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SOP for isolating subcellular fractions for 'omics analyses provided to WP2

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PUBLISHABLE SUMMARY

The development of Standard Operating Procedures (SOP) for the isolation of subcellular fractions for Omics analyses has proven to be a challenging task. Not only are the requirements for the following analytical workflows at the different molecular levels very different, but working with clinical samples and highly optimized tissue model systems come with a number of severe limitations. The dominant limitation for the development and validation of protocols is the scarce amount of biological starting material, which severely limits the options for establishing SOPs. At all molecular levels of DNA, RNA, proteins, and metabolites, the major efforts have been devoted to develop procedures that are not only highly reproducible and result in high quality molecular isolates, but that deliver this with only minimal numbers of cells as samples.

For all molecular classes, protocols have first been tested and experimentally validated for their ability to isolate high quality material for Omics analysis. In the following steps, significant efforts have been devoted to the downscaling of input amounts while maintaining comparable quality levels. As a result of the efforts and output of Deliverable 7.1, all partners dispose of SOPs for their respective molecular species. Omics-scale generation of analytical data will be possible for all molecular levels as has already been possible to validate for the proteome and is currently under way for the transcriptome, methylome, and metabolome. Selected metabolites have already proven the sensitivity and validity of the metabolome analysis protocols.

For the isolation of subcellular compartments, extensive efforts have been devoted to the testing and subsequent analysis of the fractions in respect to efficiency and specificity. Optimized and tested SOPs for subcellular fractionation are available to the partners as output of Deliverable 7.1. The further application of the SOPs will depend on the achievable amounts of starting materials, as in contrast to the cellular level samples and analyses, the respective input amounts are significantly higher and thereby currently restricted by the available tissue sizes.

OBJECTIVE

The objective of Deliverable 7.1 is to develop a Standard Operating Procedure (SOP) for the isolation of subcellular fractions for 'omics analyses and to provide the respective output to WP2.

As part of the overall Task T7.1, the development and testing of a SOP for isolating subcellular fractions for 'omics analyses from WP5 samples of in vitro 3D assays and WP6 samples from patients, aims at the isolation of cellular molecular components in a first step and subcellular compartments, including nucleus, cytosol, monoribosomes/polyribosomes and mitochondria in a second step.

INTRODUCTION

The development of Standard Operating Procedures (SOP) for the isolation of subcellular fractions for Omics analyses has proven to be a challenging task. Not only are the requirements for the following analytical workflows at the different molecular levels very different, but working with clinical samples and highly optimized tissue model systems come with a number of severe limitations. The dominant limitation for the development and validation of protocols is the scarce amount of biological starting material, which severely limits the options for establishing SOPs. At all molecular levels, DNA, RNA, proteins, and metabolites, the major efforts must therefore be devoted to develop procedures that are not only highly reproducible and result in high quality molecular isolates, but that deliver this with only minimal numbers of cells as samples. While the analysis of global molecular species at the cellular level are already a challenge, the isolation and analysis of subcellular fractions is even more difficult to achieve as it requires significantly higher input amounts. For this reason, downscaling of protocols and increasing the analytical sensitivity of the methods for Omics analysis, are essential parts of the development and validation of a respective SOP.

1. RESULTS

1.1. Development and testing of SOP for transcriptomics

1.1.1. Isolating RNA

Since the biopsies from patient (from WP6) are not yet available, we started by developing our SOP on *in vitro* 3d models first. Shephoids models being small (around one thousand cell per microtissue, we needed to optimize RNA extraction. Several techniques to isolate total and small RNA were investigated. Table 1 contains the RNA yields from the isolation protocols that can be used within the HeCaToS project. The RIN of total RNA samples in the table were 8.5 - 9.

Used kit	Used MTs	Yield		Yield /MT	
		total RNA (ng)	miRNA (ng)	total RNA (ng)	miRNA (ng)
ReliaPrep (Promega)	89	544	-	6.1	-
ReliaPrep (second time)	82	639	-	7.8	-
QIAamp miRNA kit (Qiagen)	89	-	75	-	0.8
mirVANA (Ambion)	84	1250	111	14.9	1.3

Table 1: RNA yields of microtissues from multiple protocols.

Detection of RNAs from subcellular compartments will require very high amount of starting cell material in order to obtain enough material for sequencing. Because of the low amount of cell material available from 3D cell cultures and organ biopsies, it is not (yet) feasible to investigate transcriptomics in subcellular compartments.

In the pilot experiment further described in the annual work package report referring to Task T7.2, ReliaPrep was used to isolate total RNA (not including miRNA). The RNA yield was highly variable between different pools of spheroids, and therefore, the amounts presented here should be used with caution. We are in the process to initiate the same range of tests on heart biopsies, using pig samples as a surrogate to human, for assessing the efficiency of our SOPs on *in vivo* material. No major difficulties are expected.

1.1.2. Subcellular compartments

A lot of effort was produced this year to try to optimize subcellular compartments protocol, notably by trying to adapt existing protocol to very low amount of biological material. Indeed, both in vitro spheroids and human liver and heart biopsies have in common the limitation of sample sizes. Notably, several protocol and extraction methodology were investigated to isolate mitochondrial compartment (Table 2)

Isolation method	Extraction	Nbr Cells	µg
Qproteome standard	All Prep	5 x 10 ⁶	4.5
Qproteome pure	All Prep	5 x 10 ⁶	3.6
Qproteome pure	PCI	5 x 10 ⁶	18.7
Miltenyi Biotec (MB)	All Prep	1 x 10 ⁶	0.6
Fischer	All Prep	1 x 10 ⁶	0.6
Qproteome standard	All Prep	1 x 10 ⁶	0.3
MB	All Prep	1 x 10 ⁶	1.3
Ultracentrifugation	All Prep	~15 x10 ⁶	0.6
Total DNA Raptor	All Prep	1 x 10 ⁶	6.2
Total DNA MACS	All Prep	1 x 10 ⁶	5.9

Table 2: Optimization of subcellular compartments protocols.

If some protocol were clearly more efficient in the purified mitochondrial material (notably the Qproteome pure isolation kit, extracted with phenol chloroform), we wanted to be certain of the purity of mitochondrial DNA obtain with each method. Indeed, since the planed experiments are mostly high-through-put sequencing, we cannot allow losing a high percentage of our sequenced reads on genomic DNA.

