Study N°.	160400	Study Plan Amendment N°1	

Short Title	**Adhesion Study – Fresh Direct Protocol with 3R4F and THS2.2 – MP**

Study Director	Carine Poussin	Date issued	24.02.14

Previous Text Version

We are adding additional information to the SP: https://disco.app.pmi/disco/drl/objectId/0901d4ec80427a58.

This amendment includes:

1) The design of the main phases of the adhesion study:
   - Fresh Direct protocol testing 3R4F vs ZRH sbPBS fractions
   - Indirect/Direct protocols testing: conditioned-media generated without or with MM6 cells exposed to 3R4F or ZRH sbPBS fractions
2) The links to the metadatasheet and the randomization plan for:
   - mRNA extraction phase
   - hybridization phase
3) Protocols to generate transcriptomics data
   - mRNA extraction protocol

Current Text Version

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1 Main phase (MP) experimental design for the adhesion assay using the Fresh Direct protocol

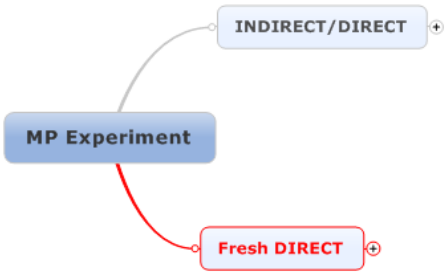
1.1 Conventional cigarette and MRTP

ZRH sticks (THS2.2) used in this main phase are coming from the following batch B08164.

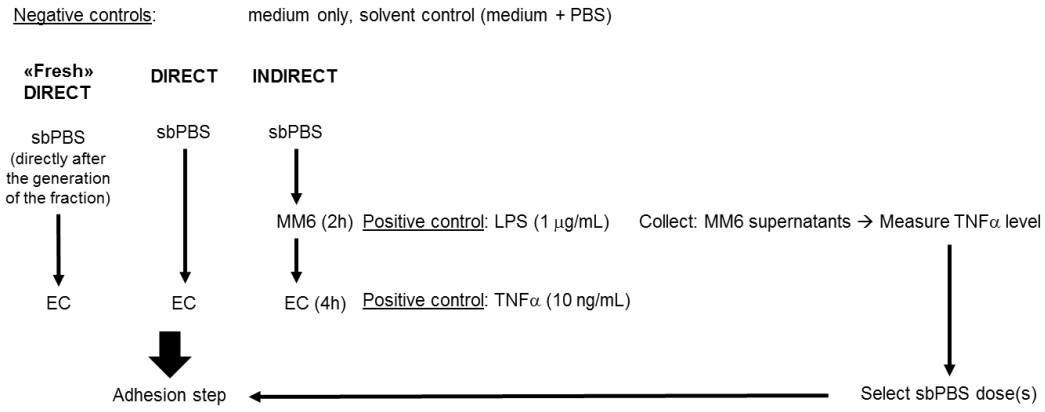
https://disco.app.pmi/disco/drl/objectId/0901d4ec80441c7c

1.2 Exposure design

Main phase (part 1) - Fresh Direct protocol to test 3R4F vs ZRH
Main phase (part 2) – Indirect and Direct protocol to test 3R4F vs ZRH



The adhesion assay can be conducted in direct (Direct and “Fresh” Direct) or indirect treatment conditions.



