Genome scan

Next Generation Epigenetics

Illumina BeadChip Technology

Guidelines

genomescan.nl

GenomeScan's Guidelines for Successful Methylation Experiments

Using the Illumina Infinium® HumanMethylation BeadChip

Dear customer,

As of the beginning of 2015 ServiceXS became a trademark of GenomeScan B.V. GenomeScan focuses exclusively on Molecular Diagnostics whereas our ServiceXS trademark is intended for your R&D projects.

GenomeScan is dedicated to help you perform methylation profiling experiments that generate high-quality, statistically sound, and biologically relevant results. This guide provides information, resources and tools for your Illumina Infinium Methylation Assay service at GenomeScan, enabling you to derive optimal results from your project.

Please read this guide carefully! This document accompanies the quotation of your methylation project and will inform you about the various stages in our service process (what you may expect from us and what we expect from you), enabling us to tune mutual expectations.
GenomeScan has extensive experience in performing microarray experiments. Therefore, we are able to advise you during every step of the project. Please do not hesitate to contact us if you have any questions after reading this document.

Our Methylation service on Illumina BeadChips falls under the scope of our ISO/IEC 17025 accreditation. GenomeScan is also an official Illumina CSPro® Service Provider for Infinium Genotyping and Direct Hybridization Gene Expression, both ensuring delivery of the highest-quality data available.

We are looking forward to a pleasant and successful collaboration!

On behalf of the GenomeScan team,

Floor Pepers
Laboratory Coordinator

Document Outline

6.7 Turnaround Time 6.8 Trend Analysis 6.9 Sample Storage Terms 6.10 Data Storage Terms	

Changes to previous version (3.1):
-Addition of Hydroxymethylation Service
-Addition of Restoration (for FFPE samples) Service
-Bisulfite QC criteria are changed

Changes to previous version (4.0):
-Lower input concentrations

Changes to previous version (4.1):
-Accreditation logo clarified. Now connected to GenomeScan B.V.
-Lay-out changes
-Added MethylAid data QC Tool

Changes to previous version (5.0):
-Introduction of the MethylationEPIC BeadChip
-Discontinuation of the Methylation450 BeadChip

Chapter 1 Service description

1.1 Introduction

DNA methylation is an epigenetic modification that plays an important role in the regulation of gene expression and has become an important avenue of research in our quest to gain a better understanding of human development and disease.
Methylation of cytosine is a covalent modification of DNA, in which hydrogen H5 of cytosine is replaced by a methyl group. In mammals, 60% - 90% of all CpGs are methylated. Methylation adds information not encoded in the DNA sequence, but it does not interfere with the Watson-Crick pairing of DNA - the methyl group is positioned in the major groove of the DNA. The pattern of methylation controls protein binding to target sites on DNA, affecting changes in gene expression and in chromatin organization, often silencing genes, which physiologically orchestrates processes like differentiation, and pathologically leads to cancer. Figure 1 is illustrating this.

Cytosine hydroxymethylation was recently discovered as another important epigenetic modification on DNA in mammalian cells. Similar to methylation, hydroxymethylation replaces, at the C5-position in cytosine, the hydrogen atom by a hydroxymethyl group. It has been demonstrated that cytosine hydroxymethylation is also involved in gene regulation. For example, the hydroxymethylation level has been found to be associated with pluripotency of stem cells. Disturbed hydroxymethylation of DNA cytosine can result in disordered cell functions, causing different types of cancers, e.g., myeloid cancer.

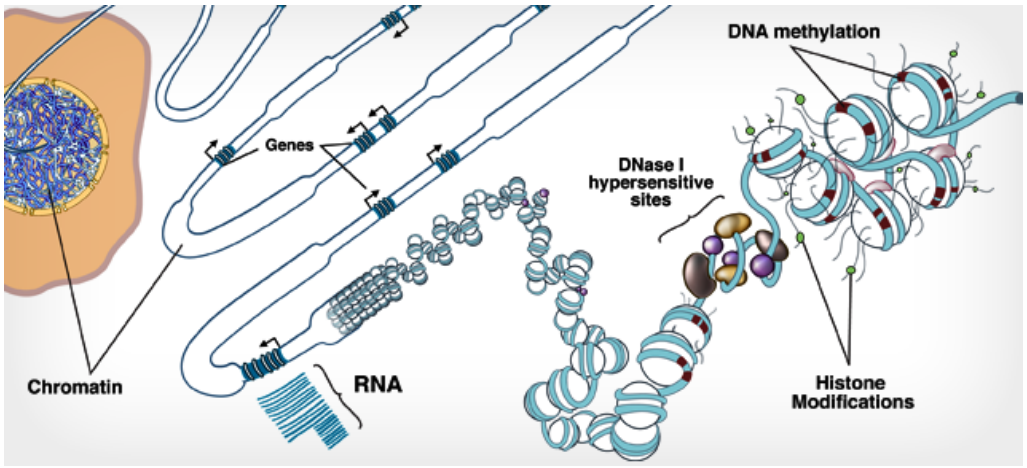


Fig. 1. Epigenomics Roadmap. Source: NIH Roadmap Epigenomics Mapping Consortium

1.2 Your project at GenomeScan

Successful methylation studies start before DNA samples are submitted to GenomeScan: experimental design, sample preparation, sample purity and concentration assessment all contribute to the overall success of the methylation experiment. To provide you from high quality data GenomeScan has a strict policy concerning sample preparation and sample shipment and their timelines.

GenomeScan offers three different types of Methylation services using the Illumina platform for array-based methylation studies:

1.2.1 DNA Methylation Service

Here we perform all steps starting from gDNA to data extraction including Quality Control steps at each phase of the service. The workflow of the DNA Methylation Service is briefly: Sample Quality Control (QC) (quantity and quality assessment), Bisulfite Conversion and QC, methylation assay execution, raw data extraction and data QC using the R script MethylAid . You simply deliver your genomic DNA and we will perform all the above mentioned steps for you. You will be kept up to date by mailings about the QC results and progress reports.

1.2.2 DNA Hydroxymethylation Service

To differentiate between hydroxymethylated loci and truly methylated loci, GenomeScan also offers oxidative treatment in addition to bisulfite treatment to the genomic DNA samples.

1.2.3 DNA FFPE Methylation Service

To analyze the methylation status of your formalin fixed paraffin embedded (FFPE) genomic DNA samples, GenomeScan performs a DNA restoration step prior to the execution of the Methylation Assay.

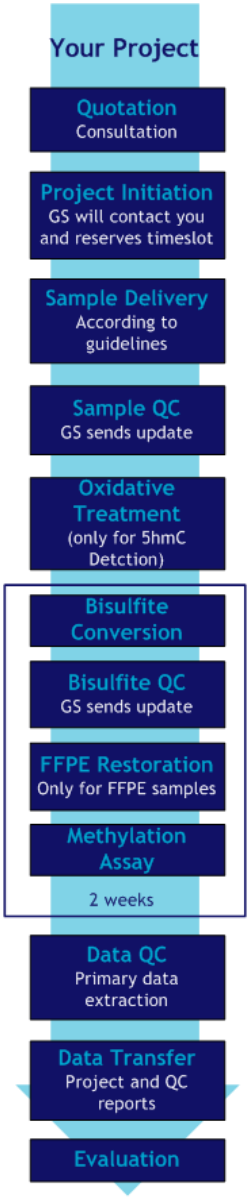
Methylation studies are interesting follow-up studies for (microarray) gene expression experiments to better understand gene regulation.

We have ample experience with Illumina's methylation BeadChips. The HumanMethylation BeadChip is an excellent tool for quantitative methylation measurement of large sample sets at single CpG site level. To study DNA methylation, GenomeScan offers the worldwide accepted and extensively validated MethylationEPIC BeadChip of Illumina. The minimal project size is 24 samples and multiples thereof.

The workflow of the DNA Methylation Services is described in chapter 1.3.

1.3 DNA Methylation Plus Workflow

When you accept our offer, the project will be initiated when the Purchase Order Form is returned to GenomeScan and is filled out completely and signed. Our administration office will confirm the project by sending you a confirmation email containing the project reference number (6 digits).



A Project Manager (PM) will be responsible for your project and monitors the workflow closely. The PM will contact you as soon as possible to discuss project details. GenomeScan will order the necessary reagents and BeadChips when your signed quotation form is received. Delivery of these reagents by Illumina may take up to six weeks. Keep this in mind when planning your experiments.

When all reagents and BeadChips have been received by GenomeScan, the Project Manager will contact you to discuss when the experiments can be performed.

Send your gDNA samples to GenomeScan on cold condition (e.g. cold packs). GenomeScan will visually inspect the package and confirm sample receipt by email. Performance of the first step of the workflow (Entry QC) is scheduled and communicated to you.

After execution and discussion of the entry QC results, GenomeScan performs the DNA treatments according to the service that you ordered (see chapter 1.2). The results of the bisulfite QC are discussed with you prior to the start of the methylation assay. Because of the short shelf-life of the bisulfite converted DNA (2 weeks) it might be possible that we go ahead with the workflow without discussing the bisulfite QC with you when all samples have passed this QC.

Reporting of the data generally takes 1-2 weeks after completion of the experiments. You will receive a sample QC Report, Project Report, a data QC Report, a bisulfite QC Report, exported data in .txt files and the raw data, as described in chapter 5.

Approximately one month after data transfer, GenomeScan will invite you to evaluate the project. These evaluations are important to GenomeScan to continuously improve our services.

Fig. 2. Schematic overview our DNA Methylation analysis workflows

Chapter 2 DNA Sample Preparation and Sample QC

High quality pure DNA samples are necessary for good bisulfite conversion results and to obtain reliable methylation results. For the DNA isolation GenomeScan recommends any commercial available column-based DNA isolation kit.
Please follow the guidelines to deliver DNA samples described in chapter 2.1.

2.1 DNA requirements

Before sending the gDNA samples we ask you to:

- Determine the concentration of the DNA samples. The most accurate and required method is the PicoGreen assay (Invitrogen/Molecular Probes, cat. # P11496). PicoGreen is an ultra-sensitive fluorescent nucleic acid stain used for quantification of double stranded DNA. Do not use the NanoDrop because results could be less accurate due to single-stranded DNA, oligonucleotides, RNA and/or proteins in the solution, all of which interfere with the dsDNA measurement. The presence of these compounds may lead to an overestimation of the actual DNA concentration.

