Differential methylation analysis of reduced representation bisulfite sequencing experiments using edgeR [version 1; referees: 2 approved, 1 approved with reservations]

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Abstract

Studies in epigenetics have shown that DNA methylation is a key factor in regulating gene expression. Aberrant DNA methylation is often associated with DNA instability, which could lead to development of diseases such as cancer. DNA methylation typically occurs in CpG context. When located in a gene promoter, DNA methylation often acts to repress transcription and gene expression. The most commonly used technology of studying DNA methylation is bisulfite sequencing (BS-seq), which can be used to measure genomewide methylation levels on the single-nucleotide scale. Notably, BS-seq can also be combined with enrichment strategies, such as reduced representation bisulfite sequencing (RRBS), to target CpG-rich regions in order to save per-sample costs. A typical DNA methylation analysis involves identifying differentially methylated regions (DMRs) between different experimental conditions. Many statistical methods have been developed for finding DMRs in BS-seq data. In this workflow, we propose a novel approach of detecting DMRs using edgeR. By providing a complete analysis of RRBS profiles of epithelial populations in the mouse mammary gland, we will demonstrate that differential methylation analyses can be fit into the existing pipelines specifically designed for RNA-seq differential expression studies. In addition, the edgeR generalized linear model framework offers great flexibilities for complex experimental design, while still accounting for the biological variability. The analysis approach illustrated in this article can be applied to any BS-seq data that includes some replication, but it is especially appropriate for RRBS data with small numbers of biological replicates.

Keywords

Methylation, BS-seq, differential methylation analysis, Bioconductor
This article is included in the Bioconductor gateway.

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Introduction

Studies in the past have shown that DNA methylation, as an important epigenetic factor, plays a vital role in genomic imprinting, X-chromosome inactivation and regulation of gene expression. Aberrant DNA methylation is often correlated with DNA instability, which leads to development of diseases including imprinting disorders and cancer.

In mammals, DNA methylation almost exclusively occurs at CpG sites, i.e. regions of DNA where a cytosine (C) is linked by a phosphate (p) and bond to a guanine (G) in the nucleotide sequence from 5' to 3'. It has been found that 70% - 80% of CpG cytosines are methylated in mammals, regardless of the cell type. Unmethylated CpGs usually group together in clusters of regions known as CpG islands, which cover about 2% of the entire genome. Around 40% of mammalian genes and 70% of human genes have CpG islands enriched in their promoter regions. CpG methylation in gene promoters is generally associated with repression of transcription, and hence silencing of gene expression. When occurring at the promoters of tumor suppressor gene, DNA methylation could repress the tumour suppressors, leading to oncogenesis. In contrast, high levels of methylation have been observed in the gene body of highly expressed genes, which implies positive correlation between gene body methylation and gene expression.

Among numerous existing technologies, the most widely used method to investigate DNA methylation is bisulfite sequencing (BS-seq), which produces data on the single-nucleotide scale. Unmethylated cytosines (C) are converted to Uracils (U) by sodium bisulfite and then deaminated to thymines (T) during PCR amplification. Methylated Cs, on the other hand, remain intact after bisulfite treatment. The BS-seq technique can be used to measure genome-wide single-cytosine methylation levels by sequencing the entire genome. This strategy produces whole genome bisulfite sequencing (WGBS) data. However, the WGBS approach could be cost-prohibitive for species, such as human, with large genome. In addition, the fact that CpG islands reside in only 2% of the entire genome makes the WGBS approach inefficient when comparing a large number of samples.

To improve the efficiency and bring down the scale and cost of WGBS, enrichment strategies have been developed and combined with BS-seq to target a specific fraction of the genome. A common targeted approach is reduced representation bisulfite sequencing (RRBS) that targets CpG-rich regions. Under the RRBS strategy, small fragments that compose only 1% of the genome are generated using MspI digestion, which means fewer reads are required to obtain accurate sequencing. The RRBS approach can capture approximately 70% of gene promoters and 85% of CpG islands, while requiring only small quantities of input sample. In general, RRBS has great advantages in cost and efficiency when dealing with large scale data, whereas WGBS is more suitable for studies where all CpG islands or promoters across the entire genome are of interest.

The first step of analyzing BS-seq data is to align short reads to genome. The number of C-to-T conversions are then counted for all the mapped reads. A number of software tools have been developed for the purposes of read mapping and methylation calling of BS-seq data. Popular ones include Bismark, MethylCoder, BRAT, B-Smooth and BSMAP. Most of the software tools rely on existing short read aligners, such as Bowtie.

Typical downstream DNA methylation studies often involve finding differentially methylated regions (DMRs) between different experimental conditions. A number of statistical methods and software packages have been developed for detecting DMRs using the BS-seq technology. methylkit and RnBeads implement Fisher’s Exact Test, which is a popular choice for two-group comparisons with no replicates. In the case of complex experimental designs, regression methods are widely used to model methylation levels or read counts. RnBeads offers a linear regression approach based on the moderated t-test and empirical Bayes method implemented in limma. BSmooth is another analysis pipeline that uses linear regression and empirical Bayes together with a local likelihood smoother. methylkit also has an option to apply logistic regression with overdispersion correction. Some other methods have been developed based on beta-binomial distribution to achieve better variance modelling. For example, DSS fits a Bayesian hierarchical beta-binomial model to BS-seq data and uses Wald tests to detect DMRs. Other software using beta-binomial model include BiSeq and RADMeth.

In this workflow, we demonstrate an edgeR approach of differential methylation analysis. edgeR is one of the most popular Bioconductor packages for assessing differential expression in RNA-seq data. It is based on the negative binomial (NB) distribution and it models the variation between biological replicates through the NB
dispersion parameter. Unlike other approaches to methylation sequencing data, the analysis explained in this workflow keeps the counts for methylated and unmethylated reads as separate observations. *edgeR* linear models are used to fit the total read count (methylated plus unmethylated) at each genomic locus, in such a way that the proportion of methylated reads at each locus is modelled indirectly as an over-dispersed binomial distribution. This approach has a number of advantages. First, it allows the differential methylation analysis to be undertaken using existing *edgeR* pipelines developed originally for RNA-seq differential expression analyses. The *edgeR* generalized linear model (GLM) framework offers great flexibility for analysing complex experimental designs while still accounting for the biological variability. Second, keeping methylated and unmethylated read count as separate data observations allows the inherent variability of the data to be modeled more directly and perhaps more realistically. Differential methylation is assessed by likelihood ratio tests so we do not need to assume that the log-fold-changes or other coefficient estimators are normally distributed.

This article presents an analysis of an RRBS data set generated by the authors containing replicated RRBS profiles of basal and luminal cell populations from the mouse mammary epithelium. As with other articles in the Bioconductor Gateway series, our aim is to provide an example analysis with complete start to finish code. As with other Bioconductor workflow articles, we illustrate one analysis strategy in detail rather than comparing different pipelines. The analysis approach illustrated in this article can be applied to any BS-seq data that includes some replication, but is especially appropriate for RRBS data with small numbers of biological replicates. The results shown in this article were generated using Bioconductor Release 3.6.

### The NB linear modeling approach to BS-seq data

#### A small example

To introduce the *edgeR* linear modeling approach to BS-seq data, consider a genomic locus that has $m_A$ methylated and $u_A$ unmethylated reads in condition A and $m_B$ methylated and $u_B$ unmethylated reads in condition B. Our approach is to model all four counts as NB distributed with the same dispersion but different means. Suppose the data is as given in Table 1. If this were a complete dataset, then it could be analyzed in *edgeR* as follows.

```r
> counts <- matrix(c(2,12,11,0),1,4)
> dimnames(counts) <- list("Locus", c("A_Me","A_Un","B_Me","B_Un"))
> counts
  A_Me A_Un B_Me B_Un
Locus  2   12   11    0
> design <- cbind(Sample1 = c(1,1,0,0),
                   Sample2 = c(0,0,1,1),
                   A_MvsU = c(1,0,1,0),
                   BvsA_MvsU = c(0,0,1,0))
> fit <- glmFit(counts, design, lib.size=c(100,100,100,100), dispersion=0.0247)
> lrt <- glmLRT(fit, coef="BvsA_MvsU")
> topTags(lrt)
Coefficient: BvsA_MvsU
    logFC logCPM   LR   PValue      FDR
Locus  8.99   16.3 20.7 5.27e-06 5.27e-06
```

<table>
<thead>
<tr>
<th>Sample</th>
<th>Condition</th>
<th>Methylated Count</th>
<th>Unmethylated Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>11</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. A small example data set.
In this analysis, the first two coefficients are used to model the total number of reads (methylated or unmethylated) for samples 1 and 2, respectively. Coefficient 3 ($A_MvsU$) estimates the log ratio of methylated to unmethylated reads for sample 1, a quantity that can also be viewed as the logit proportion of methylated reads in sample 1. Coefficient 4 ($BvsA_MvsU$) estimates the difference in logit proportions of methylated reads between conditions B and A. The difference in logits is estimated here as 8.99 on the log2 scale. The P-value for differential methylation (B vs A) is $P = 5.27 \times 10^{-6}$.

