CYPs protein is the highest (while cytotoxicity is monitored, high dose of TCDD can be toxic)

5.2.3 Part 3

- Design (BR=60):

Substance	Dose	Exposure (4h/24h/48h)	N	Measurements	Post-exposure* (24h/48h/72h)
Whole Smoke	3 (0%,8%,15%)	3	3	CYP1A1 activity CYP1B1 activity	3
TCDD	30nM	3	3	CYP1A1 + CYP1B1 protein	3
PBS	1X	3	3	Total protein	3
Triton-X100	1%	3	3	LDH assay	3
Whole Smoke	2 (0%,15%)	1(48h)	3	Histology	1(72h)

*same replicate measured at different post-exposure time points

- Objective: find the combination of exposure time and post-exposure time which shows the for which the ratio of CYPs active over CYPs protein is the highest (normalized by total protein), while cytotoxicity is monitored (high dose of TCDD can be toxic)

5.2.4 Main Phase

- (Reduced to MAP, CYP's assay)

Substance	Dose (Sham,high,low)	Post-exposure (24h/48h/72h)	N	Measurements
3R4F	3	4	6	MAP
THS2.2	3	4	6	CYP's Assay

- Objective: Compare 3R4F to THS2.2 for all MAP analyses and CYPs.

5.3 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.

5.4 Exploratory Analysis

Since very few knowledge is available from this kind 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,
- Exposure time, Dose, Post-exposure time points, will be tested and included in models if statistically relevant.
- A correction for non-equal variance, and an heterogenous variance structure will be used when applicable.

- The response to THS2.2 compared to 3R4F could be evaluated by substracting to the response the SHAM level.

In addition, the correlation between MAP analyte (or partial correction, controlling for treatment, exposure/post-exposure time) could be explored.

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.

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The transcriptomics data analysis that will be conducted will be further detailed in a separate document "Computational Analysis and Interpretation plan" articulating the biological questions and computational approaches that will be used.

6 Administrative Aspects

6.1 Study Timelines

Proposed experimental starting date:	16.09.2013
Proposed experimental completion date:	11.11.2013
Proposed date for draft study report:	21.11.2013

6.2 Names and Addresses

Study Director	Carole Mathis Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Deputy Study Director	Stefan Frentzel Quai Jeanrenaud 5 2000 Neuchâtel Switzerland

6.3 Quality Assurance

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

7 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.

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

8 References & Sources

8.1 Literature

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Pezzulo AA, Starner TD, Scheetz TE, Traver GL, Tilley AE, Harvey BG, et al. The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. American journal of physiology Lung cellular and molecular physiology. 2011;300(1):L25-31. doi: 10.1152/ajplung.00256.2010. PubMed PMID: 20971803; PubMed Central PMCID: PMC3023285.

8.2 PMI Internal Documents

PMI Internal Document No.1, 2012. Unpublished data. 2012_Batch Release Certificate 030 THS2.2 APOE I Regular signed.pdf. //disco.app.pmi/disco/drl/objectId/0901d4ec80417627

PMI Internal Document No. 2, 2013. Unpublished data. 2013_Previous results on matching doses of 3R4F smoke and THS2.2 aerosol.
//disco.app.pmi/disco/component/drl?objectId=0b01d4ec8042d56a&Reload=1379408123936

PMI Internal Document No. 3. Unpublished data. 2013_Exposure Plan_AcuteTHS2.2_Part2.xlsx.
//disco.app.pmi/disco/drl/objectId/0901d4ec8043306d

PMI Internal Document No. 4, 2013. Unpublished data. 2013_Metadatasheet_AcuteTHS2.2_Part2.xlsx. //disco.app.pmi/disco/drl/objectId/0901d4ec80434163

PMI Internal Document No. 5. Unpublished data. Protocol for CYPs protein amount determination per tissue insert 1309_02_3DOrganoCYP_DraftProtocol.docx. //disco.app.pmi/disco/drl/objectId/0901d4ec8042df39

PMI Internal Document No. 6, 2013. Unpublished data. 2013_randomized_Metadata_P170900.xlsx. //disco.app.pmi/disco/drl/objectId/0901d4ec804323ec

PMI Internal Document No.7. Unpublished data. Cytotox-ONE™ homogenous membrane integrity assay, from Promega //disco.app.pmi/disco/drl/objectId/0901d4ec803860da

PMI Internal Document No.8. Unpublished data. BioVision Bioluminescence Cytotoxicity Assay kit.pdf //disco.app.pmi/disco/drl/objectId/0901d4ec8042df3d

PMI Internal Document No. 9, 2013. Unpublished data. 2013_Sept_Project Agreement no 3_Mattek.docx. //disco.app.pmi/disco/drl/objectId/0901d4ec8042aa4b

PMI Internal Document No. 10. Unpublished data. Protocol for the purification of total RNA, including miRNA from tissues. //disco.app.pmi/disco/drl/objectId/0901d4ec8036ea84

PMI Internal Document No. 11. Unpublished data. Protocol for total protein amount determination per tissue insert WKI_EZQ.pdf. //disco.app.pmi/disco/drl/objectId/0901d4ec8042df3a

PMI Internal Document No.12. Unpublished data. Protocol for CYP1A1 and CYP1B1 activity measurement: P450 Glo Assays Protocol.pdf. //disco.app.pmi/disco/drl/objectId/0901d4ec8042df3e

PMI Internal Document No.13. Unpublished data. PMI OPS Glossary//qsmp.app.pmi/data/HQ-CH/Glossary_5.0.pdf

PMI Internal Document No.14. Unpublished data. PMI RD Glossary. //disco.app.pmi/disco/drl/objectId/0901d4ec801912d9/versionLabel/CURRENT

9 Abbreviations

Abbreviation	
ALI	Air Liquid Interface
CC	Conventional cigarettes
CS	Cigarette Smoke
EDMS	Electronic Document Management System
IHC	Immunohistochemistry
LDH	Lactate dehydrogenase
NGPs	New generation products
RIN	RNA integrity Number
RNA	RiboNucleic Acid
SDMS	Scientific Data Management System
SP	Study plan
TEER	Trans epithelial electrical resistance
THS2.2	Zurich

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

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