STUDY PLAN 150082

90 DAYS OECD (+) ZURICH STUDY GENOMICS APPROACH

Program Name	SBAV
Project Name	Animal Clinic
PUC Number	
Work Package Name	OECD Zurich - Genomics
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Date	April 18th, 2013

Table of Contents

Table of Contents 2
1 Review and Approval: 4
2 Introduction 5
3 Objectives 5
3.1 Primary Objective(s) 5
3.2 Secondary Objective(s) 5
4 Experimental Design 6
4.1 Test and Reference Items 6
4.2 Test System 6
4.3 Experimental Groups and Dosing 6
4.4 Procedures 8
4.4.1 Sample Generation 8
4.4.2 Rationale for Analysis Prioritization and Detailed Scientific Questions 13
4.4.2.1 Respiratory endpoints 13
4.4.2.2 Liver 17
4.4.2.3 Blood samples 20
4.4.2.4 Trachea/Larynx samples 20
4.4.3 Sample retrieval from Singapore and biobanking 22
4.4.4 Blood samples processing and profiling 22
4.4.5 Liver samples processing and profiling. 23
4.4.6 Nasal epithelium processing and profiling 23
4.4.7 Trachea/Larynx samples processing and profiling 24
4.4.8 Lung samples processing and profiling 25
4.4.9 Sample randomization protocol 26
4.4.10 RNA extraction from tissue 26
4.4.11 Quantity determination of Isolated RNA 27
4.4.12 Quality control of the Isolated RNA 27
4.4.13 mRNA microarray 27
4.4.13.1 High Throughput 3' In Vitro Transcription (HT 3' IVT) 27
4.4.13.2 Ovation® RNA Amplification System V2 and Encore® Biotin Module (Blood RNA Samples) 28
4.4.13.3 Hybridization, Wash, Stain, and Scan using the Fluidic Station FS450 30
4.4.13.4 Monitoring the Quality Check of 3' IVT Expression Arrays 31
4.4.14 miRNA microarray 31
4.4.14.1 RNA Sample and Quantitation 32

4.4.14.2 FlashTag™ Biotin HSR RNA Labeling Procedure 32
4.4.14.3 Poly (A) Tailing 32
4.4.14.4 FlashTag™ Biotin HSR Ligation 33
4.4.14.5 Hybridization 33
4.4.14.6 Washing and Staining 34
4.4.14.7 Scanning 34
4.4.15 DNA methylation analysis (blood samples) 35
4.4.15.1 DNA isolation 35
4.4.15.2 DNA methylation analysis 35
4.4.16 Data Processing 36
4.4.17 Sample Processing Prioritization (Transcriptomics) 37
5 Statistical Methods 38
6 Administrative Aspects 38
6.1 Names and Addresses 38
7 Archiving 38
8 References & Sources 38
8.1 Literature 38
8.2 Philip Morris International's List of Standard Operating Procedures (SOPs) and Work Instructions (WKIs) 39
8.3 Philip Morris International's Internal Documents 39
9 Appendix 39

1 Review and Approval

This document has been approved by:

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2 Introduction

In order to determine the hazard of a harmful effect to individuals or sub-populations resulting from an exposure to the Zurich platform, a battery of chemical and toxicological assays will be conducted. Among these, a 90-day inhalation study will be conducted as described in the OECD guideline 412(OECD 2009). Its aim is to characterize adverse effects following repeated daily inhalation exposure to different doses of the Zurich product. In addition, different doses of the reference cigarette 3R4F will be tested, and a sham group will be used as control.

Data derived from this subacute inhalation study will aim to characterize the toxicity of aerosol from a Zurich product prototype by the inhalation route, and will provide safety data for the regulatory toxicological assessment and to support the exploratory clinical studies.
Furthermore, additional molecular endpoints will be added in order to complement the classical OECD approach with a systems toxicology approach (OECD+). This additional part described in the PMI internal business case *SBDAV_ACL_OECD (+)*, 2012, is the focus of this study plan.

3 Objectives

3.1 Primary Objective(s)

1. To generate gene expression data from liver, nasal epithelium, blood, and lung samples in the Zurich OECD+ study in order to answer the following scientific questions:
    a. Can a dose response for the Zurich product be determined in gene expression profiles?
    b. Can a time response for the Zurich product be determined in gene expression profiles?
    c. What biological networks/ toxicity pathways are perturbed by all planned exposures?
    d. How close are the perturbations caused by Zurich compared to standard cigarette 3R4F?
    e. To which extent the molecular profiles return to sham after a 42 day-period of recovery after both Zurich and 3R4F exposure?
    f. Do the molecular patterns correspond with histopathological findings (nasopharyngeal tissues, and lungs (including main bronchi))?

3.2 Secondary Objective(s)

1. To provide a systems biology picture of diverse organs of the rats in order to propose a mechanistic explanation of the data obtained from classical endpoints in the OECD part:
    a. Correlation with body weight / food consumption (especially liver gene expression).
    b. Correlation with mediators' concentrations measured in BALF.

2. To provide a systems biology picture of diverse organs of the rats in order to propose a mechanistic explanation of the data obtained from other endpoints in the OECD (+) part.
    a. Correlation with immunohistochemical staining to analyze proliferation and apoptosis in lung. There may also be immunohistochemical staining of nasal epithelium (to be confirmed).
    b. Correlation with proteomics profiles in blood, nasal epithelium, and lung.

3. To estimate whether there is a difference in gene expression between the different right lung lobes in male rats after 28 days of exposure. To compare right and left lung lobes at 90 days.

4. To compare Zurich and conventional cigarette smoke effects between male and female rats.

5. To establish a reference with 3R4F at 28 day to be used to confirm the validity of the tests for the Nike study (PMI internal report *150122*, 2012)

4 Experimental Design

4.1 Test and Reference Items

Test items are described in detail in the section 4.1 of the main study plan (15006 THS SP):

Shortly, reference cigarette 3R4F will be purchased from the University of Kentucky. Test item is Zurich FPD 4.2 sensorial medium (batch number B-00829) abbreviated ZRH.

Test and reference items will be administered by nose-only inhalation to ensure maximum reproducibility.

Both 3R4F and ZRH will be smoked at 3 target nicotine concentrations. Test atmosphere will be characterized as detailed in the main study plan.

4.2 Test System

Experiments are conducted on male Sprague Dawley rats bred under specific pathogen-free conditions purchased from Charles River, USA (at 8-week age). For more details see the main study plan (15006 THS SP), section 4.2:

4.3 Experimental Groups and Dosing

All experimental groups included in the main study are described in the main study plan (15006 THS SP), section 4.3:

Only some of the groups are subjects of the analyses included in this particular omics study plan (OECD+ part). They are summarized in Table 1 and Figure 1.

Table 1: Experimental groups and dosing

Short Name (main study plan)	Group Code	Target nicotine (µg/L)	Time Point (days)					
			28		90		90+42	
			male	female	male	female	male	female
SHAM	11	0	6	-	6	6	5	5
3R4F – low	12	8	6	-	6	6	6	6
3R4F – medium	13	15	6	-	6	6	6	6
3R4F – high	14	23	-	-	6	6	6	6
ZRH – low	15	15	6	-	6	6	6	6
ZRH – medium	16	23	6	-	6	6	6	6
ZRH – high	17	50	-	-	6	6	-	-

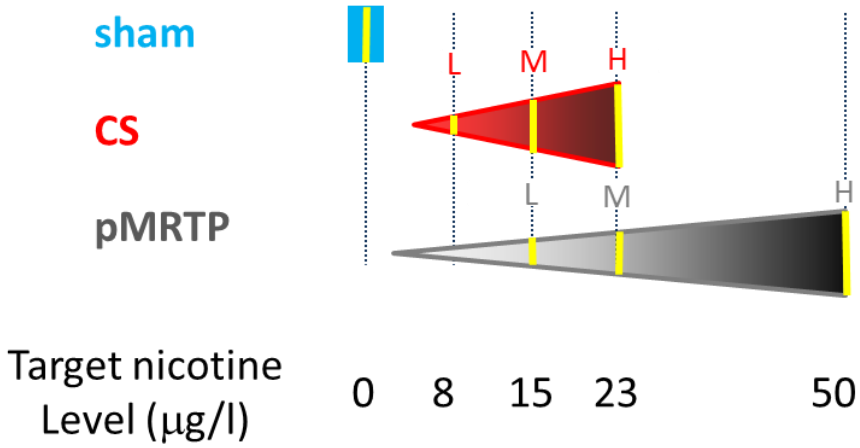


Figure 1: Schematic Representation of Groups Included in the Study with their Target Nicotine Level

In order for the final report to have a unified style, the conventions detailed in Table 2 will be used for the groups included in this study plan.

Table 2: Group naming and plotting conventions for the endpoints included in this study plan.

