

# Biseq: A package for analyzing targeted bisulfite sequencing data

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# 1 Introduction

The *BiSeq* package provides useful classes and functions to handle and analyze targeted bisulfite sequencing (BS) data such as reduced representation bisulfite sequencing (RRBS) data. In particular, it implements an algorithm to detect differentially methylated regions (DMRs), as described in detail in [1]. The package takes already aligned BS data from one or multiple samples. Until now, it was used for the analysis of CpG methylation of human and mouse samples only.

## 2 Data import and classes

### 2.1 Sample data

As sample data we use a small part of a recently published data set, see [2]. It comprises RRBS data from 10 samples of CpG sites from genomic regions on p arms of chromosome 1 and 2 covered in at least one sample. Data was obtained from 5 bone marrow probes of patients with acute promyelocytic leukemia (APL) and 5 control samples (APL in remission). RRBS data was preprocessed with the Bismark software version 0.5 [3].

### 2.2 Import of Bismark's methylation output files

Bismark [3] a bisulfite read mapper and methylation caller. *BiSeq* allows the import of Bismark output files.

```
> library(BiSeq)
```

`readBismark` imports the CpG context output files created by the methylation extractor of Bismark:

```
> readBismark(files, colData)
```

The argument `files` point to files created by Bismark's `methylation_extractor` and `bismark2bedGraph` (see the man page of `readBismark` for details on how to retrieve the right input files). `colData` specifies the sample names and additional phenotype information. This method returns a *BSraw* object.

### 2.3 The *BSraw* and *BSrel* classes

The *BiSeq* package contains the classes *BSraw* and *BSrel*, both derived from *RangedSummarizedExperiment*.

#### 2.3.1 The *BSraw* class

The *BSraw* class is a container for 'raw' RRBS data. It comprises sample information together with CpG positions and numbers of reads spanning the CpG positions as well as the number of methylated reads.

A *BSraw* object consists of four slots:

1. A `list` of arbitrary content describing the overall experiment, accessible with `metadata`.
2. A `GRanges` of the positions of CpG-sites covered by BS in at least one sample, accessible with `rowRanges`.
3. A `DataFrame` of samples and the values of variables measured on those samples, accessible with `colData`.
4. An `assays` slot containing a `SimpleList` of two matrices, one containing the numbers of reads (accessible with `totalReads`) and the other the numbers of methylated reads (accessible with `methReads`).

A new *BSraw* object can also be created by hand:

```
> metadata <- list(Sequencer = "Instrument", Year = "2013")
> rowRanges <- GRanges(seqnames = "chr1",
                        ranges = IRanges(start = c(1,2,3), end = c(1,2,3)))
> colData <- DataFrame(group = c("cancer", "control"),
                       row.names = c("sample_1", "sample_2"))
> totalReads <- matrix(c(rep(10L, 3), rep(5L, 3)), ncol = 2)
> methReads <- matrix(c(rep(5L, 3), rep(5L, 3)), ncol = 2)
> BSraw(metadata = metadata,
        rowRanges = rowRanges,
        colData = colData,
        totalReads = totalReads,
        methReads = methReads)
```

Nevertheless, users will most likely create *BSraw* objects when use `readBismark` to load data.

We load and show the APL data:

```
> data(rrbs)
> rrbs

class: BSraw
dim: 10502 10
metadata(0):
assays(2): totalReads methReads
rownames(10502): 1456 1457 ... 4970981 4970982
```

```
rowData names(0):
colnames(10): APL1 APL2 ... APL11624 APL5894
colData names(1): group
```

We show the sample characteristics slot:

```
> colData(rrbs)

DataFrame with 10 rows and 1 column
      group
  <factor>
APL1      APL
APL2      APL
APL3      APL
APL7      APL
APL8      APL
APL10961  control
APL11436  control
APL11523  control
APL11624  control
APL5894   control
```

The first CpG sites on chromosome 1 which were covered:

```
> head(rowRanges(rrbs))

GRanges object with 6 ranges and 0 metadata columns:
      seqnames      ranges strand
      <Rle> <IRanges>  <Rle>
1456      chr1      870425      +
1457      chr1      870443      +
1458      chr1      870459      +
1459      chr1      870573      +
1460      chr1      870584      +
1461      chr1      870599      +
-----
seqinfo: 25 sequences from an unspecified genome; no seqlengths
```

The coverage of the first CpG sites per sample:

```
> head(totalReads(rrbs))
```

|      | APL1 | APL2 | APL3 | APL7 | APL8 | APL10961 | APL11436 | APL11523 |
|------|------|------|------|------|------|----------|----------|----------|
| 1456 | 39   | 6    | 10   | 0    | 0    | 48       | 31       | 65       |
| 1457 | 39   | 6    | 10   | 0    | 0    | 48       | 31       | 65       |
| 1458 | 39   | 6    | 10   | 0    | 0    | 48       | 27       | 65       |
| 1459 | 20   | 26   | 49   | 48   | 39   | 27       | 23       | 34       |
| 1460 | 20   | 26   | 49   | 48   | 39   | 27       | 23       | 34       |
| 1461 | 20   | 26   | 49   | 48   | 39   | 27       | 22       | 34       |

|      | APL11624 | APL5894 |
|------|----------|---------|
| 1456 | 39       | 29      |
| 1457 | 39       | 29      |
| 1458 | 39       | 29      |
| 1459 | 29       | 15      |
| 1460 | 28       | 15      |
| 1461 | 29       | 15      |

The number of methylated reads of the first CpG sites per sample:

```
> head(methReads(rrbs))
```

|      | APL1 | APL2 | APL3 | APL7 | APL8 | APL10961 | APL11436 | APL11523 |
|------|------|------|------|------|------|----------|----------|----------|
| 1456 | 32   | 6    | 7    | 0    | 0    | 15       | 23       | 16       |
| 1457 | 33   | 6    | 7    | 0    | 0    | 18       | 10       | 19       |
| 1458 | 33   | 6    | 10   | 0    | 0    | 20       | 10       | 19       |
| 1459 | 13   | 20   | 34   | 41   | 32   | 3        | 8        | 8        |
| 1460 | 14   | 18   | 35   | 37   | 33   | 2        | 4        | 4        |
| 1461 | 14   | 16   | 35   | 40   | 31   | 5        | 5        | 5        |

|      | APL11624 | APL5894 |
|------|----------|---------|
| 1456 | 7        | 7       |
| 1457 | 2        | 7       |
| 1458 | 2        | 3       |
| 1459 | 6        | 4       |
| 1460 | 3        | 0       |
| 1461 | 1        | 2       |

### 2.3.2 The BSrel class

The *BSrel* is a container for 'relative' methylation levels of BS data. It comprises sample information together with CpG positions and the relative methylation val-

ues (between 0 and 1).