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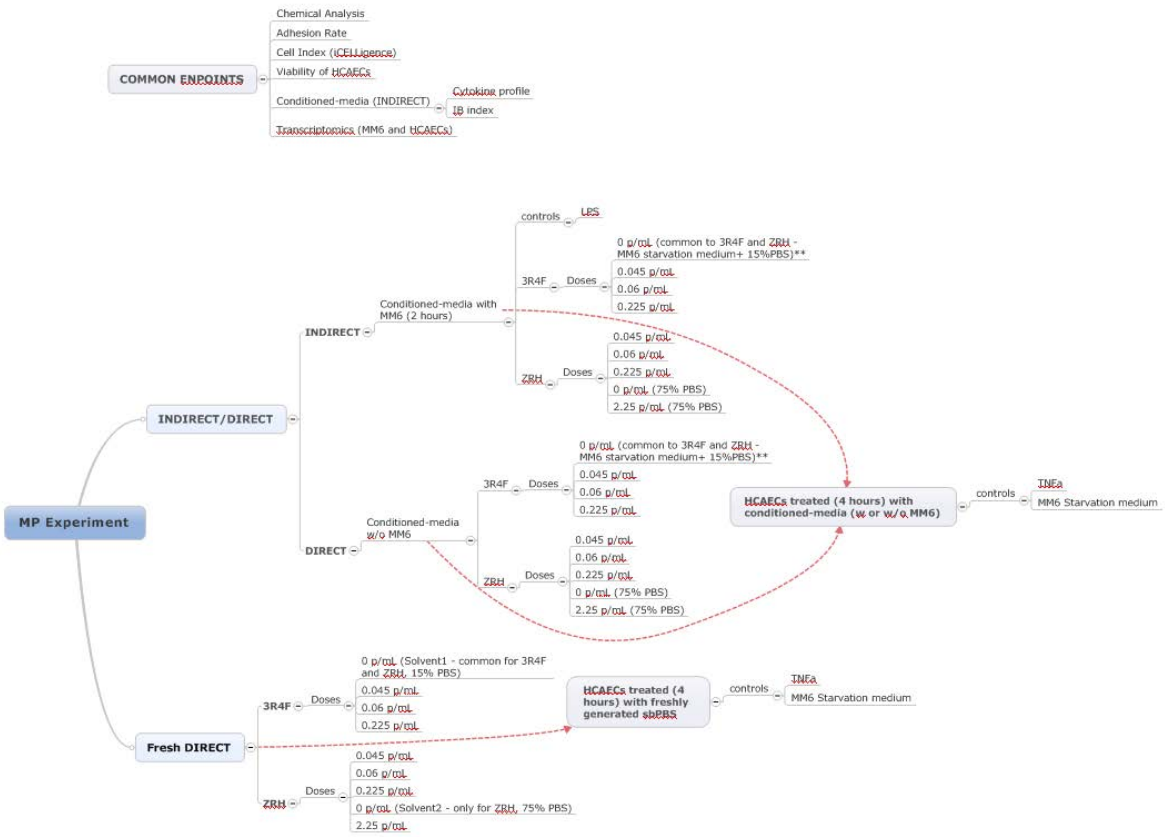
In Direct protocol, the sbPBS medium is processed the same way as for the Indirect protocol in order to have a proper control, however without using MM6 cells (sbPBS is diluted in MM6 starvation medium, incubated for 2 hours at 37°C, frozen and thawed, incubated with HCAECs for 4 hours at 37°C). The sbPBS fraction contains volatile and highly reactive molecules among other CS constituents. The effect of sbPBS fraction on HCAECs might be different if the chemical content changes upon Direct and “Fresh” Direct protocols. Therefore, the adhesion assay has been also performed using the “Fresh” Direct protocol for which freshly generated sbPBS is rapidly used to treat HCAECs for 4 hours.



The fractions tested will be 3R4F and ZRH sbPBS.

HCAECs will be exposed 4 hours to the sbPBS fractions (Fresh Direct protocol) or to conditioned-media (Indirect and Direct protocols) as defined in former experiments (Report on the adhesion assay using HCAECs: https://disco.app.pmi/disco/drl/objectId/0901d4ec80460162).

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The results of our former experiments suggest that the adhesion of monocytic cells to endothelial cells occurs through two different mechanisms.

- at low/middle doses: 0.045 / 0.06 puff/mL in Indirect conditions.

at high doses: beyond 0.18 puff/mL in Fresh Direct conditions.

For the coming data analysis, it will be important to have common doses while using the Indirect/Direct and Fresh Direct protocols.

The low dose was chosen on the basis that a maximum adhesion rate has been observed in Indirect conditions. Since we do not have the results of the MP of the second experimental arm (Indirect/Direct) yet, two doses 0.045 and 0.06 puff/mL were selected, however only one dose maximizing the adhesion rate will be chosen for the final hybridization of mRNA samples to generate transcriptomics data.