- Check the size of the DNA fragments on a 1% agarose gel. High quality genomic DNA should give a major band at 10 – 20 kb on gel. The fragments must be at least 2 kb. Please send an image of the size check on gel together with your samples. This step is optional, but it is advisable to have an indication of the quality of the samples by running a subset of the samples on gel.

- Dilute the DNA samples in Ultra Pure Water. This is of utmost importance for the oxidative reaction in the hydroxymethylation service. For the other services, the samples can also be diluted in 10mM Tris/0.1mM EDTA pH=8 (Low TE). Samples need to be delivered in a concentration ranges from 50-100 ng/µl based on a fluorescent concentration measurement.

- Be able to provide us with at least **1 µg** of DNA per sample for standard Methylation Analysis. For FFPE samples a minimum of **0.5 µg** is required and for Hydroxymethylation **1.5 µg** is required. Though lower minimum input quantities are required for the methylation assay, GenomeScan requires additional material due to plate format, robot processing and potential sample repeats. If you cannot deliver the required amount of DNA per sample, please contact us upfront.

Notes on DNA requirements

- Contact us upfront for DNA concentrations lower than 50 ng/µl. **Do not** precipitate the DNA samples: this will cause loss of DNA, both in quantity as well as quality. Instead, we advise you to use a vacuum concentrator (*e.g.* SpeedVac, Thermo Scientific) to increase the sample concentration, if necessary. Deviation from the recommended concentration is at your own risk.
- GenomeScan advises against the use of whole genome amplified (WGA) material. Our experience indicates that amplifying input DNA results in lower call rates, lower sample success rates and lower reproducibility of results. If you insist on using WGA DNA, please indicate this before sample shipment.

- DNA carrying large deletions or other genomic aberrations will typically give rise to reduced call rates, which can be 10-15% lower than those of the normal controls. In those cases, it is especially important to include one or more normal control sample for which high call rates are expected.

- For hydroxymethylation, the oxidant solution in the kit is reactive with a range of compounds, such as alcohols (ethanol, isopropanol, phenol), alcohol-containing compounds (Tris, EB buffer, TE buffer). The recommended elution solution from DNA purification method is Ultra Pure Water rather than EB or TE. A buffer exchange step is included to the workflow but the effectiveness of this exchange is dependent on the type and concentration of contaminating species in the solution.

If you cannot fulfil one or more of the requirements listed above, please contact us in advance of your project.

2.2 Sample Delivery Format

Per 96-wells plate, GenomeScan includes 1 control sample. The GenomeScan control sample (Human Brain) will be added at GenomeScan to the sample plate. For the Hydroxy Methylation Service, this control (Human Brain) will be bisulfite treated and the other aliquot of the Human Brain sample will be oxidative AND bisulfite treated.

When delivering follow-up sample plates, the position of the control sample is different for each plate, to serve as orientation control as well as identity control for each plate to be processed. The position of the empty wells will be indicated in the sample submission form by GenomeScan. The control sample(s) will be added at the GenomeScan location.

2.3 DNA sample shipment

DNA samples shipped to GenomeScan need to be free of possible biological contaminants: GenomeScan is not licensed to work with samples restricted by specific biological safety regulations. In general, DNA isolated from cells or tissue is not restricted in use.

When shipping your samples, please follow the instructions below:

- Deliver DNA samples in a thoroughly sealed 96-well skirted PCR plate (clear wells). **Do not** use plates with flat or U-bottom wells! The correct plate type is available from 4Titude (www.bioke.com, cat.# 4Ti-0740 or 4Ti-0960). Our preferred method of sealing plates for shipment is using heat-sealing (peel-seals). Samples need to be pipetted and labelled according to the SXS-sample codes as listed in the sample submission form.

- Make sure the plates used for sample delivery are **rigid and insensitive to warping or distortion** (e.g. as a result of heat-sealing). This is important since we use robotics for pipetting the DNA samples from the plates during the Infinium procedure.

- Make sure your samples do not contain any visual precipitates (e.g. protein pellets). The robot which we use for pipetting the DNA samples from the plates during the Infinium procedure will aspire the sample from only a short distance to the bottom of each well.

- Please include all sample information that you can provide on the sample submission form. If your samples are dedicated to a specific group, please mention this in the appropriate column.

- Randomize sample order, also across and within plates to make sure biological effects can be distinguished from potential batch processing effects.
- Include the results of the quality controls (picture of the gDNA on gel).
- Upon shipment, please send a notification, the shipment tracking number and the electronic versions of the sample submission forms by e-mail to us.

N.B. DNA samples shipped to GenomeScan need to be free of possible biological contaminants: GenomeScan is not licensed to work with samples restricted by specific biological safety regulations. In general, DNA isolated from cells or tissue is not restricted in use.

2.4 Entry QC

Upon arrival of the genomic DNA and prior to the start of the bisulfite conversion, the samples are subjected to the sample QC that consists of two steps:

PicoGreen concentration measurement: Determination of the concentration of the DNA samples by using the in-house validated PicoGreen assay (Invitrogen/Molecular Probes, cat.# P11496).

Gel electrophoresis: Size assessment of the DNA fragments on a 1% agarose gel. High quality genomic DNA should give a major band at 10-20 kb on gel. The fragments must be at least 2 kb. Degraded DNA samples are not suitable for processing, except for the FFPE Methylation service.

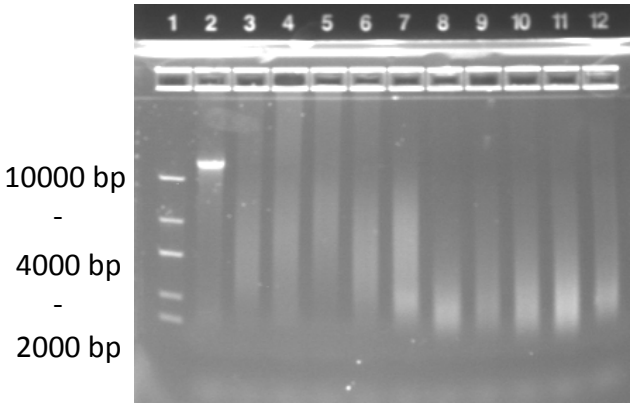


Figure 3: Degraded FFPE samples.
Lane 1: E-gel High Range ladder
Lane 2: intact genomic DNA
Lane 3-7: recent FFPE material (year 2008, 2009)
Lane 8-12: older FFPE material (year 1988)

Sample QC criteria

- DNA quantity ≥ 50ng/µl
- DNA fragment size ≥ 2kb for intact DNA. For FFPE projects, all samples can be processed, but very fragmented samples can have a negative impact on the number of detected CpG's.

When the sample QC criteria are not met, GenomeScan will contact you to discuss the continuation of the project. You can choose one of the following options:

- The customer agrees to continue and process the rejected samples at the customer's own risk. When this attempt fails and work needs to be repeated, GenomeScan will charge the customer for the additional work. The samples are marked as "Own Risk" until the final step of the project.
- The customer sends replacement samples, this will of course delay the project. When choosing this option, additional costs will be charged for extra sample QC.
- The customer agrees to leave out the failed sample(s) and will point out another sample that will be processed in duplicate.

Chapter 3 DNA Treatment and Conversion QC

Bisulfite modification of DNA is the most commonly used, "gold standard" method for DNA methylation studies. Since bisulfite treatment introduces specific changes in the DNA sequence depending on the methylation status of individual cytosine residues, single nucleotide resolution information can be obtained and is the most prominent advantage of this method. See chapter 3.1.

Traditional bisulfite based protocols discriminates between methylated and unmethylated cytosine bases within the genome but cannot differentiate between 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC). **Oxidative bisulfite chemistry** is developed to adapt bisulfite-based 5mC profiling for 5hmC detection. To study hydroxymethylation, a sample is split between a bisulfite (BS) conversion and a oxidative bisulfite (oxBS) conversion. See chapter 3.2.

To check whether the conversion(s) were performed successfully, GenomeScan uses the **restriction QC tool** for the (ox)BS converted samples to determine if the samples are correctly oxidized (chapter 3.2). GenomeScan has developed a **conversion QC tool** to determine the success rate of the bisulfite conversion (chapter 3.3).

Methylation analysis in formaline-fixed Paraffin Embedded (FFPE) DNA samples is not possible without a **restoration step** that repairs the damaged DNA. The fixation process and storage of the FFPE samples frequently leads to DNA degradation. Because of the small DNA fragments, the whole genome amplification is inefficient, resulting in poor performance of the methylation assay. The restoration process is performed after the BS conversion and after the conversion QC and is described in chapter 3.4.

3.1 Bisulfite Conversion

This chapter is only applicable for the DNA and FFPE Methylation Services.
Good results are obtained using the Gold and EZ DNA Methylation™ kit from ZYMO research (www.zymoresearch.com, cat no D5001 or D5002), with the adapted protocol for Infinium assays. Any other methylation kits are not recommended for use with this assay (Illumina FAQs).

Unmethylated cytosines are chemically deaminated to uracil in the presence of bisulfite, while methylated cytosines are refractory to the effects of bisulfite and remain cytosine.

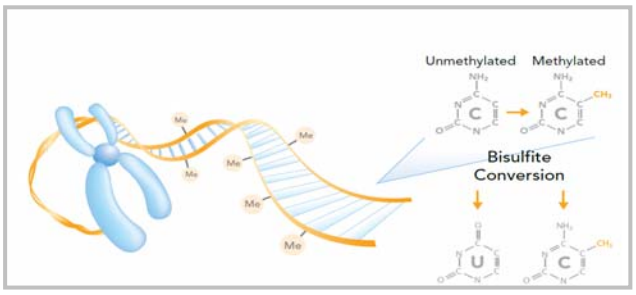


Fig. 4. A methyl group is covalently attached to cytosine C5. When DNA is treated with bisulfite, unmethylated cytosines are converted to uracil, but methylated cytosines are protected.

Prior to the bisulfite conversion we will normalize the gDNA samples (which are quantity assessed by PicoGreen) enabling us to use a consistent amount of input DNA (500 ng). In parallel to the experimental samples, GenomeScan includes a positive control for the bisulfite conversion (commercial Human Brain sample from BioChain) using the same input concentration.

