CS-E5875 High-Throughput Bioinformatics
DNA methylation analysis

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Contents

- DNA methylation
- Bisulfite sequencing (BS-seq) protocol
- Alignment and quantification of BS-seq data
- Statistical analysis of BS-seq data
- Other details

DNA methylation: what?

- Epigenetic changes are reversible modifications on DNA, or “on top of DNA”, which do not change the DNA sequence itself
- DNA methylation is an epigenetic modification where methyl group is added to the 5 position of a cytosine in DNA
- Methyl group is added enzymatically by DNA methyl transferases (DNMT)
- By far the most extensively studied epigenetic modification on DNA

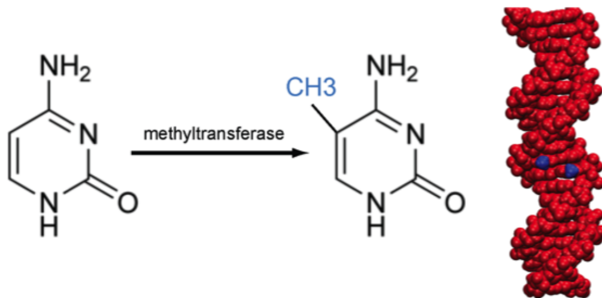

Figure from http://www.ks.uiuc.edu/Research/methylation/

DNA methylation: where?

- In mammaling genomes, DNA methylation primarily occurs in the context of CpG dinucleotides
- Non-CpG methylation found e.g. in stem cells and brain
- CpGs occur with a smaller frequency than expected
  - Human genome GC content is 42%
  - CpGs are expected to occur 4.41% of the time
  - The frequency of CpG dinucleotides is 1%
  - Methylated CpGs are prone to spontaneous deamination to thymines

Figure from (Schubeler, 2009)

DNA methylation: how?

- Two general classes of enzymatic methylation activities
  - De novo methylation (mainly) by DNMT3
  - Maintenance methylation during cell division (mainly) by DNMT1

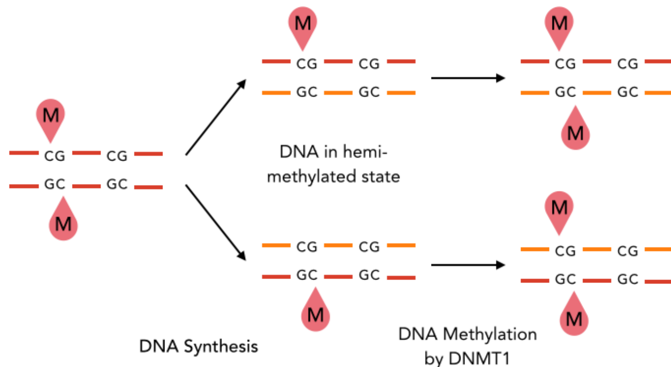

Figure from `http://2014.igem.org/Team:Heidelberg/Project/PCR_2.0`

DNA methylation in gene regulation

- CpG islands (C+G dense $\gtrsim$500 long regions) are present in the 5' regulatory regions of many genes (5' = "beginning")
- Hypermethylation (=overmethylation) of CpG islands near gene promoters contributes to transcriptional silencing by
  - Affecting binding of transcription factors (DNA binding protein that regulate gene transcription)
  - Binding proteins with methyl-CpG-binding domains (MBDs), and recruiting e.g. histone deacetylases and other chromatin remodellers

DNA methylation in gene regulation

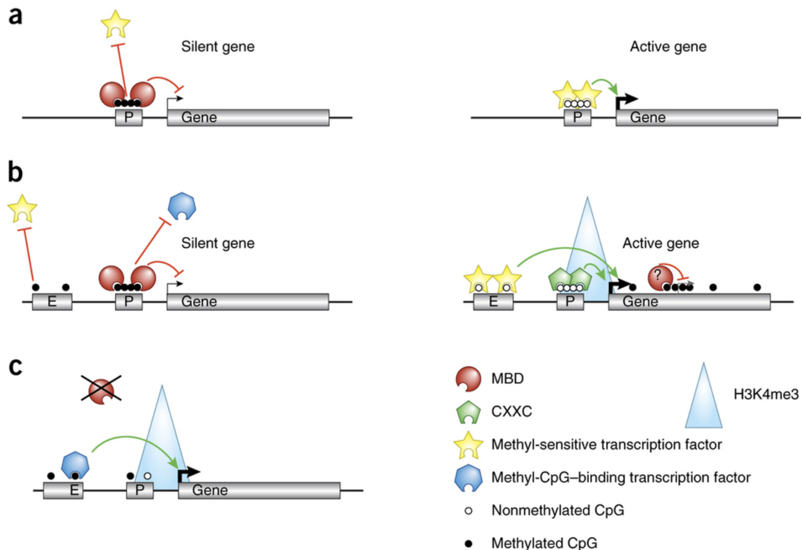

Figure from (Spruijt & Vermeulen, 2014)