We then checked the specificity of these protocol to extract pure mitochondrial DNA by quantitative PCR of two genes ND1 (NADH dehydrogenase 1), a mitochondrial encoded genes and LPL (lipoprotein lipase), an autosomal gene. Figure 1 presents the ND1/LPL ratio for the different mitochondrial isolation methods:

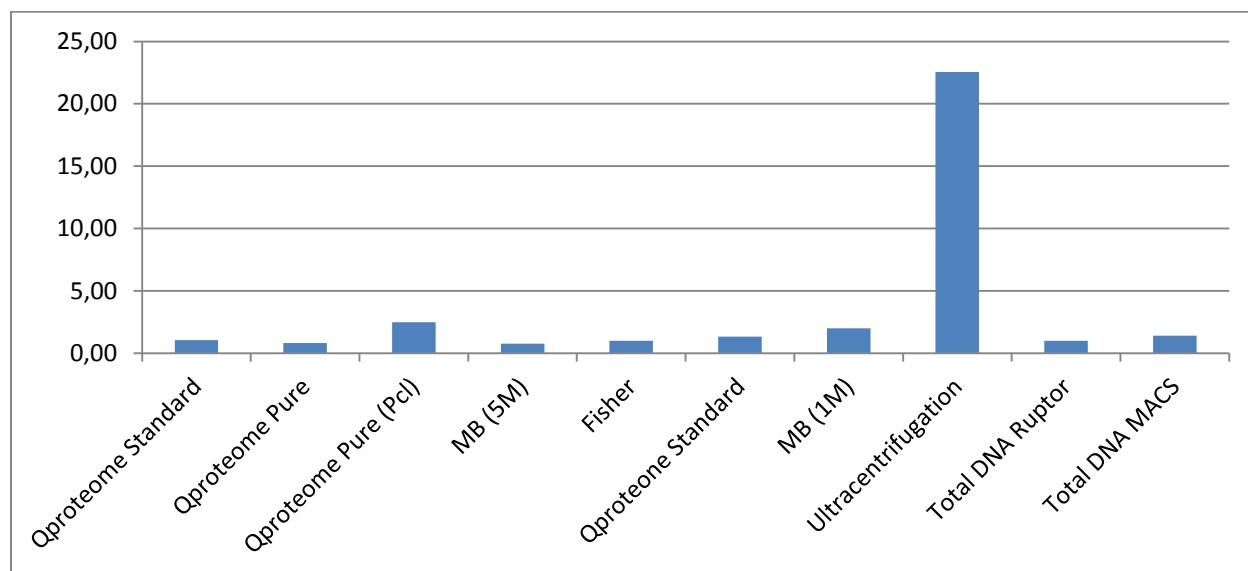


Figure 1: Specificity of mitochondrial isolation methods

Our results show that the only highly specific isolation meeting is ultracentrifugation. However, this method will not be applicable on either spheroids or tissue biopsies, because the initial amount of material required to perform any “omics” experiments would require unrealistic amount of starting material. The same conclusions were obtained from polysomes fractionation

1.1.3. SOP for Transcriptomics (miRNA and mRNA)

Isolation RNA for transcriptomics: to isolate miRNA and mRNA of whole cells, the *mirVana* miRNA Isolation kit (Ambion, life technologies) will be used. The *mirVana* miRNA Isolation procedure combines the advantages of organic extraction and solid-phase extraction, while avoiding the disadvantages of both. High yields of ultra-pure, high quality, small RNA Molecules and total RNA can be prepared (Figure 2) [1].

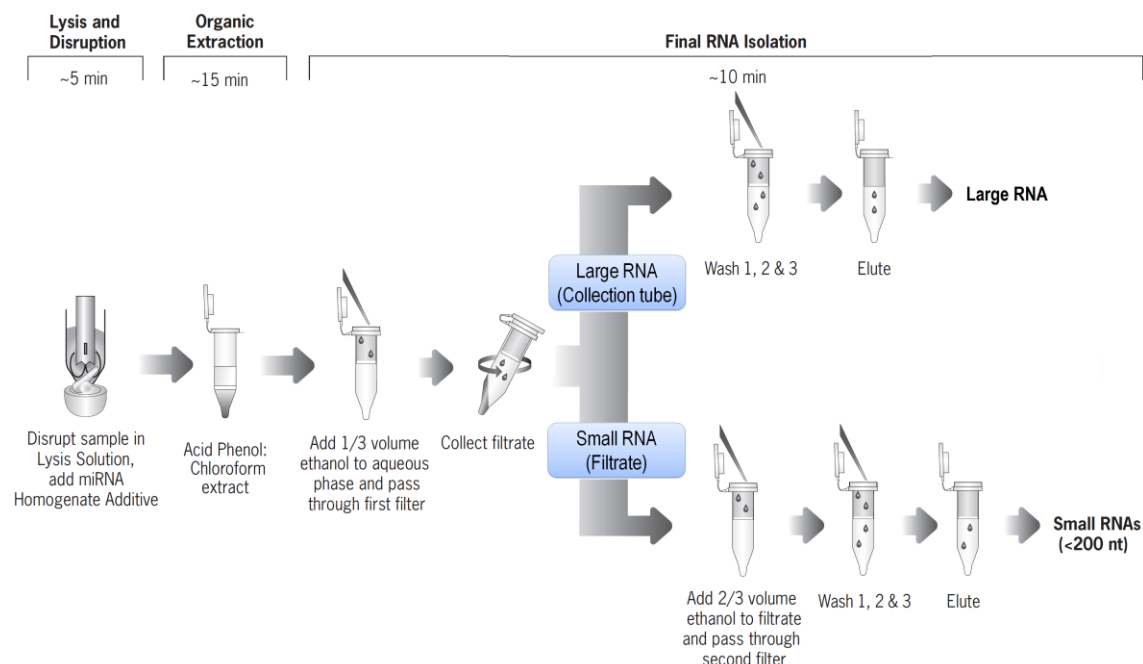


Figure 2: Overview of the mirVana™ miRNA Isolation Kit Procedure

The sample is first lysed in a denaturing lysis solution which stabilizes RNA and inactivates RNases. The lysate is then extracted once with Acid-Phenol:Chloroform which removes most of the other cellular components, leaving a semi-pure RNA sample. This is further purified over a glass-fiber filter by one of two procedures to yield either total RNA or a size fraction enriched in miRNAs. The glass-fiber filter procedure uses solutions formulated specifically for miRNA retention to avoid the loss of small RNAs that is typically seen with glass-fiber filter methods.

The mirVANA miRNA isolation kit Ambion on 84 pooled liver spheroids yielded 1.25 µg total RNA (RIN 8.8) and 111ng miRNA. This correlates with approximately 15ng total RNA and 1.2ng miRNA per spheroid. However, the first pilot experiment showed that RNA yield was highly variable between different pools of spheroids and therefore possible variation should be considered in the future.

1.1.4. RNA sequencing

The TruSeq Stranded total RNA sample preparation kit with Ribo-Zero™ Human/Mouse/Rat (Illumina) will be used for library preparation of total RNAs before sequencing on the HiSeq 2000. This procedure

uses 0.1-1µg total RNA (including miRNA) per sample and will allow us to study the complete transcriptome, including non-coding RNAs.