3.2 Oxidative treatment and Restriction QC

This chapter is only applicable for the Hydroxy Methylation Service.
GenomeScan uses the TrueMethyl™ Kit from CEGX for the Oxidative treatment followed by the Bisulfite treatment (by using the same kit).

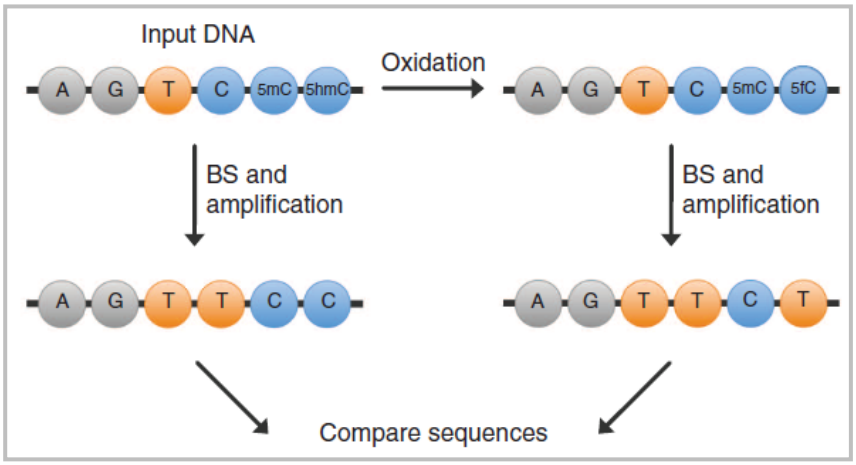


Fig. 5. Schematic overview of the input DNA is splitting up; one part is undergoing an oxidation step prior to the bisulfite conversion.

When a loci is hydroxymethylated, the 5hmC nucleotide will only be converted into a T when an oxidative treatment is perform prior to the bisulfite conversion. In table 1 this is schematically depicted.

Table 1. Differentiation between sequences after oxidative and bisulfite treatment

Base	Sequence	BS Sequence	oxBS Sequence
C	C	T	T
5mC	C	C	C
5hmC	C	C	T

Please note, the oxidant solution in the kit is reactive with a range of compounds, such as alcohols (ethanol, isopropanol, phenol), alcohol-containing compounds (Tris, EB buffer, TE buffer). The recommended elution solution from DNA purification method is Ultra Pure Water rather than EB or TE. A buffer exchange step is included to the workflow but the effectiveness of this exchange is dependent on the type and concentration of contaminating species in the solution.

Restriction QC analysis was performed to assess both the oxBS and BS conversion accurately. Without the oxidative treatment, both methylated and hydroxymethylated cytosines are protected against conversion by bisulfite to uracil. The oxidative treatment deprotects 5-hydroxymethylated Cytosines (5hmC) from bisulfite conversion by first converting them to 5-formyl Cytosine. This changes the restriction site, making it unable for the restriction enzyme to cut.

The restriction mix is subjected to Agarose gel analysis to check for the expected restriction patterns (Figure 6).

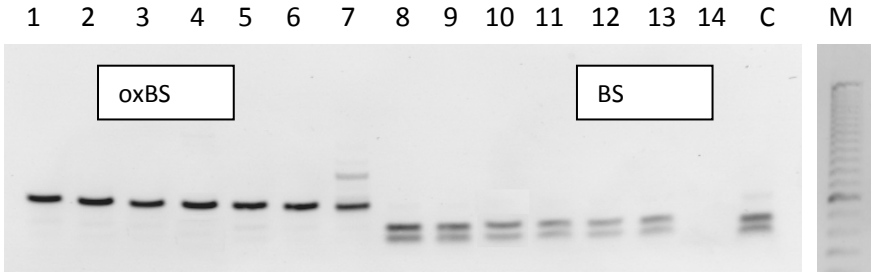


Figure 6: Taq$^{\alpha}$I digestion products of samples processed through the TrueMethyl workflow.
Samples 1-7: oxBS samples; Samples 8-14: BS samples; C: Cutting control (100 ng input); M: 25 bp marker
Digestion of the oxBS samples after incomplete conversion or oxidation failure will yield two bands of ~40 and 60 bp, as will digestion of BS samples.

3.3 Bisulfite QC

QC of bisulfite converted gDNA is an important step when performing a methylation study and will be performed on all services. To check the conversion efficiency of the bisulfite converted samples a qPCR reaction is performed, followed by melting curve analysis. Below the details of the QC steps are described. The outcome of the bisulfite QC result will be "pass" or "fail". When a sample fails bisulfite QC it does not necessarily mean that the assay will fail, but the methylation profiles are possibly less reliable and must be interpreted with extra caution.

qPCR

GenomeScan determines the bisulfite conversion efficiency by qPCR on the LC480 with primers specific for the IC2 region. These primers do not contain any C-nucleotides, ensuring that irrespective of the conversion state, the primers will always successfully bind.
Next to the experimental samples, GenomeScan includes several controls (positive and negative) enabling to check the technical performance of the qPCR reaction and the melting curve analysis. Also, commercial genomic human brain DNA will be used as a positive control for the bisulfite conversion. Finally, a dilution series from the converted universal human brain DNA sample above is included, ½, ¼, 1/8, 1/16, to assess the success of the q-PCR reaction.

Melting Curve analysis

Melting Curve Analysis (MCA) is based on the ability to detect variation in melting profiles of as little as 1 bp between fragments. Therefore, MCA can be used to detect and quantify differences in amplicons derived from bisulfite-modified DNA. The advantage of this method is that it is fast and cost effective.

As visualized in Fig. 7 and 8, bisulfite converted PCR product has a lower melting temperature (Tm), than untreated DNA. In our region of interest a maximum of 27 C-nucleotides can be converted, therefore the difference in Tm between bisulfite converted and unconverted will be substantial.

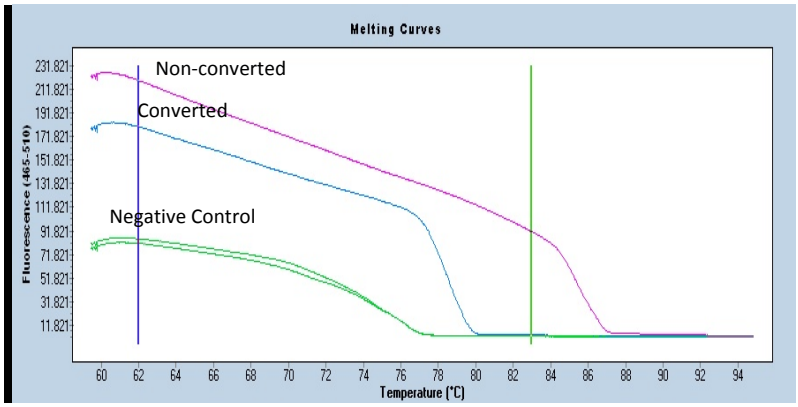


Figure 7: Shift in melting temperature (decrease in fluorescence) as determined by melting curve analysis
Pink: ZYMO non-converted control; Blue: ZYMO converted control; Green: NTC; water controls

Table 2: The expected Tm values

Type of samples	Tm1
Non-converted sample (or control)	86
Converted samples	77-79
No template/primer dimer	75-77

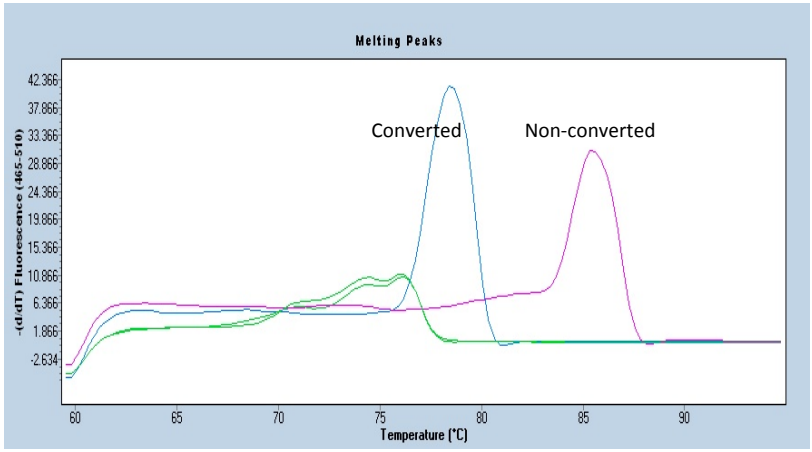


Figure 8: The plateau of the melting curve indicates the portion of the DNA that is converted.
Pink: ZYMO non-converted control; Blue: ZYMO converted control; Green: NTC; water controls

Bisulfite QC criteria

- The Ct value of the experimental samples as calculated by the Roche LC480 should be within 4 cycles of that for the in-house converted human brain DNA control sample from the same conversion.
- The samples must melt within 1°C on either side of the control Tm, which should melt between 77 and 80°C.
- Ideally, only one melting peak is visible. If a primer dimer is formed, an extra peak will appear. This extra peak may not be higher than a third of the specific signal peak.

The bisulfite QC results are presented in a so-called “Bisulfite Quality Control report” after the project is finished. Due to the short expiry time of the converted DNA, we need to continue almost immediately with the Infinium assay.

When we see a general issue with the bisulfite converted samples, you will be contacted by us to discuss further steps. You can choose one of the following options:

- The customer agrees to continue and process the rejected samples at the customer’s risk. When this attempt fails and work needs to be repeated, GenomeScan will charge the customer for the additional work. The samples are marked as “Own Risk” until the final step of the project.
- The customer sends replacement samples. This will of course delay the project but due to the short shelf-life all samples need to be replaced and the project has to start from scratch. When choosing this option, extra costs will be charged.
- The customer agrees to leave out the bad sample and will point out another sample that will be processed in duplicate.

3.4 Restoration

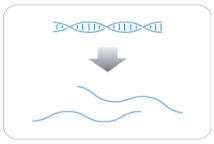
This extra step in the workflow is only part of the FFPE methylation projects. GenomeScan uses the Restoration kit from Illumina and the FFPE adapted protocol for FFPE input DNA. The restoration step is performed after the bisulfite QC step. No QC step will be performed to assess the restoration was successful.

