RNA-seq

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Transcriptomics and the analysis of RNA-Seq data

```
install.packages("BiocManager")   BiocManager::install()   BiocManager::install("DESeq2")
BiocManager::install("AnnotationDbi") BiocManager::install("org.Hs.eg.db")
```

```
library(BiocManager)
library(DESeq2)
```

```
## Loading required package: S4Vectors

## Loading required package: stats4

## Loading required package: BiocGenerics

##
## Attaching package: 'BiocGenerics'

## The following objects are masked from 'package:stats':
##
##     IQR, mad, sd, var, xtabs

## The following objects are masked from 'package:base':
##
##     anyDuplicated, append, as.data.frame, basename, cbind, colnames,
##     dirname, do.call, duplicated, eval, evalq, Filter, Find, get, grep,
##     grepl, intersect, is.unsorted, lapply, Map, mapply, match, mget,
##     order, paste, pmax, pmax.int, pmin, pmin.int, Position, rank,
##     rbind, Reduce, rownames, sapply, setdiff, sort, table, tapply,
##     union, unique, unsplit, which.max, which.min

##
## Attaching package: 'S4Vectors'

## The following objects are masked from 'package:base':
##
##     expand.grid, I, unname

## Loading required package: IRanges
```

```
## Loading required package: GenomicRanges

## Loading required package: GenomeInfoDb

## Loading required package: SummarizedExperiment

## Loading required package: MatrixGenerics

## Loading required package: matrixStats

##
## Attaching package: 'MatrixGenerics'

## The following objects are masked from 'package:matrixStats':
##
##     colAlls, colAnyNAs, colAnys, colAvgsPerRowSet, colCollapse,
##     colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
##     colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
##     colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
##     colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
##     colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
##     colWeightedMeans, colWeightedMedians, colWeightedSds,
##     colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAvgsPerColSet,
##     rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
##     rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
##     rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
##     rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
##     rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs, rowVars,
##     rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
##     rowWeightedSds, rowWeightedVars

## Loading required package: Biobase

## Welcome to Bioconductor
##
##     Vignettes contain introductory material; view with
##     'browseVignettes()'. To cite Bioconductor, see
##     'citation("Biobase")', and for packages 'citation("pkgname")'.

##
## Attaching package: 'Biobase'

## The following object is masked from 'package:MatrixGenerics':
##
##     rowMedians

## The following objects are masked from 'package:matrixStats':
##
##     anyMissing, rowMedians
```

```
counts <- read.csv("airway_scaledcounts.csv", row.names=1)
metadata <-  read.csv("airway_metadata.csv")
```

```
head(metadata)
```

```
##           id     dex celltype     geo_id
## 1 SRR1039508 control   N61311 GSM1275862
## 2 SRR1039509 treated   N61311 GSM1275863
## 3 SRR1039512 control  N052611 GSM1275866
## 4 SRR1039513 treated  N052611 GSM1275867
## 5 SRR1039516 control  N080611 GSM1275870
## 6 SRR1039517 treated  N080611 GSM1275871
```

```
head(counts)
```

```
##                 SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
## ENSG00000000003        723        486        904        445       1170
## ENSG00000000005          0          0          0          0          0
## ENSG00000000419        467        523        616        371        582
## ENSG00000000457        347        258        364        237        318
## ENSG00000000460         96         81         73         66        118
## ENSG00000000938          0          0          1          0          2
##                 SRR1039517 SRR1039520 SRR1039521
## ENSG00000000003       1097        806        604
## ENSG00000000005          0          0          0
## ENSG00000000419        781        417        509
## ENSG00000000457        447        330        324
## ENSG00000000460         94        102         74
## ENSG00000000938          0          0          0
```

Q1. How many genes are in this dataset?

```
nrow(counts)
```

```
## [1] 38694
```

Q2. How many 'control' cell lines do we have?

```
sum(metadata$dex == 'control')
```

```
## [1] 4
```

##3. Toy differential gene expression Lets perform some exploratory differential gene expression analysis. Note: this analysis is for demonstration only. NEVER do differential expression analysis this way!

```
control <- metadata[metadata[,"dex"]=="control",]
control.counts <- counts[ ,control$id]
control.mean <- rowSums( control.counts )/4
head(control.mean)
```

```
## ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
##          900.75            0.00          520.50          339.75           97.25
## ENSG00000000938
##            0.75
```