The first step of the protocol involves the removal of ribosomal RNA (rRNA) using biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal beads. Following purification, RNA is fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers. This is followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments have then an addition of a single 'A' base and subsequent ligation of the adapter. The products are purified and enriched with PCR to create the final cDNA library [2].

Furthermore, TruSeq Small RNA sample preparation kit (Illumina) will be used for library preparation of small RNAs before sequencing on the HiSeq 2000. This procedure uses small RNAs isolated from 1µg total RNA. Most mature miRNAs have a 5'-phosphate and a 3'-hydroxyl group as a result of the cellular pathway used to create them. Because of this, the Illumina adapters in the kit are directly and specifically ligated to miRNAs. The RNA 3' adapter is specifically modified to target microRNAs and other small RNAs that have a 3' hydroxyl group resulting from enzymatic cleavage by Dicer or other RNA processing enzymes. The adapters are ligated to each end of the RNA molecule and an RT reaction is used to create single stranded cDNA. The cDNA is then PCR amplified using a common primer and a primer containing one of 48 index sequences [3]. Thereafter, the sample is ready to be sequenced.

1.1.5. Development and testing of SOP for proteomics

To enable the detection of proteins from subcellular compartments, specific enrichment strategies like differential centrifugation can be applied which concentrate and enrich organelles and subcompartments from whole cell extracts. However, these biochemical approaches require high amount of starting cell material ($> 10^6$ cells) and are not suitable when multiple perturbations are investigated. Therefore we proposed an alternative approach which combines advanced analytical separation strategies, state-of-the art mass spectrometers and bioinformatic processing. Thus higher sensitivity and better proteome coverage of the molecular space is provided, particularly when low amount of cell material is available like in the case of 3D cells (i.e. spheroids) or organ biopsies.

To investigate the minimum number of liver spheroids required to perform reproducible protein extraction for proteomics analysis, several protein extraction and digestions protocols were tested and described in MS6. Two approaches namely the freeze/thaw method – combined with in-solution digestion and the high intensity focused ultrasound (HIFU) combined with filter-aided sample preparation (FASP) were evaluated in terms of reproducibility, sensitivity and robustness. In general, both approaches enabled high reproducibility in protein extraction, however a higher protein yield was obtained with the HIFU-FASP protocol. Furthermore we demonstrated that 12 liver spheroids delivered enough material to perform multiple proteomics-based experiments and enabled in depth-coverage of the liver proteome. The different steps of our analytical pipeline are described in Figure 4. Briefly, spheroids were lysed using the HIFU technology and their protein content was submitted to the FASP protocol for detergent removal, cysteine alkylation and protein digestion. The resulting peptides were submitted to the mass spectrometer for sequencing and the processed raw data were subjected to database search for protein identification. Confidently identified proteins were classified according to their cellular localization (i.e. nucleus, cytosol, mitochondria) based on the Gene Ontology Annotation database [5]. The detailed steps of the extraction and digestion protocols are described below and were also applied to the cardiac cells provided by WP5.

1.1.6. Culture and harvesting of the liver and cardiac spheroids

Liver and cardiac spheroids were cultivated at InSphero according to their Standard Operation Protocol. After washing them twice with cold phosphate buffer saline (PBS), spheroids were harvested by aspiration using a single channel pipette. After complete removal of PBS, spheroids were flash frozen in liquid nitrogen and were stored by -80°C until they were processed for further protein extraction procedures.

1.1.7. Cell lysis and protein isolation via high intensity focused ultrasound

Liver and cardiac spheroids were resuspended in Sodium Dodecyl Sulfate (SDS) -lysis buffer containing 4% SDS, 0.1 mM dithiothreitol and 100 mM Tris HCl. The detergent facilitates membrane disruption and protein denaturation by breaking protein-protein interactions. High intensity focused ultrasound (HIFU) was performed to lyse the cells, where pulsed and high frequency sound waves were applied. The ultrasonic amplitude was set to 65% for all the protein replicates. To avoid heating of the cell material, the ultrasonic treatment was applied in 3 cycles of 3 min each, and samples were kept in an ice bath during the entire process. After centrifugation, the supernatant was used to estimate the protein concentration by fluorometric assay and yielded reproducible extraction of the protein isolates.

1.1.8. Filter-aided sample preparation (FASP) for proteomics analysis

After the cell lysis by the HIFU approach, protein extracts were subjected to ultrafiltration for detergent removal, cysteine alkylation and protein digestion [6]. In the first step, SDS-containing buffer was replaced by urea buffer using several centrifugations steps. A filter device with relative molecular mass (Mr) cut-offs of 10000 (10k filter) was used to efficiently retain proteins and allow removal of impurities. Cysteines were subsequently reduced and alkylated with iodacetamide. Briefly 200 µl of urea in 100mM Tris/HCl pH 8.2 were added and the samples were centrifuged at 14'000g for 20 min at room temperature. This step was repeated once. Then 100 µl of 0.05 M iodacetamide was added to the filters and centrifuged at 14'000g for 20 min at room temperature. Filters were washed three times with urea buffer and followed by two washes with 0.5 M NaCl. Proteins were digested in 120 µl of TEAB using trypsin at enzyme to protein ratio of 1:50 in wet-cell chamber for 18 hours. The peptides were collected by centrifugation at 14'000g for 20 min and were desalted using a reverse phase chromatography.

1.1.9. Protein database search and Gene Ontology Analysis

LC-MS/MS analysis identified around 3000 proteins in each tissue type, with 1% false discovery rate and at least with 2 peptides per protein using the Scaffold algorithms [7] (Figure 3). The overlap of proteins identified in both tissues was more than 90%, demonstrating that liver and cardiac spheroids reflected similar dynamic range of protein concentrations. Gene Ontology analysis was performed in order to classify the proteins according to their cellular component and their biological process based on the information available in the Gene Ontology Annotation database [8]. The proteins were classified as followed for the cellular component: membrane associated 28%, nucleus associated 24%, mitochondrion associated 16% and cytosol associated 23%. Regarding the biological processes, 50% were associated to metabolic processes and 30 % to the regulation of biological processes.

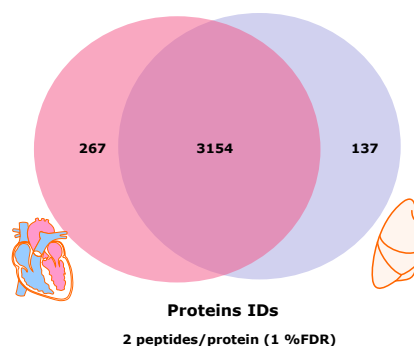


Figure 3: Overlap in protein identification between the cardiac and the liver spheroids using the HIFU-FASP protocol.