The dispersion parameter controls the degree of biological variability. If we had set $\text{dispersion}=0$ in the above code, then the above analysis would be exactly equivalent to a logistic binomial regression, with the methylated counts as responses and the total counts as sizes, and with a likelihood ratio test for a difference in proportions between conditions A and B. Positive values for the dispersion produce over-dispersion relative to the binomial distribution. We have set the dispersion here equal to the value that is estimated below for the mammary epithelial data.

In the above code, the two library sizes for each sample should be equal. Otherwise, the library size values are arbitrary and any settings would have lead to the same P-value.

**Relationship to beta-binomial modeling**

It is interesting to compare this approach with beta-binomial modeling. It is well known that if $m$ and $u$ are independent Poisson random variables with means $\mu_m$ and $\mu_u$, then the conditional distribution of $m$ given $m + u$ is binomial with success probability $p = \mu_m / (\mu_m + \mu_u)$. If the Poisson means $\mu_m$ and $\mu_u$ themselves follow gamma distributions, then the marginal distributions of $m$ and $u$ are NB instead of Poisson. If the two NB distributions have different dispersions, and have expected values in inverse proportion to the dispersions, then the conditional distribution of $m$ given $m + u$ follows a beta-binomial distribution. The approach taken in this article is closely related to the beta-binomial approach but makes different and seemingly more natural assumptions about the NB distributions. We instead assume the two NB distributions to have the same dispersion but different means. The NB linear modeling approach allows the means and dispersions of the two NB distributions to be estimated separately, in concordance with the data instead of being artificially linked.

**Description of the biological experiment**

**Aim of the study**

The epithelium of the mammary gland exists in a highly dynamic state, undergoing dramatic morphogenetic changes during puberty, pregnancy, lactation, and regression. Characterization of the lineage hierarchy of cells in the mammary epithelium is an important step toward understanding which cells are predisposed to oncogenesis.

In this study, we profiled the methylation status of the two major functionally distinct epithelial compartments: basal and luminal cells. The basal cells were further divided into those showing high or low expression of the surface marker Itga5 as part of our investigation of heterogeneity within the basal compartment. We carried out global RRBS DNA methylation assays on two biological replicates of each of the three cell populations to determine whether the epigenetic machinery played a potential role in (i) differentiation of luminal cells from basal and (ii) any compartmentalization of the basal cells associated with Itga5.

**Sample preparation**

Inguinal mammary glands (minus lymph node) were harvested from FVB/N mice. All animal experiments were conducted using mice bred at and maintained in our animal facility, according to the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee guidelines. Epithelial cells were suspended and fluorescence-activated cell sorting (FACS) was used to isolate basal and luminal cell populations. Genomic DNA (gDNA) was extracted from freshly sorted cells using the Qiagen DNeasy kit. Around 25ng gDNA input was subjected to DNA methylation analysis by BS-seq using the Ovation RRBS Methyl-seq kit from NuGEN. The process includesMspI digestion of gDNA, sequencing adapter ligation, end repair, bisulfite conversion, and PCR amplification to produce the final sequencing library. The Qiagen EpiTect Bisulfite kit was used for bisulfite-mediated conversion of unmethylated cytosines.
Experimental design

There are three groups of samples: luminal population, Itga5- basal population and Itga5+ basal population. Two biological replicates were collected for each group. This experimental design is summarized in the table below.

```r
> targets <- read.delim("targets.txt", stringsAsFactors=FALSE)
> targets

       Sample Population Description
    1     P6_1       P6          Luminal
    2     P6_4       P6          Luminal
    3     P7_2       P7  Basal_Itga5_neg
    4     P7_5       P7  Basal_Itga5_neg
    5     P8_3       P8  Basal_Itga5_pos
    6     P8_6       P8  Basal_Itga5_pos
```

The experiment has a simple one-way layout with three groups. A single grouping factor is made as follows:

```r
> Group <- factor(targets$Population)
> Group

[1] P6 P6 P7 P7 P8 P8
Levels: P6 P7 P8
```

The sequencing was carried out on the Illumina NextSeq 500 platform. About 30 million 75bp paired-end reads were generated for each sample.

Differential methylation analysis at CpG loci
Processing bisulfite sequencing FASTQ files

The first step of the analysis is to map the sequencing reads from the FASTQ files to the mouse genome and then perform methylation calls. Though many options are available, we use Bismark for read alignment and methylation calling. Bismark is one of the most popular software tools to perform alignments of bisulfite-treated sequencing reads to a genome of interest and perform methylation calls. It maps sequencing reads using the short read aligner Bowtie 1 or alternatively Bowtie 2.

To increase alignment rates and reduce false methylation calls, it is recommended to trim poor quality reads on sequence ends and remove adapters that can be potentially sequenced prior to the alignment. This is done using trim_galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). After that, Bismark version v0.13.0 is used to align the reads to the mouse genome mm10. The final methylation calls are made using bismark_methylation_extractor.

Downloading the data

The Bismark outputs include one coverage bed file of the methylation in CpG context for each sample. The coverage outputs from Bismark are available at http://bioinf.wehi.edu.au/edgeR/F1000Research2017/. Readers wishing to reproduce the analysis presented in this article can download the zipped coverage bed files produced by Bismark from the above link.

Bed files can be read into R using read.delim as for txt files. Each of the bed files has the following format:

```r
> P6_1 <- read.delim("P6_1.bismark.cov.gz", header=FALSE)
> head(P6_1)

   V1  V2  V3  V4  V5  V6
1 chr6 3052156 3052156  87.9  51  7
2 chr6 3052157 3052157  85.7  6  1
3 chr6 3052246 3052246  100.0  7  0
4 chr6 3052415 3052415  100.0 57  0
5 chr6 3052416 3052416  100.0  7  0
6 chr6 3052434 3052434  94.7  54  3
```
The columns in the bed file represent: V1: chromosome number; V2: start position of the CpG site; V3: end position of the CpG site; V4: methylation proportion; V5: number of methylated Cs; V6: number of unmethylated Cs.

Reading in the data
Since the start and end positions in the coverage outputs are identical for each CpG site, only one of them is needed for marking the location of each. We also ignore the methylation proportion as it can be directly calculated from the number of methylated and unmethylated Cs. The data can then be read into a list in R.

```r
> Sample <- targets$Sample
> fn <- paste0(Sample, ".bismark.cov.gz")
> data <- list()
> for(i in 1:length(Sample)) {
+   data[[i]] <- read.delim(file=fn[i], header=FALSE)[-3:4]
+   names(data[[i]]) <- c("Chr", "Position", "Meth", "Un")
+ }
```

The `data` object is a list containing six data frames, each of which represents one sample. The first and second columns of each data frame are the chromosome numbers and positions of all the CpG loci observed in that sample. The last two columns contain the numbers of methylated and unmethylated Cs detected at those loci. Since the number of reported CpG loci varies across different samples, care is required to combine the information from all the samples. We first obtain all unique CpG loci observed in at least one of the six samples. This is done by combining the chromosome number and position of each CpG site. Then we extract read counts of methylated and unmethylated Cs at these locations across all the samples and combine them into a count matrix.

```r
> position <- sapply(data, function(x) paste(x[1], x[2], sep="-"))
> position_all <- unique(unlist(position))
> counts <- matrix(0L, nrow=length(position_all), ncol=2*length(Sample))
> for(i in 1:length(Sample)) {
+   m <- match(position[[i]], position_all)
+   counts[m, c(2*i-1,2*i)] <- as.matrix(data[[i]][,3:4])
+ }
```

The `counts` object is a matrix of integer counts with 12 columns, two for each sample. The odd number of columns contain the numbers of methylated Cs, whereas the even number of columns contain the numbers of unmethylated Cs. The genomic positions are used as the row names of the count matrix.

```r
> rownames(counts) <- position_all
> Sample2 <- rep(Sample, each=2)
> Meth <- rep(c("Me","Un"), length(Sample))
> Meth <- factor(Meth, levels=c("Un","Me"))
> colnames(counts) <- paste(Sample2, Meth, sep="-")
> head(counts)
```

<table>
<thead>
<tr>
<th></th>
<th>P6_1-Me</th>
<th>P6_1-Un</th>
<th>P6_4-Me</th>
<th>P6_4-Un</th>
<th>P7_2-Me</th>
<th>P7_2-Un</th>
<th>P7_5-Me</th>
<th>P7_5-Un</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr6-3052156</td>
<td>51</td>
<td>7</td>
<td>62</td>
<td>13</td>
<td>48</td>
<td>3</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>chr6-3052157</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>chr6-3052246</td>
<td>57</td>
<td>0</td>
<td>75</td>
<td>1</td>
<td>50</td>
<td>1</td>
<td>36</td>
<td>1</td>
</tr>
<tr>
<td>chr6-3052416</td>
<td>7</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>chr6-3052434</td>
<td>54</td>
<td>3</td>
<td>72</td>
<td>4</td>
<td>48</td>
<td>3</td>
<td>36</td>
<td>1</td>
</tr>
<tr>
<td>chr6-3052415</td>
<td>46</td>
<td>1</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>chr6-3052416</td>
<td>47</td>
<td>0</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>chr6-3052434</td>
<td>40</td>
<td>10</td>
<td>28</td>
<td>10</td>
<td>28</td>
<td>10</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>chr6-3052415</td>
<td>46</td>
<td>1</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>chr6-3052416</td>
<td>47</td>
<td>0</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
We then proceed to the *edgeR* analysis of the methylation data. The *edgeR* package stores data in a simple list-based data object called a *DGEList*. We first create a *DGEList* object using the count matrix generated before. The information of CpG sites is converted into a data frame and stored in the *genes* component of the *DGEList* object.

```r
library(edgeR)
options(digits=3)
Chr <- gsub("-.*$", "", position_all)
Position <- gsub("^.*-", "", position_all)
Genes <- data.frame(Chr=Chr, Position=Position)
y <- DGEList(counts, genes=Genes, group=rep(Group, each=2))
```