Side-note: An alternative way to do this same thing using the dplyr package from the tidyverse is shown below. Which do you prefer and why?

```
library(dplyr)
```

```
##
## Attaching package: 'dplyr'

## The following object is masked from 'package:Biobase':
##
##     combine

## The following object is masked from 'package:matrixStats':
##
##     count

## The following objects are masked from 'package:GenomicRanges':
##
##     intersect, setdiff, union

## The following object is masked from 'package:GenomeInfoDb':
##
##     intersect

## The following objects are masked from 'package:IRanges':
##
##     collapse, desc, intersect, setdiff, slice, union

## The following objects are masked from 'package:S4Vectors':
##
##     first, intersect, rename, setdiff, setequal, union

## The following objects are masked from 'package:BiocGenerics':
##
##     combine, intersect, setdiff, union

## The following objects are masked from 'package:stats':
##
##     filter, lag

## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union
```

```
control <- metadata %>% filter(dex=="control")
control.counts <- counts %>% select(control$id)
control.mean <- rowSums(control.counts)/4
head(control.mean)
```

```
## ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
##          900.75            0.00          520.50          339.75           97.25
## ENSG00000000938
##            0.75
```

Q3. How would you make the above code in either approach more robust?

```
control <- metadata[metadata$dex=="control",]
control.counts <- counts[ ,control$id]
control.mean <- rowSums( control.counts )/ncol(control.counts)
head(control.mean)
```

```
## ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
##          900.75            0.00          520.50          339.75           97.25
## ENSG00000000938
##            0.75
```

Q4. Follow the same procedure for the treated samples (i.e. calculate the mean per gene across drug treated samples and assign to a labeled vector called treated.mean)

```
treated.counts <- counts[ ,metadata[metadata$dex=="treated",]$id]
treated.mean <- rowSums( treated.counts )/ ncol(treated.counts)
head(treated.mean)
```

```
## ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
##          658.00            0.00          546.00          316.50           78.75
## ENSG00000000938
##            0.00
```

We will combine our meancount data for bookkeeping purposes.

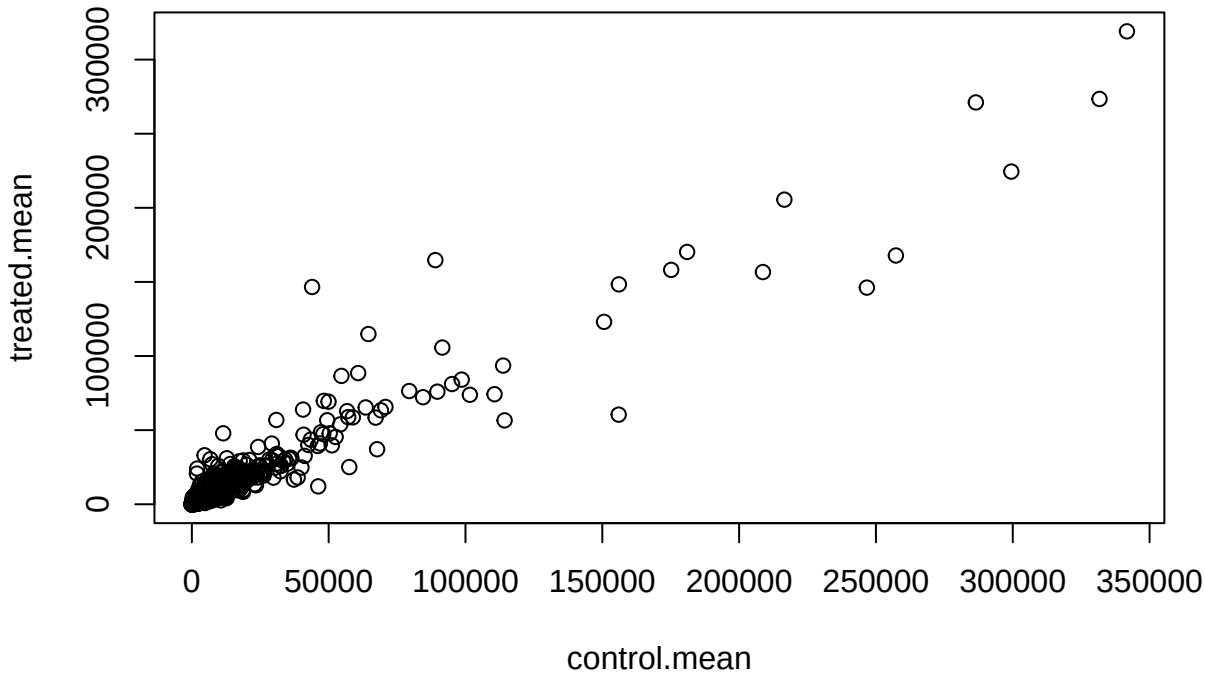
```
meancounts <- data.frame(control.mean, treated.mean)
```

```
colSums(meancounts)
```

```
## control.mean treated.mean
##     23005324     22196524
```

