STUDY PLAN 176500

90 DAYS OECD (+) THS2.2.M OMICS ENDPOINTS

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1 Review and Approval

This document has been approved by:

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Ulrike Kogel	Approved by Work package leader OECD+ part	24Sept14

* ~~Actual concentration was different~~

2 Introduction / Background

The objective of the present study is to characterize the toxicity of mainstream aerosol from the THS2.2 menthol (THS2.2M) in a 90-day inhalation study on Sprague-Dawley rats. In line with the OECD Test Guideline 413 (OECD 2009), the mainstream aerosol from THS2.2M was generated and diluted to three target concentrations of 15, 23, and 50 µg/l nicotine. The observed effects following repeated daily inhalation exposure from THS2.2M mainstream aerosol will be compared to those inherent to 3R4F cigarettes. Additionally, DDA3 1XMIS and DDA3 2XMIS mentholated combustible cigarettes were included in the study to enable equal comparisons based on menthol content. DDA3 1XMIS and DDA3 2XMIS are designed to produce approximately 1.2 mg and 2.4 mg menthol in smoke (MIS) respectively when smoked according to ISO standard 3308 (2012). The 3R4F, DDA3 1XMIS and DDA3 2XMIS cigarettes were only smoked to a single target nicotine concentration of 23 µg/l.

Rats were nose-only exposed to mainstream aerosol/smoke or filtered and conditioned air (sham exposure group) for 6 h/day, 5 days/week for 13 weeks, followed by a 42-day post-inhalation (recovery) period. An exposure time adaptation phase was included during the first 7 days of exposure to acclimatize the rats to the target aerosol concentration (1.5 h exposure for first and second day, 3 h exposure for third and fourth day and 4.5 h exposure for days 5 to 7).

General health conditions and aerosol uptake/exposure are monitored. The endpoints for analysis are outlined in the OECD TG 413 for subchronic inhalation studies. These include in-life observations during and following exposure suc as health status, body weight, ophthalmoscopy and food consumption, pathology (post-mortem gross necropsy and histopathological assessment of both respiratory and non-respiratory organs and tissues), blood analysis (clinical chemistry, complete blood counts), as well as bronchoaveolar lavage fluid (BALF) analysis. To monitor aerosol exposure, and in addition to online monitoring of carbon monoxide, 4 daily samples from the breathing zone of the exposure chambers were prepared for analysis and determination of nicotine concentration and total particulate matter (TPM). The intake of aerosol was quantified through blood carboxyhemoglobin measurement and through the quantification of nicotine metabolites in urine and blood samples. Menthol levels will also be quantified in blood samples. The breathing patterns of the rats during exposure are monitored using head-out plethysmography.

The 3R4F cigarettes, DDA3 1XMIS, DDA3 2XMIS, and THS2.2M sticks were smoked in conformity with Health Canada standards with slight changes on 30-port smoking machines. The test aerosol was diluted to the target nicotine concentrations with filtered and conditioned air. The nicotine concentration for 3R4F cigarettes has been selected according to previous results from PMRL BVBA, Leuven (Belgium) (PMI Internal Report *12694* Part1, 2010), and results from PMI-RL Singapore (15006 THS SR Part 1) which showed that 23 µg/l nicotine concentration is suitable for detecting possible differences in effects throughout the respiratory tract. For the THS2.2M, 50 µg/l nicotine concentration was selected as high dose based on the results of the previous 15006 study. However, due to the fact that high levels of added menthol are used in

the test and reference item, a dose range finding study (PMI Internal Report *15024*, 2014) was conducted before this study that aimed to determine the maximal tolerable concentration (MTC) of mainstream aerosol generated from THS2.2M as well as the MTC of mainstream smoke generated from DDA3 2XMIS. Results of that study suggested to use 50 ug/l nicotine as maximal dose.

The study is conducted in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1997). The study will run in an AVA-licensed (Agri-Food & Veterinary Authority of Singapore) facility where care and use of the rats will be in accordance with the National Advisory Committee for Laboratory Animal Research (NACLAR) Guideline 2004.

At the time this study plan is written, the inhalation phase of the 90 days' time point is finalized. The 90 +42 days recovery period is ongoing. This study plan will describe the part after the dissection and the shipment of the samples to Neuchatel. Shipped samples are only form "the plus" part that are additional molecular endpoints that are added in order to complement the classical OECD approach with a systems toxicology approach (OECD+). This additional part is described further in the following PMI internal business case STA_VIVO_*15025*_BC_THS2.2M_rat_90d and the experimental conductance is the focus of this study plan. The part till the dissection is described in the overall inhalation study plan: 15025 THS SP.

3 Objective

Objective is to generate OMICS data from the following tissues:

Blood/ tissues	Planned Endpoint
Whole blood	Transcriptomics
Serum	Lipidomics
White blood cells	Transcriptomics
Serum	Proteomics
Left lung	Transcriptomics/Proteomics/
Right lung	Lipidomics
Liver	Transcriptomics/ Lipidomcis / Proteomics
Kidneys	Transcriptomics
Left RNE	Transcriptomics
Right RNE	Proteomics
Trachea	Transcriptomics

Priority for each tissue will be defined in here.

4 Scientific questions

What is the biological impact of THS2.2M aerosol in rats after 13 weeks of exposure that are measureable via omics endpoints?

Which biological impact of THS2.2M aerosol is reversible in the 42 day recovery period and which impact is not reversible?

