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## METHOD ARTICLE

**REVISED** A cross-package Bioconductor workflow for analysing methylation array data [version 3; referees: 4 approved]Jovana Maksimovic <sup>1</sup>, Belinda Phipson <sup>1</sup>, Alicia Oshlack <sup>1-3</sup><sup>1</sup>Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne, Australia<sup>2</sup>School of BioSciences, University of Melbourne, Melbourne, Australia<sup>3</sup>School of Physics, University of Melbourne, Melbourne, Australia**v3** First published: 08 Jun 2016, 5:1281 (doi: [10.12688/f1000research.8839.1](https://doi.org/10.12688/f1000research.8839.1))Second version: 26 Jul 2016, 5:1281 (doi: [10.12688/f1000research.8839.2](https://doi.org/10.12688/f1000research.8839.2))Latest published: 05 Apr 2017, 5:1281 (doi: [10.12688/f1000research.8839.3](https://doi.org/10.12688/f1000research.8839.3))**Abstract**

Methylation in the human genome is known to be associated with development and disease. The Illumina Infinium methylation arrays are by far the most common way to interrogate methylation across the human genome. This paper provides a Bioconductor workflow using multiple packages for the analysis of methylation array data. Specifically, we demonstrate the steps involved in a typical differential methylation analysis pipeline including: quality control, filtering, normalization, data exploration and statistical testing for probe-wise differential methylation. We further outline other analyses such as differential methylation of regions, differential variability analysis, estimating cell type composition and gene ontology testing. Finally, we provide some examples of how to visualise methylation array data.



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**REVISED** Amendments from Version 2

We have fixed bugs in the workflow code that were introduced with changes to the arguments required by the “cpg.annotate” and “DMR.plot” functions in the *DMRcate* package. [Figure 11](#) once again shows the top ranked DMR, as in version 1 of the workflow. A bug present in the *minfi* “preprocessQuantile” function during the generation of version 2 necessitated us to plot DMR 11 in order to show all of the features we were trying to display; this has now been fixed by the *minfi* authors. We have changed the links to the data on figshare to a modified version of the dataset which now includes CpG islands and DNase I hypersensitive sites for chr17 instead of chr22, to reflect the change to the DMR that is being shown in [Figure 11](#). We have also included a link to a live (as opposed to static) version of this workflow on the Bioconductor website.

**See referee reports**

## Introduction

DNA methylation, the addition of a methyl group to a CG dinucleotide of the DNA, is the most extensively studied epigenetic mark due to its role in both development and disease ([Bird, 2002](#); [Laird, 2003](#)). Although DNA methylation can be measured in several ways, the epigenetics community has enthusiastically embraced the Illumina HumanMethylation450 (450k) array ([Bibikova et al., 2011](#)) as a cost-effective way to assay methylation across the human genome. More recently, Illumina has increased the genomic coverage of the platform to >850,000 sites with the release of their MethylationEPIC (850k) array. As methylation arrays are likely to remain popular for measuring methylation for the foreseeable future, it is necessary to provide robust workflows for methylation array analysis.

Measurement of DNA methylation by Infinium technology (Infinium I) was first employed by Illumina on the HumanMethylation27 (27k) array ([Bibikova et al., 2009](#)), which measured methylation at approximately 27,000 CpGs, primarily in gene promoters. Like bisulfite sequencing, the Infinium assay detects methylation status at single base resolution. However, due to its relatively limited coverage the array platform was not truly considered “genome-wide” until the arrival of the 450k array. The 450k array increased the genomic coverage of the platform to over 450,000 gene-centric sites by combining the original Infinium I assay with the novel Infinium II probes. Both assay types employ 50bp probes that query a [C/T] polymorphism created by bisulfite conversion of unmethylated cytosines in the genome, however, the Infinium I and II assays differ in the number of beads required to detect methylation at a single locus. Infinium I uses two bead types per CpG, one for each of the methylated and unmethylated states ([Figure 1a](#)). In contrast, the Infinium II design uses one bead type and the methylated state is determined at the single base extension step after hybridization ([Figure 1b](#)). The 850k array also uses a combination of the Infinium I and II assays but achieves additional coverage by increasing the size of each array; a 450k slide contains 12 arrays whilst the 850k has only 8.

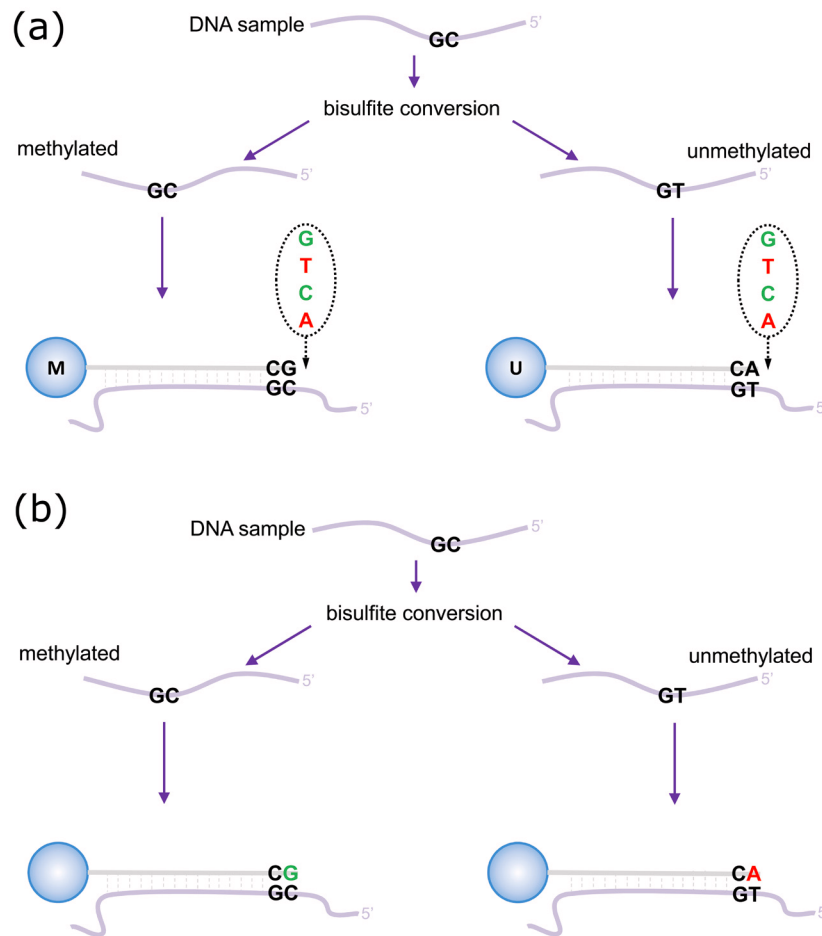
Regardless of the Illumina array version, for each CpG, there are two measurements: a methylated intensity (denoted by  $M$ ) and an unmethylated intensity (denoted by  $U$ ). These intensity values can be used to determine the proportion of methylation at each CpG locus. Methylation levels are commonly reported as either beta values ( $\beta = M/(M + U)$ ) or M-values ( $M\text{ value} = \log_2(M/U)$ ). For practical purposes, a small offset,  $\alpha$ , can be added to the denominator of the  $\beta$  value equation to avoid dividing by small values, which is the default behaviour of the `getBeta` function in *minfi*. The default value for  $\alpha$  is 100. It may also be desirable to add a small offset to the numerator and denominator when calculating M-values to avoid dividing by zero in rare cases, however the default `getM` function in *minfi* does not do this. Beta values and M-values are related through a logit transformation. Beta values are generally preferable for describing the level of methylation at a locus or for graphical presentation because percentage methylation is easily interpretable. However, due to their distributional properties, M-values are more appropriate for statistical testing ([Du et al., 2010](#)).

In this workflow, we will provide examples of the steps involved in analysing methylation array data using R ([R Core Team, 2014](#)) and Bioconductor ([Huber et al., 2015](#)), including: quality control, filtering, normalisation, data exploration and probe-wise differential methylation analysis. We will also cover other approaches such as differential methylation analysis of regions, differential variability analysis, gene ontology analysis and estimating cell type composition. Finally, we will provide some examples of useful ways to visualise methylation array data.

## Differential methylation analysis

### Obtaining the data

All of the data used in this workflow can be downloaded and extracted in R using the `download.file` and `untar` functions, as shown below. Alternatively, the data can be manually downloaded from: [https://figshare.com/articles/methylAnalysisDataV3\\_tar\\_gz/4800970](https://figshare.com/articles/methylAnalysisDataV3_tar_gz/4800970).



**Figure 1. Illumina Infinium HumanMethylation450 assay, reproduced from Maksimovic *et al.*, 2012.** (a) Infinium I assay. Each individual CpG is interrogated using two bead types: methylated (M) and unmethylated (U). Both bead types will incorporate the same labeled nucleotide for the same target CpG, thereby producing the same color fluorescence. The nucleotide that is added is determined by the base downstream of the "C" of the target CpG. The proportion of methylation can be calculated by comparing the intensities from the two different probes in the same color. (b) Infinium II assay. Each target CpG is interrogated using a single bead type. Methylation state is detected by single base extension at the position of the "C" of the target CpG, which always results in the addition of a labeled "G" or "A" nucleotide, complementary to either the "methylated" C or "unmethylated" T, respectively. Each locus is detected in two colors, and methylation status is determined by comparing the two colors from the one position.

```
# the URL for the data download
url <- "https://ndownloader.figshare.com/files/7896205"
# download the data
if(!file.exists("methylAnalysisDataV3.tar.gz")) {
  download.file(url, destfile="methylAnalysisDataV3.tar.gz", method="auto")
}
# extract the data
if(!file.exists("./data")) {
  untar("methylAnalysisDataV3.tar.gz", exdir=".", compressed="gzip")
}
```

Once the data has been downloaded and extracted, there should be a folder called `data` that contains all the files necessary to execute the workflow.

```

# set up a path to the data directory
dataDirectory <- "./data"
# list the files
list.files(dataDirectory, recursive=TRUE)

## [1] "48639-non-specific-probes-Illumina450k.csv"
## [2] "5975827018/5975827018_R06C02_Grn.idat"
## [3] "5975827018/5975827018_R06C02_Red.idat"
## [4] "6264509100/6264509100_R01C01_Grn.idat"
## [5] "6264509100/6264509100_R01C01_Red.idat"
## [6] "6264509100/6264509100_R01C02_Grn.idat"
## [7] "6264509100/6264509100_R01C02_Red.idat"
## [8] "6264509100/6264509100_R02C01_Grn.idat"
## [9] "6264509100/6264509100_R02C01_Red.idat"
## [10] "6264509100/6264509100_R02C02_Grn.idat"
## [11] "6264509100/6264509100_R02C02_Red.idat"
## [12] "6264509100/6264509100_R03C01_Grn.idat"
## [13] "6264509100/6264509100_R03C01_Red.idat"
## [14] "6264509100/6264509100_R03C02_Grn.idat"
## [15] "6264509100/6264509100_R03C02_Red.idat"
## [16] "6264509100/6264509100_R04C01_Grn.idat"
## [17] "6264509100/6264509100_R04C01_Red.idat"
## [18] "6264509100/6264509100_R04C02_Grn.idat"
## [19] "6264509100/6264509100_R04C02_Red.idat"
## [20] "6264509100/6264509100_R05C01_Grn.idat"
## [21] "6264509100/6264509100_R05C01_Red.idat"
## [22] "6264509100/6264509100_R05C02_Grn.idat"
## [23] "6264509100/6264509100_R05C02_Red.idat"
## [24] "6264509100/6264509100_R06C01_Grn.idat"
## [25] "6264509100/6264509100_R06C01_Red.idat"
## [26] "6264509100/6264509100_R06C02_Grn.idat"
## [27] "6264509100/6264509100_R06C02_Red.idat"
## [28] "ageData.RData"
## [29] "human_c2_v5.rdata"
## [30] "model-based-cpg-islands-hg19-chr17.txt"
## [31] "SampleSheet.csv"
## [32] "wgEncodeRegDnaseClusteredV3chr17.bed"

```

To demonstrate the various aspects of analysing methylation data, we will be using a small, publicly available 450k methylation dataset (GSE49667) (Zhang *et al.*, 2013). The dataset contains 10 samples in total: there are 4 different sorted T-cell types (naive, rTreg, act\_naive, act\_rTreg, collected from 3 different individuals (M28, M29, M30). For details describing sample collection and preparation, see Zhang *et al.* (2013). An additional birth sample (individual VIC5-72098-18-B) is included from another study (GSE51180) (Cruickshank *et al.*, 2013) to illustrate approaches for identifying and excluding poor quality samples.

There are several R Bioconductor packages available that have been developed for analysing methylation array data, including *minfi* (Aryee *et al.*, 2014), *missMethyl* (Phipson *et al.*, 2016), *wateRmelon* (Pidsley *et al.*, 2013), *methylumi* (Davis *et al.*, 2015), *ChAMP* (Morris *et al.*, 2014) and *charm* (Aryee *et al.*, 2011). Some of the packages, such as *minfi* and *methylumi* include a framework for reading in the raw data from IDAT files and various specialised objects for storing and manipulating the data throughout the course of an analysis. Other packages provide specialised analysis methods for normalisation and statistical testing that rely on either *minfi* or *methylumi* objects. It is possible to convert between *minfi* and *methylumi* data types, however, this is not always trivial. Thus, it is advisable to consider the methods that you are interested in using and the data types that are most appropriate before you begin your analysis. Another popular method for analysing methylation array data is *limma* (Ritchie *et al.*, 2015), which was originally developed for gene expression microarray analysis. As *limma* operates on a matrix of values, it is easily applied to any data that can be converted to a matrix in R. For a complete list of Bioconductor packages for analysing DNA methylation data, one can search for “DNAMethylation” in BiocViews ([https://www.bioconductor.org/packages/release/BiocViews.html#\\_\\_\\_DNAMethylation](https://www.bioconductor.org/packages/release/BiocViews.html#___DNAMethylation)) on the Bioconductor website.

We will begin with an example of a **probe-wise** differential methylation analysis using *minfi* and *limma*. By **probe-wise** analysis we mean each individual CpG probe will be tested for differential methylation for the comparisons of interest and p-values and moderated t-statistics (Smyth, 2004) will be generated for each CpG probe.

### Loading the data

It is useful to begin an analysis in R by loading all the packages that are likely to be required.

```
# load packages required for analysis
library(limma)
library(minfi)
library(IlluminaHumanMethylation450kanno.ilmn12.hg19)
library(IlluminaHumanMethylation450kmanifest)
library(RColorBrewer)
library(missMethyl)
library(matrixStats)
library(minfiData)
library(Gviz)
library(DMRcate)
library(stringr)
```

The *minfi*, *IlluminaHumanMethylation450kanno.ilmn12.hg19*, *IlluminaHumanMethylation450kmanifest*, *missMethyl*, *minfiData* and *DMRcate* are methylation specific packages, while *RColorBrewer* and *Gviz* are visualisation packages. We use *limma* for testing differential methylation, and *matrixStats* and *stringr* have functions used in the workflow. The *IlluminaHumanMethylation450kmanifest* package provides the Illumina manifest as an R object which can easily be loaded into the environment. The manifest contains all of the annotation information for each of the CpG probes on the 450k array. This is useful for determining where any differentially methylated probes are located in a genomic context.

```
# get the 450k annotation data
ann450k = getAnnotation(IlluminaHumanMethylation450kanno.ilmn12.hg19)
head(ann450k)

## DataFrame with 6 rows and 33 columns
##           chr      pos      strand      Name      AddressA
##           <character> <integer> <character> <character> <character>
## cg00050873      chrY    9363356      -      cg00050873    32735311
## cg00212031      chrY    21239348      -      cg00212031    29674443
## cg00213748      chrY    8148233      -      cg00213748    30703409
## cg00214611      chrY    15815688      -      cg00214611    69792329
## cg00455876      chrY    9385539      -      cg00455876    27653438
## cg01707559      chrY    6778695      +      cg01707559    45652402
##           AddressB      ProbeSeqA
##           <character>      <character>
## cg00050873    31717405 ACAAAAAACAACACACAACACTATAATAATTTTAAAAATAAATAAACCCCA
## cg00212031    38703326 CCCAATTAACCGCAAAAACTAAACAAATTATACAAATCAAAAAACATACA
## cg00213748    36767301 TTTTAACACCTAACACCATTTTAAACAATAAAAAATCTACAAAAAAAACA
## cg00214611    46723459 CTAACCTCCAAACCACACTTTATATACTAACTACAATATAACGCAACA
## cg00455876    69732350 AACTCTAAACTACCGACACAACTCCAAAACTCTCAAAAAAACTCA
## cg01707559    64689504 ACAAATTAAAAACTAAACAAACACAACACTACAACAACAAAAACA
##           ProbeSeqB      Type
##           <character> <character>
## cg00050873 ACGAAAAACAACGCACAACACTATAATAATTTTAAAAATAAATAAACCCCG      I
## cg00212031 CCCAATTAACCGCAAAAACTAAACAAATTATACGATCGAAAAACGTACG      I
## cg00213748 TTTTAACGCCTAACACCGTTTAAACGATAAAAAATCTACAAAAAAAACG      I
## cg00214611 CTAACCTCCGAACCGCGCTTTATATACTAACTACAATATAACGCGAACG      I
## cg00455876 AACTCTAAACTACCGACACAACTCCAAAACTCTCGAAAAAACTCG      I
## cg01707559 GCGAATTAAAAACTAAACGAACGCGACGACTACAACGACAAAAACG      I
```

```

##          NextBase      Color      Probe_rs Probe_maf      CpG_rs
##          <character> <character> <character> <numeric> <character>
## cg00050873          A          Red          NA          NA          NA
## cg00212031          T          Red          NA          NA          NA
## cg00213748          A          Red          NA          NA          NA
## cg00214611          A          Red          NA          NA          NA
## cg00455876          A          Red          NA          NA          NA
## cg01707559          A          Red          NA          NA          NA
##          CpG_maf      SBE_rs      SBE_maf      Islands_Name
##          <numeric> <character> <numeric>      <character>
## cg00050873          NA          NA          NA      chrY:9363680-9363943
## cg00212031          NA          NA          NA      chrY:21238448-21240005
## cg00213748          NA          NA          NA      chrY:8147877-8148210
## cg00214611          NA          NA          NA      chrY:15815488-15815779
## cg00455876          NA          NA          NA      chrY:9385471-9385777
## cg01707559          NA          NA          NA      chrY:6778574-6780028
##          Relation_to_Island
##          <character>
## cg00050873          N_Shore
## cg00212031          Island
## cg00213748          S_Shore
## cg00214611          Island
## cg00455876          Island
## cg01707559          Island
##
##          Forward_Sequence
##          <character>
## cg00050873 TATCTCTGTCTGGCGAGGAGGCAACGCACAACCTGTGGTGGTTTTTGGAGTGGGTGGACCC[CG]
## cg00212031 CCATTGGCCCGCCCCAGTTGGCCGCAGGGACTGAGCAAGTTATGCGGTGCGGAAGACGTG[CG]
## cg00213748 TCTGTGGGACCATTTTAACGCCTGGCACCGTTTAAACGATGGAGGTTCTGCAGGAGGGGG[CG]
## cg00214611 GCGCCGGCAGGACTAGCTTCCGGGCCGCGCTTTGTGTGCTGGGCTGCAGTGTGGCGCGGG[CG]
## cg00455876 CGCGTGTGCCTGGACTCTGAGCTACCCGGCACAAGCTCCAAGGGCTTCTCGGAGGAGGCT[CG]
## cg01707559 AGCGGCCGCTCCCAGTGGTGGTCAACGCCAGTGCCAATCCCTTGCGCCCGCGTGCAGTCC[CG]
##
##          SourceSeq Random_Loci
##          <character> <character>
## cg00050873 CGGGGTCCACCCACTCCAAAAACCACCACAGTTGTGCGTTGCCTCCTCGC
## cg00212031 CGCACGTCTTCCCGACCGCATAACTTGCTCAGTCCCTGCGGCCAACTGGG
## cg00213748 CGCCCCCTCCTGCAGAACCTCCATCGTTAAAACGGTGCCAGGCGTTAAAA
## cg00214611 CGCCCGCGCCACACTGCAGCCAGCACACAAAGCGCGGCCCGGAAGCTAG
## cg00455876 GACTCTGAGCTACCCGGCACAAGCTCCAAGGGCTTCTCGGAGGAGGCTCG
## cg01707559 CGCCCTCTGTGCTGCAGCCGCGCGCCGCTCCAGTGCCCCCAATTTCG
##          Methyl27_Loci UCSC_RefGene_Name      UCSC_RefGene_Accession
##          <character>      <character>      <character>
## cg00050873          TSPY4;FAM197Y2          NM_001164471;NR_001553
## cg00212031          TTTY14          NR_001543
## cg00213748
## cg00214611          TMSB4Y;TMSB4Y          NM_004202;NM_004202
## cg00455876
## cg01707559          TBL1Y;TBL1Y;TBL1Y NM_134259;NM_033284;NM_134258
##          UCSC_RefGene_Group      Phantom      DMR      Enhancer
##          <character> <character> <character> <character>
## cg00050873          Body;TSS1500
## cg00212031          TSS200
## cg00213748
## cg00214611          1stExon;5'UTR
## cg00455876
## cg01707559 TSS200;TSS200;TSS200