**Filtering to remove low counts**

We first sum up the read counts of both methylated and unmethylated Cs at each CpG site within each sample.

```r
> counts_total <- t(rowsum(t(counts), Sample2))
> head(counts_total)

P6_1 P6_4 P7_2 P7_5 P8_3 P8_6
chr6-3052156 58 75 51 39 49 38
chr6-3052157  7  5  0  4  0  2
chr6-3052246  1  0  0  2  2  2
chr6-3052415 57 76 51 37 47 36
chr6-3052416  7  5  0  4  0  2
chr6-3052434 57 76 51 37 47 36
```

CpG loci that have very low counts across all the samples shall be removed prior to downstream analysis as they provide little information for assessing methylation levels. As a rule of thumb, we require a CpG site to have a total count (both methylated and unmethylated) of at least 10 across all the samples before it is considered in the study.

```r
> keep <- rowSums(counts_total >= 10) == 6
> table(keep)

keep
FALSE  TRUE
3139160  398926
```

The *DGEList* object is subsetted to retain only the non-filtered loci:

```r
> y <- y[keep, , keep.lib.sizes=FALSE]
```

The option `keep.lib.sizes=FALSE` causes the library sizes to be recomputed after the filtering. This is generally recommended, although the effect on the downstream analysis is usually small.

**Normalization**

A key difference between BS-seq and other sequencing data is that the pair of libraries holding the methylated and unmethylated reads for a particular sample are treated as a unit. To ensure that the methylated and unmethylated reads for the same sample are treated on the same scale, we need to set the library sizes to be equal for each pair of libraries. We set the library sizes for each sample to be the average of the total read counts for the methylated and unmethylated libraries:

```r
> TotalReadCount <- colMeans(matrix(y$samples$lib.size, nrow=2, ncol=6))
> y$samples$lib.size <- rep(TotalReadCount, each=2)
> y$samples
```
Other normalization methods developed for RNA-seq data, such as TMM\cite{vo2010}, are not required for BS-seq data.

**Exploring differences between samples**

In DNA methylation studies, methylation levels are of most interest. For Illumina methylation assay, two common measurements of methylation levels are \( \beta \)-values and M-values, which are defined as \( \beta = \frac{M}{M+U} \) and \( M\text{-value} = \log_2(M/U) \) where \( M \) and \( U \) denote the methylated and unmethylated intensity\cite{buenrostro2014}. Here we adopt the same idea and extend the two measurements to BS-seq data. That is, denote the methylated and unmethylated Cs by \( M \) and \( U \) respectively, and define the \( \beta \)-values and M-values in the same way as above.

In practice, for a particular CpG site in one sample, the M-value can be computed by subtracting the log2 count-per-million (CPM) of the unmethylated Cs from that of the methylated Cs. This is equivalent to the calculation of the defined M-values as the library sizes are set to be the same for each pair of methylated and unmethylated columns and they cancel each other out in the subtraction. A prior count of 2 is added to the calculation of log2-CPM to avoid undefined values and to reduce the variability of M-values for CpG sites with low counts. The calculation of \( \beta \)-value is straight-forward though a small offset may also be added to the calculation.

```r
> Beta <- y$counts[, Meth=="Me"] / counts_total[keep, ]
> logCPM <- cpm(y, log=TRUE, prior.count=2)
> M <- logCPM[, Meth=="Me"] - logCPM[, Meth=="Un"]
> colnames(Beta) <- colnames(M) <- Sample
```

The outputs \( \beta \) and \( M \) are numeric matrices with six columns, each of which contains the \( \beta \)-values or M-values calculated at each CpG site in one sample. Then we can generate multi-dimensional scaling (MDS) plots to explore the overall differences between the methylation levels of the different samples. Here we decorate the MDS plots to indicate the cell groups:

```r
> par(mfrow=c(1,2))
> plotMDS(Beta, col=rep(1:3, each=2), main="Beta-values")
> plotMDS(M, col=rep(1:3, each=2), main="M-values")
```

**Figure 1** shows the resulting plots. In these plots, the distance between each pair of samples represents the average log-fold change between the samples for the top most differentially methylated CpG loci between that pair of samples. (We call this average the leading log-fold change.) The two replicate samples from the luminal population (P6) are seen to be well separated from the four basal samples (populations P7 and P8).
Design matrix

One aim of this study is to identify differentially methylated regions (DMRs) between different groups. In \textit{edgeR}, this can be done by fitting linear models under a specified design matrix and testing for corresponding coefficients or contrasts. Here, a design matrix is constructed as follows:

\begin{verbatim}
> design <- model.matrix(~ Sample2 + Meth)
> colnames(design) <- gsub("Sample2", "", colnames(design))
> colnames(design) <- gsub("Meth", "", colnames(design))
> colnames(design)[1] <- "Int"
> design <- cbind(design,
+   Me2=c(0,0,0,0,1,0,1,0,0,0,0,0),
+   Me3=c(0,0,0,0,0,0,1,0,1,0,1,0))
> design

    Int P6_4 P7_2 P7_5 P8_3 P8_6 Me Me2 Me3
  1    1  0  0  0  0  0  0  1  0
  2    1  0  0  0  0  0  0  0  0
  3    1  1  0  0  0  0  1  0  0
  4    1  1  0  0  0  0  0  0  0
  5    1  0  1  0  0  0  1  1  0
  6    1  0  1  0  0  0  0  0  0
  7    1  0  0  1  0  0  1  1  0
  8    1  0  0  1  0  0  0  0  0
  9    1  0  0  0  1  0  1  0  1
 10    1  0  0  0  1  0  0  0  0
 11    1  0  0  0  0  1  1  0  1
 12    1  0  0  0  0  0  1  0  0
\end{verbatim}

The first six columns represent the sample effect. It accounts for the fact that each pair of columns of the count matrix are from one of the six samples. The 7th column “Me” represents the methylation level (in M-value).
in the P6 group. The 8th column “Me2” represents the difference in methylation level between the P7 and P6 groups. Finally, the last column “Me3” represents the difference in methylation level between the P8 and P6 groups.

**Dispersion estimation**

With the design matrix specified, we can now proceed to the standard `edgeR` pipeline and analyze the data in the same way as for RNA-seq data. Similar to the RNA-seq data, the variability between biological replicates has also been observed in bisulfite sequencing data. This variability can be captured by the NB dispersion parameter under the generalized linear model (GLM) framework in `edgeR`.

The mean-dispersion relationship of BS-seq data has been studied in the past and no apparent mean-dispersion trend was observed\(^1\). This is also verified through our own practice. Therefore, we would not consider a mean-dependent dispersion trend as we normally would for RNA-seq data. A common dispersion estimate for all the loci, as well as an empirical Bayes moderated dispersion for each individual locus, can be obtained from the `estimateDisp` function in `edgeR`:

```r
> y <- estimateDisp(y, design=design, trend="none")
> y$common.dispersion

[1] 0.0247
```

```r
> summary(y$prior.df)

 Min. 1st Qu.  Median    Mean 3rd Qu.    Max.  
   Inf   Inf   Inf     Inf   Inf   Inf
```

This returns a `DGEList` object with additional components (common.dispersion and tagwise.dispersion) added to hold the estimated dispersions. Here the estimation of trended dispersion has been turned off by setting `trend="none"`. For this data, the estimated prior degrees of freedom (df) are infinite for all the loci, which implies all the CpG-wise dispersions are exactly the same as the common dispersion. A BCV plot is often useful to visualize the dispersion estimates, but it is not informative in this case.

**Testing for differentially methylated CpG loci**

We first fit NB GLMs for all the CpG loci using the `glmFit` function in `edgeR`.

```r
> fit <- glmFit(y, design)
```

Then we can proceed to testing for differentially methylated CpG sites between different populations. One of the most interesting comparisons is between the basal (P7 and P8) and luminal (P6) groups. The contrast corresponding to any specified comparison can be constructed conveniently using the `makeContrasts` function:

```r
> contr <- makeContrasts(BvsL=0.5*(Me2+Me3), levels=design)
```

The actual testing is performed using likelihood ratio tests (LRT) in `edgeR`.

```r
> lrt <- glmLRT(fit, contrast=contr)
```

The top set of most differentially methylated (DM) CpG sites can be viewed with `topTags`:

```r
> topTags(lrt)

Coefficient:  0.5*Me2  0.5*Me3  
              Chr  Position  logFC   logCPM  LR  PValue     FDR  
chr13-45709467 chr13   45709467  -7.60   3.06 342 2.71e-76 9.62e-71  
chr16-76326604 chr16    76326604   8.87   2.60 341 4.82e-76 9.62e-71  
chr10-40387375 chr10   40387375   8.13   2.85 333 2.48e-74 3.30e-69  
```
Here positive log-fold changes represent CpG sites that have higher methylation level in the basal population compared to the luminal population. The Benjamini-Hochberg multiple testing correction is applied to control the false discovery rate (FDR).