DNA methylation in health and disease

- DNA methylation is necessary for normal development, e.g. in
  - Cell differentiation
  - Protection of DNA from transposable elements
  - Genomic imprinting
  - X chromosome inactivation
- Environmental and lifestyle factors may impact DNA methylation, even in-utero
- DNA methylation patterns have been associated with several diseases

Heritability of DNA methylation patterns

- DNA methylation patterns are not directly inheritable
  - Paternal and maternal DNA methylation patterns are almost completely erased during first cell divisions after fertilization in mammals (epigenetic reprogramming)
- However, genomic variants can influence DNA methylation
  - So-called methylation quantitative trait loci (mQTL) effects
- DNA methylation patterns correlate between family members to some extent
  - For example in genome-wide human leukocyte DNA methylation profiles, correlation coefficients between 0.24 and 0.30 have been reported between first-degree relatives, compared to 0.03 between unrelated individuals (Tremblay et al. 2016)
  - These seem to be explained by both genetic inheritance and environmental factors

DNA demethylation

- Until recently, it was believed that methylated DNA can be unmethylated only by dilution during cell differentiation/DNA replication, i.e., incomplete DNA maintenance methylation
- Recently, TET family proteins were shown to be dioxygenases that converted 5mC to 5hmC, 5fC and 5caC, which can be further converted back to unmethylated C
- TETs thus contribute to active demethylation, but 5hmC, 5fC and 5caC can also have multiple functions

DNA demethylation

Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1

Mamta Tahiliani,[1] Kian Peng Koh,[1] Yinghua Shen,[2] William A. Pastor,[1] Hozefa Bandukwala,[1] Yevgeny Brudno,[2] Suneet Agarwal,[3] Lakshminarayan M. Iyer,[4] David R. Liu,[2]* L. Aravind,[4]* Anjana Rao[1]*

15 MAY 2009 VOL 324 SCIENCE www.sciencemag.org

a

Unknown decarboxylase?

TDG and BER

DNMT enzymes?

NH_2 / N / O / N / R — Cytosine

DNMT

NH_2 / N / O / N / R — 5mC

TET protein

NH_2 / OH / N / O / N / R — 5hmC

TET protein

NH_2 / O / N / O / N / R — 5fC

TET protein

NH_2 / O / OH / N / O / N / R — 5caC

AID or APOBEC?

O / OH / HN / O / N / R — 5hmU

TDG or SMUG1 and BER

Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine

Shinsuke Ito,[1,2]* Li Shen,[1,2]* Qing Dai,[3] Susan C. Wu,[1,2] Leonard B. Collins,[4] James A. Swenberg,[2,4] Chuan He,[3] Yi Zhang[1,2]†

2 SEPTEMBER 2011 VOL 333 SCIENCE www.sciencemag.org

BER := base excision repair
TDG := thymine DNA glycosylase
AID := activation-induced deaminase
APOBEC := apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

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Bisulfite sequencing (BS-seq) protocol

- Bisulfite treatment of genomic DNA converts unmethylated cytosines to uracils which are read as thymine during sequencing
- Methylated (and hydroxymethylated) cytosines are resistant to the conversion and are read as cytosine

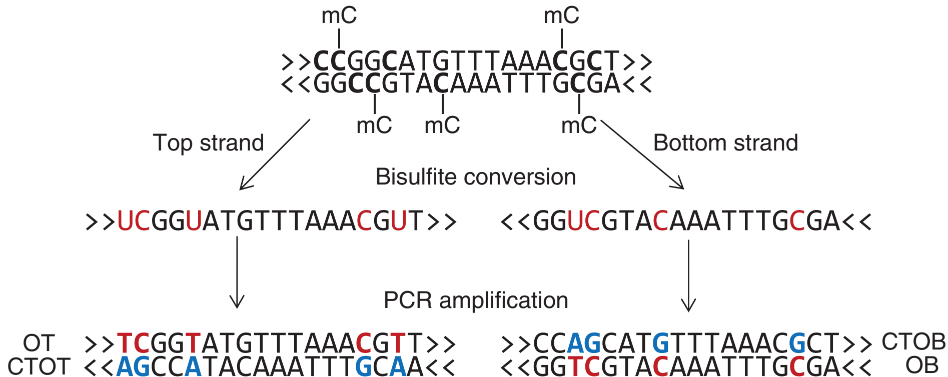

Figure from (Krueger et al, 2012)