```

```
##           HMM_Island Regulatory_Feature_Name
##           <character>           <character>
## cg00050873   Y:9973136-9976273
## cg00212031 Y:19697854-19699393
## cg00213748   Y:8207555-8208234
## cg00214611 Y:14324883-14325218   Y:15815422-15815706
## cg00455876   Y:9993394-9995882
## cg01707559   Y:6838022-6839951
##           Regulatory_Feature_Group           DHS
##           <character> <character>
## cg00050873
## cg00212031
## cg00213748
## cg00214611 Promoter_Associated_Cell_type_specific
## cg00455876
## cg01707559
```

As for their many other BeadArray platforms, Illumina methylation data is usually obtained in the form of Intensity Data (IDAT) Files. This is a proprietary format that is output by the scanner and stores summary intensities for each probe on the array. However, there are Bioconductor packages available that facilitate the import of data from IDAT files into R (Smith *et al.*, 2013). Typically, each IDAT file is approximately 8MB in size. The simplest way to import the raw methylation data into R is using the *minfi* function `read.metharray.sheet`, along with the path to the IDAT files and a sample sheet. The sample sheet is a CSV (comma-separated) file containing one line per sample, with a number of columns describing each sample. The format expected by the `read.metharray.sheet` function is based on the sample sheet file that usually accompanies Illumina methylation array data. It is also very similar to the targets file described by the *limma* package. Importing the sample sheet into R creates a `data.frame` with one row for each sample and several columns. The `read.metharray.sheet` function uses the specified path and other information from the sample sheet to create a column called `Baseline` which specifies the location of each individual IDAT file in the experiment.

```
# read in the sample sheet for the experiment
targets <- read.metharray.sheet(dataDirectory, pattern="SampleSheet.csv")
```

```
## [read.metharray.sheet] Found the following CSV files:
```

```
## [1] "./data/SampleSheet.csv"
```

```
targets
```

```
##   Sample_Name Sample_Well Sample_Source Sample_Group Sample_Label
## 1           1           A1           M28         naive         naive
## 2           2           B1           M28          rTreg          rTreg
## 3           3           C1           M28    act_naive    act_naive
## 4           4           D1           M29         naive         naive
## 5           5           E1           M29    act_naive    act_naive
## 6           6           F1           M29    act_rTreg    act_rTreg
## 7           7           G1           M30         naive         naive
## 8           8           H1           M30          rTreg          rTreg
## 9           9           A2           M30    act_naive    act_naive
## 10          10          B2           M30    act_rTreg    act_rTreg
## 11          11          H06 VICS-72098-18-B         birth         birth
##   Pool_ID Array      Slide Basename
## 1    <NA> R01C01 6264509100 ./data/6264509100/6264509100_R01C01
## 2    <NA> R02C01 6264509100 ./data/6264509100/6264509100_R02C01
## 3    <NA> R03C01 6264509100 ./data/6264509100/6264509100_R03C01
## 4    <NA> R04C01 6264509100 ./data/6264509100/6264509100_R04C01
```

```
## 5      <NA>   R05C01 6264509100 ./data/6264509100/6264509100_R05C01
## 6      <NA>   R06C01 6264509100 ./data/6264509100/6264509100_R06C01
## 7      <NA>   R01C02 6264509100 ./data/6264509100/6264509100_R01C02
## 8      <NA>   R02C02 6264509100 ./data/6264509100/6264509100_R02C02
## 9      <NA>   R03C02 6264509100 ./data/6264509100/6264509100_R03C02
## 10     <NA>   R04C02 6264509100 ./data/6264509100/6264509100_R04C02
## 11     <NA>   R06C02 5975827018 ./data/5975827018/5975827018_R06C02
```

Now that we have imported the information about the samples and where the data is located, we can read the raw intensity signals into R from the IDAT files using the `read.metharray.exp` function. This creates an `RGChannelSet` object that contains all the raw intensity data, from both the red and green colour channels, for each of the samples. At this stage, it can be useful to rename the samples with more descriptive names.

```
# read in the raw data from the IDAT files
rgSet <- read.metharray.exp(targets=targets)
rgSet

## RGChannelSet (storageMode: lockedEnvironment)
## assayData: 622399 features, 11 samples
## element names: Green, Red
## An object of class 'AnnotatedDataFrame'
## sampleNames: 6264509100_R01C01 6264509100_R02C01 ...
## 5975827018_R06C02 (11 total)
## varLabels: Sample_Name Sample_Well ... filenames (10 total)
## varMetadata: labelDescription
## Annotation
## array: IlluminaHumanMethylation450k
## annotation: ilmn12.hg19

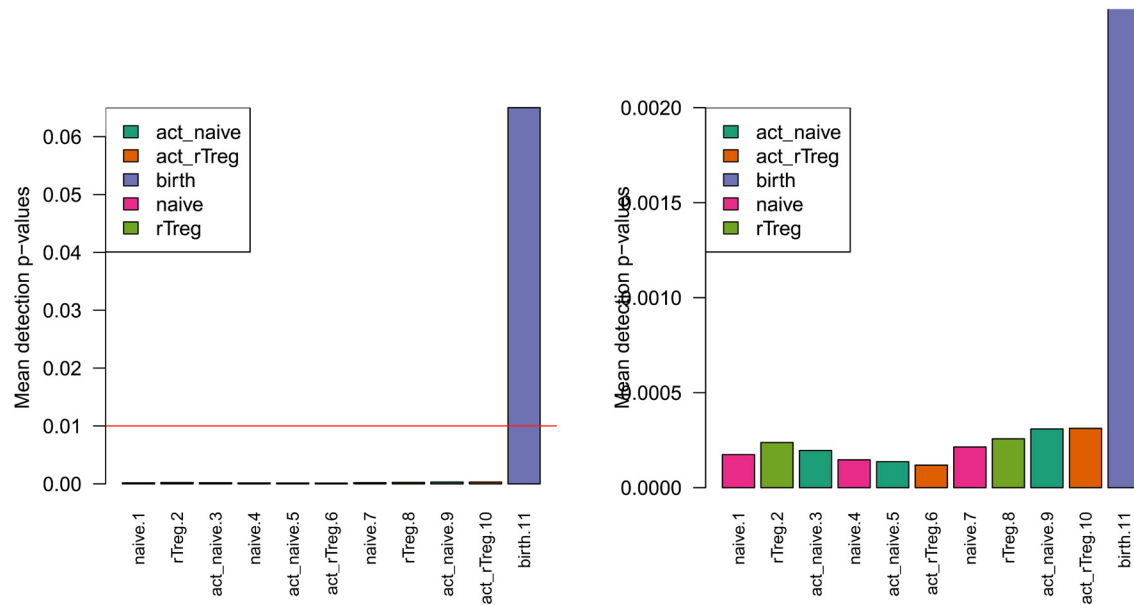
# give the samples descriptive names
targets$ID <- paste(targets$Sample_Group, targets$Sample_Name, sep=".")
sampleNames(rgSet) <- targets$ID
rgSet

## RGChannelSet (storageMode: lockedEnvironment)
## assayData: 622399 features, 11 samples
## element names: Green, Red
## An object of class 'AnnotatedDataFrame'
## sampleNames: naive.1 rTreg.2 ... birth.11 (11 total)
## varLabels: Sample_Name Sample_Well ... filenames (10 total)
## varMetadata: labelDescription
## Annotation
## array: IlluminaHumanMethylation450k
## annotation: ilmn12.hg19
```

### Quality control

Once the data has been imported into R, we can evaluate its quality. Firstly, we need to calculate detection p-values. We can generate a detection p-value for every CpG in every sample, which is indicative of the quality of the signal. The method used by *minfi* to calculate detection p-values compares the total signal ( $M + U$ ) for each probe to the background signal level, which is estimated from the negative control probes. Very small p-values are indicative of a reliable signal whilst large p-values, for example  $>0.01$ , generally indicate a poor quality signal.

Plotting the mean detection p-value for each sample allows us to gauge the general quality of the samples in terms of the overall signal reliability (Figure 2). Samples that have many failed probes will have relatively large mean detection p-values.



**Figure 2.** Mean detection p-values summarise the quality of the signal across all the probes in each sample. The plot on the right is a zoomed in version of the plot on the left.

```
# calculate the detection p-values
detP <- detectionP(rgSet)
head(detP)

##          naive.1 rTreg.2  act_naive.3 naive.4 act_naive.5 act_rTreg.6
## cg00050873      0      0 0.000000e+00      0 0.000000e+00      0
## cg00212031      0      0 0.000000e+00      0 0.000000e+00      0
## cg00213748      0      0 1.181832e-12      0 8.21565e-15      0
## cg00214611      0      0 0.000000e+00      0 0.000000e+00      0
## cg00455876      0      0 0.000000e+00      0 0.000000e+00      0
## cg01707559      0      0 0.000000e+00      0 0.000000e+00      0
##          naive.7  rTreg.8 act_naive.9 act_rTreg.10 birth.11
## cg00050873      0 0.000000e+00      0 0.000000e+00 0.0000000
## cg00212031      0 0.000000e+00      0 0.000000e+00 0.0000000
## cg00213748      0 1.469801e-05      0 1.365951e-08 0.6735224
## cg00214611      0 0.000000e+00      0 0.000000e+00 0.7344451
## cg00455876      0 0.000000e+00      0 0.000000e+00 0.0000000
## cg01707559      0 0.000000e+00      0 0.000000e+00 0.0000000

# examine mean detection p-values across all samples to identify any failed
samples
pal <- brewer.pal(8,"Dark2")
par(mfrow=c(1,2))
barplot(colMeans(detP), col=pal[factor(targets$Sample_Group)], las=2,
        cex.names=0.8,ylab="Mean detection p-values")
abline(h=0.01,col="red")
legend("topleft", legend=levels(factor(targets$Sample_Group)), fill=pal,
        bg="white")

barplot(colMeans(detP), col=pal[factor(targets$Sample_Group)], las=2,
        cex.names=0.8, ylim = c(0,0.002), ylab="Mean detection p-values")
legend("topleft", legend=levels(factor(targets$Sample_Group)), fill=pal,
        bg="white")
```

The *minfi* `qcReport` function generates many other useful quality control plots. The *minfi* vignette describes the various plots and how they should be interpreted in detail. Generally, samples that look poor based on mean detection p-value will also look poor using other metrics and it is usually advisable to exclude them from further analysis.

```
qcReport(rgSet, sampNames=targets$ID, sampGroups=targets$Sample_Group,
         pdf="qcReport.pdf")
```

Poor quality samples can be easily excluded from the analysis using a detection p-value cutoff, for example  $>0.05$ . For this particular dataset, the birth sample shows a very high mean detection p-value, and hence it is excluded from subsequent analysis (Figure 2).

```
# remove poor quality samples
keep <- colMeans(detP) < 0.05
rgSet <- rgSet[,keep]
rgSet

## RGChannelSet (storageMode: lockedEnvironment)
## assayData: 622399 features, 10 samples
## element names: Green, Red
## An object of class 'AnnotatedDataFrame'
## sampleNames: naive.1 rTreg.2 ... act_rTreg.10 (10 total)
## varLabels: Sample_Name Sample_Well ... filenames (10 total)
## varMetadata: labelDescription
## Annotation
## array: IlluminaHumanMethylation450k
## annotation: ilmn12.hg19

# remove poor quality samples from targets data
targets <- targets[keep,]
targets[,1:5]

##      Sample_Name Sample_Well Sample_Source Sample_Group Sample_Label
## 1             1           A1           M28         naive         naive
## 2             2           B1           M28          rTreg          rTreg
## 3             3           C1           M28    act_naive    act_naive
## 4             4           D1           M29         naive         naive
## 5             5           E1           M29    act_naive    act_naive
## 6             6           F1           M29    act_rTreg    act_rTreg
## 7             7           G1           M30         naive         naive
## 8             8           H1           M30          rTreg          rTreg
## 9             9           A2           M30    act_naive    act_naive
## 10            10           B2           M30    act_rTreg    act_rTreg

# remove poor quality samples from detection p-value table
detP <- detP[,keep]
dim(detP)

## [1] 485512 10
```

## Normalisation

To minimise the unwanted variation within and between samples, various data normalisations can be applied. Many different types of normalisation have been developed for methylation arrays and it is beyond the scope of this workflow to compare and contrast all of them (Fortin *et al.*, 2014; Maksimovic *et al.*, 2012; Mancuso *et al.*, 2011; Pidsley *et al.*, 2013; Sun *et al.*, 2011; Teschendorff *et al.*, 2013; Touleimat & Tost, 2012; Triche *et al.*, 2013; Wang *et al.*, 2012; Wu *et al.*, 2014). Several methods have been built into *minfi* and can be directly applied within its framework (Fortin *et al.*, 2014; Maksimovic *et al.*, 2012; Triche *et al.*, 2013; Touleimat & Tost, 2012), whilst others are *methyumi*-specific or require custom data types (Mancuso *et al.*, 2011; Pidsley *et al.*, 2013; Sun *et al.*, 2011; Teschendorff *et al.*, 2013; Wang *et al.*, 2012; Wu *et al.*, 2014). Although there is no single normalisation method that is universally considered best, a recent study by Fortin *et al.* (2014) has suggested that a good rule of thumb within the *minfi* framework is that the `preprocessFunnorm` (Fortin *et al.*, 2014) function is most appropriate for datasets with global methylation

differences such as cancer/normal or vastly different tissue types, whilst the `preprocessQuantile` function (Touleimat & Tost, 2012) is more suited for datasets where you do not expect global differences between your samples, for example a single tissue. Further discussion on appropriate choice of normalisation can be found in (Hicks & Irizarry, 2015), and the accompanying *quantro* package includes data-driven tests for the assumptions of quantile normalisation. As we are comparing different blood cell types, which are globally relatively similar, we will apply the `preprocessQuantile` method to our data (Figure 3). This function implements a stratified quantile normalisation procedure which is applied to the methylated and unmethylated signal intensities separately, and takes into account the different probe types. Note that after normalisation, the data is housed in a `GenomicRatioSet` object. This is a much more compact representation of the data as the colour channel information has been discarded and the *M* and *U* intensity information has been converted to M-values and beta values, together with associated genomic coordinates. Note, running the `preprocessQuantile` function on this dataset produces the warning: **‘An inconsistency was encountered while determining sex’**; this can be ignored as it is due to all the samples being from male donors.

```
# normalize the data; this results in a GenomicRatioSet object
mSetSq <- preprocessQuantile(rgSet)

## [preprocessQuantile] Mapping to genome.

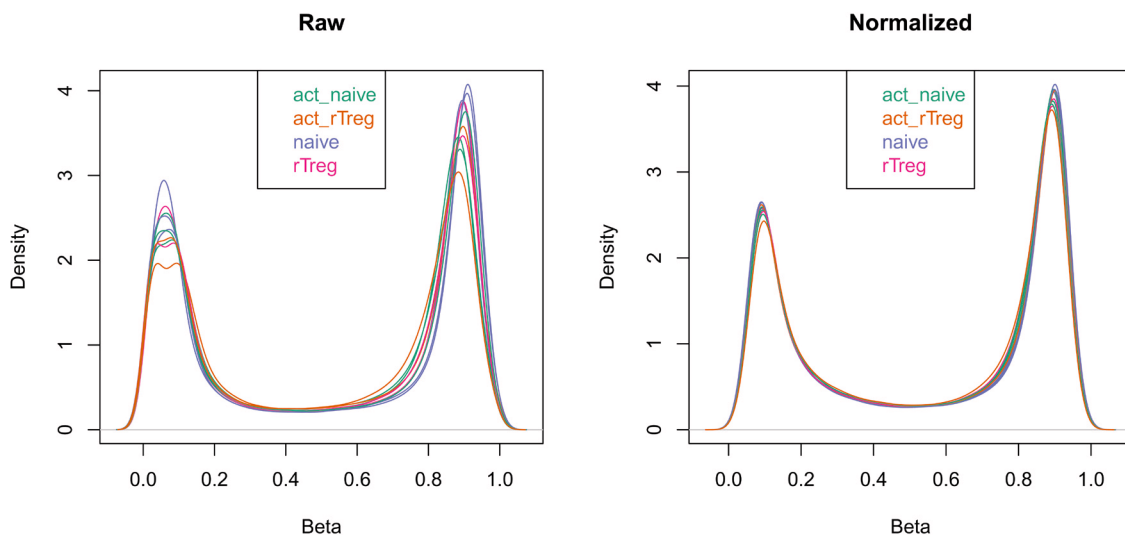
## [preprocessQuantile] Fixing outliers.

## Warning in .getSex(CN = CN, xIndex = xIndex, yIndex = yIndex, cutoff
## = cutoff): An inconsistency was encountered while determining sex. One
## possibility is that only one sex is present. We recommend further checks,
## for example with the plotSex function.

## [preprocessQuantile] Quantile normalizing.

# create a MethylSet object from the raw data for plotting
mSetRaw <- preprocessRaw(rgSet)

# visualise what the data looks like before and after normalisation
par(mfrow=c(1,2))
densityPlot(rgSet, sampGroups=targets$Sample_Group,main="Raw", legend=FALSE)
legend("top", legend = levels(factor(targets$Sample_Group)),
      text.col=brewer.pal(8,"Dark2"))
densityPlot(getBeta(mSetSq), sampGroups=targets$Sample_Group,
            main="Normalized", legend=FALSE)
legend("top", legend = levels(factor(targets$Sample_Group)),
      text.col=brewer.pal(8,"Dark2"))
```



