STUDY PLAN

HPHC (HARMFUL / POTENTIALLY HARMFUL CONSTITUENTS) SCREENING (PART II) (STUDY NUMBER 167320)

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Study Director	Ignacio Gonzalez Suarez
Author(s)	Ignacio Gonzalez Suarez
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1 Review and Approval

This document has been approved by:

Name	Justification	Date / Signature
Ignacio Gonzalez Suarez	Approved by Study Director / WP Leader	25/11/13
Emmanuel Guedj	Approved by Supervisor Gene Expression	29.11.13
Stefan Frentzel	Approved by Manager Cellular Research	29. 11. 13
Didier Goedertier	Approved by Aerosol Engineer	02 DEC 2013
Florian Martin	Approved by a senior mathematician	28/11/13
Marescotti, Diego	Approved by deputy Study Director	29/11/13
Mathis, Carole	Approved by Cellular Systems Biology Project Leader	25/11/13
Hoeng, Julia	Approved by Systems Toxicology Program Leader	4.12.2013

2 Introduction

Cigarette smoke is a complex mixture of chemicals including gases, volatile compounds contained in the gas-vapor portion (GVP) of the smoke aerosol and microscopic solid particles suspended in the smoke (total particulate matter, TPM). Overall, it is estimated that there are more than 6000 chemical constituents in tobacco smoke [1]. For obvious reasons, it is not feasible to quantify the levels of all smoke constituents, especially when there are not validated testing methods for many of them. Several regulatory agencies (e.g. Food and Drug Administration (FDA), Health Canada, etc.) have established different lists of harmful/potentially harmful constituents (HPHCs). These are compounds present in tobacco products and

tobacco smoke which cause or may cause serious health problems including cancer, lung disease, and addiction to tobacco products. Nevertheless, it is not clear how the toxic effects of individual HPHCs compare to whole smoke or smoke fractions.

Traditional toxicological risk assessment relies on the use of *in vivo* animal studies, which are often time-consuming and unable to provide high-throughput information and efficiently identify global biological perturbations [2]. In the recent years, a clear shift in the strategy of toxicological assessment of environmental agents has been started. Critical toxicity pathways perturbed upon exposure to such agents should be identified and quantified using modern tools and technologies including medium and high-throughput *in vitro* screening assays, computational toxicology, systems biology, and pharmacokinetic modeling.

This new strategy responds also to the need of finding alternative to animal testing. Although conducted *in vitro*, this new approach is based on human cell lines and thus better represents human biology in certain aspects (especially when primary cell models are used).

We will use the NHBE cells (primary culture of normal human bronchial epithelial cells obtained from a healthy adult non-smoker donor) as epithelial cells make up an efficient barrier against pathogens and aggressive molecules. These cells have been shown after exposure to cigarette smoke and pathogens, to release a variety of pro-inflammatory mediators, including chemokines, cytokines, and growth factors through various signaling pathways.

The HPHC screening study (part I) evaluated the toxic effects of 15 HPHCs on NHBE cells. The HPHC screening study (part II) will evaluate 34 additional HPHCs included in the FDA list and present in CC smoke and pMRTP aerosols at different doses and exposure times. In addition, the effects of different smoke fractions (TPM, GVP, sbPBS) from the reference cigarette 3R4F and pMRTP products (ZRH, SMAR_NAD) will be also tested.

3 Objectives

The objective is to generate robust transcriptomics data completed by classical screening assays in order to assess, *in vitro,* the biological impact of individual HPHCs and smoke fractions from CC and pMRTP at different doses and exposure times.

4 Experimental Design

4.1 Test items

4.1.1 Smoke constituents (HPHCs)

A summary of the HPHCs that will be tested is included in ***Table 1,*** together with the levels found in CC smoke and pMRTP aerosol. Some HPHCs (chromium, lead, mercury and selenium) present more than one oxidation state in nature. In these cases, the oxidation state(s) for which more toxicological data is available in the literature have been selected ***Table 1*** and has been selected as based on literature review of the respective toxic effects [3-9]. In the case of arsenic, there are two oxidation states associated with toxicity (arsenite and arsenate) [7, 10, 11] and both of them will be tested.

	HPHC	CONCENTRATION						Comments
		Units	3R4F	CC	ZRH	SMAR	EHCSS	
1	1-Aminonaphthalene	ng/L	35.7-39.4	-	0.12-0.13	-	-	
2	2-Nitropropane	ng/L	58-80	4.8-26.5	-	-	5.5-32.1	
4	5-Methylchrysene	ng/L	<1.2-2	0.4-3	-	-	0.09-0.02	
5	Acetamide	µg/L	23.8	1-21.9	6-6.5	-	1.3-5	
6	Acetone	µg/L	1111-1262	-	40.4-61.7	-	-	
7	Acrylamide	µg/L	8.3	-	2.6-2.8	-	-	
8	Arsenic	ng/L	11.4-14.6	0.9-6.7	<1.9	-	<4	8.a. Sodium arsenate 8.b. Sodium arsenite
9	Benz[a]anthracene	ng/L	48	5.2-38.5	2.2-3.7	-	0.2-1.1	
10	Benzene	µg/L	165	9.7-106	0.8-1	1.9-2.8	0.6-3.4	
11	Benzo[a]pyrene	ng/L	26.2	3.2-23.8	2	-	0.05-0.64	
12	Benzo[b]fluoranthene	ng/L	15.8-26.7	2.4-19.3	-	-	0.05-0.54	
13	Benzo[k]fluoranthene	ng/L	6.4 -10.8	1.7-6.9	-	-	0.03-0.19	
14	Chromium	ng/L	<0.9	<0.5-3.1	<0.83-2.8	-	<1.2-3.2	Sodium Chromate
15	m-cresol	(m-) ng/L	5.2	-	0.02-0.04	-	-	
16	o-cresol	(o-) ng/L	7.7	-	0.04-0.14	-	-	
17	p-cresol	(p-) ng/L	15.7	-	0.04-0.13	-	-	
18	Crotonaldehyde	µg/L	118-166	<2.6-36	4.9-6.2	-	<1.2-3.7	
19	Dibenz[a,h]anthracene	ng/L	2.9	<0.9-1.4	<0.15	-	<0.03-0.06	
20	Dibenzo[a,e]pyrene	ng/L	0.5-1.7	<0.31-0.86	-	-	<0.03-0.12	
21	Dibenzo[a,h]pyrene	ng/L	<0.7-1.2	<0.2-0.36	-	-	<0.07-0.24	
22	Dibenzo[a,i]pyrene	ng/L	<0.7-1.1	<0.2-0.73	-	-	<0.05-0.2	
23	Dibenzo[a,l]pyrene	ng/L	<0.6-1	<0.2-0.74	-	-	<0.03-0.1	
24	Indeno[1,2,3-cd]pyrene	ng/L	9.1-14.9	1.4-11	-	-	<0.02-0.34	
25	Lead	ng/L	52-63	1.3-44	<5.1	-	<3.9-6.4	Lead (II) nitrate
26	Mercury	ng/L	8.2	-	1.3-2	-	-	Mercury (II) chloride
27	Methyl ethyl ketone	µg/L	321	-	10.6	-	-	
28	Naphthalene	µg/L	2.8	-	0.01	-	-	
29	Nickel	ng/L	0.9-2.4	-	<0.8	-	3.81	Nickel (II) chloride
30	Nitrobenzene	ng/L	14.8	-	<0.3-0.5	-	-	
31	o-Anisidine	ng/L	8.5	0.9-6.2	0.07	-	0.06-0.5	
32	Phenol	µg/L	23	1-47.3	0.7-2.4	4.7-8.7	0.02-4	
33	Quinoline	µg/L	0.85	-	<0.02	-	-	
34	Selenium	ng/L	2.8	-	<1-1.2	-	-	Sodium selenite
35	Toluene	µg/L	322	12-184	3.6	1.3-1.6	1.6-7.2	

Table 1. Concentration of HPHCs in CC and pMRTPs. *The oxidation states that will be tested for arsenic, chromium, lead, mercury, nickel and selenium are included under the comments column.*

4.1.2 Smoke / aerosol fractions

All smoke fractions (TPM, GVP, sbPBS) will be generated according to Health Canada Intense (HCI) smoking profile. The items to be tested within the study are the reference cigarette 3R4F and the pMRTP products ZRH 4.2 and SMAR_NAD. (***Table 2***).

