

# DMRcate for bisulfite sequencing

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## Summary

Worked example to find DMRs from whole genome bisulfite sequencing data.

```
if (!require("BiocManager"))  
  install.packages("BiocManager")  
BiocManager::install("DMRcate")
```

Load DMRcate into the workspace:

```
library(DMRcate)
```

Bisulfite sequencing assays are fundamentally different to arrays, because methylation is represented as a pair of methylated and unmethylated reads per sample, instead of a single beta value. Although we could simply take the logit-transformed fraction of methylated reads per CpG, this removes the effect of varying read depth across the genome. For example, a sampling depth of 30 methylated reads and 10 unmethylated reads is a much more precise estimate of the methylation level of a given CpG site than 3 methylated and 1 unmethylated. Hence, we take advantage of the fact that the overall effect can be expressed as an interaction between the coefficient of interest and a two-level factor representing methylated and unmethylated reads [1].

The example shown here will be performed on a BSseq object containing bisulfite sequencing of regulatory T cells from various tissues as part of the **tissueTreg** package[2], imported using ExperimentHub. First, we will import the data:

```
library(ExperimentHub)  
eh <- ExperimentHub()  
bis_1072 <- eh[["EH1072"]]  
bis_1072  
  
## An object of type 'BSseq' with  
## 21867550 methylation loci
```

```
## 15 samples
## has been smoothed with
## BSmooth (ns = 70, h = 1000, maxGap = 100000000)
## All assays are in-memory

colnames(bis_1072)

## [1] "Fat-Treg-R1"      "Fat-Treg-R2"      "Fat-Treg-R3"      "Liver-Treg-R1"
## [5] "Liver-Treg-R2"    "Liver-Treg-R3"    "Skin-Treg-R1"     "Skin-Treg-R2"
## [9] "Skin-Treg-R3"     "Lymph-N-Tcon-R1"  "Lymph-N-Tcon-R2"  "Lymph-N-Tcon-R3"
## [13] "Lymph-N-Treg-R1" "Lymph-N-Treg-R2" "Lymph-N-Treg-R3"
```

The data contains 15 samples: 3 (unmatched) replicates of mouse Tregs from fat, liver, skin and lymph node, plus a group of 3 CD4+ conventional lymph node T cells (Tcon). We will annotate the BSseq object to reflect this phenotypic information:

```
bsseq::pData(bis_1072) <- data.frame(replicate=gsub(".*-", "", colnames(bis_1072)),
                                     tissue=substr(colnames(bis_1072), 1,
                                                    nchar(colnames(bis_1072))-3),
                                     row.names=colnames(bis_1072))
colData(bis_1072)$tissue <- gsub("-", "_", colData(bis_1072)$tissue)
as.data.frame(colData(bis_1072))

##           replicate      tissue
## Fat-Treg-R1         R1   Fat_Treg
## Fat-Treg-R2         R2   Fat_Treg
## Fat-Treg-R3         R3   Fat_Treg
## Liver-Treg-R1        R1  Liver_Treg
## Liver-Treg-R2        R2  Liver_Treg
## Liver-Treg-R3        R3  Liver_Treg
## Skin-Treg-R1         R1   Skin_Treg
## Skin-Treg-R2         R2   Skin_Treg
## Skin-Treg-R3         R3   Skin_Treg
## Lymph-N-Tcon-R1      R1 Lymph_N_Tcon
## Lymph-N-Tcon-R2      R2 Lymph_N_Tcon
## Lymph-N-Tcon-R3      R3 Lymph_N_Tcon
## Lymph-N-Treg-R1      R1 Lymph_N_Treg
## Lymph-N-Treg-R2      R2 Lymph_N_Treg
## Lymph-N-Treg-R3      R3 Lymph_N_Treg
```

For standardisation purposes (and for `DMR.plot` to recognise the genome) we will change the chromosome naming convention to UCSC:

```
bis_1072 <- renameSeqlevels(bis_1072, mapSeqlevels(seqlevels(bis_1072), "UCSC"))
```

For demonstration purposes, we will retain CpGs on chromosome 19 only:

```
bis_1072 <- bis_1072[seqnames(bis_1072)=="chr19",]
bis_1072

## An object of type 'BSseq' with
## 558056 methylation loci
## 15 samples
## has been smoothed with
## BSmooth (ns = 70, h = 1000, maxGap = 100000000)
## All assays are in-memory
```