**Figure 3.** The density plots show the distribution of the beta values for each sample before and after normalisation.

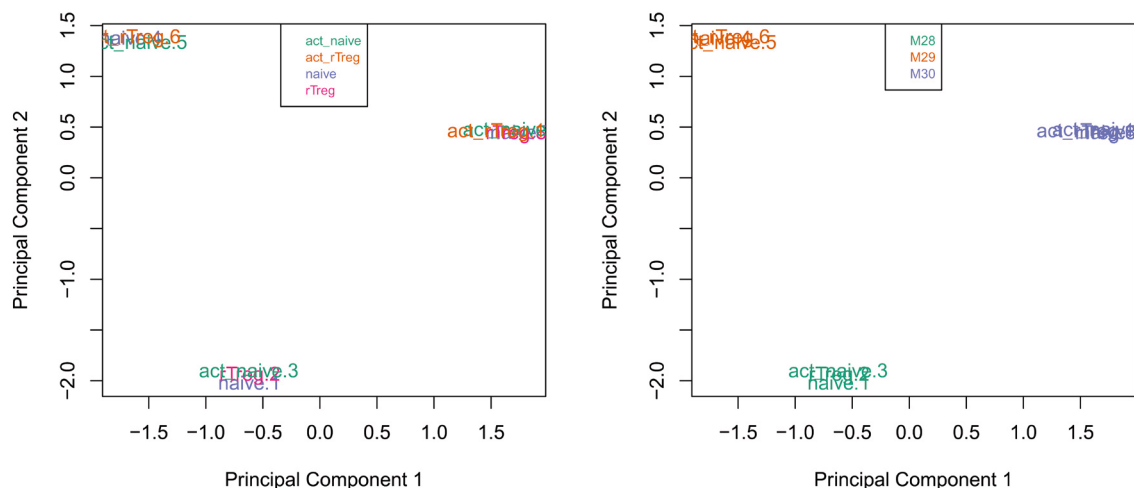
### Data exploration

Multi-dimensional scaling (MDS) plots are excellent for visualising data, and are usually some of the first plots that should be made when exploring the data. MDS plots are based on principal components analysis and are an unsupervised method for looking at the similarities and differences between the various samples. Samples that are more similar to each other should cluster together, and samples that are very different should be further apart on the plot. Dimension one (or principal component one) captures the greatest source of variation in the data, dimension two captures the second greatest source of variation in the data and so on. Colouring the data points or labels by known factors of interest can often highlight exactly what the greatest sources of variation are in the data. It is also possible to use MDS plots to decipher sample mix-ups.

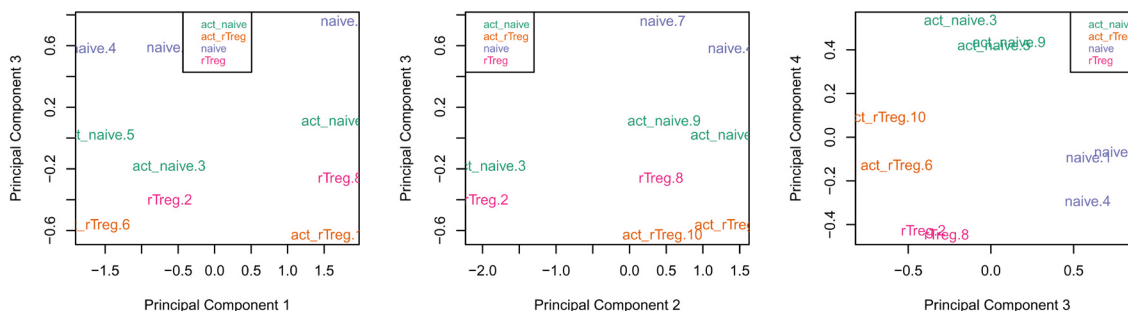
```
# MDS plots to look at largest sources of variation
par(mfrow=c(1,2))
plotMDS(getM(mSetSq), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Group)])
legend("top", legend=levels(factor(targets$Sample_Group)), text.col=pal,
      bg="white", cex=0.7)

plotMDS(getM(mSetSq), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Source)])
legend("top", legend=levels(factor(targets$Sample_Source)), text.col=pal,
      bg="white", cex=0.7)
```

Examining the MDS plots for this dataset demonstrates that the largest source of variation is the difference between individuals (Figure 4). The higher dimensions reveal that the differences between cell types are largely captured by the third and fourth principal components (Figure 5). This type of information is useful in that it can inform downstream analysis. If obvious sources of unwanted variation are revealed by the MDS plots, we can include them in our statistical model to account for them. In the case of this particular dataset, we will include individual to individual variation in our statistical model.



**Figure 4.** Multi-dimensional scaling plots are a good way to visualise the relationships between the samples in an experiment.



**Figure 5.** Examining the higher dimensions of an MDS plot can reveal significant sources of variation in the data.

```
# Examine higher dimensions to look at other sources of variation
par(mfrow=c(1,3))
plotMDS(getM(mSetSq), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Group)], dim=c(1,3))
legend("top", legend=levels(factor(targets$Sample_Group)), text.col=pal,
      cex=0.7, bg="white")

plotMDS(getM(mSetSq), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Group)], dim=c(2,3))
legend("topleft", legend=levels(factor(targets$Sample_Group)), text.col=pal,
      cex=0.7, bg="white")

plotMDS(getM(mSetSq), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Group)], dim=c(3,4))
legend("topright", legend=levels(factor(targets$Sample_Group)), text.col=pal,
      cex=0.7, bg="white")
```

### Filtering

Poor performing probes are generally filtered out prior to differential methylation analysis. As the signal from these probes is unreliable, by removing them we perform fewer statistical tests and thus incur a reduced multiple testing penalty. We filter out probes that have failed in one or more samples based on detection p-value.

```
# ensure probes are in the same order in the mSetSq and detP objects
detP <- detP[match(featureNames(mSetSq), rownames(detP)),]

# remove any probes that have failed in one or more samples
keep <- rowSums(detP < 0.01) == ncol(mSetSq)
table(keep)

## keep
## FALSE TRUE
## 977 484535

mSetSqFilt <- mSetSq[keep,]
mSetSqFilt

## class: GenomicRatioSet
## dim: 484535 10
## metadata():
## assays(2): M CN
## rownames(484535): cg13869341 cg14008030 ... cg08265308 cg14273923
## rowData names(0):
## colnames(10): naive.1 rTreg.2 ... act_naive.9 act_rTreg.10
## colData names(11): Sample_Name Sample_Well ... filenames
## predictedSex
```

```
## Annotation
##   array: IlluminaHumanMethylation450k
##   annotation: ilmn12.hg19
## Preprocessing
##   Method: Raw (no normalization or bg correction)
##   minfi version: 1.20.2
##   Manifest version: 0.4.0
```

Depending on the nature of your samples and your biological question you may also choose to filter out the probes from the X and Y chromosomes or probes that are known to have common SNPs at the CpG site. As the samples in this dataset were all derived from male donors, we will not be removing the sex chromosome probes as part of this analysis, however example code is provided below. A different dataset, which contains both male and female samples, is used to demonstrate a [Differential Variability](#) analysis and provides an example of when sex chromosome removal is necessary ([Figure 13](#)).

```
# if your data includes males and females, remove probes on the sex
# chromosomes
keep <- !(featureNames(mSetSqFilt) %in% ann450k$Name[ann450k$chr %in%
                                                    c("chrX", "chrY")])
table(keep)
mSetSqFilt <- mSetSqFilt[keep,]
```

There is a function in *minfi* that provides a simple interface for the removal of probes where common SNPs may affect the CpG. You can either remove all probes affected by SNPs (default), or only those with minor allele frequencies greater than a specified value.

```
# remove probes with SNPs at CpG site
mSetSqFilt <- dropLociWithSnps(mSetSqFilt)
mSetSqFilt

## class: GenomicRatioSet
## dim: 467351 10
## metadata(0):
## assays(2): M CN
## rownames(467351): cg13869341 cg14008030 ... cg08265308 cg14273923
## rowData names(0):
## colnames(10): naive.1 rTreg.2 ... act_naive.9 act_rTreg.10
## colData names(11): Sample_Name Sample_Well ... filenames
##   predictedSex
## Annotation
##   array: IlluminaHumanMethylation450k
##   annotation: ilmn12.hg19
## Preprocessing
##   Method: Raw (no normalization or bg correction)
##   minfi version: 1.20.2
##   Manifest version: 0.4.0
```

We will also filter out probes that have shown to be cross-reactive, that is, probes that have been demonstrated to map to multiple places in the genome. This list was originally published by [Chen \*et al.\* \(2013\)](#) and can be obtained from the authors' [website](#).

```
# exclude cross reactive probes
xReactiveProbes <- read.csv(file=paste(dataDirectory,
                                       "48639-non-specific-probes-Illumina450k.csv",
                                       sep="/"), stringsAsFactors=FALSE)
keep <- !(featureNames(mSetSqFilt) %in% xReactiveProbes$TargetID)
table(keep)

## keep
## FALSE TRUE
## 27433 439918
```

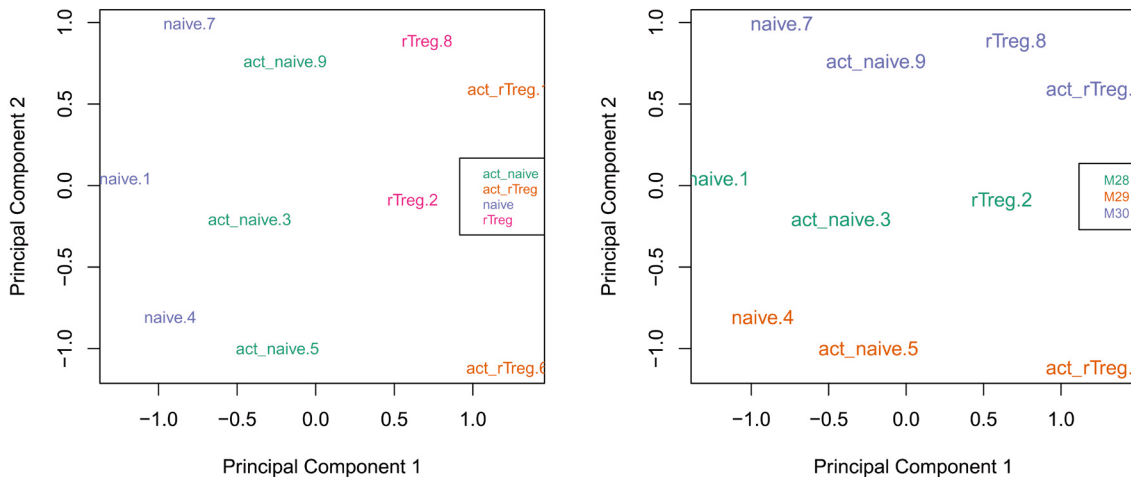
```

mSetSqFlt <- mSetSqFlt[keep,]
mSetSqFlt

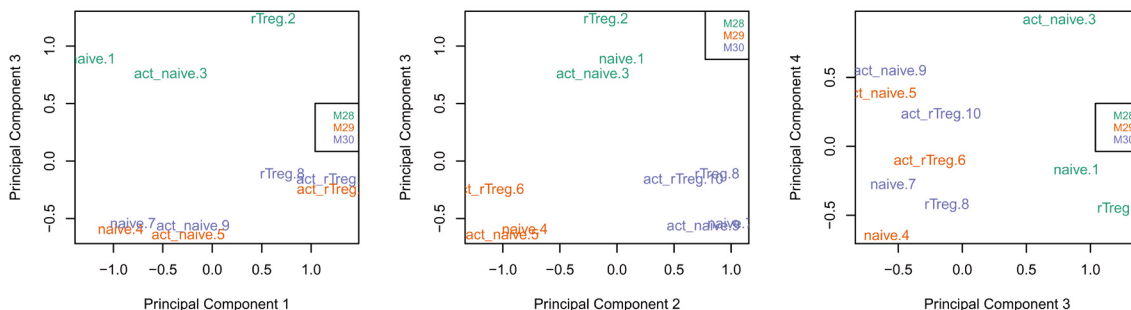
## class: GenomicRatioSet
## dim: 439918 10
## metadata(0):
## assays(2): M CN
## rownames(439918): cg13869341 cg24669183 ... cg08265308 cg14273923
## rowData names(0):
## colnames(10): naive.1 rTreg.2 ... act_naive.9 act_rTreg.10
## colData names(11): Sample_Name Sample_Well ... filenames
##   predictedSex
## Annotation
##   array: IlluminaHumanMethylation450k
##   annotation: ilmn12.hg19
## Preprocessing
##   Method: Raw (no normalization or bg correction)
##   minfi version: 1.20.2
##   Manifest version: 0.4.0

```

Once the data has been filtered and normalised, it is often useful to re-examine the MDS plots to see if the relationship between the samples has changed. It is apparent from the new MDS plots that much of the inter-individual variation has been removed as this is no longer the first principal component (Figure 6), likely due to the removal of the SNP-affected CpG probes. However, the samples do still cluster by individual in the second dimension (Figure 6 and Figure 7) and thus a factor for individual should still be included in the model.



**Figure 6.** Removing SNP-affected CpGs probes from the data changes the sample clustering in the MDS plots.



**Figure 7.** Examining the higher dimensions of the MDS plots shows that significant inter-individual variation still exists in the second and third principal components.

```

par(mfrow=c(1,2))
plotMDS(getM(mSetSqFlt), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Group)], cex=0.8)
legend("right", legend=levels(factor(targets$Sample_Group)), text.col=pal,
       cex=0.65, bg="white")

plotMDS(getM(mSetSqFlt), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Source)])
legend("right", legend=levels(factor(targets$Sample_Source)), text.col=pal,
       cex=0.7, bg="white")

par(mfrow=c(1,3))
# Examine higher dimensions to look at other sources of variation
plotMDS(getM(mSetSqFlt), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Source)], dim=c(1,3))
legend("right", legend=levels(factor(targets$Sample_Source)), text.col=pal,
       cex=0.7, bg="white")

plotMDS(getM(mSetSqFlt), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Source)], dim=c(2,3))
legend("topright", legend=levels(factor(targets$Sample_Source)), text.col=pal,
       cex=0.7, bg="white")

plotMDS(getM(mSetSqFlt), top=1000, gene.selection="common",
        col=pal[factor(targets$Sample_Source)], dim=c(3,4))
legend("right", legend=levels(factor(targets$Sample_Source)), text.col=pal,
       cex=0.7, bg="white")

```

The next step is to calculate M-values and beta values (Figure 8). As previously mentioned, M-values have nicer statistical properties and are thus better for use in statistical analysis of methylation data whilst beta values are easy to interpret and are thus better for displaying data. A detailed comparison of M-values and beta values was published by [Du et al. \(2010\)](#).

```

# calculate M-values for statistical analysis
mVals <- getM(mSetSqFlt)
head(mVals[,1:5])

##           naive.1    rTreg.2 act_naive.3    naive.4    act_naive.5
## cg13869341  2.421276  2.515948    2.165745    2.286314    2.109441
## cg24669183  2.169414  2.235964    2.280734    1.632309    2.184435
## cg15560884  1.761176  1.577578    1.597503    1.777486    1.764999
## cg01014490 -3.504268 -3.825119    -5.384735   -4.537864   -4.296526
## cg17505339  3.082191  3.924931    4.163206    3.255373    3.654134
## cg11954957  1.546401  1.912204    1.727910    2.441267    1.618331

bVals <- getBeta(mSetSqFlt)
head(bVals[,1:5])

##           naive.1    rTreg.2 act_naive.3    naive.4    act_naive.5
## cg13869341  0.84267937 0.85118462    0.8177504    0.82987650    0.81186174
## cg24669183  0.81812908 0.82489238    0.8293297    0.75610281    0.81967323
## cg15560884  0.77219626 0.74903910    0.7516263    0.77417882    0.77266205
## cg01014490  0.08098986 0.06590459    0.0233755    0.04127262    0.04842397
## cg17505339  0.89439216 0.93822870    0.9471357    0.90520570    0.92641305
## cg11954957  0.74495496 0.79008516    0.7681146    0.84450764    0.75431167

```

```

par(mfrow=c(1,2))
densityPlot(bVals, sampGroups=targets$Sample_Group, main="Beta values",
            legend=FALSE, xlab="Beta values")
legend("top", legend = levels(factor(targets$Sample_Group)),
      text.col=brewer.pal(8,"Dark2"))
densityPlot(mVals, sampGroups=targets$Sample_Group, main="M-values",
            legend=FALSE, xlab="M values")
legend("topleft", legend = levels(factor(targets$Sample_Group)),
      text.col=brewer.pal(8,"Dark2"))

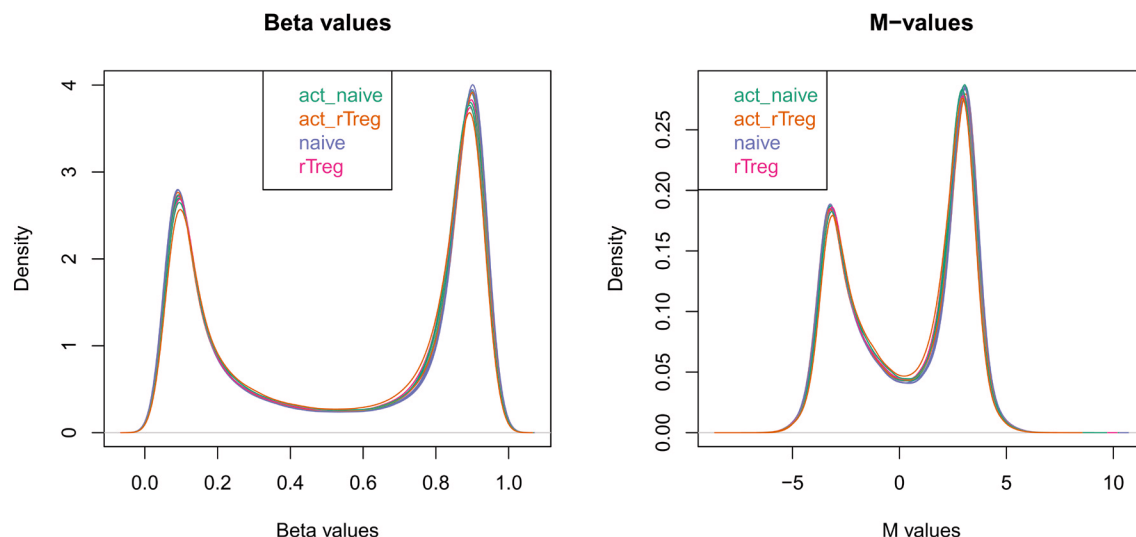
```

### Probe-wise differential methylation analysis

The biological question of interest for this particular dataset is to discover differentially methylated probes between the different cell types. However, as was apparent in the MDS plots, there is another factor that we need to take into account when we perform the statistical analysis. In the `targets` file, there is a column called `Sample_Source`, which refers to the individuals that the samples were collected from. In this dataset, each of the individuals contributes more than one cell type. For example, individual M28 contributes `naive`, `rTreg` and `act_naive` samples. Hence, when we specify our design matrix, we need to include two factors: individual and cell type. This style of analysis is called a paired analysis; differences between cell types are calculated *within* each individual, and then these differences are averaged *across* individuals to determine whether there is an overall significant difference in the mean methylation level for each CpG site. The *limma* [User's Guide](#) extensively covers the different types of designs that are commonly used for microarray experiments and how to analyse them in R.