The total number of DM CpG sites identified at an FDR of 5% can be shown with `decideTestsDGE`. There are in fact more than 50,000 differentially methylated CpGs in this comparison:

```r
> summary(decideTests(lrt))

  0.5*Me2  0.5*Me3
-1     35891
 0  344846
 1 18189
```

The differential methylation results can be visualized with an MD plot (see Figure 2):

```r
> plotMD(lrt)
```

![MD plot](image)

**Figure 2.** MD plot showing the log-fold change of the methylation level and average abundance of each CpG site. Significantly up and down methylated CpGs are highlighted in red and blue, respectively.
The logFC of the methylation level for each CpG site is plotted against the average abundance in log2-CPM. Significantly differentially methylated CpGs are highlighted.

**Differential methylation in gene promoters**

**Pre-defined gene promoters**

The majority of CpGs are methylated in mammals. On the other hand, unmethylated CpGs tend to group into clusters of CpG islands, which are often enriched in gene promoters. CpG methylation in promoter regions is often associated with silencing of transcription and gene expression\(^5\). Therefore it is of great biological interest to examine the methylation level within the gene promoter regions.

For simplicity, we define the promoter of a gene as the region from 2kb upstream to 1kb downstream of the transcription start site of that gene. The genomic locations and their associated annotations of the promoters can be obtained using the `TxDb.Mmusculus.UCSC.mm10.knownGene` package.

```r
> library(TxDB.Mmusculus.UCSC.mm10.knownGene)
> genes_Mm <- genes(TxDB.Mmusculus.UCSC.mm10.knownGene)
> pr <- promoters(genes_Mm, upstream=2000, downstream=1000)
> pr
```

GRanges object with 24044 ranges and 1 metadata column:

<table>
<thead>
<tr>
<th>seqnames</th>
<th>ranges</th>
<th>strand</th>
<th>gene_id</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr9</td>
<td>[21074497, 21077496]</td>
<td>-</td>
<td>100009600</td>
</tr>
<tr>
<td>chr7</td>
<td>[84963010, 84966009]</td>
<td>-</td>
<td>100009609</td>
</tr>
<tr>
<td>chr10</td>
<td>[77709446, 77712445]</td>
<td>+</td>
<td>100009614</td>
</tr>
<tr>
<td>chr11</td>
<td>[45806083, 45809082]</td>
<td>+</td>
<td>100009664</td>
</tr>
<tr>
<td>chr4</td>
<td>[144161652, 144164651]</td>
<td>-</td>
<td>100012</td>
</tr>
</tbody>
</table>

...   ...   ...   ...   ...

99889  chr3  [85886519, 85889518]  -  | 99889
99890  chr3  [110250000, 110252999]  -  | 99890
99899  chr3  [151748960, 151751959]  -  | 99899
99929  chr3  [65526447, 65529446]  +  | 99929
99982  chr4  [136601724, 136604723]  -  | 99982

seqinfo: 66 sequences (1 circular) from mm10 genome

Here, `pr` is a GRanges class object that contains the genomic ranges of the promoters of all the known mouse genes in the annotation package.

**Summarizing counts in promoter regions**

We create another GRanges class object `sites`, which contains the genomic locations of all the observed CpG sites.

```r
> Position <- as.numeric(Position)
> sites <- GRanges(seqnames=Chr, ranges=IRanges(start=Position, end=Position))
```

Then we find the overlaps between the gene promoter regions and all the CpG sites in the data using `findOverlaps`. 
> olap <- findOverlaps(query=pr, subject=sites)
> olap

Hits object with 1522464 hits and 0 metadata columns:

<table>
<thead>
<tr>
<th>queryHits</th>
<th>subjectHits</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;integer&gt;</td>
<td>&lt;integer&gt;</td>
</tr>
<tr>
<td>[1]</td>
<td>3</td>
</tr>
<tr>
<td>[2]</td>
<td>3</td>
</tr>
<tr>
<td>[3]</td>
<td>3</td>
</tr>
<tr>
<td>[4]</td>
<td>3</td>
</tr>
<tr>
<td>[5]</td>
<td>6</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>[1522460]</td>
<td>24044</td>
</tr>
<tr>
<td>[1522461]</td>
<td>24044</td>
</tr>
<tr>
<td>[1522462]</td>
<td>24044</td>
</tr>
<tr>
<td>[1522463]</td>
<td>24044</td>
</tr>
<tr>
<td>[1522464]</td>
<td>24044</td>
</tr>
</tbody>
</table>

-------
queryLength: 24044 / subjectLength: 3538086

The queryHits component of olap marks the indices of the promoter region as in pr, whereas the subjectHits component contains the indices of the CpG sites as in sites that overlap with the corresponding promoter regions.

The numbers of methylated and unmethylated CpGs overlapping with gene promoters are summed up for each promoter.

> counts2 <- counts[subjectHits(olap),]
> counts2 <- rowsum(counts2, queryHits(olap))

The integer matrix counts2 contains the total numbers of methylated and unmethylated CpGs observed within the promoter of each gene. Same as before, counts2 has 12 columns, two for each sample. The odd number of columns contain the numbers of methylated Cs, whereas the even number of columns contain the numbers of unmethylated Cs. The only difference is that each row of counts2 now represents a gene promoter instead of an individual CpG site.

The gene symbol information can be added to the annotation using the org.Mm.eg.db package. A DGEList object is created for the downstream edgeR analysis.

> ind <- as.numeric(rownames(counts2))
> rownames(counts2) <- pr$gene_id[ind]
> library(org.Mm.eg.db)
> anno <- select(org.Mm.eg.db, keys=pr$gene_id, columns="SYMBOL",
 + keytype="ENTREZID")
> anno <- data.frame(Symbol=anno$SYMBOL[ind])
> y2 <- DGEList(counts2, genes=anno, group=rep(Group, each=2))

We sum up the read counts of both methylated and unmethylated Cs at each CpG sites within each sample.

> counts2_total <- t(rowsum(t(counts2), Sample2))
**Filtering to remove low counts**

Filtering is performed in the same way as before. Since each row represents a 3,000-bp-wide promoter region that contains multiple CpG sites, we would expect less filtering than before.

```r
> keep2 <- rowSums(counts2_total >= 10) == 6
> table(keep2)

keep2  FALSE  TRUE
        1754 16790
```

> y2 <- y2[keep2,, keep.lib.sizes=FALSE]

Same as before, we do not perform normalization but set the library sizes for each sample to be the average of the total read counts for the methylated and unmethylated libraries.

```r
> TotalReadCount2 <- colMeans(matrix(y2$samples$lib.size, nrow=2, ncol=6))
> y2$samples$lib.size <- rep(TotalReadCount2, each=2)
```

<table>
<thead>
<tr>
<th>Group</th>
<th>Library</th>
<th>Mean Read Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6_1-Me</td>
<td>P6</td>
<td>12474999</td>
</tr>
<tr>
<td>P6_1-Un</td>
<td>P6</td>
<td>12474999</td>
</tr>
<tr>
<td>P6_4-Me</td>
<td>P6</td>
<td>12579436</td>
</tr>
<tr>
<td>P6_4-Un</td>
<td>P6</td>
<td>12579436</td>
</tr>
<tr>
<td>P7_2-Me</td>
<td>P7</td>
<td>5110397</td>
</tr>
<tr>
<td>P7_2-Un</td>
<td>P7</td>
<td>5110397</td>
</tr>
<tr>
<td>P7_5-Me</td>
<td>P7</td>
<td>11189796</td>
</tr>
<tr>
<td>P7_5-Un</td>
<td>P7</td>
<td>11189796</td>
</tr>
<tr>
<td>P8_3-Me</td>
<td>P8</td>
<td>4123987</td>
</tr>
<tr>
<td>P8_3-Un</td>
<td>P8</td>
<td>4123987</td>
</tr>
<tr>
<td>P8_6-Me</td>
<td>P8</td>
<td>3562239</td>
</tr>
<tr>
<td>P8_6-Un</td>
<td>P8</td>
<td>3562239</td>
</tr>
</tbody>
</table>

**Exploring differences between samples**

Same as before, we measure the methylation levels of gene promoter regions using both $\beta$-values and M-values. A prior count of 2 is added to the calculation of log2-CPM to avoid undefined values and to reduce the variability of M-values for gene promoters with low counts. Then MDS plots are produced to examine the overall differences between the methylation levels of the different samples.

```r
> Beta2 <- y2$count[, Meth=="Me"] / counts2_total[keep2, ]
> logCPM2 <- cpm(y2, log=TRUE, prior.count=2)
> M2 <- logCPM2[, Meth=="Me"] - logCPM2[, Meth="Un"]
> colnames(Beta2) <- colnames(M2) <- Sample
> par(mfrow=c(1,2))
> plotMDS(Beta2, col=rep(1:3, each=2), main="Beta-values")
> plotMDS(M2, col=rep(1:3, each=2), main="M-values")
```