Description	Pack Code	Type	Batch number	Short Name
ZRH/DDA1/C3/FR1/CAST LEAF-CL/Flavor/Reynaldo	-	test	B-05879	ZRH_4.2
SMAR_non Alu Disk	-	test	https://disco.app.pmi/disco/drl/objectId/0901d4ec8033cec1	SMAR_NAD
3R4F	3R4F	reference	NA	3R4F_HC

Table 2. *Test and reference items.*

ZRH test item is composed of a device (CH FDP4.2) and a sensorial media (Sens Med-1684). ZRH_4.2 test item will be provided by PHILIP MORRIS International, Neuchâtel, Switzerland, with the production of 2000 sensorial media. The sensorial media will be packed in cartons which will contain a bag with sensorial media shrink-wrapped in aluminium foil. The aluminium bag will be labelled with the production lot. No. and the quantity and the production date.

Four boxes of SMAR_NAD test item, each box containing 4000 items, were provided by PHILIP MORRIS Neuchatel in May 2012. SMAR_NAD is generated in one production batch and taken from the 5th batch of the COPD II study (Batch Release Certificate 007 SMAR-5410 COPD II.pdf, http://disco.app.pmi/disco/drl/objectId/0901d4ec8033d012).

Reference Cigarettes 3R4F (30.000 cigarettes) were purchased from the University of Kentucky, Kentucky Tobacco Research and Development Center (for specifications see ***Table 3*** and http://www.beitraege-bti.de/pdfs/2012-25-01-316.pdf). 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.

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

Table 3. Specifications for the reference cigarette 3R4F. *The smoke analysis data were generated under Federal Trade Commission (FTC) conditions*

4.1.2.1 Generation of smoke samples

As state above, the sensorial media for ZRH, SMAR_NAD and the Reference Cigarette 3R4F will be conditioned according to ISO standard 3402 (1999), i.e., for at least 48h at target conditions of 22±1°C and a relative humidity of 60±3% before being used for smoke generation. The test atmosphere for smoke generation will be 22±2°C and 60±5% relative humidity. The mainstream smoke for test and reference item will be generated under HC smoking regimen. For the HC smoking protocol, 3R4F cigarettes will be 100% vent-blocked by taping (SOP AC 226). The sensorial media as well as the SMAR_NAD test items will not be taped because there are no ventilation holes in the filter region. The smoking regimens are given in ***Table 4***

Short Name	Puff Volume (ml)	Puff Duration (s)	Puff Frequency (min^{-1})	Puff Count
3R4F_HC	55	2	2	to a butt length of 35±1 mm = X puffs
SMAR _NAD	55	2	2	12
ZRH_4.2	55	2	2	12 (predefined)

Table 4. Smoking regimens

The 3R4F cigarettes will be smoked on the 20-port rotary smoking machine Borgwaldt RM20H. For the HC smoking regimen, the machine will be used in the 10-port mode due to technical reasons. The smoking machine settings are summarized in ***Table 5*** and ***Table 6***.

Short Name	Set No.	Puff Profile No.	Puff Volume (ml)	Puff Time [1] (s)	Piston Sweep Time (s)	Puff Frequency (min^{-1})	Period Duration (s)	Pause Time (s)	Volume Correction Factor
3R4F_HC	2	0 [2]	55	2	1.9	2	30	28	variable [3]

***Table 5. Smoking Machine Settings for RM20H**, Puff Parameter Sets. [1] The following phrases may be used in parallel: Puff Time = Duration; Piston Sweep Time = Intermission; Puff Frequency = Time. [2] Zero corresponds to the bell-shaped profile. [3] The volume correction factor will be adjusted to meet the puff volume (see SOP-G_AW_004).*

Short Name	Set No.	Cigarette length (mm)	Butt length (min^{-1})	Ligther Capacity (W)	Light Twice	Puff Count
3R4F_HC	0	84	35	70	NO	To a but length according to ISO (35mm)

***Table 6. Smoking Machine Settings for RM20H**, Cigarette Parameter Set.*

Aerosols derived from Zurich 4.2 test items with the tobacco heating system (THS™, FPD4.2) are generated using 30-port rotary smoking machines (Type: SM_SDDS_ZRH) equipped with a Programmable Dual Syringe Pump (PDSP). The smoking machine is equipped with up to 30 Functional Prototype Devices (type FPD4.2) of the cigarette holder and corresponding smoking device docking station as an electrical interface between the cigarette holder and the smoking machine. The smoking machine is controlled by a programmable logic control system allowing either an automated operation of the smoking machine. The SM_SDDS_ZRH machines will be equipped with a temperature-controlled insulation kit (surface warming system) in the concentrated aerosol path, with the goal of reducing aerosol condensation. This comprises

water warmed surfaces for all components (i.e. tubes, PDSP valve, pump unit, and manifold to dilution) from the suction port of the smoking machine up to a manifold assembled to the dilution system. The sensorial media (Sens Med-1684) of the ZRH test article will be smoked with a bell-shaped puff profile under HC smoking profile. The sensorial media will be electrically heated in the cigarette holder with a defined heating pattern. In order to obtain a good and steady level of smoke, the smoking machine will need to be run for 1h prior to the smoke generation. The predefined puff count for ZRH is 12 puffs, however, the puff count can be less due to some technical reasons (e.g. batteries may be empty before). Therefore, for the calculation of nicotine / water content it is important to calculate with the total puff count and not with the amount of sticks. In addition, the smoking profile may give some slight variation due to differences in delivery between the different puffs of a ZRH test item (first puff a ZHR is very different from a later puff).

SMAR_NAD items will be smoked on 30-port rotary smoke machines (type PMRL-G, SM2000/ SMAR) used in a 15-port mode with active side stream smoke exhaust equipped with PDSP. The SM2000/SMAR is equipped with 2 lighters (heat coils) for the lighting of the SMAR. The smoking machine cabinet is supplied with conditioned air and connected to an exhaust ventilation system to stabilize the temperature conditions in the handling area of the SMAR_NAD. The items will be electrically lit using heating coil lighters. A pneumatic actuator will automatically move the SMAR with its charcoal heat source into the heating coil lighter during the lighting period (insertion depth 7mm= charcoal end line with first coil spiral) The SMAR will be lit for 25s at 50W and immediately inserted into the carrousel during 1 rotation, i.e., complete loading of the carrousel needs 8 rotations when 15 ports are used. All samples from the SMAR are collected immediately before the pump outside of the carrousel at a distance of 60cm from the suction port for technical reasons.

4.1.2.2 Generation of smoke fractions

TPM samples will be generated from each test item in independent smoke runs. TPM will be collected on glass fiber filters (44mm diameter). TPM is extracted by syringe extraction method with 5ml of ethanol in a glass vessel. For 3R4F, mainstream smoke from a total of 6 cigarettes, divided in two batches of 3 items, will be trapped on two separate glass fiber filters followed by ethanol extraction. Hereby the first filter is extracted with 5ml of ethanol. The second filter is extracted with the first crude extract. For ZRH and SMAR_AD, mainstream smoke from a total of 10 sticks, divided in two batches of 5 sticks, will be trapped on 2 separate glass fiber filters followed by extraction. Hereby the first filter is extracted with 5ml of ethanol. The second filter is extracted with the first crude extract.