A *BSrel* object consists of four slots:

1. A list of arbitrary content describing the overall experiment, accessible with `metadata`.
2. A *GRanges* of the positions of CpG-sites covered by BS in at least one sample, accessible with `rowRanges`.
3. A *DataFrame* of samples and the values of variables measured on those samples, accessible with `colData`.
4. An `assays` slot containing a *SimpleList* of a matrix with the relative methylation levels (between 0 and 1), accessible with `methLevel`.

A new *BSraw* object can be created by:

```
> methLevel <- matrix(c(rep(0.5, 3), rep(1, 3)), ncol = 2)
> BSrel(metadata = metadata,
        rowRanges = rowRanges,
        colData = colData,
        methLevel = methLevel)
```

We can convert a *BSraw* object to a *BSrel* object easily:

```
> rrbs.rel <- rawToRel(rrbs)
> rrbs.rel

class: BSrel
dim: 10502 10
metadata(0):
assays(1): methLevel
rownames(10502): 1456 1457 ... 4970981 4970982
rowData names(0):
colnames(10): APL1 APL2 ... APL11624 APL5894
colData names(1): group
```

The relative methylation values of the first CpG sites:

```
> head(methLevel(rrbs.rel))
```



|      | APL1      | APL2      | APL3      | APL7      | APL8      |
|------|-----------|-----------|-----------|-----------|-----------|
| 1456 | 0.8205128 | 1.0000000 | 0.7000000 | NaN       | NaN       |
| 1457 | 0.8461538 | 1.0000000 | 0.7000000 | NaN       | NaN       |
| 1458 | 0.8461538 | 1.0000000 | 1.0000000 | NaN       | NaN       |
| 1459 | 0.6500000 | 0.7692308 | 0.6938776 | 0.8541667 | 0.8205128 |
| 1460 | 0.7000000 | 0.6923077 | 0.7142857 | 0.7708333 | 0.8461538 |
| 1461 | 0.7000000 | 0.6153846 | 0.7142857 | 0.8333333 | 0.7948718 |

|      | APL10961   | APL11436  | APL11523  | APL11624   | APL5894   |
|------|------------|-----------|-----------|------------|-----------|
| 1456 | 0.31250000 | 0.7419355 | 0.2461538 | 0.17948718 | 0.2413793 |
| 1457 | 0.37500000 | 0.3225806 | 0.2923077 | 0.05128205 | 0.2413793 |
| 1458 | 0.41666667 | 0.3703704 | 0.2923077 | 0.05128205 | 0.1034483 |
| 1459 | 0.11111111 | 0.3478261 | 0.2352941 | 0.20689655 | 0.2666667 |
| 1460 | 0.07407407 | 0.1739130 | 0.1176471 | 0.10714286 | 0.0000000 |
| 1461 | 0.18518519 | 0.2272727 | 0.1470588 | 0.03448276 | 0.1333333 |

## 2.4 Data handling

All methods for *RangedSummarizedExperiment* objects are applicable for *BSraw* and *BSrel* objects:

```
> dim(rrbs)

[1] 10502    10

> colnames(rrbs)

[1] "APL1"      "APL2"      "APL3"      "APL7"      "APL8"
[6] "APL10961" "APL11436" "APL11523" "APL11624" "APL5894"
```

We can return subsets of samples or CpG sites:

```
> rrbs[, "APL2"]
> ind.chr1 <- which(seqnames(rrbs) == "chr1")
> rrbs[ind.chr1,]
```

We can also subset by overlaps with a *GRanges* object:

```
> region <- GRanges(seqnames="chr1",
                     ranges=IRanges(start = 875200,
                                     end = 875500))
```

```
> findOverlaps(rrbs, region)
> subsetByOverlaps(rrbs, region)
```

We can sort *BSraw* and *BSrel* objects into ascending order of CpG sites positions on chromosomes:

```
> sort(rrbs)
```

*BSraw* and *BSrel* objects can be combined and splitted:

```
> combine(rrbs[1:10, 1:2], rrbs[1:1000, 3:10])
> split(rowRanges(rrbs),
        f = as.factor(as.character(seqnames(rrbs))))
```

### 3 Quality control

Via two very simple methods it is possible to compare the sample's coverages. `covStatistics` lists the number of CpG sites that were covered per sample together with the median of the coverage of these CpG sites. `covBoxplots` represent the coverage distributions per sample.

```
> covStatistics(rrbs)
```

```
$Covered_CpG_sites
```

| APL1     | APL2     | APL3     | APL7    | APL8 | APL10961 |
|----------|----------|----------|---------|------|----------|
| 5217     | 4240     | 4276     | 3972    | 3821 | 5089     |
| APL11436 | APL11523 | APL11624 | APL5894 |      |          |
| 5169     | 6922     | 6483     | 7199    |      |          |

```
$Median_coverage
```

| APL1     | APL2     | APL3     | APL7    | APL8 | APL10961 |
|----------|----------|----------|---------|------|----------|
| 12       | 5        | 12       | 15      | 11   | 10       |
| APL11436 | APL11523 | APL11624 | APL5894 |      |          |
| 6        | 8        | 4        | 5       |      |          |

```
> covBoxplots(rrbs, col = "cornflowerblue", las = 2)
```