What are the biological impact differences between THS2.2M aerosol and 3R4F/3R4F-like mentholated aerosol?

Can we detect a difference on the biological impact between 3R4F aerosol and 3R4F-like menthol aerosol?

Are the biological mechanics (pathways, networks) different in the exposure groups or is the amplitude different?

Can we detect a gender effect in the different exposure groups after 90 days of exposure?

5 Experimental Design

OECD Endpoints Groups 1-11	Non-GLP 'Plus' Endpoints Groups 12-18
GLP 1. Histopathology 2. Clinical chemistry 3. Hematology 4. Inflammatory cells in BALF Non-GLP 1. MAP of BALF 2. Biomarkers of Exposure in urine and blood	1. Proteomics 2. Transcriptomics 3. Lipidomics

This study plan focus on the non-GLP "plus' endpoints (see right side).

Lipidomics analysis will be defined in another document as this is an outsourced activity.

5.1 Test and Reference Items

Test items are described in detail in the section 4.1of the study plan 15025 THS SP:

- Reference item 1 = 3R4F

- Reference item 2 = DDA3 1XMIS and DDA3 2XMIS are mentholated combustible cigarettes designed to meet the Tar, Nicotine, CO, cigarette diameter specifications of 3R4F conventional cigarette from University of Kentucky (for specifications, see batch release documents in PMF), but with menthol added (approximately 1.2 mg and 2.4 mg MIS respectively, when smoked according to ISO 3308 standard; (for specifications, analysis report A-0011876 and A-0011877)
- Test item = THS2.2M (for specifications, batch release B-11130)

5.2 Test System

Experiments are conducted on male and female Sprague Dawley rats bred under specific pathogen-free conditions purchased from Charles River, USA (at 6-7 weeks of age -week age). For more details see the main study plan (15025 THS SP), section 4.2:

5.3 Experimental Groups and Dosing

All experimental groups included in the main study are described in the main study plan (15025 THS SP), section 4.5:

The groups are included in this particular omics study plan (OECD+ part). They are summarized in Table 1.

Table 1. Exposure Groups designated for exploratory 'Plus' groups

			90d	90+42d
Group Code	**Long Name**	**Target nicotine (µg/L)**	**M/F**	**M/F**
012	Sham PLUS	0	6/6	0/6
013	3R4F PLUS	23	6/5	0/6
014	DDA3 1XMIS PLUS	23	6/6	0/0
015	DDA3 2XMIS PLUS	23	6/6	0/6
016	THS2.2M Low PLUS	15	6/6	0/0
017	THS2.2M Medium PLUS	23	6/6	0/0
018	THS2.2M High PLUS	50	6/6	0/6
	Total		42/41	0/24

In total 108 animals. 7 groups, 2 genders, 2 time points for female, 6 biological replicates. F = female, M = male

To note: as we got after the 90 days exposure all the reserve animals which were not needed anymore, the actual sample size increased as follows: There are 11 additional animals in the PLUS groups. The final distribution will be as follows (Table 2):

Table 2: **Actual sample number receive after 90 days dissection**

Group Code	Long Name	M/F
012	Sham PLUS	6/6
013	3R4F PLUS	6/ 5
014	DDA3 1XMIS PLUS	6/8
015	DDA3 2XMIS PLUS	6/6
016	THS2.2M Low PLUS	8/8
017	THS2.2M Medium PLUS	7/8
018	THS2.2M High PLUS	7/6

Please note, only 6 planned samples per group will be used and the others will be kept as reserve.
If e.g. the RNA quality of the RNE is not sufficient we can prepare the other samples of the group.
However, in the lung slicing table (see randominisation for slicing.csv) we will define the animal number to be used and which are the reserve animals. All other organs e.g. liver will be used exactly from the same animals.
As we do not have the 90+42 animals yet, final animal number may change as well.

5.4 Procedures

5.4.1 **Sample Generation**

The animal dissection and sample collection is described in detail in the study plan (15025 THS SP) and in the table below:

Blood/ tissues	Planned Endpoint	Collection/ Processing	Collection	Storage
Whole blood	Transcriptomics	RNAprotect animal blood tube	RT	-80°C
Whole blood	Lipidomics	EDTA tubes	RT	-80°C
White blood cells	Transcriptomics	CPT tubes (in addition white blood cell count)	RT	-80°C
Serum	Proteomics	Microfuge tube	RT	-80°C
Left lung	Transcriptomics/Proteomics/	Perfused with saline (whole body perfused)	Liq N2	-80°C
Right lung	Lipidomics	Perfused with saline (whole body perfused)	Liq N2	-80°C
Liver	Transcriptomics/ Lipidomcis /	Whole body perfused) Freeze in different pieces	Liq N2	-80°C
Kidneys	Transcriptomics	Freeze right and left separately (whole body perfused)	Liq N2	-80°C
Left RNE	Transcriptomics	Collection after whole body perfusion	Liq N2	-80°C
Right RNE	Proteomics	Collection after whole body perfusion	Liq N2	-80°C
Trachea	Transcriptomics	Trimmed and rinsed with PBS (via syringe)	Liq N2	-80°C

5.4.2 **Analysis Prioritization**

Analysis will be start with the **left lung of the 90 days** that will be sliced in a randomized manner (randominisation for slicing.csv) over all groups for proteomics, transcriptomics and genomics. After slicing the 90 days samples the slicing will be continued with the **90+42 days** samples. Afterwards a new randomization will be performed for the protein isolation and RNA isolation. Samples for genomics will be kept on hold and not processed.

