The qsmooth user's guide

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Contents

1 Introduction

Global normalization methods such as quantile normalization [1, 2] have become a standard part of the analysis pipeline for high-throughput data to remove unwanted technical variation. These methods and others that rely solely on observed data without external information (e.g. spike-ins) are based on the assumption that only a minority of genes are expected to be differentially expressed (or that an equivalent number of genes increase and decrease across biological conditions [3]). This assumption can be interepreted in different ways leading to different global normalization procedures. For example, in one normalization procedure, the method assumes the mean expression level across genes should be the same across samples [4]. In contrast, quantile normalization assumes the only difference between the statistical distribution of each sample is technical variation. Normalization is achieved by forcing the observed distributions to be the same and the average distribution, obtained by taking the average of each quantile across samples, is used as the reference [1].

While these assumptions may be reasonable in certain experiments, they may not always be appropriate [5, 6]. For example, mRNA content has been shown to fluctuate significantly during zebrafish early developmental stages [3]. Similarily, cells expressing high levels of c-Myc undergo transcriptional amplification causing a 2 to 3 fold change in global gene expression compared to cells expressiong low c-Myc [5].

Recently, an R/Biocoductor package (`quantro`) [6] has been developed to test for global differences between groups of distributions to evaluate whether global normalization methods such as quantile normalization should be applied. If global differences are found between groups of distributions, these changes may be of technical or biological of interest. If these changes are of technical interest (e.g. batch effects), then global normalization methods should be applied. If these changes are related to a biological covariate (e.g. normal/tumor or two tissues), then global normalization methods should not be applied because the methods will remove the interesting biological variation (i.e. differentially expressed genes) and artifically induce differences between genes that were not differentially expressed. In the cases with global differences between groups of distributions between biological conditions, quantile normalization is not an appropriate normalization method. In these cases, we can consider a more relaxed assumption about the data, namely that the statistical distribution of each sample should be the same within biological conditions or groups (compared to the more stringent assumption of quantile normalization, which states the statistical distribution is the same across all samples).

In this vignette we introduce a generalization of quantile normalization, referred to as **smooth quantile normalization** (**qsmooth**), which is a weighted average of the two types of assumptions about the data. The `qsmooth` R-package contains the `qsmooth()` function, which computes a weight at every quantile that compares the variability between groups relative to within groups. In one extreme, quantile normalization is applied and in the other extreme quantile normalization within each biological condition is applied. The weight shrinks the group-level quantile normalized data towards the overall reference quantiles if variability between groups is sufficiently smaller than the variability within groups. The algorithm is described in Figure 1 below.

Let `gene(g)` denote the g^{th} row after sorting each column in the data. For each row, `gene(g)`, we compute the weight $w_{(g)} \in [0, 1]$, where a weight of 0 implies quantile normalization within groups is applied and a weight of 1 indicates quantile normalization is applied. The weight at each row depends on the between group sum of squares $\text{SSB}_{(g)}$ and total sum of squares $\text{SST}_{(g)}$, as follows:

$$w_{(g)} = \text{median}\{1 - \text{SSB}_{(i)}/\text{SST}_{(i)} |\ i = g - k, \dots, g, \dots, g + k\}, \tag{1}$$

where k = floor(Total number of genes * 0.05). The number 0.05 is a flexible parameter that can be altered to change the window of the number of genes considered. In this way, we can use a rolling median to borrow information from neighboring genes in the weight.