We are interested in pairwise comparisons between the four cell types, taking into account individual to individual variation. We perform this analysis on the matrix of M-values in *limma*, obtaining moderated t-statistics and associated p-values for each CpG site. A convenient way to set up the model when the user has many comparisons of interest that they would like to test is to use a contrasts matrix in conjunction with the design matrix. A contrasts matrix will take linear combinations of the columns of the design matrix corresponding to the comparisons of interest.

Since we are performing hundreds of thousands of hypothesis tests, we need to adjust the p-values for multiple testing. A common procedure for assessing how statistically significant a change in mean levels is between two groups when a very large number of tests is being performed is to assign a cut-off on the false discovery rate ([Benjamini & Hochberg, 1995](#)), rather than on the unadjusted p-value. Typically 5% FDR is used, and this is interpreted as the researcher willing to accept that from the list of significant differentially methylated CpG sites, 5% will be false discoveries. If the p-values are not adjusted for multiple testing, the number of false discoveries will be unacceptably high. For this dataset, assuming a Type I error rate of 5%, we would expect to see  $0.05 \times 439918 = 21996$  statistical significant results for a given comparison, even if there were truly no differentially methylated CpG sites.



**Figure 8.** The distributions of beta and M-values are quite different; beta values are constrained between 0 and 1 whilst M-values range between  $-\infty$  and  $\infty$ .

Based on a false discovery rate of 5%, there are 3021 significantly differentially methylated CpGs in the naive vs rTreg comparison, while rTreg vs act\_rTreg doesn't show any significant differential methylation.

```
# this is the factor of interest
cellType <- factor(targets$Sample_Group)
# this is the individual effect that we need to account for
individual <- factor(targets$Sample_Source)

# use the above to create a design matrix
design <- model.matrix(~0+cellType+individual, data=targets)
colnames(design) <- c(levels(cellType), levels(individual)[-1])

# fit the linear model
fit <- lmFit(mVals, design)
# create a contrast matrix for specific comparisons
contMatrix <- makeContrasts(naive-rTreg,
                           naive-act_naive,
                           rTreg-act_rTreg,
                           act_naive-act_rTreg,
                           levels=design)

contMatrix

##           Contrasts
## Levels      naive - rTreg naive - act_naive rTreg - act_rTreg
## act_naive           0             -1             0
## act_rTreg           0              0            -1
## naive              1              1             0
## rTreg             -1              0             1
## M29                0              0             0
## M30                0              0             0
##           Contrasts
## Levels      act_naive - act_rTreg
## act_naive              1
## act_rTreg             -1
## naive                  0
## rTreg                  0
## M29                    0
## M30                    0

# fit the contrasts
fit2 <- contrasts.fit(fit, contMatrix)
fit2 <- eBayes(fit2)

# look at the numbers of DM CpGs at FDR < 0.05
summary(decideTests(fit2))

##      naive - rTreg naive - act_naive rTreg - act_rTreg act_naive - act_rTreg
## -1           1618           400             0             559
## 0           436897          439291          439918          438440
## 1            1403            227             0             919
```

We can extract the tables of differentially expressed CpGs for each comparison, ordered by B-statistic by default, using the `topTable` function in *limma*. The B-statistic is the log-odds of differential methylation, first published by Lonnstedt and Speed (Lonnstedt & Speed, 2002). To order by p-value, the user can specify `sort.by="p"`; and in most cases, the ordering based on the p-value and ordering based on the B-statistic will be identical. The results of the analysis for the first comparison, naive vs. rTreg, can be saved as a `data.frame` by setting `coef=1`. The `coef` parameter explicitly refers to the column in the contrasts matrix which corresponds to the comparison of interest.

```
# get the table of results for the first contrast (naive - rTreg)
```

```
ann450kSub <- ann450k[match(rownames(mVals),ann450k$Name),  
  c(1:4,12:19,24:ncol(ann450k))]
```

```
DMPs <- topTable(fit2, num=Inf, coef=1, genelist=ann450kSub)
```

```
head(DMPs)
```

| ## | chr        | pos                                                  | strand | Name                    | Probe_rs | Probe_maf | CpG_rs |
|----|------------|------------------------------------------------------|--------|-------------------------|----------|-----------|--------|
| ## | cg07499259 | chr1 12188502                                        | +      | cg07499259              | <NA>     | NA        | <NA>   |
| ## | cg26992245 | chr8 29848579                                        | -      | cg26992245              | <NA>     | NA        | <NA>   |
| ## | cg09747445 | chr15 70387268                                       | -      | cg09747445              | <NA>     | NA        | <NA>   |
| ## | cg18808929 | chr8 61825469                                        | -      | cg18808929              | <NA>     | NA        | <NA>   |
| ## | cg25015733 | chr2 99342986                                        | -      | cg25015733              | <NA>     | NA        | <NA>   |
| ## | cg21179654 | chr3 114057297                                       | +      | cg21179654              | <NA>     | NA        | <NA>   |
| ## |            | CpG_maf SBE_rs SBE_maf                               |        | Islands_Name            |          |           |        |
| ## | cg07499259 | NA <NA> NA                                           |        |                         |          |           |        |
| ## | cg26992245 | NA <NA> NA                                           |        |                         |          |           |        |
| ## | cg09747445 | NA <NA> NA                                           |        | chr15:70387929-70393206 |          |           |        |
| ## | cg18808929 | NA <NA> NA                                           |        | chr8:61822358-61823028  |          |           |        |
| ## | cg25015733 | NA <NA> NA                                           |        | chr2:99346882-99348177  |          |           |        |
| ## | cg21179654 | NA <NA> NA                                           |        |                         |          |           |        |
| ## |            | Relation_to_Island                                   |        |                         |          |           |        |
| ## | cg07499259 | OpenSea                                              |        |                         |          |           |        |
| ## | cg26992245 | OpenSea                                              |        |                         |          |           |        |
| ## | cg09747445 | N_Shore                                              |        |                         |          |           |        |
| ## | cg18808929 | S_Shelf                                              |        |                         |          |           |        |
| ## | cg25015733 | N_Shelf                                              |        |                         |          |           |        |
| ## | cg21179654 | OpenSea                                              |        |                         |          |           |        |
| ## |            | UCSC_RefGene_Name                                    |        |                         |          |           |        |
| ## | cg07499259 | TNFRSF8;TNFRSF8                                      |        |                         |          |           |        |
| ## | cg26992245 |                                                      |        |                         |          |           |        |
| ## | cg09747445 | TLE3;TLE3;TLE3                                       |        |                         |          |           |        |
| ## | cg18808929 |                                                      |        |                         |          |           |        |
| ## | cg25015733 | MGAT4A                                               |        |                         |          |           |        |
| ## | cg21179654 | ZBTB20;ZBTB20;ZBTB20;ZBTB20;ZBTB20;ZBTB20;ZBTB20     |        |                         |          |           |        |
| ## |            |                                                      |        |                         |          |           |        |
| ## |            | UCSC_RefGene_Accession                               |        |                         |          |           |        |
| ## | cg07499259 | NM_152942;NM_001243                                  |        |                         |          |           |        |
| ## | cg26992245 |                                                      |        |                         |          |           |        |
| ## | cg09747445 |                                                      |        |                         |          |           |        |
| ## | cg18808929 |                                                      |        |                         |          |           |        |
| ## | cg25015733 |                                                      |        |                         |          |           |        |
| ## | cg21179654 | NM_001164343;NM_001164346;NM_001164345;NM_001164342; |        |                         |          |           |        |
| ## |            | UCSC_RefGene_Group Phantom DMR Enhancer              |        |                         |          |           |        |
| ## | cg07499259 | 5'UTR;Body                                           |        |                         |          |           |        |
| ## | cg26992245 |                                                      |        |                         |          |           | TRUE   |
| ## | cg09747445 | Body;Body;Body                                       |        |                         |          |           |        |
| ## | cg18808929 |                                                      |        |                         |          |           | TRUE   |
| ## | cg25015733 | 5'UTR                                                |        |                         |          |           |        |
| ## | cg21179654 | 3'UTR;3'UTR;3'UTR;3'UTR;3'UTR;3'UTR;3'UTR            |        |                         |          |           |        |
| ## |            | HMM_Island Regulatory_Feature_Name                   |        |                         |          |           |        |
| ## | cg07499259 | 1:12111023-12111225                                  |        |                         |          |           |        |
| ## | cg26992245 |                                                      |        |                         |          |           |        |
| ## | cg09747445 |                                                      |        |                         |          |           |        |
| ## | cg18808929 |                                                      |        |                         |          |           |        |
| ## | cg25015733 |                                                      |        |                         |          |           |        |
| ## | cg21179654 | 3:114057192-114057775                                |        |                         |          |           |        |

```
##           Regulatory_Feature_Group DHS      logFC      AveExpr
## cg07499259                        3.654104  2.46652171
## cg26992245                        4.450696 -0.09180715
## cg09747445                       -3.337299 -0.25201484
## cg18808929                       -2.990263  0.77522878
## cg25015733                       -3.054336  0.83280190
## cg21179654 Unclassified_Cell_type_specific 2.859016  1.32460816
##           t      P.Value      adj.P.Val      B
## cg07499259 18.73131 7.267204e-08 0.005067836 7.453206
## cg26992245 18.32674 8.615461e-08 0.005067836 7.359096
## cg09747445 -18.24438 8.923101e-08 0.005067836 7.339443
## cg18808929 -17.90181 1.034217e-07 0.005067836 7.255825
## cg25015733 -17.32615 1.333546e-07 0.005067836 7.108231
## cg21179654 17.27804 1.362674e-07 0.005067836 7.095476
```

The resulting `data.frame` can easily be written to a CSV file, which can be opened in Excel.

```
write.table(DMPs, file="DMPs.csv", sep=";", row.names=FALSE)
```

It is always useful to plot sample-wise methylation levels for the top differentially methylated CpG sites to quickly ensure the results make sense (Figure 9). If the plots do not look as expected, it is usually an indication of an error in the code, or in setting up the design matrix. It is easier to interpret methylation levels on the beta value scale, so although the analysis is performed on the M-value scale, we visualise data on the beta value scale. The `plotCpg` function in *minfi* is a convenient way to plot the sample-wise beta values stratified by the grouping variable.

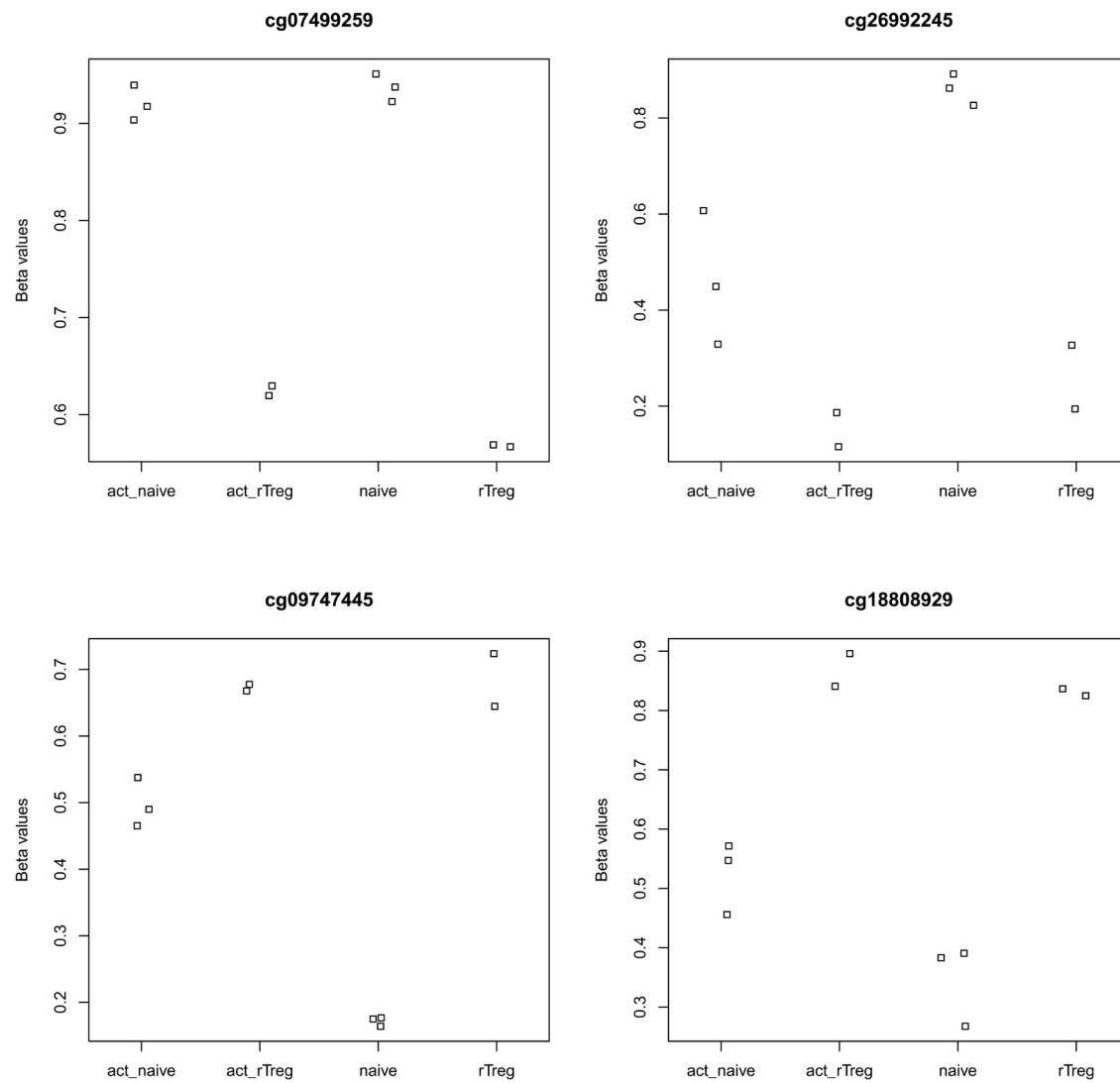
```
# plot the top 4 most significantly differentially methylated CpGs
par(mfrow=c(2,2))
sapply(rownames(DMPs)[1:4], function(cpg){
  plotCpg(bVals, cpg=cpg, pheno=targets$Sample_Group, ylab = "Beta values")
})

## $cg07499259
## NULL
##
## $cg26992245
## NULL
##
## $cg09747445
## NULL
##
## $cg18808929
## NULL
```

### Differential methylation analysis of regions

Although performing a *probe-wise* analysis is useful and informative, sometimes we are interested in knowing whether several proximal CpGs are concordantly differentially methylated, that is, we want to identify differentially methylated *regions*. There are several Bioconductor packages that have functions for identifying differentially methylated regions from 450k data. Some of the most popular are the `dmrFind` function in the *charm* package, which has been somewhat superseded for 450k arrays by the `bumphunter` function in *minfi* (Aryee *et al.*, 2014; Jaffe *et al.*, 2012), and, the recently published `dmrcate` in the *DMRcate* package (Peters *et al.*, 2015). They are each based on different statistical methods. In our experience, the `bumphunter` and `dmrFind` functions can be somewhat slow to run unless you have the computer infrastructure to parallelise them, as they use permutations to assign significance. In this workflow, we will perform an analysis using the `dmrcate`. As it is based on *limma*, we can directly use the `design` and `contMatrix` we previously defined.

Firstly, our matrix of M-values is annotated with the relevant information about the probes such as their genomic position, gene annotation, etc. By default, this is done using the `ilmn12.hg19` annotation, but this can be substituted for any argument compatible with the interface provided by the *minfi* package. The *limma* pipeline is then used for differential methylation analysis to calculate moderated t-statistics.