Q5 (a). Create a scatter plot showing the mean of the treated samples against the mean of the control samples. Your plot should look something like the following.

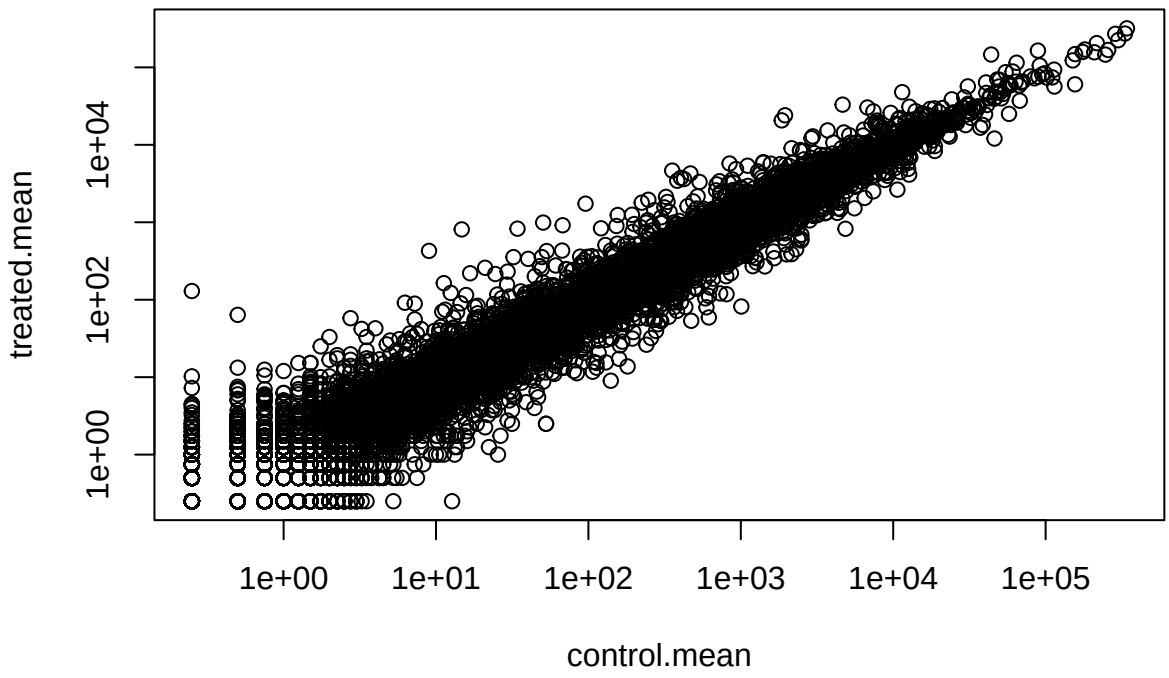
```
plot(meancounts)
```




```
plot(meancounts, log='xy')
```

```
## Warning in xy.coords(x, y, xlabel, ylabel, log): 15032 x values <= 0 omitted
## from logarithmic plot
```

```
## Warning in xy.coords(x, y, xlabel, ylabel, log): 15281 y values <= 0 omitted
## from logarithmic plot
```