All other samples (e.g., RNE, liver) will be randomized when the 90+42 days dissection is finalized. The respiratory tract organs will have priority over other tissues

1) Left **RNE** will be processes as soon as the 90+42 days samples arrive for transcriptomics (miRNA optional). For proteomics right RNE will be processed.
2) After the slicing is finalized **lung** samples will be processed for transcriptomics (miRNA optional) and for iTRAQ.
3) **Trachea** will be processed for transcriptomics
4) **Liver** will be processed for transcriptomics and proteomics
5) **White blood** cells will be processed for transcriptomics
6) Whole blood, serum and kidney will be kept frozen until further notice

5.4.3 **Sample randomization protocol**

As we want to compare t

a) The effect of THS vs references
b) The effect of a recovery period
c) The gender effect

All samples (=107 per tissue) need to be randomized and processed together.

For cryoslicing, 90 days and 90+42 will be randomized in two batches.

For transcriptomics randominzation, potential cryoslicing batch effects, RNA extraction batch effect and different Affymetrix fluidics stations will be taken into account. Randominization plan will be provided by bioinformatics team.

For iTraq, samples will be randomized according to the plan provided by bioinformatics team (Bjorn Titz).

5.4.4 **Lung sample cryoslicing**

The Tissue Research Laboratory will cryosection the left lung lobe into 40um slices. To ensure a proper representation of the full lung tissue, serial sections from the three areas of the organ (represented in the Figure 1 as Parenchyma, Whole Lung and Main Bronchus) will be collected in one tube.

Serial sections will be collected for transcriptomics, for proteomics (iTraq analysis) and also for genomics analysis (just to be frozen, scope of a different study plan).

The requirement for the **transciptomics** processing is that approximately 20 slices will be needed. More tissue will interfere with the downstream RNA isolation. Less tissue would result in a RNA yield which might be too low for transcriptomics and miRNA chips. The **proteomics** analysis would require approximately 40 slices to have enough protein amount for further analysis. To obtain some slices more would not be an issue.

To cope with the different requirements, the following slicing plan was developed:

In total 4 tubes (for tubes specifications see below) will be filled

1. for genomics analysis / DNA isolation (1 slice)
2. for transciptomics /RNA isolation (1 slice)
3. for Proteomics / protein isolation (2 slices)
4. Back up (2 slices)

Repeat 1-4 till the main bronchus is reached.

This plan is based on the estimation that a whole lung will contain approximately 120 of 40 um slices, then transcriptomics requirement of 20 slices will be met.

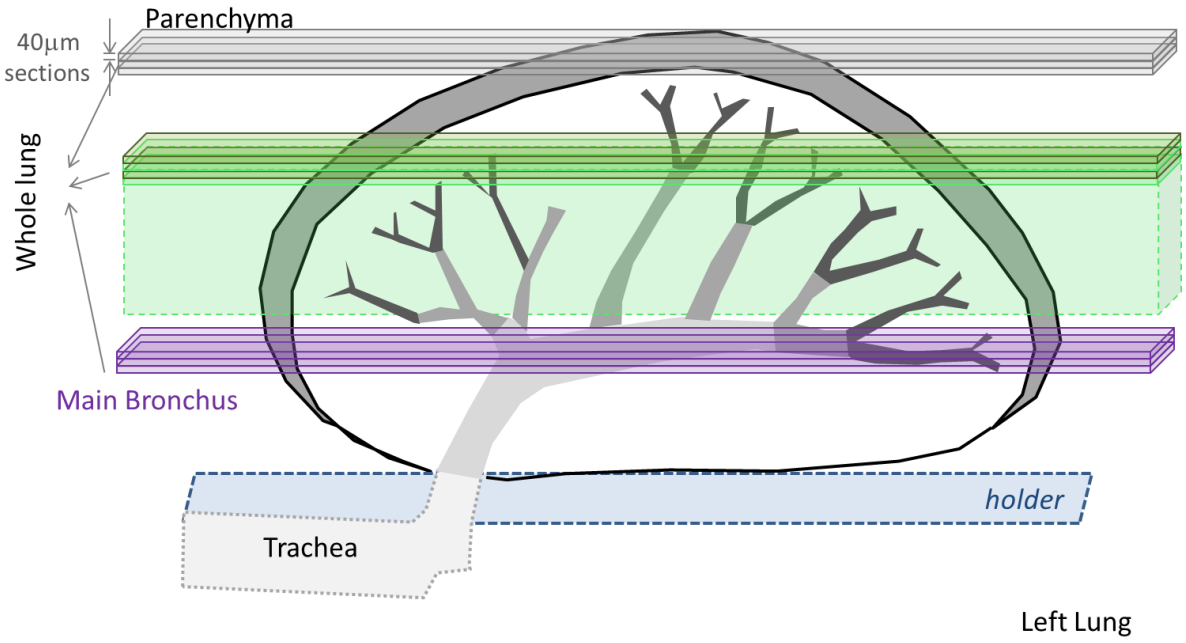


Figure 1: **Schematic lung cryosection**

- Slices (optimal 20) collected for **transcriptomics** will be placed in a **2 ml MagNA Lyser Green Beads** (Roche ref. 03 358 941 001) tube containing ceramic beads. For homogenization, 600ul RLT buffer (Qiagen ref. 79216) containing 1% β-mercaptoethanol (Sigma) will be added and the tube will be immediately centrifuge for 45 sec at 6000rpm using MagNA Lyser centrifuge (Roche).
- Slices for **proteomics** will be collected in **2ml safe-lock tube** (Eppendorf ref. 0030 120.094) without any buffer.
- Slices for **genomics** will be collected in **2 ml MagNA Lyser Green Beads** (Roche ref. 03 358 941 001) without any buffer.