Now we can prepare the model to be fit for `sequencing.annotate()`. The arguments are equivalent to `cpg.annotate()` but for a couple of exceptions:

- There is an extra argument `all.cov` giving an option whether to retain only CpGs where *all* samples have non-zero coverage, or whether to retain CpGs with only partial sample representation.
- The design matrix should be constructed to reflect the 2-factor structure of methylated and unmethylated reads. Fortunately, `edgeR::modelMatrixMeth()` can take a regular design matrix and transform it into the appropriate structure ready for model fitting.

```
tissue <- factor(pData(bis_1072)$tissue)
tissue <- relevel(tissue, "Liver_Treg")

#Regular matrix design
design <- model.matrix(~tissue)
colnames(design) <- gsub("tissue", "", colnames(design))
colnames(design)[1] <- "Intercept"
rownames(design) <- colnames(bis_1072)
design
```

	Intercept	Fat_Treg	Lymph_N_Tcon	Lymph_N_Treg	Skin_Treg
## Fat-Treg-R1	1	1	0	0	0
## Fat-Treg-R2	1	1	0	0	0
## Fat-Treg-R3	1	1	0	0	0
## Liver-Treg-R1	1	0	0	0	0
## Liver-Treg-R2	1	0	0	0	0
## Liver-Treg-R3	1	0	0	0	0
## Skin-Treg-R1	1	0	0	0	1
## Skin-Treg-R2	1	0	0	0	1
## Skin-Treg-R3	1	0	0	0	1

```

## Lymph-N-Tcon-R1      1      0      1      0      0
## Lymph-N-Tcon-R2      1      0      1      0      0
## Lymph-N-Tcon-R3      1      0      1      0      0
## Lymph-N-Treg-R1      1      0      0      1      0
## Lymph-N-Treg-R2      1      0      0      1      0
## Lymph-N-Treg-R3      1      0      0      1      0
## attr("assign")
## [1] 0 1 1 1 1
## attr("contrasts")
## attr("contrasts")$tissue
## [1] "contr.treatment"

#Methylation matrix design
methdesign <- edgeR::modelMatrixMeth(design)
methdesign

##      Sample1 Sample2 Sample3 Sample4 Sample5 Sample6 Sample7 Sample8 Sample9
## 1          1      0      0      0      0      0      0      0      0
## 2          1      0      0      0      0      0      0      0      0
## 3          0      1      0      0      0      0      0      0      0
## 4          0      1      0      0      0      0      0      0      0
## 5          0      0      1      0      0      0      0      0      0
## 6          0      0      1      0      0      0      0      0      0
## 7          0      0      0      1      0      0      0      0      0
## 8          0      0      0      1      0      0      0      0      0
## 9          0      0      0      0      1      0      0      0      0
## 10         0      0      0      0      1      0      0      0      0
## 11         0      0      0      0      0      1      0      0      0
## 12         0      0      0      0      0      1      0      0      0
## 13         0      0      0      0      0      0      1      0      0
## 14         0      0      0      0      0      0      1      0      0
## 15         0      0      0      0      0      0      0      1      0
## 16         0      0      0      0      0      0      0      1      0
## 17         0      0      0      0      0      0      0      0      1
## 18         0      0      0      0      0      0      0      0      1
## 19         0      0      0      0      0      0      0      0      0
## 20         0      0      0      0      0      0      0      0      0
## 21         0      0      0      0      0      0      0      0      0
## 22         0      0      0      0      0      0      0      0      0
## 23         0      0      0      0      0      0      0      0      0
## 24         0      0      0      0      0      0      0      0      0
## 25         0      0      0      0      0      0      0      0      0
## 26         0      0      0      0      0      0      0      0      0
## 27         0      0      0      0      0      0      0      0      0
## 28         0      0      0      0      0      0      0      0      0
## 29         0      0      0      0      0      0      0      0      0