Short Name (Omics)	Long Name (main study plan)	Symbol (pch)	Line (Lty)	Color (greyscale)	Color (color-scale)
SHAM	Sham Plus	1	3	#F7F7F7	#F7F7F7
CS_LOW	3R4F- Low Plus	2	3	#D9D9D9	#FEE0B6
CS_MED	3R4F- Medium Plus	22	2	#BDBDBD	#F1A340
CS_HI	3R4F- High Plus	23	4	#969696	#B35806
pMRTP_LOW	ZRH- Low Plus	19	6	#737373	#D8DAEB
pMRTP_MED	ZRH- Medium Plus	17	5	#525252	#998EC3
pMRTP_HI	ZRH- High Plus	15	1	#252525	#542788

4.4 Procedures

4.4.1 Sample Generation

The animal dissection and sample collection is described in detail in the amendment of the study plan as presented in 15006 THS SR Part 3 (Amendment 4, pages 27-37):

In short the organs that will be collected, **16 to 24 hours after the last exposure and under non-GLP conditions**, for the purpose of this study plan are:

- Blood
- Lung
- Trachea and larynx
- Liver
- Respiratory nasal epithelium (RNE).

More precisely, the allocation of the different tissues from the dissected animals is summarized in Figure 2. In addition to the endpoints considered in this study plan, additional endpoints collected from the same and other organs is also summarized as they will be correlated with the data obtained from this study plan.

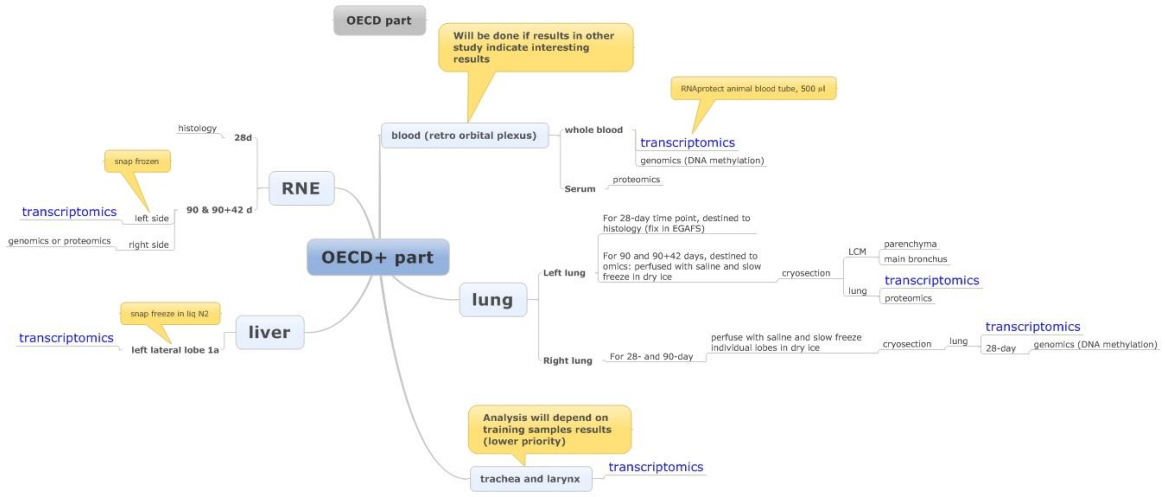


Figure 2: Tissues allocation and processing summary**.**

The number of potentially available replicates for each tissue and treatment group is given in Table 3 for transcriptomics analysis.

The number of samples that have been prioritized for analysis will depend partially on the results obtained in the right lung lobes comparison at 28 day. Therefore, the 28 day samples will be processed first and analysed before proceeding with the 90 and 90+42 day sample processing and analysis.

Depending on when the results have to be available for the lung samples and on the resources in the tissue research lab, LCM may not be doable. As an impact, a correlation with gene expression and histological phenotype in different lung area will not be possible. LCM is not part of this study plan, and should there be resources, it may be processed as an amendment later on. Lung parts dedicated to LCM should be kept in the freezers until further notice.

Possible miRNA profiling and DNA methylation analysis will depend on method set-up and results in other experiments and may be the subject of an amendment of this study plan.

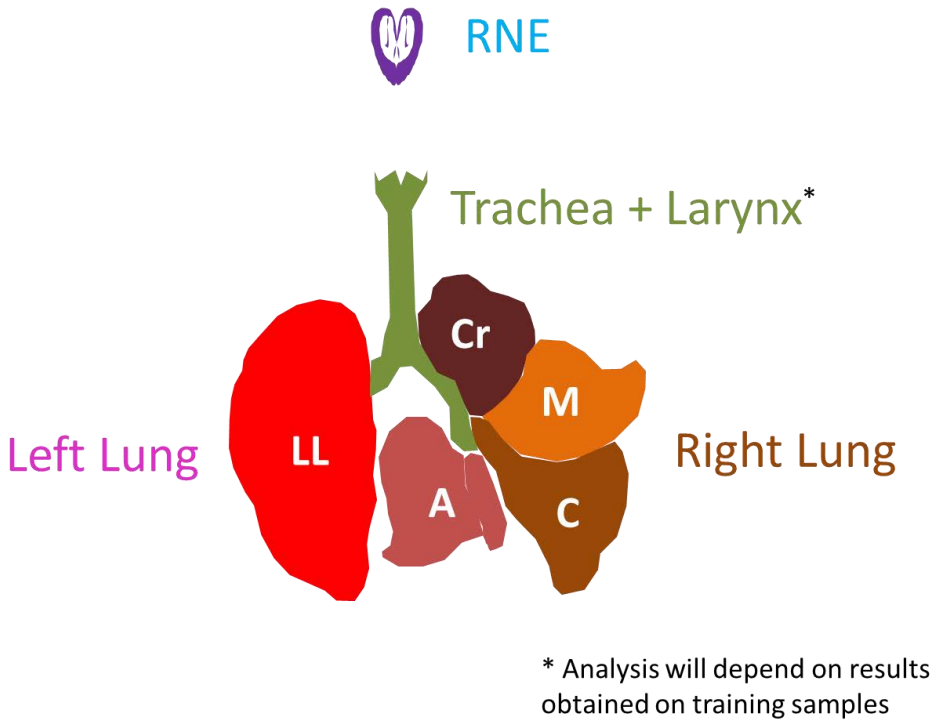


Figure 3: Cartoon representation of rat respiratory system, with lung lobes designation

Table 3: All samples potentially available for analysis

Group	Time point (days)	Gender	Lung								RNE	Blood	Liver	Trachea + Larynx
			Right Lobe					Left Lobe						
			Cr	M	C	A	pool 4 lobes	"whole" lung	LCM					
									Paren-chyma	Main bronch				
Sham	28	M	6	6	6	6	6	NA	NA	NA	NA	6	6	6
	90	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
	90+42	M	5	5	5	5	5	5	5	5	5	5	5	5
		F	5	5	5	5	5	5	5	5	5	5	5	5
CS_LOW	28	M	6	6	6	6	6	NA	NA	NA	NA	6	6	6
	90	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
	90+42	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
CS_MED	28	M	6	6	6	6	6	NA	NA	NA	NA	6	6	6
	90	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
	90+42	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
CS_HI	90	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
	90+42	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
pMRTP_LOW	28	M	6	6	6	6	6	NA	NA	NA	NA	6	6	6
	90	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
	90+42	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
pMRTP_MED	28	M	6	6	6	6	6	NA	NA	NA	NA	6	6	6
	90	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
	90+42	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
pMRTP_HI	90	M	6	6	6	6	6	6	6	6	6	6	6	6
		F	6	6	6	6	6	6	6	6	6	6	6	6
TOTAL	**2088**		**184**	**184**	**184**	**184**	**184**	**154**	**154**	**154**	**154**	**184**	**184**	**184**