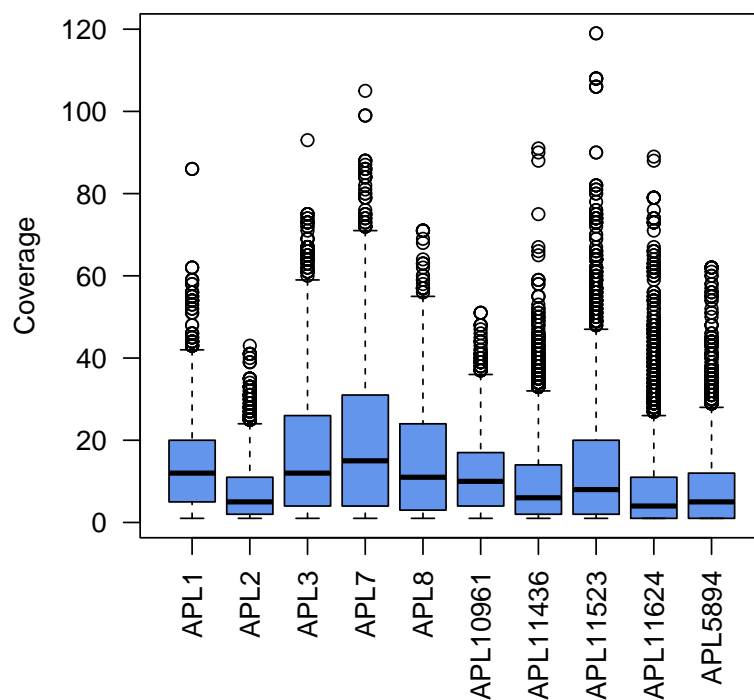


Figure 1: Sample wise coverage distributions

## 4 Detection of DMRs within groups of samples

The algorithm to detect differentially methylated regions (DMRs) within two groups of samples (e.g. cancer and control) is described in detail in [1]. To better understand this User's guide it is helpful to know the rough procedure. The DMR detection is a five-steps approach:

1. Definition of CpG clusters
2. Smooth methylation data within CpG clusters
3. Model and test group effect for each CpG site within CpG clusters
4. Apply hierarchical testing procedure:
  - (a) Test CpG clusters for differential methylation and control weighted FDR on cluster
  - (b) Trim rejected CpG clusters and control FDR on single CpGs
5. Define DMR boundaries

Please see [1] for more details.

### 4.1 Definition of CpG clusters

In order to smooth the methylation data we first have to detect CpG clusters (regions with a high spatial density of covered CpG sites). Within a *BSraw* object `clusterSites` searches for agglomerations of CpG sites across all samples. In a first step the data is reduced to CpG sites covered in `round(perc.samples*ncol(object))` samples (here: 4 samples), these are called 'frequently covered CpG sites'. In a second step regions are detected where not less than `min.sites` frequently covered CpG sites are sufficiently close to each other (`max.dist`). Note, that the frequently covered CpG sites are considered to define the boundaries of the CpG clusters only. For the subsequent analysis the methylation data of all CpG sites within these clusters are used.

We perform the analysis on a subset of our data to save time:

```
> rrbs.small <- rrbs[1:1000,]  
> rrbs.clust.unlim <- clusterSites(object = rrbs.small,  
                                   groups = colData(rrbs)$group,
```

```
perc.samples = 4/5,
min.sites = 20,
max.dist = 100)
```

`rrbs.clust.unlim` is a again *BSraw* object but restricted to CpG sites within CpG clusters. Each CpG site is assigned to a cluster:

```
> head(rowRanges(rrbs.clust.unlim))
```

GRanges object with 6 ranges and 1 metadata column:

|        | seqnames | ranges    | strand | cluster.id  |
|--------|----------|-----------|--------|-------------|
|        | <Rle>    | <IRanges> | <Rle>  | <character> |
| 1513   | chr1     | 872335    | *      | chr1_1      |
| 1514   | chr1     | 872369    | *      | chr1_1      |
| 401911 | chr1     | 872370    | *      | chr1_1      |
| 1515   | chr1     | 872385    | *      | chr1_1      |
| 401912 | chr1     | 872386    | *      | chr1_1      |
| 1516   | chr1     | 872412    | *      | chr1_1      |

-----

seqinfo: 25 sequences from an unspecified genome; no seqlengths

The underlying CpG clusters can also be converted to a *GRanges* object with the start and end positions:

```
> clusterSitesToGR(rrbs.clust.unlim)
```

GRanges object with 6 ranges and 1 metadata column:

|     | seqnames | ranges        | strand | cluster.id  |
|-----|----------|---------------|--------|-------------|
|     | <Rle>    | <IRanges>     | <Rle>  | <character> |
| [1] | chr1     | 872335-872616 | *      | chr1_1      |
| [2] | chr1     | 875227-875470 | *      | chr1_2      |
| [3] | chr1     | 875650-876028 | *      | chr1_3      |
| [4] | chr1     | 876807-877458 | *      | chr1_4      |
| [5] | chr1     | 877684-877932 | *      | chr1_5      |
| [6] | chr2     | 45843-46937   | *      | chr2_1      |

-----

seqinfo: 25 sequences from an unspecified genome; no seqlengths

## 4.2 Smooth methylation data

In the smoothing step CpG sites with high coverages get high weights. To reduce bias due to unusually high coverages we limit the coverage, e.g. to the 90% quantile:

```
> ind.cov <- totalReads(rrbs.clust.unlim) > 0
> quant <- quantile(totalReads(rrbs.clust.unlim)[ind.cov], 0.9)
> quant
```

```
90%
 32
```

```
> rrbs.clust.lim <- limitCov(rrbs.clust.unlim, maxCov = quant)
```

We then smooth the methylation values of CpG sites within the clusters with the default bandwidth  $h = 80$  base pairs. It is possible - and recommended - to parallelize this step by setting `mc.cores`, to 6 cores for instance, if there are 6 available.

```
> predictedMeth <- predictMeth(object = rrbs.clust.lim)
```

`predictedMeth` is a *BSrel* object with smoothed relative methylation levels for each CpG site within CpG clusters:

```
> predictedMeth
```

```
class: BSrel
dim: 344 10
metadata(0):
assays(1): methLevel
rownames(344): 1 2 ... 343 344
rowData names(1): cluster.id
colnames(10): APL1 APL2 ... APL11624 APL5894
colData names(1): group
```

The effect of the smoothing step can be shown with the `plotMeth` function:

```
> covBoxplots(rrbs.clust.lim, col = "cornflowerblue", las = 2)
```