1.1.10. Increase Proteome coverage

In order to increase the detection of proteins from subcellular cell compartments, the peptide fractionation by hydrophilic interaction (HILIC) chromatography is an excellent approach to decrease sample complexity, and also required low amount of starting material [9]. The protocol used for peptide fractionation is illustrated in the scheme below (Figure 4). Proteins were isolated from pooled spheroids (> 30 spheroids) using the HIFU approach and were further processed using the FASP approach. The resulting peptides were then subjected to HILIC chromatography using a 30 min gradient (0-50 %, 5 % acetonitrile, 100 mM KH_2PO_4 pH 4.5) followed by a 5 min gradient (50-100 %, 5 % acetonitrile, 100 mM KH_2PO_4 pH 4.5). In total eight fractions were collected and analysed twice by LC-MS/MS. Overall, 7000 proteins were identified with 1% false discovery rate and at least with 2 peptides per protein using the Scaffold algorithms [7].

Gene Ontology analysis revealed that for all investigated cellular components a higher number of proteins were annotated in the HILIC-fractionated samples compared to non-fractionated samples. Indeed, 1849 proteins were annotated to membrane, 1612 to nucleus, 845 to mitochondria and 1073 to cytosol for the fractionated samples. In contrast, 881 proteins were annotated to membrane, 742 to nucleus, 504 to mitochondria and 716 to cytosol for the unfractionated samples. Thus the peptide fractionation by HILIC increased the detection of proteins associated to subcellular cell compartment such as mitochondria, an organelle essential in the regulation of hepatotoxicity. Proteins were also mapped on the representative metabolic and regulatory protein networks available for the KEGG human pathway database (<http://pathways.embl.de/iTuby>) and revealed higher coverage of the metabolic and regulatory pathways for the HILIC fractionated samples compared the non-fractionated samples.

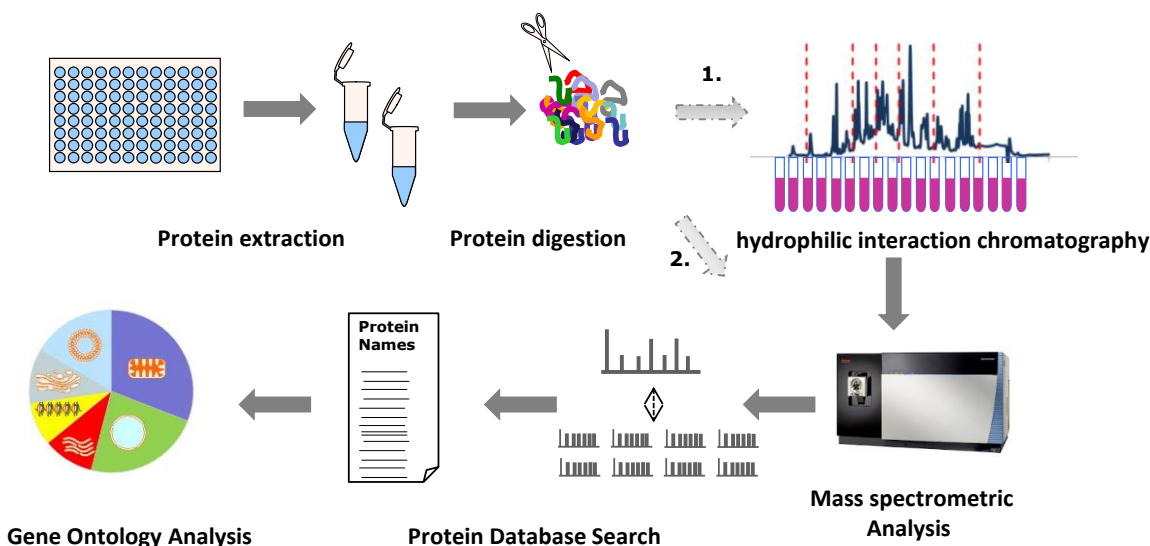


Figure 4: Analytical workflow for the proteomics analysis of liver and cardiac spheroids with the fractionation step by HILIC (1) and without HILIC fractionation (2).

1.2. Development and testing of SOP for methylation analysis

1.2.1. Whole-genome methylation analysis

In the reporting period MPIMG has worked on adaptation and improvements of an existing SOP for genome-wide methylation analysis using MeDIP-seq [10, 11], in particular with respect to experiments with low input samples which is crucial for the experiments with spheroids that will be carried out under HeCaToS. MeDIP is a technology capable of targeting the vast majority of the methylome. It involves antibodies directed against mC (methylated cytosines) or mCG (methylated cytosines within CpG dinucleotides) to precipitate methylated DNA fragments. MeDIP is able to detect methylated cytosines in both mC and mCG contexts. Combining MeDIP with next-generation sequencing (MeDIP-seq) provides high-quality methylomes at typically 100- to 300-bp resolution (depending on chosen insert size) at costs comparable to other capture-based techniques. The developed protocol builds on recent published work [12] and is suited for low-input experiments down to 50ng DNA per sample.

1) DNA isolation
2) Sonication
3) Verification with Agilent Bioanalyzer
4) Library preparation steps
5) DNA purification
6) MeDIP
7) Library size selection
8) Gel DNA extraction
9) Quality control steps
10) Next-generation sequencing of DNA
11) Bioinformatics

Table 3: The different steps of the SOP

Detailed descriptions of the different steps are given in the Appendix. The SOP is currently been tested with DNA derived from liver spheroids treated with acetaminophen (Figure 5; together with INSPHERO and UM). First results are expected by the end of November 2014.

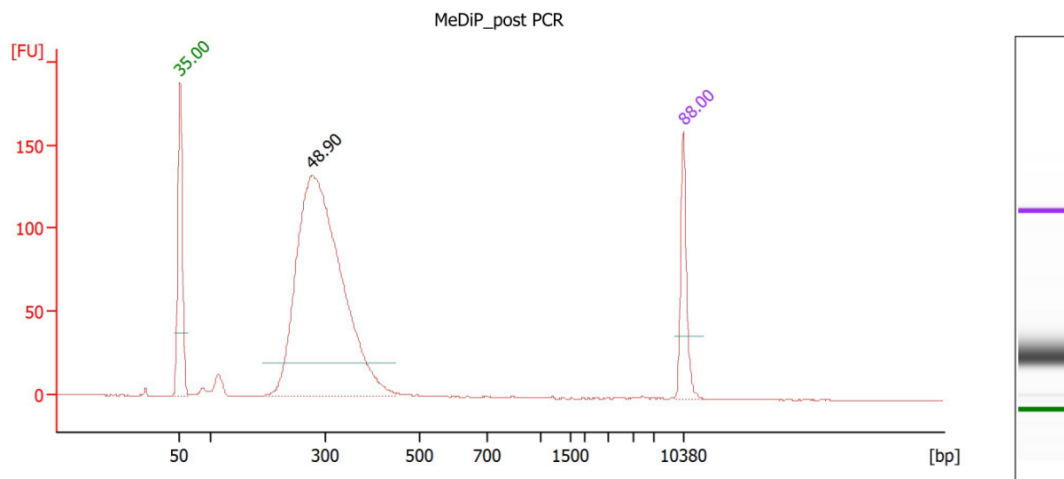


Figure 5: Bioanalyzer profile of genomic DNA after being processed in the MeDIP-pipeline. The DNA was sheared to 100-200bp. DNA ends were repaired and adapter oligonucleotides were ligated. After MeDIP the enriched DNA library has undergone a final PCR step resulting in a ready-to-be-sequenced library of 320-420bp after gel extraction.