```

## 30	0	0	0	0	0	0	0	0	0	0
##	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Intercept	Fat_Treg		
## 1	0	0	0	0	0	0		1	1	
## 2	0	0	0	0	0	0		0	0	
## 3	0	0	0	0	0	0		1	1	
## 4	0	0	0	0	0	0		0	0	
## 5	0	0	0	0	0	0		1	1	
## 6	0	0	0	0	0	0		0	0	
## 7	0	0	0	0	0	0		1	0	
## 8	0	0	0	0	0	0		0	0	
## 9	0	0	0	0	0	0		1	0	
## 10	0	0	0	0	0	0		0	0	
## 11	0	0	0	0	0	0		1	0	
## 12	0	0	0	0	0	0		0	0	
## 13	0	0	0	0	0	0		1	0	
## 14	0	0	0	0	0	0		0	0	
## 15	0	0	0	0	0	0		1	0	
## 16	0	0	0	0	0	0		0	0	
## 17	0	0	0	0	0	0		1	0	
## 18	0	0	0	0	0	0		0	0	
## 19	1	0	0	0	0	0		1	0	
## 20	1	0	0	0	0	0		0	0	
## 21	0	1	0	0	0	0		1	0	
## 22	0	1	0	0	0	0		0	0	
## 23	0	0	1	0	0	0		1	0	
## 24	0	0	1	0	0	0		0	0	
## 25	0	0	0	1	0	0		1	0	
## 26	0	0	0	1	0	0		0	0	
## 27	0	0	0	0	1	0		1	0	
## 28	0	0	0	0	1	0		0	0	
## 29	0	0	0	0	0	1		1	0	
## 30	0	0	0	0	0	1		0	0	
##	Lymph_N_Tcon	Lymph_N_Treg	Skin_Treg							
## 1	0	0	0							
## 2	0	0	0							
## 3	0	0	0							
## 4	0	0	0							
## 5	0	0	0							
## 6	0	0	0							
## 7	0	0	0							
## 8	0	0	0							
## 9	0	0	0							
## 10	0	0	0							
## 11	0	0	0							
## 12	0	0	0							

```
## 13      0      0      1
## 14      0      0      0
## 15      0      0      1
## 16      0      0      0
## 17      0      0      1
## 18      0      0      0
## 19      1      0      0
## 20      0      0      0
## 21      1      0      0
## 22      0      0      0
## 23      1      0      0
## 24      0      0      0
## 25      0      1      0
## 26      0      0      0
## 27      0      1      0
## 28      0      0      0
## 29      0      1      0
## 30      0      0      0
```

Just like for `cpg.annotate()`, we can specify a contrast matrix to find our comparisons of interest.

```
cont.mat <- limma::makeContrasts(treg_vs_tcon=Lymph_N_Treg-Lymph_N_Tcon,
                                fat_vs_ln=Fat_Treg-Lymph_N_Treg,
                                skin_vs_ln=Skin_Treg-Lymph_N_Treg,
                                fat_vs_skin=Fat_Treg-Skin_Treg,
                                levels=methdesign)

cont.mat
```

##	Contrasts				
## Levels	treg_vs_tcon	fat_vs_ln	skin_vs_ln	fat_vs_skin	
## Sample1	0	0	0	0	
## Sample2	0	0	0	0	
## Sample3	0	0	0	0	
## Sample4	0	0	0	0	
## Sample5	0	0	0	0	
## Sample6	0	0	0	0	
## Sample7	0	0	0	0	
## Sample8	0	0	0	0	
## Sample9	0	0	0	0	
## Sample10	0	0	0	0	
## Sample11	0	0	0	0	
## Sample12	0	0	0	0	
## Sample13	0	0	0	0	
## Sample14	0	0	0	0	
## Sample15	0	0	0	0	

##	Intercept	0	0	0	0
##	Fat_Treg	0	1	0	1
##	Lymph_N_Tcon	-1	0	0	0
##	Lymph_N_Treg	1	-1	-1	0
##	Skin_Treg	0	0	1	-1

Say we want to find DMRs between the regulatory and conventional T cells from the lymph node. First we would fit the model, where `sequencing.annotate()` transforms counts into log2CPMs (via `limma::voom()`) and uses `limma` under the hood to generate per-CpG *t*-statistics, indexing the FDR at 0.05:

```
seq_annot <- sequencing.annotate(bis_1072, methdesign, all.cov = TRUE,
                                contrasts = TRUE, cont.matrix = cont.mat,
                                coef = "treg_vs_tcon", fdr=0.05)

## Filtering out all CpGs where at least one sample has zero coverage...
## Processing BSseq object...
## Transforming counts...
## Fitting model...
## Your contrast returned 157 individually significant CpGs. We recommend
## the default setting of pcutoff in dmrcate().

seq_annot

## CpGannotated object describing 506908 CpG sites, with independent
## CpG threshold indexed at fdr=0.05 and 157 significant CpG sites.
```