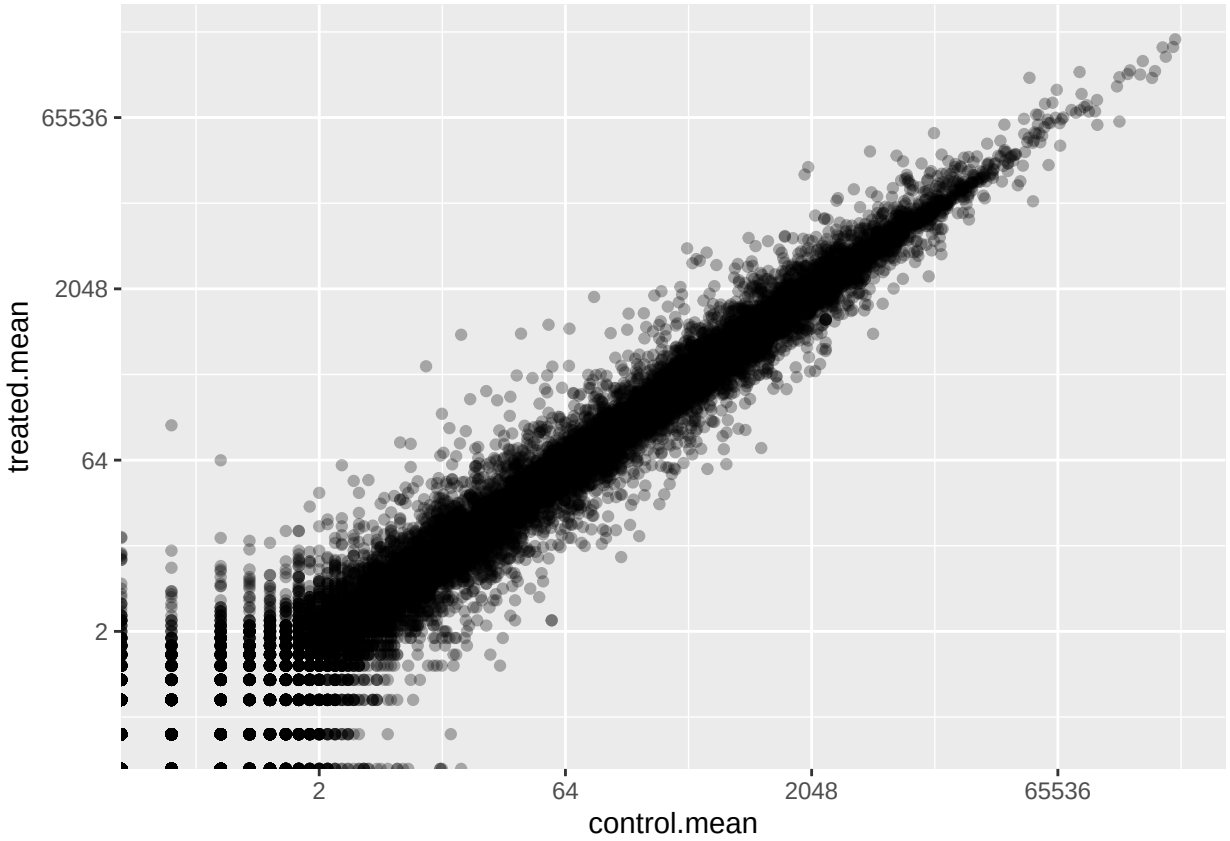


Q5 (b).You could also use the ggplot2 package to make this figure producing the plot below. What geom_?() function would you use for this plot?

```
library(ggplot2)
ggplot(meancounts, aes(control.mean, treated.mean)) + geom_point(alpha = 0.3) + scale_x_continuous(trans
```

```
## Warning: Transformation introduced infinite values in continuous x-axis
```

```
## Warning: Transformation introduced infinite values in continuous y-axis
```



Here we calculate log2foldchange, add it to our meancounts data.frame and inspect the results either with the head() or the View() function for example.

```
meancounts$log2fc <- log2(meancounts[,"treated.mean"]/meancounts[,"control.mean"])
head(meancounts)
```

```
##                 control.mean treated.mean      log2fc
## ENSG00000000003       900.75       658.00 -0.45303916
## ENSG00000000005         0.00         0.00         NaN
## ENSG00000000419       520.50       546.00  0.06900279
## ENSG00000000457       339.75       316.50 -0.10226805
## ENSG00000000460        97.25        78.75 -0.30441833
## ENSG00000000938         0.75         0.00        -Inf
```

There are a couple of "weird" results. Namely, the NaN ("not a number") and -Inf (negative infinity) results.

