SERVICE GUIDE

Methyl-Seq Service

CustomerCare@agrf.org.au
1300 247 301
www.agrf.org.au

SVG2403MET

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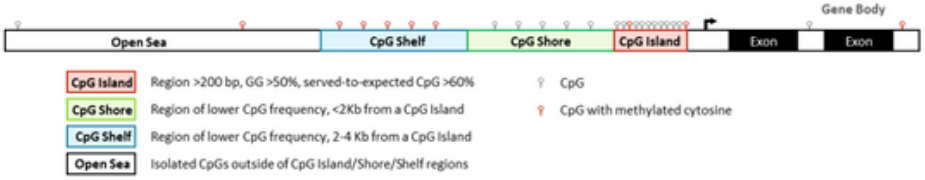
1.0 Overview

DNA methylation is an important epigenetic mechanism influencing the expression of genes and the structure of chromatin. DNA methylation plays an essential role in development, through regulation of gene expression, genomic imprinting and preservation of chromosome stability. Dysregulation of DNA methylation plays a role in several human diseases including cancer and inherited disorders such as Prader–Willi syndrome.

5-methylcytosine (5mC) is the most common and widely studied form of DNA methylation in eukaryotes.
In mammals, cytosine methylation is found almost exclusively in a CpG dinucleotides, where a methylated cytosine is immediately followed by guanine. In vertebrate genomes, CpG dinucleotides occur at lower than expected frequencies, with uneven distribution across the genome.CpG islands are regions with a high frequency of CpG dinucleotides, often associated with the promotorregions of genes.

Figure 1: Schematic representation of the distribution of CpG in vertebrate genomes.



In other organisms, including plants, cytosine methylation can also occur in CHG< or CHH contexts (where H corresponds to either A, T or C).

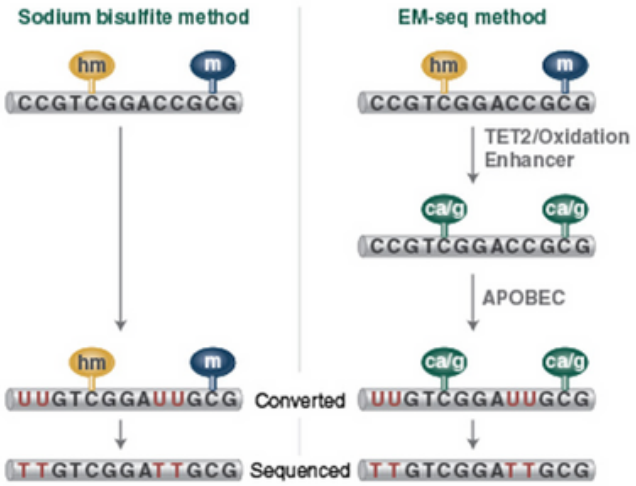
2.0 Methylated Sequencing (Methyl-Seq)

Methylated cytosines can be detected using the process of sodium bisulfite conversion or enzymatic (EM-seq) conversion (Figure 2).

Bisulfite treatment of DNA leads to the deamination of cytosines resulting in a conversion to uracil – whereas methylated cytosines remain unchanged. Following bisulfite conversion, PCR amplification converts uracils to thymines.

Enzymatic treatment of DNA uses a two-step enzymatic cocktail to detect methylated cytosines. The first step uses TET2 and an oxidation enhancer to protect 5mC/5hmC from deamination, followed by step two which uses APOBEC to deaminate cytosine to uracil but 5mC/5hmC are not deaminated.

Figure 2: Summary of methylation conversion mechanisms for both sodium bisulphite and EM-seq. (NEB Information here.)



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AGRF offers Whole Genome Enzymatic Methylation Sequencing (WGEMS) and Reduced Representation Bisulfite Sequencing (RRBS) services providing single-base resolution of DNA methylation.