The high doses were chosen on the basis that a maximum adhesion rate with a minimum HCAECs death (< 20%) has been observed in Fresh Direct conditions. It was important to have a common high dose (0.225 puff/mL) for 3R4F and ZRH. However, an additional high dose has been added for ZRH only (2.25 puff/mL) at which an adhesion effect is observed. This dose is too toxic for HCAECs when using 3R4F.

Endpoints measured:

- Adhesion rate (Cellomics)
- Cell index over time (iCELLigence)
- Chemical analysis (Carbonyls)
- Cell viability (Resazurin assay)
- Transcriptomics (Affymetrix)

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Layout for "Fresh" Direct protocol

Plate layout for Transcriptomic

		Doses sbPBS (p/mL)									
		15% PBS	75% PBS	3R4F - 15% PBS			ZRH - 15% PBS			ZRH - 75% PBS	
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25	
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25	
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25	
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25	
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25	
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25	
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Seeding HCAEC: 30k cells/well											

CTL - : MM6 starvation medium
CTL + : TNFa (10ng/ml)

Layout for Indirect/Direct protocol

Generation of conditioned media with MM6 cells

Plate layout

Plate 1	**sbPBS 3R4F**					
			15% PBS			
puff/ml	**0 (15% PBS)**	**0 (75% PBS)**	**0.045**	**0.06**	**0.225**	
1 million MM6/well	Replicat 1	Replicat 1	Replicat 1	Replicat 1	Replicat 1	
	Replicat 2	Replicat 2	Replicat 2	Replicat 2	Replicat 2	
	Replicat 3	Replicat 3	Replicat 3	Replicat 3	Replicat 3	

Plate 2	**sbPBS ZRH**					
	15% PBS			75% PBS		
puff/ml	**0.045**	**0.06**	**0.225**	**2.25**	**LPS (1µg/ml)**	**Triton**
1 million MM6/well	Replicat 1	Replicat 1	Replicat 1	Replicat 1	Replicat 1	Replicat 1
	Replicat 2	Replicat 2	Replicat 2	Replicat 2	Replicat 2	
	Replicat 3	Replicat 3	Replicat 3	Replicat 3	Replicat 3	

Plate 1 + 2 : 31 million MM6 necessary

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Generation of conditioned-media without MM6 cells

Plate 3	**sbPBS 3R4F**					
			15% PBS			
puff/ml	**0 (15% PBS)**	**0 (75% PBS)**	**0.045**	**0.06**	**0.225**	
No MM6 cells	Replicat 1	Replicat 1	Replicat 1	Replicat 1	Replicat 1	
	Replicat 2	Replicat 2	Replicat 2	Replicat 2	Replicat 2	
	Replicat 3	Replicat 3	Replicat 3	Replicat 3	Replicat 3	

Plate 4	**sbPBS ZRH**					
	15% PBS			75% PBS		
puff/ml	**0.045**	**0.06**	**0.225**	**2.25**		
No MM6 cells	Replicat 1	Replicat 1	Replicat 1	Replicat 1		
	Replicat 2	Replicat 2	Replicat 2	Replicat 2		
	Replicat 3	Replicat 3	Replicat 3	Replicat 3		

Plate 1 + 2 : No MM6 necessary

Treatment of HCAECs with conditioned-media for 4 hours

		15% PBS	75%PBS	3R4F			ZRH					
		0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		Indirect
		0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
		0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
		0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
		0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
		0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		

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		15% PBS	75%PBS	3R4F			ZRH					
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		Direct
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
CTL-	CTL +	0	0	0.045	0.06	0.225	0.045	0.06	0.225	2.25		
Seeding HCAEC: 30k cells/well												

CTL-: MM6 starvation medium
CTL+:TNFa (10 ng/mL) (diluted in MM6 Starvation medium + PBS 15%