All the tubes containing slices must be maintain on dry ice or in cryostat chamber (-20ºC) to avoid melting. All tubes are stored in -80ºC freezer in a box labelled with relevant details (Study ID, organ, application).

The whole process will be performed according to the following SOP's for tissue cryoslicing (PMI_RD_WKI_001250).

5.4.5 Nasal epithelium processing (i.e. RNA and protein isolation)

For **transcriptomics**, left Nasal epithelium (Respiratory Nasal Epithelia: RNE) samples are in 2.0ml MagNa Lyser tubes and frozen at -80C. Samples will be thawed and 700 ul Qiazol buffer will be added and vortex for 30s. Further RNA isolation will be done with the miRNAeasy extraction kit (Qiagen, Hilden, Germany) according to the PMI_RD_WKI_001117. Purified RNA will be eluted in 30uL of RNAse free water.

For **proteomics**, the homogenized mixture will be thawed and split into 2 aliquots (1 for iTRAQ LC-MS/MS and 1 for the verification experiments) and each aliquot is subjected to acetone precipitation by adding 4 x volume of pre-chilled acetone for 2 hour in -20 °C followed by centrifugation at 16,000 x g at 4 °C. The precipitates will be resuspended in 200 µl of 0.5 M triethylammonium bicarbonate (TEAB) buffer containing 1 M urea and 0.1% SDS. Procedures are described in PMI_RD_WKI_001043:

5.4.6 Lung samples processing (i.e. RNA and protein isolation)

Lung samples will be collected as stated in 5.4.4.

For **transcriptomics,** the cell lysate will be thawed and further processed with the miRNeasy extraction kit (Qiagen, Hilden, Germany) according to the PMI_RD_WKI_001115. Purified RNA will be eluted in 30uL of RNAse free water.

For **proteomics** the homogenized mixture will be subjected to the same procedure described in **5.4.5**.

5.4.7 Trachea processing (i.e. RNA isolation)

Pre-washed trachea samples will be thawed. Trachea will be filled with 700µL RLT+1%Beta-mercaptoethanol lysis buffer in order to lyse cells from the lumen of the trachea. The rinsed buffer is collected in a 2 ml tubes and vortexed. Further processing follows the miRNeasy extraction kit (Qiagen, Hilden, Germany) handbook according to the PMI_RD_WKI_001135. Purified RNA will be eluted in 20 to 30 uL of RNAse free water depending on the size of the tissue.

5.4.8 Liver samples processing (i.e. RNA and protein)

The piece liver A 1 (tissue segment indicated with “A” was cut into 2 pieces for lipidomics and RNA isolation) (see: 15025 THS SP part 10.2.7) will be thawed.

For **transcriptomics**, liver samples are grinded in 700uL Qiazol buffer in a 2.0ml MagNa Lyser tubes.

Further processing follows the miRNeasy extraction kit (Qiagen, Hilden, Germany) handbook following the PMI_RD_WKI_001117. Purified RNA will be eluted in 30 to 80uL of RNAse free water depending on the size of the tissue.

For **proteomics** actually no liver piece was planned. However, a “big” liver piece “liver B” – (see 15025 THS SP part 10.2.7) is frozen for e.g. Oil Red O staining.

A piece of B can be used for proteomics. A method, how to cut and how much to cut needs to be developed and will be described in an amendment. The best would be using a cryostat to maintain the liver frozen.

5.4.9 White Blood cells (i.e. RNA)

Samples will be grinded in 350ul Trizol LS+93.3ul chloroform.

For the RNA extraction step, miRNeasy micro Kit (according to the manufacturer instructions) will be used and elution will be done in 20uL.

5.4.10 Blood samples

Provided interesting results are acquired in other running studies, and the proof of concept is made, blood samples may be analyzed in order to look for potential biomarkers and to help in translatability issues. For now, serum for proteomics and whole blood for transcriptomics (the study could include also miRNA profiling) **will be stored** at -80C until further notice.

5.4.11 Kidney samples

Kidney samples will be stored till further notice.

5.4.12 Quantity determination of Isolated RNA and proteins

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 needs to amount to at least 50 ng/µl. If the concentration is too low, the sample will be concentrated using vacuum centrifugation (Eppendorf speedvac). The RNA will be dissolved in RNAse free water in an appropriate volume.

Protein concentrations of the samples are determined using the Bradford assay according to supplier’s instructions. Procedures are described in PMI_RD_WKI_001043 .

5.4.13 Quality control of the Isolated RNA and protein

RNA quality will be measured using Agilent 2100 Bioanalyzer. Minimal acceptance criteria for RNA samples, which will be analyzed for gene expression is RIN score greater than 6.0.

The quality control procedure to check the sample **proteins'** stability before performing the experimental analyses will consist of 2 steps: (i) determination of protein concentrations and (ii) determine sample integrity by checking the protein-band profile using 1D-PAGE according to PMI_RD_WKI_001104, PMI_RD_WKI_001103, PMI_RD_WKI_001131.

