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Genetica
Genome Analysis Facility

Title: Guidelines DNA Quantity and Quality for Methylation projects

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1. Introduction

These guidelines are intended for experiments based on Illumina Human Methylation 450K beadchips and Sequenom EpiTYPER methylation assays performed by the Genome Analysis Facility.

To ensure successful and rapid processing of the DNA samples, the DNA quality needs to meet a few criteria. There are also special demands about the format of the sample delivery. All the required information for the preparation of the DNA samples is described in this document.

2. DNA isolation

To isolate genomic DNA out of multiple blood samples the Qiagen QiaAmp DNA 96 blood kit is recommended.

Alternatively many other DNA isolation methods can be used. The quality of DNA isolated by most isolation methods usually meets the criteria for a successful Methylation assay. Methylation assays do not work on amplified DNA.

The DNA should be dissolved in Elution buffer or in 10 mM Tris-HCL pH 8.0, 1 mM EDTA

3. Applications and limitations of the techniques

Genome Wide Methylation Analysis with the Illumina's Human Methylation 450K beadchip

The 450K Methylation assay is an Illumina array-based assay intended to measure genome-wide DNA methylation across many samples. The 450K methylation assay uses the Infinium technology, a reliable technology frequently used for genotyping experiments. It is based on highly multiplexed genotyping of bisulfite-converted genomic DNA. First, genomic DNA is treated with a bisulfite conversion kit. This is followed by a PCR-free whole genome amplification and hybridization. After hybridization, allele-specific single-base extension incorporates a fluorescent label for detection.

The content of the Human-Methylation 450 beadchip has been selected by experts and provides quantitative methylation measurement at the single-CpG-site level. It covers 99% of RefSeq genes, with an average of 17 CpG sites per gene region distributed across the promoter, 5'UTR, first exon, gene body, and 3'UTR. It covers 96% of CpG islands, with additional coverage in island shores and the regions flanking them. Beyond gene and CpG island regions, methylation experts requested multiple additional content categories, which were also included:

- CpG sites outside of CpG islands
- Non-CpG methylated sites identified in human stem cells
- Differentially methylated sites identified in tumor versus normal (multiple forms of cancer) and across several tissue types
- FANTOM 4 promoters
- DNase hypersensitive sites
- miRNA promoter regions
- ~ 90% of content contained on the Illumina HumanMethylation27 BeadChip

Focussed Methylation analysis using Sequenom's EpiTyper Assay

The EpiTYPER assay is a tool for the detection and quantitative analysis of DNA methylation in a particular DNA fragment using base-specific cleavage and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). The Sequenom system uses the speed and accuracy of this method to identify differentially methylated sites through this quantitative analysis.

A well designed custom made EpiTyper assay demonstrates how base-specific cleavage of bisulfite-treated DNA results in clear differences between the mass signal patterns of methylated and non-methylated DNA.

To consider prior to starting the experiment

1. Some important pitfalls of genome-wide association studies using the 450K beadchip are:
 - The methylation status of DNA is tissue specific and depending of the age of the tissue.
 - Data analysis requires thorough quality control and is complicated. Please ensure to arrange proper knowledge and guidance with these analyses.
 - In addition to CpGs, there are SNPs present on the 450K beadchip. However, it is not possible to generate genotypes using the standard analysis with the methylation module of GenomeStudio. Also, it is not possible to do a gender estimation using the standard Infinium approach.
 - For genome-wide association studies statistical power is crucial for the success of the study. Ensure the power is sufficient at the start of the study.
 - Availability of a cohort to replicate associations. Be aware that replication problems between studies may occur due to diversity of study designs.
2. Sequenom EpiTyper Assays are custom designed. The assay is intended to study the methylation status of the CpG sites in a specific DNA fragment (such as an CpG island) of approximately 500 bp. For each fragment one or more assays are custom designed using special software. Validation of the design is part of the procedure. Contact the Genome Analysis Facility to discuss the design and the EpiTyper procedure.
3. Practical aspects:
 - DNA isolation and quantification: Use the same method throughout the study to prevent clustering of samples per method.
 - Avoid clustering of samples due to batch differences or technical effects. Randomize the samples, but prevent sample mix-ups.
 - Biological replicates add value, as well as technical replicates.
4. Limitations of the assays:

The bisulphite conversion step as well as the 450K beadchip hybridisation or EpiTyper assay is sensitive for batch effects:

 - It is possible to see beadchip-to-beadchip variation
 - It is possible to see section-to-section variation
5. Points to consider in the design of the study and data analyses:
 - To account for beadchip-to-beadchip or plate to plate variation it is important to take along control DNA samples on every beadchips or plate. For this you can use 1 male and 1 female DNA sample. Do not place these samples on fixed positions on the 96-well microplate, rather let the samples 'walk' over the beadchips/plates. While supplies last, the Genome Analysis Facility can provide you with control samples that are used UMCG-wide.
 - In order to assess if the bisulfite conversion was successful, it is important to take along two commercially obtained human DNA samples, <5% and 100% methylated (Zymo research; Human Methylated & Non-methylated DNA Set DNA w/ primers; 5 µg/20 µl of each; cat. # D5014). In

450K methylation experiments these are positioned on a fixed position on every 96-well microplate; the non-methylated sample are placed on position H12 of odd-numbered plates, the methylated sample is placed on position H12 of the even-numbered plates. After bisulfite conversion the Human (non-)methylated samples will be extracted from the plates. These samples will be used to check if the bisulfite conversion was successful. The created empty positions can be filled with either a biological replicate emerging from the same plate or a technical replicate.

For EpiTYPER experiments it is also essential to incorporate at least one blank water sample per sample-plate to verify the PCR reaction.

In EpiTYPER experiments, the bisulfite conversion control samples and blank 'walk' over the plate. After bisulfite conversion, some of the samples will be extracted from the plates for bisulfite conversion validation, but the remainder will stay on the plate and be part of the experiment.

- Normalization of the data is necessary. However, there is no consensus on what method to use for this. Processing of control DNA samples together with the test-samples gives extra normalization possibilities.
- There is no consensus on how to analyze the generated methylation data

4. DNA quantity and quality

In the preparation of the genomic DNA samples used for methylation array studies, *it is extremely important to handle the samples as equally as possible*. This is to prevent batch differences being seen in the final data instead of biological differences.

The quantity and quality of the isolated DNA should be determined with a spectrophotometer, ideally by the Nanodrop spectrophotometer. We recommend to also check a subset of the samples using agarose gel electrophoresis. The quality criteria are:

- Concentration should be between 50 ng/μl and 100 ng/μl
- The OD 260/280 ratio should be between 1.8 and 2.0
- The OD 260/230 ratio should be above 1.5

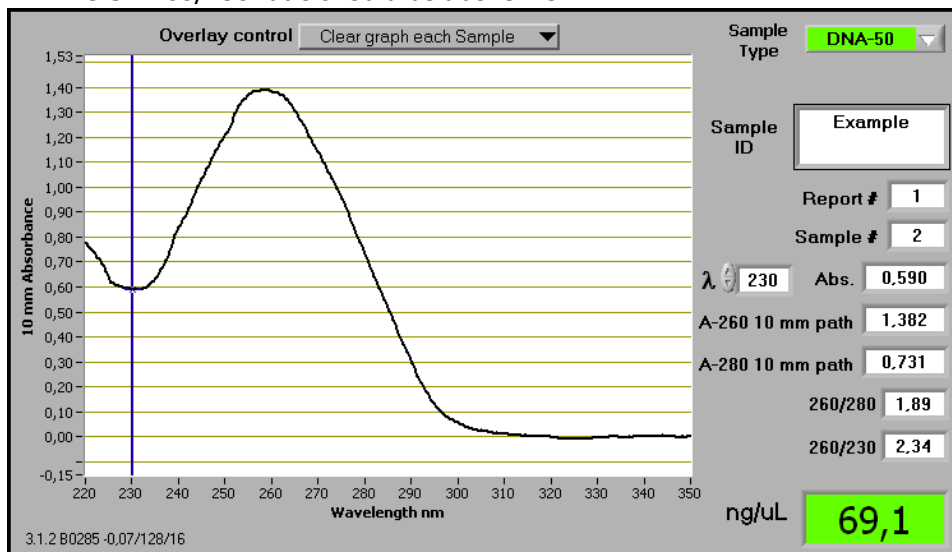


Figure 1: Example of a nanodrop measurement of a good quality DNA sample

- The DNA should be high molecular fragments of > 5kb.
- In total 500-1000 ng of DNA per sample is needed for proper methylation profiling (based on nanodrop measurements)
- The quality measurement of DNA samples with the Nanodrop can be performed by employees of the Genome Analysis Facility, but the final sample preparation and randomisation remains the responsibility of the researcher. Please contact the Genome Analysis Facility to discuss this if necessary.