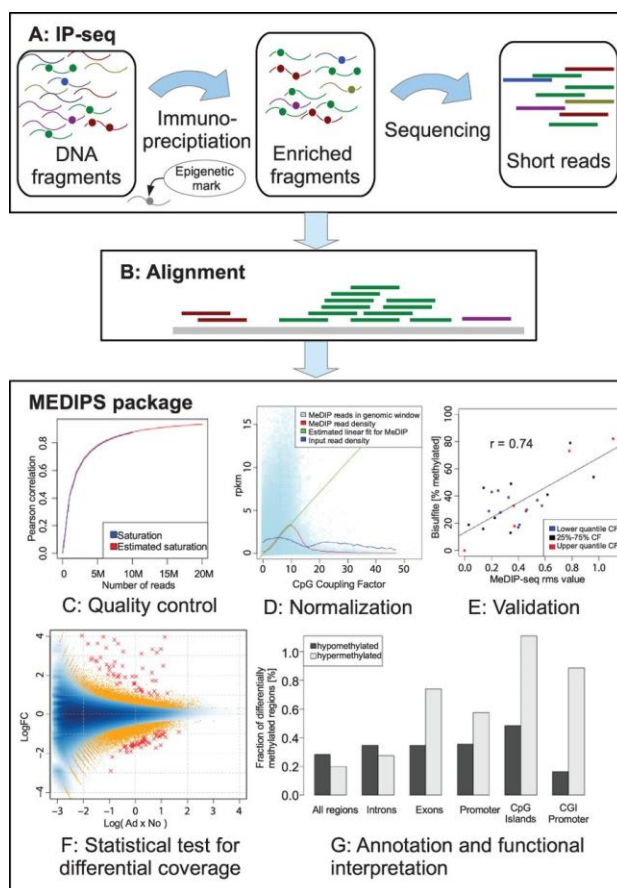


Figure 6: MEDIPS workflow.

Furthermore, MPIMG has developed the MEDIPS software for the analysis of MeDIP-seq data. MEDIPS is a full computational pipeline including mapping of single- or paired-end sequencing reads, preparation of annotation files, quality control procedures, CpG density normalization, differential coverage analysis and annotation of differentially methylated regions. MEDIPS allows for an arbitrary number of replicates per group and integrates sophisticated statistical methods for the detection of differential coverage between experimental conditions. MEDIPS has been implemented in R and is integrated as a package in the publicly available R/BioConductor software (<http://www.bioconductor.org/packages/release/bioc/html/MEDIPS.html>) [13].

1.2.2. Development and testing of SOP for metabolomics

Metabolomics experiments are always limited by the sample biomass since no amplification of metabolites exists as for RNA and DNA. Given the very low biomass of the 3D cultures and tissue that we would likely be working with, we took the strategy of focusing on metabolomics characterisation of the culture media. This approach would provide information on metabolite utilisation and production in key metabolic pathways/processes, using material readily generated but typically discarded by other omics experiments. In this way metabolomics could be efficiently incorporated into experiments within the project.

Our standard protocol uses ^1H NMR spectroscopy to measure precisely key metabolites in cell culture media. For media metabolite composition to provide meaningful information we need to have sufficient concentration changes from the initial starting point during the timeframe of the experiment to determine uptake or production rates. Hence it was necessary to understand the time course of accumulated changes in the metabolism and extracellular metabolome of our culture systems. To achieve this we conducted a series of experiments with the liver microtissue spheroids (as these were the only available test system):

1. to test the effect on viability of long term culture without media replacement and how the rate of change in viability changes as we pooled microtissues;
2. to test the performance of NMR/GC-MS in pooling and extraction of a large number of microtissues for intracellular metabolome analysis;
3. Seeing how the effect on the extracellular metabolome varies with altered frequency of media changes.

Details of the experimental design are given below (Figure 7).

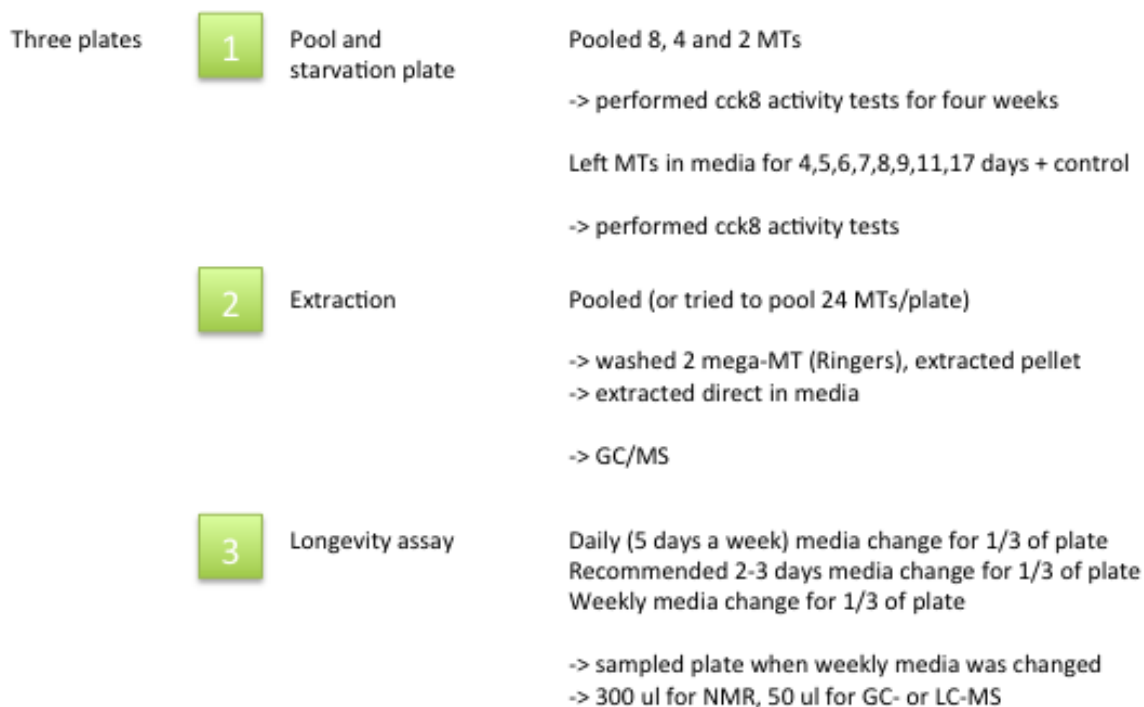


Figure 7: Experimental design to explore how metabolite levels and viability change over long term culture.