The resulting Figure 3 shows that the two replicate samples from the luminal population (P6) are well separated from the four replicate samples from the basal population (P7 and P8).
Dispersion estimation
We estimate the NB dispersions using the `estimateDisp` function in `edgeR`. For the same reason, we do not consider a mean-dependent dispersion trend as we normally would for RNA-seq data.

```r
> y2 <- estimateDisp(y2, design=design, trend="none", robust=TRUE)
```

```r
> y2$common.dispersion
[1] 0.0301
```

```r
> summary(y2$prior.df)
         Min. 1st Qu.  Median    Mean 3rd Qu.    Max. 
10.2 10.2    10.3    10.3 10.4    10.4
```

The dispersion estimates can be visualized with a BCV plot (see Figure 4):

```r
> plotBCV(y2)
```

Testing for differential methylation in gene promoters
We first fit NB GLMs for all the gene promoters using `glmFit`.

```r
> fit2 <- glmFit(y2, design)
```

Then we can proceed to testing for differentially methylation in gene promoter regions between different populations. Suppose the comparison of interest is same as before. The same contrast can be used for the testing.

```r
> lrt2 <- glmLRT(fit2, contrast=contr)
```
The top set of most differentially methylated gene promoters can be viewed with `topTags`:

```
> topTags(lrt2)
```

<table>
<thead>
<tr>
<th>Symbol</th>
<th>logFC</th>
<th>logCPM</th>
<th>LR</th>
<th>PValue</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lnx1</td>
<td>6.85</td>
<td>5.24</td>
<td>314</td>
<td>2.49e-70</td>
<td>4.18e-66</td>
</tr>
<tr>
<td>Akap6</td>
<td>5.26</td>
<td>4.06</td>
<td>249</td>
<td>4.19e-56</td>
<td>3.52e-52</td>
</tr>
<tr>
<td>Popdc2</td>
<td>-4.91</td>
<td>5.84</td>
<td>226</td>
<td>4.07e-51</td>
<td>2.28e-47</td>
</tr>
<tr>
<td>Angpt2</td>
<td>-5.58</td>
<td>3.30</td>
<td>209</td>
<td>4.23e-47</td>
<td>9.37e-44</td>
</tr>
<tr>
<td>Cldn4</td>
<td>5.56</td>
<td>5.16</td>
<td>190</td>
<td>2.23e-47</td>
<td>1.28e-39</td>
</tr>
<tr>
<td>Tas2r143</td>
<td>-4.52</td>
<td>4.49</td>
<td>178</td>
<td>1.39e-40</td>
<td>3.88e-37</td>
</tr>
</tbody>
</table>

Here positive log-fold changes represent gene promoters that have higher methylation level in the basal population compared to the luminal population. The Benjamini-Hochberg multiple testing correction is applied to control the false discovery rate (FDR).

**Figure 4.** Scatterplot of the BCV against the average abundance of CpG sites in each gene promoter. The plot shows the square-root estimates of the common and tagwise NB dispersions.
The total number of DM gene promoters identified at an FDR of 5% can be shown with `decideTestsDGE`. There are in fact about 1,200 differentially methylated gene promoters in this comparison:

```r
> summary(decideTests(lrt2))

  0.5*Me2  0.5*Me3  -1             817
  0      15617
  1      356
```

The differential methylation results can be visualized with an MD plot (see Figure 5):

```r
> plotMD(lrt2)
```

### Correlate with RNA-seq profiles

**RNA-seq profiles of mouse epithelium**

To show that DNA methylation (particularly in the promoter regions) represses gene expression, we relate the differential methylation results to the gene expression profiles of the RNA-Seq data. The RNA-seq data used here is from a study of the epithelial cell lineage in the mouse mammary gland\(^3\), in which the expression profiles of basal stem-cell enriched cells and committed luminal cells in the mammary glands of virgin, pregnant and lactating mice were examined. The complete differential expression analysis of the data is described in Chen et al.\(^3\).

**Figure 5.** MD plot showing the log-fold change of the methylation level and average abundance of CpG sites in each gene promoter. Significantly up and down methylated gene promoters are highlighted in red and blue, respectively.
The RNA-seq data is stored in the format of a DGEList object \texttt{y\_rna} and saved in a RData file \texttt{rna.RData}. The object \texttt{y\_rna} contains the count matrix, sample information, gene annotation, design matrix and dispersion estimates of the RNA-seq data. The gene filtering, normalization and dispersion estimation were performed in the same way as described in Chen \textit{et al.}\textsuperscript{35}. The DE analysis between the basal and luminal in the virgin mice was performed using \texttt{glmTreat} with a fold-change threshold of 3. The results are saved in the spread sheet \texttt{BvsL-fc3.csv}. Both \texttt{rna.RData} and \texttt{BvsL-fc3.csv} are available for download at \url{http://bioinf.wehi.edu.au/edgeR/F1000Research2017/}.

We load the \texttt{RData} file and read in the DE results from the spread sheet.

```r
> load("rna.RData")
> y_rna

An object of class "DGEList"
$counts
   MCL1.DG MCL1.DH MCL1.DI MCL1.DJ MCL1.DK MCL1.DL MCL1.LA MCL1.LB MCL1.LC
497097  438   300   65   237   354   287    0    0    0
20671  106   182   82   105    43    82   16    25   18
27395  309   234  337   300   290   270  560   464   489
18777  652   515  948   935   928   791   826   862   668
21399 1604  1495 1721 1317  1159  1066 1334 1258 1068
  MCL1.LD MCL1.LE MCL1.LF
497097    0    0    0
20671    8    3    10
27395   328   307   342
18777   646   544   581
21399   926   508   500
15636 more rows ...

$samples
   group     lib.size norm.factors
MCL1.DG   B.virgin 23137472  1.23
MCL1.DH   B.virgin 21687755  1.21
MCL1.DI   B.pregnant 23974787  1.13
MCL1.DJ   B.pregnant 22545375  1.07
MCL1.DK   B.lactating 21420532  1.04
17 more rows ...

$genes
   Length Symbol
497097   3634 Xkr4
20671   3130 Sox17
27395   4203 Mrpl15
18777   2433 Lypla1
21399   2847  Tcea1
15636 more rows ...

$design
   B.lactating B.pregnant B.virgin L.lactating L.pregnant L.virgin
1     0      0      1      0      0      0
2     0      0      1      0      0      0
3     0      1      0      0      0      0
```
$common.dispersion
[1] 0.0134

$trended.dispersion
[1] 0.02086 0.03012 0.01303 0.01007 0.00957
15636 more elements ...

$tagwise.dispersion
[1] 0.13795 0.08336 0.01387 0.00678 0.00631
15636 more elements ...

$AveLogCPM
[1] 2.58 1.32 4.00 5.06 5.64
15636 more elements ...

$trend.method
[1] "locfit"

$prior.df
[1] 4.68 6.08 6.77 6.77 6.77
15636 more elements ...

$prior.n
[1] 0.78 1.01 1.13 1.13 1.13
15636 more elements ...

$span
[1] 0.292

> rna_DE <- read.csv("BvsL-fc3.csv", row.names="GeneID")
> head(rna_DE)

<table>
<thead>
<tr>
<th>Length</th>
<th>Symbol</th>
<th>logFC</th>
<th>unshrunk.logFC</th>
<th>logCPM</th>
<th>PValue</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>24117</td>
<td>2242</td>
<td>9.15</td>
<td>9.18</td>
<td>6.77</td>
<td>1.79e-15</td>
<td>1.67e-11</td>
</tr>
<tr>
<td>69538</td>
<td>5264</td>
<td>7.35</td>
<td>7.36</td>
<td>7.66</td>
<td>2.67e-15</td>
<td>1.67e-11</td>
</tr>
<tr>
<td>55987</td>
<td>3506</td>
<td>8.15</td>
<td>8.18</td>
<td>6.01</td>
<td>3.20e-15</td>
<td>1.67e-11</td>
</tr>
<tr>
<td>12293</td>
<td>7493</td>
<td>8.30</td>
<td>8.31</td>
<td>7.54</td>
<td>1.14e-14</td>
<td>3.51e-11</td>
</tr>
<tr>
<td>12560</td>
<td>7493</td>
<td>8.30</td>
<td>8.31</td>
<td>7.54</td>
<td>1.14e-14</td>
<td>3.51e-11</td>
</tr>
<tr>
<td>110308</td>
<td>2190</td>
<td>8.94</td>
<td>8.94</td>
<td>10.27</td>
<td>1.35e-14</td>
<td>3.51e-11</td>
</tr>
</tbody>
</table>

Correlation between the two datasets
We select the genes of which the promoters are significantly DM (FDR < 0.05) and examine their expression level in the RNA-Seq data. A data frame object lfc is created to store the gene information, log-fold change of methylation level and log-fold change of gene expression of the selected genes.