2 Metadatasheet and randomization plan for RNA extraction

mRNA extraction

https://disco.app.pmi/disco/drl/objectId/0901d4ec80474a0e
Metadatasheet_S160400_Exp055_CW12_Adhesion_Assay.xlsx

https://disco.app.pmi/disco/drl/objectId/0901d4ec80474a0d

Metadatasheet_S160400_Exp056_CW13_Adhesion_Assay_MM6.xlsx

https://disco.app.pmi/disco/drl/objectId/0901d4ec80474a10

Metadatasheet_S160400_Exp056_CW13_Adhesion_Assay_HCAEC.xlsx

mRNA reverse transcription and hybridization

https://disco.app.pmi/disco/drl/objectId/0901d4ec80474a0f

Metadatasheet_S160400_Exp055+056_Adhesion_Assay_MM6+HCAEC_AFFYMETRIX_WITH_IDs.xlsx

3 Protocols for transcriptomics

3.1 Extraction of mRNA

3.1.1 Extraction of Human Coronary Artery Endothelial Cells: HCAEC

Total RNA will be extracted from cells using the QIAgen **RNeasy® Micro Kit (http://www.qiagen.com/Products/Catalog/Sample-Technologies/RNA-Sample-Technologies/Total-RNA/RNeasy-Micro-Kit).** The protocol for RNA extraction is detailed below:

a. Cells are washed with cold PBS 1X twice.
b. PBS is removed.
c. Cells from pooled wells are resuspended in RLT buffer (containing 1% βmercapto-ethanol) and transfer to labeled tubes.

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d. If samples need to be pooled, the same amount of RLT will be used per pooled well and transferred from well to well: 350 uL

e. Vortex.

f. Samples are frozen in dry ice before storage at -80 C.

1. RNA will be extracted from the frozen samples manually or using the Qiacube robot, following the instructions of the manufacturer and using 20µl as an elution volume.
2. All samples will be extracted on the same day.
3. After extraction, RNA samples will be QC (RIN measurement via Bioanalyzer) and selected for hybridization if good quality/yield are obtained.

3.1.2 Extractions of the Mono Mac 6 Human Monocytic Cell: MM6

Total RNA will be extracted from cells using the QIAgen **RNeasy® Mini Kit (http://www.qiagen.com/products/catalog/sample-technologies/rna-sample-technologies/total-rna/rneasy-mini-kit).** The Qiagen miRNeasy mini kit may be used at this step according to the manufacturer instructions.

The protocol for RNA extraction is detailed below:

a. Cells are washed with cold PBS 1X.
b. Centrifuge 7 min at 400g
c. Repeat steps a and b
d. PBS is removed.
e. Cells from pooled wells are resuspended in RLT buffer (containing 1% βmercapto-ethanol) and transfer to labeled tubes.
f. Vortex.
g. Samples are frozen in dry ice before storage at -80 C.

4. RNA will be extracted from the frozen samples manually or using the Qiacube robot, following the instructions of the manufacturer and using 30µl as an elution volume.
5. All samples will be extracted on the same day.
6. After extraction, RNA samples will be QC (RIN measurement via Bioanalyzer) and selected for hybridization if good quality/yield are obtained.

1.1.1 Sample randomization

In order to minimize batch effects, we will apply a randomization protocol at the time of RNA extraction and at the time of hybridization (see paragraph 2).

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1.1.2 Quantity determination of isolated RNA

The quantity of the isolated RNA will be determined with the Nanodrop 1000 or 8000 spectrophotometer (Thermo Scientific). For the subsequent RNA amplification protocol (Ovation RNA Amplification System V2) an amount of 50ng RNA is required. To carry out the Affymetrix IVT express labeling, a minimum of 100ng in 3uL will be required. The RNA will be normalized in RNAse-DNAse free water in the appropriate volume.

1.1.3 Quality control of the RNA

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

1.1.4 DNA microarray

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

Case N°1: If yields are below 33.3 ng/uL, the Ovation RNA Amplification System V2 may be used (Nugen, The Netherland). The Ovation® RNA Amplification System V2 provides a 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.

All RNA samples will be analyzed with GeneChip Human Genome U133 Plus 2.0 Array (Ref. number 900467, Affymetrix). A randomization of the samples will be determined for Affymetrix hybridization

. Also only one chip lot will be used (lot number will be provided in the final report). Afterwards, the analysis of this raw data will be performed by the Computational Sciences and Bioinformatics team within the Systems Biology Development, Application & Verification program.