1.3. Results

In experiment 1 individual microtissues remained viable for over 2 weeks in continuous culture, however a drift in CCK-8 reduction indicated a drift in metabolic behaviour (Figure 8). We also observed that pooling microtissues lead to an increase in the rate of metabolic drift and some loss of viability, consistent with exhaustion of components in the media. There was also evidence for greater loss of viability on the outer wells of the plate, i.e. edge effects.

In experiment 2 we observed very few clearly discernable signals over background in intracellular extracts of 24 pooled microtissues by ^1H NMR spectroscopy (Figure 9). Similar results were obtained by GC-MS (data not shown). Interestingly we observed a large number of additional signals compared to the media profile if microtissues were not washed but analysed together with media in very close proximity to the microtissue ('deep media') not normally harvested in a standard media replacement. This pericellular fluid may thus yield additional metabolic information, but further work is required to be confident of extracting this reliably.

In experiment 3 we were able to define more precisely the relationship between media changes and metabolic alterations in the media. We observed that microtissues produced lactate and alanine but consumed little glucose (Figure 10), appearing to prefer glutamine as a substrate (net glutamine production is observed because the cells are cultured with glutamax from which glutamine is released). This is precisely the same behaviour that we have observed in primary rodent hepatocytes and very distinct from the hepatoma cell line HepG2 (Figure 11). The metabolic transformation suggested by experiment 1 could be observed to be greatest when media changes were infrequent, i.e. weekly. We also determined that changes according to the manufacturer's protocol (2-3 days) was a sufficient duration to observe metabolite consumption or release.

In conclusion we assert that it will be possible to incorporate metabolomic analysis into other “omics” experiments on microtissues by profiling the media that is normally discarded in these experiments. For NMR analysis we require 300ul of media, which can be obtained by pooling from a small number of wells. Media exposed to the cells for durations recommended in the manufacturer’s protocol is sufficient for the detection of metabolite consumption or release. Given that extraction of intracellular metabolites in whole microtissues still poses a challenge it does not appear that isolation of subcellular fractions is a possibility at this stage.

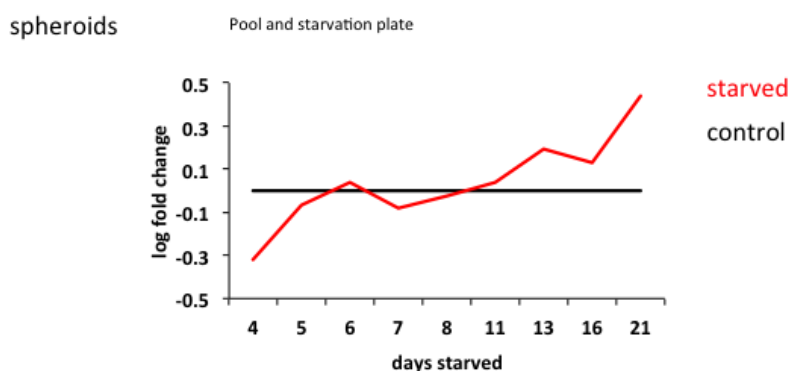


Figure 8: Individual microtissues are resistant to continuous culture without media replacement.

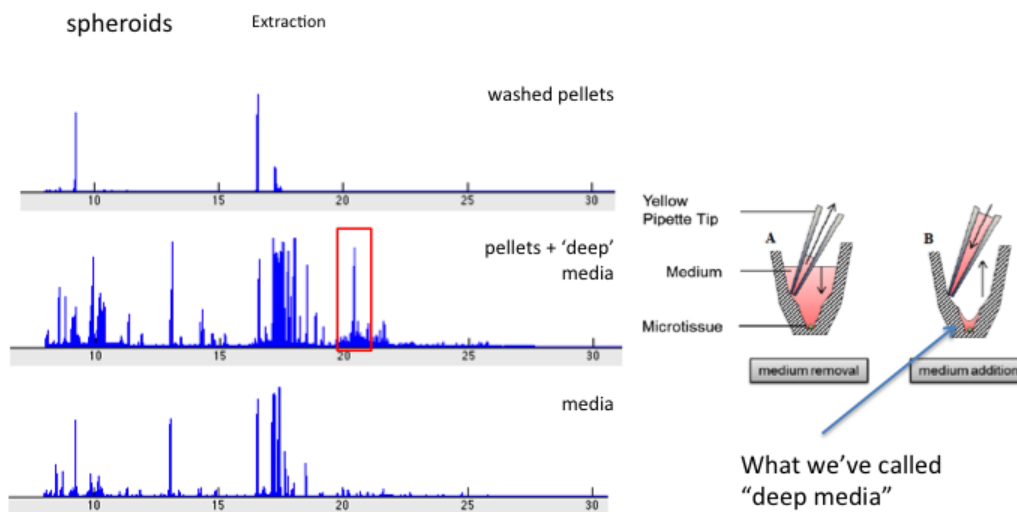


Figure 9: ^1H NMR spectroscopy of extracted pooled microtissues.

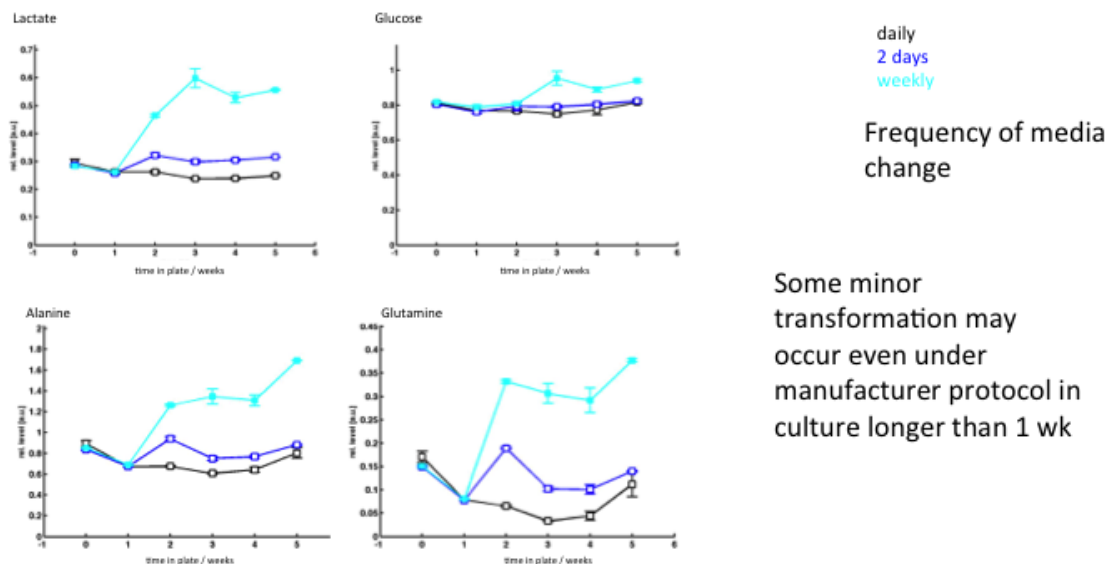


Figure 10: Metabolic transformation over long term culture is dependent on the frequency of media changes.