The NaN is returned when you divide by zero and try to take the log. The -Inf is returned when you try to take the log of zero. It turns out that there are a lot of genes with zero expression. Let's filter our data to remove these genes. Again inspect your result (and the intermediate steps) to see if things make sense to you

```
zero.vals <- which(meancounts[,1:2]==0, arr.ind=TRUE)

to.rm <- unique(zero.vals[,1]) # get all row index where 0s are
mycounts <- meancounts[-to.rm,]
head(mycounts)
```

```
##                 control.mean treated.mean      log2fc
## ENSG00000000003       900.75       658.00 -0.45303916
## ENSG00000000419       520.50       546.00  0.06900279
## ENSG00000000457       339.75       316.50 -0.10226805
## ENSG00000000460        97.25        78.75 -0.30441833
## ENSG00000000971      5219.00      6687.50  0.35769358
## ENSG00000001036      2327.00      1785.75 -0.38194109
```

Q7. What is the purpose of the arr.ind argument in the which() function call above? Why would we then take the first column of the output and need to call the unique() function?

A: which() returns the indices of all positions that evaluate to true. we take the fist column because it records all the row numbers where O's are. There could be duplicates since we have 2 columns in the dataset. calling unique eliminates the duplicates (where the gene in both treated and control is 0) so that we can delete all rows with 0's in the dataset.

A common threshold used for calling something differentially expressed is a log2(FoldChange) of greater than 2 or less than -2. Let's filter the dataset both ways to see how many genes are up or down-regulated.

```
# the percentage of log2fc > 2
round((sum(mycounts$log2fc > 2)/nrow(mycounts))*100, 2)
```

```
## [1] 1.15
```

```
up.ind <- mycounts$log2fc > 2
down.ind <- mycounts$log2fc < (-2)
```

Q8. Using the up.ind vector above can you determine how many up regulated genes we have at the greater than 2 fc level?

```
sum(up.ind)
```

```
## [1] 250
```

Q9. Using the down.ind vector above can you determine how many down regulated genes we have at the greater than 2 fc level?

```
sum(down.ind)
```

```
## [1] 367
```

Q10. Do you trust these results? Why or why not?

A: No, we did not do any statistical tests to show the significance of these changes, thus it is not appropriate to draw any conclusion.

4. DESeq2 analysis

```
citation("DESeq2")
```

```
##
##   Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change
##   and dispersion for RNA-seq data with DESeq2 Genome Biology 15(12):550
##   (2014)
##
## A BibTeX entry for LaTeX users is
##
##   @Article{,
##     title = {Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2},
##     author = {Michael I. Love and Wolfgang Huber and Simon Anders},
##     year = {2014},
##     journal = {Genome Biology},
##     doi = {10.1186/s13059-014-0550-8},
##     volume = {15},
##     issue = {12},
##     pages = {550},
##   }
```

```
dds <- DESeqDataSetFromMatrix(countData=counts,
                              colData=metadata,
                              design=~dex)
```

```
## converting counts to integer mode
```

```
## Warning in DESeqDataSet(se, design = design, ignoreRank): some variables in
## design formula are characters, converting to factors
```

```
dds
```

```
## class: DESeqDataSet
## dim: 38694 8
## metadata(1): version
## assays(1): counts
## rownames(38694): ENSG00000000003 ENSG00000000005 ... ENSG00000283120
##   ENSG00000283123
## rowData names(0):
## colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
## colData names(4): id dex celltype geo_id
```

#DESeq analysis Next, let's run the DESeq analysis pipeline on the dataset, and reassign the resulting object back to the same variable. Note that before we start, dds is a bare-bones DESeqDataSet. The DESeq() function takes a DESeqDataSet and returns a DESeqDataSet, but with additional information filled in (including the differential expression results we are after). Notice how if we try to access these results before running the analysis, nothing exists.