GVP, defined as the substance which passes through the glass fiber filter, will be collected by bubbling the smoke / aerosol through ice-cold PBS in a gas wash bottle. For 3R4F, mainstream smoke from a total of 6 cigarettes, divided in two batches of 3 items, will be trapped in 36ml of PBS. For ZRH, mainstream smoke from a total of 10 cigarettes, divided in two batches of 5 items, will be trapped in 40ml of PBS. For SMAR_NAD, mainstream smoke from a total of 10 cigarettes, divided in two batches of 5 items, will be trapped in 25ml of PBS.

In a separate smoke run, **sbPBS** will be generated by bubbling whole smoke through ice-cold PBS in a gas wash bottle. The number of sticks and volume of PBS for each item are the same as in the case of GVP.

The amount of sensorial media / sticks to be smoked for the generation of the different fractions, as well as the amount of solvent is detailed in ***Table 7.***

Short Name	TPM		GVP		TPM	
	Items	EtOH	Items	PBS	Items	PBS
ZRH	2 X 5	5 ml	2 X 5	40ml	10	40 ml
SMAR_NAD	2 X 5	5 ml	2 X 5	25 ml	10	25 ml
3R4F	2 X 3	5 ml	2 X 3	36 ml	6	36 ml

Table 7. Experimental details for the preparation of TPM, GVP and sbPBS

NOTE: During the validation test of the ZRH smoking machine the smoke collection protocols were optimized, thus allowing better sample to sample reproducibility. The changes are detailed in ***Table 8.*** The conditions for the 3R4F and SMAR_NAD have not changed.

Short Name	TPM		GVP		sb PBS	
	Items	EtOH	Items	PBS	Items	PBS
ZRH (current)	2 X 5	5 ml	2 X 5	40 ml	10	40 ml
ZRH (previous)	2 X 15	5 ml	2 X 15	25 ml	2 X 15	25 ml

Table 8. Modifications in the preparation of TPM, GVP and sbPBS from ZRH

These conditions will be different from those used in previous studies (e.g. FIAS study http://disco.app.pmi /disco/drl/objectId/0901d4ec80370f2a). The toxicity of ZRH fractions under the new experimental conditions will be tested for the first time as part of this study.

4.1.2.3 Analytical determination of Nicotine and Carbonyls in TPM, GVP and sbPBS

Nicotine will be determined in TPM samples by gas chromatography with FID detection using isoquinolin as internal standard. Water will be also determined by gas chromatography with TCD detection using methanol as internal standard.

Carbonyls (formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, methyl-ethyl-ketone and butyraldehyde) will be determined in GVP and sbPBS samples. For this purpose, an aliquot of 400µl of the bubbled PBS will be derivatized with 600µl of DNPH solution for 30 min and then quenched with 50µl of pyridine. The concentration of all 8 carbonyls will be measured by LC-ESI-MS/MS using deuterated species as internal standards.

4.2 Exposure and cellular analysis

There is limited information available on the toxicity of the selected HPHC in NHBE cells. In the case of the smoke fractions, there is data available from previous studies (e.g. FIAS), however, we will be testing new experimental conditions (e.g. generation of ZRH aerosol fractions) as well as novel technologies (xCELLigence, High Content Screening) which have not been systematically applied internally to CC or pMRTPs. Therefore, initial dose-range finding experiments will be necessary in order to define the most appropriate doses for the HPHCs and smoke fractions. The experimental part of the study will be divided in 3 phases:

1. Phase I: Dose-range finding experiments using the xCELLigence system.

2. Phase II: Cellular analysis using High Content Screening (HCS).
3. Phase III: RNA extraction and transcriptomics.

4.2.1 In vitro system: NHBE cells

The use of NHBE cells with a limited capacity of cell divisions avoids immortalization-related artifacts usually present in cell lines; however, the inter-individual differences cause larger batch-to-batch variations than those within an immortalized cell line.

The NHBE cells used within this study are primary cells obtained from a 60-year-old male Caucasian, an alcohol-using (i.e. used to alcohol but not in a diseased state), non-smoking donor and were purchased from Lonza (CC-2540). **Lot no. 140733 of cells from this donor will be used in the study** (same donor used in the HPHC screening, FIAS and HPHC mixture assessment studies)

From this primary cell line, stock cultures were prepared (passage 2). The stock cultures are stored in liquid nitrogen with 10% (v/v) DMSO. A stock culture (passage 3-8 will be used) comprising approximately 10^6 cells in 1ml will be quick-thawed, diluted with 20ml bronchial epithelial cell medium (Lonza, bullet kit CC-3170), plated in a 75cm^2 culture flask, and incubated at 37.0±1°C in a humidified incubator with 5.0±0.5% CO_2 for 24h±1 h. After this time, the medium will be changed and the cells will be maintained in culture, changing the media every other day in order to obtain enough cells for the experimental phase. When cells cultured in a flask reaches 80-90% confluence, cells will be split into two 75cm^2 culture flasks.

For harvesting, cells will be trypsinized (Lonza, reagent pack CC-5034), neutralized with trypsin-neutralization solution, centrifuged at 1000 rpm for 5min, and re-suspended in bronchial epithelial cell culture medium. The cell concentration will be adjusted according to the plate/ flask format for subsequent analysis. Cellular morphology will be checked routinely to display stable characteristics (microscopic check). Only mycoplasma-free cell cultures showing the typical morphology of the NHBE cells will be used[1]

4.2.2 *Phase I*: Dose-range finding (xCELLigence)

The aim of *Phase I* is to evaluate the toxicity of the HPHCs and the smoke fractions in NHBE cells and determine the appropriate doses to be investigated in more detail. The experiments in Phase I will be performed in the xCELLigence system.

The xCELLigence system is a real-time cellular analysis platform based on the multi-electrode array technology. The system uses tissue culture plates with sensor micro-elecrodes covering approximately 70% of the area of each well bottom. The presence of the cells on top of the electrodes will affect the local ionic environment at the electrode/solution interface, leading to an increase in the electrode impedance. The more cells are attached on the electrodes, the larger the increases in electrode impedance. In addition, the impedance depends on the quality of the cell interaction with the electrodes. For example, increased cell

[1] All cell cultures used in team CRL are routinely checked for absence of mycoplasma contamination according to SOP CRS 4.

adhesion or spreading will lead to a larger change in electrode impedance. Thus, electrode's impedance, can be used to monitor cell viability and cell number (adapted from Bucher Biotec, http://www.bucher.ch/en/products/ acea/technology.html). The cellular lab at PMI Neuchatel is equipped with an xCELLigence RTCA MP station (ACEA biosciences), which can analyse up to 6 different 96-well plates independently.

4.2.2.1 Smoke constituents (HPHCs)

All compounds will be dissolved in the appropriate vehicle (ethanol or PBS) and diluted to a final concentration of 2% ethanol or 12.5% PBS in cell culture medium. In a previous in-house study ethanol showed toxicity (more than 20% decrease in cell viability after 24h exposure) at concentrations in the culture media above 2% (v/v). A similar result was observed with concentrations of PBS above 12.5%. The maximum concentration to be tested for each HPHC will be 20mM (twenty times higher than ICH recommendations for testing genotoxicity in mammalian cell assays [12]). If no toxicity is observed at the highest dose, higher concentrations could be tested, providing that the final concentration of vehicle is maintained below 2% ethanol and 12.5% PBS. On the contrary, if solubility is a limiting factor, we will use the lowest HPHC concentration at which minimal precipitate is observed. The remaining doses will be prepared by serial dilution.

NHBE cells will be seeded in an uncoated ACEA 96-well tissue culture plate at a density of 7000 cells in 100µl of culture media per well and incubated for 24h. Cells will be then exposed (in three replicates) to 7 different doses of the HPHC synthetic mixture and 3R4F whole-smoke condensate for additional 24h. Appropriate positive controls (staurosporine) and negative controls (ethanol, PBS, culture medium) will be incorporated into each plate. The plate layout is detailed in ***Figure 1***.