5.4.14 **Affymetrix gene chips processing**

All RNA samples from the whole lung, nasal epithelium, liver and white blood cells will be analysed with Affymetrix gene expression arrays. RNA randomization will be performed as described in the report.

Depending on the extraction protocol, the yields and the quality of RNA, 3 main protocols may be used to perform the Gene Expression Profiling of these samples:

Case 1 (if concentrations are below 33.3 ng/ul)***:***

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

All RNA samples will be analyzed with GeneChip GeneChip® Rat Genome 230 2.0 Array). A randomization of the samples will be determined for Affymetrix hybridization. Also only one chip lot will be used (lot number will be provided in the final report). Afterwards, the analysis of this raw data will be performed by the Computational Sciences and Bioinformatics team within the Systems Biology Development, Application & Verification program.

<u>Ovation® RNA Amplification System V2 and Encore® Biotin Module</u>

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. 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 2.

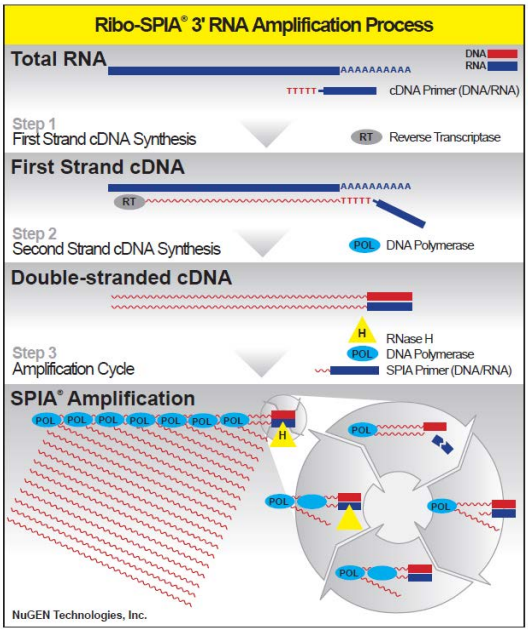


Figure 2: The Ribo-SPIA Amplification Process used in the Ovation RNA Amplification System V2

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 3). 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.

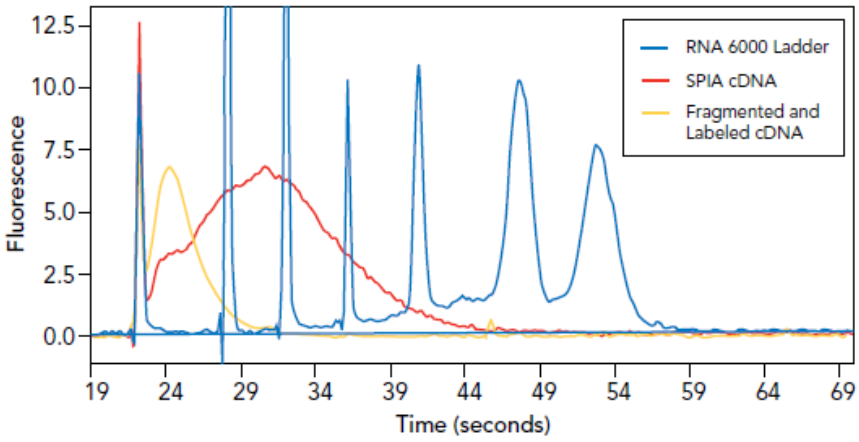


Figure 3. Bionalyzer trace of Amplified, Un-fragmented and Fragmented cDNA

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. The labeled product is ready for hybridization to the arrays.

Procedures are also captured in the following WKIs:
PMI_RD_WKI_001239, PMI_RD_FOR_000765, PMI_RD_WKI_000815, PMI_RD_WKI_000814, and PMI_RD_FOR_000761.

Case 2 (If concentrations are greater than 33.3 ng/uL)**:**

If concentrations are greater than 33.3 ng/uL High Throughput 3' In Vitro Transcription PLUS (HT 3' IVTPLUS) kit will be used.

High Throughput 3' In Vitro Transcription plus (HT 3' IVTPLUS)

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 PLUS Kit User Manual (Affymetrix). The GeneChip® HT 3’ IVT PLUS Kit represents the newest high-throughput target generation assay for 3’-based arrays. The configuration of the HT 3’ IVT PLUS Kit is optimized for use with liquid-handling robotics and generates a high-quality, array-ready target and requires minimal manual intervention. As starting material 100ng total RNA gained from the different samples will be used. All samples will be processed in one series after all RNA preparations have been completed. A positive and negative control will be added to the sample preparation in order to have a positive control and check potential cross contamination issues. This whole reaction will be pipetted automatically by using the robotic technology of the Beckman Biomek® FXp Target Prep Express (TPE) System. After the amplification step, the quantity of the aRNA will be measured as A260 determined with the Spectramax. The accurate calculation of the yield is necessary so that the correct amount of aRNA will be added to the fragmentation reaction. If necessary, aRNA will be concentrated by vacuum centrifugation (Eppendorf speedvac). The quality of the aRNA will be determined by assessing the size of the unfragmentated aRNA by using the Agilent Bioanalyzer electropherogram. The expected aRNA profile is a distribution of sizes from 250–5500 nt with most of the aRNA between 500–1500 nt. Average aRNA size may vary slightly depending on RNA quality and total RNA input amount. Average sizes below 500 nt could hint for partially degraded input RNA.

