RNA-Seq differential expression work flow using DESeq2

Tools

This analysis was performed using R (ver. 3.1.0).

Introduction

One of the aim of RNAseq data analysis is the detection of differentially expressed genes. The package DESeq2 provides methods to test for differential expression analysis.

This document presents an RNAseq differential expression workflow. We will start from the FASTQ files, align to the reference genome, prepare gene expression values as a count table by counting the sequenced fragments, perform differential gene expression analysis, and visually explore the results.

Input data

We will use publicly available data from the article by **Felix Haglund et al., J Clin Endocrin Metab 2012**. The purpose of the experiment was to investigate the role of the estrogen receptor in parathyroid tumors. The investigators derived primary cultures of parathyroid adenoma cells from 4 patients. These primary cultures were treated with diarylpropionitrile (DPN), an estrogen receptor beta agonist, or with 4-hydroxytamoxifen (OHT). RNA was extracted at 24 hours and 48 hours from cultures under treatment and control.

Part of the data from this experiment is provided in the Bioconductor data package **parathyroidSE**.

Aligning reads to a reference

What we get from the sequencing machine is a set of FASTQ files that contain the nucleotide sequence of each read and a quality score at each position. These reads must first be aligned to a reference genome or transcriptome. It is important to know if the sequencing experiment was single-end or paired-end, as the alignment software will require the user to specify both FASTQ files for a paired-end experiment. The output of this alignment step is commonly stored in a file format called BAM.

Here we use the TopHat2 spliced alignment software in combination with the Bowtie index available at the Illumina iGenomes.

For example, the paired-end RNA-Seq reads for the parathyroidSE package were aligned using TopHat2 with 8 threads, with the call:

```
 tophat2 -o file_tophat_out -p 8 path/to/genome file_1.fastq file_2.fastq samtools sort -n fi
le_tophat_out/accepted_hits.bam _sorted
```

The second line sorts the reads by name rather than by genomic position, which is necessary for counting paired-end reads within Bioconductor . This command uses the SAMtools software .

The BAM files for a number of sequencing runs can then be used to generate count matrices, as described in the following section.

Example BAM files

We will use BAM files from **parathyroidSE** package to demonstrate how a count table can be constructed from BAM files.

```
library( "parathyroidSE" )
#Find extdata
extDataDir <- system.file("extdata", package = "parathyroidSE", mustWork = TRUE)
list.files( extDataDir )
```

```
## [1] "conversion.txt"                  "GSE37211_series_matrix.txt"   "SRR479052.bam"
"SRR479053.bam"
## [5] "SRR479054.bam"
```

Typically, we have a table with experimental meta data for our samples. For these three files, it is as follows:

```
#Sample tables
sampleTable <- data.frame(
   sampleName = c( "Ctrl_24h_1", "Ctrl_48h_1", "DPN_24h_1" ),
   fileName = c( "SRR479052.bam", "SRR479053.bam",  "SRR479054.bam" ),
   treatment =  c( "Control", "Control", "DPN" ),
   time = c( "24h", "48h", "24h" ) )
#This is how the sample table should look like
sampleTable
```

```
##   sampleName      fileName treatment time
## 1 Ctrl_24h_1 SRR479052.bam   Control  24h
## 2 Ctrl_48h_1 SRR479053.bam   Control  48h
## 3  DPN_24h_1 SRR479054.bam       DPN  24h
```

Construct the full paths to the files we want to perform the counting operation on:

```
bamFiles <- file.path( extDataDir, sampleTable$fileName )
bamFiles
```

```
##                                                                                   [1]
"/Library/Frameworks/R.framework/Versions/3.1/Resources/library/parathyroidSE/extdat
a/SRR479052.bam"
##                                                                                   [2]
"/Library/Frameworks/R.framework/Versions/3.1/Resources/library/parathyroidSE/extdat
a/SRR479053.bam"
##                                                                                   [3]
"/Library/Frameworks/R.framework/Versions/3.1/Resources/library/parathyroidSE/extdat
a/SRR479054.bam"
```

We can peek into one of the BAM files to see the naming style of the sequences (chromosomes). Here we use the **BamFile** function from the **Rsamtools** package.

```
library( "Rsamtools" )
seqinfo( BamFile( bamFiles[1] ) )
```

```
## Seqinfo of length 25
## seqnames seqlengths isCircular genome
## 1         249250621       <NA>   <NA>
## 2         243199373       <NA>   <NA>
## 3         198022430       <NA>   <NA>
## 4         191154276       <NA>   <NA>
## 5         180915260       <NA>   <NA>
## ...             ...        ...    ...
## 21         48129895       <NA>   <NA>
## 22         51304566       <NA>   <NA>
## X         155270560       <NA>   <NA>
## Y          59373566       <NA>   <NA>
## MT            16569       <NA>   <NA>
```

✓

We want to make sure that these sequence names are the same style as that of the gene models we will obtain in the next section.

Counting reads in genes

To count how many read map to each gene, we need transcript annotation. Download the current GTF file with human gene annotation from Ensembl.

From this file, the function **makeTranscriptDbFromGFF** from the **GenomicFeatures** package constructs a database of all annotated transcripts.

For this lab you can use the truncated version of this file, called Homo_sapiens.GRCh37.75.subset.gtf.gz.

Preparing gene models from GFF files

```
library( "GenomicFeatures" )
#hse <- makeTranscriptDbFromGFF( "/path/to/your/genemodel_file.GTF", format="gtf" )
hse <- makeTranscriptDbFromGFF( "Homo_sapiens.GRCh37.75.subset.gtf", format="gtf" )
#Extract exons for each gene
exonsByGene <- exonsBy( hse, by="gene" )
exonsByGene
```

```
## GRangesList of length 100:
## $ENSG00000000003
## GRanges with 13 ranges and 2 metadata columns:
##        seqnames               ranges strand   |   exon_id   exon_name
##           <Rle>            <IRanges>  <Rle>   | <integer> <character>
##     [1]        X [99883667, 99884983]      -  |      3038        <NA>
##     [2]        X [99885756, 99885863]      -  |      3039        <NA>
##     [3]        X [99887482, 99887565]      -  |      3040        <NA>
##     [4]        X [99887538, 99887565]      -  |      3041        <NA>
##     [5]        X [99888402, 99888536]      -  |      3042        <NA>
##     ...      ...                  ...    ... ...       ...         ...
##     [9]        X [99890555, 99890743]      -  |      3046        <NA>
##    [10]        X [99891188, 99891686]      -  |      3047        <NA>
##    [11]        X [99891605, 99891803]      -  |      3048        <NA>
##    [12]        X [99891790, 99892101]      -  |      3049        <NA>
##    [13]        X [99894942, 99894988]      -  |      3050        <NA>
##
## ...
## <99 more elements>
## ---
## seqlengths:
##    7 12  2  6 16  4  3  1 17  8 19  X 11  9 20
##   NA NA NA NA NA NA NA NA NA NA NA NA NA NA NA
```

- Note that gene models can also be prepared directly from BioMart :

```r
library( "GenomicFeatures" )
# takes ~10 min
txdb <- makeTranscriptDbFromBiomart( biomart="ensembl",
                                    dataset="hsapiens_gene_ensembl" )
exonsByGene <- exonsBy( txdb, by="gene" )
```

Use **saveDb()** to only do this once. Use **loadDb()** to load the database next time.

library(TxDb.Hsapiens.UCSC.hg19.knownGene) is also an ready to go option for gene models.

- To convert from `chrX` to `X` and back:

```r
seqlevelsStyle(gr) <- "NCBI"
seqlevelsStyle(gr) <- "UCSC"
```

Reads counting

The function **summarizeOverlaps** from the **GenomicAlignments** package will do this.

```
library( "GenomicAlignments" )
se <- summarizeOverlaps( exonsByGene, BamFileList( bamFiles ), mode="Union",
  singleEnd=FALSE, ignore.strand=TRUE, fragments=TRUE )
```

✓ We use the counting mode "Union", which indicates that those reads which overlap any portion of exactly one feature are counted. As this experiment produced paired-end reads, we specify singleEnd =FALSE. As protocol was not strand-specific, we specify ignore.strand = TRUE. fragments = TRUE indicates that we also want to count reads with unmapped pairs. This last argument is only for use with paired-end experiments.

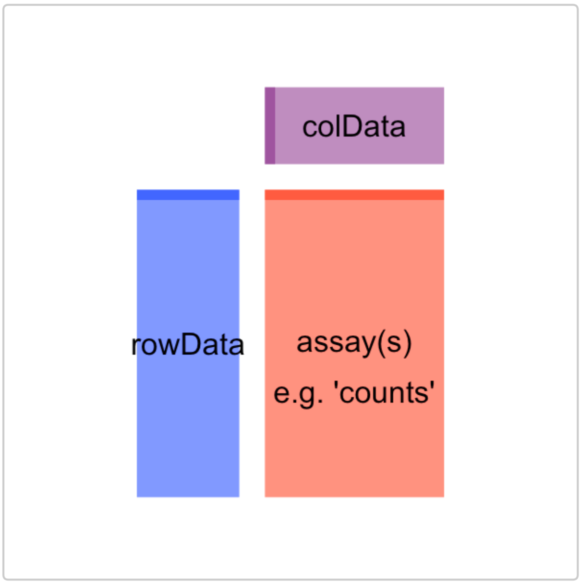
Details on how to read from the BAM files can be specified using the **BamFileList** function. For example, to control the memory, we could have specified that batches of **2 000 000** reads should be read at a time:

```
BamFileList( bamFiles, yieldSize = 2000000 )
```

SummarizedExperiment object : Output of counting

We investigate the resulting **SummarizedExperiment class** by looking at the counts in the **assay** slot, the phenotypic data about the samples in **colData** slot (in this case an empty **DataFrame**), and the data about the genes in the **rowData** slot.

