Methylation data analysis

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Methylation technologies

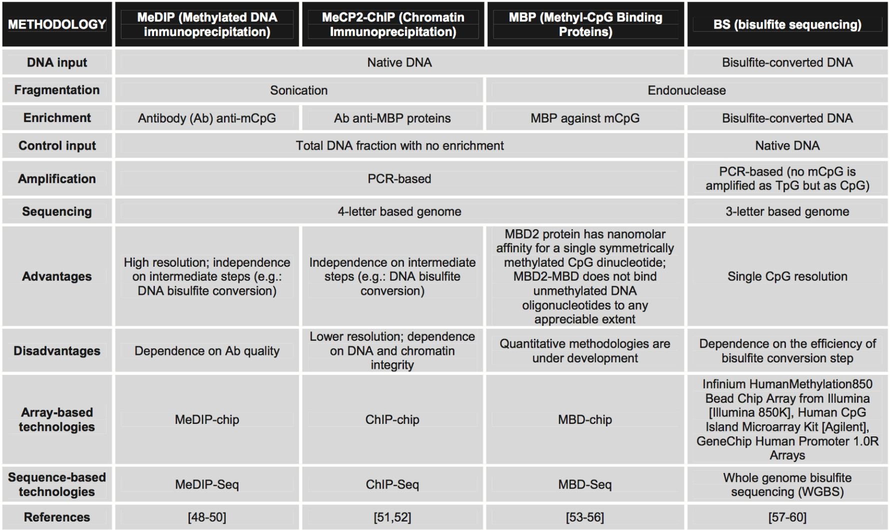
Three categories:

1. Methylation-specific enzyme digestion
2. Affinity enrichment
3. Chemical treatment with bisulphite (BS)

Techniques have been used in combination (e.g., enzyme digestion then BS, commonly known as RRBS)

Differ by cost, resolution, scalability, amount of starting DNA

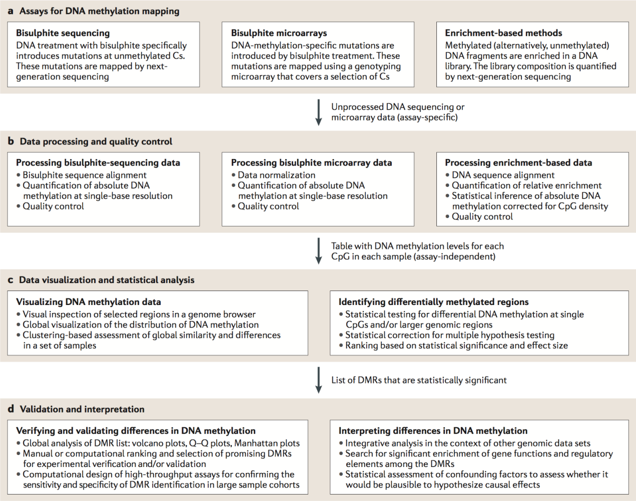
High-throughput DNA methylation techniques



METHODOLOGY	MeDIP (Methylated DNA immunoprecipitation)	MeCP2-ChIP (Chromatin Immunoprecipitation)	MBP (Methyl-CpG Binding Proteins)	BS (bisulfite sequencing)
DNA input	Native DNA			Bisulfite-converted DNA
Fragmentation	Sonication		Endonuclease	
Enrichment	Antibody (Ab) anti-mCpG	Ab anti-MBP proteins	MBP against mCpG	Bisulfite-converted DNA
Control input	Total DNA fraction with no enrichment			Native DNA
Amplification	PCR-based			PCR-based (no mCpG is amplified as TpG but as CpG)
Sequencing	4-letter based genome			3-letter based genome
Advantages	High resolution; independence on intermediate steps (e.g.: DNA bisulfite conversion)	Independence on intermediate steps (e.g.: DNA bisulfite conversion)	MBD2 protein has nanomolar affinity for a single symmetrically methylated CpG dinucleotide; MBD2-MBD does not bind unmethylated DNA oligonucleotides to any appreciable extent	Single CpG resolution
Disadvantages	Dependence on Ab quality	Lower resolution; dependence on DNA and chromatin integrity	Quantitative methodologies are under development	Dependence on the efficiency of bisulfite conversion step
Array-based technologies	MeDIP-chip	ChIP-chip	MBD-chip	Infinium HumanMethylation850 Bead Chip Array from Illumina [Illumina 850K], Human CpG Island Microarray Kit [Agilent], GeneChip Human Promoter 1.0R Arrays
Sequence-based technologies	MeDIP-Seq	ChIP-Seq	MBD-Seq	Whole genome bisulfite sequencing (WGBS)
References	[48-50]	[51,52]	[53-56]	[57-60]

https://academic.oup.com/bfg/article/doi/10.1093/bfgp/elx018/4082035/Epigenetic-regulation-of-gene-expression-in-cancer