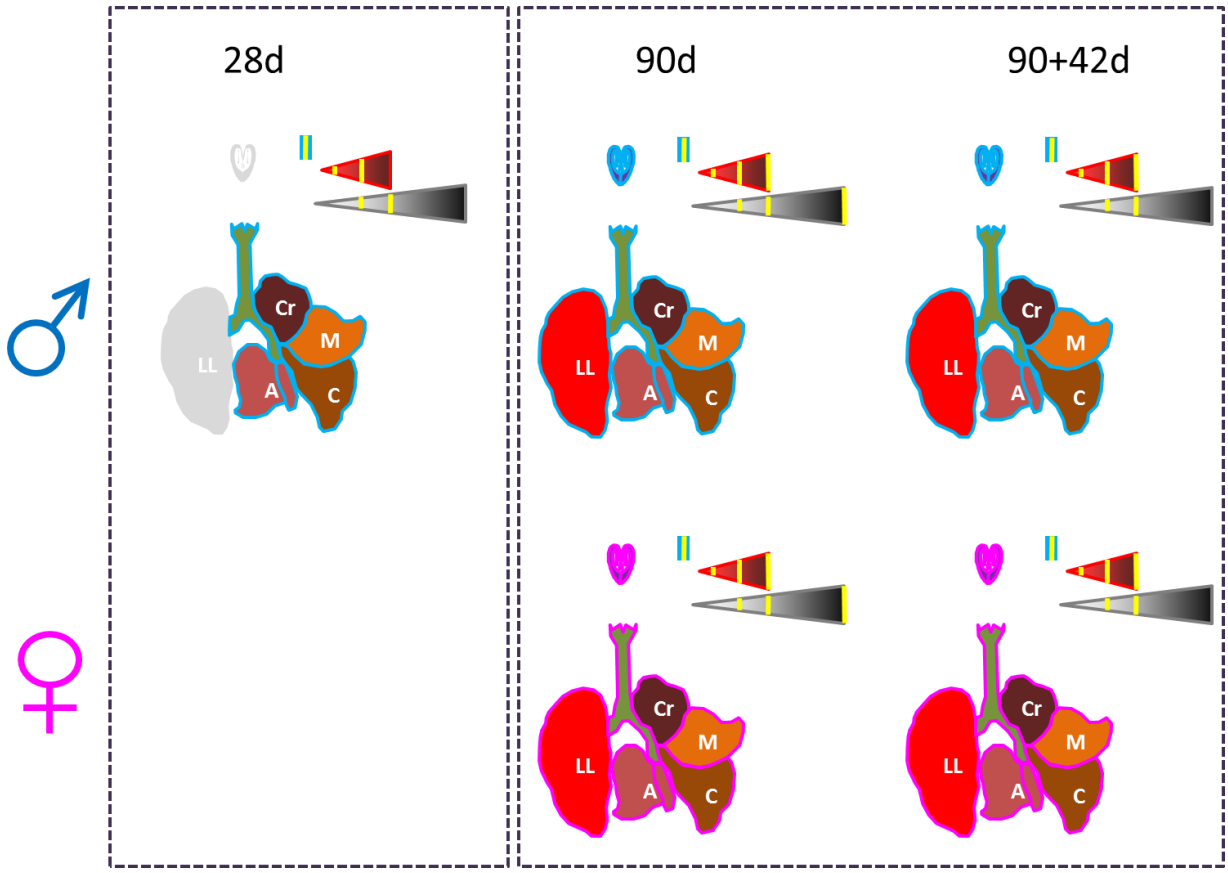


Figure 4: Schematic Representation of All Samples Available for Omics Analyses in the Respiratory System

4.4.2 Rationale for Analysis Prioritization and Detailed Scientific Questions

4.4.2.1 Respiratory endpoints

Analysis of the respiratory system of rats exposed to Zurich product has the top priority in this study. Lung lobes will be cryosectioned to give more reproducible results and to allow acquiring data not only for transcriptomics but also proteomics Figure 3 and Figure 4.

4.4.2.1.1 28-day time point

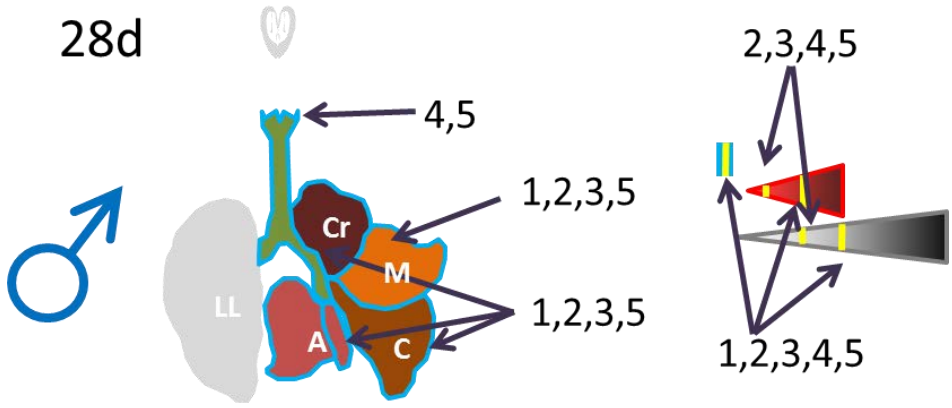


Figure 5: Respiratory Sample Selection at 28-day time point

Please to refer Figure 5 and Table 4

1. Is there a significant difference in gene expression in the 4 lobes of the right lung (using only sham and medium doses)?
2. Is there a dose-response to CS (3R4F) or to ZRH after 28d exposure in the right lung (pool of 4 lobes)?
3. How do the exposures to CS (3R4F) and ZRH compare in the right lung?
4. Trachea/Larynx gene expression: is there a dose response to CS or to ZRH? How do the exposures to CS and ZRH compare?
5. What are the similarities and differences between lung and trachea/larynx gene expression responses?

Table 4: Summary of samples analyzed and scientific questions for which they are needed in brackets.

Group	Right Lung					Trachea + Larynx
	Pool 4 lobes	M	Cr	A	C	
Sham	6 (1,3,5)	6 (1,3,5)	6 (1,3,5)	6 (1,3,5)	6 (1,2,3,5)	6 (4,5)
CS_LOW	-	-	-	-	6 (1,2,3,5)	6 (4,5)
CS_MED	6 (1,3,5)	6 (1,3,5)	6 (1,3,5)	6 (1,3,5)	6 (1,2,3,5)	6 (4,5)
pMRTP_LOW	-	-	-	-	6 (1,2,3,5)	6 (4,5)
pMRTP_MED	6 (1,3,5)	6 (1,3,5)	6 (1,3,5)	6 (1,3,5)	6 (1,2,3,5)	6 (4,5)
TOTAL	**18**	**18**	**18**	**18**	**30**	**30**

4.4.2.1.2 90 and 90+42 time points

4.4.2.1.2.1 Nasal epithelium

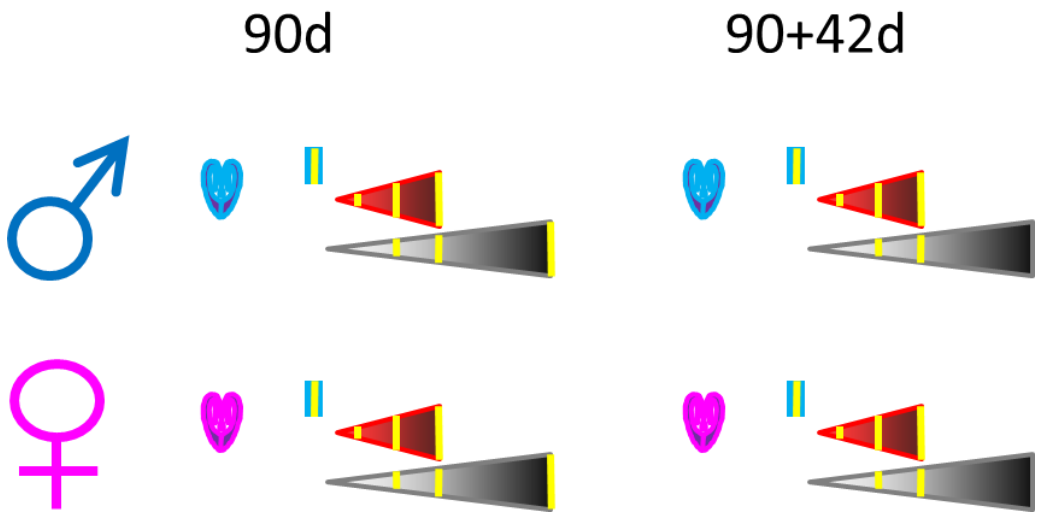


Figure 6: Respiratory Nasal Epithelium Samples Selection at 90 and 90+42day time points

All available RNE samples should be analyzed (Figure 6) to answer the following scientific questions:

6. Is there a dose-response to ZRH in nasal epithelium of male rats?
7. Is there a dose-response to ZRH in nasal epithelium in female rats?
8. How do the dose-responses to ZRH and CS compare in male rats?
9. How do the dose-responses to ZRH and CS compare in female rats?
10. How do the dose-responses to CS in nasal epithelium compare between male and female rats?
11. How do the dose-responses to ZRH in nasal epithelium compare between male and female rats?

12. Are there still gene expression changes after 42days without exposure in nasal epithelium of male rats exposed to ZRH for 90 days? Is this dose-dependent?
13. Are there still gene expression changes after 42days without exposure in nasal epithelium of female rats exposed to ZRH for 90 days? Is this dose-dependent?
14. How does post exposure compare in CS vs. ZRH-exposed male rats?
15. How does post exposure compare in CS vs. ZRH-exposed female rats?
16. Are there gender-related effects in the recovery process (in ZRH or CS)?
17. Are the same biological pathways/mechanisms activated in male rats' RNE at 90 and 90+42? If yes, is the direction changing?
18. Are the same biological pathways/mechanisms activated in female rats' RNE at 90 and 90+42? If yes, is the direction changing?
19. Are there gender-specificities?