Chapter 4 Illumina's Infinium Methylation Assay and Data QC

4.1 Infinium Methylation Assay Overview

The Infinium methylation assay uses beads displaying long, target specific probes designed to interrogate individual CpG sites within a given DNA sample. DNA methylation is measured using quantitative "genotyping" of bisulfite-converted genomic DNA.

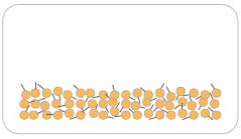
Below, an overview of the Infinium Assay is shown. GenomeScan performs the experiments according to Illumina's specifications and our in-house validated SOPs.



DNA Amplification
The DNA samples are denatured and neutralized to prepare them for amplification.

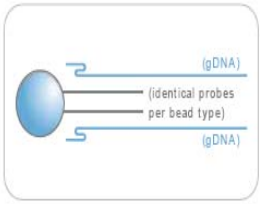
DNA Incubation
The denatured DNA is isothermally amplified in an overnight step. The whole-genome amplification uniformly increases the amount of the DNA sample by several thousand fold without introducing large amounts of amplification bias.

DNA Fragmentation
The amplified product is fragmented by a controlled enzymatic process that does not require gel electrophoresis. The process uses end-point fragmentation to avoid over-fragmenting the sample.

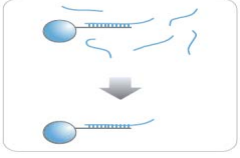


DNA Precipitation
After an isopropanol precipitation, the fragmented DNA is collected by centrifugation at 4ºC.

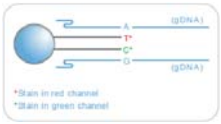
DNA Resuspension
The precipitated DNA is resuspended in hybridization buffer.



Hybridization to the HumanMethylation BeadChip
The BeadChip is prepared for hybridization. Samples are applied to the BeadChip and divided by an IntelliHyb® seal. The loaded BeadChip is incubated overnight in the Illumina Hybridization Oven. The amplified and fragmented DNA samples anneal to locus-specific 50-mers (covalently linked to one of up to 200,000 bead types) during hybridization. One bead type corresponds to each allele per SNP locus.



Washing of the BeadChip
Unhybridized and non-specifically hybridized DNA is washed away, and the BeadChip is prepared for staining and extension.



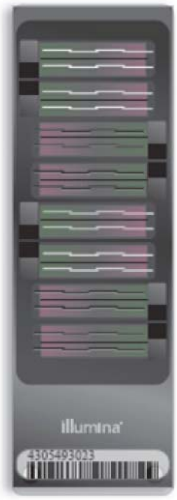
Extension and Staining (X-Stain) BeadChip
Single-base extension of the oligos on the BeadChip, using the captured DNA as a template, incorporates detectable labels on the BeadChip and determines the genotype call for the sample.

Scanning the BeadChip using the Illumina iScan
Finally, the BeadChips are scanned using the high-resolution (submicron) Illumina iScan Reader, which generates images of the light emitted from the fluorophores.

Fig. 9. DNA methylation assay overview

4.2 Infinium MethylationEPIC BeadChip

Powered by Illumina's revolutionary Infinium Methylation Assay, this BeadChip allows researchers to interrogate > 850,000 methylation sites per sample at single-nucleotide resolution. The Infinium MethylationEPIC BeadChip (Figure 10) builds upon the industry-leading Infinium HUmanMethylation450 BeadChip with > 90% of the original CpGs plus an additional 350,000 CpGs in enhancer regions. This Methylation beadChip is a pan-enhancer and coding region view of the methylome that can be used for epigenome-wide association studies on various human tissues and includes the following content categories requested by methylation experts:



- Non-CpG methylated sites identified in human stem cells (CHH)
- CpG sites outside of CPG islands
- Differentially methylated sites identified in tumor versus normal (multiple forms of cancer) and across several tissue types
- FANTOM5 enhancers
- ENCODE open chromatin and enhancers
- DNase hypersensitivity sites
- miRNA promoter regions
- > 90% of content contained on the Illumina HumanMethylation450 BeadChip

Fig. 10. The Infinium Human MethylationEPIC BeadChip

The HumanMethylation BeadChip combines Infinium I and Infinium II assay chemistry technologies. Infinium I and II offer complementary strengths that benefit the array's breadth of coverage.

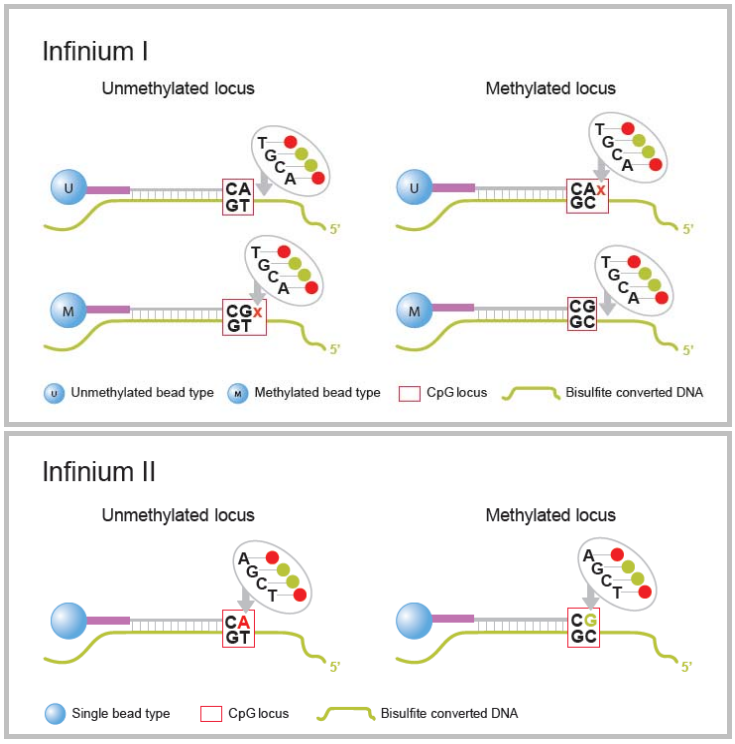


Fig. 11. The HumanMethylation BeadChip employs both Infinium I and II assays, enhancing its breadth of coverage.

Infinium I

The Infinium I assay employs two probes per CpG locus: one "unmethylated" and one "methylated" query probe. The 3' terminus of each probe is designed to match either the protected cytosine (methylation design) or the thymine base resulting from bisulfite conversion and whole-genome amplification (unmethylated design). Probe designs for Infinium I assays are based on the assumption that methyaltion is regionally correlated within a 50 bp span and, thus, underlying CpG sites are treated as in phase with the "methylated" (C) or "unmethylated" (T) query sites.

Infinium II

The Infinium II assay design requires only one probe per locus. The 3' terminus of the probe complements the base directly upstream of the query site while a single base extension results in the addition of a labelled G or A base, complementary to either the "methylated" C or "unmethylated" T. The Infinium II design uses one bead type, with the methylated state determined at the single base extension step after the hybridization.

Due to their different chemistries, the Infinium I and II assays each have distinct advantages. Differences between the two chemistries have been observed that result in distinct beta values distributions within data sets. Figure 11 shows histograms of the beta values in bins of 0.02 and categorized by Infinium design type. In general, the peaks at the extreme ends of the beta distribution tend to be further out for Infinium I probes than for Infinium II probes, capturing the full spectrum of methylation.

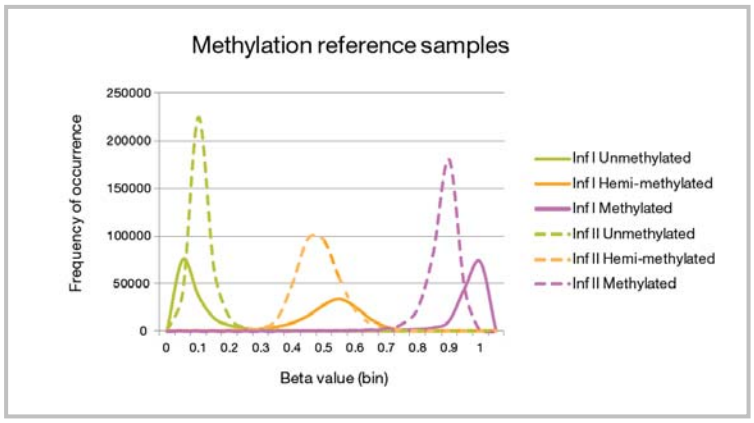


Fig. 12. The different chemistries of Infinium I and II assays results in distinct beta values distributions.

For more information about the content and the chemistry of the Infinium I and II assay on the HumanMethylation BeadChip we refer to the Technical Notes: Epigenetics and Epigenetic Analysis, respectively which can be found on www.illumina.com

4.3 Data QC

After execution of the Infinium Methylation Assay, the scanned BeadChips are checked for the correct placing of the grid by the scanning software. Other overall quality criteria are assessed by Illumina software GenomeStudio Methylation Module, such as the average background and gene signals and the number of significant detected CpG's. Finally, we will assess the quality of the methylation data by the Illumina assay controls. These controls and how they are expected to perform are explained in Chapter 5.1. You will receive a copy of the data QC Report together with the data.

Chapter 5 Delivery of Results

5.1 Infinium Methylation Assay Controls

The Illumina Infinium Methylation Assay has a number of built-in assay controls, which are analysed by the R script MethylAid. GenomeScan closely monitors the quality control parameters. The results of the quality control parameters are listed in a Quality Report, which is delivered with the methylation data.

Controls include sample-independent and sample-dependent controls. Sample-independent controls are staining controls to examine the efficiency of the staining step in both red and green channels, hybridization controls to test overall performance of the assay using five different concentrations of synthetic targets, target removal controls to check the stripping of the captured DNA fragments after the extension reaction, and extension controls to test the extension efficiency.

Sample-dependent controls are bisulfite conversion controls, stringency controls, non-specific binding controls and non-polymorphic controls. The Infinium Assay includes redundant, built-in, bisulfite conversion quality controls that measure the conversion rate of non-CpG cytosines and background signal.

Sample-independent controls

Sample-independent controls evaluate the performance of specific steps in the process flow.