> tp <- topTags(lrt2, n=Inf, p=0.05)
> m <- match(row.names(tp), row.names(rna_DE))
> lfc <- tp[,c("Symbol","logFC")]
> names(lfc)[2] <- "ME"
> lfc$RNA <- rna_DE$logFC[m]
The Pearson correlation coefficient between the two log-fold changes of the selected genes is estimated. The result shows high negative correlation between gene expression and methylation in gene promoters.

```
> cor(lfc$ME, lfc$RNA)
[1] -0.47
```

The log-fold changes of the selected genes from the two datasets are plotted against each other for visualization (see Figure 6):

```
> plot(lfc$ME, lfc$RNA, main="Basal vs Luminal", xlab="log-FC Methylation", +     ylab="log-FC Gene Expression", pch=16, cex=0.8, col="gray30")
> abline(h=0, v=0, col="gray10", lty=2, lwd=2)
```

![Figure 6. Scatter plot of the log-fold changes of methylation levels in gene promoters (x-axis) vs the log fold-changes of gene expression (y-axis).](image)
The plot shows results for the genes of which the promoters are significantly differentially methylated between basal and luminal. The red line shows the least squares line with zero intercept. A strong negative correlation is observed.
The horizontal axis of the scatterplot shows the log-fold change in methylation level for each gene while the vertical axis shows the log-fold change in expression. To assess the correlation, we fit a least squares regression line through the origin and compute the p-value:

```r
> u <- lm(lfc$RNA ~ 0 + lfc$ME)
> coef(summary(u))

                  Estimate Std. Error t value Pr(>|t|)
    lfc$ME     -0.739     0.0473   -15.6  1.08e-47
```

The negative association is highly significant ($P = 10^{-47}$). The last line of code adds the regression line to the plot (Figure 6).

**Gene set testing**

A rotation gene set test can be performed to further examine the relationship between gene expression and methylation in gene promoters. This is to test whether the set of genes (i.e., genes of which the promoters are differentially methylated) are differentially expressed (DE) and in which direction they are DE.

The indices are made by matching the Entrez Gene Ids between the two datasets. The log-fold changes of methylation level in gene promoters are used as weights for those genes. The test is conducted using the `fry` function in `edgeR`. The contrast is set to compare basal with luminal in virgin mice.

```r
> ME <- data.frame(GeneID=row.names(lfc), weights=lfc$ME)
> fry(y_rna, index=ME, design=y_rna$design, contrast=c(0,0,1,0,0,-1))

<table>
<thead>
<tr>
<th>NGenes</th>
<th>Direction</th>
<th>PValue</th>
<th>PValue.Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>731</td>
<td>Down</td>
<td>1.51e-09</td>
<td>7.17e-11</td>
</tr>
</tbody>
</table>
```

The small **PValue** indicates the significant testing result. The result **Down** in the **Direction** column indicates negative correlation between the methylation and gene expression.

We can visualize the gene set results with a barcode plot (see Figure 7):

```r
> m <- match(row.names(rna_DE), row.names(tp))
> gw <- tp$logFC[m]
> gw[is.na(gw)] <- 0
> barcodeplot(rna_DE$logFC, gene.weights=gw, labels=c("Luminal","Basal"),
+             main="Basal vs Luminal")
> legend("topright", col=c("red","blue"), lty=1, lwd=2,
+        legend=c("Up-methylation in Basal", "Up-methylation in Luminal") )
```

In the barcode plot, genes are sorted left to right according to expression changes. Genes up-regulated in luminal are on the left and genes up-regulated in basal are on the right. The x-axis shows the expression log2-fold change between basal and luminal. The vertical red bars indicate genes up-methylated in basal and vertical blue bars indicate genes down-methylated in basal. The variable-height vertical bars represent the methylation log-fold changes. The red and blue worms measure relative enrichment, showing that increased methylation is associated with decreased regulation and down-methylation is associated with up-regulation. In other words, there is a negative association between methylation of promoter regions and expression of the corresponding gene.
Packages used
This workflow depends on various packages from version 3.6 of the Bioconductor project, running on R version 3.4.0 or higher. Most of the workflow also works with Bioconductor 3.5, but the code in the last section (Correlate with RNA-seq samples) requires some minor changes for use with Bioconductor 3.5 because the earlier version of `topTags` did not preserve row names in the output table. A complete list of the packages used for this workflow is shown below:

```r
> sessionInfo()
```

R version 3.4.2 (2017-09-28)
Platform: x86_64-w64-mingw32/x64 (64-bit)
Running under: Windows 10 x64 (build 15063)

Matrix products: default

locale:
[1] LC_COLLATE=English_Australia.1252  LC_CTYPE=English_Australia.1252
[3] LC_MONETARY=English_Australia.1252 LC_NUMERIC=C
[5] LC_TIME=English_Australia.1252

attached base packages:
[1] stats4 parallel stats graphics grDevices utils datasets methods base
other attached packages:
[1] org.Mm.eg.db_3.4.2
[2] TxDb.Mmusculus.UCSC.mm10.knownGene_3.4.0
[3] GenomicFeatures_1.30.0
[4] AnnotationDbi_1.40.0
[5] Biobase_2.38.0
[6] GenomicRanges_1.30.0
[8] tRanges_2.12.0
[9] S4Vectors_0.16.0
[10] BiocGenerics_0.24.0
[12] limma_3.34.0
[13] knitr_1.17

loaded via a namespace (and not attached):
[1] Rcpp_0.12.13               compiler_3.4.2
[3] highr_0.6                  XVector_0.18.0
[5] prettyunits_1.0.2          bitops_1.0-6
[7] tools_3.4.2                zlibbioc_1.24.0
[9] progress_1.1.2             statmod_1.4.30
[11] biomaRt_2.34.0             digest_0.6.12
[13] bit_1.1-12                 RSQLite_2.0
[15] evaluate_0.10.1            memoise_1.1.0
[17] tibble_1.3.4               lattice_0.20-35
[19] pkgconfig_2.0.1            rlang_0.1.4
[21] Matrix_1.2-11              DelayedArray_0.4.1
[23] DBI_0.7                    GenomeInfoDbData_0.99.1
[25] rtracklayer_1.38.0         stringr_1.2.0
[27] Biostrings_2.46.0          locfit_1.5-9.1
[29] bit64_0.9-7                grid_3.4.2
[31] R6_2.2.2                   BiocParallel_1.12.0
[33] XML_3.98-1.9               RMySQL_0.10.13
[35] blob_1.1.0                  magrittr_1.5
[37] matrixStats_0.52.2         GenomicAlignments_1.14.0
[39] Rsamtools_1.30.0           SummarizedExperiment_1.8.0
[41] assertthat_0.2.0           stringi_1.1.5
[43] RCurl_1.95-4.8

Data and software availability
All data and supporting files used in this workflow are available from: http://bioinf.wehi.edu.au/edgeR/
F1000Research2017

Archived code/data as at time of publication: http://doi.org/10.5281/zenodo.1052871

All software used is publicly available as part of Bioconductor 3.6.

Competing interests
No competing interests were disclosed.
Grant information

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Acknowledgments

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References


Open Peer Review

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Version 1

Referee Report 20 December 2017

https://doi.org/10.5256/f1000research.14317.r28485

Peter F. Hickey
Department of Biostatistics, Johns Hopkins University, Baltimore, MD, USA

Chen et al. propose a novel use of negative binomial generalized linear models (GLMs), as implemented in the edgeR software, to test for differential methylation from bisulfite-sequencing data, particularly reduced representation bisulfite-sequencing (RRBS). By leveraging the existing edgeR software, a popular tool in the analysis of differential gene expression analysis from RNA-seq data, this method is immediately able to handle complex experimental designs and integrates with downstream analysis tools such as gene set tests provided by the limma software. The paper is well-written and I was able to reproduce the authors’ analysis. It will be a useful workflow for people needing to develop an analysis of RRBS data.

Like Simon Andrews, I initially struggled a little with some of the detail of the method itself. The method’s elegance and power, like all those based on (generalized) linear models, is driven by careful formulation of the design matrix and the choice of contrasts. Necessarily, the design matrix for analysing bisulfite-sequencing data is more complex than that used to analyse RNA-seq data from an identical experimental design. As I know the authors are well aware, getting the design matrix and contrasts correct is 95% of the battle for most people analysing data with edgeR and limma. I will explain my concerns below (many are the same as raised by Simon in his review).

Main points

- p4: The initial example has no replicates. Since the method is designed for “any BS-seq data that includes some replication”, should this example include replicates? I appreciate the desire to keep the initial example simple (especially in light of my next comment).
- p4: I initially found the design matrix confusing. In fact, I had the same reaction/interpretation as Simon Andrews 4th comment: “In the small example description you say that A_MvsU estimates the log ratio for Sample1, but it wasn’t clear to me why this would apply to only Sample 1 since the factor has a 1 against the meth count for both samples 1 and 2”. I had to manually check a few quantities to convince myself, e.g., to rounding error, `.coef(fit)[, "A_MvsU"]` is `logit((2 + prior.count) / (2 + prior.count + 12 + prior.count))`, where `prior.count = 0.125`. Because so much depends on constructing the appropriate design matrix, this description/section may warrant further explanation (e.g., comparing to some manually computed quantities).
- Like James MacDonald, although the code was clearly written, I was a little surprised that it didn’t use more consistent integration with existing Bioconductor packages and data structures. To add to his example, almost all the work in the section ‘Reading in the data’ can be achieved with `bsseq::read.bismark(fn)` , which will: read in an arbitrary number of Bismark `.cov.gz` files, appropriately combine samples with different sets of CpGs, and return a `SummarizedExperiment`
appropriately combine samples with different sets of CpGs, and return a `SummarizedExperiment`-derived object (a `BSseq` instance) which could readily be used to construct the `DGEList` used in the analysis. In my experience, loading the data and combining different sets of loci is a step fraught with danger of hard-to-track-down errors, so it may be better to advise workflow users to use a fairly well-tested function. Full disclosure: I am the author of `bsseq::read.bismark`

- p14: The aggregation of CpGs to promoters may lead to surprising results. An (extreme) example: the first half of a promoter is methylated in one condition and unmethylated in the other, and vice versa for the second half of the promoter. In aggregate over the promoter the proportion of methylated CpGs may be similar in both conditions, yet this promoter is clearly differentially methylated. I think a note encouraging workflow users to think carefully about their hypothesis when doing this form of aggregation is warranted.