**Figure 9.** Plotting the top few differentially methylated CpGs is a good way to check whether the results make sense.

```
myAnnotation <- cpg.annotate(object = mVals, datatype = "array", what = "M",
                             analysis.type = "differential", design = design,
                             contrasts = TRUE, cont.matrix = contMatrix,
                             coef = "naive - rTreg", arraytype = "450K")

## Your contrast returned 3021 individually significant probes.
## We recommend the default setting of pcutoff in dmrcate().

str(myAnnotation)

## List of 7
## $ ID      : Factor w/ 439918 levels "cg00000029","cg00000108",...: 232388 391918 260351 ...
## $ stat    : num [1:439918] 0.0489 -2.0773 0.7711 -0.0304 -0.764 ...
## $ CHR     : Factor w/ 24 levels "chr1","chr10",...: 1 1 1 1 1 1 1 1 1 ...
## $ pos     : int [1:439918] 15865 534242 710097 714177 720865 758829 763119 779995 ...
## $ betafc  : num [1:439918] 0.00039 -0.04534 0.01594 0.00251 -0.00869 ...
```

```
## $ indfdr: num [1:439918] 0.994 0.565 0.872 0.997 0.873 ...
## $ is.sig: logi [1:439918] FALSE FALSE FALSE FALSE FALSE ...
## - attr(*, "row.names")= int [1:439918] 1 2 3 4 5 6 7 8 9 10 ...
## - attr(*, "class")= chr "annot"
```

Once we have the relevant statistics for the individual CpGs, we can then use the `dmrcate` function to combine them to identify differentially methylated regions. The main output table `DMRs$results` contains all of the regions found, along with their genomic annotations and p-values.

```
DMRs <- dmrcate(myAnnotation, lambda=1000, C=2)
head(DMRs$results)
```

```
##          coord no.cpgs          minfdr      Stouffer
## 452   chr17:57915665-57918682          12  4.957890e-91  6.639928e-10
## 723   chr3:114012316-114012912           5  1.622885e-180  1.515378e-07
## 464   chr17:74639731-74640078           6  9.516873e-90  1.527961e-07
## 1053  chrX:49121205-49122718           6  6.753751e-84  2.936984e-07
## 487   chr18:21452730-21453131           7  5.702319e-115  7.674943e-07
## 186  chr10:135202522-135203200          6  1.465070e-65  7.918224e-07
##          maxbetafc meanbetafc
## 452    0.3982862    0.3131611
## 723    0.5434277    0.4251622
## 464   -0.2528645   -0.1951904
## 1053    0.4529088    0.3006242
## 487   -0.3867474   -0.2546089
## 186    0.2803157    0.2293419
```

As for the probe-wise analysis, it is advisable to visualise the results to ensure that they make sense. The regions can easily be viewed using the `DMR.plot` function provided in the *DMRcate* package (Figure 10).

```
# convert the regions to annotated genomic ranges
data(dmrdata)
results.ranges <- extractRanges(DMRs, genome = "hg19")

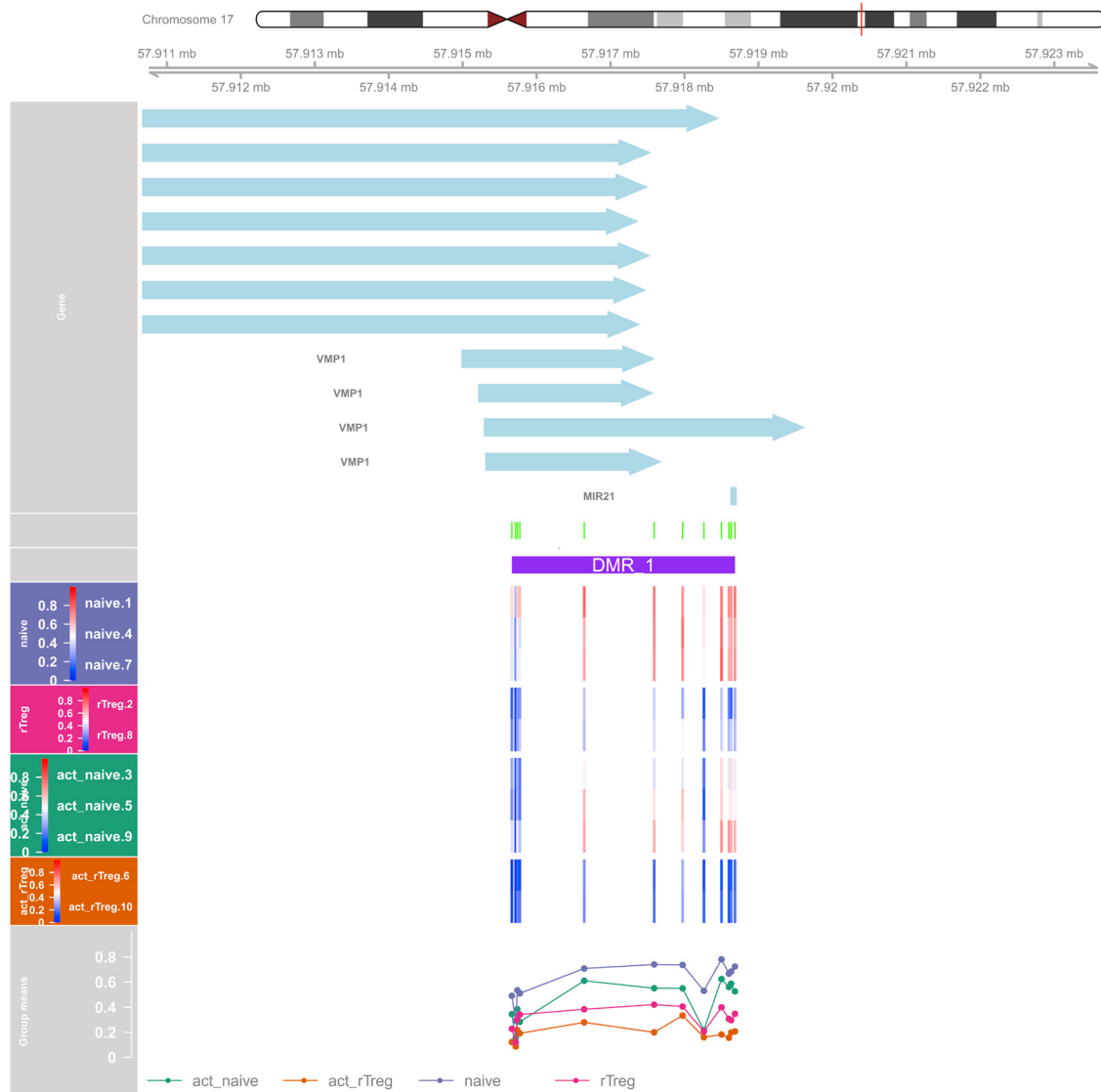
# set up the grouping variables and colours
groups <- pal[1:length(unique(targets$Sample_Group))]
names(groups) <- levels(factor(targets$Sample_Group))
cols <- groups[as.character(factor(targets$Sample_Group))]
samps <- 1:nrow(targets)

# draw the plot for the top DMR
par(mfrow=c(1,1))
DMR.plot(ranges=results.ranges, dmr=1, CpGs=bVals, phen.col=cols, what = "Beta",
         arraytype = "450K", pch=16, toscale=TRUE, plotmedians=TRUE,
         genome="hg19", samps=samps)
```

### Customising visualisations of methylation data

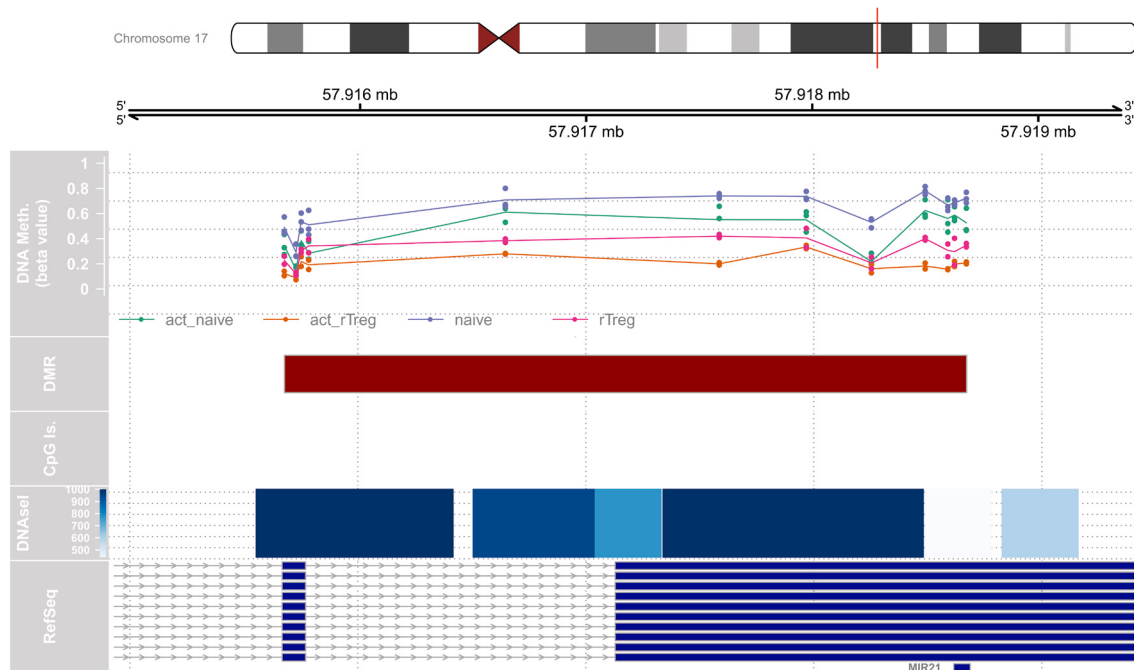
The *Gviz* package offers powerful functionality for plotting methylation data in its genomic context. The package *vignette* is very extensive and covers the various types of plots that can be produced using the *Gviz* framework. We will plot one of the differentially methylated regions from the *DMRcate* analysis to demonstrate the type of visualisations that can be created (Figure 11).

We will first set up the genomic region we would like to plot by extracting the genomic coordinates of the top differentially methylated region.



**Figure 10.** The DMRcate “DMR.plot” function allows you to quickly visualise DMRs in their genomic context. By default, the plot shows the location of the DMR in the genome, the position of any genes that are nearby, the base pair positions of the CpG probes, the methylation levels of the individual samples as a heatmap and the mean methylation levels for the various sample groups in the experiment. This plot shows the top ranked DMR identified by the DMRcate analysis.

```
# indicate which genome is being used
gen <- "hg19"
# the index of the DMR that we will plot
dmrIndex <- 1
# extract chromosome number and location from DMR results
coords <- strsplit2(DMRs$results$coord[dmrIndex], ":")
chrom <- coords[1]
start <- as.numeric(strsplit2(coords[2], "-")[1])
end <- as.numeric(strsplit2(coords[2], "-")[2])
# add 25% extra space to plot
minbase <- start - (0.25*(end-start))
maxbase <- end + (0.25*(end-start))
```



**Figure 11.** The Gviz package provides extensive functionality for customising plots of genomic regions. This plot shows the top ranked DMR identified by the DMRcate analysis.

Next, we will add some genomic annotations of interest such as the locations of CpG islands and DNaseI hypersensitive sites; this can be any feature or genomic annotation of interest that you have data available for. The CpG islands data was generated using the method published by [Wu \*et al.\* \(2010\)](#); the DNaseI hypersensitive site data was obtained from the [UCSC Genome Browser](#).

```
# CpG islands
islandHMM <- read.csv(paste(dataDirectory,
                             "model-based-cpg-islands-hg19-chr17.txt", sep="/"),
                      sep="\t", stringsAsFactors=FALSE, header=FALSE)

head(islandHMM)

##           V1      V2      V3      V4      V5      V6      V7      V8
## 1 chr17_ctg5_hap1  8935  10075  1141   129   815  0.714  0.887
## 2 chr17_ctg5_hap1 64252  64478   227    30   165  0.727  1.014
## 3 chr17_ctg5_hap1 87730  89480  1751   135  1194  0.682  0.663
## 4 chr17_ctg5_hap1 98265  98591   327    29   226  0.691  0.744
## 5 chr17_ctg5_hap1 120763 125451  4689   359  3032  0.647  0.733
## 6 chr17_ctg5_hap1 146257 146607   351    19   231  0.658  0.500

islandData <- GRanges(seqnames=Rle(islandHMM[,1]),
                      ranges=IRanges(start=islandHMM[,2], end=islandHMM[,3]),
                      strand=Rle(strand(rep("*", nrow(islandHMM)))))

islandData

## GRanges object with 3456 ranges and 0 metadata columns:
##           seqnames      ranges strand
##           <Rle>          <IRanges> <Rle>
##      [1] chr17_ctg5_hap1  [ 8935, 10075]      *
##      [2] chr17_ctg5_hap1  [ 64252, 64478]      *
##      [3] chr17_ctg5_hap1  [ 87730, 89480]      *
##      [4] chr17_ctg5_hap1  [ 98265, 98591]      *
```

```
##      [5] chr17_ctg5_hap1      [120763, 125451]      *
##      ...      ...      ...      ...
##      [3452]      chr17 [81147380, 81147511]      *
##      [3453]      chr17 [81147844, 81148321]      *
##      [3454]      chr17 [81152612, 81153665]      *
##      [3455]      chr17 [81156194, 81156512]      *
##      [3456]      chr17 [81162945, 81165532]      *
##      -----
##      seqinfo: 5 sequence from an unspecified genome; no seqlengths

# DNaseI hypersensitive sites
dnase <- read.csv(paste(dataDirectory,"wgEncodeRegDnaseClusteredV3chr17.bed",
                        sep="/"),
                 sep="\t",stringsAsFactors=FALSE,header=FALSE)

head(dnase)

##      V1      V2      V3 V4      V5 V6      V7
## 1 chr17    125    335  7 444  7      84,83,88,90,77,87,89,
## 2 chr17    685    835  1 150  1      80,
## 3 chr17   2440   2675 13 410 13 0,30,102,104,38,47,61,68,122,1,51,73,75,
## 4 chr17   3020   3170  1 247  1      120,
## 5 chr17   3740   3890  2 161  2      71,73,
## 6 chr17   5520   6110  4 241  5      17,19,25,16,16,
##
##                                     V8
## 1                                     328,208,444,218,109,171,191,
## 2                                     150,
## 3 204,410,301,206,46,48,84,164,85,12,98,215,146,
## 4                                     247,
## 5                                     108,161,
## 6                                     241,185,239,26,52,

dnaseData <- GRanges(seqnames=dnase[,1],
                     ranges=IRanges(start=dnase[,2], end=dnase[,3]),
                     strand=Rle(rep("+",nrow(dnase))),
                     data=dnase[,5])

dnaseData

## GRanges object with 74282 ranges and 1 metadata column:
##      seqnames      ranges strand |      data
##      <Rle>      <IRanges> <Rle> | <integer>
##      [1]      chr17      [ 125,  335]      * |      444
##      [2]      chr17      [ 685,  835]      * |      150
##      [3]      chr17     [2440, 2675]      * |      410
##      [4]      chr17     [3020, 3170]      * |      247
##      [5]      chr17     [3740, 3890]      * |      161
##      ...      ...      ...      ...      ...
##      [74278]      chr17 [81153140, 81153350]      * |      574
##      [74279]      chr17 [81153580, 81153810]      * |      208
##      [74280]      chr17 [81185540, 81185750]      * |      326
##      [74281]      chr17 [81188880, 81189090]      * |      209
##      [74282]      chr17 [81194900, 81195115]      * |      185
##      -----
##      seqinfo: 1 sequence from an unspecified genome; no seqlengths
```

Now, set up the ideogram, genome and RefSeq tracks that will provide context for our methylation data.

```
iTrack <- IdeogramTrack(genome = gen, chromosome = chrom, name="")
gTrack <- GenomeAxisTrack(col="black", cex=1, name="", fontcolor="black")
rTrack <- UcscTrack(genome=gen, chromosome=chrom, track="refGene",
  from=minbase, to=maxbase, trackType="GeneRegionTrack",
  rstarts="exonStarts", rends="exonEnds", gene="name",
  symbol="name2", transcript="name", strand="strand",
  fill="darkblue", stacking="squish", name="RefSeq",
  showId=TRUE, geneSymbol=TRUE)
```

Ensure that the methylation data is ordered by chromosome and base position.

```
ann450kOrd <- ann450kSub[order(ann450kSub$chr,ann450kSub$pos),]
head(ann450kOrd)
```

```
## DataFrame with 6 rows and 22 columns
##           chr      pos      strand      Name      Probe_rs
##           <character> <integer> <character> <character> <character>
## cg13869341      chr1      15865          + cg13869341      NA
## cg24669183      chr1      534242         - cg24669183      rs6680725
## cg15560884      chr1      710097          + cg15560884      NA
## cg01014490      chr1      714177         - cg01014490      NA
## cg17505339      chr1      720865         - cg17505339      NA
## cg11954957      chr1      758829          + cg11954957      rs115498424
##           Probe_maf      CpG_rs      CpG_maf      SBE_rs      SBE_maf
##           <numeric> <character> <numeric> <character> <numeric>
## cg13869341      NA      NA      NA      NA      NA
## cg24669183      0.108100      NA      NA      NA      NA
## cg15560884      NA      NA      NA      NA      NA
## cg01014490      NA      NA      NA      NA      NA
## cg17505339      NA      NA      NA      NA      NA
## cg11954957      0.029514      NA      NA      NA      NA
##           Islands_Name Relation_to_Island UCSC_RefGene_Name
##           <character>      <character>      <character>
## cg13869341      OpenSea      WASH5P
## cg24669183      chr1:533219-534114      S_Shore
## cg15560884      chr1:713984-714547      N_Shelf
## cg01014490      chr1:713984-714547      Island
## cg17505339      OpenSea
## cg11954957      chr1:762416-763445      N_Shelf
##           UCSC_RefGene_Accession UCSC_RefGene_Group      Phantom
##           <character>      <character> <character>
## cg13869341      NR_024540      Body
## cg24669183
## cg15560884
## cg01014490
## cg17505339
## cg11954957
##           DMR      Enhancer      HMM_Island Regulatory_Feature_Name
##           <character> <character>      <character>      <character>
## cg13869341
## cg24669183      1:523025-524193
## cg15560884
## cg01014490      1:703784-704410      1:713802-715219
## cg17505339
## cg11954957
##           Regulatory_Feature_Group      DHS
##           <character> <character>
## cg13869341
## cg24669183
## cg15560884
## cg01014490      Promoter_Associated
## cg17505339
## cg11954957
```

```

bValsOrd <- bVals[match(ann450kOrd$Name, rownames(bVals)),]
head(bValsOrd)

##           naive.1    rTreg.2 act_naive.3    naive.4 act_naive.5
## cg13869341 0.84267937 0.85118462 0.8177504 0.82987650 0.81186174
## cg24669183 0.81812908 0.82489238 0.8293297 0.75610281 0.81967323
## cg15560884 0.77219626 0.74903910 0.7516263 0.77417882 0.77266205
## cg01014490 0.08098986 0.06590459 0.0233755 0.04127262 0.04842397
## cg17505339 0.89439216 0.93822870 0.9471357 0.90520570 0.92641305
## cg11954957 0.74495496 0.79008516 0.7681146 0.84450764 0.75431167
##           act_rTreg.6    naive.7    rTreg.8 act_naive.9 act_rTreg.10
## cg13869341 0.8090798 0.8891851 0.88537940 0.90916748 0.88334231
## cg24669183 0.8187838 0.7903763 0.85304116 0.80930568 0.80979554
## cg15560884 0.7721528 0.7658623 0.75909061 0.78099397 0.78569274
## cg01014490 0.0644404 0.0245281 0.02832358 0.07740468 0.04640659
## cg17505339 0.9286016 0.8889361 0.87205348 0.90099782 0.93508348
## cg11954957 0.8116911 0.7832207 0.84929777 0.84719430 0.83350220