Staining Controls

Staining controls are used to examine the efficiency of the staining step in both the red and green channels. Staining controls have dinitrophenyl (DNP) or biotin attached to the beads. These controls are independent of the hybridization and extension steps. DNP and biotin monitor the sensitivity and efficiency of the staining step. Both red and green channels are evaluated using the staining controls.

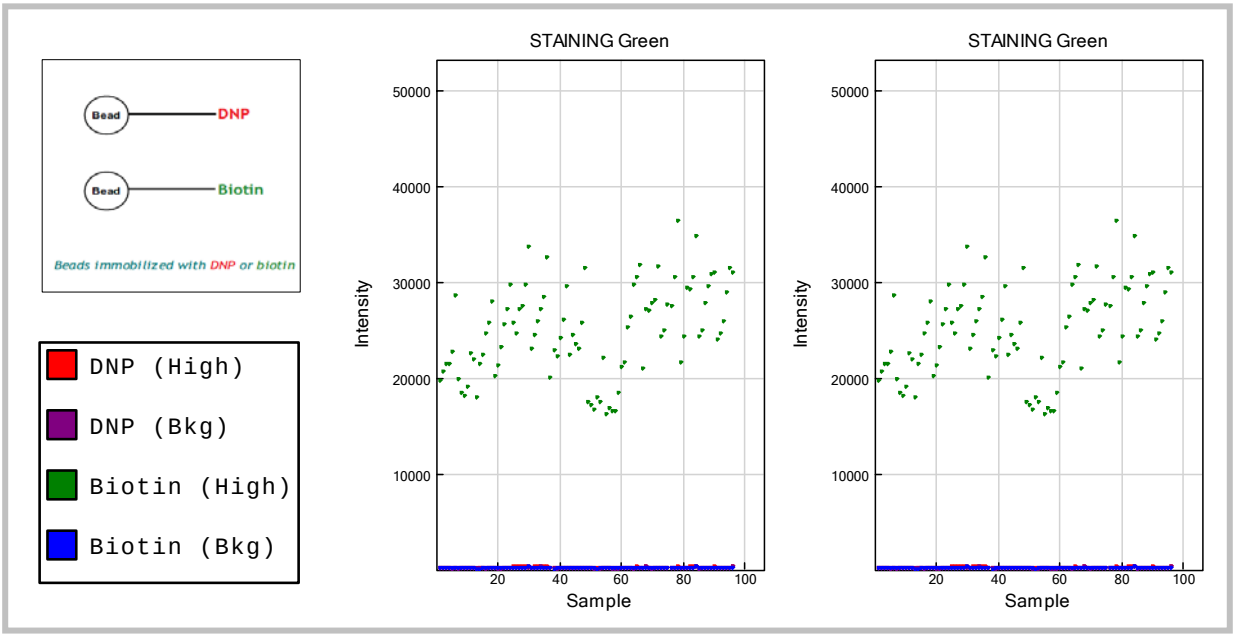


Fig. 13. Staining controls

Hybridization Controls

The hybridization controls test the overall performance of the entire assay using synthetic targets instead of amplified DNA. These synthetic targets complement the sequence on the array perfectly, allowing the probe to extend on the synthetic target as template. The synthetic targets are present in the hybridization buffer at three levels, monitoring the response from high concentration targets (5pM), medium (1pM), and low concentrations (0.2pM). All bead type IDs should result in signal with various intensities, corresponding to the concentrations of the initial synthetic target concentrations. Performance of hybridization controls is only monitored in the green channel.

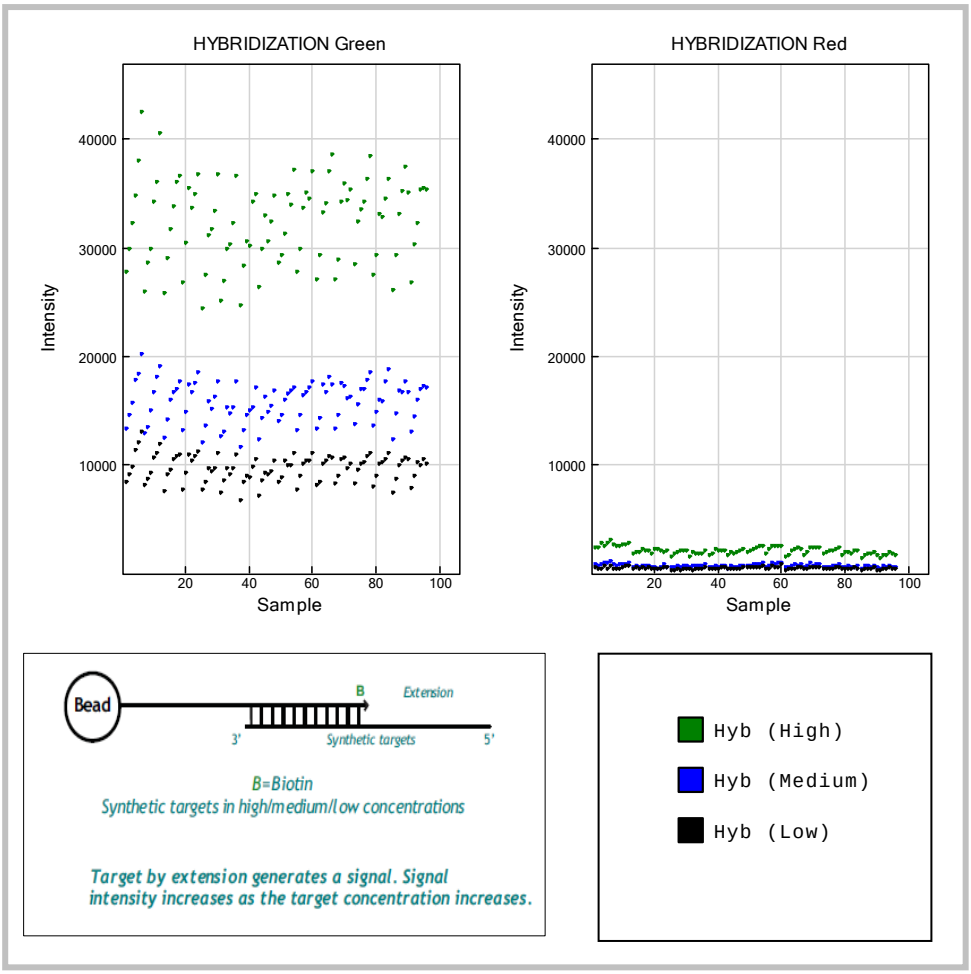


Fig. 14. Hybridization controls

Target Removal Controls

Target removal controls test the efficiency of the stripping step after the extension reaction. In contrast to allele specific extension, the control oligos are extended using the probe sequence as template. This process generates labeled targets. The probe sequences are designed such that extension from the probe does not occur. All target removal controls should result in a low signal compared to the hybridization controls, indicating that the targets were removed efficiently after extension. Performance of target removal controls is only monitored in the green channel.

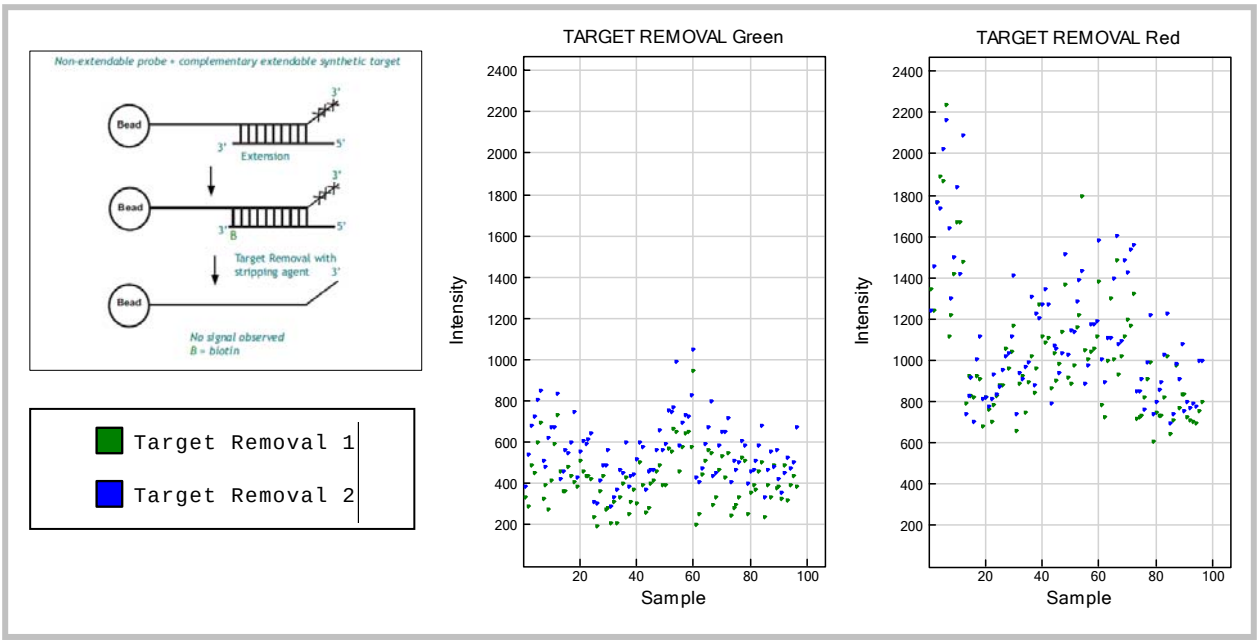


Fig. 15. Target removal controls

Extension Controls

Extension controls test the extension efficiency of nucleotides A, T, C, and G nucleotides from a hairpin probe, and are therefore sample-independent. Both red (A,T) and green (C,G) channels are monitored.