Minor points

- p1: “The most commonly used technology of studying DNA methylation is bisulfite sequencing (BS-seq)”. The Illumina 27k/450k/EPIC microarrays are the most commonly used ‘genome-wide’ assays for studying DNA methylation. However, (whole genome) BS-seq is arguably the gold standard genome-wide assay.
- p3: I think there’s some confusion about CpGs and CpG islands (CGI). Approximately 0.9% of dinucleotides in the human genome (hg19) are CpGs, and approximately 0.7% of the genome is a CGI (using UCSC CGIs, which is not the only definition but perhaps the standard); see code below:

```R
library(BSgenome.Hsapiens.UCSC.hg19)

hg19_size <- sum(as.numeric(seqlengths(BSgenome.Hsapiens.UCSC.hg19)[
paste0("chr", c(1:22, "X", "Y"))])

# CpGs on chr1-22,chrX,chrY in hg19
n_CpGs <- Reduce(sum, bsapply(BSParms = new("BSParams",
    X = BSgenome.Hsapiens.UCSC.hg19,
    FUN = countPattern,
    exclude = c("M", "."),
    pattern = "CG"))
100 * n_CpGs / hg19_size

# CGIs in hg19
library(rtracklayer)

my_session <- browserSession("UCSC")
genome(my_session) <- "hg19"
cgi <- track(ucscTableQuery(my_session, track = "cpgIslandExt"))
sum(width(cgi)) / hg19_size
```

- p3: Possible type, “with a large genome”
- p3: “WGBS is more suitable for studies where all CpG islands or promoters across the entire genome are of interest.” Might also add ‘distal regulatory elements’ and CG-poor regions (RRBS targets CG-rich regions of the genome).
- p3: `BSSmooth` (implemented in `bsseq`) doesn’t use Empirical Bayes although it does use `limma` for linear regression
- p4: Missing a `library(edgeR)` in order for the code to work
- p4: There’s an extra parenthesis at the end of line 2 when constructing `dimnames(counts)`
- p4: The authors note that the method is “especially appropriate for RRBS data”. Is the main challenge for running on WGBS data that of computational resources?
Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Gordon Smyth, Walter and Eliza Hall Institute of Medical Research, Australia

Thank you for your thoughtful and positive comments on our article. We particularly wish to thank you for your comments on the introductory material, which we have rewritten to take account of all your suggestions. We have specified the version of Bowtie, adopted the “hyper” and “hypo” terminology and made all the other corrections. In this revision we have added a lot of new material to the expository section, including an example with replicates, while retaining the first example without replicates. The purpose of the first example is to introduce the key mathematical idea that underlies our article, which is the use of linear model terms to account for total read coverage at each CpG, and to relate this to the more familiar beta-binomial modeling approach. This idea is already manifest in the very small dataset without
replicates, and so that is the most fundamental context in which to exposit the idea. The need for replicates relates to dispersion estimation, which is a separate issue. For pedagogic purposes it is best to isolate these concepts separately.

We now demonstrate in the first example how the logFC is computed in complete arithmetic detail. We have switched the toy examples to use the group-mean parametrization instead of the treatment-parametrization so that the computation of logFC is explicit rather than implicit. See also our reply to Simon Andrews.

We have introduced a new function modelMatrixMeth() to automate the construction of the design matrix. From a user point of view, the design matrix is now made exactly as it would be for an RNA-seq analysis, with the sample effects added automatically. We have also added a new section “Analogy with paired-samples expression analyses” to show that the entire linear modeling approach is equivalent to an (already well understood) type of RNA-seq experiment.

The purpose of our article is to show that methylation data can be analyzed exactly like RNA-seq and we use the same packages and data structures as our earlier RNA-seq workflow article (Chen et al, 2017). Adding extra packages and data structures would complicate rather than help our workflow.

We are aware of your read.bismark() function in the bbseq package. When run on our data, read.bismark() produces a number of alarming warnings suggesting that incorrect genome annotation has been used for some of the samples. These warnings are false alarms, but would worry users, so we would have to either suppress them or explain them to readers, were we to adopt read.bismark.

We are also aware of the readBismark() function in the BiSeq package and the methRead() function in the methylKit package. It seems that every Bioconductor package that analyses BS-seq has its own read function. The functions read the methylation data into a binomial format, whereas the whole point of our article is to show that the methylated and unmethylated counts can be analysed as they are, instead of doing a binomial analysis. All produce data structures that are incompatible with edgeR. We could wrangle the data formats back to a DGEList, but the end result would be code that is no shorter or simpler than our original plain base R code. In fact the code would be harder to follow because of the extra packages and data structures involved.

It seems unfortunate that the Bioconductor methylation packages all create their own read functions from scratch in order to create their own specialist S4 objects. Each defines a complex S4 class, unique to that one package. If Bioconductor provided a fast, light-weight, base-R style function to read Bismark files, then all the packages could use that as building block instead of re-writing from scratch.

In this revision, we have decided to create a new function readBismark2DGE() to read the Bismark files directly into a DGEList object, substantially simplifying our workflow for users. The new function is faster than any existing options and over 3 times as fast as read.bismark() on the workflow data. The new function creates simple list output with minimal processing and so serves the purpose of a light-weight base-R style function.

You comment on aggregation over promoters and guidance about hypotheses. We have added more text to clarify that aggregation of counts over gene promoters tests for overall changes only, but we think that users would have expected this already and shouldn’t be surprised.

The thought example you give is indeed extreme and does not match real that we have seen. Even if such a phenomenon could occur, our workflow includes a CpG-level analysis, so readers can easily check for CpGs changing in different directions within a promoter if that is of interest to them. We did this check ourselves and found that for 99% of genes whose promoters contain any DM CpGs, all the DM CpGs change in the same direction. In almost all the remaining cases, only one CpG changes in the “wrong” direction. The only exception is Mir1298 on Chromosome X that has two CpGs changing in the other direction to the majority.
Even if there were opposing effects within a few gene promoters, it wouldn’t invalidate our aggregate analysis. It would simply mean that exceptional genes would have to be analyzed and interpreted individually to understand the fully story, and we see no problem with that. We think there is a role for both aggregate analyses and high-resolution analyses of DNA methylation. However we think that aggregate analyses are under-used in the literature and should be encouraged more than at present. We think that genomic analysts sometimes try to accommodate too much complexity, leading to analyses that lack statistical power. This in turn leads to the danger of cherry picking, possibly over-interpreting results that might not be reproducible. Aggregate analyses ignore some of the complexity, but can lead to more reliable and interpretable “big picture” messages. In this article, our promoter region aggregated analysis leads to a clean and reliable correlation of methylation with RNA-seq results, a correlation that is more difficult to achieve by other means.

Our remark that our method is especially appropriate for RRBS is prompted by the thought that researchers engaged in a WGBS study may have slightly different aims, and this goes back to your comment about hypotheses. In particular, it may be important in a WGBS analysis to discover DMRs in a de novo fashion, pooling results for consecutive genomic loci without the use of gene de novo annotation. We have added remarks in the Introduction and the Discussion to clarify our aims and to briefly indicate possible extensions. There is no problem with computational resources – our pipeline is reasonably efficient.

In this revision, we have relaxed the filtering threshold to 8 instead of 10 reads per CpG, although it makes no material difference to our analysis. While the filtering does indeed remove 85% of the CpGs, it keeps more than half the counts. The number of CpGs retained in the analysis is plenty for our purposes.

In this revision, we form the MDS plot in a slightly better way and P8_6 is no longer an outlier. In Figure 2, the meaning of “average abundance” is explained by the axis label as log count per million, i.e., coverage for that CpG divided by sequencing depth. In effect, it is the coverage for each CpG relative to other CpGs. It is independent of sequencing depth.

**Competing Interests:** No competing interests were disclosed.

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Referee Report 18 December 2017

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James W. MacDonald

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This is primarily a software pipeline article, showing how to use the Bioconductor edgeR package to analyze RRBS data, but to a certain extent is also a methods paper, as to my knowledge this is the first proposal for directly analyzing count data rather than converting to either ratios and using a beta-binomial, or to logits and using conventional linear modeling. This is an interesting idea, and should be explored further, but for this manuscript the main goal is to present the software pipeline.