```

Create the data tracks using the appropriate track type for each data type.

```

# create genomic ranges object from methylation data
cpgData <- GRanges(seqnames=Rle(ann450kOrd$chr),
                   ranges=IRanges(start=ann450kOrd$pos, end=ann450kOrd$pos),
                   strand=Rle(rep("...", nrow(ann450kOrd))),
                   betas=bValsOrd)

# extract data on CpGs in DMR
cpgData <- subsetByOverlaps(cpgData, results.ranges[dmrIndex])

# methylation data track
methTrack <- DataTrack(range=cpgData, groups=targets$Sample_Group, genome = gen,
                      chromosome=chrom, ylim=c(-0.05,1.05), col=pal,
                      type=c("a","p"), name="DNA Meth.\n(beta value)",
                      background.panel="white", legend=TRUE, cex.title=0.8,
                      cex.axis=0.8, cex.legend=0.8)

# CpG island track
islandTrack <- AnnotationTrack(range=islandData, genome=gen, name="CpG Is.",
                              chromosome=chrom, fill="darkgreen")

# DNaseI hypersensitive site data track
dnaseTrack <- DataTrack(range=dnaseData, genome=gen, name="DNaseI",
                       type="gradient", chromosome=chrom)

# DMR position data track
dmrTrack <- AnnotationTrack(start=start, end=end, genome=gen, name="DMR",
                           chromosome=chrom, fill="darkred")

```

Set up the track list and indicate the relative sizes of the different tracks. Finally, draw the plot using the `plotTracks` function (Figure 11).

```

tracks <- list(iTrack, gTrack, methTrack, dmrTrack, islandTrack, dnaseTrack,
              rTrack)
sizes <- c(2,2,5,2,2,2,3) # set up the relative sizes of the tracks
plotTracks(tracks, from=minbase, to=maxbase, showTitle=TRUE, add53=TRUE,
           add35=TRUE, grid=TRUE, lty.grid=3, sizes=sizes, length(tracks))

```

## Additional analyses

### Gene ontology testing

Once you have performed a differential methylation analysis, there may be a very long list of significant CpG sites to interpret. One question a researcher may have is, “which gene pathways are over-represented for differentially methylated CpGs?” In some cases it is relatively straightforward to link the top differentially methylated CpGs to

genes that make biological sense in terms of the cell types or samples being studied, but there may be many thousands of CpGs significantly differentially methylated. In order to gain an understanding of the biological processes that the differentially methylated CpGs may be involved in, we can perform gene ontology or KEGG pathway analysis using the `gometh` function in the *missMethyl* package (Phipson *et al.*, 2016).

Let us consider the first comparison, naive vs rTreg, with the results of the analysis in the `DMPs` table. The `gometh` function takes as input a character vector of the names (e.g. `cg20832020`) of the significant CpG sites, and optionally, a character vector of all CpGs tested. This is recommended particularly if extensive filtering of the CpGs has been performed prior to analysis. For gene ontology testing (default), the user can specify `collection="GO"`. For testing KEGG pathways, specify `collection="KEGG"`. In the `DMPs` table, the `Name` column corresponds to the CpG name. We will select all CpG sites that have adjusted p-value of less than 0.05.

```
# Get the significant CpG sites at less than 5% FDR
sigCpGs <- DMPs$Name[DMPs$adj.P.Val<0.05]
# First 10 significant CpGs
sigCpGs[1:10]

## [1] "cg07499259" "cg26992245" "cg09747445" "cg18808929" "cg25015733"
## [6] "cg21179654" "cg26280976" "cg16943019" "cg10898310" "cg25130381"

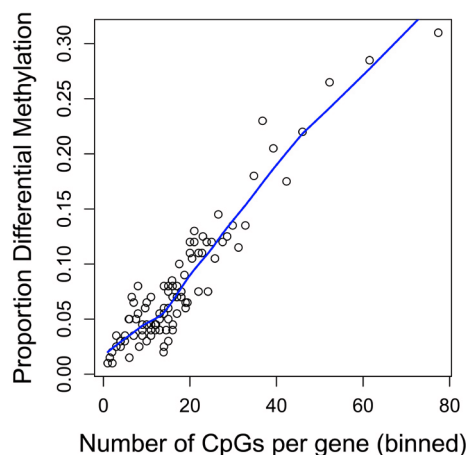
# Total number of significant CpGs at 5% FDR
length(sigCpGs)

## [1] 3021

# Get all the CpG sites used in the analysis to form the background
all <- DMPs$Name
# Total number of CpG sites tested
length(all)

## [1] 439918
```

The `gometh` function takes into account the varying numbers of CpGs associated with each gene on the Illumina methylation arrays. For the 450k array, the numbers of CpGs mapping to genes can vary from as few as 1 to as many as 1200. The genes that have more CpGs associated with them will have a higher probability of being identified as differentially methylated compared to genes with fewer CpGs. We can look at this bias in the data by specifying `plot=TRUE` in the call to `gometh` (Figure 12).



**Figure 12.** Bias resulting from different numbers of CpG probes in different genes.



the top 20 pathways, topGSA can be called. gsmeth accepts a single gene set, or a list of gene sets. The gene identifiers in the gene set must be Entrez Gene IDs. To demonstrate gsmeth, we are using the curated genesets (C2) from the Broad Institute Molecular signatures [database](#). These can be downloaded as an RData object from the WEHI Bioinformatics [website](#).

```
# load Broad human curated (C2) gene sets
load(paste(dataDirectory, "human_c2_v5.rdata", sep="/"))
# perform the gene set test(s)
gsa <- gsmeth(sig.cpg=sigCpGs, all.cpg=all, collection=Hs.c2)

## Warning in alias2SymbolTable(flat$symbol): Multiple symbols ignored for one
## or more aliases

# top 10 gene sets
topGSA(gsa, number=10)
```

|                                     | N    | DE  | P.DE                              |
|-------------------------------------|------|-----|-----------------------------------|
| REACTOME_IMMUNE_SYSTEM              | 933  | 127 | 0.000000000000000000000000        |
| DACOSTA_UV_RESPONSE_VIA_ERCC3_DN    | 855  | 147 | 0.000000000000000000000000        |
| ZHENG_BOUND_BY_FOXP3                | 491  | 138 | 0.000000000000000000000000        |
| MARSON_BOUND_BY_FOXP3_UNSTIMULATED  | 1229 | 169 | 0.000000000000000000000000        |
| CHEN_METABOLIC_SYNDROM_NETWORK      | 1210 | 162 | 0.000000000000000000000000        |
| MARTENS_BOUND_BY_PML_RARA_FUSION    | 456  | 105 | 0.000000000000000000000000        |
| PILON_KLF1_TARGETS_DN               | 1972 | 262 | 0.000000000000000000000000        |
| JAATINEN_HEMATOPOIETIC_STEM_CELL_DN | 226  | 59  | 0.000000000000000000004151028     |
| SMID_BREAST_CANCER_NORMAL_LIKE_UP   | 476  | 92  | 0.0000000000000000002674784772    |
| LEE_EARLY_T_LYMPHOCYTE_DN           | 57   | 25  | 0.000000000000000000825660086765  |
|                                     |      |     | FDR                               |
| REACTOME_IMMUNE_SYSTEM              |      |     | 0.000000000000000000000000        |
| DACOSTA_UV_RESPONSE_VIA_ERCC3_DN    |      |     | 0.000000000000000000000000        |
| ZHENG_BOUND_BY_FOXP3                |      |     | 0.000000000000000000000000        |
| MARSON_BOUND_BY_FOXP3_UNSTIMULATED  |      |     | 0.000000000000000000000000        |
| CHEN_METABOLIC_SYNDROM_NETWORK      |      |     | 0.000000000000000000000000        |
| MARTENS_BOUND_BY_PML_RARA_FUSION    |      |     | 0.000000000000000000000000        |
| PILON_KLF1_TARGETS_DN               |      |     | 0.000000000000000000000000        |
| JAATINEN_HEMATOPOIETIC_STEM_CELL_DN |      |     | 0.00000000000000000002451701      |
| SMID_BREAST_CANCER_NORMAL_LIKE_UP   |      |     | 0.0000000000000000001404262005    |
| LEE_EARLY_T_LYMPHOCYTE_DN           |      |     | 0.0000000000000000000390124390997 |

While gene set testing is useful for providing some biological insight in terms of what pathways might be affected by aberrant methylation, care should be taken not to over-interpret the results. Gene set testing should be used for the purpose of providing some biological insight that ideally would be tested and validated in further laboratory experiments. It is important to keep in mind that we are not observing gene level activity such as in RNA-Seq experiments, and that we have had to take an extra step to associate CpGs with genes.

### Differential variability

Rather than testing for differences in mean methylation, we may be interested in testing for differences between group variances. For example, it has been hypothesised that highly variable CpGs in cancer may contribute to tumour heterogeneity ([Hansen et al., 2011](#)). Hence we may be interested in CpG sites that are consistently methylated in one group, but variably methylated in another group.

Sample size is an important consideration when testing for differentially variable CpG sites. In order to get an accurate estimate of the group variances, larger sample sizes are required than for estimating group means. A good rule of thumb is to have at least ten samples in each group ([Phipson & Oshlack, 2014](#)). To demonstrate testing for differentially variable CpG sites, we will use a publicly available dataset on ageing GSE30870, where whole blood samples were collected from 18 centenarians and 18 newborns and profiled for methylation on the 450k array ([Heyn et al., 2012](#)). The data (`age.rgSet`) and sample information (`age.targets`) have been included as an R data object in the data archive you previously downloaded from [figshare](#). We can load the data using the `load` command, after which it needs to be normalised and filtered as previously described.

```

# load data
load(paste(dataDirectory, "ageData.RData", sep="/"))

# calculate detection p-values
age.detP <- detectionP(age.rgSet)

# pre-process the data after excluding poor quality samples
age.mSetSq <- preprocessQuantile(age.rgSet)

## [preprocessQuantile] Mapping to genome.

## [preprocessQuantile] Fixing outliers.

## [preprocessQuantile] Quantile normalizing.

# add sex information to targets information
age.targets$Sex <- getSex(age.mSetSq)$predictedSex

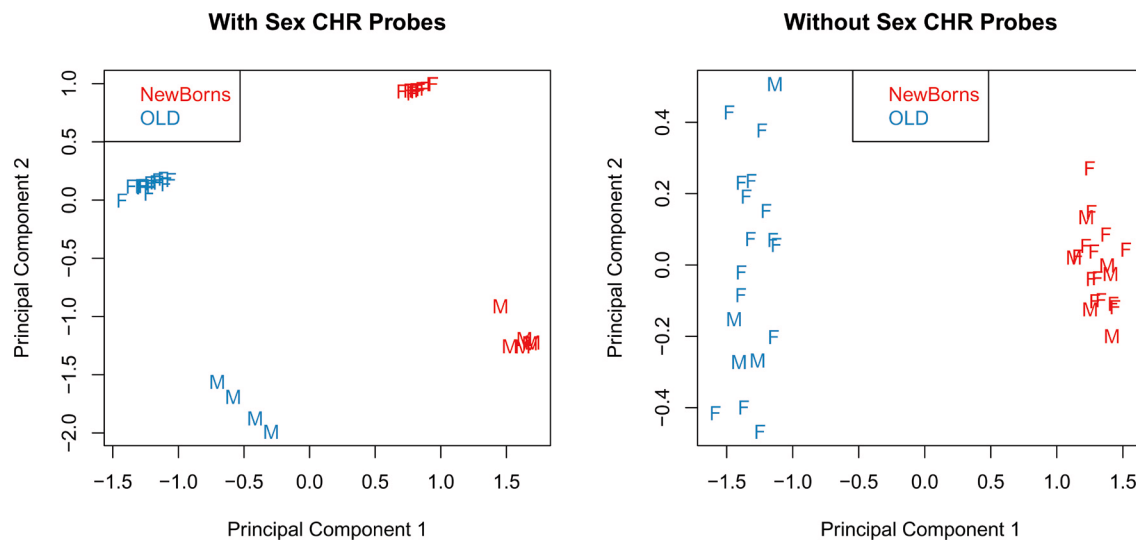
# ensure probes are in the same order in the mSetSq and detP objects
age.detP <- age.detP[match(featureNames(age.mSetSq), rownames(age.detP)),]
# remove poor quality probes
keep <- rowSums(age.detP < 0.01) == ncol(age.detP)
age.mSetSqFilt <- age.mSetSq[keep,]

# remove probes with SNPs at CpG or single base extension (SBE) site
age.mSetSqFilt <- dropLociWithSnps(age.mSetSqFilt, snps = c("CpG", "SBE"))

# remove cross-reactive probes
keep <- !(featureNames(age.mSetSqFilt) %in% xReactiveProbes$TargetID)
age.mSetSqFilt <- age.mSetSqFilt[keep,]

```

As this dataset contains samples from both males and females, we can use it to demonstrate the effect of removing sex chromosome probes on the data. The MDS plots below show the relationship between the samples in the ageing dataset before and after sex chromosome probe removal (Figure 13). It is apparent that before the removal of sex chromosome probes, the sample cluster based on sex in the second principal component. When the sex chromosome probes are removed, age is the largest source of variation present and the male and female samples no longer form separate clusters.



**Figure 13.** When samples from both males and females are included in a study, sex is usually the largest source of variation in methylation data.

```

# tag sex chromosome probes for removal
keep <- !(featureNames(age.mSetSqFilt) %in% ann450k$Name[ann450k$chr %in%
                                                    c("chrX", "chrY")])

age.pal <- brewer.pal(8, "Set1")
par(mfrow=c(1,2))
plotMDS(getM(age.mSetSqFilt), top=1000, gene.selection="common",
        col=age.pal[factor(age.targets$Sample_Group)], labels=age.targets$Sex,
        main="With Sex CHR Probes")
legend("topleft", legend=levels(factor(age.targets$Sample_Group)),
      text.col=age.pal)

plotMDS(getM(age.mSetSqFilt[keep,]), top=1000, gene.selection="common",
        col=age.pal[factor(age.targets$Sample_Group)], labels=age.targets$Sex,
        main="Without Sex CHR Probes")
legend("top", legend=levels(factor(age.targets$Sample_Group)),
      text.col=age.pal)

# remove sex chromosome probes from data
age.mSetSqFilt <- age.mSetSqFilt[keep,]

```

We can test for differentially variable CpGs using the `varFit` function in the *missMethyl* package. The syntax for specifying which groups we are interested in testing is slightly different to the standard way a model is specified in *limma*, particularly for designs where an intercept is fitted (see *missMethyl* vignette for further details). For the ageing data, the design matrix includes an intercept term, and a term for age. The `coef` argument in the `varFit` function indicates which columns of the design matrix correspond to the intercept and grouping factor. Thus, for the ageing dataset we set `coef=c(1,2)`. Note that design matrices without intercept terms are permitted, with specific contrasts tested using the `contrasts.varFit` function.

```

# get M-values for analysis
age.mVals <- getM(age.mSetSqFilt)

design <- model.matrix(~factor(age.targets$Sample_Group))
# Fit the model for differential variability
# specifying the intercept and age as the grouping factor
fitvar <- varFit(age.mVals, design = design, coef = c(1,2))

# Summary of differential variability
summary(decideTests(fitvar))

##      (Intercept) factor(age.targets$Sample_Group)OLD
## -1             0                               1325
##  0             11441                           393451
##  1             417787                           34452

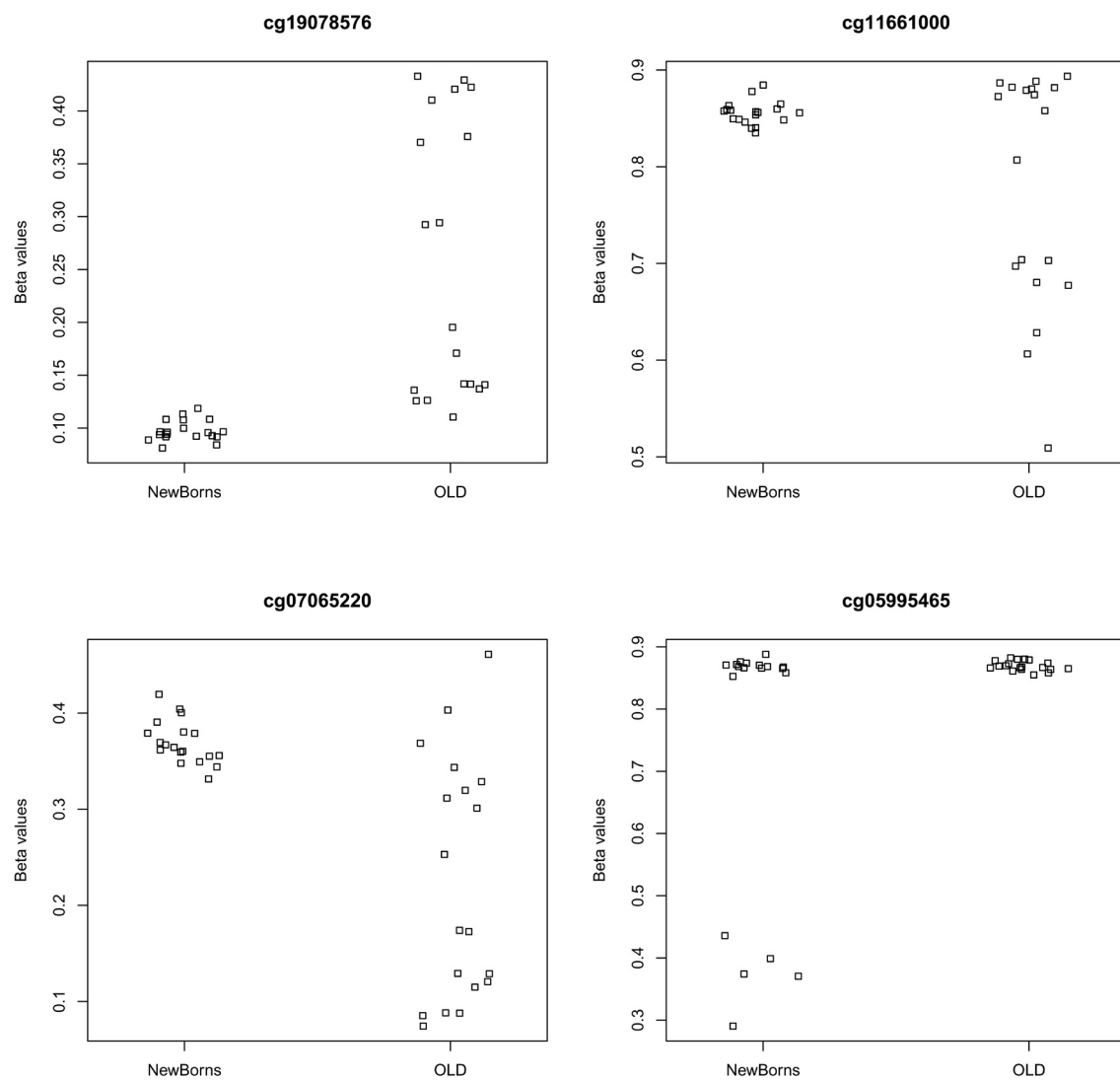
topDV <- topVar(fitvar, coef=2)
# Top 10 differentially variable CpGs between old vs. newborns
topDV

##      SampleVar LogVarRatio DiffLevene      t      P.Value
## cg19078576 1.1128910      3.746586 0.8539180 7.006476 0.0000000006234780
## cg11661000 0.5926226      3.881306 0.8413614 6.945711 0.0000000008176807
## cg07065220 1.0111380      4.181802 0.9204407 6.840327 0.0000000013069867
## cg05995465 1.4478673     -5.524284 -1.3035981 -6.708321 0.0000000023462074
## cg18091046 1.1121511      3.564282 1.0983340 6.679920 0.0000000026599570
## cg05491001 0.9276904      3.869760 0.7118591 6.675892 0.0000000027077013
## cg05542681 1.0287320      3.783637 0.9352814 6.635588 0.0000000032347355
## cg02726803 0.3175570      4.063650 0.6418968 6.607508 0.0000000036608219
## cg08362283 1.0028907      4.783899 0.6970960 6.564472 0.0000000044240941

```

```
## cg18160402 0.5624192 3.716228 0.5907985 6.520508 0.0000000053665347
## Adj.P.Value
## cg19078576 0.0001754857
## cg11661000 0.0001754857
## cg07065220 0.0001869984
## cg05995465 0.0001937035
## cg18091046 0.0001937035
## cg05491001 0.0001937035
## cg05542681 0.0001964159
## cg02726803 0.0001964159
## cg08362283 0.0002109939
## cg18160402 0.0002303467
```

Similarly to the differential methylation analysis, is it useful to plot sample-wise beta values for the differentially variable CpGs to ensure the significant results are not driven by artifacts or outliers (Figure 14).