	1	2	3	4	5	6	7	8	9	10	11	12
A	HPHC 1Dose 1	HPHC 1Dose 1	HPHC 1Dose 1	HPHC 2 Dose 1	HPHC 2 Dose 1	HPHC 2 Dose 1	HPHC 3 Dose 1	HPHC 3 Dose 1	HPHC 3 Dose 1	HPHC 4 Dose 1	HPHC 4 Dose 1	HPHC 4 Dose 1
B	HPHC 1Dose 2	HPHC 1Dose 2	HPHC 1Dose 2	HPHC 2 Dose 2	HPHC 2 Dose 2	HPHC 2 Dose 2	HPHC 3 Dose 2	HPHC 3 Dose 2	HPHC 3 Dose 2	HPHC 4 Dose 2	HPHC 4 Dose 2	HPHC 4 Dose 2
C	HPHC 1Dose 3	HPHC 1Dose 3	HPHC 1Dose 3	HPHC 2 Dose 3	HPHC 2 Dose 3	HPHC 2 Dose 3	HPHC 3 Dose 3	HPHC 3 Dose 3	HPHC 3 Dose 3	HPHC 4 Dose 3	HPHC 4 Dose 3	HPHC 4 Dose 3
D	HPHC 1Dose 4	HPHC 1Dose 4	HPHC 1Dose 4	HPHC 2 Dose 4	HPHC 2 Dose 4	HPHC 2 Dose 4	HPHC 3 Dose 4	HPHC 3 Dose 4	HPHC 3 Dose 4	HPHC 4 Dose 4	HPHC 4 Dose 4	HPHC 4 Dose 4
E	HPHC 1Dose 5	HPHC 1Dose 5	HPHC 1Dose 5	HPHC 2 Dose 5	HPHC 2 Dose 5	HPHC 2 Dose 5	HPHC 3 Dose 5	HPHC 3 Dose 5	HPHC 3 Dose 5	HPHC 4 Dose 5	HPHC 4 Dose 5	HPHC 4 Dose 5
F	HPHC 1Dose 6	HPHC 1Dose 6	HPHC 1Dose 6	HPHC 2 Dose 6	HPHC 2 Dose 6	HPHC 2 Dose 6	HPHC 3 Dose 6	HPHC 3 Dose 6	HPHC 3 Dose 6	HPHC 4 Dose 6	HPHC 4 Dose 6	HPHC 4 Dose 6
G	HPHC 1Dose 7	HPHC 1Dose 7	HPHC 1Dose 7	HPHC 2 Dose 7	HPHC 2 Dose 7	HPHC 2 Dose 7	HPHC 3 Dose 7	HPHC 3 Dose 7	HPHC 3 Dose 7	HPHC 4 Dose 7	HPHC 4 Dose 7	HPHC 4 Dose 7
H	Medium	Medium	Medium	Vehicle 1	Vehicle 1	Vehicle 1	Vehicle 2	Vehicle 2	Vehicle 2	Positive Control	Positive Control	Positive Control

Figure 1. Plate layout for cytotoxicity assay (xCELLigence). *4 HPHCs and 7 doses will be tested per plate. Positive and negative controls (medium alone and vehicles) will be included in each plate.*

The exposure will be performed by adding 25µl of culture medium containing the different doses, positive and negative controls at a 5X concentration. Impedance will be measured every hour during the 24h pre-exposure, and every 15 minutes for 24h after exposure (in order to accurately capture cytotoxicity). The doses to be tested are detailed below:

1. *Medium*: Cells will be incubated in culture medium alone. This control will allow us to monitor basal cell viability in NHBE cells.
2. *Vehicle (1&2)*: Cells will be exposed to 2% ethanol or 12.5% PBS.
3. *Positive control*: Staurosporine 300µM.
4. *HPHC doses:*

Dose 1: 20mM *Dose 2:* 2mM *Dose 3*: 200µM

Dose 4: 20µM *Dose 5*: 2µM *Dose 6*: 200nM

Dose 7: 20nM

The experiment will be repeated three times on different plates. Based on the results from the first experiment, the doses for a given HPHC may be slightly modified in order to better define the toxic dose ranges. All concentrations tested will be included in the final report. Based on the current design the estimated number of NHBE cells required will be:

- **7,000cells X 96 wells X 3 experimental repeats X 9 plates= 18.15x10^6 cells**

4.2.2.2 Smoke fractions (TPM, GVP, sbPBS)

Previous in-house studies investigated *in vitro* the toxicity of different smoke fractions from 3R4F and pMRTPs in NHBE cells using the resazurin assay (***Table 9***). The data will be used as a guideline for the dose range finding experiments, in order to bridge past results to the new experimental conditions that will be tested in this study.

	Item	EC50	
		Cig / L	Puff/ml
TMP	3R4F	2.1	0.021
	ZRH	57.5	0.66
	SMAR_NAD	N/A	N/A
GVP	3R4F	>6	>0.06
	ZRH	60	0.72
	SMAR_NAD	N/A	N/A
sbPBS	3R4F	6	0.06
	ZRH	65	0.78
	SMAR_NAD	N/A	N/A

Table 9. Estimated EC50 values for TPM, GVP and sbPBS (based on the FIAS study) *EC50 values for SMAR_NAD are not available. The highest dose tested resulted in less than 50% toxicity compared to vehicle control).*

The maximum dose to be tested for each item and fraction will be the maximum that can be achieved while maintaining a final concentration of vehicle in the culture medium of 2% ethanol and 12.5% PBS. The calculations are detailed in ***Table 10***.

	Item	Vehicle (max amount)	Fraction generation		Max dose
			items	vehicle	Puff/mL
TMP	3R4F	2% (ethanol)	6	5ml	0.24
	ZRH	2% (ethanol)	10	5ml	0.48
	SMAR_NAD	2% (ethanol)	10	5ml	0.48
GVP	3R4F	12.5% (PBS)	6	36ml	0.22
	ZRH	12.5% (PBS)	10	40ml	0.37
	SMAR_NAD	12.5% (PBS)	10	25ml	0.6
sbPBS	3R4F	12.5% (PBS)	6	36ml	0.22
	ZRH	12.5% (PBS)	10	40ml	0.37
	SMAR_NAD	12.5% (PBS)	10	25ml	0.6

Table 10. Maximum doses to be tested for each item and smoke fractions. *The maximum doses have been calculated based on the number of items and volume of vehicle used for fraction collection and assuming 12 puffs/item for ZRH and SMAR and 10 puffs/item for 3R4F.*

NHBE cells will be seeded in an uncoated ACEA 96-well tissue culture plate at a density of 7000 cells in 100µl of culture media per well and incubated for 24h. Cells will be then exposed (in three replicates) to 7 different doses of the HPHC synthetic mixture and 3R4F whole-smoke condensate for additional 24h. Appropriate positive controls (3R4F TPM, Staurosporine) and negative controls (ethanol, PBS, culture medium) will be incorporated into each plate. The plate layout is detailed in ***Figure 2*** and will be the same for all fractions.