4.4.2.1.2.2 Lung

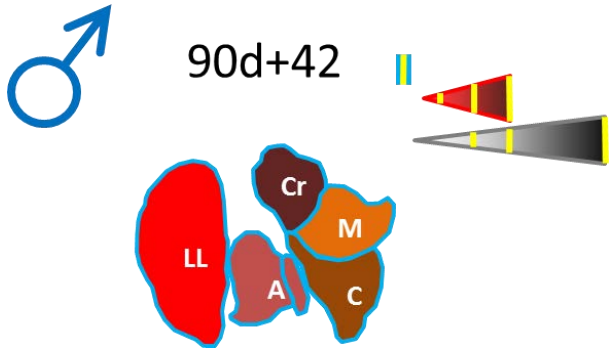


Figure 7 Lung Samples Selection at 90 and 90+42day time points

The question whether all, a few, or only one right lung lobe should be analyzed at the 90 and 90+42 day time point will depend on the results obtained with the 28day time point.

If no significant difference is seen (scenario 1 below), then the C lobe (Figure 7) only would be used to see how right and left lung compare. Otherwise, all right lung lobes should be analyzed again. This is reflected in the scenario 2 below.

The scientific questions addressed by the samples at 90 and 90+42 days are the following:

20. Is there a dose-response to ZRH right lung of male rats?
21. Is there a dose-response to ZRH in left “whole lung” in male rats?
22. How do the high dose responses to ZRH and CS compare in right lobe of male rats?
23. How do the dose-responses to ZRH and CS compare in left lobe (whole lung) of male rats?
24. How do the high doses to ZRH and CS compare in right vs. left lobe (whole lung) of male rats?
25. Are there still gene expression changes after 42days without exposure in right lung lobe of male rats exposed to ZRH for 90 days?
26. Are there still gene expression changes after 42days without exposure in left lung lobe (whole lung) of male rats exposed to ZRH for 90 days?
27. Are there differences between the different lobes/lung parts?
28. Is there any significant time-progression from the 28 to 90 days’ time points (both CS and ZRH) in the right lobe?
29. Are the same biological pathways/mechanisms activated in male rats’ right lung lobe at 90 and 90+42? If yes, is the direction changing?
30. Are the same biological pathways/mechanisms activated in male rats’ left lung lobe (whole lung) at 90 and 90+42? If yes, is the direction changing?

4.4.2.2 Liver

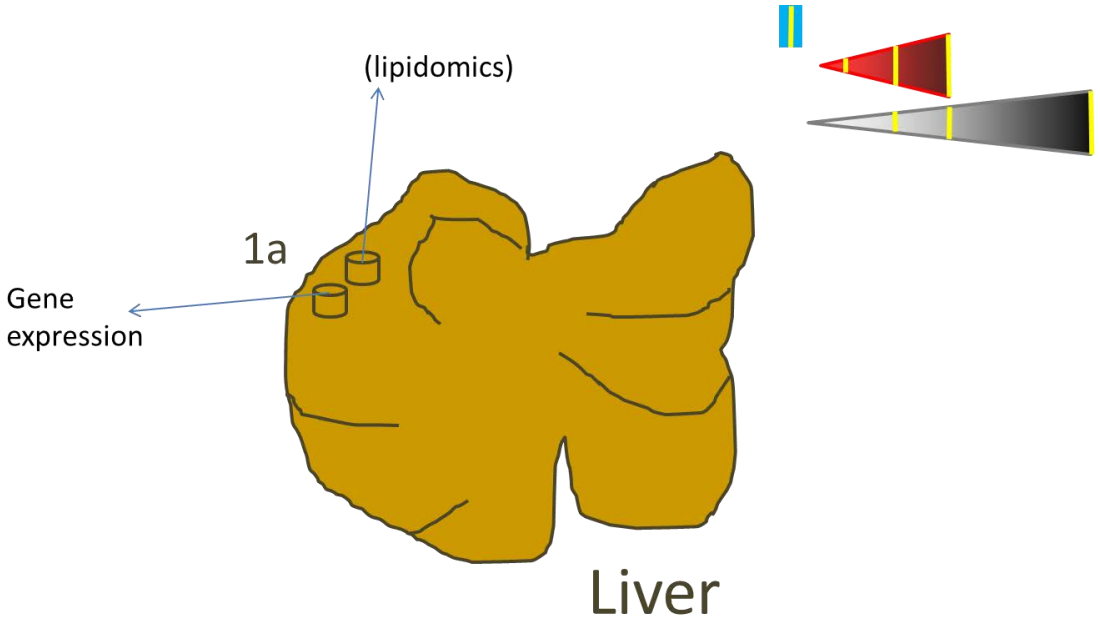


Figure 8: Liver Sample Selection

The scientific questions that should be addressed by the transcriptomics profiling of the liver samples (Figure 8) are the following:

31. What is the impact of ZRH on general metabolism in male rats? Is a dose-response identifiable?
32. What is the impact of ZRH on general metabolism in female rats? Is a dose-response identifiable?
33. How do ZRH and 3R4F exposures compare? In male and in female?
34. Can liver gene expression gives clues about in life observations, and more specifically food consumption and body weight progression?
35. Can the gene expression in liver be correlated with lipid profiles (including lipoprotein profile)?

A summary of the number of samples that may then be analyzed is given in Table 5 and Table 6.

Table 5: High Priority Samples for Transcriptomics – Scenario 1: Analysis of Right Lung Lobes does not show Significant Differences.

Group	Time point (days)	Gender	Lung						RNE	Liver
			Right Lobe					Left Lobe		
			pool 4 lobes	M	Cr	A	C	"whole" lung		
Sham	28	M	**6**	**6**	**6**	**6**	**6**	NA	NA	**6**
	90	M	0	0	0	0	**6**	**6**	**6**	**6**
		F	0	0	0	0	**6**	**6**	**6**	**6**
	90+42	M	0	0	0	0	**5**	**5**	**5**	**5**
		F	0	0	0	0	**5**	**5**	**5**	**5**
CS_LOW	28	M	**6**	0	0	0	**6**	NA	NA	**6**
	90	M	0	0	0	0	**6**	0	**6**	**6**
		F	0	0	0	0	0	0	**6**	**6**
	90+42	M	0	0	0	0	**6**	0	**6**	**6**
		F	0	0	0	0	0	0	**6**	**6**
CS_MED	28	M	**6**	**6**	**6**	**6**	**6**	NA	NA	**6**
	90	M	0	0	0	0	**6**	**6**	**6**	**6**
		F	0	0	0	0	0	**6**	**6**	**6**
	90+42	M	0	0	0	0	**6**	0	**6**	**6**
		F	0	0	0	0	0	0	**6**	**6**
CS_HI	90	M	0	0	0	0	**6**	**6**	**6**	**6**
		F	0	0	0	0	**6**	**6**	**6**	**6**
	90+42	M	0	0	0	0	**6**	**6**	**6**	**6**
		F	0	0	0	0	**6**	**6**	**6**	**6**
pMRTP_LOW	28	M	**6**	0	0	0	**6**	NA	NA	**6**
	90	M	0	0	0	0	**6**	**6**	**6**	**6**
		F	0	0	0	0	0	**6**	**6**	**6**
	90+42	M	0	0	0	0	**6**	0	**6**	**6**
		F	0	0	0	0	0	0	**6**	**6**
pMRTP_MED	28	M	**6**	**6**	**6**	**6**	**6**	NA	NA	**6**
	90	M	0	0	0	0	**6**	**6**	**6**	**6**
		F	0	0	0	0	0	**6**	**6**	**6**
	90+42	M	0	0	0	0	**6**	**6**	**6**	**6**
		F	0	0	0	0	0	**6**	**6**	**6**
pMRTP_HI	90	M	0	0	0	0	**6**	**6**	**6**	**6**
		F	0	0	0	0	**6**	**6**	**6**	**6**
TOTAL	**770**		**30**	**18**	**18**	**18**	**136**	**106**	**154**	**184**

Table 6: High Priority Samples for Transcriptomics – Scenario 2: Analysis of Right Lung Lobes shows Significant Differences.

Group	Time point (days)	Gender	Lung						RNE	Liver
			Right Lobe					Left Lobe		
			pool 4 lobes	M	Cr	A	C	"whole" lung		
Sham	28	M	**6**	**6**	**6**	**6**	**6**	NA	NA	**6**
	90	M	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
		F	0	0	0	0	**6**	**6**	**6**	**6**
	90+42	M	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
		F	0	0	0	0	**5**	**5**	**5**	**5**
CS_LOW	28	M	**6**	0	0	0	**6**	NA	NA	**6**
	90	M	0	0	0	0	**6**	0	**6**	**6**
		F	0	0	0	0	0	0	**6**	**6**
	90+42	M	0	0	0	0	**6**	0	**6**	**6**
		F	0	0	0	0	0	0	**6**	**6**
CS_MED	28	M	0	0	0	0	**6**	NA	NA	**6**
	90	M	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
		F	0	0	0	0	0	**6**	**6**	**6**
	90+42	M	0	0	0	0	**6**	0	**6**	**6**
		F	0	0	0	0	0	0	**6**	**6**
CS_HI	90	M	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
		F	0	0	0	0	**6**	**6**	**6**	**6**
	90+42	M	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
		F	0	0	0	0	**6**	**6**	**6**	**6**
pMRTP_LOW	28	M	**6**	0	0	0	**6**	NA	NA	**6**
	90	M	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
		F	0	0	0	0	0	**6**	**6**	**6**
	90+42	M	**6**	**6**	**6**	**6**	**6**	0	**6**	**6**
		F	0	0	0	0	0	0	**6**	**6**
pMRTP_MED	28	M	**6**	**6**	**6**	**6**	**6**	NA	NA	**6**
	90	M	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
		F	0	0	0	0	0	**6**	**6**	**6**
	90+42	M	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
		F	0	0	0	0	0	**6**	**6**	**6**
pMRTP_HI	90	M	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
		F	0	0	0	0	**6**	**6**	**6**	**6**
TOTAL	**982**		**83**	**71**	**71**	**71**	**136**	**106**	**154**	**184**

4.4.2.3 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 (the study would include miRNA profiling) and to help in translatability issues.