DNA methylation analysis methods



a Assays for DNA methylation mapping

Bisulphite sequencing
DNA treatment with bisulphite specifically introduces mutations at unmethylated Cs. These mutations are mapped by next-generation sequencing

Bisulphite microarrays
DNA-methylation-specific mutations are introduced by bisulphite treatment. These mutations are mapped using a genotyping microarray that covers a selection of Cs

Enrichment-based methods
Methylated (alternatively, unmethylated) DNA fragments are enriched in a DNA library. The library composition is quantified by next-generation sequencing

Unprocessed DNA sequencing or microarray data (assay-specific)

b Data processing and quality control

Processing bisulphite-sequencing data
- Bisulphite sequence alignment
- Quantification of absolute DNA methylation at single-base resolution
- Quality control

Processing bisulphite microarray data
- Data normalization
- Quantification of absolute DNA methylation at single-base resolution
- Quality control

Processing enrichment-based data
- DNA sequence alignment
- Quantification of relative enrichment
- Statistical inference of absolute DNA methylation corrected for CpG density
- Quality control

Table with DNA methylation levels for each CpG in each sample (assay-independent)

c Data visualization and statistical analysis

Visualizing DNA methylation data
- Visual inspection of selected regions in a genome browser
- Global visualization of the distribution of DNA methylation
- Clustering-based assessment of global similarity and differences in a set of samples

Identifying differentially methylated regions
- Statistical testing for differential DNA methylation at single CpGs and/or larger genomic regions
- Statistical correction for multiple hypothesis testing
- Ranking based on statistical significance and effect size

List of DMRs that are statistically significant

d Validation and interpretation

Verifying and validating differences in DNA methylation
- Global analysis of DMR list: volcano plots, Q–Q plots, Manhattan plots
- Manual or computational ranking and selection of promising DMRs for experimental verification and/or validation
- Computational design of high-throughput assays for confirming the sensitivity and specificity of DMR identification in large sample cohorts

Interpreting differences in DNA methylation
- Integrative analysis in the context of other genomic data sets
- Search for significant enrichment of gene functions and regulatory elements among the DMRs
- Statistical assessment of confounding factors to assess whether it would be plausible to hypothesize causal effects

www.nature.com/doifinder/10.1038/nbt.1681

Methylation assays

Sensitivity of restriction enzymes for methylated CpG sites

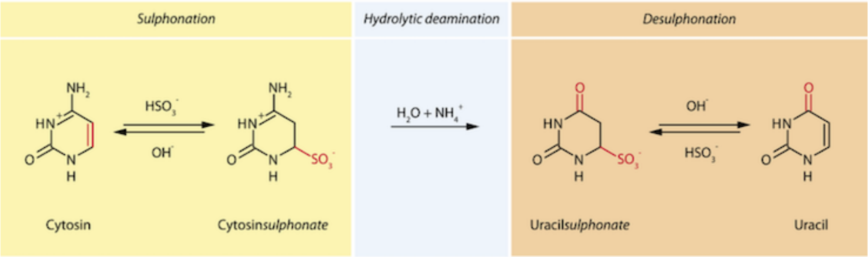
MeDIP (Methylated DNA immuno-precipitation) - capture based, same as ChIP-seq, but uses antibody against methylated DNA

- Anti-methylcytidine Ab to Me-C => ChIP–chip or ChIP-seq
- Analysis methods are the same as ChIP-seq
- Resolution is low: can roughly quantify the amount of DNA methylation in a few hundred bps.

Sodium Bisulfite conversion

- Modifies non-methylated cytosines to uracil (methylation is protective from conversion)
- Differentiation of methylated and non-methylated cytosines at base-pair resolution
- $C \rightarrow U$ - which reads as **T** during sequencing
- $C^M \rightarrow C$ - which reads as **C** during sequencing

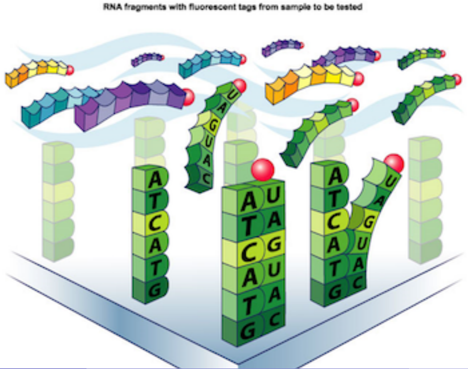
Bisulfite-mediated conversion of cytosine to uracil



Tollefsbol T (ed.): Handbook of Epigenetics: The New Molecular and Medical Genetics. 1st edition. London, San Diego: Academic Press, 2011.