	1	2	3	4	5	6	7	8	9	10	11	12
A	3R4F Dose 1	3R4F Dose 1	3R4F Dose 1	ZRH Dose 1	ZRH Dose 1	ZRH Dose 1	SMAR Dose 1	SMAR Dose 1	SMAR Dose 1			
B	3R4F Dose 2	3R4F Dose 2	3R4F Dose 2	ZRH Dose 2	ZRH Dose 2	ZRH Dose 2	SMAR Dose 2	SMAR Dose 2	SMAR Dose 2			
C	3R4F Dose 3	3R4F Dose 3	3R4F Dose 3	ZRH Dose 3	ZRH Dose 3	ZRH Dose 3	SMAR Dose 3	SMAR Dose 3	SMAR Dose 3			
D	3R4F Dose 4	3R4F Dose 4	3R4F Dose 4	ZRH Dose 4	ZRH Dose 4	ZRH Dose 4	SMAR Dose 4	SMAR Dose 4	SMAR Dose 4			
E	3R4F Dose 5	3R4F Dose 5	3R4F Dose 5	ZRH Dose 5	ZRH Dose 5	ZRH Dose 5	SMAR Dose 5	SMAR Dose 5	SMAR Dose 5			
F	3R4F Dose 6	3R4F Dose 6	3R4F Dose 6	ZRH Dose 6	ZRH Dose 6	ZRH Dose 6	SMAR Dose 6	SMAR Dose 6	SMAR Dose 6			
G	3R4F Dose 7	3R4F Dose 7	3R4F Dose 7	ZRH Dose 7	ZRH Dose 7	ZRH Dose 7	SMAR Dose 7	SMAR Dose 7	SMAR Dose 7			
H	Medium	Medium	Medium	Vehicle 1	Vehicle 1	Vehicle 1	Positive Control	Positive Control	Positive Control			

Figure 2. Plate layout for cytotoxicity assay (xCELLigence). *3 test items and 7 doses will be tested per plate. Positive and negative controls (medium alone and vehicle) will be included in each plate.*

The exposure will be performed by adding 25µl of culture medium containing the different doses, positive and negative controls at a 5X concentration. Impedance will be measured every hour during the 24h pre-exposure, and every 15 minutes for 24h after exposure (in order to accurately capture cytotoxicity). The doses to be tested are detailed below:

1. *Medium*: Cells will be incubated in culture medium alone. This control will allow us to monitor basal cell viability in NHBE cells.
2. *Vehicle*: Cells will be exposed to 2% ethanol or 12.5% PBS at the same concentration used as vehicle in the stimuli.
3. *Positive control*: Staurosporine 300µM.
4. *Fraction doses (GVP / sbPBS):*

   3R4F: 0.031, 0.062, 0.093, 0.124, 0.155, 0.186, 0.22 puffs/ml

   ZRH: 0.053; 0.106; 0.159; 0.212; 0.265; 0.318; 0.370 puffs/ml

   SMAR_NAD: 0.086; 0.172; 0.258; 0.344; 0.430; 0.516; 0.600 puffs/ml

   Fraction doses (TPM)

   3R4F: 0.034, 0.068, 0.102, 0.136, 0.170, 0.204, 0.24 puffs/ml

   ZRH: 0.069; 0.138; 0.207; 0.276; 0.345; 0.414; 0.48 puffs/ml

   SMAR_NAD: 0.069; 0.138; 0.207; 0.276; 0.345; 0.414; 0.48 puffs/ml

The experiment will be repeated three times on different plates. Based on the results from the first experiment, the doses for a given item / fraction may be slightly modified in order to better define the toxic dose ranges. All concentrations tested will be included in the final report. Based on the current design the estimated number of NHBE cells required will be:

- **7,000cells X 72 wells X 3 experimental repeats X 3 fractions= $4.5x10^6$ cells**

4.2.3 *Phase II*: Toxicological evaluation (HCS)

A total of 13 multi-parametric indicators of toxicity, grouped in 7 different assays (cytotoxicity screening panel, DNA damage, oxidative stress, glutathione content, apoptosis / necrosis, proliferation and stress kinase) will be measured in NHBE cells using a high content screening (HCS) platform with automated fluorescence imaging system (Cellomics Arrayscan VTI, Thermo Fisher). HCS is an automated method used to identify substances that alter the phenotype of a cell in a particular manner. This technology results from a combination of fluorescence or I microscopy, a high-throughput image acquisition system and a series of algorithms and software tools that allows for image processing and analysis. Using a combination of antibodies and fluorescent dyes, the HCS technology can detect changes in target intensity and localization, and cellular morphology in live or fixed cells [13]. The cellular lab at PMI Neuchatel is equipped with two *Cellomics* Array Scan VTI readers (Thermo scientific). Seven assays covering 13 different biological endpoints have been established in NHBE cells by our external collaborator Cyprotex in the context of the HPHC impact assessment study (https://disco.app.pmi/disco/ drl/objectId/0901d4ec8036b1cc.

The list of the endpoints is detailed in **Table 11:**

Assay	#	Biological endpoint	Description
Included in all assays	1	Cell count	A decreasing number of cells per well indicates toxicity due to necrosis, apoptosis or a reduction in cellular proliferation
	2	Nuclear area	An increase in nuclear size can indicate necrosis or G2 cell cycle arrest and a decrease can indicate apoptosis
	3	DNA structure	An increase in DNA structure can indicate chromosomal instability and DNA damage
Cytotoxicity screening panel	4	Mitochondrial mass	A decrease in mitochondrial mass indicates loss of total mitochondria and an increase implies mitochondrial swelling or an adaptive response to cellular energy demands
	5	Mitochondrial membrane potential	A decrease indicates mitochondrial toxicity, as well as a potential role in apoptosis signaling, an increase in mitochondrial membrane potential indicates an adaptive response to cellular energy demands
	6	Cell membrane permeability	An increase in cell membrane permeability is a general indicator of cell death
	7	Cytochrome C release	An increase in cytochrome c release is one of the hallmarks of the apoptosis signaling cascade
DNA damage	8	phospho-H2AX	H2AX phosphorylation (detected by immunostaining) occurs following the induction of DNA double strand breaks (correlates with neutral comet assay – PMID: 17064697).
Oxidative stress	9	DHE	Dihydroethidium (DHE) is a non-fluorescent cell-permeable ROS-sensitive probe. It allows the detection of intracellular superoxide anion production by turning fluorescent upon oxidation.
Gluthatione content	10	GSH	Monochlorobimane added to the culture medium readily enters cells to form a fluorescent GSH-monochlorobimane adduct that can be measured fluorometrically. This reaction is catalyzed by glutathione S-transferase and allows the quantitative measurement of intracellular glutathione that is the principal intracellular low-molecular-weight thiol and plays a critical role in the cellular defense against agents that impose oxidative stress.
Apoptosis / necrosis	11	Caspase 3/7	An increase in caspase 3 / 7 activity indicates the onset of the cell signaling cascade leading to cell death by apoptosis.
	12	Cell membrane permeability	An increase in cell membrane permeability is a general indicator of cell death
Proliferation	13	phospho-H3	An increase in the mitotic marker phospho histone H3 (pH3) indicates an increase of cells in prophase due either to increase proliferation or to growth arrest. A decrease in pH3 indicates a decrease of cells undergoing mitosis.
Stress kinase	14	Phospho-cJun	An increase in phosphorylated-cJun indicates the upregulation of the stress kinase pathway, which includes downstream targets such as cell differentiation and apoptosis.

Table 11. List of HCS-based toxicity endpoints.