A summary of the number of samples that may then be analyzed is given in Table 7.

4.4.2.4 Trachea/Larynx samples

Provided training samples deliver good quality results, trachea/larynx samples may be analyzed to answer the following questions:

36. Is there a dose-response to ZRH in larynx/trachea of male rats?
37. Is there a dose-response to ZRH in larynx/trachea of female rats?
38. How do the responses compare in male and female rats?
39. How do the dose-responses to ZRH and CS compare in male rats?
40. How do the dose-responses to ZRH and CS compare in female rats?
41. Are there still gene expression changes after 42days without exposure in nasal epithelium of male rats exposed to ZRH for 90 days? Is this dose-dependent?
42. Are there still gene expression changes after 42days without exposure in nasal epithelium of female rats exposed to ZRH for 90 days? Is this dose-dependent?
43. How does post exposure compare in CS vs. ZRH-exposed male rats?
44. How does post exposure compare in CS vs. ZRH-exposed female rats?

A summary of the number of samples that may then be analyzed is given in Table 7.

Table 7: Lower Priority Samples for Transcriptomics: their processing will depend on the results obtained in other studies.

Group	Time point (days)	Gender	Blood	Trachea + Larynx
Sham	28	M	**6**	**6**
	90	M	**6**	**6**
		F	**6**	**6**
	90+42	M	**5**	**5**
		F	**5**	**5**
CS_LOW	28	M	**6**	**6**
	90	M	**6**	**6**
		F	**6**	**6**
	90+42	M	**6**	**6**
		F	**6**	**6**
CS_MED	28	M	**6**	**6**
	90	M	**6**	**6**
		F	**6**	**6**
	90+42	M	**6**	**6**
		F	**6**	**6**
CS_HI	90	M	**6**	**6**
		F	**6**	**6**
	90+42	M	**6**	**6**
		F	**6**	**6**
pMRTP_LOW	28	M	**6**	**6**
	90	M	**6**	**6**
		F	**6**	**6**
	90+42	M	**6**	**6**
		F	**6**	**6**
pMRTP_MED	28	M	**6**	**6**
	90	M	**6**	**6**
		F	**6**	**6**
	90+42	M	**6**	**6**
		F	**6**	**6**
pMRTP_HI	90	M	**6**	**6**
		F	**6**	**6**
TOTAL	**368**		**184**	**184**

4.4.3 Sample retrieval from Singapore and biobanking

Samples will be shipped directly from Singapore to Neuchâtel without passing for registration to Biostorage, due to urgent needs of getting the samples and results, and because the OECD+ part is not performed under GLP.

Samples will be sorted after randomization, using Liquid Nitrogen and Dry ice to avoid any thawing of the samples.

Unique labelling will be given to each sample and will be entered in the LIMS biobanking database.

4.4.4 Blood samples processing and profiling

Blood samples will be collected and analysed for their mRNA profile by microarray, their miRNA profile by Affymetrix array, and their DNA methylation profile. Steps are summarized in Figure 9.

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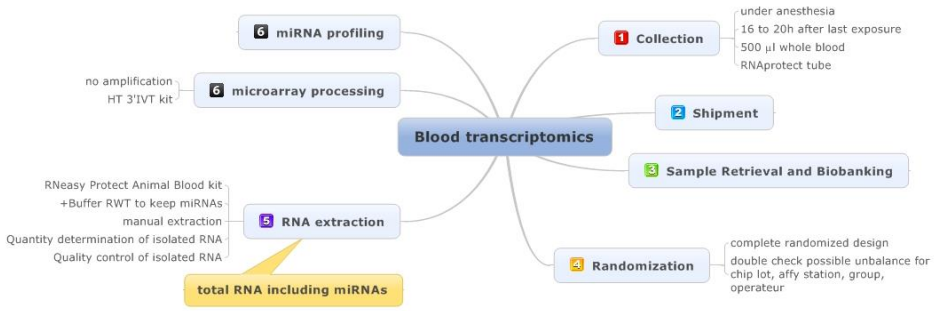


Figure 9: Schematic processing of blood samples

500µl of whole blood collected from retro-orbital plexus in RNAprotect animal blood tubes using uncoated (= not containing any heparin) capillary tubes will be processed for RNA extraction (total RNA + miRNAs) using the Qiagen RNeasy Protect Animal Blood Kit and Buffer RWT (Figure 10).

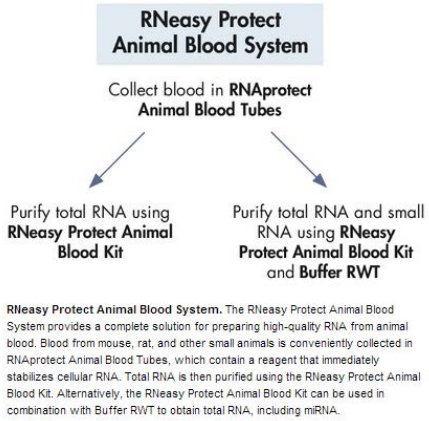


Figure 10: RNeasy Protect Animal Blood System (Qiagen)

500µl of whole blood collected from retro-orbital plexus in EDTA tubes (to reduce DNA degradation) will be processed for DNA extraction.

4.4.5 Liver samples processing and profiling.

Liver samples will be collected and analysed for their mRNA profile by microarray. Steps are summarized in Figure 11.

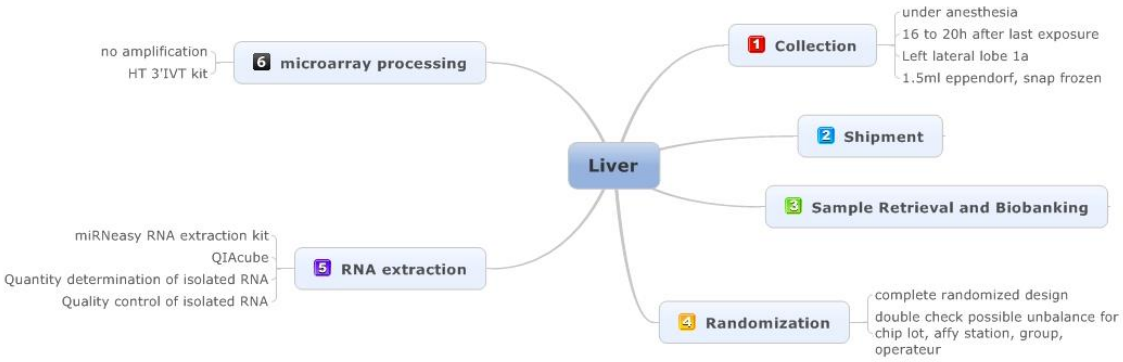


Figure 11: Schematic processing of liver samples

4.4.6 Nasal epithelium processing and profiling

Nasal epithelium samples will be collected and analysed for their mRNA profile by microarray. Steps are summarized in Figure 12.

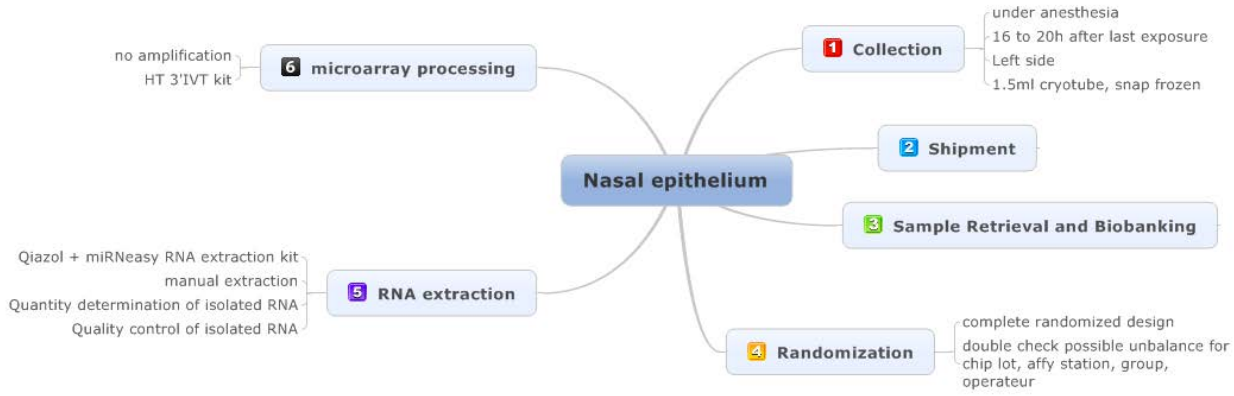


Figure 12: Schematic processing of nasal epithelium samples.