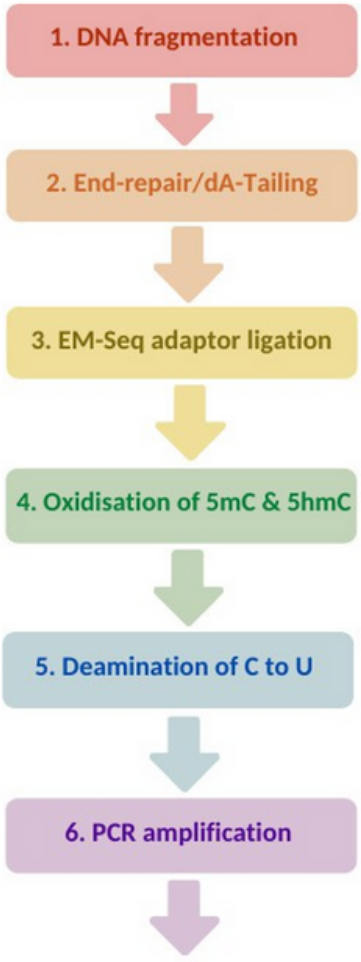
3.0 Whole Genome Enzymatic Methylation Sequencing (WGEMS)

Shotgun sequencing of EM converted genomic DNA enables single-base and strand-specific resolution of methylated cytosines throughout the whole genome. EM-seq can detect cytosine methylation patterns in an entire genome, including CpG, CHH and CHG contexts.

AGRF uses the NEBNext Enzymatic Methyl-seq library preparation system from New England Biolabs (NEB) for our Whole Genome Enzymatic Methylation Sequencing (WGEMS) service. The NEB protocol uses enzymatic conversion of DNA instead of sodium bisulphite conversion. Benefits of this workflow include:

- Superior sensitivity of detection of 5-mC and 5-hmC
- Greater mapping efficiency
- More uniform GC coverage
- Detection of more CpGs with fewer sequence reads
- Uniform dinucleotide distribution
- High-efficiency librarypreparation

Figure 3: Workflow for Whole Genome Enzymatic Methylation Sequencing (WGEMS).

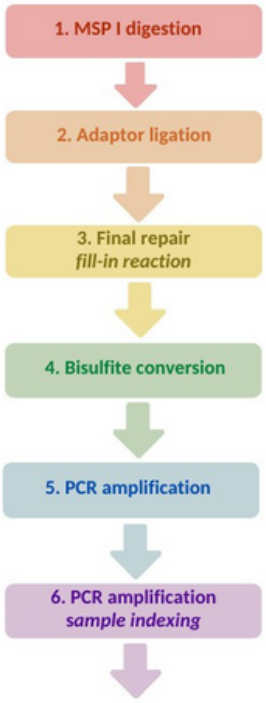


4.0 Reduced Representation Bisulfite Sequencing (RRBS)

Reduced Representation Bisulfite Sequencing (RRBS) provides an option to sequence the CpG rich region of the genome. This is achieved by digesting genomic DNA with the restriction enzyme MspI, which recognises and cleaves the sequence CCGG regardless of the methylation state. Following the digestion with MspI, sequence adapters with complementary overhang are ligated to the DNA, followed by bisulfite conversion. MspI digestion greatly reduces the proportion of the genome sequenced, while enriching for CpG-rich regions.

AGRF employs the Ovation RRBS Methyl-Seq system from NuGEN for our RRBS service. This protocol includes novel sequencing adapters to mitigate the challenges inherent with RRBS protocols, maximising data yield on Illumina sequencing platforms.

Figure 4: Workflow for Reduced Representation Bisulfite Sequencing (RRBS).



5.0 Sequencing Recommendations for Methyl-Seq

5.1 Whole Genome Enzymatic Methylation Sequencing (WGEMS)

WGEMS will provide complete coverage of the genome. A full DNA methylome should have at least 30x coverage of the genome. Due to strand specificity of methylation sequencing data, 30x coverage is equivalent to 15x per strand of the genome. Paired-end sequencing with 150bp reads is recommend to maximise genome coverage.

5.2 Reduced Representation Bisulfite Sequencing (RRBS)

Reduced Representation Bisulfite Sequencing (RRBS) enriches for CpG rich regions and greatly reduces the representation of the genome. Generally AGRF recommends a sequencing depth of 30 million reads (100bp single read) to provide high coverage of up to 4 millionCpG loci.

Table 1: General recommendations for sequence depth and the read length for WGEMS and RRBS.

Application	Coverage/Sequence Depth	Read Type	Output for Human
WGEMS	30x	150bp paired-end	110-125Gb
RRBS	30 million reads	100bp single-read	3Gb

6.0 Data Outputs

All Methyl-Seq projects will undergo QC analysis to assess the quality of both the sequencing and library preparation. AGRF then provides untrimmed and unfiltered Illumina sequencing data in FASTQ format.