Figure 1 explains the basic structure of the **SummarizedExperiment** class.



```r
# SummarizedExperiment
se
```

```
## class: SummarizedExperiment
## dim: 100 3
## exptData(0):
## assays(1): counts
##     rownames(100):     ENSG00000000003     ENSG00000000005     ...     ENSG00000005469
ENSG00000005471
## rowData metadata column names(0):
## colnames(3): SRR479052.bam SRR479053.bam SRR479054.bam
## colData names(0):
```

```r
# Count data
head( assay(se) )
```

```
##                 SRR479052.bam SRR479053.bam SRR479054.bam
## ENSG00000000003             0             0             1
## ENSG00000000005             0             0             0
## ENSG00000000419             0             0             0
## ENSG00000000457             0             1             1
## ENSG00000000460             0             0             0
## ENSG00000000938             0             0             0
```

```r
#Column sum
colSums( assay(se) )
```

```
## SRR479052.bam SRR479053.bam SRR479054.bam
##            31            21            30
```

```
#Phenotypic data
colData(se)
```

```
## DataFrame with 3 rows and 0 columns
```

```
#Data about genes
rowData(se)
```

```
## GRangesList of length 100:
## $ENSG00000000003
## GRanges with 13 ranges and 2 metadata columns:
##        seqnames               ranges strand   |   exon_id   exon_name
##           <Rle>            <IRanges>  <Rle>   | <integer> <character>
##    [1]        X [99883667, 99884983]      -   |      3038        <NA>
##    [2]        X [99885756, 99885863]      -   |      3039        <NA>
##    [3]        X [99887482, 99887565]      -   |      3040        <NA>
##    [4]        X [99887538, 99887565]      -   |      3041        <NA>
##    [5]        X [99888402, 99888536]      -   |      3042        <NA>
##    ...      ...                  ...    ... ...       ...         ...
##    [9]        X [99890555, 99890743]      -   |      3046        <NA>
##   [10]        X [99891188, 99891686]      -   |      3047        <NA>
##   [11]        X [99891605, 99891803]      -   |      3048        <NA>
##   [12]        X [99891790, 99892101]      -   |      3049        <NA>
##   [13]        X [99894942, 99894988]      -   |      3050        <NA>
##
## ...
## <99 more elements>
## ---
## seqlengths:
##   7 12  2  6 16  4  3  1 17  8 19  X 11  9 20
##  NA NA NA NA NA NA NA NA NA NA NA NA NA NA NA
```

✓ Note that the **rowData** slot is a **GRangesList**, which contains all the information about the exons for each gene, i.e., for each row of the count table.

The **colData** slot, so far empty, should contain all the meta data. We hence assign our sample table to it:

```
#Column data
colData(se) <- DataFrame( sampleTable )
```

We can extract columns from the **colData** using the **$** operator, and we can omit the **colData** to avoid extra keystrokes.

```
colData(se)$treatment
```

```
## [1] Control Control DPN
## Levels: Control DPN
```

```
se$treatment
```

```
## [1] Control Control DPN
## Levels: Control DPN
```

We can also use the sampleName table to name the columns of our data matrix:

```
colnames(se) <- sampleTable$sampleName
head( assay(se) )
```

```
##                 Ctrl_24h_1 Ctrl_48h_1 DPN_24h_1
## ENSG00000000003          0          0         1
## ENSG00000000005          0          0         0
## ENSG00000000419          0          0         0
## ENSG00000000457          0          1         1
## ENSG00000000460          0          0         0
## ENSG00000000938          0          0         0
```

✓ This SummarizedExperiment object se is then all we need to start our analysis.

The DESeqDataSet, column metadata, and the design formula

The data object class in **DESeq2** is the **DESeqDataSet**, which is built on top of the **SummarizedExperiment** class. One main differences is that the **assay** slot is instead accessed using the **count** accessor, and the values in this matrix must be non-negative integers.

A second difference is that the **DESeqDataSet** has an associated "design formula". The design formula tells which variables in the column metadata table **colData** specify the experimental design and how these factors should be used in the analysis.

The simplest design formula for differential expression would be `~ condition`, where **condition** is a column in `colData(dds)` which specifies which of two (or more groups) the samples belong to.

For the parathyroid experiment, we will specify `~ patient + treatment`, which means that we want to test for the effect of treatment (the last factor), controlling for the effect of patient (the first factor).

We now use R's **data** command to load a prepared **SummarizedExperiment** that was generated from the publicly available sequencing data files associated with the Haglund et al. paper, described on page 1. The steps we used to produce this object were equivalent to those you worked through in the previous Section, except that we used the complete set of samples and all reads.

```
#Run data
data( "parathyroidGenesSE" )
se <- parathyroidGenesSE
colnames(se) <- se$run
# Check column data
colData(se)[1:5,1:4]
```

```
## DataFrame with 5 rows and 4 columns
##                     run experiment  patient treatment
##             <character>   <factor> <factor>  <factor>
## SRR479052    SRR479052   SRX140503        1   Control
## SRR479053    SRR479053   SRX140504        1   Control
## SRR479054    SRR479054   SRX140505        1       DPN
## SRR479055    SRR479055   SRX140506        1       DPN
## SRR479056    SRR479056   SRX140507        1       OHT
```

Here we see that this object already contains an informative **colData slot.**

When you work with your own data, you will have to add the pertinent sample / phenotypic information for the experiment at this stage. We highly recommend keeping this information in a comma-separated value (CSV) or tab-separated value (TSV) file, which can be exported from an Excel spreadsheet, and the assign this to the **colData** slot, as shown in the previous section.

Make sure that the order of rows in your column data table matches the order of columns in the assay data slot.

A bonus about the workflow we have shown above is that information about the gene models we used is included without extra effort. The **str** R function is used to compactly display the structure of the data in the list.

```
str( metadata( rowData(se) ) )
```

```
## List of 1
##  $ genomeInfo:List of 20
##   ..$ Db type                                  : chr "TranscriptDb"
##   ..$ Supporting package                       : chr "GenomicFeatures"
##   ..$ Data source                              : chr "BioMart"
##   ..$ Organism                                 : chr "Homo sapiens"
##   ..$ Resource URL                             : chr "www.biomart.org:80"
##   ..$ BioMart database                         : chr "ensembl"
##    ..$  BioMart  database  version                   :  chr  "ENSEMBL  GENES  72  (SANGER
UK)"
##   ..$ BioMart dataset                          : chr "hsapiens_gene_ensembl"
##     ..$  BioMart  dataset  description                     :  chr  "Homo  sapiens  genes
(GRCh37.p11)"
##   ..$ BioMart dataset version                  : chr "GRCh37.p11"
##   ..$ Full dataset                             : chr "yes"
##   ..$ miRBase build ID                         : chr NA
##   ..$ transcript_nrow                          : chr "213140"
##   ..$ exon_nrow                                : chr "737783"
##   ..$ cds_nrow                                : chr "531154"
##   ..$ Db created by                           : chr "GenomicFeatures package from
Bioconductor"
##   ..$ Creation time                           : chr "2013-07-30 17:30:25 +0200
(Tue, 30 Jul 2013)"
##   ..$ GenomicFeatures version at creation time: chr "1.13.21"
##   ..$ RSQLite version at creation time        : chr "0.11.4"
##   ..$ DBSCHEMAVERSION                         : chr "1.0"
```

Once we have our fully annotated **SummerizedExperiment** object, we can construct a DESeqDataSet object from it, which will then form the staring point of the actual **DESeq2** package

```
library( "DESeq2" )
ddsFull <- DESeqDataSet( se, design = ~ patient + treatment )
```

Note that there are two alternative functions, **DESeqDataSetFromMatrix** and **DESeqDataSetFromHTSeq**, which allow you to get started in case you have your data not in the form of a **SummarizedExperiment** object, but either as a simple matrix of count values or as output files from the **htseq-count** script from the **HTSeq** Python package.

Collapsing technical replicates

There are a number of samples which were sequenced in multiple runs. For example, sample **SRS308873** was sequenced twice.

```
#as.data.frame forces R to show us the full list
head(as.data.frame( colData( ddsFull )[ ,c("sample","patient","treatment","time") ] ), 12)
```

```
##              sample patient treatment time
## SRR479052 SRS308865       1   Control  24h
## SRR479053 SRS308866       1   Control  48h
## SRR479054 SRS308867       1       DPN  24h
## SRR479055 SRS308868       1       DPN  48h
## SRR479056 SRS308869       1       OHT  24h
## SRR479057 SRS308870       1       OHT  48h
## SRR479058 SRS308871       2   Control  24h
## SRR479059 SRS308872       2   Control  48h
## SRR479060 SRS308873       2       DPN  24h
## SRR479061 SRS308873       2       DPN  24h
## SRR479062 SRS308874       2       DPN  48h
## SRR479063 SRS308875       2       OHT  24h
```

A convenience function has been implemented to collapse, which can take an object, either **SummarizedExperiment** or **DESeqDataSet**, and a grouping factor, in this case the sample name, and return the object with the counts summed up for each unique sample. Optionally, we can provide a third argument, run, which can be used to paste together the names of the runs which were collapsed to create the new object.