4.4.7 Trachea/Larynx samples processing and profiling

Trachea samples will be collected and analysed for their mRNA profile by microarray. Steps are summarized in Figure 13. As no prior experience exists in processing of larynx samples and no training samples are available, it was decided that 1 to 2 samples per group could be used for training and setting-up purposes. Two methods will be investigated for this purpose: a) filling from inside with RNA extraction buffer b) scraping with a scalpel the epithelial cells.

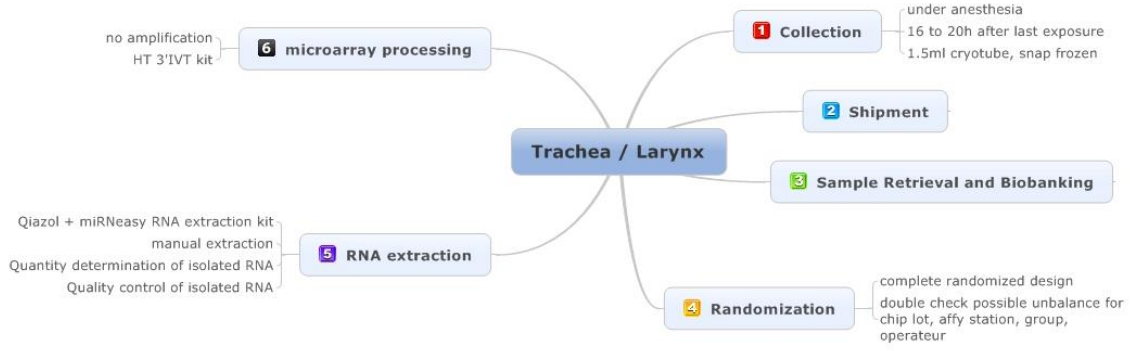


Figure 13: Schematic processing of trachea samples.

4.4.8 Lung samples processing and profiling

Lung samples will be collected and analysed for their mRNA profile by microarray and their miRNA profile by Affymetrix chip. Steps are summarized in Figure 14.

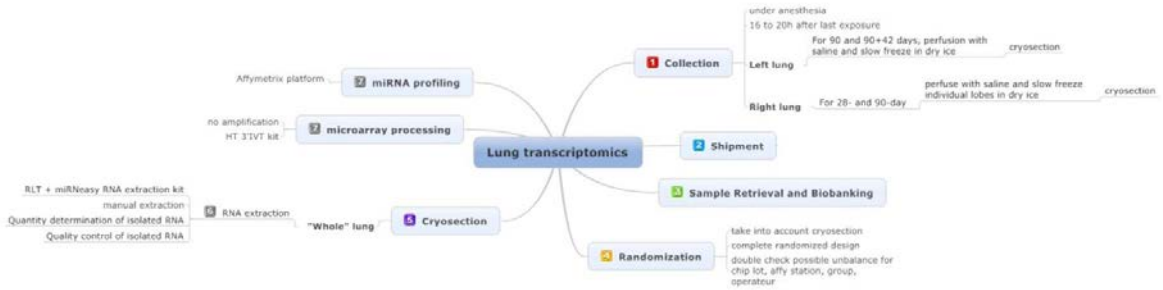


Figure 14: Schematic processing of lung samples.

The Tissue Research (TR) Laboratory will cryoslice the right lung lobes or the left lung lobe into 40μm slices.
For 28 day time point, slices from the right lung lobes will all go for transcriptomics: they will either go in tubes for lobe-specific profiling or in a tube for the pooled profile of the right lung lobes. In detail, each lobe will be sliced in serial sections according to the following ratios: 4 slices for lobe-specific profiling and 1 slice for pooled right lung lobes.
For 90 and 90+42 days, serial sections of the inner region of the lungs (in green in Figure 15) will be allocated to transcriptomics, targeted proteomics and untargeted proteomics according to 1:1:1 ratios. Slices from parenchyma and main bronchus (in gray and violet in Figure 15) will be used for LCM only. As LCM is not planned yet, samples should be kept until further processing.

Slices for transcriptomics will be placed in a 2 ml MagNA Lyser Green Beads (Roche CatN°03 358 941 001) tube containing ceramic beads for homogenization in 600ul RLT buffer (Qiagen CatN°79216) + B-ME (Sigma), vortexed 30 sec and frozen on dry ice until RNA extraction.

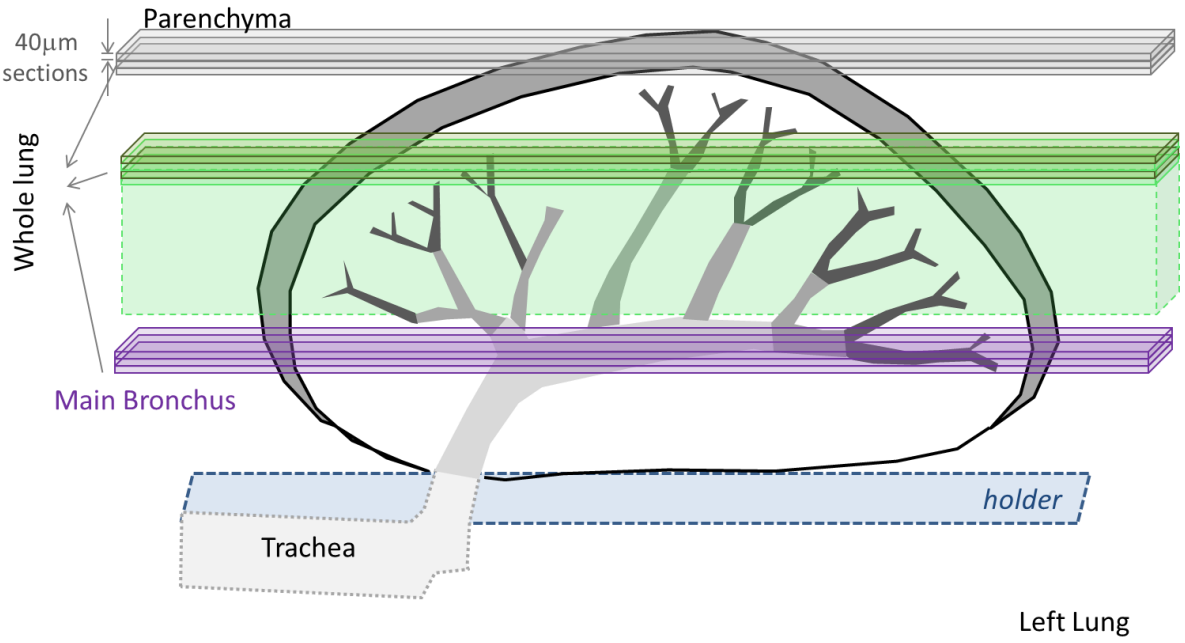


Figure 15: Schematic lung cryosection.

The whole process will be performed according to the company's SOPs for tissue cryoslicing and laser capture microdissection (Preparation of cryosections with cryostat).

4.4.9 Sample randomization protocol

For each tissue, the full set of samples will be randomized together.

Only one restriction on randomization will be included for all tissues except for lung tissues, ensuring a balanced assignment within/across experimental groups to the hybridization stations (four stations will be used for this study, all the same model).

For the lung, all samples will be randomized, balanced on Affy station, cryoslicing batches, and RNA extraction batches.

4.4.10 RNA extraction from tissue

- miRNeasy® RNA extraction from tissue (for liver, nasal epithelium, larynx, and lung)

  RNA extraction from nasal epithelium and liver will be done using qiazol and miRNeasy® Mini Kit (QIAGEN cat #217004). The miRNeasy kits are designed for purification of total RNA, including miRNA and other small RNA molecules, from small amounts of cultured cells and various animal and human tissues. Phase Lock Gel Heavy 2ml tubes (5Prime cat #2302830) were used for better Qiazol/Chloroform separation according to manufacturer recommendation. Lung samples RNA will be isolated using RLT+B-ME and miRNeasy mini kit. The modified protocol allows the preservation of miRNA species (see below). The QIAcube robot can process 12 samples simultaneously and the procedure will be adjusted for isolation of mRNA as well as miRNA. The qiacube will be used for liver and larynx samples. Nasal epithelium and lung samples will be processed manually to avoid loss of material due to dead

volume of the Qiacube (miRNeasy Mini Handbook; Modified Protocol: Purification of Total RNA from Lung cryoslices including microRNAs).