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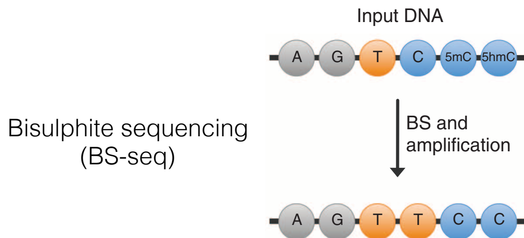

Cytosine modification

	C	5mC	5hmC
BS-seq	T	C	C

Figure from (Booth et al, 2012)

Reduced representation BS-seq (RRBS-seq)

- BS-seq provides an accurate map of methylation state at single nucleotide resolution
- Whole genome analysis is expensive since only about 1% of the human genome contains CpGs
- Reduced representation BS-seq (RRBS-seq) uses restriction enzymes prior to bisulfite sequencing focus on CpG regions
  - MspI digests genomic DNA in a methylation-insensitive manner
  - MspI targets 5'CCGG3' sequences and cleaves the phosphodiester bonds upstream of CpG dinucleotide.
  - → Each fragment will have a CpG at each end
- RRBS-seq will cover majority of promoters and GC rich regions

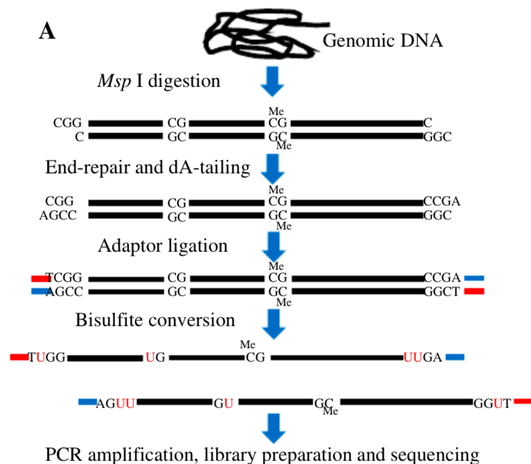

Figure from (Lianga et al, 2014)

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RRBS data analysis workflow example

1. RRBS data preprocessing

- TrimGalore (trimming of low-quality bases, end repair biases, adapter sequences, and overlaps between paired-end reads), fastQC before and after TrimGalore
- Bismark-alignment
- Bismark methylation extractor to extract numbers of methylated and unmethylated reads at each CpG site in each sample
- Excluding of samples that have low bisulfite conversion efficiency (estimated as proportion of Cs converted to Ts in the completely unmethylated lambda phage genome)
- Trimming of M-biases

2. SNP detection (e.g. bis-SNP or bs-SNPer)

- SNPs removed from each sample

3. Coverage filtering

- CpG sites with extremely high coverages within each sample removed to avoid PCR duplicates
- Low-coverage CpG sites, e.g. with coverage < 10 reads in more than 50 % of the samples removed

4. Differential methylation analysis

- E.g. beta-binomial models are suitable for read count data:

$$m \sim \mathrm{Bin}(p,n)$$
$$p \sim \mathrm{Beta}(\alpha, \beta)$$

5. Interpretation

- Annotation to genomic parts, nearest genes, regulatory regions
- Known mQTL and eQTM effects

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Aligning BS-seq reads

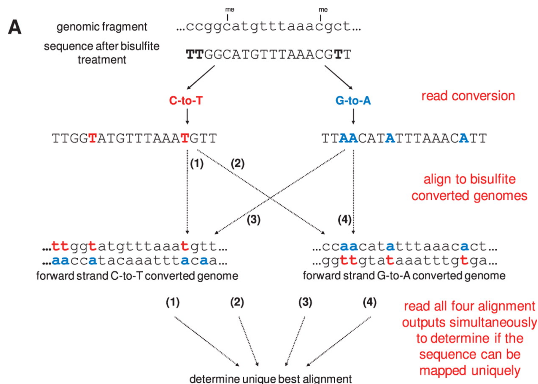
Bisulfite treatment introduces mutations into genomic DNA in a methylation dependent manner