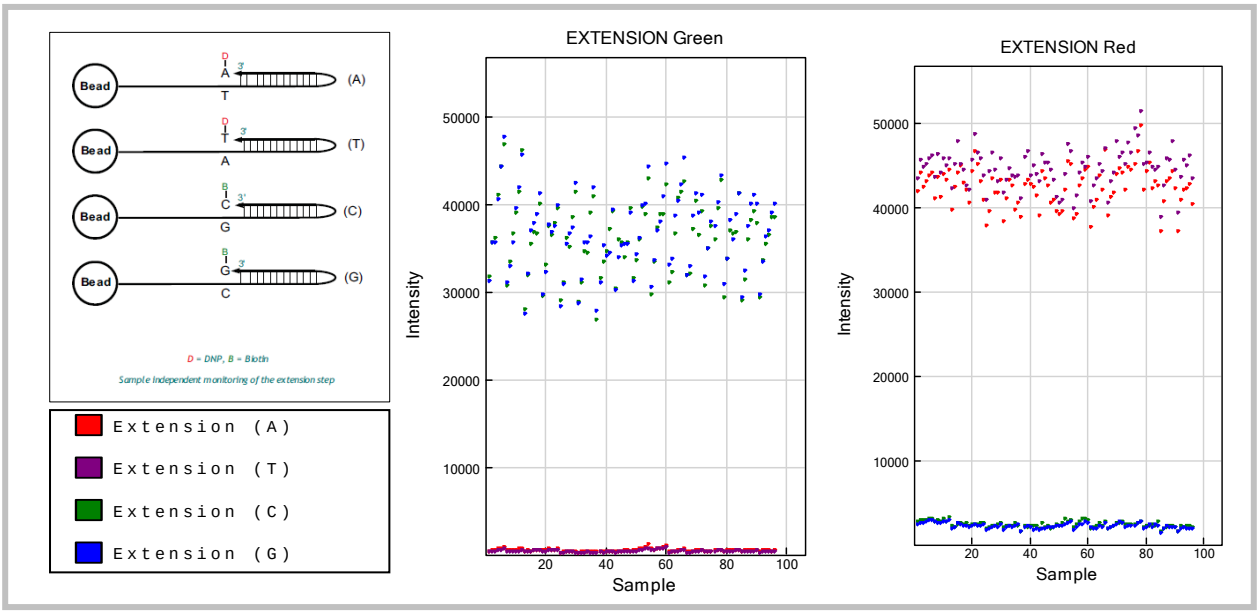


Fig. 16. Extension controls

Sample-dependent controls

The sample-dependent controls are used to evaluate performance across samples. These control oligos are designed for bisulfite converted human genomic DNA sequences. Because target sequences do not contain CpG dinucleotides, the performance of the control oligos does not depend on the methylation status of the template DNA.

Bisulfite Conversion Controls

These controls assess the efficiency of bisulfite conversion of the genomic DNA. The Infinium Methylation probes query a [C/T] polymorphism created by bisulfite conversion of non-CpG cytosines in the genome.

Bisulfite Conversion I

These controls use the Infinium I probe design and allele-specific single base extension to monitor efficiency of bisulfite conversion. If the bisulfite conversion reaction was successful, the "C" (Converted) probes will match the converted sequence and will be extended. If the sample has unconverted DNA, the "U" (Unconverted) probes will be extended. The “C” probes must have a signal higher than 4000, see red line.

There are no underlying C bases in the primer landing sites, except for the query site itself. Performance of bisulfite conversion controls C1, C2 and C3 should be monitored in the green channel, and controls C4, C5 and C6 should be monitored in the red channel.

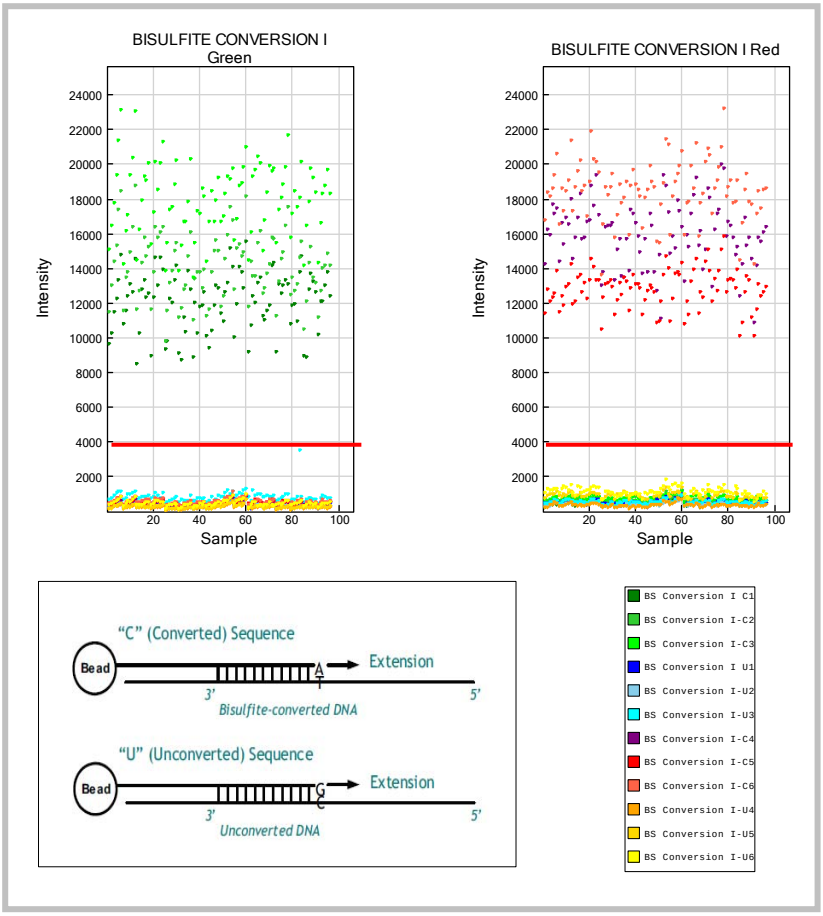


Fig. 17. Bisulfite Conversion I controls

Bisulfite Conversion II

These controls use the Infinium II design and single base extension to monitor the efficiency of bisulfite conversion. If the bisulfite conversion reaction was successful, the "A" base will be incorporated and the probe will have intensity in the red channel. The probes in the red channel must have a signal higher than 4000, see red line. If the sample has unconverted DNA, the "G" base will be incorporated across the unconverted cytosine, and the probe will have elevated signal in the green channel.

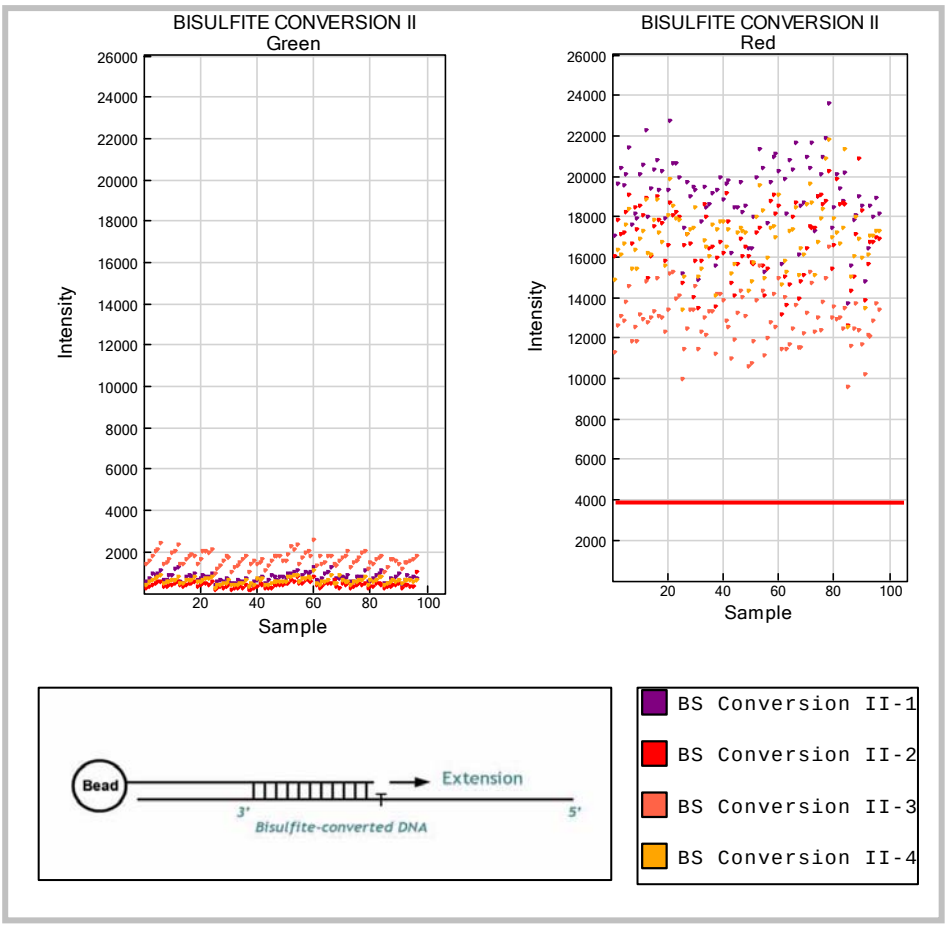


Fig. 18. Bisulfite Conversion II controls

Specificity Controls

Specificity controls are designed to monitor potential non-specific primer extension for Infinium I and II assay probes. Specificity controls are designed against non-polymorphic T sites.

Specificity I

These controls are designed to monitor allele-specific extension for Infinium I probes. The methylation status of a particular cytosine is carried out following bisulfite treatment of DNA through the use of query probes for unmethylated and methylated state of each CpG locus. In the assay oligo design, the A/T match (PM) corresponds to the unmethylated status of the interrogated C, and the G/C match (PM) corresponds to the methylated status of C. G/T mismatch (MM) controls check for non-specific detection of methylation signal over unmethylated background. PM controls correspond to A/T perfect match and should give high signal. MM controls correspond to G/T mismatch and should give low signal. Performance of GT Mismatch controls should be monitored in

both green and red channels. The Controls dashboard table lists expected outcome for controls probes.