Ovation® RNA Amplification System V2 and Encore® Biotin Module

Affymetrix 3' expression arrays have probe sets targeting the 3' end of the transcript. Targets for these arrays are prepared by using amplification and labeling methodologies that generated labeled targets initially primed from the Poly-A tail of the transcript. The procedure will be carried out as described 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

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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.

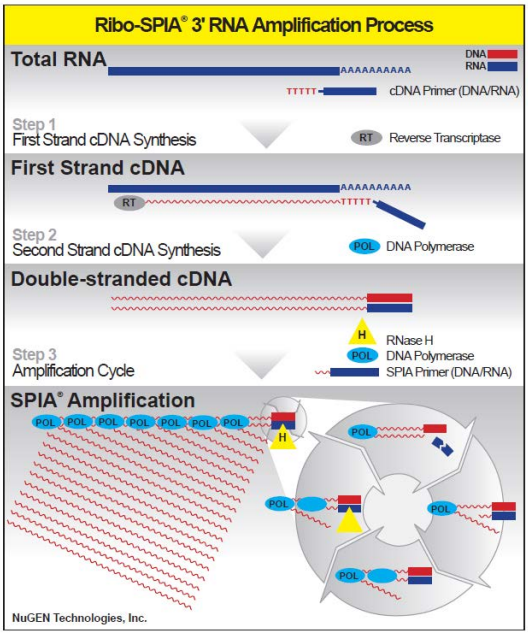

Figure 1: The Ribo-SPIA Amplification Process used in the Ovation RNA Amplification System V2

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.

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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 2**). 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.

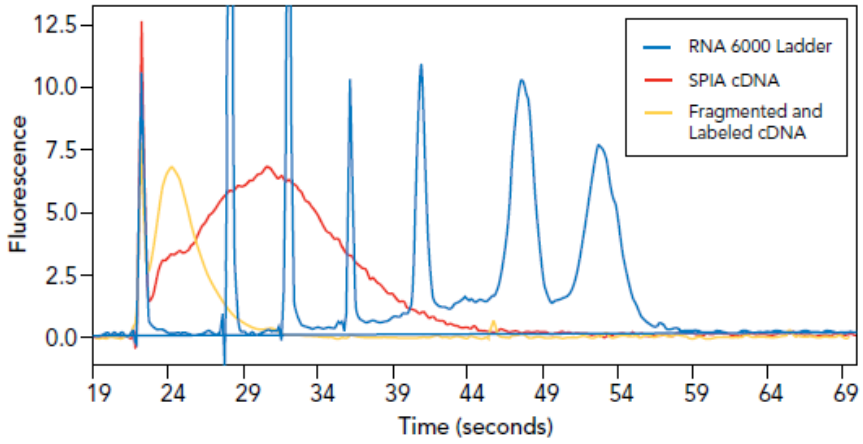

Figure 2. Bionalyzer trace of Amplified, Un-fragmented and Fragmented cDNA

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.

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

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

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with Fluidic Stations FS450 using AGCC software and the protocol FS450_0004. In the last step, the arrays are moved into the GeneChip® Scanner 3000 7G and each array will be scanned.

1.1.4.2 Monitoring the Quality Check of 3' IVT Expression Arrays

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

This image will be monitored for:

- Artifacts.
- Overall intensity and intensity distribution.
- Checkerboards at the corners.
- Readability of the array name.
- Central cross.

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

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

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

Case N°2: If yields are greater than 33.3 ng/uL:

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

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

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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.

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

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

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Monitoring the Quality Check of 3’ IVT Expression Arrays

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

This image will be monitored for

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

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

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

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

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

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

Reason for Change

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The design of this main phase could only be done once the adhesion assay was established with HCAECs, and the dose range finding experiments were performed.

Actions Required

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Approval	Name	Date and Signature
Mandatory		
Study Director, WP and Project Leader	Carine Poussin	08/04/14
Program Leader	Julia Hoeng	22.4.2014
Project Leader	Carole Mathis	08.04.14
WP Leader	Hector De Leon	8.4.14
Cellular Research Lab Manager	Stefan Frentzel	Apr. 8, Stefan Frentzel
Supervisor Gene Expression	Emmanuel Guedj	10.04.2014
Computational Scientist	Carine Poussin	08/04/14

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