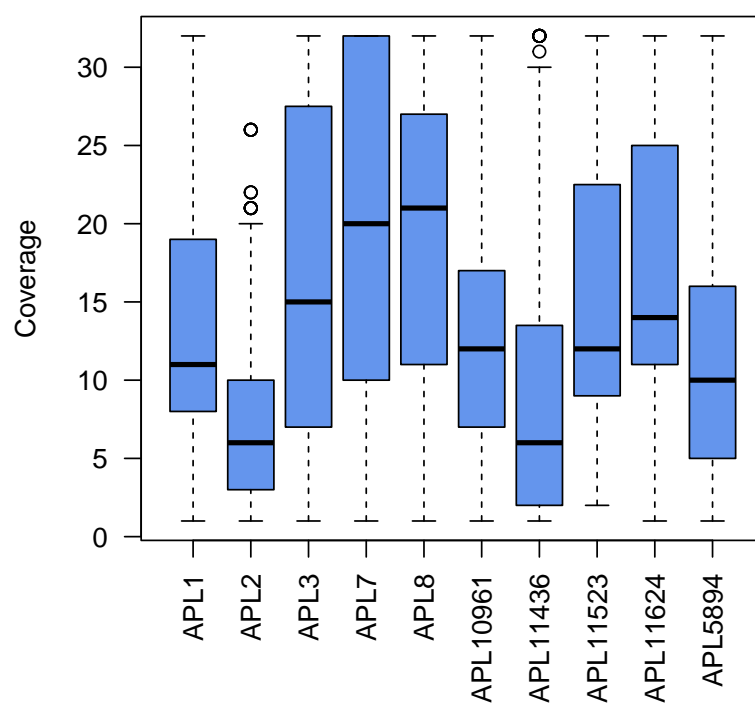


Figure 2: Sample wise coverage distributions after coverage limitation



```
> plotMeth(object.raw = rrbs[,6],
  object.rel = predictedMeth[,6],
  region = region,
  lwd.lines = 2,
  col.points = "blue",
  cex = 1.5)
```



Figure 3: Raw data together with smoothed methylation levels

### 4.3 Model and test group effect

We observe a differential methylation between cancer and control for some CpG sites:

```

> cancer <- predictedMeth[, colData(predictedMeth)$group == "APL"]
> control <- predictedMeth[, colData(predictedMeth)$group == "control"]
> mean.cancer <- rowMeans(methLevel(cancer))
> mean.control <- rowMeans(methLevel(control))
> plot(mean.control,
       mean.cancer,
       col = "blue",
       xlab = "Methylation in controls",
       ylab = "Methylation in APLs")

```



Figure 4: Smoothed methylation levels in APL and control samples

To detect the CpG sites where the DNA methylation differs between APL and control samples we model the methylation within a beta regression with the group as explanatory variable and use the Wald test to test if there is a group effect:

```

> ## To shorten the run time set mc.cores, if possible!
> betaResults <- betaRegression(formula = ~group,
                                link = "probit",
                                object = predictedMeth,
                                type = "BR")

> ## OR:
> data(betaResults)

```

betaResults is a *data.frame* containing model and test information for each CpG site:

```

> head(betaResults)

```

|        | chr  | pos    | p.val        | meth.group1 | meth.group2 |
|--------|------|--------|--------------|-------------|-------------|
| chr1.1 | chr1 | 872335 | 0.0011317652 | 0.9525098   | 0.8635983   |
| chr1.2 | chr1 | 872369 | 0.0007678027 | 0.9414368   | 0.8444060   |
| chr1.3 | chr1 | 872370 | 0.0008347451 | 0.9414314   | 0.8448725   |
| chr1.4 | chr1 | 872385 | 0.0010337477 | 0.9412217   | 0.8521862   |
| chr1.5 | chr1 | 872386 | 0.0010975571 | 0.9410544   | 0.8526863   |
| chr1.6 | chr1 | 872412 | 0.0035114839 | 0.9378250   | 0.8718024   |

|        | meth.diff  | estimate   | std.error | pseudo.R.sqr |
|--------|------------|------------|-----------|--------------|
| chr1.1 | 0.08891149 | -0.5730618 | 0.1760266 | 0.6304051    |
| chr1.2 | 0.09703074 | -0.5542171 | 0.1647422 | 0.6291904    |
| chr1.3 | 0.09655890 | -0.5522164 | 0.1652843 | 0.6246474    |
| chr1.4 | 0.08903549 | -0.5192564 | 0.1582531 | 0.6108452    |
| chr1.5 | 0.08836810 | -0.5156622 | 0.1579728 | 0.6090794    |
| chr1.6 | 0.06602261 | -0.4018161 | 0.1376551 | 0.5851744    |

|        | cluster.id |
|--------|------------|
| chr1.1 | chr1_1     |
| chr1.2 | chr1_1     |
| chr1.3 | chr1_1     |
| chr1.4 | chr1_1     |
| chr1.5 | chr1_1     |
| chr1.6 | chr1_1     |

By setting `type = "BR"` the maximum likelihood with bias reduction is called. This is especially useful, when the sample size is small, see [4]. The mean of the response (methylation) is linked to a linear predictor described by  $\sim x_1 +$

$x_2$  using a link function while the precision parameter is assumed to be constant. Sometimes the variance of DNA methylation is dependent on the group factor, e.g. the methylation variance in cancer samples is often higher than in normal samples. These additional regressors can be linked to the precision parameter within the formula of type  $\sim x_1 + x_2 \mid y_1 + y_2$  where the regressors in the two parts can be overlapping, see the documentation in the *betareg* package.

## 4.4 Test CpG clusters for differential methylation

The aim is to detect CpG clusters containing at least one differentially methylated location. To do so the P values  $p$  from the Wald tests are transformed to  $Z$  scores:  $z = \Phi^{-1}(1 - p)$ , which are normally distributed under Null hypothesis (no group effect). As cluster test statistic a standardized  $Z$  score average is used. To estimate the standard deviation of the  $Z$  scores we have to estimate the correlation and hence the variogram of methylation between two CpG sites within a cluster. The estimation of the standard deviation requires that the distribution of the  $Z$  scores follows a standard normal distribution. However, if methylation in both groups differs for many CpG sites the density distribution of P values shows a peak near 0. To ensure that the P values are roughly uniformly distributed to get a variance of the  $Z$  scores that is Gaussian with variance 1 we recommend to estimate the variogram (and hence the correlation of  $Z$  scores) under the null hypothesis. To do so we model the beta regression again for resampled data:

```
> ## Both resampled groups should have the same number of
> ## cancer and control samples:
> predictedMethNull <- predictedMeth[,c(1:4, 6:9)]
> colData(predictedMethNull)$group.null <- rep(c(1,2), 4)
> ## To shorten the run time, please set mc.cores, if possible!
> betaResultsNull <- betaRegression(formula = ~group.null,
                                   link = "probit",
                                   object = predictedMethNull,
                                   type="BR")

> ## OR:
> data(betaResultsNull)
```

We estimate the variogram for the  $Z$  scores obtained for the resampled data:

```
> vario <- makeVariogram(betaResultsNull)
```

```
> ## OR:  
> data(vario)
```