**Figure 14.** As for DMPs, it is useful to plot the top few differentially variable CpGs to check that the results make sense.

```
# get beta values for ageing data
age.bVals <- getBeta(age.mSetSqFlt)

par(mfrow=c(2,2))
sapply(rownames(topDV)[1:4], function(cpg) {
  plotCpg(age.bVals, cpg=cpg, pheno=age.targets$Sample_Group,
          ylab = "Beta values")
})
```

An example of testing for differential variability when the design matrix does not have an intercept term is detailed in the *missMethyl* [vignette](#).

### Cell type composition

As methylation is cell type specific and methylation arrays provide CpG methylation values for a population of cells, biological findings from samples that are comprised of a mixture of cell types, such as blood, can be confounded with cell type composition ([Jaffe & Irizarry, 2014](#)). The *minfi* function `estimateCellCounts` facilitates the estimation of the level of confounding between phenotype and cell type composition in a set of samples. The function uses a modified version of the method published by [Houseman \*et al.\* \(2012\)](#) and the package `FlowSorted.Blood.450k`, which contains 450k methylation data from sorted blood cells, to estimate the cell type composition of blood samples.

```
# load sorted blood cell data package
library(FlowSorted.Blood.450k)
# ensure that the "Slide" column of the rgSet pheno data is numeric
# to avoid "estimateCellCounts" error
pData(age.rgSet)$Slide <- as.numeric(pData(age.rgSet)$Slide)
# estimate cell counts
cellCounts <- estimateCellCounts(age.rgSet)

## [estimateCellCounts] Combining user data with reference (flow sorted) data.

## [estimateCellCounts] Processing user and reference data together.

## [preprocessQuantile] Mapping to genome.

## [preprocessQuantile] Fixing outliers.

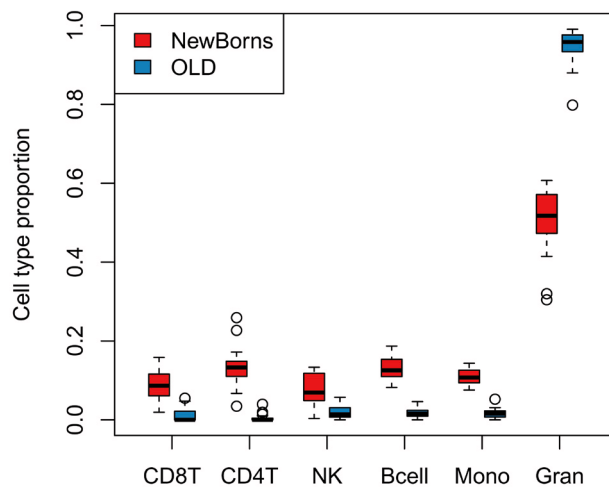
## [preprocessQuantile] Quantile normalizing.

## [estimateCellCounts] Picking probes for composition estimation.

## [estimateCellCounts] Estimating composition.

# plot cell type proportions by age
par(mfrow=c(1,1))
a = cellCounts[age.targets$Sample_Group == "NewBorns",]
b = cellCounts[age.targets$Sample_Group == "OLD",]
boxplot(a, at=0:5*3 + 1, xlim=c(0, 18), ylim=range(a, b), xaxt="n",
        col=age.pal[1], main="", ylab="Cell type proportion")
boxplot(b, at=0:5*3 + 2, xaxt="n", add=TRUE, col=age.pal[2])
axis(1, at=0:5*3 + 1.5, labels=colnames(a), tick=TRUE)
legend("topleft", legend=c("NewBorns", "OLD"), fill=age.pal)
```

As reported by [Jaffe & Irizarry \(2014\)](#), the preceding plot demonstrates that differences in blood cell type proportions are strongly confounded with age in this dataset ([Figure 15](#)). Performing cell composition estimation can alert you to potential issues with confounding when analysing a mixed cell type dataset. Based on the results, some type of adjustment for cell type composition may be considered, although a naive cell type adjustment is not recommended. [Jaffe & Irizarry \(2014\)](#) outline several strategies for dealing with cell type composition issues.



**Figure 15.** If samples come from a population of mixed cells e.g. blood, it is advisable to check for potential confounding between differences in cell type proportions and the factor of interest.

## Discussion

Here we present a commonly used workflow for methylation array analysis based on a series of Bio-conductor packages. While we have not included all the possible functions or analysis options that are available for detecting differential methylation, we have demonstrated a common and well used workflow that we regularly use in our own analysis. Specifically, we have not demonstrated more complex types of analyses such as removing unwanted variation in a differential methylation study (Leek *et al.*, 2012; Maksimovic *et al.*, 2015; Teschendorff *et al.*, 2011), block finding (Aryee *et al.*, 2014; Hansen *et al.*, 2011) or A/B compartment prediction (Fortin & Hansen, 2015). Our differential methylation workflow presented here demonstrates how to read in data, perform quality control and filtering, normalisation and differential methylation testing. In addition we demonstrate analysis for differential variability, gene set testing and estimating cell type composition. One important aspect of exploring results of an analysis is visualisation and we also provide an example of generating region-level views of the data.

## Software versions

The R markdown file and/or R script for this version workflow can be downloaded from: <https://github.com/Oshlack/MethylationAnalysisWorkflow/tree/version3/scripts>. A live version of this workflow that is regularly built using updated Bioconductor packages can be accessed on the Bioconductor website: <https://www.bioconductor.org/help/workflows/methylationArrayAnalysis/>. The version of the workflow presented here uses the following packages available from Bioconductor (release 3.4):

```
sessionInfo()
```

```
## R version 3.3.1 (2016-06-21)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: CentOS release 6.7 (Final)
##
## locale:
##   [1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C
##   [3] LC_TIME=en_US.UTF-8      LC_COLLATE=en_US.UTF-8
##   [5] LC_MONETARY=en_US.UTF-8  LC_MESSAGES=en_US.UTF-8
##   [7] LC_PAPER=en_US.UTF-8     LC_NAME=C
##   [9] LC_ADDRESS=C             LC_TELEPHONE=C
##  [11] LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C
##
```

```

## attached base packages:
## [1] splines      grid        stats4      parallel    stats        graphics    grDevices
## [8] utils        datasets   methods     base
##
## other attached packages:
## [1] stringr_1.2.0
## [2] DMRcate_1.10.8
## [3] DMRcatedata_1.10.1
## [4] DSS_2.14.0
## [5] bsseq_1.10.0
## [6] Gviz_1.18.2
## [7] minfiData_0.20.0
## [8] matrixStats_0.51.0
## [9] missMethyl_1.8.0
## [10] RColorBrewer_1.1-2
## [11] IlluminaHumanMethylation450kmanifest_0.4.0
## [12] IlluminaHumanMethylation450kanno.ilmn12.hg19_0.6.0
## [13] minfi_1.20.2
## [14] bumpHunter_1.14.0
## [15] locfit_1.5-9.1
## [16] iterators_1.0.8
## [17] foreach_1.4.3
## [18] Biostrings_2.42.1
## [19] XVector_0.14.1
## [20] SummarizedExperiment_1.4.0
## [21] GenomicRanges_1.26.4
## [22] GenomeInfoDb_1.10.3
## [23] IRanges_2.8.2
## [24] S4Vectors_0.12.2
## [25] Biobase_2.34.0
## [26] BiocGenerics_0.20.0
## [27] limma_3.30.13
##
## loaded via a namespace (and not attached):
## [1] colorspace_1.3-2
## [2] siggenes_1.48.0
## [3] mclust_5.2.3
## [4] rprojroot_1.2
## [5] biovizBase_1.22.0
## [6] htmlTable_1.9
## [7] base64enc_0.1-3
## [8] dichromat_2.0-0
## [9] base64_2.0
## [10] interactiveDisplayBase_1.12.0
## [11] AnnotationDbi_1.36.2
## [12] IlluminaHumanMethylationEPICanno.ilm10b2.hg19_0.6.0
## [13] codetools_0.2-15
## [14] R.methodsS3_1.7.1
## [15] methylumi_2.20.0
## [16] knitr_1.15.1
## [17] Formula_1.2-1
## [18] Rsamtools_1.26.1
## [19] annotate_1.52.1
## [20] cluster_2.0.5
## [21] GO.db_3.4.0
## [22] R.oo_1.21.0
## [23] shiny_1.0.0
## [24] httr_1.2.1
## [25] backports_1.0.5

```

```
## [26] assertthat_0.1
## [27] Matrix_1.2-8
## [28] lazyeval_0.2.0
## [29] acepack_1.4.1
## [30] htmltools_0.3.5
## [31] tools_3.3.1
## [32] gtable_0.2.0
## [33] doRNG_1.6
## [34] Rcpp_0.12.9
## [35] multtest_2.30.0
## [36] preprocessCore_1.36.0
## [37] nlme_3.1-131
## [38] rtracklayer_1.34.2
## [39] mime_0.5
## [40] ensemblDb_1.6.2
## [41] rngtools_1.2.4
## [42] gtools_3.5.0
## [43] statmod_1.4.29
## [44] XML_3.98-1.5
## [45] beanplot_1.2
## [46] org.Hs.eg.db_3.4.0
## [47] AnnotationHub_2.6.5
## [48] zlibbioc_1.20.0
## [49] MASS_7.3-45
## [50] scales_0.4.1
## [51] BSgenome_1.42.0
## [52] VariantAnnotation_1.20.3
## [53] BiocInstaller_1.24.0
## [54] GEOquery_2.40.0
## [55] yaml_2.1.14
## [56] memoise_1.0.0
## [57] gridExtra_2.2.1
## [58] ggplot2_2.2.1
## [59] pkgmaker_0.22
## [60] biomaRt_2.30.0
## [61] rpart_4.1-10
## [62] reshape_0.8.6
## [63] latticeExtra_0.6-28
## [64] stringi_1.1.2
## [65] RSQLite_1.1-2
## [66] highr_0.6
## [67] genefilter_1.56.0
## [68] permute_0.9-4
## [69] checkmate_1.8.2
## [70] GenomicFeatures_1.26.3
## [71] BiocParallel_1.8.1
## [72] bitops_1.0-6
## [73] nor1mix_1.2-2
## [74] evaluate_0.10
## [75] lattice_0.20-34
## [76] ruv_0.9.6
## [77] GenomicAlignments_1.10.1
## [78] htmlwidgets_0.8
## [79] plyr_1.8.4
## [80] magrittr_1.5
## [81] R6_2.2.0
## [82] Hmisc_4.0-2
## [83] DBI_0.6
## [84] foreign_0.8-67
```

```
## [85] survival_2.40-1
## [86] RCurl_1.95-4.8
## [87] nnet_7.3-12
## [88] tibble_1.2
## [89] rmarkdown_1.3
## [90] data.table_1.10.4
## [91] digest_0.6.12
## [92] xtable_1.8-2
## [93] httpuv_1.3.3
## [94] illuminaio_0.16.0
## [95] R.utils_2.5.0
## [96] openssl_0.9.6
## [97] munsell_0.4.3
## [98] registry_0.3
## [99] BiasedUrn_1.07
## [100] quadprog_1.5-5
```

### Author contributions

JM and BP designed the content and wrote the paper. AO oversaw the project and contributed to the writing and editing of the paper.

### Competing interests

No competing interests were disclosed.

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*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

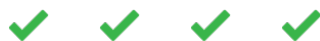
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# Open Peer Review

Current Referee Status:



## Version 2

Referee Report 31 October 2016

doi:10.5256/f1000research.9951.r17311



**Timothy J. Peters**

Garvan Institute of Medical Research, Darlinghurst, NSW, Australia

I am happy with the amendments made, and approve of the publication.

**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.**

**Competing Interests:** I am the primary author of the DMRcate package

Referee Report 03 August 2016

doi:10.5256/f1000research.9951.r15416



**Peter Hickey**

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

Thank you for making the data easily available and re-running the workflow with the latest release version of Bioconductor, Jovanna. My minor comments on the initial submission have also been addressed to my satisfaction. Therefore, I happily approve the revised manuscript. I believe it will be a helpful resource to the Bioconductor community and those wishing to learn best practises for analysing DNA methylation array data.

Minor comments I have on the revised manuscript:

- I got slightly different numbers and output when running the workflow (see below). I believe this is due to a bug being fixed in in v1.18.4 (see <https://github.com/Bioconductor-mirror/minfi/commit/c16b28e2f4c7127696cbea807f668d0a10c54t>); I tested using minfi v1.18.6. These differences were first observable when running This affects all subsequent output such as which probes are identified as DMPs. Unfortunately, such changes in output are par for the course in bioinformatics software. These differences are outside the author's control, and I do not believe these affect the quality and usefulness of the workflow. I'm not sure anything can be done except to point out that such differences may exist due to different software versions.

```
> head(mVals[,1:5])
      naive.1  rTreg.2 act_naive.3  naive.4  act_naive.5
cg13869341  2.205808  2.205808   2.167697  2.173122   2.106660
```

```
cg24669183 2.169414 2.235964 2.280734 1.632309 2.184435
cg15560884 1.761176 1.577578 1.597503 1.777486 1.764999
cg01014490 -2.918305 -2.979692 -4.042347 -3.448734 -3.189924
cg17505339 3.082191 3.924931 4.163206 3.255373 3.654134
cg11954957 1.546401 1.912204 1.727910 2.441267 1.618331
```

- There is a typo (extra whitespace) in the BiocViews URL
- I was unable to get the Gviz plot to correctly render. Unsure what's going on here as the Gviz version on my machine matches that in the workflow (v1.16.1)  
> plotTracks(tracks, from=minbase, to=maxbase, showTitle=TRUE, add53=TRUE,  
+ add35=TRUE, grid=TRUE, lty.grid=3, sizes=sizes, length(tracks))  
Error in valid.viewport(x, y, width, height, just, gp, clip, xscale, yscale, :  
invalid 'yscale' in viewport  
In addition: Warning messages:  
1: In min(x) : no non-missing arguments to min; returning Inf  
2: In max(x) : no non-missing arguments to max; returning -Inf

**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.**

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

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## Version 1

Referee Report 22 June 2016

doi:10.5256/f1000research.9514.r14247



### Davide Risso

Division of Biostatistics, School of Public Health, University of California, Berkeley, Berkeley, CA, USA

As someone who has experience with R/Bioconductor and with genomics data, but not direct experience analyzing methylation array data, I found the workflow very useful and I would suggest it to anyone wanting to start analyzing this type of data.

I do agree with the other reviewers that the value of the workflow will be greatly increased if the dataset used was available as an R object. The authors should consider submitting an experiment data package to Bioconductor to accompany the workflow. Alternatively, they could provide the dataset as a supplementary file.

As for the analysis itself, I only have one major question. Note that I do not have direct experience analyzing methylation array, so this is a genuine question rather than a criticism.

In gene expression analysis, we tend to perform filtering prior to normalization, while the authors here first normalize the data by quantile normalization and then filter out probes that are low quality and/or affected by SNPs. Wouldn't it be safer to perform filtering before normalization? I understand that given the few probes affected, the order has likely very little effect in this dataset. But I naively imagine that if there are

many problematic probes and, say, the quality of the samples is confounded with the biology, there could be issues in using low quality probes for normalization.

Other minor points:

- I agree that the code should be re-run with the latest release of R and Bioconductor.
- In the definition of  $\beta$ ,  $\alpha$  should be defined, and its default value in `getBeta()` should be specified.
- Spelling: most of the article uses British English spelling, but the word "normalization" is sometimes (but not always) spelled in American English.
- A sentence describing what is the procedure implemented in `preprocessQuantile()` is needed for people not familiar with normalization.
- I agree that it would be useful to provide a brief description of what is a contrasts matrix as this section could be confusing for people unfamiliar with statistical models.
- For the same reason, the authors should add a brief explanation of the problem of multiple testing and what is the false discovery rate. Or at least provide references to the appropriate literature.

**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.**

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

Author Response 14 Jul 2016

**Jovana Maksimovic**, Murdoch Childrens Research Institute, Australia

Thanks for your review, Davide.