Note that `dds$variable` is equivalent to `colData(dds)$variable`.

```
ddsCollapsed <- collapseReplicates( ddsFull,
                                    groupby = ddsFull$sample,
                                    run = ddsFull$run )
head( as.data.frame( colData(ddsCollapsed)[ ,c("sample","runsCollapsed") ] ), 12 )
```

```
##              sample       runsCollapsed
## SRS308865 SRS308865           SRR479052
## SRS308866 SRS308866           SRR479053
## SRS308867 SRS308867           SRR479054
## SRS308868 SRS308868           SRR479055
## SRS308869 SRS308869           SRR479056
## SRS308870 SRS308870           SRR479057
## SRS308871 SRS308871           SRR479058
## SRS308872 SRS308872           SRR479059
## SRS308873 SRS308873 SRR479060,SRR479061
## SRS308874 SRS308874           SRR479062
## SRS308875 SRS308875 SRR479063,SRR479064
## SRS308876 SRS308876           SRR479065
```

We can confirm that the counts for the new object are equal to the summed up counts of the columns that had the same value for the grouping factor:

```
original <- rowSums( counts(ddsFull)[ , ddsFull$sample == "SRS308873" ] )
all( original == counts(ddsCollapsed)[ ,"SRS308873" ] )
```

```
## [1] TRUE
```

Running the DESeq2 pipeline

Here we will analyze a subset of the samples, namely those taken after 48 hours, with either control, DPN or OHT treatment, taking into account the multifactor design.

Preparing the data object for the analysis of interest

First we subset the relevant columns from the full dataset:

```
dds <- ddsCollapsed[ , ddsCollapsed$time == "48h" ]
```

Sometimes it is necessary to drop levels of the factors, in case that all the samples for one or more levels of a factor in the design have been removed. If time were included in the design formula, the following code could be used to take care of dropped levels in this column.

```
dds$time <- droplevels( dds$time )
```

It will be convenient to make sure that **Control** is the **first** level in the treatment factor, so that the default log2 fold changes are calculated as treatment over control and not the other way around. The function **relevel** achieves this:

```
dds$treatment <- relevel( dds$treatment, "Control" )
```

A quick check whether we now have the right samples:

```
as.data.frame( colData(dds) )
```

```
##                         run  experiment  patient  treatment  time  submission      study
sample       runsCollapsed
##  SRS308866  SRR479053    SRX140504           1     Control    48h    SRA051611   SRP012167
SRS308866             SRR479053
##  SRS308868  SRR479055    SRX140506          1          DPN    48h    SRA051611   SRP012167
SRS308868             SRR479055
##  SRS308870  SRR479057    SRX140508          1          OHT    48h    SRA051611   SRP012167
SRS308870             SRR479057
##  SRS308872  SRR479059    SRX140510           2     Control    48h    SRA051611   SRP012167
SRS308872             SRR479059
##  SRS308874  SRR479062    SRX140512          2          DPN    48h    SRA051611   SRP012167
SRS308874             SRR479062
##  SRS308876  SRR479065    SRX140514          2          OHT    48h    SRA051611   SRP012167
SRS308876             SRR479065
##  SRS308878  SRR479067    SRX140516           3     Control    48h    SRA051611   SRP012167
SRS308878             SRR479067
##  SRS308880  SRR479069    SRX140518          3          DPN    48h    SRA051611   SRP012167
SRS308880             SRR479069
##  SRS308882  SRR479071    SRX140520          3          OHT    48h    SRA051611   SRP012167
SRS308882             SRR479071
##  SRS308883  SRR479072    SRX140521           4     Control    48h    SRA051611   SRP012167
SRS308883             SRR479072
##  SRS308885  SRR479074    SRX140523          4          DPN    48h    SRA051611   SRP012167
SRS308885 SRR479074,SRR479075
##  SRS308887  SRR479077    SRX140525          4          OHT    48h    SRA051611   SRP012167
SRS308887 SRR479077,SRR479078
```

In order to speed up some annotation steps below, it makes sense to remove genes which have zero counts for all samples. This can be done by simply indexing the **dds** object:

```
dds <- dds[ rowSums( counts(dds) ) > 0 , ]
```

Running the pipeline

Let’s recall what design we have specified:

```
#design
design(dds)
```

```
## ~patient + treatment
```

```
#Run DESeq : Modeling counts with patient and treatment effects
dds <- DESeq(dds)
```

✓ This function will print out a message for the various steps it performs. Briefly these are: the estimation of size factors (which control for differences in the library size of the sequencing experiments), the estimation of dispersion for each gene, and fitting a generalized linear model.

A **DESeqDataSet** is returned which contains all the fitted information within it, and the following section describes how to extract out results tables of interest from this object.

Inspecting the results table

Calling **results** without any arguments will extract the estimated log2 fold changes and **p** values for the last variable in the design formula. If there are more than 2 levels for this variable - as is the case in this analysis - **results** will extract the results table for a comparison of the last level over the first level. The following section describes how to extract other comparisons.

```
res <- results( dds )
res
```

```
## log2 fold change (MAP): treatment OHT vs Control
## Wald test p-value: treatment OHT vs Control
## DataFrame with 32082 rows and 6 columns
##                  baseMean log2FoldChange     lfcSE      stat    pvalue      padj
##                 <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003  613.8196      -0.044799   0.08787  -0.50983  0.610173    0.9837
## ENSG00000000005    0.5502      -0.568326   1.08754  -0.52258  0.601268        NA
## ENSG00000000419  304.0482       0.116118   0.09623   1.20668  0.227555        NA
## ENSG00000000457  183.5157       0.007441   0.12314   0.06042  0.951819        NA
## ENSG00000000460  207.4336       0.470836   0.14493   3.24867  0.001159        NA
## ...                   ...            ...       ...       ...       ...       ...
## ENSG00000271699   0.17125       -1.09753    0.9097  -1.20646   0.22764        NA
## ENSG00000271704   0.10233       -0.01414    0.7157  -0.01975   0.98424        NA
## ENSG00000271707   9.21112       -0.84445    0.4832  -1.74774   0.08051        NA
## ENSG00000271709   0.08257       -0.01108    0.6782  -0.01633   0.98697        NA
## ENSG00000271711   0.74835       -0.95188    1.0655  -0.89335   0.37167        NA
```

As **res** is a **DataFrame** object, it carries metadata with information on the meaning of the columns:

```
mcols(res, use.names=TRUE)
```

```
## DataFrame with 6 rows and 2 columns
##                        type                                  description
##                 <character>                                  <character>
## baseMean       intermediate                   the base mean over all rows
## log2FoldChange      results log2 fold change (MAP): treatment OHT vs Control
## lfcSE               results         standard error: treatment OHT vs Control
## stat                results         Wald statistic: treatment OHT vs Control
## pvalue              results      Wald test p-value: treatment OHT vs Control
## padj                results                         BH adjusted p-values
```

The first column, **baseMean**, is a just the average of the normalized count values, dividing by size factors, taken over all samples. The remaining four columns refer to a specific **contrast**, namely the comparison of the levels **DPN** versus **Control** of the factor variable **treatment**.

See the help page for **results** (by typing `?results`) for information on how to obtain other contrasts.

The column **log2FoldChange** is the effect size estimate. It tells us how much the gene' s expression seems to have changed due to treatment with DPN in comparison to control. This value is reported on a logarithmic scale to base 2: for example, a log2 fold change of 1.5 means that the gene's expression is increased by a multiplicative factor of $2^{1.5} \approx 2.82$.

Of course, this estimate has an uncertainty associated with it, which is available in the column **lfcSE**, the standard error estimate for the log2 fold change estimate. The column *p* value indicates wether the observed difference between treatment and control is significantly different.

We note that a subset of the *p* values in **res** are **NA** ("notavailable"). This is **DESeq**'s way of reporting that all counts for this gene were zero, and hence not test was applied. In addition, *p* values can be assigned **NA** if the gene was excluded from analysis because it contained an extreme count outlier. For more information, see the outlier detection section of the advanced vignette.

We can examine the counts and normalized counts for the gene with the smallest p value:

```
# SmallestPvalue
idx <- which.min(res$pvalue)
counts(dds)[idx, ]
```

```
##  SRS308866  SRS308868  SRS308870  SRS308872  SRS308874  SRS308876  SRS308878  SRS308880 
SRS308882 SRS308883 SRS308885 SRS308887 
##         95         62         30         24         13         21        276        357 
112       388       404       296 
```

```
#Normalization
counts(dds, normalized=TRUE)[ idx, ]
```

```
##  SRS308866  SRS308868  SRS308870  SRS308872  SRS308874  SRS308876  SRS308878  SRS308880 
SRS308882 SRS308883 SRS308885 SRS308887 
##      86.81      61.43      37.42      35.25      15.96      25.85     314.96     201.94 
136.92    461.46    269.09    172.00 
```

Other comparisons

The results for a comparison of any two levels of a variable can be extracted using the **contrast** argument to **results**. The user should specify three values: The name of the variable, the name of the level in the numerator, and the name of the level in the denominator.

Here we extract results for the log2 of the fold change of DPN/Control:

```
#Save result table
resOHT <- res
#Other comparisons
res <- results( dds, contrast = c("treatment", "DPN", "Control") )
res
```

```
## log2 fold change (MAP): treatment DPN vs Control
## Wald test p-value: treatment DPN vs Control
## DataFrame with 32082 rows and 6 columns
##                  baseMean log2FoldChange     lfcSE      stat    pvalue      padj
##                 <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003  613.8196       -0.01722   0.08665  -0.19868   0.84252    0.9775
## ENSG00000000005    0.5502       -0.10344   1.09363  -0.09458   0.92465        NA
## ENSG00000000419  304.0482       -0.01695   0.09517  -0.17807   0.85867    0.9811
## ENSG00000000457  183.5157       -0.09654   0.12138  -0.79533   0.42642    0.8945
## ENSG00000000460  207.4336        0.35004   0.14375   2.43497   0.01489    0.2762
## ...                   ...            ...       ...       ...       ...       ...
## ENSG00000271699   0.17125        -1.1391    0.9251   -1.2313    0.2182        NA
## ENSG00000271704   0.10233         0.5925    0.7547    0.7851    0.4324        NA
## ENSG00000271707   9.21112        -0.5099    0.4579   -1.1136    0.2655        NA
## ENSG00000271709   0.08257         0.5156    0.7112    0.7250    0.4685        NA
## ENSG00000271711   0.74835        -0.6649    1.0511   -0.6326    0.5270        NA
```

Adding gene names

Our result table only uses Ensembl gene IDs, but gene names may be more informative. Bioconductor ’s annotation packages help with mapping various ID schemes to each other .