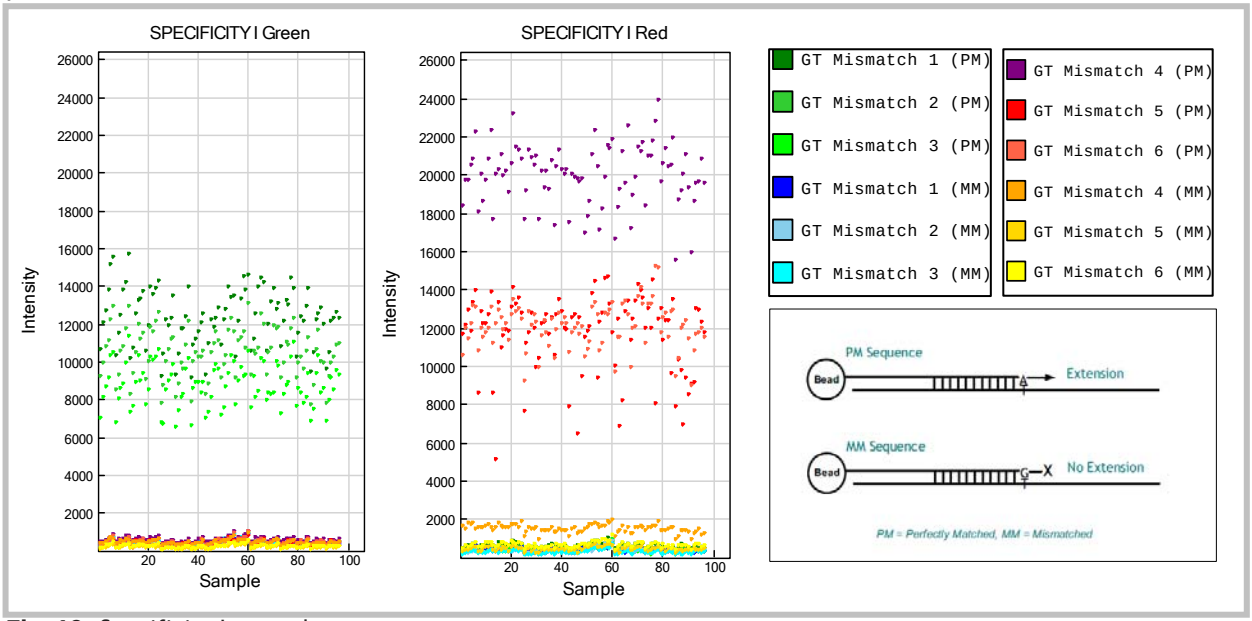


Fig. 19. Specificity I controls

Specificity II

These controls are designed to monitor extension specificity for Infinium II probes and check for potential non-specific detection of methylation signal over unmethylated background. Specificity II probes should incorporate the "A" base across the nonpolymorphic T and have intensity in the red channel. In case of non-specific incorporation of the "G" base, the probe will have elevated signal in the green channel.

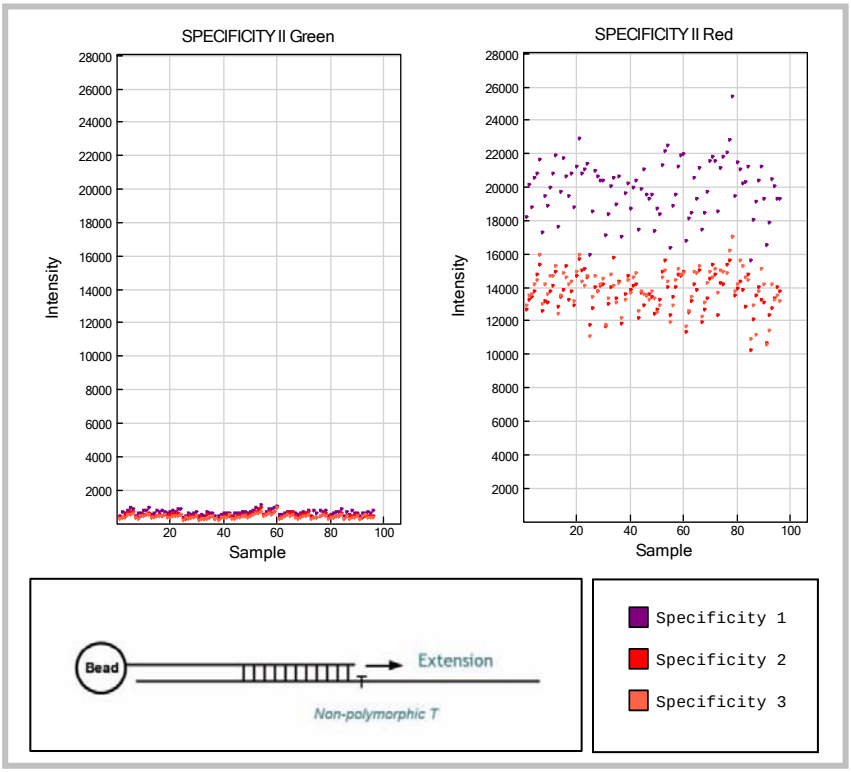


Fig. 20. Specificity II controls

Negative controls

Negative control probes are randomly permutated sequences that should not hybridize to the DNA template. Negative controls are particularly important for methylation studies because of a decrease in sequence complexity after bisulfite treatment. The mean signal of these probes defines the system background. This is a comprehensive measurement of background, including signal resulting from cross-hybridization, as well as non-specific extension and imaging system background. The GenomeStudio application uses the signal and standard deviation of these probes to establish detection limits for the methylation probes. Performance of negative controls should be monitored in both green and red channels.

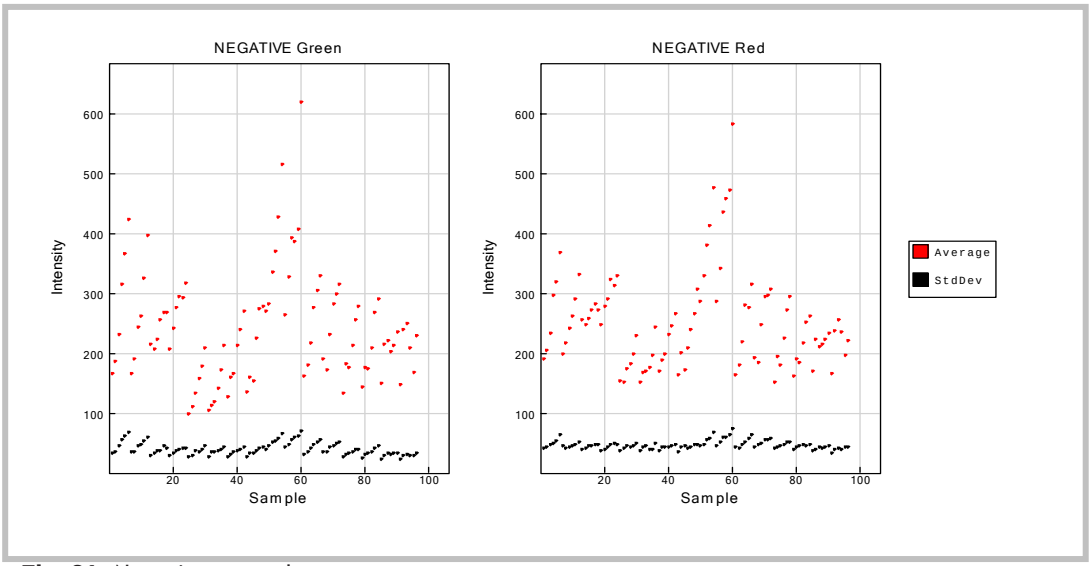


Fig. 21. Negative controls

Non-Polymorphic Controls

Non-polymorphic controls test the overall performance of the assay, from amplification to detection, by querying a particular base in a non-polymorphic region of the bisulfite genome. They let you compare assay performance across different samples. One non-polymorphic control has been designed to query each of the four nucleotides (A,T, C, and G). The target with the C base results from querying the opposite whole genome amplified strand generated from the converted strand.

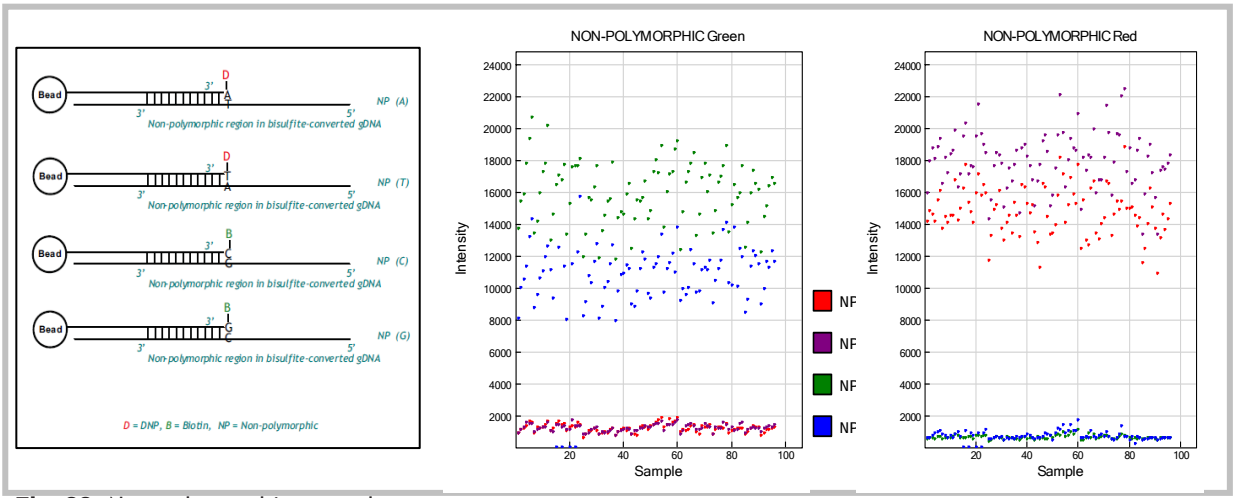


Fig. 22. Non-polymorphic controls

Restoration Controls

The restoration controls test the performance of the restoration step preceding the Infinium assay.