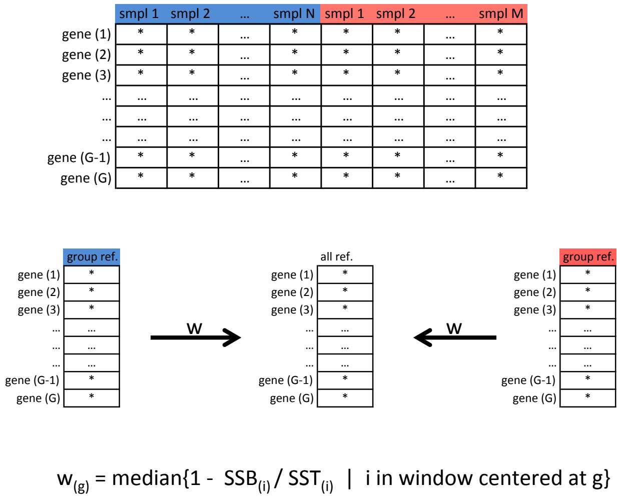


Figure 1: The qsmooth algorithm

2 Getting Started

Load the package in R

```
> library(qsmooth)
```

3 Data

The **bodymapRat** package contains an `ExpressionSet` of 652 RNA-Seq samples from a comprehensive rat transcriptomic BodyMap study. This data was derived from the raw FASTQ files obtained from Yu et al. (2014) [7]. It contains expression levels from 11 organs in male and female rats at 4 developmental stages. We will use a subset of this data in this vignette.

The R-package bodymapRat can be installed from GitHub using the R package **devtools**.

```
> library(devtools)
> install_github("stephaniehicks/bodymapRat")
```

3.1 bodymapRat Example 1 - Comparing male and female rats in one tissue

The first example is based a dataset which contains lung samples from 21 week old male and female rats. Four samples are from males and four samples are from females.

```
> library(Biobase)
> library(bodymapRat)
> data(bodymapRat)
> # select lung samples, stage 21 weeks, and only bio reps
> keepColumns = (pData(bodymapRat)$organ == "Lung") &
+     (pData(bodymapRat)$stage == 21) & (pData(bodymapRat)$techRep == 1)
> keepRows = rowMeans(exprs(bodymapRat)) > 10 # Filter out low counts
> bodymapRatE1 <- bodymapRat[keepRows,keepColumns]
> bodymapRatE1

ExpressionSet (storageMode: lockedEnvironment)
assayData: 18629 features, 8 samples
  element names: exprs
protocolData: none
phenoData
  sampleNames: SRR1170173 SRR1170175 ... SRR1170213 (8 total)
  varLabels: sraExperiment sraRun ... ytile (22 total)
  varMetadata: labelDescription
featureData: none
experimentData: use 'experimentData(object)'
Annotation:
```

3.2 bodymapRat Example 2 - Comparing two tissues

The second example is based a dataset which contains brain and liver tissue samples from 21 week old male and female rats. eight samples are from males and eight samples are from females.

```
> # select brain and liver samples, stage 21 weeks, and only bio reps
> keepColumns = (pData(bodymapRat)$organ %in% c("Brain", "Liver")) &
+          (pData(bodymapRat)$stage == 21) & (pData(bodymapRat)$techRep == 1)
> keepRows = rowMeans(exprs(bodymapRat)) > 10 # Filter out low counts
> bodymapRatE2 <- bodymapRat[keepRows,keepColumns]
> bodymapRatE2

ExpressionSet (storageMode: lockedEnvironment)
assayData: 18629 features, 16 samples
  element names: exprs
protocolData: none
phenoData
  sampleNames: SRR1169975 SRR1169977 ... SRR1170277 (16 total)
  varLabels: sraExperiment sraRun ... ytile (22 total)
  varMetadata: labelDescription
featureData: none
experimentData: use 'experimentData(object)'
Annotation:
```

4 Using the qsmooth() function

4.1 Input for qsmooth()

The qsmooth() function must have two objects as input:

- object: a data frame or matrix with observations (e.g. probes or genes) on the rows and samples as the columns. qsmooth() can accept objects that inherit eSets such as an ExpressionSet or MethylSet. In this case, the groupFactor must still be provided.

- groupFactor: a continuous or categorial covariate that represents the group level biological variation about each sample. For example if the samples represent two different tissues, provide qsmooth() with a covariate representing which columns in the object are different tissue samples.

- batch: **optional** batch covariate (multiple batches are not allowed). If batch covariate is provided, ComBat() from sva is used prior to qsmooth normalization to remove batch effects. See ComBat() for more details.