We load the annotation package **org.Hs.eg.db**:

```
library( "org.Hs.eg.db" )
```

This is the organism annotation package (“org”“) for **Homo sapiens** (”Hs“”), organized as an **AnnotationDbi** package (“db”“), using Entrez Gene IDs (”eg“) as primary key.

To get a list of all available key types, use

```
columns(org.Hs.eg.db)
```

```
##   [1] "ENTREZID"       "PFAM"           "IPI"            "PROSITE"        "ACCNUM"
"ALIAS"        "CHR"
##   [8] "CHRLOC"         "CHRLOCEND"      "ENZYME"         "MAP"            "PATH"
"PMID"         "REFSEQ"
## [15] "SYMBOL"         "UNIGENE"        "ENSEMBL"        "ENSEMBLPROT"    "ENSEMBLTRANS"
"GENENAME"     "UNIPROT"
## [22] "GO"             "EVIDENCE"       "ONTOLOGY"       "GOALL"          "EVIDENCEALL"
"ONTOLOGYALL"  "OMIM"
## [29] "UCSCKG"
```

Converting IDs with the native functions from the **AnnotationDbi** package is currently a bit cumbersome, so we provide the following convenience function (without explaining how exactly it works):

```
#ids = list of IDS
#fromKey = key type; toKey = key type we want to convert to
#db = the AnnotationDb object to use.
#ifMultiple = the argument specifies what to do if one source ID maps to several target IDs:
  #should the function return an NA or simply the first of the multiple IDs?
convertIDs <- function( ids, fromKey, toKey, db, ifMultiple=c( "putNA", "useFirst" ) ) {
   stopifnot( inherits( db, "AnnotationDb" ) )
   ifMultiple <- match.arg( ifMultiple )
   suppressWarnings( selRes <- AnnotationDbi::select(
      db, keys=ids, keytype=fromKey, columns=c(fromKey,toKey) ) )
   if( ifMultiple == "putNA" ) {
      duplicatedIds <- selRes[ duplicated( selRes[,1] ), 1 ]
      selRes <- selRes[ ! selRes[,1] %in% duplicatedIds, ] }
   return( selRes[ match( ids, selRes[,1] ), 2 ] )
}
```

To convert the Ensembl IDs in the rownames of **res** to gene symbols and add them as a new column, we use:

```
res$hgnc_symbol <- convertIDs( row.names(res), "ENSEMBL", "SYMBOL", org.Hs.eg.db )
res$entrezid <- convertIDs( row.names(res), "ENSEMBL", "ENTREZID", org.Hs.eg.db )
head(res, 4)
```

```
## log2 fold change (MAP): treatment DPN vs Control
## Wald test p-value: treatment DPN vs Control
## DataFrame with 4 rows and 8 columns
##                  baseMean  log2FoldChange     lfcSE       stat     pvalue       padj
hgnc_symbol    entrezid
##                 <numeric>       <numeric> <numeric>  <numeric>  <numeric>  <numeric>
<character> <character>
## ENSG00000000003   613.8196        -0.01722   0.08665   -0.19868     0.8425     0.9775
TSPAN6         7105
## ENSG00000000005     0.5502        -0.10344   1.09363   -0.09458     0.9246         NA
TNMD        64102
## ENSG00000000419   304.0482        -0.01695   0.09517   -0.17807     0.8587     0.9811
DPM1           8813
## ENSG00000000457   183.5157        -0.09654   0.12138   -0.79533     0.4264     0.8945
SCYL3         57147
```

✓ This conversion can be done from Ensembl BioMart web site.

Further points

Multiple testing

DESeq2 uses the so-called Benjamini-Hochberg (BH) adjustment for multiple testing problem; in brief, this method calculates for each gene an **adjusted *p* value** which answers the following question: if one called significant all genes with a *p* value less than or equal to this gene's *p* value threshold, what would be the fraction of false positives (the **false discovery rate**, FDR) among them (in the sense of the calculation outlined above)? These values, called the BH-adjusted *p* values, are given in the column **padj** of the **results** object.

Hence, if we consider a fraction of 10% false positives acceptable, we can consider all genes with an **adjusted** *p* value below 10%=0.1 as significant. How many such genes are there?

```
sum( res$padj < 0.1, na.rm=TRUE )
```

```
## [1] 249
```

We subset the results table to these genes and then sort it by the log2 fold change estimate to get the significant genes with the strongest down-regulation:

```
resSig <- subset(res, res$padj < 0.1 )
head( resSig[ order( resSig$log2FoldChange ), ], 4)
```

```
## log2 fold change (MAP): treatment DPN vs Control
## Wald test p-value: treatment DPN vs Control
## DataFrame with 4 rows and 8 columns
##                    baseMean  log2FoldChange      lfcSE       stat     pvalue       padj
hgnc_symbol    entrezid
##                  <numeric>       <numeric> <numeric> <numeric> <numeric> <numeric>
<character> <character>
## ENSG00000163631      233.3         -0.9307    0.2842     -3.275  1.058e-03    0.05817
ALB           213
## ENSG00000119946      151.6         -0.6903    0.1566     -4.408  1.041e-05    0.00246
CNNM1         26507
## ENSG000000419 82    1377.0         -0.6859    0.1845     -3.717  2.018e-04    0.02045
TNC          3371
## ENSG00000155111      530.9         -0.6756    0.2109     -3.203  1.359e-03    0.06850
CDK19         23097
```

and with the strongest upregulation :

```
head( resSig[ order( -resSig$log2FoldChange ), ], 4)
```

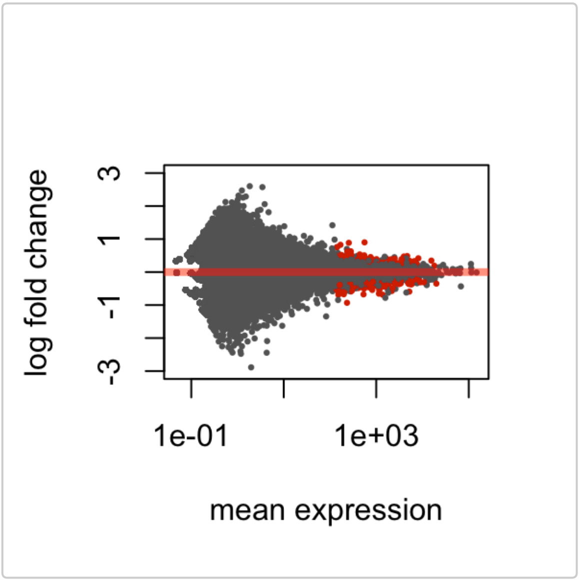
```
## log2 fold change (MAP): treatment DPN vs Control
## Wald test p-value: treatment DPN vs Control
## DataFrame with 4 rows and 8 columns
##                   baseMean  log2FoldChange      lfcSE       stat     pvalue       padj
hgnc_symbol    entrezid
##                  <numeric>       <numeric>  <numeric>  <numeric>  <numeric>  <numeric>
<character> <character>
## ENSG00000092621      558.5          0.9002     0.1258      7.158  8.191e-13  2.628e-09
PHGDH          26227
## ENSG00000101255      255.1          0.8860     0.1694      5.229  1.706e-07  2.346e-04
TRIB3          57761
## ENSG00000103257      168.5          0.8259     0.1644      5.024  5.064e-07  3.249e-04
SLC7A5          8140
## ENSG00000156414      142.8          0.7582     0.1661      4.564  5.025e-06  1.547e-03
TDRD9         122402
```

Diagnostic plot

MA plot

A so-called MA plot provides a useful overview for an experiment with a two-group comparison:

```
plotMA( res, ylim = c(-3, 3) )
```



The MA-plot represents each gene with a dot. The *x* axis is the average expression over all samples, the *y* axis the log2 fold change of normalized counts (i.e the average of counts normalized by size factor) between treatment and control. Genes with an adjusted *p* value below a threshold (here 0.1, the default) are shown in red.

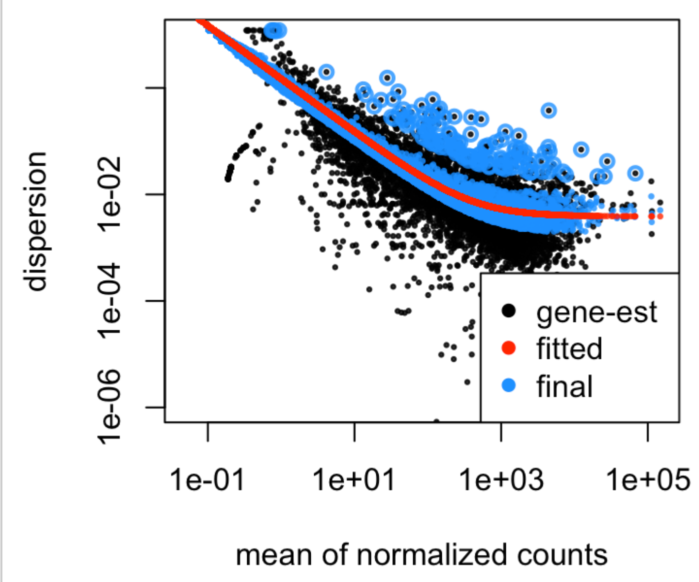
✓ This plot demonstrates that only genes with a large average normalized count contain sufficient information to yield a significant call.