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

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

See also:
PMI_RD_WKI_001118, PMI_RD_FOR_000763, PMI_RD_WKI_000813, PMI_RD_WKI_000816, PMI_RD_FOR_000761.

Case 3 (*if the concentration of samples is below the detection limit)*:

If the concentration of samples is below the detection limit (5ng/uL) but still visible on the 2100 Bioanalyzer using the Pico chips (RNA 6000 Pico Kit – PN: 5067-1513) the Nugen Ovation Pico WTA System V2 will be used. This kit will very likely be used following the WBC collection and RNA isolation as the obtained yields are usually very low. The Pico WTA System V2 is a whole transcriptome amplification solutions for preparing cDNA from a range of 500 picograms to 50 nanograms of total RNA. Each protocol can be used for qPCR, sample archiving and microarray gene expression studies. After amplification, the cDNA product can be used directly for downstream qPCR analysis. Alternatively, to use the amplified product for gene expression profiling on microarrays, simply use either the Encore™ Biotin Module, or follow a validated NuGEN protocol to fragment and label the target. The labeled product is ready for hybridization to the arrays.

Hybridization, Wash, Stain, and Scan using the Fluidic Station FS450 (for case 1-3)

The Biomek FXp TPE System will proceed to make the hybridization ready sample by mixing appropriate amounts of fragmented aRNA and hybridization cocktail master mix according to the manufacturer's manual. The final array format will be selected and this option will produce approximately 230µl of hybridization cocktail per well including the fragmented aRNA and the controls (processed poly-A RNA controls, hybridization controls, control oligonucleotide B2). Each sample is hybridized to an Affymetrix GeneChip® Rat Genome 230 2.0 Array. All the arrays are moved to the hybridization oven for 16 hours at 45°C with 60 rpm (IVT Protocol) and for 16-18 hours at 45°C with 60 rpm (Nugen Protocol). After hybridization arrays will be washed several times and then stained with Fluidic Stations FS450 using AGCC Software with the protocol FS450_0001 (following the IVT protocol) and with the protocol FS450_0004 (following the Nugen protocol). In the last step, the arrays will be moved to GeneChip Scanner 3000 7G and each array will be scanned.

Monitoring the Quality Check of 3' IVT Expression Arrays (for case 1-3)

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

5.4.15 **Data Processing Gene expression**

The data will be processed through standard pipeline. Briefly, raw data files will be read by the ReadAffy function of the affy package (Gautier 2004) belonging to Bioconductor suite of microarray analysis tools (Gentleman 2004) available for the R statistical environment (R Development Core Team 2007). Quality will be controlled by generating and examining RNA degradation plots (AffyRNAdeg function of the affy package), NUSE and RLE plots (affyPLM package (Brettschneider 2008)), calculating the MA(RLE) values.

Arrays that would fall below a set of thresholds on the quality control checks will be excluded from further analysis. Robust Microarray Analysis (RMA) background correction and quantile normalization will be used to generate microarray expression values (Irizarry 2003) from all arrays passing quality control checks.

An overall linear model will be fit to the and specific contrasts of interest will be evaluated to generate raw *p*-values for each probe set on the expression array (Smyth 2004). The Benjamini-Hochberg False Discovery Rate (FDR) method will then be used to correct for multiple testing effects.

5.4.16 **iTRAQ LC MS/MS procedure**

Labeling of peptides and MS/MS analysis.

1. Labeling with iTRAQ reagents. An amount of 50 µg of each iTRAQ aliquot prepared according to 4.5.3 will be subjected to reduction of cysteines using tris-(2- carboxyethyl) phosphine (TCEP) and blocking using methyl methane-thiosulfonate (MMTS). iTRAQ labeling procedure is described in PMI_RD_WKI_001038, PMI_RD_WKI_001034, PMI_RD_WKI_001036.
2. The samples will be digested with trypsin (Promega) to break down the proteins into small peptides followed by labeling with iTRAQ reagents.
3. After labeling, the samples will be mixed together.
4. The labeled tryptic peptides will be separated by 1D LC column separation and analyzed by Q-Exactive ESI-MS/MS (Thermo Scientific) to obtain the mass lists as described in PMI_RD_WKI_001038, PMI_RD_WKI_001034, PMI_RD_WKI_001036.
5. Xcalibur and RawMeat software is used to evaluate the spectra and mass-accuracy as described in PMI_RD_WKI_001104, PMI_RD_WKI_001103, PMI_RD_WKI_001131.

Suggested design for 8-plex iTRAQ sets

- In detail, Create **90+42d iTRAQ set** with the four 90+42d female exposure groups and the corresponding female 90d exposure groups [4+4 groups] ➔ allows comparison between the two time points and the four exposure conditions that have been included for the 90+42d time point
- Create a combined male/female iTRAQ set for the 7 exposure groups of the **90 day** time point. Randomize between male and female samples for each 8-plex set ➔ allows comparison between the exposure conditions for the 90d time point and between the genders
- Randomize the channel to sample mappings within each 8-plex set
- Note that the four 90-day sample types that overlap with the 90+42d samples types will be analyzed in duplicate, ie in the "**90-day male/female iTRAQ" and "90-day vs. 90+42d sample" sets.**