And then, just like before, we can call DMRs with `dmrcate()`:

```
dmrcate.res <- dmrcate(seq_annot, C=2, min.cpgs = 5)

## Fitting chr19...
## Demarcating regions...
## Done!

dmrcate.res

## DMResults object with 9 DMRs.
## Use extractRanges() to produce a GRanges object of these.

treg_vs_tcon.ranges <- extractRanges(dmrcate.res, genome="mm10")

## see ?DMRcatedata and browseVignettes('DMRcatedata') for documentation
## loading from cache

treg_vs_tcon.ranges
```

```
## GRanges object with 9 ranges and 8 metadata columns:
##      seqnames      ranges strand |   no.cpgs min_smoothed_fdr
##      <Rle>        <IRanges> <Rle> | <integer>      <numeric>
## [1]   chr19 29270611-29272005     * |      16      4.32351e-94
## [2]   chr19 36378257-36379597     * |      27      1.53747e-76
## [3]   chr19 40808208-40809554     * |      26      3.43873e-63
## [4]   chr19 46653280-46654180     * |      19      3.94008e-59
## [5]   chr19 26683453-26684174     * |      12      1.77927e-57
## [6]   chr19 32276886-32278089     * |      13      1.74620e-56
## [7]   chr19 29374953-29375393     * |      12      1.48257e-54
## [8]   chr19 41874401-41874895     * |      22      2.75829e-39
## [9]   chr19 57092365-57092646     * |      10      3.80468e-36
##      Stouffer      HMFDR      Fisher      maxdiff      meandiff      overlapping.genes
##      <numeric> <numeric> <numeric> <numeric> <numeric>      <character>
## [1] 7.30413e-50 1.53438e-06 9.06017e-52 -6.40482 -4.22353      Jak2
## [2] 5.11228e-45 5.32893e-06 6.32480e-49 -6.09625 -3.03550      Pcgf5
## [3] 1.57979e-50 7.52023e-05 1.44219e-45 -4.83855 -3.07494      Cc2d2b
## [4] 6.97298e-34 5.32893e-06 1.28153e-35  5.18388  2.93152      Wbp1l
## [5] 3.23045e-25 3.29036e-07 1.30324e-30 -6.40328 -3.53692      Smarca2
## [6] 2.50713e-38 5.32893e-06 1.55805e-36  5.81470  3.93201      Sgms1
## [7] 7.68507e-20 3.13417e-06 3.83714e-26 -6.10902 -3.02083      Cd274, Gm36043
## [8] 1.15140e-30 6.23899e-05 7.96953e-30  4.57011  2.56520      Rrp12
## [9] 1.28763e-24 7.32179e-06 6.36706e-23 -4.67645 -3.36472      Ablim1
## -----
##      seqinfo: 1 sequence from an unspecified genome; no seqlengths
```

Looks like the top DMR is associated with the *Jak2* locus and hypomethylated in the Treg cells (since `meandiff < 0`). We can plot it like so:

```
cols <- as.character(plyr::mapvalues(tissue, unique(tissue),
                                     c("darkorange", "maroon", "blue",
                                       "black", "magenta")))
names(cols) <- tissue

DMR.plot(treg_vs_tcon.ranges, dmr = 1,
         CpGs=bis_1072[,tissue %in% c("Lymph_N_Tcon", "Lymph_N_Treg")],
         phen.col = cols[tissue %in% c("Lymph_N_Tcon", "Lymph_N_Treg")],
         genome="mm10")
```





Now, let's find DMRs between fat and skin Tregs.

```
seq_annot <- sequencing.annotate(bis_1072, methdesign, all.cov = TRUE,
                                contrasts = TRUE, cont.matrix = cont.mat,
                                coef = "fat_vs_skin", fdr=0.05)

## Filtering out all CpGs where at least one sample has zero coverage...
## Processing BSseq object...
## Transforming counts...
## Fitting model...
## Your contrast returned 5 individually significant CpGs; a small
but real effect. Consider increasing the 'fdr' parameter using changeFDR(),
but be warned there is an increased risk of Type I errors.
```