Dispersion plot

Also note DESeq2 shrinkage estimation of log fold changes (LFCs): When count values are too low to allow an accurate estimate of the LFC, the value is “shrunken”" towards zero to avoid that these values, which otherwise would frequently be unrealistically large, dominate the top-ranked log fold change. Whether a gene is called significant depends not only on its LFC but also on its within-group variability, which DESeq2 quantifies as the **dispersion**. For strongly expressed genes, the dispersion can be understood as a squared coefficient of variation: a dispersion value of 0.01 means that the gene’s expression tends to differ by typically $\sqrt{0.01}=10\%$ between samples of the same treatment group. For weak genes, the Poisson noise is an additional source of noise, which is added to the dispersion.

The function **plotDispEsts** visualizes DESeq2’s dispersion estimates:

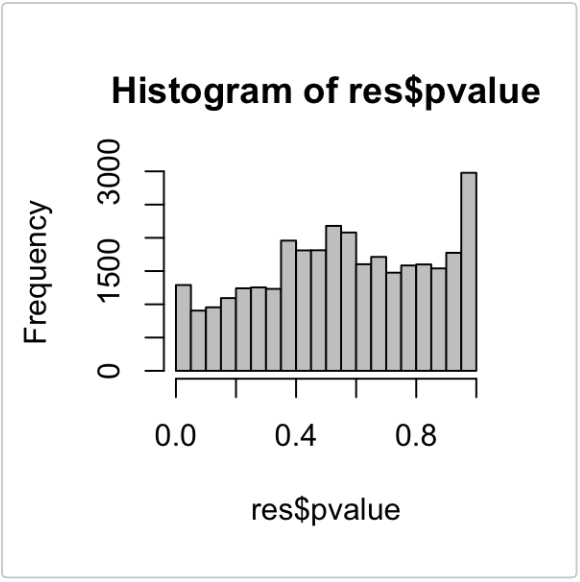
```
plotDispEsts( dds, ylim = c(1e-6, 1e1) )
```



The black points are the dispersion estimates for each gene as obtained by considering the information from each gene separately. Unless one has many samples, these values fluctuate strongly around their true values. Therefore, we fit the red trend line, which shows the dispersions’ dependence on the mean, and then shrink each gene’s estimate towards the red line to obtain the final estimates (blue points) that are then used in the hypothesis test. The blue circles above the main “cloud”" of points are genes which have high gene-wise dispersion estimates which are labelled as dispersion outliers. These estimates are therefore not shrunk toward the fitted trend line.

Histogram of the p-value

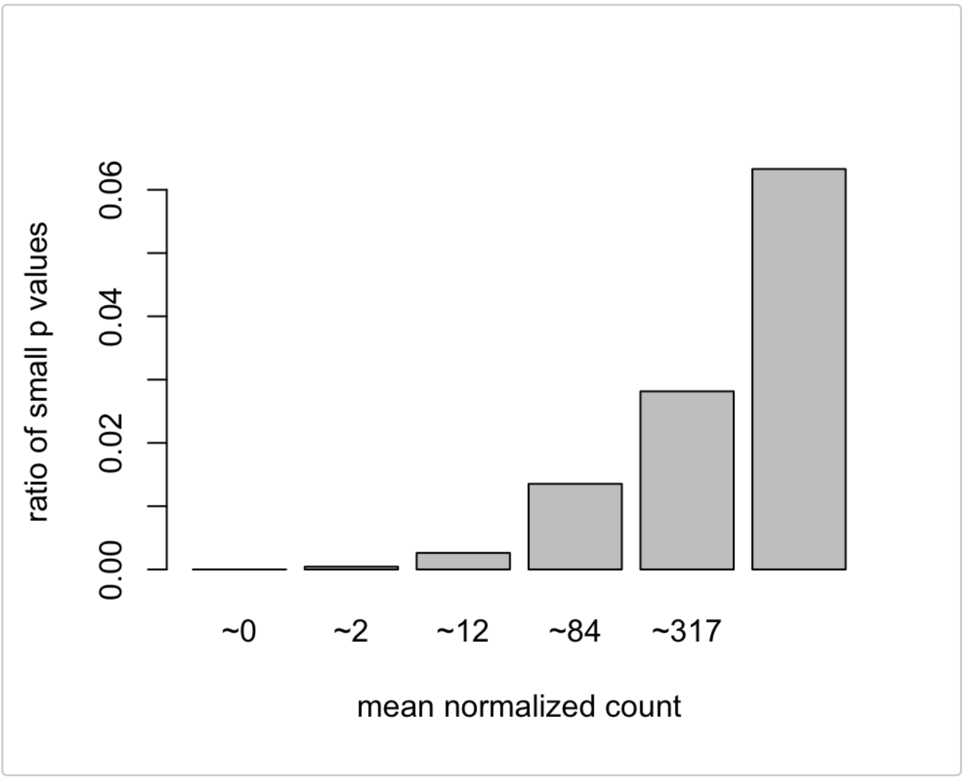
```
hist( res$pvalue, breaks=20, col="grey" )
```



Independent filtering

The MA plot highlights an important property of RNA-Seq data. **For weakly expressed genes, we have no chance of seeing differential expression**, because the low read counts suffer from so high Poisson noise that any biological effect is drowned in the uncertainties from the read counting. We can also show this by examining the ratio of small *p* values (say, less than, 0.01) for genes binned by mean normalized count:

```
# create bins using the quantile function
qs <- c( 0, quantile( res$baseMean[res$baseMean > 0], 0:7/7 ) )
# "cut" the genes into the bins
bins <- cut( res$baseMean, qs )
# rename the levels of the bins using the middle point
levels(bins) <- paste0("~",round(.5*qs[-1] + .5*qs[-length(qs)]))
# calculate the ratio of p values less than .01 for each bin
ratios <- tapply( res$pvalue, bins, function(p) mean( p < .01, na.rm=TRUE ) )
# plot these ratios
barplot(ratios, xlab="mean normalized count", ylab="ratio of small p values")
```



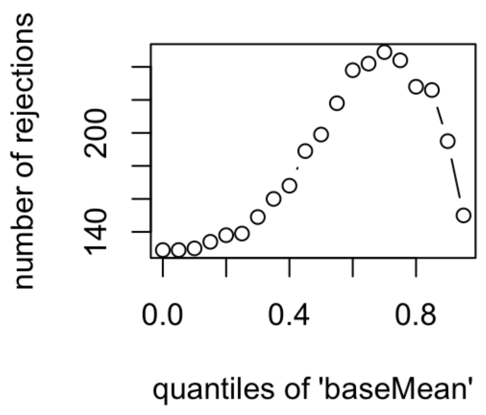
At first sight, there may seem to be little benefit in filtering out these genes. After all, the test found them to be non-significant anyway . However, these genes have an influence on the multiple testing adjustment, whose performance improves if such genes are removed. By removing the weakly-expressed genes from the input to the FDR procedure, we can find more genes to be significant among those which we keep, and so improved the power of our test. This approach is known as **independent filtering**.

The DESeq software automatically performs independent filtering which maximizes the number of genes which will have adjusted *p* value less than a critical value (by default, **alpha** is set to 0.1). This automatic independent filtering is performed by, and can be controlled by, the **results** function. We can observe how the number of rejections changes for various cutoffs based on mean normalized count. The following optimal threshold and table of possible values is stored as an **attribute** of the results object.

```
attr(res,"filterThreshold")
```

```
##   70%
## 126.9
```

```
plot(attr(res,"filterNumRej"),type="b",
     xlab="quantiles of 'baseMean'",
     ylab="number of rejections")
```



The term **independent** highlights an important caveat. Such filtering is permissible only if the filter criterion is independent of the actual test statistic. Otherwise, the filtering would invalidate the test and consequently the assumptions of the BH procedure. This is why we filtered on the average over **all** samples: this filter is blind to the assignment of samples to the treatment and control group and hence independent.

Exporting results

You can easily save the results table in a CSV file, which you can then load with a spreadsheet program such as Excel:

```
res[1:2,]
write.csv( as.data.frame(res), file="results.csv" )
```

Gene-set enrichment analysis

Do the genes with a strong up- or down-regulation have something in common? We perform next a gene-set enrichment analysis (GSEA) to examine this question.

We here present a relatively simplistic approach, to demonstrate the basic ideas, but note that a more careful treatment will be needed for more definitive results.

We use the gene sets in the R eactome database:

```
#source("http://bioconductor.org/biocLite.R")
#biocLite("reactome.db")
library( "reactome.db" )
```

This database works with Entrez IDs, so we will need the **entrezid** column that we added earlier to the **res** object.