<u>iTRAQ 8-plex sets</u>

- **90-day male/female iTRAQ sets (**2x6 replicates = 12 sets**)**
    - For each 8-plex set:
        - Include one refmix (1 channel)
        - Include one sample of each sample type (7 channels)
        - For each selected sample type, randomly pick either a male or female sample (out of the 2x6 replicates)
        - Randomize the sample to channel mapping in each set
- **90-day vs. 90+42d sample set** (6 replicates= 6 sets)
    - For each 8-plex set:
        - Include one sample for each of the four **90+42d** sample type (Sham, 3R4F, DDA3 2X, THS2.2M High; all female) (4 channels)
        - Include one corresponding **90-day** sample for each selected sample type (Sham, 3R4F, DDA3 2X, THS2.2M High; all female) (4 channels)
        - Randomize the sample to channel mapping in each set

- 18 iTRAQ sets needed

5.4.17 **Protein database searches**

Protein database searches and quantification will be performed with Proteome Discover (Thermo Scientific). The generated mass lists will be matched to protein databases to obtain identification of proteins. Changes in differences in protein abundance between samples will be calculated from the intensities of the corresponding iTRAQ-reporter ions in each sample. The procedure for database

searching for protein identifications is described in the PMI_RD_WKI_001104, PMI_RD_WKI_001103, PMI_RD_WKI_001131.

5.4.18 **Verification Experiments.**

Analysis in lung samples will be performed on protein aliquots for verification prepared according to 5.4.5. For serum, proteins will be extracted as described in PMI_RD_WKI_001043. For both sample types an amount of 100 µg will be used for these experiments.

The procedure for biomarkers verifications using selected reaction monitoring (SRM) is as follows:

1. One to four tryptic peptides representative of the selected protein in the sample mixture will be selected according to their MS properties (abundance, length and content of proline residues), uniqueness (no interfering isoforms), possible presence of post-translational modifications and/or propensity for chemically induced modifications, and susceptibility for missed cleavage sites (avoid non-cleavable and missed cleaved tryptic peptides). The peptides will be selected using the Pinpoint software (Thermo Scientific).
2. Samples will be trypsin digested as separated using 1-D LC as described in PMI_RD_WKI_001407, PMI_RD_WKI_001272, PMI_RD_WKI_001402.
3. An MS scan performed on the Q-Exactive (Thermo Scientific) will confirm the presence of the peptide(s) in the spectrum and the MS/MS will ensure that these selected peptides can be fragmented. This is the proof of feasibility check that it will work and further confirms the presence of the protein.
4. After the peptides pass the proof of feasibility check, the tryptic digested samples as described in step 2, will be injected again to perform relative quantitation. Multiple protein biomarkers can be monitored at the same time. Synthetic peptides will be ordered 2 per target protein to perform absolute quantifications.
5. The pinpoint software will confirm and verify the presence of the target of interest, and the absolute quantification as compared to synthetic peptides of the target

5.4.19 **miRNA microarrays (optional)**

All isolated RNA samples will be contain miRNA. Thus, miRNA expression can be also investigated using the same samples.

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 labelling total RNA or low molecular weight (LMW) RNA for analysis by Affymetrix® GeneChip® miRNA Arrays.

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 labelling 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 (Figure 4). 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 labelling process is complete in less than two hour.

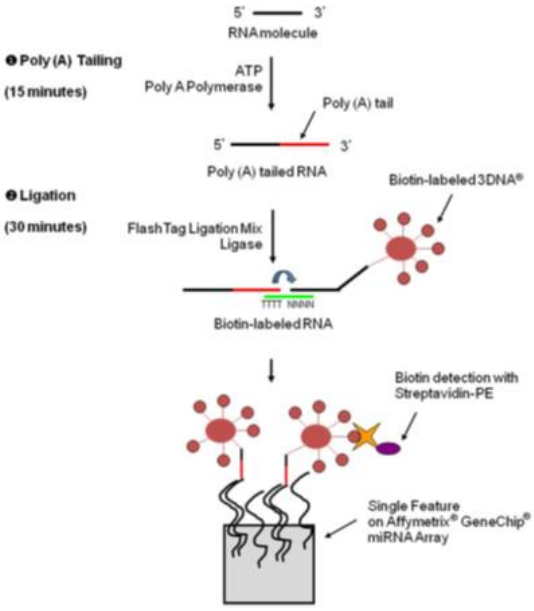


Figure 4. FlashTagTM HSR: Procedure overview

Required materials:

1. FlashTag™ Biotin HSR RNA Labeling Kit
2. FlashTag Biotin HSR RNA Labeling Kit, 10 rxn (P/N 901910) or 30 rxn (P/N 901911).
3. Additional materials, please refer to the Table 3:

Table 3: 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.

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). If after the RNA extraction, the RNA yield is acceptable, we will use 200ng as starting material of total RNA (See Table 4).

Table 4: RNA input for FlashTag Biotin HSR labelling

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

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 PolyA Tailing Master Mix in a nuclease-free tube in the order listed in Table 5. Include 10% overage to cover pipetting errors.

Table 5: Poly A Tailing Master Mix (for a single reaction)

Component	Amount
10X Reaction Buffer (Vial 1)	1.5 µL
25mM $MnCl_2$ (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.

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).
7. Mix and microfuge the 23.5 µL of ligated sample.
8. 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.
9. 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 4 to the 21.5 µL biotin-labeled sample in the order listed, to prepare the array hybridization cocktail (See Table 6):

Table 6: 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 (See Table 7).

Table 7: 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

1. Incubate at 99°C for 5 minutes, then 45°C for 5 minutes.
2. Aspirate 100 µL (400/169 format array) or 130 µL (100 format array) and inject into an array (See Figure 5).