While we agree that normalisation post-filtering makes sense, there are some practical aspects with the data objects that `minfi` uses which makes this difficult. Many (but not all) normalisation procedures in `minfi` accept an `rgSet` object, which can be thought of as a raw data object, which cannot easily be subset by CpG site. These normalisation procedures then output a different type of data object, such as `MethylSet` or `GenomicRatioSet`, which are much easier to work with in terms of filtering out problematic CpG sites. Due to the sheer number of CpG sites observed per sample (>450,000) we believe it shouldn't make too much difference for most datasets, especially if very poor quality samples are excluded prior to normalisation, although it is possible that there are exceptions to this.

Response to minor points:

- We have spent some time modifying the workflow to run with the latest R and Bioconductor.
- We have added additional details regarding beta values, M-values and the offset in the paper.
- We have changed "normalization" to "normalisation" throughout the text.
- A sentence has been added about `preprocessQuantile` in the normalisation section.
- We have included some additional explanation of contrast matrices.

- An additional paragraph was added explaining about the issues of multiple testing in very high dimensional data.

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

Referee Report 21 June 2016

doi:10.5256/f1000research.9514.r14248



**Michael I. Love**

Department of Biostatistics, Harvard TH Chan School of Public Health, Boston, MA, USA

I am not an expert in analysis of methylation data, and have not used the methylation packages mentioned in this workflow, so I reviewed the workflow as an uninitiated reader might approach it.

Major comments:

I found the workflow to be easy to follow and informative. The authors have done a good job summarizing a large and complex topic into an reasonable size for a workflow article, while still mentioning the various alternatives that are possible at each step. I appreciated the focus on EDA and checking the quality of results by eye, for example the M-values for the most significant tests and the MDS plots colored by different variables.

I did not try to run the code, and I agree with the other two reviewers that the code and datasets should be made available and linked to from this workflow.

Minor comments:

The first time “moderated t-statistics” is mentioned, it would benefit to have a citation so that a reader who hasn’t encountered this method before can read the reference, e.g. Smyth 2004.

The first or second time IDAT files are mentioned, a small description of these would be useful, a little more than just that these are the raw files. Which platforms produce IDAT files? Are they compressed files? About how large are they?

Figure 2: It wasn’t obvious at first that the plot on the right is the same as the left but zoomed in.

When discussing the choice of normalization depending on whether or not there are global changes across samples due to underlying biology, the authors might consider referencing the *quantro* article and Bioconductor package by Stephanie Hicks for determining whether there are global changes in genomic datasets across samples, and therefore whether quantile normalization is appropriate. Hicks has an example of whether or not to use quantile normalization for methylation data in the article.

- <https://www.bioconductor.org/packages/quantro>

Principal components is misspelled in the text: “principle components”

In the paragraph above the call to *makeContrasts*, it would be good to state in the text in one sentence

what it is this function does, for the benefit of someone who has never performed linear modeling before. Likewise, to explicitly state that `coef=1` is referencing the first column of the contrast matrix. It should be stated what is the B-statistic which orders the topTable.

The authors should explain a bit more what is being shown in Figure 10 in the caption.

In the text and code the authors have written DNaseI, but I believe the more common capitalization is DNase.

The authors might consider commenting on the top GO categories and the associated FDR values. How far down the list should one look? Can the authors advise the reader how GO results should be reported? Is it fair to pick out the most relevant categories from this list and only report them?

It wasn't clear to me the difference between the *gometh* and *gsameth* approaches.

It would be good to provide references to literature for "it has been hypothesised that highly variable CpGs in cancer are important for tumour progression".

## References

1. Smyth GK: Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004; **3**: Article3 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Hicks SC, Irizarry RA: quantro: a data-driven approach to guide the choice of an appropriate normalization method. *Genome Biol*. 2015; **16**: 117 [PubMed Abstract](#) | [Publisher Full Text](#)

**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.**

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

Author Response 14 Jul 2016

**Jovana Maksimovic**, Murdoch Childrens Research Institute, Australia

Thank you for reviewing our paper, Michael.

In response to your comments/suggestions we have made the following changes:

- The Smyth 2004 citations have been added the first time "moderated t-statistics" is mentioned
- A description of IDAT files has been added to the text along with a reference to a Bioconductor package that is specifically for reading IDAT files.
- We have added to the legend for Figure 2 to clarify that the plot on the right is the same as the left but zoomed in.
- There is now a reference to Hicks and quantro included in the Normalisation section.
- Spelling mistakes and typos have been fixed.
- Function of *makeContrasts* is described: See response to Davide Risso.
- We now explicitly state that `coef=1` is referencing the first column of the contrast matrix.
- Included explanation for B-statistic and citation.
- More detail about the plot has been added to the figure caption for Figure 10.
- Changed DNaseI to DNase
- Typically we would consider GO categories that have associated FDRs less than 5% as significant. Some discussion of these points has been added to the gene set testing section.

- The gometh function specifically tests only GO and KEGG pathways, whereas the gsmeth is a more general function that requires the user to supply their own gene sets for testing.
- We have changed the sentence “it has been hypothesised that highly variable CpGs in cancer are important for tumour progression” to “it has been hypothesised that highly variable CpGs in cancer may contribute to tumour heterogeneity” and included the following reference:

Hansen KD, Timp W, Bravo HC, Sabuncian S, Langmead B, McDonald OG, Wen B, Wu H, Liu Y, Diep D, Briem E, Zhang K, Irizarry RA, Feinberg AP: Increased methylation variation in epigenetic domains across cancer types. Nat Genet. 2011, 43: 768-775

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

Referee Report 17 June 2016

doi:10.5256/f1000research.9514.r14425



**Peter Hickey**

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

This paper is a well-written workflow for analysing DNA methylation microarrays using Bioconductor packages. A challenge in writing these workflows is to produce something that is opinionated enough to be useful and balanced enough to be fair to packages developed by other people; I believe the authors have struck the right balance.

However, my overall assessment is "Approved With Reservations" because the data used in the workflow is not easily available and therefore the workflow cannot be tested out by the interested reader.

I spent some time trying to compile the raw data from GEO, but to me this feels a bit too much to expect of the reader, especially when it is likely that the interested reader is a beginner or intermediate user of bioinformatics software. I strongly believe the workflow should either include code to curate/construct/download the necessary files such as SampleSheet.csv and the IDAT files or include a link to prepared example data that can be used right from the 'Loading the data' section of the workflow. For example, <http://f1000research.com/articles/4-1070/v1> uses data from the *airway* Bioconductor package that can easily be installed by the reader to follow along with the workflow.

My other main suggestion would be to re-run the code using the recently published Bioconductor version 3.3. I expect this might require some minor changes to the code, e.g., the `minfi::read.450k*` functions have been deprecated in favour of `minfi::read.metharray*` functions.

I have some additional minor comments and suggestions that I will include once I'm able to run through and review the workflow from beginning to end.

**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.**

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

Referee Response 06 Jul 2016

**Peter Hickey**, Johns Hopkins Bloomberg School of Public Health, USA

- p2:  $\beta = M / (M + U + \alpha)$ , the  $\alpha$  parameter should be explained. Also, both the definition of  $\beta$  and  $M$ -value differ slightly from that given in the cited Du, P. et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics 11, 587 (2010).
- p3: Perhaps worth mentioning that a complete list of packages for analysing DNA methylation data can be accessed using BiocViews (<https://www.bioconductor.org/packages/release/bioc/html/biocViews.html> and [https://www.bioconductor.org/packages/release/BiocViews.html#\\_\\_\\_DNAMethylation](https://www.bioconductor.org/packages/release/BiocViews.html#___DNAMethylation))
- p4: "...loading all the package libraries..." should be "...loading all the packages..."
- p4: Perhaps worth commenting on which of the loaded packages are methylation-focused and/or purpose of other packages, e.g., stringr, Gviz.
- p4: This is \*super\* pedantic (sorry!): strictly speaking the IlluminaHumanMethylation450kmanifest package provides the Illumina manifest for the 450k array, which can then be accessed by using ``minfi::getAnnotation()``
- Figure 2: Not immediately obvious that righthand plot is zoomed in version of lefthand plot. The caption could better explain this.
- p10: The code produces a warning. Would be helpful to the reader to comment on whether this is cause for concern in this case.
- Figure 9: Wondering whether helpful to have each panel with y-axis = [0, 1]
- p25: ``islandData`` apparently contains 0 ranges. This looks like a bug in the code.
- p28: "For gene ontology testing (default), the user can specific collection = "GO" for KEGG testing collection = "KEGG""; this sentence seems incomplete or is perhaps missing a word
- p29 and p30: The code produces a warning. Would be helpful to the reader to comment on whether this is cause for concern in this case.
- The workflow uses multiple packages and it's not always clear where each function comes from. This could be clarified e.g., by namespacing functions such as ``limma::plotMDS()`` instead of ``plotMDS()``

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

Author Response 14 Jul 2016

**Jovana Maksimovic**, Murdoch Childrens Research Institute, Australia

Thanks for taking the time to review our workflow, Peter.

In response to your suggestion we have made the data available and rerun the workflow using the latest R and Bioconductor.

In response to your other comments:

- p2:  $\beta = M / (M + U + \alpha)$ , the  $\alpha$  parameter should be explained. Also, both the definition of  $\beta$  and  $M$  value differ slightly from that given in the cited Du, P. et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics 11, 587 (2010).  
*This has been addressed. See response to Davide Risso.*
- p3: Perhaps worth mentioning that a complete list of packages for analysing DNA methylation data can be accessed using BiocViews (<https://www.bioconductor.org/packages/release/bioc/html/biocViews.html> and [https://www.bioconductor.org/packages/release/BiocViews.html#\\_\\_\\_DNAMethylation](https://www.bioconductor.org/packages/release/BiocViews.html#___DNAMethylation))  
*This has been added to the paper.*
- p4: "...loading all the package libraries..." should be "...loading all the packages..."  
*The text has been modified accordingly.*
- p4: Perhaps worth commenting on which of the loaded packages are methylation-focused and/or purpose of other packages, e.g., stringr, Gviz.  
*The text has been modified accordingly.*
- p4: This is \*super\* pedantic (sorry!): strictly speaking the IlluminaHumanMethylation450kmanifest package provides the Illumina manifest for the 450k array, which can then be accessed by using ``minfi::getAnnotation()``  
*The text has been modified accordingly.*
- Figure 2: Not immediately obvious that righthand plot is zoomed in version of lefthand plot. The caption could better explain this.  
*This has been clarified in the figure caption.*
- p10: The code produces a warning. Would be helpful to the reader to comment on whether this is cause for concern in this case.  
*A sentence has been included that explains the reason for the warning.*
- Figure 9: Wondering whether helpful to have each panel with y-axis = [0, 1]  
*As we are trying to highlight the differences between the groups tested for **individual** CpGs and not comparing between CpGs, we feel that the axes are appropriate for the purposes of "sanity checking" the results of the statistical analysis.*
- p25: ``islandData`` apparently contains 0 ranges. This looks like a bug in the code.  
*This was due to the fact that there were not any CpG islands present in the region being plotted; we have selected another region to plot that does have a CpG island so that islandData is no longer empty.*
- p28: "For gene ontology testing (default), the user can specific collection = "GO" for KEGG testing collection = "KEGG""; this sentence seems incomplete or is perhaps missing a word  
*This sentence has been modified.*
- p29 and p30: The code produces a warning. Would be helpful to the reader to comment on whether this is cause for concern in this case.  
*Added a sentence to the text to explain the warning.*
- The workflow uses multiple packages and it's not always clear where each function comes from. This could be clarified e.g., by namespacing functions such as ``limma::plotMDS()`` instead of ``plotMDS()``  
*We don't feel it is a particularly useful exercise to change every function to include the*

*package name. Searching the help for any of the functions will inform users which package the function comes from. For example ?plotMDS.*

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

Referee Report 15 June 2016

doi:10.5256/f1000research.9514.r14245



**Timothy J. Peters**

Garvan Institute of Medical Research, Darlinghurst, NSW, Australia

This paper describes a workflow for processing, filtering and analysis of Illumina Infinium methylation array data. It showcases a reproducible pipeline integrating a suite of tools from Bioconductor for multi-faceted genomic insights. While none of the tools individually are novel, their integration into a sensible, reproducible pipeline is. I am recommending this manuscript for indexation for 3 main reasons:

- The tools contained therein and their application are in line with, or near, best practice. The workflow itself contains all the major steps that this reviewer usually uses for their methylation array processing.
- An integrated workflow such as this will be valuable for novice and intermediate bioinformaticians who are tasked with processing methylation data. The number of caveats and sanity checks needed for appropriate biological interpretation is not trivial, and this workflow does a satisfactory job of outlining them.
- The reproducible nature of this manuscript is a strength; it is very "coalface bioinformatics". Many published methods have very poor or buggy implementations and no effort is made to contextualise them in a given pipeline. Publication may set a precedent for other authors to give worked examples and context, which in this reviewer's opinion accelerates the path to best practice.

Minor amendments needed:

1. I could not find any public links to the data files imported into this workflow. These ought to be provided.
2. a) The mathematical definition of  $\beta$  is given as  $\beta = M/(M + U + \alpha)$ . While I realise  $\alpha$  is a fudge factor for offset purposes this is not clear to the lay reader and needs to be made so.  
  
b) Why are there no offsets for  $M$  or  $U$  in the calculation of  $M$  values, especially since there is one for the calculation of  $\beta$ ? On (admittedly) rare occasions  $M$  or  $U$  will be exactly zero and hence offsets need to be put in both the numerator and denominator of the ratio to be log-transformed, else a non-number will result.
3. A justification for the preference of  $M$  values over  $\beta$  values for use in the MDS plots is needed, especially since the statement is made that "Beta values are generally preferable ... for graphical

presentation". This reviewer's experience is that  $\beta$  is much more common for use in PCA/MDS, and is certainly the standard for other methylation platforms e.g. bisulfite sequencing data.

4. Legends are needed for density plots in Figs. 3 and 8. I appreciate *minfi* annoyingly puts the default legend in the top right, obscuring the hypermethylated mode, but a custom call to `legend()` ought to fix this.
5. Appropriate Y-axis labels are needed for Figs. 9 and 14.

**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.**

**Competing Interests:** I am the primary author of the DMRcate package

Author Response 14 Jul 2016

**Jovana Maksimovic**, Murdoch Childrens Research Institute, Australia

Thanks Tim for taking the time to review our paper.

In response to your comments/suggestions:

- I could not find any public links to the data files imported into this workflow. These ought to be provided.  
*In addition to the references, we have now included links to GEO for the data used and have also made a bundle of all the data available on Figshare which can now be used directly from within R to download the data and complete the workflow.*
- a) The mathematical definition of  $\beta$  is given as  $\beta = M/(M + U + \alpha)$ . While I realise  $\alpha$  is a fudge factor for offset purposes this is not clear to the lay reader and needs to be made so.  
b) Why are there no offsets for  $M$  or  $U$  in the calculation of  $M$  values, especially since there is one for the calculation of  $\beta$ ? On (admittedly) rare occasions  $M$  or  $U$  will be exactly zero and hence offsets need to be put in both the numerator and denominator of the ratio to be log-transformed, else a non-number will result.  
*This has been clarified in the text. See also response to Davide Risso.*
- A justification for the preference of  $M$  values over  $\beta$  values for use in the MDS plots is needed, especially since the statement is made that "Beta values are generally preferable ... for graphical presentation". This reviewer's experience is that  $\beta$  is much more common for use in PCA/MDS, and is certainly the standard for other methylation platforms e.g. bisulfite sequencing data.  
*We disagree that beta values should be used in principal components analysis. While plotMDS does produce a graphic, the function is performing a statistical analysis (i.e. principal components analysis), which is based on normal distribution theory. The same reasons for not performing differential methylation analysis on the beta values apply in this case (i.e. heteroscedasticity of the beta values).*
- Legends are needed for density plots in Figs. 3 and 8. I appreciate *minfi* annoyingly puts the default legend in the top right, obscuring the hypermethylated mode, but a custom call to `legend()` ought to fix this.  
*These legends have been added as suggested.*
- Appropriate Y-axis labels are needed for Figs. 9 and 14.  
*The Y-axis labels have been added.*

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

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## Discuss this Article

### Version 2

Referee Response 31 Mar 2017

**Tim Peters**, Garvan Institute of Medical Research, Australia

Thanks again Jenny for the bugspot, and thank you Jovana for updating the workflow. Apologies for not alerting you to the changed syntax earlier. The bug should be fixed from 1.10.10 onwards.

Tim

**Competing Interests:** I am the primary author of the DMRcate package

Author Response 28 Mar 2017

**Jovana Maksimovic**, Murdoch Childrens Research Institute, Australia

Hi Jenny,

Thanks very much for the comment - I'm very glad that you have found the workflow useful. I also recently noticed the bug you described which is a result of a recent update to the DMRcate package. I will be posting an updated version of the workflow ASAP which includes the bug fix. In the meantime, thanks very much for including the fix in your comment.

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

Reader Comment 27 Mar 2017

**Jenny Drnevich**, University of Illinois, USA

Thanks for an amazing workflow! There is one place where the code no longer works as of R 3.3.3, the call to `cpg.annotate()`, but can be fixed by adding two arguments to correctly specify the values for the "what" and "arraytype":

```
myAnnotation <- cpg.annotate(mVals, datatype = "array", arraytype="450K", what = "M",
                             analysis.type="differential", design=design,
                             contrasts = TRUE, cont.matrix = contMatrix,
                             coef="naive - rTreg")
```

These also should be specified in the `DMR.plot()` function right below:

```
DMR.plot(ranges=results.ranges, dmr=1, CpGs=bVals, phen.col=cols, what = "Beta", arraytype =
"450K",
```

```
pch=16, toscale=TRUE, plotmedians=TRUE, genome="hg19", samps=samps)
```

Thanks again for writing this workflow - you just saved me a ton of time!!  
Jenny

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

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### Version 1

Author Response 11 Jul 2016

**Belinda Phipson**, Murdoch Childrens Research Institute, Australia

Dear David, thank you for your comment. When you read in the methylation data using the targets file specifically in the call:

```
rgSet <- read.450k.exp(targets=targets)
```

[or the read.metharray.exp function if using the latest Bioconductor]

this ensures that the order of the samples in the targets file and in the data objects are the same.

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

Reader Comment 07 Jul 2016

**David McGaughey**, NIH, USA

I'm using this workflow to (re)analyze a 450k cohort and I'm finding it to be a solid overview so far (I'm currently in the "Probe-wise differential methylation analysis" section). One (potentially) crucial note: when the limma call is being done, there should explicit matching of the targets data.frame with the mVals set. The workflow assumes that the samples are in the same order. Something like this line should be added to explicitly match the orders:

```
targets<- targets[match(colnames(mVals), targets$Sample),]
```

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

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