First, we subset the results table, **res**, to only those genes for which the Reactome database has data (i.e, whose Entrez ID we find in the respective key column of **reactome.db** and for which the DESeq2 test gave an adjusted *p* value that was not **NA**.

```
res2 <- res[ res$entrezid %in% keys( reactome.db, "ENTREZID" ) & !is.na( res$padj ) , ]
head(res2)
```

```
## log2 fold change (MAP): treatment DPN vs Control
## Wald test p-value: treatment DPN vs Control
## DataFrame with 6 rows and 8 columns
##                  baseMean  log2FoldChange      lfcSE       stat     pvalue       padj
hgnc_symbol     entrezid
##                 <numeric>       <numeric> <numeric>  <numeric> <numeric>  <numeric>
<character> <character>
## ENSG000000004 19      304.0       -0.016946    0.09517   -0.17807     0.8587     0.9811
DPM1          8813
## ENSG00000001084      312.0        0.039811    0.10228    0.38923     0.6971     0.9540
GCLC          2729
## ENSG000000011 67      398.8       -0.056003    0.09711   -0.57669     0.5641     0.9357
NFYA          4800
## ENSG00000001630      463.5        0.070002    0.09676    0.72348     0.4694     0.9123
CYP51A1          1595
## ENSG000000028 22      169.5       -0.001926    0.13784   -0.01397     0.9889     0.9982
MAD1L1          8379
## ENSG00000003056      995.8        0.007716    0.06785    0.11373     0.9095     0.9859
M6PR          4074
```

Using **select**, a function from **AnnotationDbi** for querying database objects, we get a table with the mapping from Entrez IDs to Reactome Path IDs :

```
reactomeTable <- AnnotationDbi::select( reactome.db,
   keys=as.character(res2$entrezid), keytype="ENTREZID",
   columns=c("ENTREZID","REACTOMEID") )
head(reactomeTable)
```

```
##   ENTREZID REACTOMEID
## 1     8813     162699
## 2     8813     163125
## 3     8813     392499
## 4     8813     446193
## 5     8813     446203
## 6     8813     446219
```

The next code chunk transforms this table into an **incidence matrix**. This is a Boolean matrix with one row for each Reactome Path and one column for each unique gene in **res2**, which tells us which genes are members of which Reactome Paths.

```
#problem with this code is that res2$entrez are not unique
#ft2mat <- function(x, y) {
  # ux = unique(x)
  # uy = unique(y)
  # im = matrix(FALSE, nrow=length(ux), ncol=length(uy), dimnames=list(ux, uy))
  # im[ cbind(x, y) ] = TRUE
  # return(im)
 #}
#incm <- with(reactomeTable, ft2mat(REACTOMEID, ENTREZID))
```

```
incm <- do.call( rbind, with(reactomeTable, tapply(
  ENTREZID, factor(REACTOMEID), function(x) res2$entrezid %in% x ) ))
colnames(incm) <- res2$entrez
str(incm)
```

```
##  logi [1:1458, 1:3400] FALSE FALSE FALSE FALSE FALSE FALSE ...
##  - attr(*, "dimnames")=List of 2
##   ..$ : chr [1:1458] "1059683" "109581" "109582" "109606" ...
##   ..$ : chr [1:3400] "8813" "2729" "4800" "1595" ...
```

We remove all rows corresponding to Reactome Paths with less than 20 or more than 80 assigned genes.

```
within <- function(x, lower, upper) (x>=lower & x<=upper)
incm <- incm[ within(rowSums(incm), lower=20, upper=80), ]
```

To test whether the genes in a Reactome Path behave in a special way in our experiment, we calculate a number of statistics, including a *t*-statistic to see whether the average of the genes' log2 fold change values in the gene set is different from zero. To facilitate the computations, we define a little helper function:

```
testCategory <- function( reactomeID ) {
  isMember <- incm[ reactomeID, ]
  data.frame(
     reactomeID  = reactomeID,
     numGenes    = sum( isMember ),
     avgLFC      = mean( res2$log2FoldChange[isMember] ),
     sdLFC       = sd( res2$log2FoldChange[isMember] ),
      zValue       = mean( res2$log2FoldChange[isMember] ) /sd( res2$log2FoldChange[isMember]
),
     strength    = sum( res2$log2FoldChange[isMember] ) / sqrt(sum(isMember)),
     pvalue      = t.test( res2$log2FoldChange[ isMember ] )$p.value,
     reactomeName = reactomePATHID2NAME[[reactomeID]],
     stringsAsFactors = FALSE ) }
```

The function can be called with a Reactome Path ID:

```
testCategory("109606")
```

```
##       reactomeID   numGenes        avgLFC        sdLFC     zValue   strength   pvalue
reactomeName
## 1       109606          31  -0.02215  0.07769  -0.2852   -0.1234  0.1228  Homo  sapiens:
Intrinsic Pathway for Apoptosis
```

As you can see the function not only performs the *t* test and returns the p value but also lists other useful information such as the number of genes in the category , the average log fold change, a “strength”" measure (see below) and the name with which R eactome describes the Path.

We call the function for all Paths in our incidence matrix and collect the results in a data frame:

```
reactomeResult <- do.call( rbind, lapply( rownames(incm), testCategory ) )
```

As we performed many tests, we should again use a multiple testing adjustment.

```
reactomeResult$padjust <- p.adjust( reactomeResult$pvalue, "BH" )
```

This is a list of Reactome Paths which are significantly differentially expressed in our comparison of DPN treatment with control, sorted according to sign and strength of the signal:

```
reactomeResultSignif <- reactomeResult[ reactomeResult$padjust < 0.05, ]
head( reactomeResultSignif[ order(-reactomeResultSignif$strength), ] )
```

```
##     reactomeID numGenes   avgLFC   sdLFC  zValue strength    pvalue
## 174     191273       21  0.10808 0.09894  1.0923   0.4953 6.786e-05
## 308     381070       41  0.07055 0.13898  0.5076   0.4517 2.341e-03
## 60     1638091       30  0.06877 0.10742  0.6402   0.3767 1.499e-03
## 150    1799339       75  0.04083 0.07791  0.5240   0.3536 2.153e-05
## 405      72689       64  0.02905 0.07284  0.3989   0.2324 2.213e-03
## 23     1169091       53 -0.02579 0.06195 -0.4162  -0.1877 3.804e-03
##                                                                    reactomeName
padjust
## 174                                     Homo  sapiens:  Cholesterol  biosynthesis
0.007838
## 308                                Homo  sapiens:  IRE1alpha  activates  chaperones
0.041597
## 60                     Homo  sapiens:  Heparan  sulfate/heparin  (HS-GAG)  metabolism
0.032968
## 150 Homo  sapiens:  SRP-dependent  cotranslational  protein  targeting  to  membrane
0.003316
## 405                      Homo  sapiens:  Formation  of  a  pool  of  free  40S  subunits
```

```
0.041130
## 23                         Homo sapiens: Activation of NF-kappaB in B cells
0.048870
```

Working with rlog-transformed data

The rlog transform

Many common statistical methods for exploratory analysis of multidimensional data, especially methods for clustering (e.g., principal-component analysis and the like), work best for (at least approximately) homoskedastic data; this means that the variance of an observable quantity (i.e., here, the expression strength of a gene) does not depend on the mean. In RNA-Seq data, however, variance grows with the mean. For example, if one performs PCA directly on a matrix of normalized read counts, the result typically depends only on the few most strongly expressed genes because they show the largest absolute differences between samples. A simple and often used strategy to avoid this is to take the logarithm of the normalized count values plus a small pseudocount; however, now the genes with low counts tend to dominate the results because, due to the strong Poisson noise inherent to small count values, they show the strongest relative differences between samples.

As a solution, **DESeq2** offers the **regularized-logarithm transformation**, or **rlog** for short. For genes with high counts, the rlog transformation differs not much from an ordinary log2 transformation. For genes with lower counts, however, the values are shrunken towards the genes' averages across all samples. Using an empirical Bayesian prior in the form of a **ridge penalty**, this is done such that the rlog-transformed data are approximately homoskedastic.

✓ Note that the rlog transformation is provided for applications other than differential testing. For differential testing we recommend the DESeq function applied to raw counts, as described earlier in this vignette, which also takes into account the dependence of the variance of counts on the mean value during the dispersion estimation step.

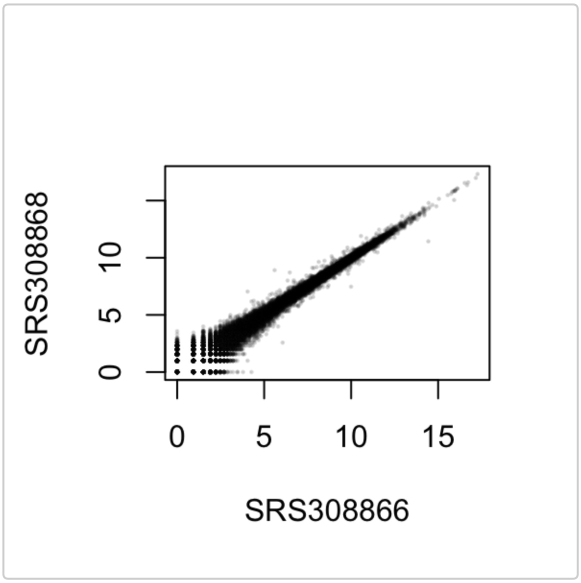
The function **rlog** returns a **SummarizedExperiment** object which contains the rlog-transformed values in its **assay** slot:

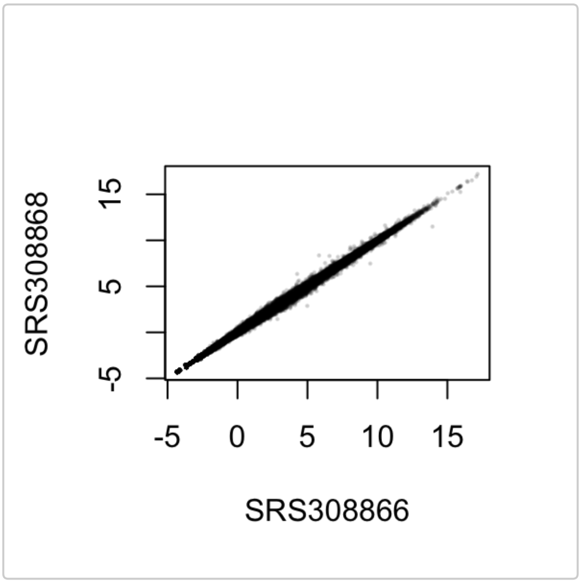
```
rld <- rlog( dds )
assay(rld)[ 1:3, 1:3]
```

```
##                  SRS308866 SRS308868 SRS308870
## ENSG00000000003    9.7614    9.7285    9.8653
## ENSG00000000005   -0.9813   -0.7408   -0.9447
## ENSG00000000419    8.0647    8.0770    8.1186
```

To show the effect of the transformation, we plot the first sample against the second, first simply using the **log2** function (after adding 1, to avoid taking the log of zero), and then using the rlog-transformed values.

```
plot( log2( 1+counts(dds, normalized=TRUE)[, 1:2] ), col="#00000020", pch=20, cex=0.3 )
plot( assay(rld)[, 1:2], col="#00000020", pch=20, cex=0.3 )
```





In Figure , we can see how genes with low counts seem to be excessively variable on the ordinary logarithmic scale, while the rlog transform compresses differences for genes for which the data cannot provide good information anyway.