Based on the variogram plot we evaluate the sill (usually near 1) of the variogram and smooth the curve:

```
> plot(vario$variogram$v)  
> vario.sm <- smoothVariogram(vario, sill = 0.9)  
> lines(vario.sm$variogram[,c("h", "v.sm")],  
        col = "red", lwd = 1.5)  
> grid()
```



Figure 5: Estimated variogram together with the smoothed curve

The `vario.sm` object is a list of two:

```

> names(vario.sm)

[1] "variogram" "pValsList"

> head(vario.sm$variogram)

  h          v.sm
1 1 0.0000000000
2 2 0.0000000000
3 3 0.0000000000
4 4 0.0004532592
5 5 0.0019789496
6 6 0.0038612920

> head(vario.sm$pValsList[[1]])

      chr    pos      p.val meth.group1 meth.group2
chr1.1 chr1 872335 0.8567258    0.9267336    0.9191601
chr1.2 chr1 872369 0.7910594    0.9180561    0.9056264
chr1.3 chr1 872370 0.7855283    0.9187319    0.9060236
chr1.4 chr1 872385 0.7462536    0.9225722    0.9080750
chr1.5 chr1 872386 0.7462667    0.9225955    0.9081423
chr1.6 chr1 872412 0.8575813    0.9165966    0.9093737
      meth.diff      estimate std.error pseudo.R.sqrt
chr1.1 0.007573505 -0.05244358 0.2904759    0.006696118
chr1.2 0.012429670 -0.07781982 0.2937315    0.013223677
chr1.3 0.012708263 -0.07993265 0.2937384    0.013863179
chr1.4 0.014497213 -0.09359431 0.2892434    0.020416968
chr1.5 0.014453228 -0.09334700 0.2884945    0.020484807
chr1.6 0.007222875 -0.04562887 0.2542651    0.006457349
      cluster.id      z.score pos.new
chr1.1      chr1_1 -1.0657239      1
chr1.2      chr1_1 -0.8101026     35
chr1.3      chr1_1 -0.7910010     36
chr1.4      chr1_1 -0.6627467     51
chr1.5      chr1_1 -0.6627876     52
chr1.6      chr1_1 -1.0695155     78

```

We replace the `pValsList` object (which consists of the test results of the re-sampled data) by the test results of interest (for group effect):

```

> ## auxiliary object to get the pValsList for the test
> ## results of interest:
> vario.aux <- makeVariogram(betaResults, make.variogram=FALSE)
> vario.sm$pValsList <- vario.aux$pValsList
> head(vario.sm$pValsList[[1]])

```

|        | chr  | pos    | p.val        | meth.group1 | meth.group2 |
|--------|------|--------|--------------|-------------|-------------|
| chr1.1 | chr1 | 872335 | 0.0011317652 | 0.9525098   | 0.8635983   |
| chr1.2 | chr1 | 872369 | 0.0007678027 | 0.9414368   | 0.8444060   |
| chr1.3 | chr1 | 872370 | 0.0008347451 | 0.9414314   | 0.8448725   |
| chr1.4 | chr1 | 872385 | 0.0010337477 | 0.9412217   | 0.8521862   |
| chr1.5 | chr1 | 872386 | 0.0010975571 | 0.9410544   | 0.8526863   |
| chr1.6 | chr1 | 872412 | 0.0035114839 | 0.9378250   | 0.8718024   |

|        | meth.diff  | estimate   | std.error | pseudo.R.sqrt |
|--------|------------|------------|-----------|---------------|
| chr1.1 | 0.08891149 | -0.5730618 | 0.1760266 | 0.6304051     |
| chr1.2 | 0.09703074 | -0.5542171 | 0.1647422 | 0.6291904     |
| chr1.3 | 0.09655890 | -0.5522164 | 0.1652843 | 0.6246474     |
| chr1.4 | 0.08903549 | -0.5192564 | 0.1582531 | 0.6108452     |
| chr1.5 | 0.08836810 | -0.5156622 | 0.1579728 | 0.6090794     |
| chr1.6 | 0.06602261 | -0.4018161 | 0.1376551 | 0.5851744     |

|        | cluster.id | z.score  | pos.new |
|--------|------------|----------|---------|
| chr1.1 | chr1_1     | 3.053282 | 1       |
| chr1.2 | chr1_1     | 3.167869 | 35      |
| chr1.3 | chr1_1     | 3.143485 | 36      |
| chr1.4 | chr1_1     | 3.080361 | 51      |
| chr1.5 | chr1_1     | 3.062480 | 52      |
| chr1.6 | chr1_1     | 2.695753 | 78      |

`vario.sm` now contains the smoothed variogram under the Null hypothesis together with the P values (and  $Z$  scores) from the Wald test, that the group has no effect on methylation. The correlation of the  $Z$  scores between two locations in a cluster can now be estimated:

```

> locCor <- estLocCor(vario.sm)

```

We test each CpG cluster for the presence of at least one differentially methylated location at  $q$  what can be interpreted as the size-weighted FDR on clusters:

```

> clusters.rej <- testClusters(locCor,
                               FDR.cluster = 0.1)

```

3 CpG clusters rejected.

```
> clusters.rej$clusters.reject
```

GRanges object with 3 ranges and 2 metadata columns:

|     | seqnames | ranges        | strand | X           |
|-----|----------|---------------|--------|-------------|
|     | <Rle>    | <IRanges>     | <Rle>  | <character> |
| [1] | chr1     | 872335-872616 | *      | chr1_1      |
| [2] | chr1     | 875227-875470 | *      | chr1_2      |
| [3] | chr2     | 45843-46937   | *      | chr2_1      |

|     | p.value     |
|-----|-------------|
|     | <numeric>   |
| [1] | 6.86716e-03 |
| [2] | 4.29518e-07 |
| [3] | 1.88108e-09 |