NHBE cells will be seeded in black, clear-bottom 96-well tissue culture plates at a density of 12000 cells in 100µl of culture media per well (3000 cells for the proliferation assay). Cells will be incubated for 24h in the culture media and then exposed (in three replicates) to HPHCs or the smoke fractions of 3R4F, ZRH or SMAR_NAD. Two different exposure times: 4h and 24h will be tested. Appropriate positive controls (specific for each endpoint) will be used. As a negative control, we will expose the cells to the appropriate vehicles. Three HPHCs can be assayed simultaneously in one plate. For TPM, GVP and sbPBS, all three items will be tested in the same plate. The plate layout is detailed in **Figure 3** and **Figure 4**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	HPHC 1 Dose 1	HPHC 1 Dose 2	HPHC 1 Dose 3	HPHC 1 Dose 4	HPHC 1 Dose 5	HPHC 1 Dose 6	HPHC 1 Dose 7	HPHC 1 Dose 8	HPHC 1 Dose 9	HPHC 3 Dose 1	HPHC 3 Dose 1	HPHC 3 Dose 1
B	HPHC 1 Dose 1	HPHC 1 Dose 2	HPHC 1 Dose 3	HPHC 1 Dose 4	HPHC 1 Dose 5	HPHC 1 Dose 6	HPHC 1 Dose 7	HPHC 1 Dose 8	HPHC 1 Dose 9	HPHC 3 Dose 2	HPHC 3 Dose 2	HPHC 3 Dose 2
C	HPHC 1 Dose 1	HPHC 1 Dose 2	HPHC 1 Dose 3	HPHC 1 Dose 4	HPHC 1 Dose 5	HPHC 1 Dose 6	HPHC 1 Dose 7	HPHC 1 Dose 8	HPHC 1 Dose 9	HPHC 3 Dose 3	HPHC 3 Dose 3	HPHC 3 Dose 3
D	HPHC 2 Dose 1	HPHC 2 Dose 2	HPHC 2 Dose 3	HPHC 2 Dose 4	HPHC 2 Dose 5	HPHC 2 Dose 6	HPHC 2 Dose 7	HPHC 2 Dose 8	HPHC 2 Dose 9	HPHC 3 Dose 4	HPHC 3 Dose 4	HPHC 3 Dose 4
E	HPHC 2 Dose 1	HPHC 2 Dose 2	HPHC 2 Dose 3	HPHC 2 Dose 4	HPHC 2 Dose 5	HPHC 2 Dose 6	HPHC 2 Dose 7	HPHC 2 Dose 8	HPHC 2 Dose 9	HPHC 3 Dose 5	HPHC 3 Dose 5	HPHC 3 Dose 5
F	HPHC 2 Dose 1	HPHC 2 Dose 2	HPHC 2 Dose 3	HPHC 2 Dose 4	HPHC 2 Dose 5	HPHC 2 Dose 6	HPHC 2 Dose 7	HPHC 2 Dose 8	HPHC 2 Dose 9	HPHC 3 Dose 6	HPHC 3 Dose 6	HPHC 3 Dose 6
G	Control Dose 1	Control Dose 1	Control Dose 1	Vehicle 2	Vehicle 2	Vehicle 2	Vehicle 1	Vehicle 1	Vehicle 1	HPHC 3 Dose 7	HPHC 3 Dose 7	HPHC 3 Dose 7
H	Control Dose 2	Control Dose 2	Control Dose 2	Control vehicle	Control vehicle	Control vehicle	HPHC 3 Dose 9	HPHC 3 Dose 9	HPHC 3 Dose 9	HPHC 3 Dose 8	HPHC 3 Dose 8	HPHC 3 Dose 8

Figure 3. Plate layout for testing HPHCs using HCS. *Three different samples, 9 doses and one time point will be assayed in one plate.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	3R4F Dose 1	3R4F Dose 2	3R4F Dose 3	3R4F Dose 4	3R4F Dose 5	3R4F Dose 6	3R4F Dose 7	3R4F Dose 8	3R4F Dose 9	SMAR Dose 1	SMAR Dose 1	SMAR Dose 1
B	3R4F Dose 1	3R4F Dose 2	3R4F Dose 3	3R4F Dose 4	3R4F Dose 5	3R4F Dose 6	3R4F Dose 7	3R4F Dose 8	3R4F Dose 9	SMAR Dose 2	SMAR Dose 2	SMAR Dose 2
C	3R4F Dose 1	3R4F Dose 2	3R4F Dose 3	3R4F Dose 4	3R4F Dose 5	3R4F Dose 6	3R4F Dose 7	3R4F Dose 8	3R4F Dose 9	SMAR Dose 3	SMAR Dose 3	SMAR Dose 3
D	ZRH Dose 1	ZRH Dose 2	ZRH Dose 3	ZRH Dose 4	ZRH Dose 5	ZRH Dose 6	ZRH Dose 7	ZRH Dose 8	ZRH Dose 9	SMAR Dose 4	SMAR Dose 4	SMAR Dose 4
E	ZRH Dose 1	ZRH Dose 2	ZRH Dose 3	ZRH Dose 4	ZRH Dose 5	ZRH Dose 6	ZRH Dose 7	ZRH Dose 8	ZRH Dose 9	SMAR Dose 5	SMAR Dose 5	SMAR Dose 5
F	ZRH Dose 1	ZRH Dose 2	ZRH Dose 3	ZRH Dose 4	ZRH Dose 5	ZRH Dose 6	ZRH Dose 7	ZRH Dose 8	ZRH Dose 9	SMAR Dose 6	SMAR Dose 6	SMAR Dose 6
G	Control Dose 1	Control Dose 1	Control Dose 1				Vehicle 1	Vehicle 1	Vehicle 1	SMAR Dose 7	SMAR Dose 7	SMAR Dose 7
H	Control Dose 2	Control Dose 2	Control Dose 2	Control vehicle	Control vehicle	Control vehicle	SMAR Dose 9	SMAR Dose 9	SMAR Dose 9	SMAR Dose 8	SMAR Dose 8	SMAR Dose 8

Figure 4. Plate layout for testing smoke fractions using HCS. *For each smoke fraction, all three items, 9 doses and one time point will be assayed in one plate.*

The exposure will be performed by adding 25µl of culture medium containing the different doses, positive and negative controls at a 5X concentration. The doses to be tested are detailed below:

1. *HPHCs:*
    - *Dose 1:* Lowest concentration in pMRTP (based on ***Table 1***)
    - *Dose 2:* Highest concentration in pMRTP (based on ***Table 1***)
    - *Dose 3:* Average concentration in 3R4F (based on ***Table 1***)
    - *Dose 4:* Bridging dose between *doses 3 and 5*
    - *Dose 5:* Second highest dose with <10% cytotoxicity (based on results from *Phase I*)
    - *Dose 6:* Highest dose with <10% cytotoxicity (based on results from *Phase I*)
    - *Dose 7:* EC20 dose (based on results from *Phase I*)
    - *Dose 8:* EC50 dose (based on results from *Phase I*)
    - *Dose 9:* EC80 dose (based on results from *Phase I*)

    The exact doses selected for each HPHC will be detailed in the final report.
2. Smoke fractions:
    - Doses ranging 0-80% cytotoxicity will be selected based on the results from *Phase I*. ***The exact doses selected for each HPHC will be detailed in the final report.***
3. Negative controls:
    - Vehicles 1&2: Ethanol or PBS.
    - Control vehicle: DMSO
4. *Positive controls*:
    - *CCCP* (Cytotoxicity panel): 50, 500µM. Vehicle: DMSO
    - *Tacrine* (Oxidative Stress): 100, 1000µM. Vehicle: DMSO
    - *Nocodazole* (proliferation): 0.1, 1µM. Vehicle (DMSO).
    - *Staurosporine* (apoptosis/necrosis): 30, 300µM. Vehicle (DMSO)
    - *Ethacrynic acid* (GSH content): 100, 1000µM. Vehicle (DMSO).
    - *Mitomicyn C* (DNA damage): 20, 200µM. Vehicle (DMSO)
    - Colchicine (Stress kinase): 1, 10µM. Vehicle (DMSO)

Each endpoint will be repeated three times using independently generated HPHC dilutions and smoke fractions. Based on the current design the estimated number of NHBE cells required will be:

6 endpoints x 96 wells x 12,000cells x 3 repeats x 2 time points x 15 plates = $622x10^6$ cells

1 endpoints x 96 wells x 3,000cells x 3 repeats x 2 time points x 15 plates = $25x10^6$ cells

The estimated total number of plates is **630**. Based on current resources, the maximum number of plates that can be processed per week is 42.

4.2.4 *Phase III*: RNA extraction.

Based on the results from the toxicological evaluation we will select one or more HPHCs and / or smoke fractions for further analysis *via* transcriptomics. For each selected item, we will test 3 doses (High, Medium and low) and two time points (4h and 24h). Dose selection will be as follows:

1. ***Doses for HPHCs:***
   - *High*: EC80 dose (based on results from *Phase I)*
   - *Medium:* Average concentration in 3R4F (based on ***Table 1***)
   - *Low:* Concentration in pMRTP (based on ***Table 1***)

   The exact doses selected for each HPHC will be detailed in the final report.

2. ***Doses for smoke fractions:*** High dose will be selected as the EC80 dose based on results from *Phase I.*if the toxicity of 3R4F, ZRH and SMAR_NAD is similar (***Figure 6*, left panel**) we will select the exact same dose for all items. If there are differences in toxicity (***Figure 6*, right panel**), doses will be selected so that the highest dose of the more toxic item is equivalent to the lowest of the less toxic item.