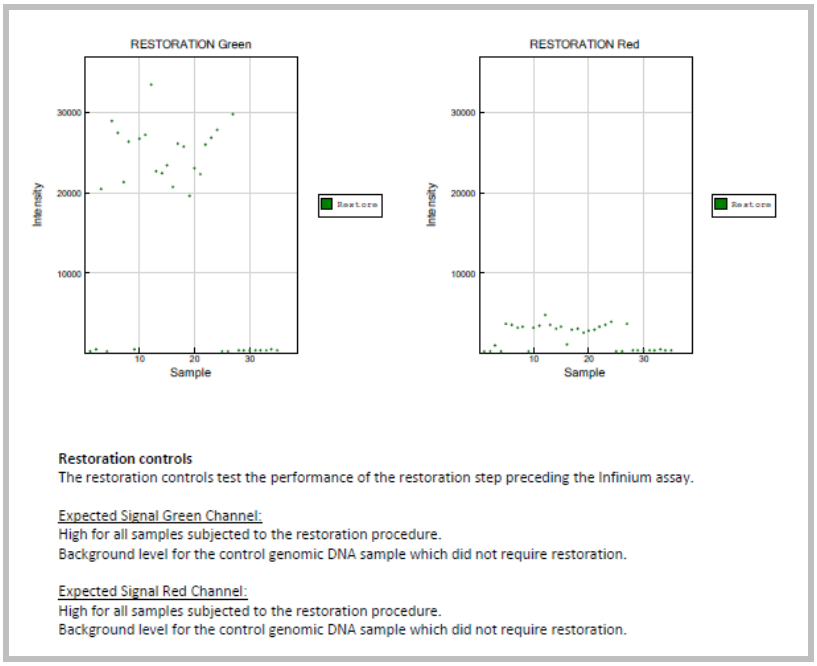


Fig. 23. Restoration controls

Hydroxymethylation Control Scatter Plot

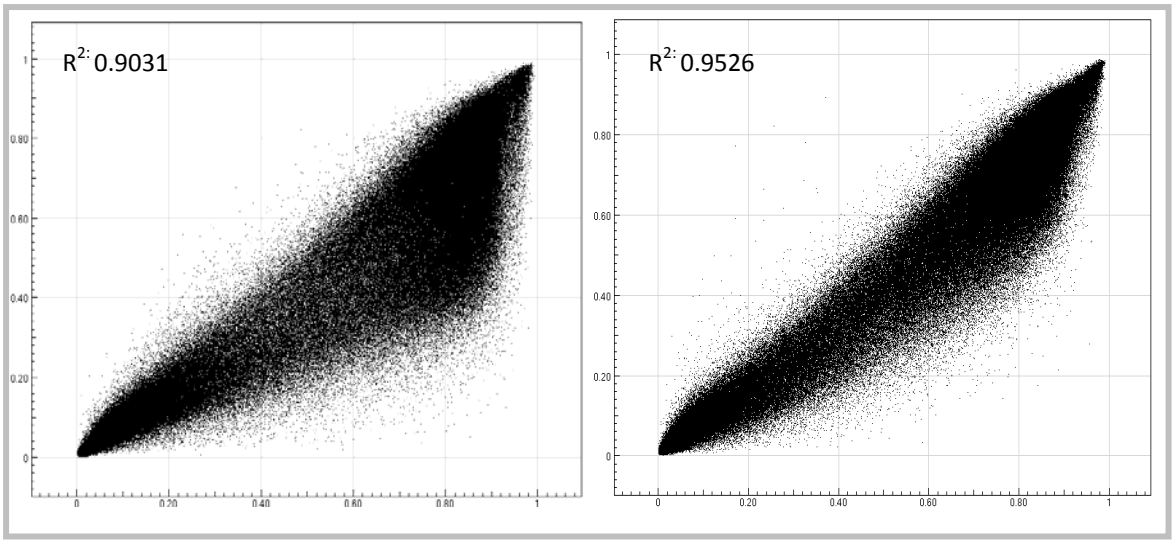


Fig. 24. Examples of BS vs oxBS comparisons of two types of brain DNA samples. Both samples were processed starting with 4 µg gDNA (now 1µg is required).

5.2 Generation of Data Output – Primary Data Analysis

The R script MethylAid is used to assess the quality of the Methylation data. The package provides a visual and interactive web application using RStudio's shiny package. Bad quality samples are detected using sample-dependent and sample-independent controls present on the array. In depth exploration of bad quality samples can be performed using several interactive diagnostic plots of the quality control probes present on the array. Furthermore, the impact of any batch effect can be explored. Our regular service does not include differential methylation analysis.

GenomeScan delivers data as idat files and sdf files, together with the raw image data. A sample sheet containing the beadChip lay-out will also be delivered for your analysis in *e.g.* R.

5.3 Deliverables

GenomeScan established the following deliverable for Illumina Infinium Methylation Analysis:

For DNA and FFPE Methylation Service

- A minimum number of detected CpGs (p0.01) of 807,500 (95% call rate) and a minimal signal value of 4000 for the Conversion Controls I and II for at least 90% of your samples using the HumanMethylation BeadChip. For FFPE projects, a 80% success rate on the restored samples is applicable.

For Hydroxy Methylation Service

- A minimum number of detected CpGs (p0.01) of 807,500 (95% call rate) for at least 90% of your samples using the HumanMethylation BeadChip. The design of the Illumina conversion controls are suboptimal for the (ox)BS converted samples and are not part of the deliverables.

The results of the Infinium Methylation Analysis strongly depend on the quality of the input material. Using the three QC phases (Entry QC, Conversion QC and Data QC), GenomeScan is able to monitor the performance of the experiments and the bisulfite conversion success. If resulting sample success rates do not meet our deliverables, GenomeScan will thoroughly inspect the Infinium assay controls and experimental performance using our sample tracking system. In case the Infinium assay controls point out that the experiments have been performed well, the lower number of detected genes is likely the result of sample quality.

If all quality criteria are met, a Project Report, a bisulfite QC Report, a data QC Report as well as the results of your experiment will be sent to you. In general, the raw data is sent on a USB or external hard disk using an overnight courier service. The disk contains the following files:

- Project Report (summary of experiments and results)
- Bisulfite QC Report
- Data QC Report
- Appendix to Project Report
- Folder with Genome Studio Methylation analysis file
- Folder with exported data files (.txt)
- Folder with sample details
- Folder with image data
- Overview of exported file types
- GenomeStudio Methylation Module v1.0 user guide

5.4 Project Evaluation

Approximately four weeks after completion of the project, a GenomeScan team member will invite you to evaluate our service. Sharing your experience allows us to optimize our services tuned to our customers needs. We would very much appreciate it if you would complete the short questionnaire!

5.5 Follow-up Research

Of all the genes represented on the HumanMethylation BeadChip, more than 20,000 are also present on the HumanHT-12 v4 Expression BeadChip, permitting combined analysis of global methylation status and gene expression levels. In addition, investigators may integrate methylation data with genotyping data from GWAS studies to better understand the interplay between genotype and methylation state in driving phenotypes of interest.

Chapter 6 Sample Policy and Requirements

In the following paragraphs a brief outline is given of what you can expect from GenomeScan regarding our sample policies.

6.1 Sample Delivery

Upon receipt of the signed PO form, we ask our customers to give us an estimation of the delivery date of the samples. If this is not known at that time, please notify us as soon as possible by sending us the completed Sample Submission Form with the correct date. We use this date to schedule the first step of the experimental workflow. When the delivery of your samples is delayed, inform us as soon as possible so we can reschedule your project. For rescheduling of your project extra costs may be charged for lost time slots.

6.2 Sample Shipment

Before sending your samples to GenomeScan, notify your Project Manager so he/she can advise you when to send your samples to ensure that the shipment is on time for the scheduled start of the project. We can also help you avoid delays such as during (Dutch) bank holidays. Do not ship samples just before the weekend, but preferably at the start of the workweek. Make sure your Project Coordinator knows the exact delivery date, so we can track the shipment if necessary.

6.3 Ordering of Project Specific Perishable Reagents

GenomeScan will order materials and reagents upon receipt of the signed PO form, just before the sample delivery date that you have indicated on the Sample Submission Form. Certain projects require materials or reagents that are solely used during your specific project. If this is the case, you will be informed of the expiration date. It is your responsibility to deliver samples well within the expiry date of these reagents. GenomeScan cannot use reagents or materials past the expiration date as part of our requirements for ISO 17025 certification.

6.4 Batch Policy

The number of batches is agreed upon and documented in the quotation. When deviating from the quotation additional costs are charged.

6.5 Spare Sample Policy

The quality of samples will be analyzed at the start of the project. In case one or more samples do not meet our QC requirements, we inform you of this result. You may send a replacement batch to GenomeScan. Extra costs may apply when the number of batches exceeds the number stated in the quotation. Furthermore, sending replacement sample(s) usually affects the turnaround time of your project.

6.6 Use of ‘Failed’ Samples

Samples, especially those consisting of RNA, are prone to fail the sample QC. You can choose to continue with these samples when you agree to process the failed samples at your own risk. GenomeScan will no longer guarantee normal data quality standards or any sample preparation results at all for those samples that are labelled ‘Own Risk’. GenomeScan will offer this option for samples for which GenomeScan deems that there is considerable chance that the processing will be successful or result in usable data. You will be asked to confirm that you agree to process the failed samples at your own risk. If no technical failure is detected, we conclude that the run is within specifications and no replacement sample is granted.

6.7 Turnaround Time

The average turnaround time for a methylation project is 4 – 6 weeks (for projects < 192 samples) starting from the date of sample arrival. Preparing the samples according to the GenomeScan' requirements must be followed enabling us to execute the project as efficient as possible

GenomeScan is dependent on its suppliers for materials (can take up to 6 weeks to deliver) and technical support for machinery. When delays are unavoidable, GenomeScan will notify you as soon as possible and keep you updated on the progress. GenomeScan reserves the right to run a project at a different location (preferably our partner: the Leiden Genome Technology Center) if there are no alternatives.

6.8 Trend Analysis

GenomeScan uses a reference Human brain sample in every sample plate to keep track of the methylation assay and reagent performance. Furthermore, several QC parameters will be monitored from each project to be certain of a constant quality within each step of the workflow.

6.9 Sample Storage Terms

Samples present at GenomeScan are stored for 6 months after completion of a project. On the Sample Submission Form sent to you at the start of the project, you can choose to receive the samples after the project is terminated or that the samples will be stored at GenomeScan and discarded after the indicated period. If the samples are returned to you by courier, additional shipment costs may apply.

6.10 Data Storage Terms

The raw data will be stored in our hard-drives for a long period of time, but we will strongly advise you to make a copy of your data after the delivery.

Additional Information

For additional information, project consultancy or a project quotation, please contact us at the address below! To learn more about our Service Portfolio, visit www.servicexs.com

Caring for your future