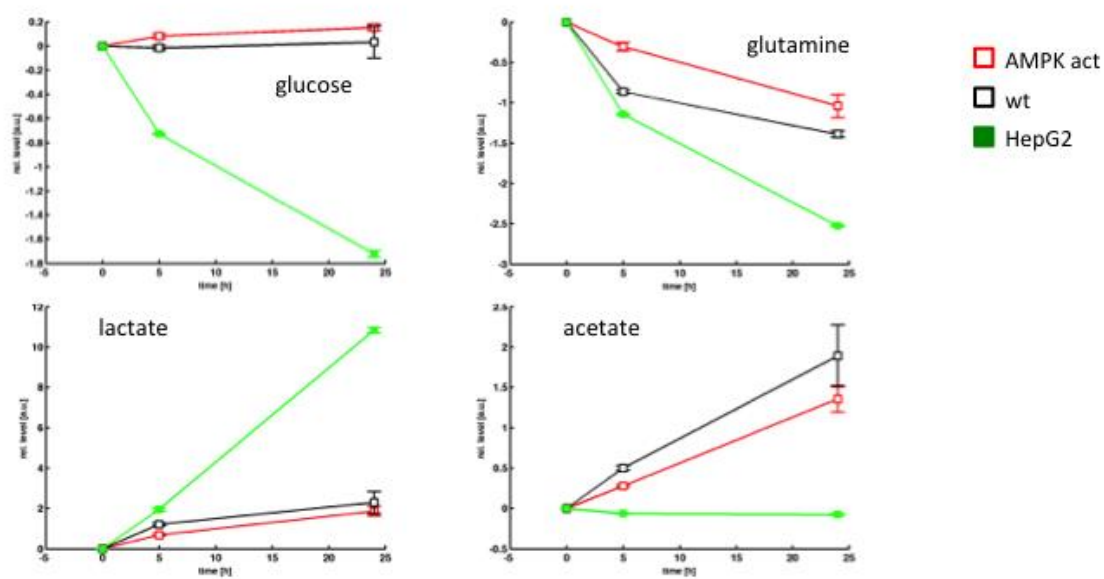


Figure 11: Comparative metabolic behaviour in primary hepatocytes and hepatoma cells.

DIFFICULTIES

For all Omics levels, protocols for the generation of high quality molecular isolates have been tested, established, validated before large efforts have been devoted to the downscaling of input sample amounts. While for the cellular fractions of the different molecular species the developed SOPs work well and reliably, the isolation of subcellular compartments and from those the molecular species has been hampered by the limited amounts of tissue that can be made available for molecular analysis. Also for this part, SOPs have been established and are available to all partners but will depend in their applicability on the scale-up of tissue resources.

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Annex: SOP for MeDIP-seq experiments

SOP for MeDIP-seq experiments with low-input DNA experiments (50-500ng DNA based on Taiwo et al. (2012) Nat Protocols 7:617-636), MPIMG (Ralf Herwig)

1. Library Preparation:

- Sonication
 - o 50-500ng in 50µl EB or water
 - o Covaris S2:
 - o Use 100µl glass tubes with AFA fibre
 - o 10% duty cycle, Intensity 5, 200 cycles/burst, 7 cycles à 1min
 - ➔ Take off 2µl for Agilent Chip analysis
 - **End repair:**
 - o Mix the following on ice in a sterile PCR tube and incubate in a thermocycler at 20°C for **1 h**
 - o NEBNext End Repair Enzyme mix: 3µl
 - o NEBNext End Repair Reaction Buffer (10X) 6 µl
 - o Fragmented DNA : 48µl
 - o Fill with water to **60 µl**
 - **Purify DNA sample** with Ampure XP purification beads or equivalent:
 - o **Add 1.8 volumes of beads**
 - o **incubate 5min at RT**
 - o **take off supernatant on magnetic rack**
 - o **wash twice with 250µl 80% EtOH each**
 - o **elute in 39 µl elution buffer** (incubate beads with elution buffer for 5minutes)
 - o Take off 2µl for Agilent Chip
 - **A Tailing:**
 - o Mix the following on ice in a sterile PCR tube and incubate in a thermal cycler for **1 h at 37°C**.
 - o Klenow Fragment (3' → 5' exo-): 3 µl
 - o NEBNext dA-tailing Reaction Buffer (10X): 5 µl
 - o Sterile H₂O: 5µl
 - o DNA: 37 µl
 - o Total Volume 50 µl
 - o Pipette up and down to mix.
 - **Purify DNA sample with Ampure XP purification beads** and elute in **28 µl** elution buffer→ take off 2µl for Bionalyser.
 - **Run Agilent Bioanalyzer** 2100 and determine molarity of the full range of DNA fragments using the instrument's software.
 - **Adapter ligation**
 - o Prior to first adapter ligation: hybridize single stranded adapters:
 - TRUSEQ_UNI_TRUNC: ACACCTCTTCCCTACACGAC**GCTCTTCCGATC***T
 - Index_SCAFFOLD: **5P-GATCGGAAGAGC**ACACGTCTGAACCTCCAGTCAC
 - ➔ Spin down lyophilized adapter oligos in a chilled (4 °C) microcentrifuge at 200g for 5 min. Resuspend each oligo in 1× STE buffer to 100 µM. Add equimolar quantities of each adapter into a 1.5-ml microcentrifuge tube. Divide into small (e.g., 200 µl) aliquots and incubate at 95 °C for 15 min, and then leave to cool down to room temperature (~1 h). Store aliquots at - 20 °C until required. Final concentration: 50 µM.
 - o Mix the following on ice in a sterile PCR tube and incubate in a thermocycler at 18°C for **2 h**.
 - Quick T4 DNA Ligase: 5 µl
 - Quick Ligation Reaction Buffer (5x): 10 µl
 - End Repaired, Blunt, dA- Tailed DNA from Step 21 25 µl
 - DNA Adaptormix: 10 µl (final conc. 5x Molarity of DNA)
 - ➔ Total Volume 50 µl
- ➔ Example: DNA concentration: 100nM→ final conc (50µl): 50nM
 - o ➔ final conc adaptormix: 250nM
 - o ➔ conc adaptormix to be used (10µl of total 50µl): 5*250nM=1250nM
- **Purify DNA sample** with Ampure XP purification beads and elute in **40 µl** elution buffer.
- Run 1 µl of eluted sample on a **Agilent Bioanalyser**
- **Spike in 2µl mix of methylated and unmethylated** control fragments (generated from lambda DNA) (final concentration ~0.01pM each): 1-10CpG methylated, 15CpG unmethylated
 - o Generate PCR fragments prior to use
 - 1CpG_F: GAGGTGATAAAATTAAGTGC
 - 1CpG_R: GGCTCTACCATATCTCCTA