5. Sample preparation

1. At least 1 µg in 90 µL DNA should be transferred to 96-well microplates (Greiner Bio-One, PCR-plate full skirted, order nr. 652270).
2. Place the control DNA samples on the plates. Make sure that on every beadchip/plate a control sample is placed.
3. For 450K experiments, place the bisulfite conversion control samples on the plate: the non-methylated DNA samples should be placed on position H12 of odd-numbered plates and the methylated samples should be placed on position H12 of the even-numbered plates. (Zymo research; Human Methylated & Non-methylated DNA Set DNA w/ primers; 5 µg/20 µl of each; cat. # D5014). For EpiTyper experiments, the plate layouts that tell where the bisulfite conversion control samples should be placed are provided by the Genome Analysis Facility
4. To avoid seeing batch differences in the final data, it is important to put the samples in the plate in a random order following the well-order A1, B1, C1, D1 etc. Don't leave any blank wells between samples.
5. Record the sample IDs in a special XLS file according to the instructions below. Note that the Genome Analysis Facility ***does not accept samples with names of individuals!*** A unique plate name should be given to each 96-well plate, using a permanent marker to annotate each plate on two sides.
6. The 96-well plates should be firmly closed preferentially with a Thermo-seal (Thermo scientific order nr. AB-0559) using a heat plate sealer. Alternatively, if a heat plate sealer is not available, adhesive PCR Foil seals (Thermo scientific order nr. AB-0626) can be used.
7. After the DNA-solution is transferred to a 96-well plate and the plate is sealed properly, the plates should be stored and transported at -20°C.
8. The quality measurement of DNA-samples with the Nanodrop spectrophotometer and the transfer to 96-well plates can be performed by employees of the facility as part of the project. Please contact the Genome Analysis Facility to discuss this if necessary/requested.
9. The Genome Analysis Facility is not responsible for long time storage of samples or intermediate products. Left overs are thrown away after finishing the project.

6. Documentation of the sample and plate layout

All sample-IDs and corresponding wells in the 96-well plate should be administrated in a XSL-file (Excel; see figure 2). In this XSL-file all required information is summarized:

- Number
- Plate name
- Well with corresponded to it a unique sample ID
- Information of the DNA quality; DNA concentration, OD 260/280 ratio, OD 260/230 ratio

- Sample-ID of the biological and/or technical replicate with corresponding to it the plate name
- For verification reasons the gender should also be administrated

nr	plate	well	sampleID	well_sampleID replicate	DNA-concentration (ng/ μ L)	OD 260/280	OD 260/230	Gender
1	UMCG-1	A1	455		68	1.81	1.65	Female
2	UMCG-1	B1	474		72	1.85	2.04	Male
3	UMCG-1	C1	331		98	1.90	1.81	Male
4	UMCG-1	D1	587		66	1.81	2.19	Female
5	UMCG-1	E1	585		69	1.81	1.50	Female
92	UMCG-1	D12	296		97	1.91	2.09	Male
93	UMCG-1	E12	254		63	1.89	2.02	Female
94	UMCG-1	F12	585		85	1.90	2.19	Male
95	UMCG-1	G12	255		89	1.92	2.14	Male
96	UMCG-1	H12	BC_0	F12_585	-	-	-	Male

Figure 2: Example of the attached XSL-file

Please email the XLS-file with the sample-information to: p.van.der.vlies@umcg.nl

7. Sample shipment

Before submitting your samples, please contact the Genome Analysis Facility!

Please deliver the samples on dry ice on the lab of the Genome Analysis Facility or send your samples by a courier-company to the laboratory of the Genome Analysis Facility of the department Genetics of the UMCG. The shipment should be carried out in boxes containing a sufficient amount of dry-ice.

To avoid any delay during the weekend, please ship the material in the beginning of the week on the following address:

Address: Genome Analysis Facility
 Dept. Genetics UMCG CB50
 Hanzeplein 1
 9713 GZ Groningen
 The Netherlands

Phone number: 050-3617100