- Alignment of BS-seq reads is more challenging
- Standard alignment methods cannot be used directly

Bismark tool uses the following approach to map BS-seq reads

- Reads from a BS-seq experiment are converted into a C-to-T version and a G-to-A version
- The same conversion for the genome
- Bowtie alignment in the genome that has reduced complexity
- A unique best alignment is determined from four parallel alignment processes (see next page)

Bismark tool



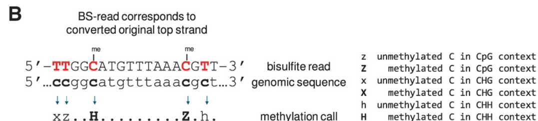


Figure from (Krueger & Andrews, 2011)

Quantifying BS-seq data

- Bismark outputs, among others, one line per read containing useful information
  - Mapping position, alignment strand, the bisulfite read sequence, its equivalent genomic sequence and a methylation call string
- Bismark automatically extracts the methylation information at individual cytosine positions
  - For different sequence contexts (CpG, CHG, CHH; where H can be either A, T or C)
  - Strand-specific or strands merged
- That is, for each cytosine Bismark outputs
  - n_i the number of reads covering the cytosine in sample i
  - m_i the number of methylated readouts (i.e., "C") for the cytosine in sample i
- One way to quantify methylation proportion is

$$\hat{p}_i = \frac{m_i}{n_i} = \frac{\text{the number of C reads overlapping the cytosine}}{\text{the number of C or T reads overlapping the cytosine}}$$

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Beta-binomial model

- At the end, one is typically interested in testing a hypothesis, e.g. is there a statistically significant difference in methylation levels between group A and group B
- Some early methods applied e.g. the t-test on the estimated methylation fractions $\hat{p}_i$ (or their logit transformations)
- We will look at RadMeth tool (Dolzhenko and Smith, 2014)
- RadMeth uses the beta-binomial regression model, where beta-binomial is a compound distribution obtained from the binomial by assuming that its probability of success parameter follows a beta distribution

Beta-binomial model

$i = 1, \ldots, s$, where s is the number of biological samples
For each cytosine in the genome we have the following model

- n_i: the number of reads that contain "C" or "T" readout at the cytosine in sample i
- m_i: the number of reads that contain "C" readout (i.e. methylated) at the cytosine in sample i ($0 \leq m_i \leq n_i$)
- If we knew the underlying methylation proportion p_i, then: $M_i \sim \mathrm{Binom}(p_i, n_i)$

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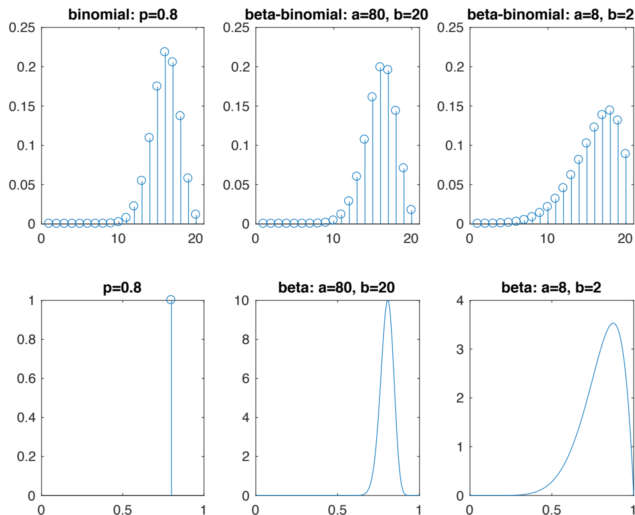
- If we knew the underlying methylation proportion p_i, then: $M_i \sim \mathrm{Binom}(p_i, n_i)$
- p_i: the unknown methylation level of the cytosine in sample i
- Instead of assuming a fixed (unknown) methylation level, assume p_i has a compounding distribution $p_i \sim \mathrm{Beta}(\alpha, \beta),\ \alpha \geq 0, \beta \geq 0$
- The probability of observing methylation level $M_i = m_i$ for a coverage n_i follows so called beta-binomial model $\mathrm{BetaBinomial}(n_i, \alpha, \beta)$