- 5CpG_F : CATGTCCAGAGCTCATTC
 - 5CpG_R: GTTTAAATCACTAGGCCGA
 - 10CpG_F: CTGACCATTTCATCATTC
 - 10CpG_R: GTAACAAACAGGAGCCG
 - 15CpG_F: ATGTATCCATTGAGCATTGCC
 - 15CpG_R: CACGAATCAGCGGTAAAGGT
 - 50µl PCR reactions:
 - Phusion Polymerase reaction mix (2x): 25µl
 - 1µl Lambda DNA
 - 2.5µl Primermix (5µM each)
 - H2O
 - In vitro methylation of 1-10CpG fragments with SssI DNA- Methyltransferase:
 - Water 9µl
 - NEB2 buffer: 2µl
 - SAM (1.6mM): 3µl
 - DNA: 5µl
 - SssI enzyme (4U/µl): 1µl
 - 2h at 37°C, 20min at 65°C
 - Purify with AMPURE beads→ 20µl EB
 - Digest methylated fragments with methylation sensitive HpyCH4IV (NEB1: 2.5µl, 1µl enzyme, 20µl DNA, 1.5µl H2O), gel purify and cut out uncut fragments
 - Purify with qiagen minelute and quantify with Agilent bioanalyser
 - Use equimolar amounts of 1-10CpG methylated and 15CPG unmethylated fragments
- **Input control: take off 5µl**

2. MEDIP:

- MagMEDIP Kit: Methylated DNA IP Module
 - Prepare beads:
 - Prepare **bead wash buffer**: 1:5 Mag Buffer A with water→ 100µl /rxn
 - 11µl **MagBeads** to new tube/rxn/4°C
 - On ice:
 - Magnet beads→ keep beads
 - On magnetic rack: + 22µl bead wash buffer
 - Magnet beads, take off wash buffer and add 22µl wash buffer to beads
 - Prepare IP incubation mix:
 - DNA: 35µl
 - 20µl MagBuffer A
 - 5µl MagBuffer B
 - 15µl water
 - 75µl
 - Incubate IP mix @95°C for 3min
 - Quickly chill in ice water
 - Prepare antibody mix:
 - Dilute antibody 1:2 with water
 - Per rxn (total 5µl):
 - 0.3µl diluted antibody
 - 0.6µl MagBufferA
 - 2.1µl water
 - 2µl MagBufferC
 - Add 5µl of antibody mix to 75µl of DNA IP mix and 20µl of washed beads.
- Place on **rotating wheel overnight @4°C**
- **Wash** beads in cold room/on ice
 - Spin down and place in magnetic rack
 - Discard buffer
 - Wash three times with 100µl ice cold MagWashBuffer 1
 - Wash with 100µl MagWashBuffer2
 - Take off all wash buffer but don't dry beads!
 - Add **1µl of proteinase K to 100µl of DIB** buffer
 - Resuspend beads in proteinase K/DIB buffer and incubate 15min@55°C
 - Incubate 15min @100°C
 - →Magnetic rack
 - Transfer supernatant to new tubes and store @-20°C (!single stranded DNA)

3. qPCR (enrichment success, recovery of unmethylated fragments):

- Use input DNA and 5µl of output DNA and dilute accordingly to obtain triplicate values:
- Primers:
 - o 1CpG_qPCR_F: ACAAGTTGTTTGATCTTTGC
 - o 1CpG_qPCR_R: CCTATGAGCAACGTGTTAG
 - o 5CpG_qPCR_F: CACTTGAATCTGTGGTTCAT
 - o 5CpG_qPCR_R: TAGAAAAAGACAACCTCTGGC
 - o 10CpG_qPCR_F: GAACTCACACACAACCA
 - o 10CpG_qPCR_R: ACTCTGAATACCGACTCAAT
 - o 15CpG_qPCR_F: TATCACTGTTGATTCTCGC
 - o 15CpG_qPCR_R: GGTAAAGAGTTTGGATTAGG
- PCR reaction:
 - o 5µl 2xKappa Mix
 - o 1.5µl Primermix (5µM each)
 - o 2.5µl H2O
 - o 1µl diluted input OR output DNA
- Roche Lightcycler: 95°C 3min, 40 cycles of 95°C 15'' and 60°C 1', melting curve
- Calculate recovery of methylated and unmethylated fragments: output of unmethylated should be <1% of input

4. Library preparation 2:

- Use 10µl of MeDIP output library to determine appropriate cycle number for final library amplification: dilute in 1:4 steps and run 17 cycles of 10µl PCR reactions followed by gel analysis:
 - o PCR TRUSEQ Primer (1µl of 10µM stock): AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
 - o Index - Primer (1µl of 10µM stock): choose one
 - Index1: CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
 - Index8: CAAGCAGAAGACGGCATACGAGATCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
 - Index10: CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
 - Index11: CAAGCAGAAGACGGCATACGAGATGAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
 - o 0.1 µl 5x Phusion High Fi
 - o 0.2µl dNTP Mix
 - o 2µl Fusion 5x
 - o DNA in 1:4, 1:16, 1:64, or 1:256 dilution
 - o Ad 10µl: H2O

Cycling: 98°C for 30'', 17 cycles of: 98°C 10'', 65°C 30'', 72°C 30''; 72°C 5', 4°C forever

- Calculate **cycle number N** from dilution where a smear is well visible
- **Amplify MeDIP enriched DNA**
 - o PCR TRUSEQ Primer (10µl of 10µM stock): AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
 - o Index - Primer (10µl of 10µM stock): choose one
 - Index1: CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
 - Index8: CAAGCAGAAGACGGCATACGAGATCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
 - Index10: CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
 - Index11: CAAGCAGAAGACGGCATACGAGATGAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
 - o 1 µl Phusion HF Polymerase (2U/µl)
 - o 2µl dNTP Mix (10mM each)
 - o 20µl Phusion HF 5x Buffer
 - o 50 µl MeDIP-DNA
 - o Ad 100µl: H2O
 - o Cycling: 98°C for 30'', **N cycles** of: 98°C 10'', 65°C 30'', 72°C 30''; 72°C 5', 4°C forever
- **Ampure XP purification** → 18µl
 - o Take off 2µl for QC
- **Size selection:** low melting agarose
 - o **Cut region:** 220-320bp
- **MinElute Gel DNA extraction: elute in 10µl**
- Determine concentration with Agilent Bionalyser: should be around 2ng/µl
- **Library ready for pooling**