Bisulfite conversion-based Microarray Analysis

- A DNA microarray is a technology that consists of thousands of spots with DNA oligonucleotides (probes) that are used to hybridize a target sequence.
- Probe-target hybridization is usually detected and quantified by detection of fluorophore-, or chemiluminescence-labeled targets.


RNA fragments with fluorescent tags from sample to be tested
A
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C
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T
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U
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A
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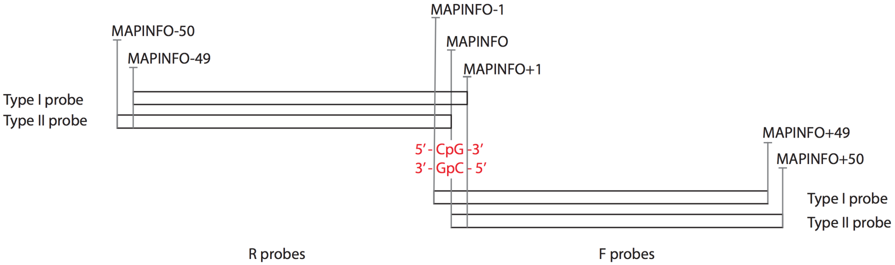
C

Illumina Infinium methylation assay

- Unmethylated **cytosines** are chemically deaminated to **uracil** in the presence of bisulfite.
- Methylated cytosines are refractory to the effects of bisulfite and remain cytosine.
- After bisulfite conversion, each sample is whole-genome amplified (WGA) and enzymatically fragmented.
- The bisulfite-converted WGA-DNA samples is purified and applied to the BeadChips.

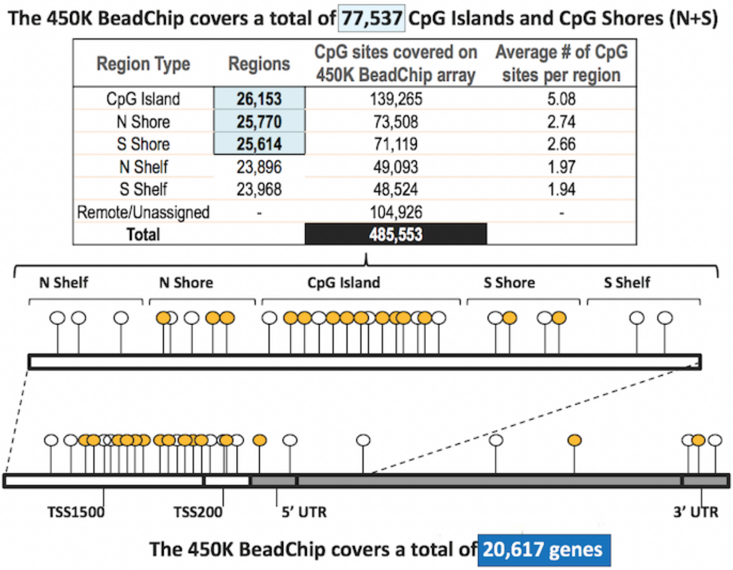
Illumina Infinium methylation assay

- Bead technology
- Each bead has oligos containing 23-base address + 100-base probe complementary to bisulfite converted DNA with the CpG site in the center



Illumina Infinium evolution

- 2008: **HumanMethylation27K**. 25,578 probes targeting CpG sites within the proximal promoter regions.
- 2011: **HumanMethylation450K**. 485,577 probes targeting additional CpG islands, shores and shelves, the 5' and 3' UTRs, gene bodies, some enhancer regions. Covers 99% of RefSeq genes.
- 2015: **MethylationEPIC**. >850,000 probes. Additional cooverage of regulatory elements. 58% of FANTOM5 enhancers, 7% distal and 27% proximal ENCODE regulatory elements.