- RNeasy Protect Animal Blood System for blood samples as per Qiagen recommendations.

4.4.11 Quantity determination of Isolated RNA

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.

4.4.12 Quality control of the Isolated RNA

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.

4.4.13 mRNA microarray

All RNA samples from the whole lung, nasal epithelium, larynx, blood and liver will be analysed with Affymetrix. Randomization of samples will be applied during Affymetrix hybridization as described in section 4.4.8.

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

4.4.13.1 High Throughput 3' In Vitro Transcription (HT 3' IVT)

Affymetrix 3' expression arrays have probe sets targeting the 3' end of the transcript. Targets for these arrays are prepared by using amplification and labeling methodologies that generate labeled targets initially primed from the poly-A tail of the transcript. The procedure will be carried out as described in the GeneChip® HT 3' IVT Express Kit User Manual (Affymetrix). The GeneChip® HT 3' IVT Express Kit represents the newest high-throughput target generation assay for 3'-based arrays. The configuration of the HT 3' IVT Express Kit is optimized for use with liquid-handling robotics and generates a high-quality, array-ready target and requires minimal manual intervention. As starting material 100ng total RNA gained from the different samples will be used. All samples will be processed in one series after all RNA preparations have been completed. The aRNA generation, labeling, and fragmentation procedure is depicted in Figure 16.

Total RNA undergoes reverse transcription to synthesize first-strand cDNA. Therefore, the RNA is primed with T7 oligo(dT) primer to synthesize cDNA which contains a T7 polymerase promoter sequence. This cDNA is then converted into a double-stranded DNA template for transcription. This reaction employs DNA polymerase and RNAse H to simultaneously degrade the RNA and synthesize the second strand.

Double-stranded cDNA is amplified in a linear fashion by In Vitro Transcription (IVT) using T7 polymerase. As the aRNA (amplified RNA also commonly referred to as cRNA) is being synthesized biotin-conjugated nucleotides are incorporated and serve as a label for the message.

The aRNA is then purified to remove unincorporated NTPs, salts, enzyme and inorganic phosphate. Fragmentation of the biotin-labeled aRNA prepares the samples to the hybridization onto GeneChip® Arrays.

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 unfragmented 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 (see Figure 16 **left**). 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.

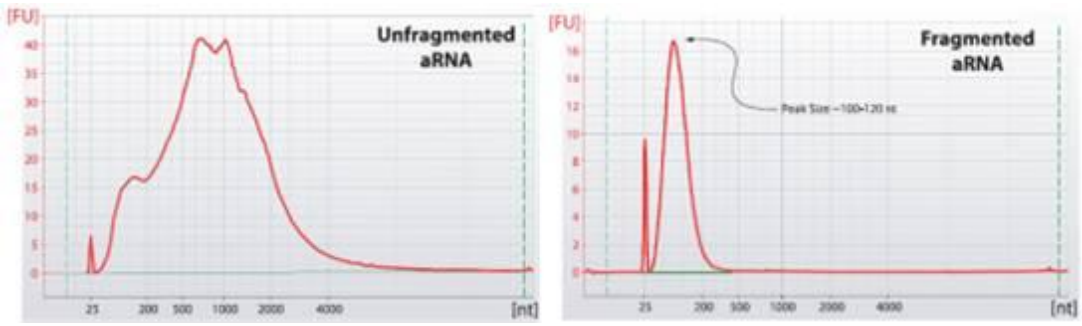


Figure 16: Examples aRNA profiles.

After the aRNA yield calculation, the Biomek FXp TPE System will automatically proceed through fragmentation and labeling 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 fragmented aRNA with a sharp peak (Figure 16 **right**).

4.4.13.2 Ovation® RNA Amplification System V2 and Encore® Biotin Module **(Blood RNA Samples)**

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 17). The Encore® Biotin Module allows the fragmentation and the labeling process via the combination of

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.

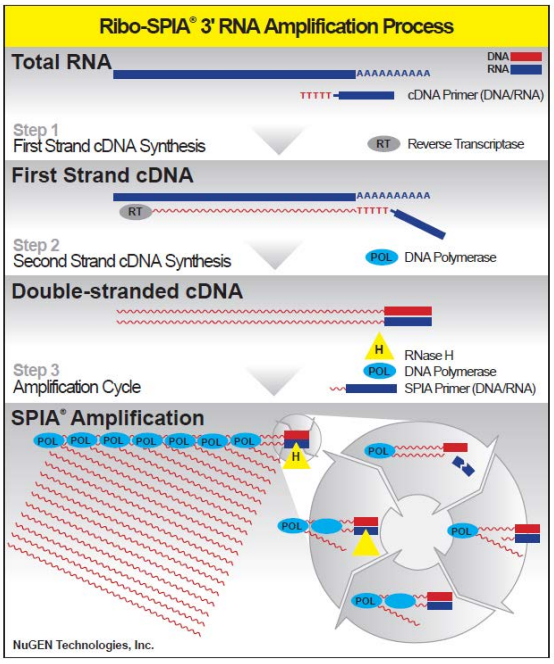


Figure 17: The Ribo-SPIA Amplification Process used in the Ovation RNA Amplification System V2

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 hetero-duplex 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 NTPs, 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 18). 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.

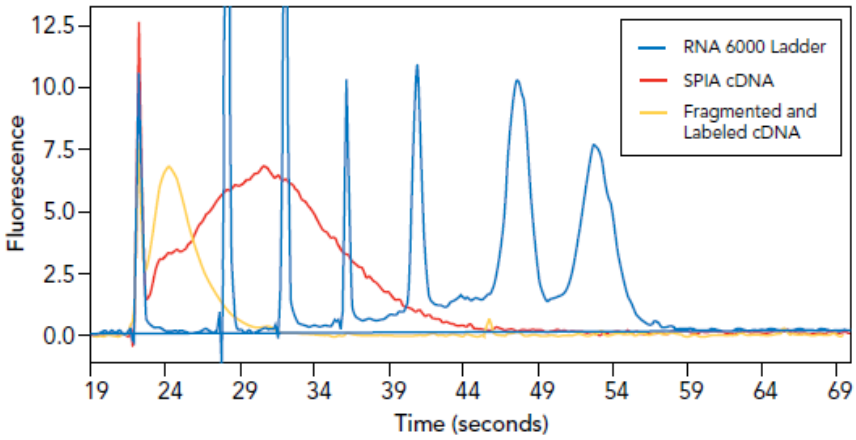


Figure 18: Bionalyzer trace of Amplified, Un-fragmented and Fragmented cDNA

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

For the HT 3'IVT, the Biomek FXp TPE System will proceed to make the hybridization ready sample by mixing appropriate amounts of fragmented aRNA and hybridization cocktail master mix according to the manufacturer's manual. The final array format will be selected and this option will produce approximately 250µl of hybridization cocktail per well including the fragmented aRNA and the controls (processed poly-A RNA controls, hybridization controls, control oligonucleotide B2). Each sample is hybridized to an array. All the arrays are moved to the hybridization oven for 16 hours at 45°C with 60 rpm. After hybridization arrays will be washed several times and then stained with Fluidic Stations FS450 using AGCC Software with the protocol FS450_0001.

For the NuGen Method, after the labeling and after checking that the fragmentation was done correctly according to the manufacturer's guidelines, the hybridization cocktails are prepared. 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 will be moved to GeneChip® Scanner 3000 7G and each array will be scanned.

4.4.13.4 Monitoring the Quality Check of 3' IVT Expression Arrays

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

This image will be monitored for

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

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

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

The results are reported in tabular and graphical formats (Expression Report (.rpt) file).

In addition, pseudo images will be calculated using R. These pseudo images provide a better means to monitor artifacts that are not visible on the original image like non-uniform distribution of signals.

4.4.14 miRNA microarray

miRNA analysis will be performed on GeneChip® miRNA 3.0 array as follow:

Total RNA or low molecular weight (LMW) RNA will be labelled with the FlashTag™ Biotin HSR Labeling Kit and analysed on Affymetrix® GeneChip® miRNA Arrays. Minimal amount of RNA input is 130ng.

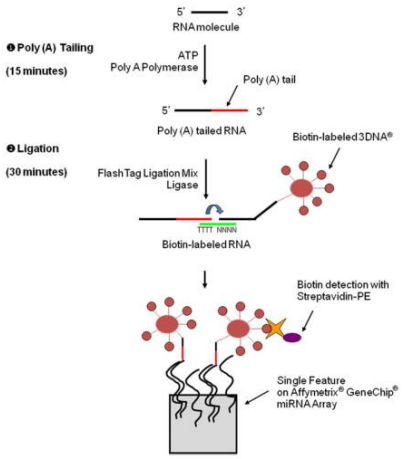


Figure 19*: FlashTag™ HSR: Procedure Overview*

4.4.14.1 RNA Sample and Quantitation

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).