- normFactors: **optional** scaling normalization factors. Default is NULL. If normFactors is not equal to NULL, the user can provide a vector of scaling factors that will be used to modify the expression data set prior to applying the qsmooth algorithm.
- window: window size for running median (defined as a fraction of the number of rows of exprs. Default is 0.05

4.2 Running qsmooth()

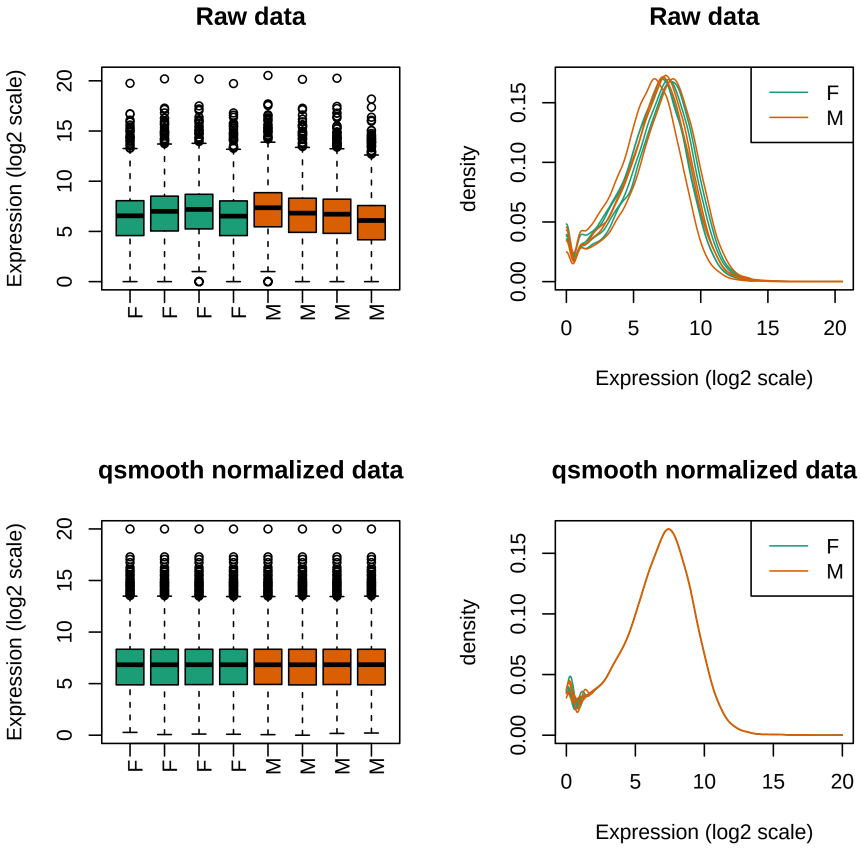
4.2.1 Using Example 1 data

In the first example, the groups we are interested in comparing are contained in the sex column in the pData(bodymapRatE1) dataset. To run the qsmooth() function, input the data object and the object containing the phenotypic data. Here we use the bodymapRatE1 data set as an example.

The first row shows the boxplots and density plot of the raw data that has been transformed on the log2() scale and added a pseudo-count of 1 (i.e. log2(counts+1)).