The 450K BeadChip covers a total of 77,537 CpG Islands and CpG Shores (N+S)

Region Type	Regions	CpG sites covered on 450K BeadChip array	Average # of CpG sites per region
CpG Island	**26,153**	139,265	5.08
N Shore	**25,770**	73,508	2.74
S Shore	**25,614**	71,119	2.66
N Shelf	23,896	49,093	1.97
S Shelf	23,968	48,524	1.94
Remote/Unassigned	-	104,926	-
Total		**485,553**	

The 450K BeadChip covers a total of 20,617 genes

Measurement of methylation level

Illumina 450K and 850K use two types of probes:

- **Type I probes** have two separate probe sequences per CpG site (one each for methylated and unmethylated CpGs). ~28% of probes. Suggested to be more stable and reproducible than the Type II probes
- **Type II probes** have just one probe sequence per CpG site. Use half of the physical space. ~ 72% of probes. Have a decreased quantitative dynamic range compared to Type I probes.

Measurement of methylation level

Beta-value - bimodal distribution within [0,1] range

$$\beta = \frac{M}{U + M}$$

- M - signal from methylated probes
- U - signal from unmethylated probes

$\beta = 0/1$ - all probes are non-methylated/fully methylated, respectively

Measurement of methylation level

Beta-value - bimodal distribution within [0,1] range

$$\beta = \frac{M}{U + M}$$

- M - signal from methylated probes
- U - signal from unmethylated probes

M-value - centered around 0, $[-\infty, +\infty]$ range

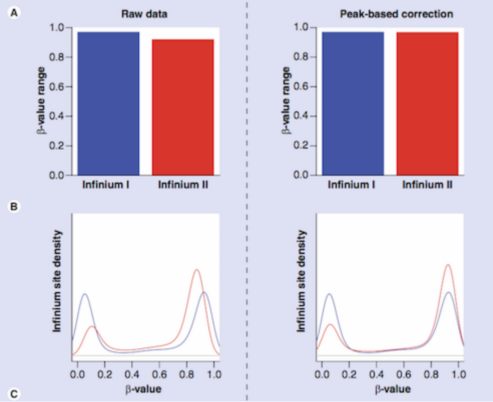
$$Mvalue = log\left(\frac{M}{U}\right) = log\left(\frac{\beta}{1-\beta}\right)$$

$M = -\infty$ - all probes are non-methylated

$M = +\infty$ - all probes are methylated

Measurement of methylation level

- β values obtained from Infinium II probes are slightly less accurate and reproducible than those obtained from Infinium I probes (Dedeurwaerder et.al. 2011)
- Peak correction methods (normalization) are available



Filter questionable probes

- Remove probes that have failed to hybridize (detection p-value)
  - Detection p-value represents the probability the target signal was distinguishable against background noise
- Drop probes that failed in n^{th} percent of samples
  - Common thresholds are 20%, 10%, 5% of probes at $>$0.05, $>$0.01
- Drop samples that failed in n^{th} percent of probes
  - Common thresholds are 50%, 20% at $>$0.05, $>$0.01

Filter questionable probes

- Probes on X and Y chromosomes
- Probes with lowest variation
- Probes with extreme methylation level (e.g. median $= 0\%$ or 100%)
- Keep only those in regions of interest (e.g. CpG islands, shores)

Filter questionable probes

- A list of potential nonspecific probes and polymorphic probes of Illumina Human 27k Methylation Array, http://braincloud.jhmi.edu/NonspecificAndPolymorphic.zip
- Data from Chen YA, et.al. "Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray." Epigenetics.
    - List of non-specific probes - 29,233 non-specific 'cg' probes, 1,736 non-specific 'ch' probes;
    - List of polymorphic CpGs - 70,899 records (66,877 unique probes) about CpGs containing SNPs at or near single base extension (SBE) position, 316,034 records (220,582 unique probes) having SNPs in probe sequences.
- More for MethylationEPIC at https://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-1066-1

My pipeline

1. Filtering non-specific, polymorphic, SNP, chromosome Y probes
2. Pre-processing and QC
   - `dasen` (background correction and quantile normalization)
   - `BIMQ` (Beta-mixture quantile normalization, correcting batch effect of Infinium I and II chemistries)
   - Principal Components Analysis to detect batch effects
   - `ComBat`, `ISVA` (removing batch effect)
3. Association analysis, or differential methylation
   - `betareg` regression model
   - Pearson correlation coefficient
   - `limma`, `minfi` for differentially methylated tegions
   - Benjamini-Hochberg adjusted p-values < 0.05
4. Functional enrichment analyses