4.4.14.2 FlashTag™ Biotin HSR RNA Labeling Procedure

An automated script is being developed and validated internally allowing a throughput of 96 samples/day. Both techniques (manual and automated) may be used for this study depending on needs. The procedures are described below and a general overview is provided in Figure 19.

4.4.14.3 Poly (A) Tailing

1. Adjust the volume of RNA to 8 µL with Nuclease-Free Water (Vial 11).
2. Transfer the 8 µL RNA to ice. Add 2 µL RNA Spike Control Oligos (Vial 8) and return to ice.
3. Dilute the ATP mix (Vial 3) in 1 mM Tris as follows:
    i. For total RNA samples, dilute the ATP Mix 1:500.
    ii. For enriched, quantitated samples, calculate the dilution factor according to the following formula:

        5000 ÷ ng input LMW RNA
4. Dilute the ATP Mix 1:50.
    i. For enriched samples that are not quantitated, calculate the dilution factor according to the following formula:

        1000 ÷ µg input total RNA
5. Assemble a Poly A Tailing Master Mix in a nuclease-free tube.

6. Add 5 µL of Master Mix to the 10 µL RNA/Spike Control Oligos (Step 2 above), for a volume of 15 µL.
7. Mix gently (do not vortex) and microfuge.
8. Incubate in a 37°C heat block for 15 minutes. Discard any unused, diluted ATP mix from Step 3.

4.4.14.4 FlashTag™ Biotin HSR Ligation

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

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

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

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

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

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

Mix and microfuge the 23.5 µL of ligated sample.

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

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

4.4.14.5 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 8 to the 21.5 µL biotin-labeled sample in the order listed, to prepare the array hybridization cocktail Table 9:

Table 8: Hybridization Cocktail (for a single reaction)

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

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

Table 9: Hybridization Cocktail Using a Hybridization Master Mix

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

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

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

6. Remove the pipet tip from the upper right septum of the array.

7. Cover both septa with 1/2" Tough-Spots® to minimize evaporation and/or prevent leaks.

8. Place the arrays into hybridization oven trays.

9. Load the trays into the hybridization oven.

10. Incubate the arrays at 48°C and 60 rpm for 16 to 18 hours.

4.4.14.6 Washing and Staining

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

1. After 16 to 18 hours of hybridization, remove the arrays from the oven. Remove the Tough-Spots from the arrays.
2. Extract the hybridization cocktail from each array and transfer it to a new tube or well of a 96-well plate in order to save the hybridization cocktail. Store on ice during the procedure, or at –80°C for long-term storage. Refer to Appendix B of Flashtag User Guide, Array Rehybridization Procedure on page 21, if necessary.
3. Fill each array completely with Array Holding Buffer.
4. Allow the arrays to equilibrate to room temperature before washing and staining.

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

5. Place vials into sample holders on the fluidics station:
   A. Place one (amber) vial containing 600 µL Stain Cocktail 1 in sample holder 1.
   B. Place one (clear) vial containing 600 µL Stain Cocktail 2 in sample holder 2.
   C. Place one (clear) vial containing 800 µL Array Holding Buffer in sample holder 3.
6. Wash and stain with Fluidics Station 450 using the appropriate fluidics script for the array format.
7. 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.

4.4.14.7 Scanning

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

Data normalization and quality control

Use Expression Console software for data summarization, normalization, and quality control (v. 1.2 and higher). Please refer to the website for Expression Console for instructions on miRNA Array analysis Figure 20. 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.

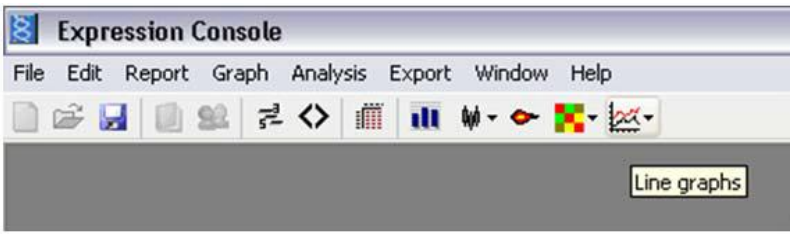


Figure 20: Expression console window

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

> spike_in-control-2_st

> spike_in-control-23_st

> spike_in-control-29_st

> spike_in-control-31_st

> spike_in-control-36_st.

Oligos 2, 23 and 29 are RNA, and confirm the poly(A) tailing and ligation. Oligo 31 is poly(A) RNA and confirms ligation. Oligo 36 is poly(dA) DNA and confirms ligation and the lack of RNases in the RNA sample. If the array hybridization, wash, stain and scan procedure was successful, the arrays should be gridded successfully and cel and chp files should be generated. The hybridization control probe sets should have signal commensurate with concentration: AFFX-r2-Ec-c1-BioB-3_at < AFFX-r2-Ec-c1-BioC-3_at < AFFX-r2-Ec-c1-BioD-3_at < AFFX-r2-Ec-c1-cre-3_at Export the data into third party software for further analysis.

4.4.15 DNA methylation analysis (blood samples)

4.4.15.1 DNA isolation

DNA will be isolated from 500 ul whole blood in EDTA tube using the Qiagen QIA amp DNA blood midi kit (ref 51183).

4.4.15.2 DNA methylation analysis

As the establishment of the method is still ongoing, an amendment to the study plan will be written at a later time point.

4.4.16 Data Processing

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.

4.4.17 Sample Processing Prioritization (Transcriptomics)

Table 10: Prioritization Table for Samples Processing and Estimated Timelines

	Processing order	Total number of samples	RNA extraction	Affymetrix	Planned date cryoslicing done	Planned date microarray done
28d – right lung	1 (highest priority)	84 lung lobes – 114 RNA samples	Manual?	3'IVT	End June 2013	End July 2013
90/90+42 right + left	2 (high priority)	TBD (depends on scenario)	Qiacube	3'IVT	TBD (depends on scenario)	TBD (depends on scenario)
90/90+42 RNE	3 (high priority)	154	Manual	3'IVT	NA	TBD
28/90/90+42 liver	4	184	Qiacube	3'IVT	NA	TBD
28/90/90+42 tranchea+larynx	6 (lower priority)	TBD	Qiacube	3'IVT	NA	TBD
28/90/90+42 blood	7 (lower priority)	TBD	Manual?	Nugen	NA	TBD

5 Statistical Methods

NA

6 Administrative Aspects

6.1 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

7 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.

8 References & Sources

8.1 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.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5(10):R80. Epub 2004/10/06. PubMed PMID: 15461798; PubMed Central PMCID: PMC545600.

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.

OECD OfEC-oaD. Test No. 412. Subacute-inhalation-toxicity-28-day-study. http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD-TG412.pdf2009.

R Development Core Team. R: A Language and Environment for Statistical Computing. 2007.

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.

8.2 Philip Morris International's List of Standard Operating Procedures (SOPs) and Work Instructions (WKIs)

Preparation of cryosections with cryostat: (PMI_RD_WKI_001250)

miRNeasy Mini Handbook: http://disco.app.pmi/disco/drl/objectId/0901d4ec80377994

Modified Protocol: Purification of Total RNA from Lung cryoslices including microRNAs:

HT 3'IVT Express Kit: http://disco.app.pmi/disco/drl/objectId/0901d4ec80377993

GeneChip® miRNA 3.0 array: http://media.affymetrix.com/support/technical/datasheets/mirna_3_datasheet.pdf

Flashtag User Guide: https://disco.app.pmi/disco/drl/objectId/0901d4ec8058591a

8.3 Philip Morris International's Internal Documents

Philip Morris International. Unpublished on file data. Internal Study Plan: STUDY PLAN 150122 - OECD NIKE-GENOMIC ENDPOINTS. Study Number 150122. 2012.

Philip Morris International. Unpublished on file data. Internal business case. SBDAV_ACL_OECD (+): 90 DAY INHALATION STUDY (OECD 413) INCLUDING ADDITIONAL ENDPOINTS (OECD+). 2012.

15006 THS SP: Philip Morris International. Unpublished on file data. Internal Study Plan; "90 DAY OECD INHALATION STUDY ON SPRAGUE-DAWLEY RATS TO CHARACTERIZE TOXICITY OF MAINSTREAM AEROSOL FROM ZURICH FPD 4.2 CIGARETTES AND COMPARISON TO 3R4F MAINSTREAM AEROSOL TOXICITY". Study number 15006. 2013.

15006 THS SR Part 3: Philip Morris International. Unpublished on file data. Internal Study Report; "STUDY REPORT 90 DAY OECD INHALATION STUDY ON SPRAGUE-DAWLEY RATS TO CHARACTERIZE TOXICITY OF MAINSTREAM AEROSOL FROM ZURICH FPD 4.2 CIGARETTES AND COMPARISON TO 3R4F MAINSTREAM AEROSOL TOXICITY STUDY NUMBER 15006 PART 3 – STUDY AMENDMENTS AND DEVIATIONS". Study number 15006. 2014.

9 Appendix

Not available