-----

seqinfo: 2 sequences from an unspecified genome; no seqlengths

## 4.5 Trim significant CpG clusters

We then trim the rejected CpG clusters that is to remove the not differentially methylated CpG sites at  $q_1$  what can be interpreted as the location-wise FDR:

```
> clusters.trimmed <- trimClusters(clusters.rej,  
                                   FDR.loc = 0.05)  
> head(clusters.trimmed)
```

|                | chr  | pos    | p.val        | meth.group1 |
|----------------|------|--------|--------------|-------------|
| chr1_1.chr1.1  | chr1 | 872335 | 0.0011317652 | 0.9525098   |
| chr1_1.chr1.2  | chr1 | 872369 | 0.0007678027 | 0.9414368   |
| chr1_1.chr1.3  | chr1 | 872370 | 0.0008347451 | 0.9414314   |
| chr1_1.chr1.4  | chr1 | 872385 | 0.0010337477 | 0.9412217   |
| chr1_1.chr1.5  | chr1 | 872386 | 0.0010975571 | 0.9410544   |
| chr1_2.chr1.21 | chr1 | 875227 | 0.0003916052 | 0.7175829   |

|               | meth.group2 | meth.diff  | estimate   | std.error |
|---------------|-------------|------------|------------|-----------|
| chr1_1.chr1.1 | 0.8635983   | 0.08891149 | -0.5730618 | 0.1760266 |
| chr1_1.chr1.2 | 0.8444060   | 0.09703074 | -0.5542171 | 0.1647422 |
| chr1_1.chr1.3 | 0.8448725   | 0.09655890 | -0.5522164 | 0.1652843 |
| chr1_1.chr1.4 | 0.8521862   | 0.08903549 | -0.5192564 | 0.1582531 |



|                |               |            |            |           |
|----------------|---------------|------------|------------|-----------|
| chr1_1.chr1.5  | 0.8526863     | 0.08836810 | -0.5156622 | 0.1579728 |
| chr1_2.chr1.21 | 0.3648251     | 0.35275781 | -0.9212669 | 0.2598282 |
|                | pseudo.R.sqrt | cluster.id | z.score    | pos.new   |
| chr1_1.chr1.1  | 0.6304051     | chr1_1     | 3.053282   | 1         |
| chr1_1.chr1.2  | 0.6291904     | chr1_1     | 3.167869   | 35        |
| chr1_1.chr1.3  | 0.6246474     | chr1_1     | 3.143485   | 36        |
| chr1_1.chr1.4  | 0.6108452     | chr1_1     | 3.080361   | 51        |
| chr1_1.chr1.5  | 0.6090794     | chr1_1     | 3.062480   | 52        |
| chr1_2.chr1.21 | 0.6818046     | chr1_2     | 3.358661   | 1         |
|                | p.li          |            |            |           |
| chr1_1.chr1.1  | 0.019767638   |            |            |           |
| chr1_1.chr1.2  | 0.019953150   |            |            |           |
| chr1_1.chr1.3  | 0.021679300   |            |            |           |
| chr1_1.chr1.4  | 0.028817819   |            |            |           |
| chr1_1.chr1.5  | 0.030594942   |            |            |           |
| chr1_2.chr1.21 | 0.004852623   |            |            |           |

`clusters.trimmed` is a *data.frame* object containing all differentially methylated CpG sites. The `p.li` column contains the P values estimated in the cluster trimming step, see [1].

## 4.6 Definition of DMR boundaries

We can now define the boundaries of DMRs as rejected CpG sites within which rejected CpG sites solely are located. Within the DMRs the distance between neighbored rejected CpG sites should not exceed `max.dist` base pairs (usually the same as for `max.dist` in `clusterSites`), otherwise, the DMR is splitted. DMRs are also splitted if the methylation difference switches from positive to negative, or vice versa, if `diff.dir = TRUE`. That way we ensure that within a DMR all CpG sites are hypermethylated, and hypomethylated respectively.

```
> DMRs <- findDMRs(clusters.trimmed,
                    max.dist = 100,
                    diff.dir = TRUE)
> DMRs
```

GRanges object with 4 ranges and 4 metadata columns:

| seqnames | ranges | strand | median.p |
|----------|--------|--------|----------|
|----------|--------|--------|----------|

|     | <Rle>              | <IRanges>     | <Rle>              |  | <numeric>   |
|-----|--------------------|---------------|--------------------|--|-------------|
| [1] | chr1               | 872335-872386 | *                  |  | 1.03375e-03 |
| [2] | chr1               | 875227-875470 | *                  |  | 6.67719e-06 |
| [3] | chr2               | 46126-46718   | *                  |  | 3.96040e-05 |
| [4] | chr2               | 46915-46937   | *                  |  | 1.48460e-02 |
|     | median.meth.group1 |               | median.meth.group2 |  |             |
|     |                    | <numeric>     |                    |  | <numeric>   |
| [1] |                    | 0.941431      |                    |  | 0.8521862   |
| [2] |                    | 0.502444      |                    |  | 0.1820820   |
| [3] |                    | 0.438629      |                    |  | 0.0776818   |
| [4] |                    | 0.136364      |                    |  | 0.0369198   |
|     | median.meth.diff   |               |                    |  |             |
|     |                    | <numeric>     |                    |  |             |
| [1] |                    | 0.0890355     |                    |  |             |
| [2] |                    | 0.3194742     |                    |  |             |
| [3] |                    | 0.3553632     |                    |  |             |
| [4] |                    | 0.0994440     |                    |  |             |

-----

seqinfo: 2 sequences from an unspecified genome; no seqlengths

## 5 Detection of DMRs between two samples

If there are two samples only to be compared we can use the `compareTwoSamples` function which determines the differences per CpG site and aggregates the sites surpassing the minimum difference `minDiff`:

```
> DMRs.2 <- compareTwoSamples(object = predictedMeth,  
                               sample1 = "APL1",  
                               sample2 = "APL10961",  
                               minDiff = 0.3,  
                               max.dist = 100)
```

Some of the DMRs detected within these two samples overlap with the group-wise DMRs:

```
> sum(overlapsAny(DMRs.2, DMRs))  
  
[1] 1
```

## 6 Testing of predefined genomic regions

There are sometimes biological and/or technical reasons for testing predefined genomic regions for differential methylation. For example, one might want to test for methylation differences in known CpG islands or promoter regions. In this scenario it is not of interest which exact CpG sites are differentially methylated. The methods of choice for testing genomic regions are BiSeq and the global test (which we also implemented in this package) [5]. Here, we want to give a short guidance on how to use the *BiSeq* package in order to test predefined genomic regions using BiSeq and the global test.

### 6.1 Testing predefined genomic regions using the BiSeq method

Testing predefined genomic regions using the BiSeq method is mostly equivalent to the procedure shown above for DMR detection. Instead of the definition of CpG clusters (which we described in section 4.1) we will just add the region information to the CpG sites in the *BSraw* object. In this example, we assume that we want to test gene promoters for differential methylation.