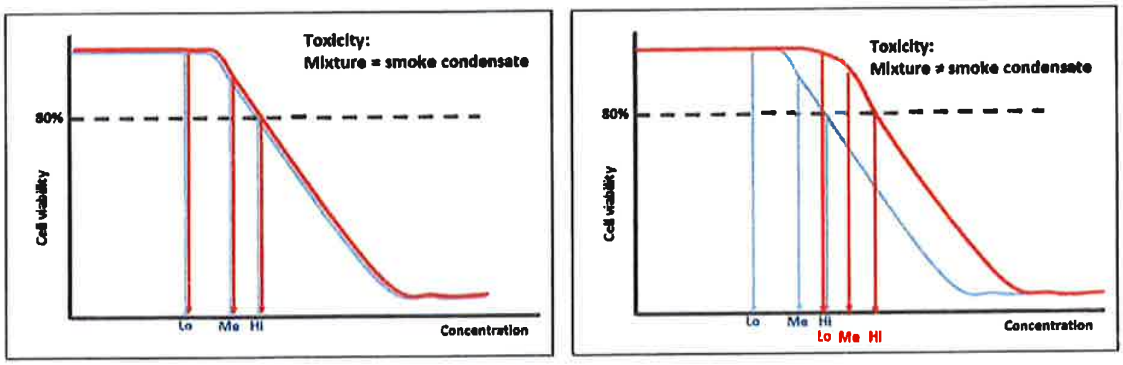

Figure 5. Dose selection for RNA extraction. *(Left) Items show similar toxicity. (Right) Two items show differences in toxicity.*

The estimated maximum number of RNA samples to be extracted will be:

- **For each HPHC selected:**

  2 (times) X 3 (replicates) X 3 (experiments) X 4 (doses: 3 + vehicle) = 72 samples

- **For each fraction selected:**

  2 (times) X 3 (replicates) X 3 (experiments) X 4 (doses: 3 + vehicle) X 3 items = 216 samples

4.2.4.1 Cellular exposure

NHBE cells will be seeded in a 96-well tissue culture treated polystyrene plate at a density of 12000 cells in 100µl of culture media per well and incubated for 24h prior to dosing. Cells will be then exposed (in triplicate) to vehicle (ethanol or PBS) or three doses of the selected HPHCs or smoke fractions. The plate layout is described in ***Figure 6*** and will be the same in all cases. For the high dose, 4 wells will be seeded for each replicate (12 wells total). For the middle dose, Low dose and vehicle, 2 wells will be seeded for each replicate (6 wells total per dose). The cells are exposed for the selected time points (4h and 24h).

Three HPHCs (or all three items for each smoke fraction) and will be exposed per 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	High Rep 1	High Rep 1	High Rep 1	High Rep 1	Medium Rep 1	Medium Rep 1	Low Rep 1	Low Rep 1	High Rep 1	High Rep 1	High Rep 1	High Rep 1
B	High Rep 2	High Rep 2	High Rep 2	High Rep 2	Medium Rep 2	Medium Rep 2	Low Rep 2	Low Rep 2	High Rep 2	High Rep 2	High Rep 2	High Rep 2
C	High Rep 3	High Rep 3	High Rep 3	High Rep 3	Medium Rep 3	Medium Rep 3	Low Rep 3	Low Rep 3	High Rep 3	High Rep 3	High Rep 3	High Rep 3
D	High Rep 1	High Rep 1	High Rep 1	High Rep 1	Medium Rep 1	Medium Rep 1	Low Rep 1	Low Rep 1	Medium Rep 1	Medium Rep 1	Low Rep 1	Low Rep 1
E	High Rep 2	High Rep 2	High Rep 2	High Rep 2	Medium Rep 2	Medium Rep 2	Low Rep 2	Low Rep 2	Medium Rep 2	Medium Rep 2	Low Rep 2	Low Rep 2
F	High Rep 3	High Rep 3	High Rep 3	High Rep 3	Medium Rep 3	Medium Rep 3	Low Rep 3	Low Rep 3	Medium Rep 3	Medium Rep 3	Low Rep 3	Low Rep 3
G	Vehicle Rep 1	Vehicle Rep 2	Vehicle Rep 3									
H	Vehicle Rep 1	Vehicle Rep 2	Vehicle Rep 3									

Figure 6. Plate layout for RNA extraction. *Three different HPHCs or test items, 3 different doses and one time point can be included in one plate. 3 different concentrations will be selected in each case.*

In order to bridge the results of RNA extraction and the toxicological evaluation from *Phases I & II*, NHBE cells will be tested in parallel for cell viability using the xCELLigence system. Briefly, NHBE cells will be seeded in an uncoated ACEA 96-well tissue culture plate at a density of 7000 cells in 100µl of culture media per well and incubated for 24h. Cells will be then exposed (in three replicates) to the selected doses of test items and positive (staurosporine) and negative controls (vehicle, culture medium), following the layout detailed in ***Figure 7.*** The exposure will be performed by adding 25µl of culture medium containing the different doses and negative controls at a 5X concentration. Impedance will be measured every hour during the 24h pre-exposure, and every 15 minutes for 24h after exposure.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Vehicle Rep 1	Vehicle Rep 2	Vehicle Rep 3	Vehicle Rep 1	Vehicle Rep 2	Vehicle Rep 3	Medium alone	Medium alone	Medium alone			
B	Low Rep 1	Low Rep 2	Low Rep 3	Low Rep 1	Low Rep 2	Low Rep 3	Low Rep 1	Low Rep 2	Low Rep 3			
C	Medium Rep 1	Medium Rep 2	Medium Rep 3	Medium Rep 1	Medium Rep 2	Medium Rep 3	Medium Rep 1	Medium Rep 2	Medium Rep 3			
D	High Rep 1	High Rep 2	High Rep 3	High Rep 1	High Rep 2	High Rep 3	High Rep 1	High Rep 2	High Rep 3			
E	Positive Control	Positive Control	Positive Control									
F												
G												
H												

Figure 7. Plate layout for xCELLigence..

4.2.4.2 RNA isolation:

Total RNA will be extracted from NHBE cells using the QIAgen **RNeasy® Micro Kit** (http://www.qiagen.com/products/catalog/sample-technologies/rna-sample-technologies/total-rna/rneasy-micro-kit). The protocol for RNA extraction is detailed below:

1. After exposure, the samples from each plate will be frozen at -80 C following these steps:
   a. Cells are washed with cold PBS 1X twice.
   b. PBS is removed.
   c. Cells from pooled wells are resuspended in RT buffer and transfer to labeled tubes.
   d. Vortex.
   e. Samples are frozen in dry ice before storage at -80 C.
2. RNA will be extracted from the frozen samples manually, following the instructions of the manufacturer and using 20µl as an elution volume.
3. All samples will be extracted on the same day.
4. After extraction, RNA samples will be QC (RIN measurement via Bioanalyzer) and selected for hybridization if good quality/yield are obtained.

4.2.5 Randomization of RNA samples

In order to minimize batch effects, we will apply a randomization protocol at the time of RNA extraction and at the time of hybridization (https://disco.app.pmi/disco/drl/objectId/0901d4ec804453ef)

4.2.6 Quantity determination of isolated RNA

The quantity of the isolated RNA will be determined with the Nanodrop 1000 or 8000 spectrophotometer (Thermo Scientific). For the subsequent RNA amplification protocol (Ovation RNA Amplification System V2) an amount of 50ng RNA is required. The concentration needs to amount to a least 16.7ng/µl. The RNA will be dissolved in RNAse-DNAse free water in the appropriate volume.

4.2.7 Quality control of the RNA

Minimal acceptance criteria for RNA samples, which can be analyzed for gene expression is a RIN score (RNA integrity number) equal or superior to 6.0. The RNA profile will be determined by using the Agilent 2100 Bioanalyzer.