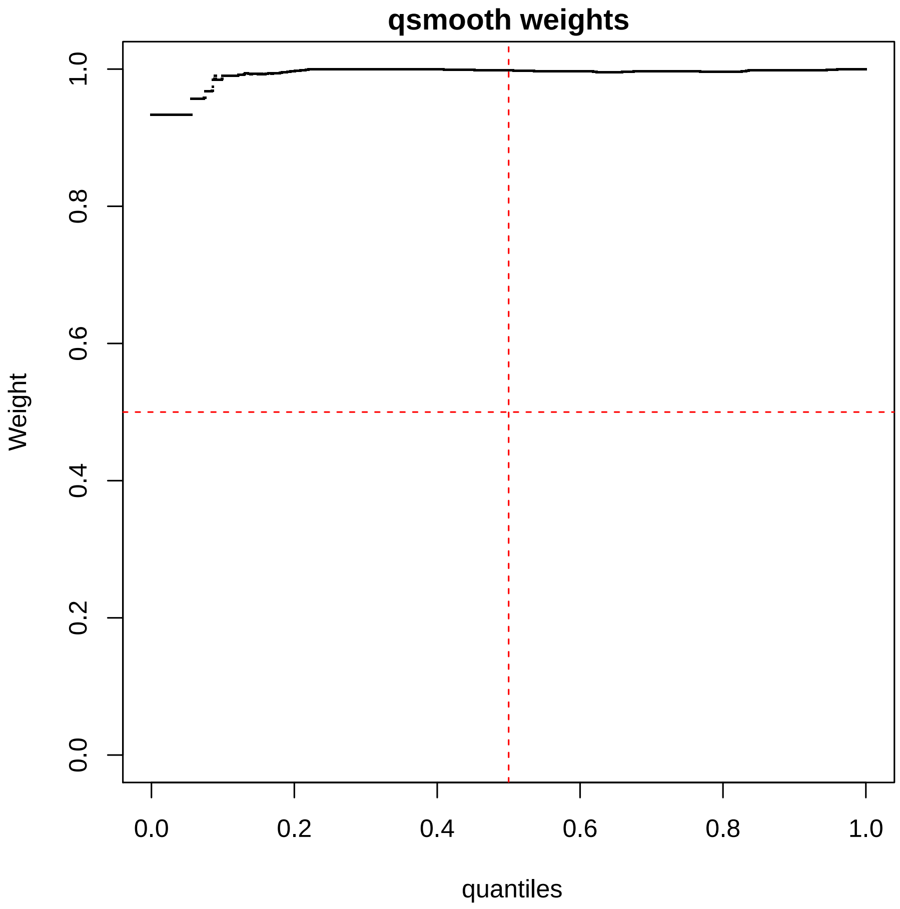
The second row shows the boxplots and density plot of the qsmooth normalized data.

```
> library(quantro)
> par(mfrow=c(2,2))
> pd1 <- pData(bodymapRatE1)
> eset1_ercc <- exprs(bodymapRatE1)[grepl("^ERCC", rownames( exprs(bodymapRatE1))), ]
> eset1 <- exprs(bodymapRatE1)[!grepl("^ERCC", rownames( exprs(bodymapRatE1))), ]
> matboxplot(log2(eset1+1), groupFactor = pd1$sex, main = "Raw data",
+            ylab = "Expression (log2 scale)", xaxt="n")
> axis(1, at=seq_len(length(pd1$sex)), labels=FALSE)
> text(seq_len(length(pd1$sex)), par("usr")[3] -1, labels = pd1$sex, srt = 90, pos = 1, xpd = TRUE)
> matdensity(log2(eset1+1), groupFactor = pd1$sex, main = "Raw data",
+            xlab = "Expression (log2 scale)", ylab = "density")
> legend('topright', levels(factor(pd1$sex)), col = 1:2, lty = 1)
> qsNormE1 <- qsmooth(object = eset1, groupFactor = pd1$sex)
> matboxplot(log2(qsmoothData(qsNormE1)+1), groupFactor = pd1$sex,
+            main = "qsmooth normalized data", ylab = "Expression (log2 scale)",
+            xaxt="n")
> axis(1, at=seq_len(length(pd1$sex)), labels=FALSE)
> text(seq_len(length(pd1$sex)), par("usr")[3] -1, labels = pd1$sex, srt = 90, pos = 1, xpd = TRUE)
> matdensity(log2(qsmoothData(qsNormE1)+1), groupFactor = pd1$sex,
+            main = "qsmooth normalized data",
+            xlab = "Expression (log2 scale)", ylab = "density")
> legend('topright', levels(factor(pd1$sex)), col = 1:2, lty = 1)
```



The smoothed quantile normalized data can be extracted using the `qsmoothData()` function (see above) and the smoothed quantile weights can plotted using the `qsmoothPlotWeights()` function (see below).

```
> qsmoothPlotWeights(qsNormE1)
```



The weights are calculated for each quantile in the data set. A weight of 1 indicates quantile normalization is applied, where as a weight of 0 indicates quantile normalization within the groups is applied. See Figure 1 for more details on the weights.