Interpretation

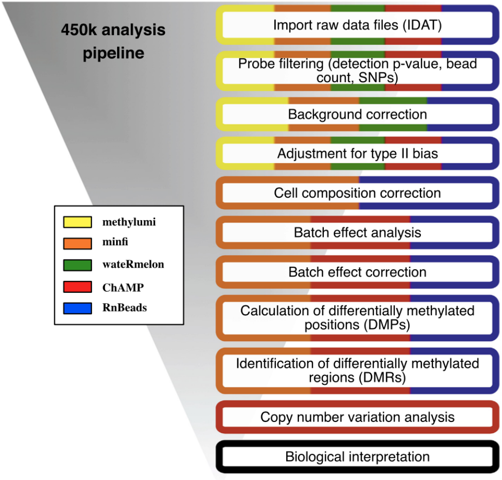
- Map CpG sites of interest to the nearby genes, analyze genes for functional enrichment
- Analyze genomic location of CpG sites, using genomic coordinates
    - **GREAT** predicts functions of cis-regulatory regions, http://bejerano.stanford.edu/great/public/html/
    - **Enrichr**, gene- and genomic regions enrichment analysis tool, http://amp.pharm.mssm.edu/Enrichr/#
    - **GenomeRunner**, Functional interpretation of SNPs (any genomic regions) within regulatory/epigenomic context, http://integrativegenomics.org/

R packages for Illumina Infinium array analysis

- **lumi** - normalization, vusualization, gene annotation https://www.bioconductor.org/packages/release/bioc/html/lumi.html
- **methylumi** - normalization and general data handling http://www.bioconductor.org/packages/release/bioc/html/methylumi.html
- **minfi** - normalization, analysis and visualization http://www.bioconductor.org/packages/release/bioc/html/minfi.html, or **ChAMP** - eight functions to run *minfi* pipelines, https://bioconductor.org/packages/release/bioc/html/ChAMP.html
- **RnBeads** - works for 450K arrays, BS-Seq, MeDIP or MBD-Seq data https://bioconductor.org/packages/release/bioc/html/RnBeads.html
- **wateRmelon** - 15 normalization methods, other QC metrics https://bioconductor.org/packages/release/bioc/html/wateRmelon.html

Morris TJ, Beck S "**Analysis pipelines and packages for Infinium HumanMethylation450 BeadChip (450k) data**" Methods. 2015 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4304832/

R packages for Illumina Infinium array analysis


450k analysis pipeline
Import raw data files (IDAT)
Probe filtering (detection p-value, bead count, SNPs)
Background correction
Adjustment for type II bias
Cell composition correction
Batch effect analysis
Batch effect correction
Calculation of differentially methylated positions (DMPs)
Identification of differentially methylated regions (DMRs)
Copy number variation analysis
Biological interpretation
methylumi
minfi
wateRmelon
ChAMP
RnBeads

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Methylation statistics packages

- **BiSeq** (K. Hebestreit et al.) Beta regression model, impractical for very large data other than RRBS or targeted BS-Seq https://bioconductor.org/packages/release/bioc/html/BiSeq.html
- **bsseq** (K.D. Hansen) Implements the BSmooth smoothing algorithm. Numerous CpG-wise t-tests and p-value cutoff to define DMRs. Outperforms Fisher's exact test. Requires biological replicates for DMR detection https://bioconductor.org/packages/release/bioc/html/bsseq.html
- **DMAP** (P. Stockwell et al.) RRBS fragment or fixed window approach, Fisher's exact test, Chi-squared or ANOVA RADMeth (C++ command line tool by E. Dolzhenko and A.D. Smith) Beta-binomial regression analysis to find DMCs or DMRs, local likelihood, adjust for neighbouring CpGs http://biochem.otago.ac.nz/research/databases-software

Methylation statistics packages, continued

- **DSS** (Feng et al., 2014) Constructs genome-wide prior distribution for beta-binomial dispersion. Bayesian hierarchical model to detect differentially methylated loci https://www.bioconductor.org/packages/3.3/bioc/html/DSS.html
- **methylKit** (A. Akalin et al.) Sliding window, Fisher's exact test or logistic regression. Adjusts p-values to q-values using SLIM method. https://github.com/al2na/methylKit
- **MOABS** (D. Sun et al.) Beta binomial hierarchical model to capture sampling and biological variation, Credible Methylation Difference (CDIF) single metric that combines biological and statistical significance https://code.google.com/archive/p/moabs/
- **methyLiftover** - map bisulfite sequencing data to the Illumina 450K methylation CpG set https://github.com/Christensen-Lab-Dartmouth/methyLiftover