4.2.8 DNA microarray

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 (see section 5). Also only one chip lot will be used. 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.

4.2.8.1 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 in the Ovation® RNA Amplification System V2 User Guide (NuGen®) and in the Encore® Biotin Module 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 (see **Figure 8**). 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 50ng 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.

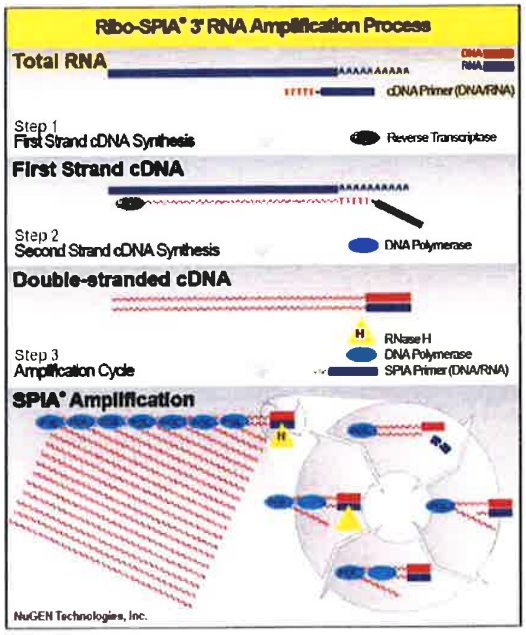

Figure 8. : *The Ribo-SPIA Amplification Process used in the Ovation RNA Amplification System V2*

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 9**). 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.

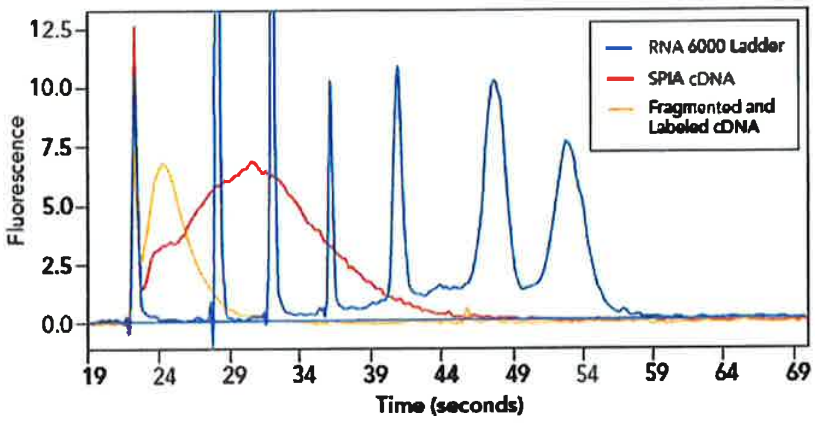

Figure 9. Bionalyzer trace of Amplified, Un-fragmented and Fragmented cDNA

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.

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

After the labeling and after to be sure that the fragmentation is perfect and according to the manufacturer's guidelines, it will be prepared the hybridization cocktails. The final array format will be selected and this option will produce approximately 220µl of hybridization cocktail per sample including the fragmented cDNA and controls (processed Poly-A controls, hybridization controls and control oligonucleotide B2). Then the cocktail is denatured (2min at 99°C, 5min to 45°C) and centrifuged 5min to Vmax. Each sample is hybridized to an array and all the arrays are moved to the hybridization oven for 18 hours ± 2 hours at 45°C with 60 rpm. After the hybridization step, arrays will be washed several times and then stained with Fluidic Stations FS450 using AGCC software and the protocol FS450_0004. In the last step, the arrays are moved into the GeneChip® Scanner 3000 7G and each array will be scanned.

4.2.8.3 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:

- Artifacts.
- 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 oligonucleotides, 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. These pseudo images provide a better means to monitor artifacts that are not visible on the original image like non-uniform distribution of signals

5 Statistical Methods

5.1 Statistical Design

Samples will be randomized for Affymetrix hybridization. Only Affymetrix stations will be used and samples will be processed in one single batch of 48. The randomization will be done in accordance. To minimize potential biases only one chip lot will be used.

5.2 General Considerations for Data Analysis

The QC pipeline implemented in the computational biology environment will be used to detect and possibly discard bad quality chip results

5.2.1 Missing Values

NA

5.2.2 Significance Level

For differentially expressed genes FDR<0.05 is usually considered and FDR<0.05 for GSEA analysis

5.3 Descriptive Statistics

Principal component analysis and Hierarchical clustering analysis will be used to detect batch effects, which in turn might be statistically corrected. Also low signal probesets will be filtered out (95%-quantile of expression values <7).

5.4 Hypothesis and Confirmatory Analysis

5.5 For contrast testing see below.Exploratory Analysis

LIMMA models will be estimated for each probeset (moderate t-statistics for the estimated contrasts). A particular focus will be on the contrasts $Compound_i$ x $Time_j$ x $Dose_k$ vs. Corresponding vehicle. It ill serves as the basis for differentially expressed gene identification, Gene Set Enrichment Analysis and Network Perturbation amplitude computation.

5.6 Sample Size Justification / Power Considerations

Three replicates per experimental group in such an in-vitro system, considering cost constraints and replicate definition (i.e experimental unit definition, see above) will lead to a satisfying signal-to-noise ratio in the gene expression profiles (*Note: ~50'000 probesets (=endpoints) are measured simultaneously*).

6 Administrative Aspects

6.1 Study Timelines

Proposed experimental starting date:	29/11/13
Proposed experimental completion date:	06/09/14
Proposed date for draft study report:	06/11/14

Phase I	Test 36 HPHCs by xCELLigence	**6 weeks**
	Test 3 smoke fractions from 3R4F, ZRH, SMAR_NAD by xCELLigence	**3 weeks**
Phase II	Test 36 HPHCs by HCS	**5 weeks**
	Test 3 smoke fractions from 3R4F, ZRH, SMAR_NAD by HCS	**15 weeks***
Phase III	RNA extraction	**1-3 weeks**
	Chip Hybridization	**2-4 weeks**
	Data interpretation	**4-8 weeks**

Estimated timelines for the study. *Number of weeks may decrease once HCS robot is installed.

6.2 Names and Addresses

Sponsor	Manuel Peitsch Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Test Facility	Cellular lab, smoke chemistry labs, Chemistry labs and Nucleic Acid Laboratories Philip Morris Products S. A Research & Development Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Study Director	Ignacio Gonzalez Suarez Quai Jeanrenaud 5 2000 Neuchâtel Switzerland

Deputy Study Director	Diego Marescotti 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

8.1 Literature references

8.2 Kit user guides and manuals

MagMAX™-96 Total RNA isolation kit (Life Technologies cat#AM1830)

MagMAX express-96 Standard plates (Life Technologies cat#4388475)

MagMAX express-96 Standard tip comb (Life Technologies cat#4388488)

MagMAX-96 RNA isolation kit Instruction Manual

Constituents and dose-ranges

http://disco.app.pmi/disco/drl/objectId/0901d4ec803577d7

Ovation®RNA Amplification System V2 User Guide

According to manufacturer instructions.

Ovation® RNA Amplification System V2 User Guide

Encore®Biotin Module User Guide

According to manufacturer instructions.

User guide

Encore® Biotin Module User Guide (NuGen®)

9 Abbreviations

Abbreviation	
AGCC	Affymetrix® GeneChip Command Console™
DHE	Dihydroethidium
FDA	Food and Drug Administration
FTC	Federal Trade Commission
GVP	Gas vapor phase
HCI	Health Canada Intense
HCS	High Content Screening
HPHC	Harmful / Potentially Harmful Constituent
NHBE	Normal Human Bronchial Epithelial
PDSP	Programmable Dual Syringe Pump
QC	Quality control
RIN	RNA integrity number
sbPBS	Smoke bubbled PBS
TPM	Total particulate matter

For complete definition, refer to PMI OPS Glossary and PMI RD Glossary.

10 Appendix

N/A

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