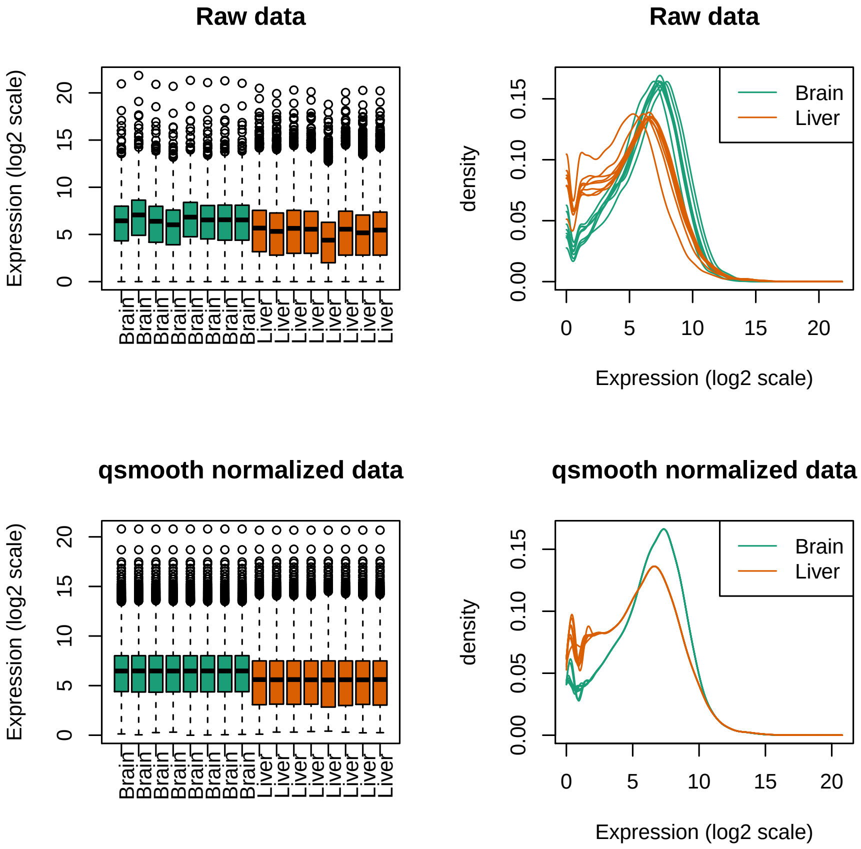
In this example, the weights are close to 1 across all the quantiles indicating that there is no major difference between the group-level quantiles in the female and male rats. Here, the `qsmooth()` normalization method returns a normalized data set that is nearly identical to the conventional quantile normalization.

4.2.2 Using Example 2 data

In the second example (`bodymapRatE2`), the groups we are interested in comparing are the two types of tissues in the 21 week old male and female rats.

Similar to the first example, the first row shows the boxplots and density plot of the raw data that has been transformed on the `log2()` scale and added a pseudo-count of 1 (i.e. `log2(counts+1)`). The second row shows the boxplots and density plot of the qsmooth normalized data.