$$\begin{aligned} P(M_i = m_i | n_i, \alpha, \beta) &= \int_0^1 \mathrm{Binom}(m_i | p_i, n_i) \mathrm{Beta}(p_i | \alpha, \beta) \mathrm{d} p_i \\ &= \binom{n_i}{m_i} \frac{\mathrm{B}(m_i + \alpha, n_i - m_i + \beta)}{\mathrm{B}(\alpha, \beta)}, \end{aligned}$$

where B is the beta function

Beta-binomial model

- An illustration of binomial / beta / beta-binomial densities



Binomial and beta-binomial densities

Beta-binomial model

- Mean and variance of the beta-binomial random variable are

$$\mu = \frac{n_i\alpha}{\alpha+\beta} \quad \text{and} \quad \sigma^2 = \frac{n_i\alpha\beta(\alpha+\beta+n_i)}{(\alpha+\beta)^2(\alpha+\beta+1)}$$

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- Reparameterization
  - $\pi = \frac{\alpha}{\alpha+\beta}$ is the the average methylation level of a set of replicate samples
  - $\gamma = \frac{1}{\alpha+\beta+1}$ is the common dispersion parameter

Beta-binomial model

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- Reparameterization
  - $\pi = \frac{\alpha}{\alpha+\beta}$ is the the average methylation level of a set of replicate samples
  - $\gamma = \frac{1}{\alpha+\beta+1}$ is the common dispersion parameter
- This allows us to write the original model $\mathrm{BetaBinomial}(n_i, \alpha, \beta)$ equivalently as

$$M_i \sim \mathrm{BetaBinomial}(n_i, \pi, \gamma)$$

  where the mean and the variance are now defined as
  - $\mathrm{E}(M_i) = n_i \pi$
  - $\mathrm{Var}(M_i) = n_i \pi (1 - \pi)(1 + (n_i - 1)\gamma)$
- That is, given π and γ, solve for α and β, and evaluate pdf using $\mathrm{BetaBinomial}(n_i, \alpha, \beta)$
- Recall that the variance of the binomial distribution is $n_i \pi (1 - \pi)$ which is smaller than $\mathrm{Var}(M_i)$ for $n_i \geq 2$

GLM with beta-binomial likelihood

- In most of the real world applications, methylation levels can be confounded by one or more factors (e.g. age and smoking)
- The generalized linear model (GLM) generalizes the linear regression to allow for response variables that have likelihood models other than a normal distribution

GLM with beta-binomial likelihood

- For each sample i (and for each cytosine), the mean methylation level π_i depends on covariates $\mathbf{x}_i = (x_{i1}, x_{i2}, \ldots, x_{ip})^T$

$$g(\pi_i) = \sum_{j=1}^{p} x_{ij}\beta_j = \mathbf{x}_i^T \boldsymbol{\beta}$$

where $\boldsymbol{\beta}$ is a $p \times 1$ parameter vector and

$$\begin{aligned} g(\pi) &= \mathrm{logit}(\pi) = \log\left(\frac{\pi}{1-\pi}\right) \\ \pi_i &= \mathrm{logit}^{-1}(\mathbf{x}_i^T \boldsymbol{\beta}) = \mathrm{logistic}(\mathbf{x}_i^T \boldsymbol{\beta}) = \frac{\exp(\mathbf{x}_i^T \boldsymbol{\beta})}{\exp(\mathbf{x}_i^T \boldsymbol{\beta}) + 1} \end{aligned}$$

- $\mathrm{logit}(\cdot) :]0,1[\to \mathbb{R}$, thus $\mathrm{logit}(\cdot)^{-1} : \mathbb{R} \to]0,1[$

Model fitting and inference

- The beta-binomial regression is fit separately for each CpG site
- The parameters $\boldsymbol{\beta}$ and γ are estimated using maximum likelihood
  - E.g. iteratively reweighted least squares algorithm using a Newton-Raphson method
- Test the differential methylation w.r.t. a covariate x_j with parameter β_j:
  - Null hypothesis: $\beta_j = 0$
  - Estimate the full model, and the reduced model without the covariate x_j
  - Compare the models using log-likelihood ratio test

$$D = -2 \ln \left(\frac{\text{likelihood of the reduced model}}{\text{likelihood of the full model}} \right)$$

- p-value from chi-square test with $d_{full} - d_{reduced}$ degrees of freedom, where d_{full} denotes the number of free parameters in the full model
- Remember multiple testing!
- Neighbouring CpGs that are detected to be statistical significant can be combined to form differentially methylated regions (DMRs)