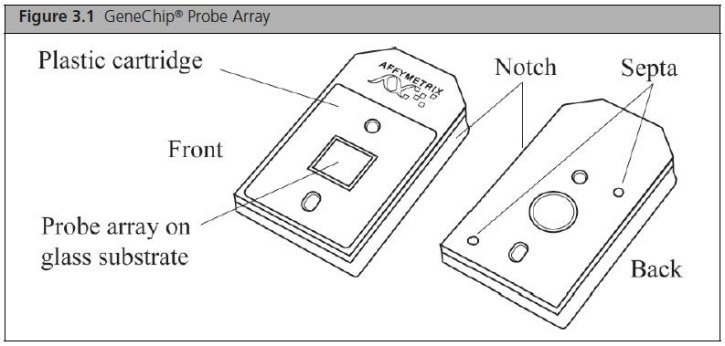


Figure 5: GeneChip® Probe Array

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

Washing and staining

For additional information about washing, staining, and scanning, refers 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.
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:
   i. Place one (amber) vial containing 600 µL Stain Cocktail 1 in sample holder 1.
   ii. Place one (clear) vial containing 600 µL Stain Cocktail 2 in sample holder 2.
   iii. Place one (clear) vial containing 800 µL Array Holding Buffer in sample holder 3

   Wash and stain with Fluidics Station 450 using the appropriate fluidics script: FS450_0002 (See Table 8).

Table 8: Fluidics Protocols

	Fluidics Station 450 Protocol FS450_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 1 (Vial Position 1) 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 4 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)

6. 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.

Scanning

The instructions for using the scanner and scanning arrays can be found in the Affymetrix® Command Console® User Manual (P/N 702569 See Table 9).

Table 9: Software version

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

Data normalization and quality control

Use Expression Console software for data summarization, normalization, and quality control (v. 1.2 and higher – See Table 9 above). 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.

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.

6 Statistical Methods

NA

7 Administrative Aspects

7.1 Study Timelines

Proposed experimental starting date:	22. September 2014
Proposed experimental completion date:	TBD
Proposed date for draft study report:	TBD

7.2 Names and Addresses

Sponsor	Manuel Peitsch Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Program Leader SBAV	Julia Hoeng Quai Jeanrenaud 5 2000 Neuchâtel Switzerland
Test Facility	Nikolai Ivanov Genomics Research and Technology Laboratories Philip Morris Products S. A Research & Development Quai Jeanrenaud 5 2000 Neuchâtel Switzerland

8 Archiving

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

9 References & Sources

9.1 Literature

Brettschneider J, Collins F, Bolstad BM. Quality Assessment for Short Oligonucleotide Microarray Data. Technometrics. 2008;50(3):241-64.

Gautier L, Cope L, Bolstad BM, Irizarry RA. affy---analysis of Affymetrix GeneChip data at the probe level. Bioinformatics. 2004;20:307-15.

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Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003;4(2):249-64. Epub 2003/08/20. PubMed PMID: 12925520.

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Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical applications in genetics and molecular biology. 2004;3:Article3. Epub 2006/05/02. PubMed PMID: 16646809.

9.2 Philip Morris International's List of Standard Operating Procedures (SOP) and Work Instructions (WKI)

Randominisation for slicing.csv: on request stored at (https://disco.app.pmi/disco/drl/objectId/0901d4ec804ab2d4)

Preparation of cryosections with cryostat: PMI_RD_WKI_001250

Nasal Epithelium and Liver RNA isolation: PMI_RD_WKI_001117

Lung RNA isolation: PMI_RD_WKI_001115

Trachea RNA isolation: PMI_RD_WKI_001135

Normalization Macro prior to Nugen Ovation Protocol: PMI_RD_WKI_001239

Tissue nugen Worksheet: PMI_RD_FOR_000765

Nugen Encore: PMI_RD_WKI_000815

Nugen Ovation WB: PMI_RD_WKI_000814

Tissue_AGCC_Upload: PMI_RD_FOR_000761.

Normalization Macro prior to IVT Protocol: PMI_RD_WKI_001118

Tissue_IVT_Worksheet: PMI_RD_FOR_000763

Affymetrix-IVT2-Express BioMek: PMI_RD_WKI_000813

Affymetrix-3'array-Cartridge-Hybridization: PMI_RD_WKI_000816

LC-MS for proteomics analysis: PMI_RD_WKI_001038

iTRAQ 8-plex: PMI_RD_WKI_001034

Sample purification for LC MS/MS: PMI_RD_WKI_001036

Protein Extraction from Murine Lung Tissue and Acetone Precipitation: PMI_RD_WKI_001043

Determination of Protein concentration using 660 nm: PMI_RD_WKI_001104

Determination of Protein concentration using Bradford assay: PMI_RD_WKI_001103

Quality control of protein samples using LabChip instrument: PMI_RD_WKI_001131

Sample purification for PRM: PMI_RD_WKI_001407

Parallel reaction monitoring: PMI_RD_WKI_001272

Creation of heavy-labeled peptide pool for PRM: PMI_RD_WKI_001402

9.3 Philip Morris International's Internal Documents

Philip Morris International. Unpublished on file data. Internal business case. STA_VIVO_15025_BC_THS2.2M_rat_90d.docx: "ZURICH THS 2.2 MENTHOL OECD 90 DAYS RAT INHALATION STUDY". 2014

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