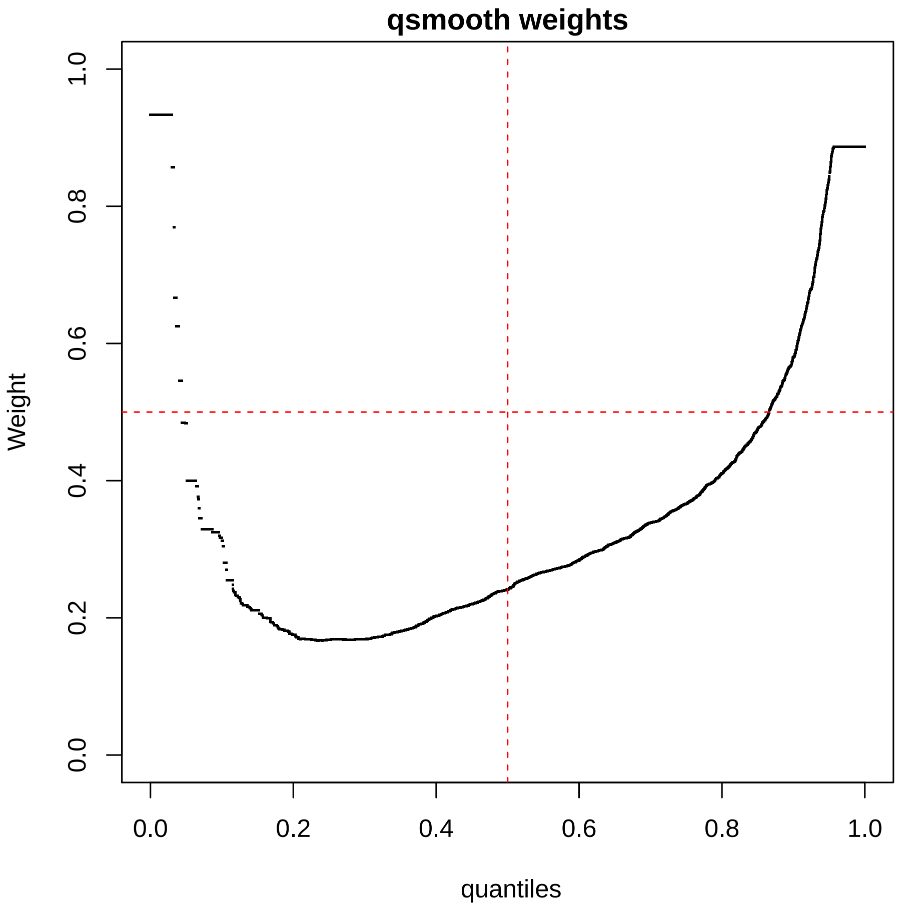
```
> par(mfrow=c(2,2))
> pd2 <- pData(bodymapRatE2)
> eset2_ercc <- exprs(bodymapRatE2)[grepl("^ERCC", rownames( exprs(bodymapRatE2))), ]
> eset2 <- exprs(bodymapRatE2)[!grepl("^ERCC", rownames( exprs(bodymapRatE2))), ]
> pd2$group <- paste(pd2$organ, pd2$sex, sep="_")
> matboxplot(log2(eset2+1), groupFactor = factor(pd2$organ), main = "Raw data",
+            ylab = "Expression (log2 scale)", xaxt="n")
> axis(1, at=seq_len(length(as.character(pd2$organ))), labels=FALSE)
> text(seq_len(length(pd2$organ)), par("usr")[3] -2, labels = pd2$organ, srt = 90, pos = 1, xpd = TRUE)
> matdensity(log2(eset2+1), groupFactor = pd2$organ, main = "Raw data",
+            xlab = "Expression (log2 scale)", ylab= "density")
> legend('topright', levels(factor(pd2$organ)), col = 1:2, lty = 1)
> qsNormE2 <- qsmooth(object = eset2, groupFactor = pd2$organ)
> matboxplot(log2(qsmoothData(qsNormE2)+1), groupFactor = pd2$organ,
+            main = "qsmooth normalized data", ylab = "Expression (log2 scale)",
+            xaxt="n")
> axis(1, at=seq_len(length(pd2$organ)), labels=FALSE)
> text(seq_len(length(pd2$organ)), par("usr")[3] -2, labels = pd2$organ, srt = 90, pos = 1, xpd = TRUE)
> matdensity(log2(qsmoothData(qsNormE2)+1), groupFactor = pd2$organ,
+            main = "qsmooth normalized data",
+            xlab = "Expression (log2 scale)", ylab = "density")
> legend('topright', levels(factor(pd2$organ)), col = 1:2, lty = 1)
```



We see there are global differences in the distributions between the two tissues (liver and brain) in the rats.