We filter out all CpG sites of the *BSraw* object that do not fall into any promoter region. The remaining CpG sites get the promoter identifier in a column named `cluster.id`:

```
> data(promoters)
> data(rrbs)
> rrbs.red <- subsetByOverlaps(rrbs, promoters)
> ov <- findOverlaps(rrbs.red, promoters)
> rowRanges(rrbs.red)$cluster.id[queryHits(ov)] <- promoters$acc_no[su
> head(rowRanges(rrbs.red))
```

GRanges object with 6 ranges and 1 metadata column:

|         | seqnames | ranges    | strand | cluster.id   |
|---------|----------|-----------|--------|--------------|
|         | <Rle>    | <IRanges> | <Rle>  | <character>  |
| 4636436 | chr2     | 46089     | +      | NM_001077710 |
| 4636437 | chr2     | 46095     | +      | NM_001077710 |
| 4636438 | chr2     | 46104     | +      | NM_001077710 |
| 4636439 | chr2     | 46111     | +      | NM_001077710 |
| 4636440 | chr2     | 46113     | +      | NM_001077710 |
| 4636441 | chr2     | 46126     | +      | NM_001077710 |

```
-----
seqinfo: 25 sequences from an unspecified genome; no seqlengths
```

From here on we can use the same pipeline as usual: Smoothing and modeling (sections 4.2 and 4.3) of the DNA methylation data and subsequent testing of the regions (section 4.4).

## 6.2 Testing predefined genomic regions using the Global Test

Goeman et al. [6] proposed a score test for testing high dimensional alternatives. The approach is implemented in the R/Bioconductor package *globaltest*. The *BiSeq* package offers a wrapper function to apply Goeman's Global Test directly to RRBS data given as a *BSrel* object:

```
> data(promoters)
> data(rrbs)
> rrbs <- rawToRel(rrbs)
> promoters <- promoters[overlapsAny(promoters, rrbs)]
> gt <- globalTest(group~1,
                    rrbs,
                    subsets = promoters)
> head(gt)
```

|   | p-value | Statistic | Expected | Std.dev | #Cov |
|---|---------|-----------|----------|---------|------|
| 1 | 0.00975 | 2.50e+01  | 11.1     | 4.89    | 206  |
| 2 | 0.05381 | 2.05e+01  | 11.1     | 5.61    | 67   |
| 3 | 0.05381 | 2.05e+01  | 11.1     | 5.61    | 67   |
| 4 | 1.00000 | 1.85e-31  | 11.1     | 14.81   | 8    |
| 5 | 1.00000 | 1.85e-31  | 11.1     | 14.81   | 8    |
| 6 | 0.06744 | 1.84e+01  | 11.1     | 4.88    | 149  |

If the parameter `subsets` is not given, the null hypothesis is that no CpG site (rather than region) is associated with the given response variable.

## 7 Further data processing

The `plotMethMap` function is helpful to evaluate DMRs graphically. Via `zlim = c(0, 1)` that is passed to the `heatmap` function we ensure that green stands for a relative methylation of 0 and red stands for a relative methylation of 1:

```
> rowCols <- c("magenta", "blue")[as.numeric(colData(predictedMeth)$group)]
> plotMethMap(predictedMeth,
               region = DMRs[3],
               groups = colData(predictedMeth)$group,
               intervals = FALSE,
               zlim = c(0, 1),
               RowSideColors = rowCols,
               labCol = "", margins = c(0, 6))
```



Figure 6: Methylation map of smoothed methylation data within a detected DMR together with hierarchical clustering of the samples

To represent the smoothed methylation curves we can use the `plotSmoothMeth`

function:

```
> plotSmoothMeth(object.rel = predictedMeth,  
  region = DMRs[3],  
  groups = colData(predictedMeth)$group,  
  group.average = FALSE,  
  col = c("magenta", "blue"),  
  lwd = 1.5)  
> legend("topright",  
  legend=levels(colData(predictedMeth)$group),  
  col=c("magenta", "blue"),  
  lty=1, lwd = 1.5)
```



Figure 7: The smoothed methylation curves for all samples within a detected DMR

We can annotate the detected DMRs by means of a *GRanges* object, e.g. a list of promoter regions. In case of an overlapping of both *GRanges* objects the DMR is marked as `TRUE`, or with the respective identifier in the promoter list:

```
> data(promoters)
> head(promoters)
```

GRanges object with 6 ranges and 1 metadata column:

|     | seqnames | ranges            | strand | acc_no       |
|-----|----------|-------------------|--------|--------------|
|     | <Rle>    | <IRanges>         | <Rle>  | <character>  |
| [1] | chr1     | 66998824-67000324 | *      | NM_032291    |
| [2] | chr1     | 8383389-8384889   | *      | NM_001080397 |
| [3] | chr1     | 16766166-16767666 | *      | NM_001145277 |
| [4] | chr1     | 16766166-16767666 | *      | NM_001145278 |
| [5] | chr1     | 16766166-16767666 | *      | NM_018090    |
| [6] | chr1     | 50489126-50490626 | *      | NM_032785    |

-----

seqinfo: 24 sequences from an unspecified genome; no seqlengths

```
> DMRs.anno <- annotateGRanges(object = DMRs,
                                regions = promoters,
                                name = 'Promoter',
                                regionInfo = 'acc_no')
```

```
> DMRs.anno
```

GRanges object with 4 ranges and 5 metadata columns:

|     | seqnames | ranges        | strand | median.p    |
|-----|----------|---------------|--------|-------------|
|     | <Rle>    | <IRanges>     | <Rle>  | <numeric>   |
| [1] | chr1     | 872335-872386 | *      | 1.03375e-03 |
| [2] | chr1     | 875227-875470 | *      | 6.67719e-06 |
| [3] | chr2     | 46126-46718   | *      | 3.96040e-05 |
| [4] | chr2     | 46915-46937   | *      | 1.48460e-02 |

|     | median.meth.group1 | median.meth.group2 |
|-----|--------------------|--------------------|
|     | <numeric>          | <numeric>          |
| [1] | 0.941431           | 0.8521862          |
| [2] | 0.502444           | 0.1820820          |
| [3] | 0.438629           | 0.0776818          |
| [4] | 0.136364           | 0.0369198          |

|  | median.meth.diff | Promoter |
|--|------------------|----------|
|--|------------------|----------|



|     | <numeric> | <character>  |
|-----|-----------|--------------|
| [1] | 0.0890355 | <NA>         |
| [2] | 0.3194742 | <NA>         |
| [3] | 0.3553632 | NM_001077710 |
| [4] | 0.0994440 | NM_001077710 |