Because this comparison is a bit more subtle, there are very few significantly differential CpGs at this threshold. So we can use `changeFDR()` to relax the

FDR to 0.25, taking into account that there is an increased risk of false positives.

```
seq_annot <- changeFDR(seq_annot, 0.25)

## Threshold is now set at FDR=0.25, resulting in 63 significantly differential CpGs.
```

```
dmrcate.res <- dmrcate(seq_annot, C=2, min.cpgs = 5)

## Fitting chr19...
## Demarcating regions...
## Done!

fat_vs_skin.ranges <- extractRanges(dmrcate.res, genome="mm10")

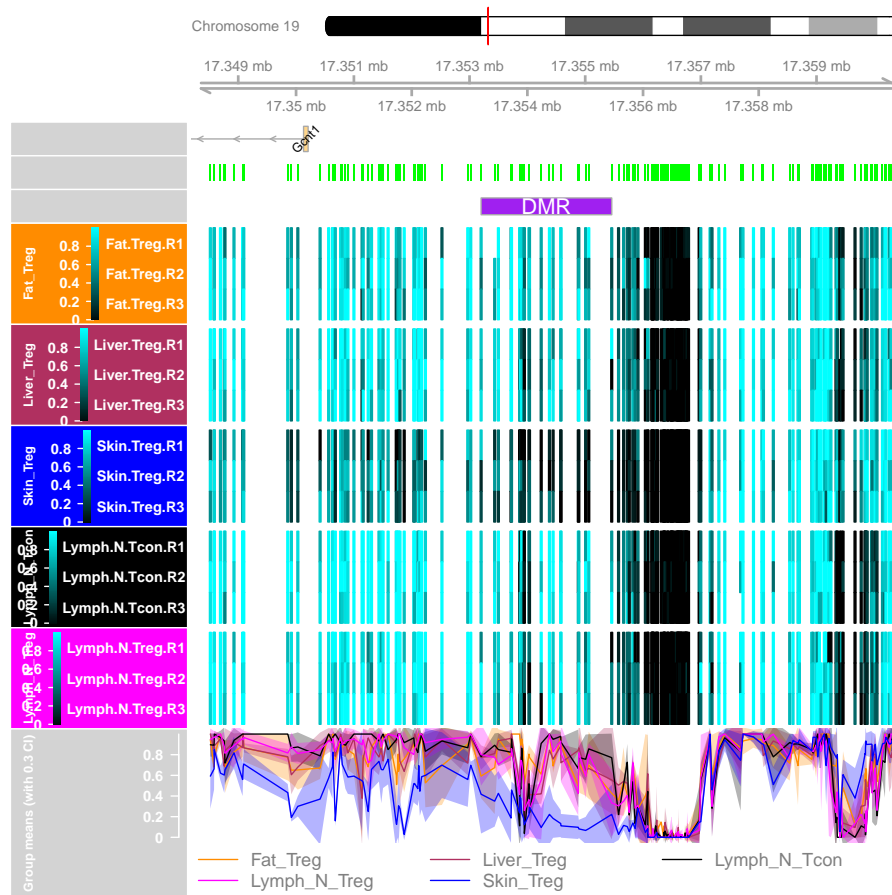
## see ?DMRcatedata and browseVignettes('DMRcatedata') for documentation
## loading from cache
```

Now let's plot the top DMR with not only fat and skin, but with all samples:

```
cols

##      Fat_Treg      Fat_Treg      Fat_Treg      Liver_Treg      Liver_Treg      Liver_Treg
## "darkorange" "darkorange" "darkorange"      "maroon"      "maroon"      "maroon"
##      Skin_Treg      Skin_Treg      Skin_Treg      Lymph_N_Tcon      Lymph_N_Tcon      Lymph_N_Tcon
##          "blue"          "blue"          "blue"          "black"          "black"          "black"
##      Lymph_N_Treg      Lymph_N_Treg      Lymph_N_Treg
##          "magenta"      "magenta"      "magenta"

DMR.plot(fat_vs_skin.ranges, dmr = 1, CpGs=bis_1072, phen.col = cols, genome="mm10")
```