The smoothed quantile normalized data can be extracted using the `qsmoothData()` function (see above) and the smoothed quantile weights can plotted using the `qsmoothPlotWeights()` function (see below).

```
> qsmoothPlotWeights(qsNormE2)
```



Recall, a weight of 1 indicates quantile normalization is applied, where as a weight of 0 indicates quantile normalization within the groups is applied.

In this example, the weights range from 0.2 to 0.8 across the quantiles, where the weights are close to 0.2 for the quantiles close to 0 and the weights are close to 0.8 for the quantiles close to 1. This plot suggests the distributions contain more variablity between the groups compared to within groups for the small quantiles (and the conventional quantile normalization is not necessarily appropriate). As the quantiles get bigger, there is less variability between groups which increases the weight closer to 0.8 as the quantiles get bigger.

5 SessionInfo

```
> sessionInfo()
```

```
R version 3.3.2 (2016-10-31)
Platform: x86_64-apple-darwin13.4.0 (64-bit)
Running under: OS X El Capitan 10.11.6

locale:
[1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8

attached base packages:
[1] parallel  stats     graphics  grDevices datasets  utils
[7] methods   base

other attached packages:
[1] quantro_1.6.2       bodymapRat_0.0.1    Biobase_2.32.0
[4] BiocGenerics_0.18.0 qsmooth_0.0.1

loaded via a namespace (and not attached):
 [1] mclust_5.2                base64_2.0
 [3] Rcpp_0.12.7               locfit_1.5-9.1
 [5] lattice_0.20-34           Rsamtools_1.24.0
 [7] Biostrings_2.40.2         digest_0.6.10
 [9] foreach_1.4.3             R6_2.1.3
[11] GenomeInfoDb_1.8.7        plyr_1.8.4
[13] chron_2.3-47              stats4_3.3.2
[15] RSQLite_1.0.0             httr_1.2.1
[17] bumphunter_1.12.0         ggplot2_2.1.0
[19] zlibbioc_1.18.0           GenomicFeatures_1.24.5
[21] data.table_1.9.6          annotate_1.50.0
[23] S4Vectors_0.10.3          Matrix_1.2-7.1
[25] preprocessCore_1.34.0     splines_3.3.2
[27] BiocParallel_1.6.6        stringr_1.1.0
[29] munsell_0.4.3             RCurl_1.95-4.8
[31] biomaRt_2.28.0            rtracklayer_1.32.2
[33] multtest_2.28.0           pkgmaker_0.22
[35] openssl_0.9.4             SummarizedExperiment_1.2.3
[37] GEOquery_2.38.4           quadprog_1.5-5
[39] IRanges_2.6.1             codetools_0.2-15
[41] matrixStats_0.50.2        XML_3.98-1.4
[43] reshape_0.8.5             GenomicAlignments_1.8.4
[45] MASS_7.3-45               bitops_1.0-6
[47] grid_3.3.2                nlme_3.1-128
[49] xtable_1.8-2              gtable_0.2.0
[51] registry_0.3              DBI_0.5-1
[53] magrittr_1.5              scales_0.4.0
[55] stringi_1.1.1             XVector_0.12.1
[57] genefilter_1.54.2         doRNG_1.6
[59] doParallel_1.0.10         limma_3.28.21
[61] minfi_1.18.6              nor1mix_1.2-2
[63] BiocStyle_2.0.3           RColorBrewer_1.1-2
```

```
  [65] iterators_1.0.8           siggenes_1.46.0
  [67] tools_3.3.2               illuminaio_0.14.0
  [69] rngtools_1.2.4            survival_2.39-5
  [71] colorspace_1.2-6          AnnotationDbi_1.34.4
  [73] GenomicRanges_1.24.3      beanplot_1.2
```

References

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stages. *Nat Commun*, 5:3230, 2014. doi:10.1038/ncomms4230.