7.0 Sample Requirements

The standard input for WGEMS and RRBS is high quality total DNA. Our recommendations for isolation of DNA are listed below:

- Please ensure the extraction protocol includes an RNase treatment to ensure removal of RNA.
- Purified DNA should be eluted/resuspended in 10mM Tris-Cl, pH 8.5 or nuclease-free water.

7.1 Sample Quality

Sample quality is a key factor for successful NGS experiments. AGRF will perform quality control prior to commencing a project, however we recommend you check the DNA before submission.

7.2 Sample Purity

Purity of nucleic acid samples can be assessed by measuring the absorbance spectra via spectrophotometer (e.g. Nanodrop). The ratio of absorbance values 260nm and 280nm or 230nm provide estimates of sample purity or the presence of common contaminants. Purified DNA is expected to have a A260/280 ratio of ~1.8.

Table 2: Recommendation for sample purity as assessed by absorbance spectra.

Ratio	Target	Low Ratio (<1.6) indications
A260/280	1.8	Residual phenol from extraction or very low concentration of RNA (<10ng/µl)
A260/A230	>1.6	Residual guanidine from the extraction protocol Carryover of carbohydrates (e.g. plant polysaccharides)

7.3 DNA Integrity

DNA Integrity can be assessed by agarose gel electrophoresis. DNA should appear as a clear, high molecular weight band. There should be no indication of RNA contamination (as evident by a faint band toward the bottom of the gel).

7.4 Sample Quantity

Quantification of gDNA by dsDNA assay such as PicoGreen or Qubit is highly recommended. The following table shows how much DNA we require for Methyl-Seq services.

Table 3: Sample Requirements of our Methyl-Seq Service.

Library Type		DNA Quality	DNA Concentration
WGEMS	Whole Genome Enzymatic Methylation Sequencing	≥200ng	≥10ng/µl
RRBS	Reduced Representation Bisulfite Sequencing	>500ng	≥20ng/µl

8.0 Sample Returns/Discards

Samples are stored with AGRF for 3 months after you receive your data except for our clinical WGS and clinical exome services which are stored for 1 year. If you wish for your samples to be returned, you must discuss this with your account manager during quoting or contact us after you receive your data. At the completion of your project, we can either:

- Return your samples by courier at ambient (please ask your account manager for a quote).
- Return samples by courier with dry ice (please ask your account manager for a quote).

If we are not notified within the specified time frame, samples will be automatically discarded.

9.0 Sample Submission

9.1 Online Submission

- In the client portal, select 'Next Generation Sequencing' from the service dropdown menu.
- Enter your species and submission format (tube or plate).
- Complete and upload the template file.
- ≤23 please complete tube submissions.
- ≥24 Please complete plate submissions, (an additional handling charge per sample will occur if tubes are used).
- We recommend shipping plates that are heat-sealed, or strip-cap sealed on dry ice.

Submit the form and print the submission receipt to be included with your sample package.

9.2 Packaging of Samples

- DNA samples can be sent at ambient temperature or on ice blocks.
- If you are sending your samples in plates, please use strip caps to seal the plates.
- Sample tubes or plates should be in a zip-lock bag or box to avoid direct contact with dry ice.

AGRF can organise dry ice shipment for your samples as part of your quoted services or you can use our free shipping between nodes once a week service. For information on this service go to Free Shipping.

Post/send/deliver samples to the addresses below:

Physical address(courier)
ATTN NEXT GENERATION SEQUENCING AGRF
VCCC LOADING DOCK 14 FLEMINGTON ROAD
NORTH MELBOURNE, VIC 3051

Postal Address (mail)
ATTN NEXT GENERATION SEQUENCING AGRF
LEVEL 13, VICTORIAN COMPREHENSIVE CANCER
CENTRE 305 GRATTAN STREET
Melbourne, VIC 3000

10.0 Quality Statement

Non-clinical works are performed following the strict requirements of ISO17025: 2005. AGRF Ltd is accredited in the field of Biological Testing (Scope: DNA Analysis) according to the ISO17025: 2005 standard by the National Association of Testing Authorities (NATA). Staff and analysis processes follow Standard Operating Procedures, which define responsibilities and quality checks to achieve reported standards. Compliance is monitored at regular reviews and during internal audits. All work is supervised by a person with relevant qualifications and is checked while in progress and upon completion to ensure that it meets the necessary ISO17025: 2005 standards.