Sample distances

A useful first step in an RNA-Seq analysis is often to assess overall similarity between samples.

We use the R function `dist` to calculate the Euclidean distance between samples. To avoid that the distance measure is dominated by a few highly variable genes, and have a roughly equal contribution from all genes, we use it on the rlog-transformed data:

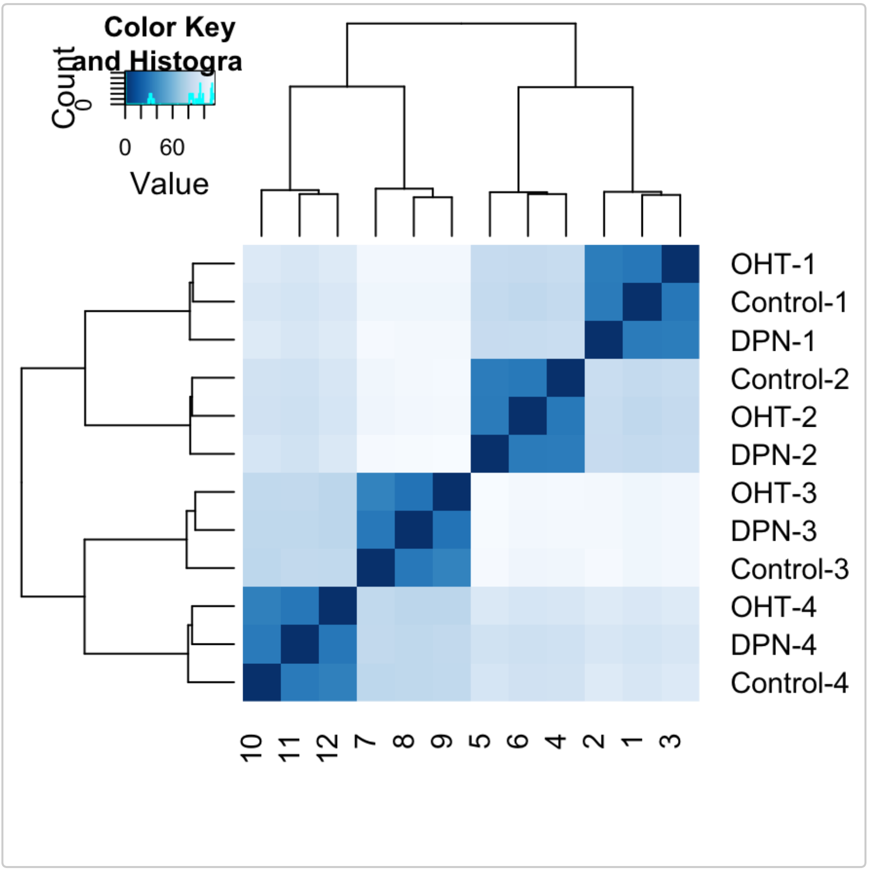
```
sampleDists <- dist( t( assay(rld) ) )
as.matrix( sampleDists )[ 1:3, 1:3 ]
```

```
##           SRS308866 SRS308868 SRS308870
## SRS308866      0.00     32.87     31.21
## SRS308868     32.87      0.00     33.73
## SRS308870     31.21     33.73      0.00
```

✓ Note the use of the function `t` to transpose the data matrix. We need this because `dist` calculates distances between data **rows** and our samples constitute the columns.

We visualize the distances in a heatmap, using the function `heatmap.2` from the **gplots** package.

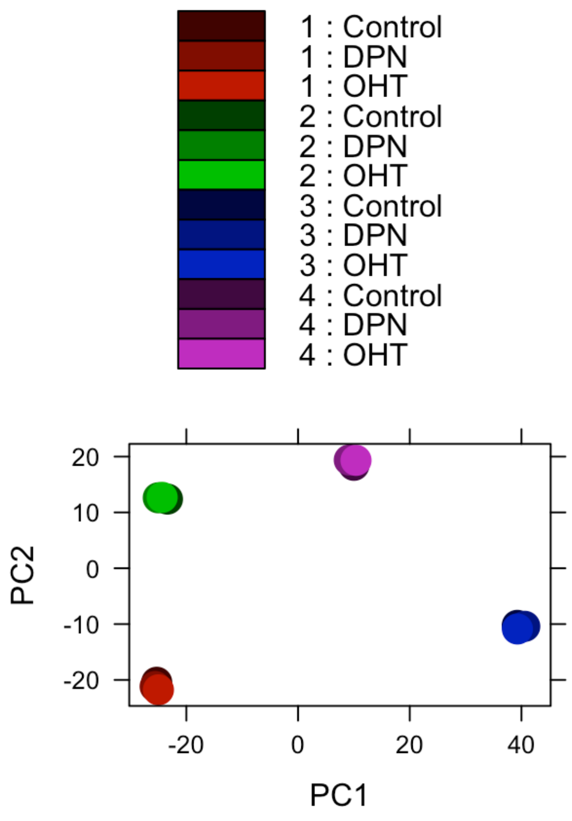
```
sampleDistMatrix <- as.matrix( sampleDists )
rownames(sampleDistMatrix) <- paste( rld$treatment,
   rld$patient, sep="-" )
colnames(sampleDistMatrix) <- NULL
library( "gplots" )
library( "RColorBrewer" )
colours = colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
heatmap.2( sampleDistMatrix, trace="none", col=colours)
```



✓ Note that we have changed the row names of the distance matrix to contain treatment type and patient number instead of sample ID, so that we have all this information in view when looking at the heatmap.

Another way to visualize sample-to-sample distances is a principal-components analysis (PCA). In this ordination method, the data points (i.e., here, the samples) are projected onto the 2D plane such that they spread out optimally.

```
colours <- c(rgb(1:3/4,0,0),rgb(0,1:3/4,0),rgb(0,0,1:3/4),rgb(1:3/4,0,1:3/4))
plotPCA( rld, intgroup = c("patient","treatment"), col=colours )
```



Here, we have used the function `plotPCA` which comes with **DESeq2**. The two terms specified as `intgroup` are column names from our sample data; they tell the function to use them to choose colours.

From both visualizations, we see that the differences between patients is much larger than the difference between treatment and control samples of the same patient. This shows why it was important to account for this paired design (``paired”, because each treated sample is paired with one control sample from the **same** patient).

We did so by using the design formula ~ `patient + treatment` when setting up the data object in the beginning. Had we used an un-paired analysis, by specifying only , we would not have found many hits, because then, the patient-to-patient differences would have drowned out any treatment effects.

Gene clustering

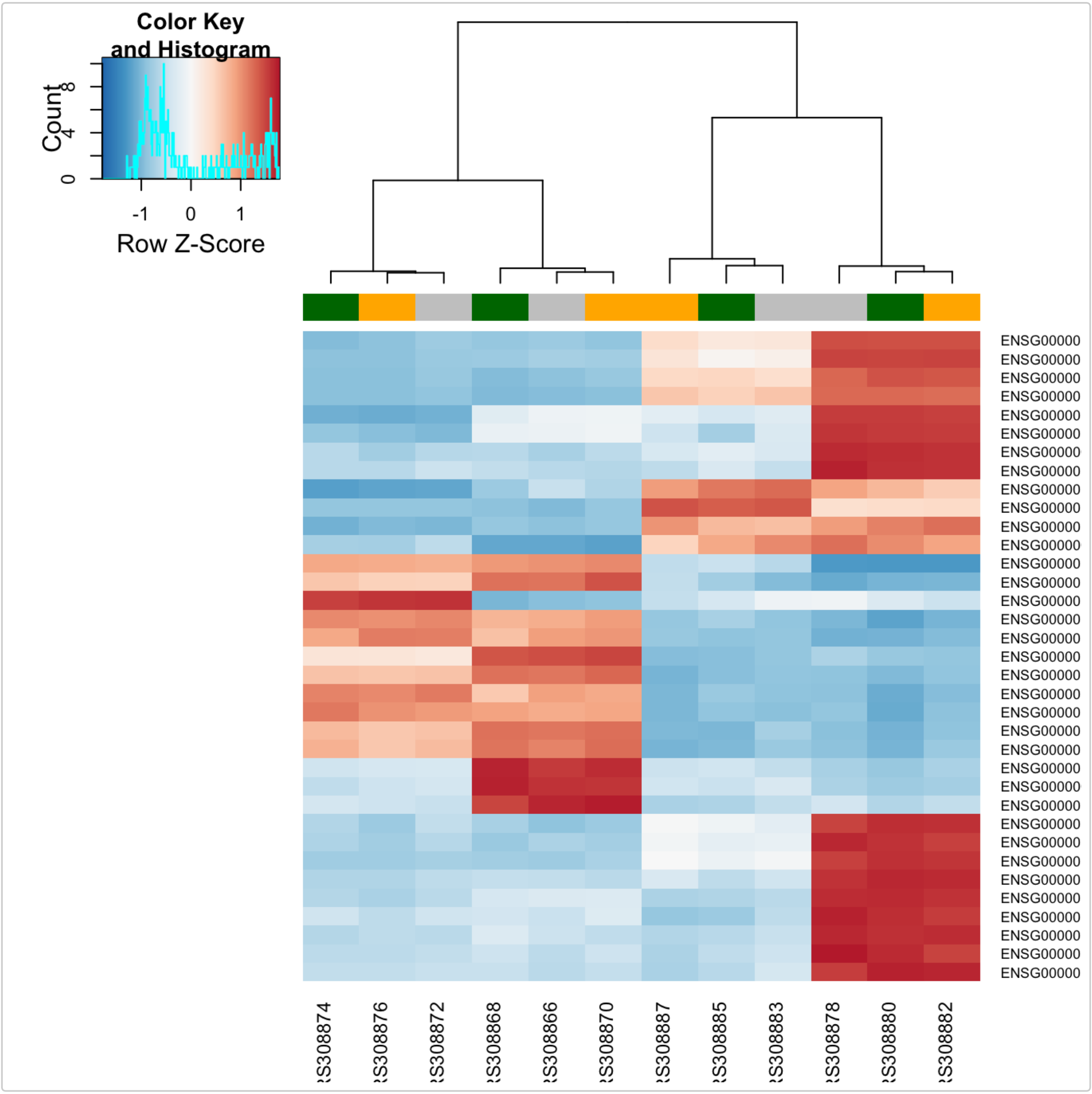
In the above heatmap, the dendrogram at the side shows us a hierarchical clustering of the samples. Such a clustering can also be performed for the genes.