The authors progress through each step of the pipeline, clearly describing each step as well as providing code (and links to the underlying data), so readers can easily understand the process and get some hands on experience as well.
The code is clearly written, and as straightforward as one could expect for a relatively complex analysis. However, I would prefer to see more consistent integration with other Bioconductor packages. In particular, when reading in the raw data, the authors use a clever trick to account for the fact that not all samples have reads for the same genomic positions. This step could just as easily be accomplished using the Bioconductor GenomicRanges package, which is intended for manipulating genomic data. In fact, the authors use GenomicRanges later in the pipeline to subset the methylation data to just gene promoter sites, so it would be more natural to start with a GRanges if you will need one later anyway.

Otherwise this is a good article that clearly shows how one could use an innovative method to analyze RRBS data using the edgeR package.

Typos:
Under a small example section, (BvsA_MvsU) estimates the difference in logit proportions of methylated

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 01 Oct 2018
Gordon Smyth, Walter and Eliza Hall Institute of Medical Research, Australia

Thank you for your positive report on our article. You’re right, there is a novel methods proposal in our article, which we will seek to justify in more detail in a methodological article elsewhere, but the current article concentrates on the software pipeline. In this revision, we have introduced new functions readBismark2DGE() and nearestTSS() to simplify the workflow. These functions allow us to eliminate from the workflow any code chunks that involve substantial programming, making the workflow considerably easier for users. We felt the original code chunk to find CpGs in promoters in particular was too complex for the purposes of our article and assumed too much user knowledge.
I have used GenomicRanges for other projects (https://f1000research.com/articles/4-1080) but it doesn't help with the current workflow. The file reading code would be similar in length and speed with or without GenomicRanges, so we tend to give preference to R base. In any case, we have eliminated that code entirely, as explained in our discussion with Peter Hickey. The new nearestTSS() function is more informative than the old code, as well as being fast. It has the added advantage of using the Bioconductor organism package, which has more up-to-date TSS annotation than the transcript package.

**Competing Interests:** No competing interests were disclosed.

### Referee Report 07 December 2017

https://doi.org/10.5256/f1000research.14317.r28483

**Simon Andrews**

Bioinformatics Group, Babraham Institute, Cambridge, UK

Chen et al present an interesting re-application of the EdgeR analysis package to the analysis of bisulphite sequencing data. The method they propose would utilise the existing negative biomial models within EdgeR and would potentially provide the power which comes with the linear model framework to bisulphite data. The method described requires no changes to EdgeR itself, and merely describes a suitable formulation of design matrix to allow this to be applied to bisulphite data.

The article is generally well written and the authors go to great lengths to break down and describe the method. They also provide a page from which all of the underlying data and code can be obtained and I was able to reproduce the results, and independently verify them in a parallel analysis.

The main thing which I struggled with was some of the detail in the description of the method itself. There were some parts which I wasn't clear on, and some nomenclature which didn't help in understanding the explanation. I'll try to lay out my concerns below:

1) In the small example I completely understand that the authors wanted to keep this as simple as possible, but it might have helped to have had 2 samples per condition so that the full complexity of the method is visible.

2) There is a typo in the code for the small example so it doesn't run as is. The list function on line 2 has an extra bracket at the end.

3) The nomenclature in the small example is inconsistent. You have samples 1 and 2, but (in the table) also conditions 1 and 2, but in the code the conditions are A and B. If you had Samples 1,2,3,4 in conditions A and B this might help to alleviate some of the confusion.

4) In the small example description you say that A_MvsU estimates the log ratio for Sample1, but it wasn't clear to me why this would apply to only Sample 1 since the factor has a 1 against the meth count for both samples 1 and 2.

In the expanded examples there were also some points on which I wasn't clear.
5) You calculate a single dispersion parameter for all data points and say that in contrast to RNA-Seq there is no global trend to follow. It wasn't clear to be exactly why this is since read count and methylation level would all affect the dispersion - is it simply because these factors are explicitly accounted for in the linear model?

6) In the design matrix for the RRBS it wasn't clear why the first column was all 1s, whereas the rest obviously matched the condition from which they came. This also contrasted with the simple example where the structure wasn't like this. Is this because you were comparing both P7 and P8 to P6?

7) I think this is possibly the same thing as point 4, but you say that the Me column represents the methylation level in P6, but again this highlights the methylated values in all samples, so why only P6?

For the final results obtained it would have been nice to show the general level of concordance with running the same analysis through one of the beta-distribution models to either show general agreement, or to generally explain any major differences.

Minor points:

In the introduction you say that "40% of mammalian genes and 70% of human genes have CpG islands enriched in their promoter regions". Enriched probably isn't the right word to use (or you need to say that CpGs are enriched rather than islands). The difference between 'humans' and 'mammals' is also somewhat contentious - non-human mammals certainly have weaker CpG islands which get missed by CpG island prediction tools, but for example in mouse Illingworth et al showed that if you use CpG binding protein ChIP that you can see about the same number of islands in both species.

It's also not really fair to say that CpG methylation in promoters is "generally" associated with repression of transcription. There is a categorical expression level shift associated with the presence/absence of CpG islands, but you can make a Dnmt1 knockout which removes pretty much all methylation from the genome and for the vast majority of genes their transcription is completely unaffected.

P3 "with large genome" should be "with large genomes"
P5 "mythylated" should be "methylated"

Is the rationale for developing the new method (or application) clearly explained? Yes

Is the description of the method technically sound? Partly

Are sufficient details provided to allow replication of the method development and its use by others? Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility? Yes
Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**Gordon Smyth, Walter and Eliza Hall Institute of Medical Research, Australia**

Thank you for your thoughtful comments on our article. We have rewritten the Introduction to take account of your comments and have fixed all typos. We have also expanded the expository section on the NB linear modeling approach to five pages instead of one.

The purpose of the first toy example (Table 1) is to introduce the key mathematical idea that underlies our article, which is the use of linear model terms to account for read coverage at each CpG, and to relate this to the more familiar beta-binomial modeling approach. This idea is already manifest in the very small dataset without replicates, making the one-sample example the most fundamental and effective context in which to introduce the idea. We agree however that the condition labels in Table 1 should be A and B.

Following your remarks, we have included a second example with 2 samples per condition (Table 2). Keep in mind though that the edgeR approach to methylation however is very powerful and flexible and its full complexity is still far from being visible in a single locus example like this. To see more of the features in play, one has to work through the full data example. We have however taken the opportunity to preview more ideas in the expanded linear modeling introduction, including dispersion estimation and the use of contrasts.

You asked about the A_MvsU coefficient computation. This is a standard linear modeling result in R but one that does confuse a lot of people when they see it for the first time. You will see analogous results explained in the limma and edgeR User’s Guides. Anyway, we have rewritten the first example in a different way so that the coefficients are defined more explicitly and their calculation is explained in full arithmetic detail.

The original implicit formulation of the logFC is now presented in the section “Another way to make the design matrix”. Consider the last two columns of the design matrix in that section, which are now called “Intercept” and “ConditionB”, and write $\beta_A$ and $\beta_{BvsA}$ for the corresponding two coefficients. The interpretation of these coefficients becomes clear by multiplying the coefficients by the design matrix. For samples corresponding to condition A, the methylation level is represented by $\beta_A$ alone because the last column of the design matrix is zero (rows 1 and 3). When condition B is applied, the methylation is modelled by $\beta_A + \beta_{BvsA}$ (rows 5 and 7). So it is apparent that $\beta_A$ must represent the methylation status for condition A while $\beta_{BvsA}$ must be the difference in methylation between B and A. Users don’t need to follow this mathematics, we just present it here for completeness.

You also asked about the first column of the design matrix. It doesn’t matter whether the first column is all 1s or not, as long as the leading columns of the design matrix span the sample effects. We have added a small example on pages 3-4 to demonstrate this. The first few columns of the design matrix model the sample coverages, and these columns are quite independent of the
remaining columns which model the treatment conditions. We can compare the treatment conditions in any way we wish regardless of how the design matrix is parametrized. We have added some material to the introduction to try to explain this but, in any case, it isn’t something that a user needs to worry about.

In this revision, we have introduced a new function modelMatrixMeth to create the design matrix automatically, so users now only need to focus on the treatment conditions, not on how to adjust for the sample effects.

There is no reason why the dispersion should be a function of read count or of methylation level. The purpose of the NB statistical model is to capture the technical mean-variance trends so that the dispersion parameter can be interpreted independently of these things. We have added a sub-section on dispersion estimation to page 7 to try to make this clear.

Regarding concordance with other methylation analysis software, we do not know of any beta-binomial modeling software that is able to conduct an equivalent analysis to that presented in our article. We have formed a contrast between luminal cells and the two basal populations, and no other software can do that as far as we know. This is an example of the extra flexibility provided by our approach. The DSS Bioconductor package is documented to be able to form general contrasts between treatment conditions, but this is not effective in practice because it does not distinguish hyper from hypo methylation for a contrast.

Our experience is that edgeR gives better DM results than beta-binomial software but it would be unfair for us to claim that in our article without undertaking a full comparison study with rigorous benchmarking. Our plan is to publish such a comparison elsewhere.

**Competing Interests:** No competing interests were disclosed.