RadMeth application

- Neuron and non-neuron RRBS-seq samples from mouse frontal cortex: $x_{i1} \in \{0, 1\}$
- 6 samples: $s = 6$
- Two additional factors: age ($x_{i2} \in \mathbb{R}_+$), sex ($x_{i3} \in \{0, 1\}$)
- 72 000 differentially methylated regions (DMRs) between neuron and non-neuron samples that contain at least 10 CpGs
- DMRs with minimum methylation difference above 0.55
  - 1708 lowly methylated (active) regions in neurons
  - These regions are associated with (located close to) 1089 genes
  - GO enrichment analysis by DAVID found a strong association of these genes with various aspects of neuronal development and function

RadMeth application

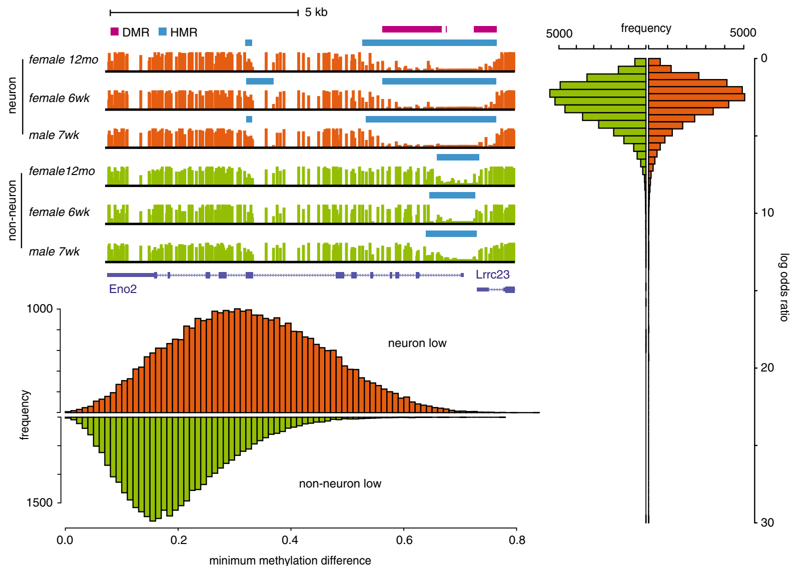

Figure from (Dolzhenko and Andrew, 2014)

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1. Preprocessing

MspI digest

5′ CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNC −3′
3′ − CNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC −5′

end repair + A-tailing

5′ − CGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGA −3′
3′ − AGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGC −5′

adapter ligation (X = adapter sequence)

5′ −XXXXXXXTCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGAXXXXXXXXXX−3′ OT
3′ −XXXXXXXAGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCTXXXXXXXXXX−5′ OB

bisulfite treatment

PCR amplification of the OT strand

5′ −XXXXXXXTCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGAXXXXXXXXXX−3′ OT
3′ −XXXXXXXAGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCTXXXXXXXXXX−5′ CTOT

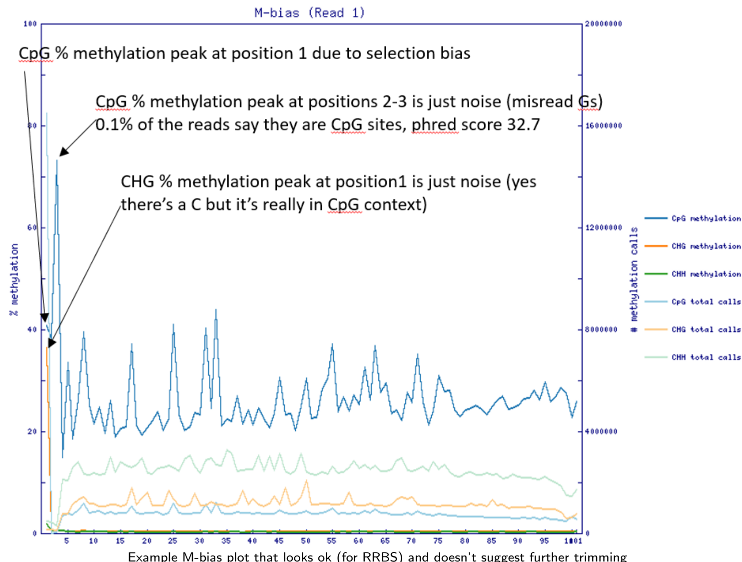
PCR amplification of the OB strand

5′ −XXXXXXXTCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCGAXXXXXXXXXX−3′ CTOB
3′ −XXXXXXXAGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCTXXXXXXXXXX−5′ OB

Figure from (Krueger 2013).