Since the clustering is only relevant for genes that actually carry signal, one usually carries it out only for a subset of most highly variable genes. Here, for demonstration, let us select the 35 genes with the highest variance across samples:

```r
library( "genefilter" )
topVarGenes <- head( order( rowVars( assay(rld) ), decreasing=TRUE ), 35 )
```

The heatmap becomes more interesting if we do not look at absolute expression strength but rather at the amount by which each gene deviates in a specific sample from the gene’s average across all samples. Hence, we center and scale each genes’ values across samples, and plot a heatmap.

```
heatmap.2( assay(rld)[ topVarGenes, ], scale="row",
     trace="none", dendrogram="column",
     col = colorRampPalette( rev(brewer.pal(9, "RdBu")) )(255),
     ColSideColors = c( Control="gray", DPN="darkgreen", OHT="orange" )[
        colData(rld)$treatment ] )
```



We can now see on the heatmap, blocks of genes which covary across patients. Often, such a heatmap is insightful, even though here, seeing these variations across patients is of limited value because we are rather interested in the effects between the treatments from each patient.

Going further

Analyze more datasets: use the function defined in the following code chunk to download a processed count matrix from the ReCount website .

The following function takes a name of the dataset from the ReCount website, e.g. **“hammer”**, and returns a **SummarizedExperiment** object.

```
recount2SE <- function(name) {
  filename <- paste0(name,"_eset.RData")
  if (!file.exists(filename)) download.file(paste0(
    "http://bowtie-bio.sourceforge.net/recount/ExpressionSets/",
    filename),filename)
  load(filename)
  e <- get(paste0(name,".eset"))
  se <- SummarizedExperiment(SimpleList(counts=exprs(e)),
                             colData=DataFrame(pData(e)))
  se
}
```

See also

For a more in-depth explanation of the advanced details, we advise you to proceed to the vignette of the DESeq2 package package, Differential analysis of count data. For a treatment of exon-level differential expression, we refer to the vignette of the DEXSeq package, Analyzing RN-seq data for differential exon usage with the DEXSeq package.

`DEXSeq` for differential exon usage. See the accompanying vignette, **Analyzing RNA-seq data for differential exon usage with the DEXSeq package**, which is similar to the style of this tutorial.

- Other Bioconductor packages for RNA-Seq differential expression:

edgeR, **limma**, **DSS**, **BitSeq** (transcript level), **EBSeq**, **cummeRbund** (for importing and visualizing Cufflinks results), **monocle** (single-cell analysis). More at http://bioconductor.org/packages/release/BiocViews.html#___RNASeq

- Methods for gene set testing: **romer** and **roast** in **limma**, permutation based: **safe**
- Packages for normalizing for covariates (e.g., GC content): **cqn**, **EDASeq**
- Packages for detecting batches: **sva**, **RUVSeq**
- Generating HTML results tables with links to outside resources (gene descriptions): **ReportingTools**

Session Info

As last part of this document, we call the function , which reports the version numbers of R and all the packages used in this session. It is good practice to always keep such a record as it will help to trace down what has happened in case that an R script ceases to work because a package has been changed in a newer version.

R version 3.1.0 (2014-04-10) Platform: x86_64-apple-darwin13.1.0 (64-bit)

locale: [1] fr_FR.UTF-8/fr_FR.UTF-8/fr_FR.UTF-8/C/fr_FR.UTF-8/fr_FR.UTF-8 attached base packages: [1] parallel stats graphics grDevices utils datasets methods base

other attached packages: [1] genefilter_1.46.1 RColorBrewer_1.0-5 gplots_2.14.2 reactome.db_1.48.0
[5] org.Hs.eg.db_2.14.0 RSQLite_0.11.4 DBI_0.3.1 DESeq2_1.4.5
[9] RcppArmadillo_0.4.450.1.0 Rcpp_0.11.3 GenomicAlignments_1.0.6 BSgenome_1.32.0
[13] GenomicFeatures_1.16.2 AnnotationDbi_1.26.0 Biobase_2.24.0 Rsamtools_1.16.1
[17] Biostrings_2.32.1 XVector_0.4.0 parathyroidSE_1.2.0 GenomicRanges_1.16.4
[21] GenomeInfoDb_1.0.2 IRanges_1.22.10 BiocGenerics_0.10.0

loaded via a namespace (and not attached): [1] annotate_1.42.1 base64enc_0.1-2 BatchJobs_1.4 BBmisc_1.7 BiocParallel_0.6.1 biomaRt_2.20.0
[7] bitops_1.0-6 brew_1.0-6 caTools_1.17.1 checkmate_1.4 codetools_0.2-9 digest_0.6.4
[13] evaluate_0.5.5 fail_1.2 foreach_1.4.2 formatR_1.0 gdata_2.13.3 geneplotter_1.42.0 [19] grid_3.1.0 gtools_3.4.1 htmltools_0.2.6 iterators_1.0.7 KernSmooth_2.23-13 knitr_1.6
[25] lattice_0.20-29 locfit_1.5-9.1 RCurl_1.95-4.3 rmarkdown_0.3.3 rtracklayer_1.24.2 sendmailR_1.2-1
[31] splines_3.1.0 stats4_3.1.0 stringr_0.6.2 survival_2.37-7 tools_3.1.0 XML_3.98-1.1
[37] xtable_1.7-4 yaml_2.1.13 zlibbioc_1.10.0

References

- Michael Love, Simon Anders, Wolfgang Huber, RNA-Seq differential expression workfow : http://www.bioconductor.org/help/course-materials/2014/BioC2014/RNA-Seq-Analysis-Lab.pdf
- Courses: http://www.bioconductor.org/help/course-materials/2014/CSAMA2014/

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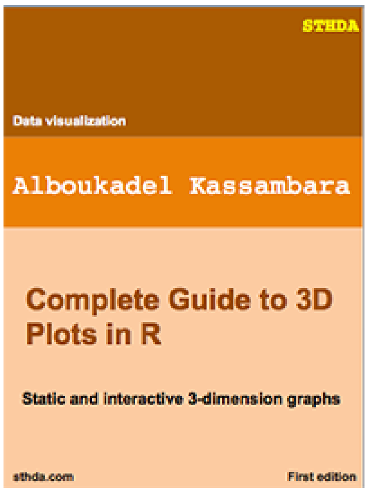
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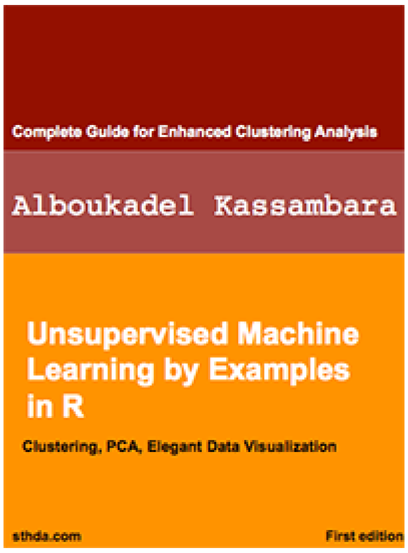
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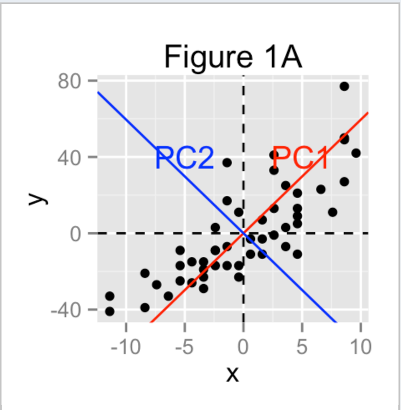

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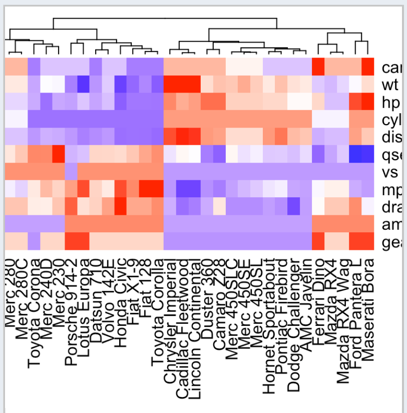

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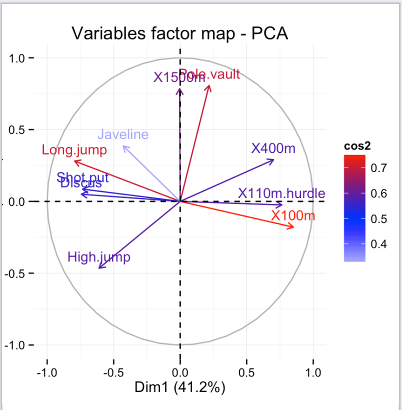

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