-----

seqinfo: 2 sequences from an unspecified genome; no seqlengths

`plotBindingSites` plots the average methylation around given genomic regions, e.g. protein binding sites. Here, we compare the methylation in and around promoter regions between APL and controls:

```

> plotBindingSites(object = rrbs,
                    regions = promoters,
                    width = 4000,
                    group = colData(rrbs)$group,
                    col = c("magenta", "blue"),
                    lwd = 1.5)
> legend("top",
        legend=levels(colData(rrbs)$group),
        col=c("magenta", "blue"),
        lty=1, lwd = 1.5)

```



Figure 8: Methylation around 1,000 promoters; Position 0 refers to the centers of the promoters

The raw and relative methylation data can also be viewed in the Integrative Ge-

nomics Viewer (IGV; freely available for download from [www.broadinstitute.org/igv](http://www.broadinstitute.org/igv)) [7]. To do so we first write the methylation information of each sample within the *BSraw* or *BSrel* object to a bed file:

```
> track.names <- paste(colData(rrbs)$group,
                        "_",
                        gsub("APL", "", colnames(rrbs)),
                        sep="")
> writeBED(object = rrbs,
            name = track.names,
            file = paste(colnames(rrbs), ".bed", sep = ""))
> writeBED(object = predictedMeth,
            name = track.names,
            file = paste(colnames(predictedMeth), ".bed", sep = ""))
```

We can load the bed files of the raw data in the IGV. The integers beneath the CpG marks represent the numbers of sequencing reads covering the CpG sites:



Figure 9: IGV snapshot of the raw data in and around a detected DMR

We can also load the smoothed methylation levels:

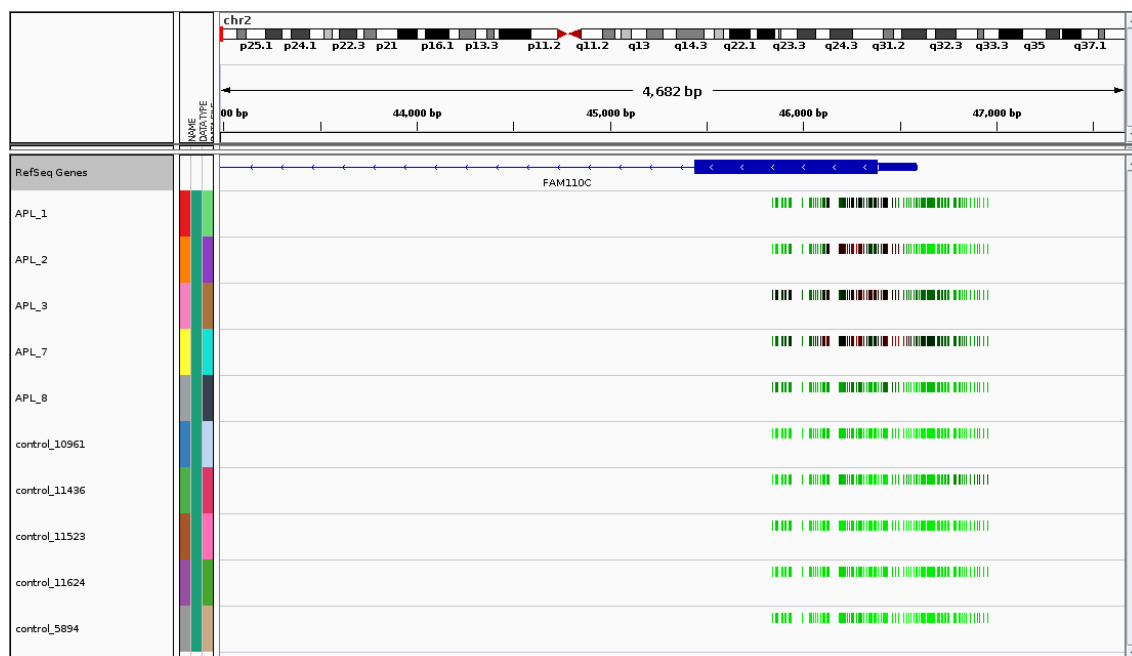


Figure 10: IGV snapshot of the smoothed data in and around a detected DMR

## References

- [1] Katja Hebestreit, Martin Dugas, and Hans-Ulrich Klein. Detection of significantly differentially methylated regions in targeted bisulfite sequencing data. *Bioinformatics*, 29(13):1647–1653, Jul 2013. URL: <http://dx.doi.org/10.1093/bioinformatics/btt263>, doi:10.1093/bioinformatics/btt263.
- [2] Till Schoofs, Christian Rohde, Katja Hebestreit, Hans-Ulrich Klein, Stefanie Göllner, Isabell Schulze, Mads Lerdrup, Nikolaj Dietrich, Shuchi Agrawal-Singh, Anika Witten, Monika Stoll, Eva Lengfelder, Wolf-Karsten Hofmann, Peter Schlenke, Thomas Büchner, Klaus Hansen, Wolfgang E Berdel, Frank Rosenbauer, Martin Dugas, and Carsten Müller-Tidow. Dna methylation changes are a late event in acute promyelocytic leukemia and coincide with loss of transcription factor binding. *Blood*, Nov 2012. URL: <http://dx.doi.org/10.1182/blood-2012-08-448860>, doi:10.1182/blood-2012-08-448860.
- [3] Felix Krueger and Simon R Andrews. Bismark: a flexible aligner and methylation caller for bisulfite-seq applications. *Bioinformatics*, 27(11):1571–1572, Jun 2011. URL: <http://dx.doi.org/10.1093/bioinformatics/btr167>, doi:10.1093/bioinformatics/btr167.
- [4] Bettina Grün, Ioannis Kosmidis, and Achim Zeileis. *Extended Beta Regression in R: Shaken, Stirred, Mixed, and Partitioned*, 2012.
- [5] Hans-Ulrich Klein and Katja Hebestreit. An evaluation of methods to test predefined genomic regions for differential methylation in bisulfite sequencing data. *Under review*.
- [6] Jelle J. Goeman, Sara A. Van De Geer, and Hans C. Van Houwelingen. Testing against a high dimensional alternative. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)*, 68(3):477–493, 2006.
- [7] Helga Thorvaldsdóttir, James T Robinson, and Jill P Mesirov. Integrative genomics viewer (igv): high-performance genomics data visualization and exploration. *Brief Bioinform*, Apr 2012. URL: <http://dx.doi.org/10.1093/bib/bbs017>, doi:10.1093/bib/bbs017.