Here we can see the methylation of skin cells over this region near the *Gent1* promoter is hypomethylated not only relative to fat, but to the other tissues as well.

```
sessionInfo()

## R version 4.4.1 (2024-06-14)
## Platform: x86_64-pc-linux-gnu
## Running under: Ubuntu 22.04.4 LTS
##
## Matrix products: default
## BLAS: /home/biocbuild/bbs-3.19-bioc/R/lib/libRblas.so
## LAPACK: /usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.10.0
##
## locale:
## [1] LC_CTYPE=en_US.UTF-8 LC_NUMERIC=C
```

```

## [3] LC_TIME=en_GB          LC_COLLATE=C
## [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
##
## time zone: America/New_York
## tzcode source: system (glibc)
##
## attached base packages:
## [1] parallel stats4 stats graphics grDevices utils datasets
## [8] methods base
##
## other attached packages:
## [1] bsseq_1.40.0
## [2] tissueTreg_1.24.0
## [3] IlluminaHumanMethylationEPICv2anno.20a1.hg38_1.0.0
## [4] DMRcatedata_2.22.0
## [5] IlluminaHumanMethylationEPICanno.ilm10b4.hg19_0.6.0
## [6] IlluminaHumanMethylationEPICmanifest_0.3.0
## [7] FlowSorted.Blood.EPIC_2.8.0
## [8] minfi_1.50.0
## [9] bumpHunter_1.46.0
## [10] locfit_1.5-9.10
## [11] iterators_1.0.14
## [12] foreach_1.5.2
## [13] Biostrings_2.72.1
## [14] XVector_0.44.0
## [15] SummarizedExperiment_1.34.0
## [16] Biobase_2.64.0
## [17] MatrixGenerics_1.16.0
## [18] matrixStats_1.3.0
## [19] GenomicRanges_1.56.1
## [20] GenomeInfoDb_1.40.1
## [21] IRanges_2.38.1
## [22] S4Vectors_0.42.1
## [23] ExperimentHub_2.12.0
## [24] AnnotationHub_3.12.0
## [25] BiocFileCache_2.12.0
## [26] dbplyr_2.5.0
## [27] BiocGenerics_0.50.0
## [28] DMRcate_3.0.8
##
## loaded via a namespace (and not attached):
## [1] splines_4.4.1

```

```
## [2] BiocIO_1.14.0
## [3] bitops_1.0-8
## [4] filelock_1.0.3
## [5] cellranger_1.1.0
## [6] tibble_3.2.1
## [7] R.oo_1.26.0
## [8] preprocessCore_1.66.0
## [9] XML_3.99-0.17
## [10] rpart_4.1.23
## [11] lifecycle_1.0.4
## [12] httr2_1.0.2
## [13] edgeR_4.2.1
## [14] base64_2.0.1
## [15] MASS_7.3-61
## [16] lattice_0.22-6
## [17] ensemblDb_2.28.1
## [18] scribe_1.3.5
## [19] backports_1.5.0
## [20] magrittr_2.0.3
## [21] limma_3.60.4
## [22] Hmisc_5.1-3
## [23] rmarkdown_2.28
## [24] yaml_2.3.10
## [25] doRNG_1.8.6
## [26] askpass_1.2.0
## [27] Gviz_1.48.0
## [28] DBI_1.2.3
## [29] RColorBrewer_1.1-3
## [30] abind_1.4-5
## [31] zlibbioc_1.50.0
## [32] quadprog_1.5-8
## [33] purrr_1.0.2
## [34] R.utils_2.12.3
## [35] AnnotationFilter_1.28.0
## [36] biovizBase_1.52.0
## [37] RCurl_1.98-1.16
## [38] nnet_7.3-19
## [39] VariantAnnotation_1.50.0
## [40] rappdirs_0.3.3
## [41] GenomeInfoDbData_1.2.12
## [42] genefilter_1.86.0
## [43] annotate_1.82.0
## [44] permute_0.9-7
## [45] DelayedMatrixStats_1.26.0
## [46] codetools_0.2-20
```

```

## [47] DelayedArray_0.30.1
## [48] xml2_1.3.6
## [49] tidyselect_1.2.1
## [50] UCSC.utils_1.0.0
## [51] beanplot_1.3.1
## [52] base64enc_0.1-3
## [53] illuminaio_0.46.0
## [54] GenomicAlignments_1.40.0
## [55] jsonlite_1.8.8
## [56] multtest_2.60.0
## [57] Formula_1.2-5
## [58] survival_3.7-0
## [59] missMethyl_1.38.0
## [60] tools_4.4.1
## [61] progress_1.2.3
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