- TrimGalore: trimming of
  - Low-quality bases
  - End repair biases
  - Adapter sequences
  - overlaps between paired-end reads

1. Preprocessing (steps after alignment and methylation extractor)



Example M-bias plot that looks ok (for RRBS) and doesn't suggest further trimming

- Excluding of samples that have low bisulfite conversion efficiency (estimated as proportion of Cs converted to Ts in the completely unmethylated lambda phage genome)
- Trimming of M-biases

2. SNP detection

- Possible effects of SNPs (Liu et al. 2012):
  - C to T SNPs at methylation sites might be misinterpreted as partially (50%) or completely unmethylated Cs

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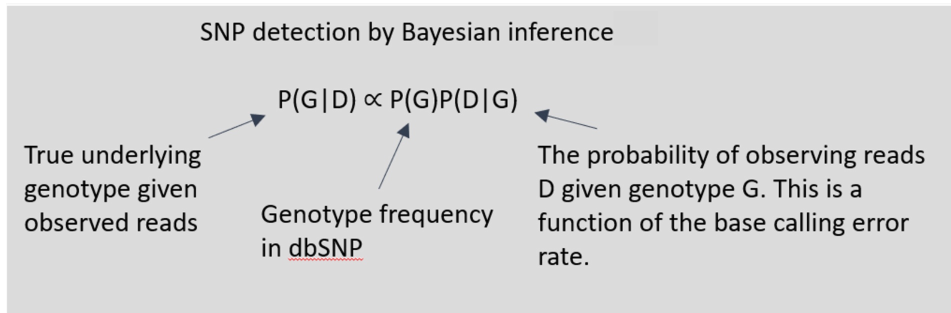
- Possible effects of SNPs (Liu et al. 2012):
  - C to T SNPs at methylation sites might be misinterpreted as partially (50%) or completely unmethylated Cs
- SNP detection: Most bisulfite sequencing protocols only convert unmethylated C, while G on the opposing strand remains unchanged

$\rightarrow$ Detect C to T mutations using the reads from the opposite strand



3. Coverage filtering

- CpG sites with extremely high coverages within each sample removed to avoid PCR duplicates in RRBS (although some number of overlaps are expected)
- For example, low-coverage CpG sites, i.e. with coverage < 10 reads in more than 50 % of the samples removed

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5. Interpretation after the differential methylation analysis

Could the differentially methylated sites/regions have an impact on the expression of some gene?

- Nearest genes
  - E.g. some GO terms enriched in the nearest genes?
- Genomic parts: intron/exon/promoter/intergenic
  - R package genomation

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- Enhancer regions
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  - TRANSFAC (a manually curated database of transcription factor binding sites)

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- Transcription factor binding sites
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- mQTL and eQTM
  - For human whole blood e.g. GoDMC mQTLdb (mqtldb.godmc.org.uk) or BIOS QTL browser (genenetwork.nl)

mQTL (methylation quantitative trait loci) and eQTM (expression quantitative trait methylation)

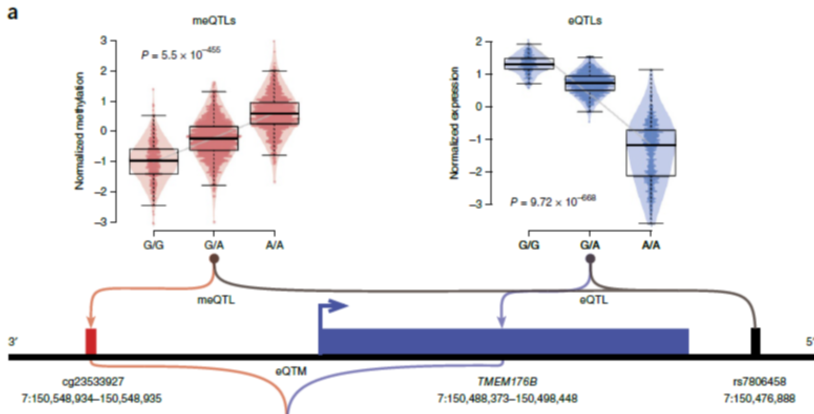

Figure from (Bonder et al. 2017)

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