Here, we're running the DESeq pipeline on the dds object, and reassigning the whole thing back to dds, which will now be a DESeqDataSet populated with all those values. Get some help on ?DESeq (notice, no "2" on the end). This function calls a number of other functions within the package to essentially run the entire pipeline (normalizing by library size by estimating the "size factors," estimating dispersion for the negative binomial model, and fitting models and getting statistics for each gene for the design specified when you imported the data).

```
dds <- DESeq(dds)
```

```
## estimating size factors

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship

## final dispersion estimates

## fitting model and testing
```

#Getting results Since we've got a fairly simple design (single factor, two groups, treated versus control), we can get results out of the object simply by calling the results() function on the DESeqDataSet that has been run through the pipeline.

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

```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 38694 rows and 6 columns
##                   baseMean log2FoldChange     lfcSE      stat    pvalue
##                  <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003  747.1942     -0.3507030  0.168246 -2.084470 0.0371175
## ENSG00000000005    0.0000             NA        NA        NA        NA
## ENSG00000000419  520.1342      0.2061078  0.101059  2.039475 0.0414026
## ENSG00000000457  322.6648      0.0245269  0.145145  0.168982 0.8658106
## ENSG00000000460   87.6826     -0.1471420  0.257007 -0.572521 0.5669691
## ...                    ...            ...       ...       ...       ...
## ENSG00000283115  0.000000             NA        NA        NA        NA
## ENSG00000283116  0.000000             NA        NA        NA        NA
## ENSG00000283119  0.000000             NA        NA        NA        NA
## ENSG00000283120  0.974916      -0.668258   1.69456 -0.394354  0.693319
## ENSG00000283123  0.000000             NA        NA        NA        NA
##                       padj
##                  <numeric>
## ENSG00000000003  0.163035
## ENSG00000000005        NA
## ENSG00000000419  0.176032
## ENSG00000000457  0.961694
## ENSG00000000460  0.815849
## ...                   ...
## ENSG00000283115        NA
## ENSG00000283116        NA
## ENSG00000283119        NA
## ENSG00000283120        NA
## ENSG00000283123        NA
```

```
summary(res)
```

```
##
## out of 25258 with nonzero total read count
## adjusted p-value < 0.1
## LFC > 0 (up)       : 1563, 6.2%
## LFC < 0 (down)     : 1188, 4.7%
## outliers [1]       : 142, 0.56%
## low counts [2]     : 9971, 39%
## (mean count < 10)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

The results function contains a number of arguments to customize the results table. By default the argument alpha is set to 0.1. If the adjusted p value cutoff will be a value other than 0.1, alpha should be set to that value:

```
res05 <- results(dds, alpha=0.05)
summary(res05)
```

```
##
## out of 25258 with nonzero total read count
## adjusted p-value < 0.05
## LFC > 0 (up)       : 1236, 4.9%
## LFC < 0 (down)     : 933, 3.7%
## outliers [1]       : 142, 0.56%
## low counts [2]     : 9033, 36%
## (mean count < 6)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

5. Adding annotation data

Our result table so far only contains the Ensembl gene IDs. However, alternative gene names and extra annotation are usually required for informative interpretation of our results. In this section we will add this necessary annotation data to our results.

We will use one of Bioconductor's main annotation packages to help with mapping between various ID schemes. Here we load the AnnotationDbi package and the annotation data package for humans org.Hs.eg.db.

```
library("AnnotationDbi")
```

```
## Warning: package 'AnnotationDbi' was built under R version 4.1.2
```

```
##
## Attaching package: 'AnnotationDbi'
```

```
## The following object is masked from 'package:dplyr':
##
##     select
```

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

```
##
```

The later of these is is the organism annotation package ("org") for Homo sapiens ("Hs"), organized as an AnnotationDbi database package ("db"), using Entrez Gene IDs ("eg") as primary key. To get a list of all available key types that we can use to map between, use the columns() function:

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

```
##  [1] "ACCNUM"       "ALIAS"        "ENSEMBL"      "ENSEMBLPROT"  "ENSEMBLTRANS"
##  [6] "ENTREZID"     "ENZYME"       "EVIDENCE"     "EVIDENCEALL"  "GENENAME"
## [11] "GENETYPE"     "GO"           "GOALL"        "IPI"          "MAP"
## [16] "OMIM"         "ONTOLOGY"     "ONTOLOGYALL"  "PATH"         "PFAM"
## [21] "PMID"         "PROSITE"      "REFSEQ"       "SYMBOL"       "UCSCKG"
## [26] "UNIPROT"
```

The main function we will use from the AnnotationDbi package is called mapIds().

We can use the mapIds() function to add individual columns to our results table. We provide the row names of our results table as a key, and specify that keytype=ENSEMBL. The column argument tells the mapIds() function which information we want, and the multiVals argument tells the function what to do if there are multiple possible values for a single input value. Here we ask to just give us back the first one that occurs in the database.

```
res$symbol <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",        # The format of our genenames
                     column="SYMBOL",          # The new format we want to add
                     multiVals="first")
```

```
## 'select()' returned 1:many mapping between keys and columns
```

```
head(res)
```

```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 6 rows and 7 columns
##                   baseMean log2FoldChange     lfcSE      stat    pvalue
##                  <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
## ENSG00000000005   0.000000             NA        NA        NA        NA
## ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
## ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
## ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
## ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
##                      padj      symbol
##                 <numeric> <character>
## ENSG00000000003  0.163035      TSPAN6
## ENSG00000000005        NA        TNMD
## ENSG00000000419  0.176032        DPM1
## ENSG00000000457  0.961694       SCYL3
## ENSG00000000460  0.815849    C1orf112
## ENSG00000000938        NA         FGR
```

Q11. Run the mapIds() function two more times to add the Entrez ID and UniProt accession and GENENAME as new columns called `res$entrez`, `res$uniprot` and `res$genename`.

```
res$entrez <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",        # The format of our genenames
                     column="ENTREZID",          # The new format we want to add
                     multiVals="first")
```

```
## 'select()' returned 1:many mapping between keys and columns
```

```
res$uniprot <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",        # The format of our genenames
                     column="UNIPROT",          # The new format we want to add
                     multiVals="first")
```

```
## 'select()' returned 1:many mapping between keys and columns
```

```
res$genename <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",        # The format of our genenames
                     column="GENENAME",          # The new format we want to add
                     multiVals="first")
```

```
## 'select()' returned 1:many mapping between keys and columns
```

```
head(res)
```

```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 6 rows and 10 columns
##                   baseMean log2FoldChange     lfcSE      stat    pvalue
##                  <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003 747.194195     -0.3507030  0.168246 -2.084470 0.0371175
## ENSG00000000005   0.000000             NA        NA        NA        NA
## ENSG00000000419 520.134160      0.2061078  0.101059  2.039475 0.0414026
## ENSG00000000457 322.664844      0.0245269  0.145145  0.168982 0.8658106
## ENSG00000000460  87.682625     -0.1471420  0.257007 -0.572521 0.5669691
## ENSG00000000938   0.319167     -1.7322890  3.493601 -0.495846 0.6200029
##                      padj      symbol      entrez     uniprot
##                 <numeric> <character> <character> <character>
## ENSG00000000003  0.163035      TSPAN6        7105  A0A024RCI0
## ENSG00000000005        NA        TNMD       64102      Q9H2S6
## ENSG00000000419  0.176032        DPM1        8813      O60762
## ENSG00000000457  0.961694       SCYL3       57147      Q8IZE3
## ENSG00000000460  0.815849    C1orf112       55732  A0A024R922
## ENSG00000000938        NA         FGR        2268      P09769
##                            genename
##                         <character>
## ENSG00000000003          tetraspanin 6
```

```
## ENSG00000000005            tenomodulin
## ENSG00000000419 dolichyl-phosphate m..
## ENSG00000000457 SCY1 like pseudokina..
## ENSG00000000460 chromosome 1 open re..
## ENSG00000000938 FGR proto-oncogene, ..
```

You can arrange and view the results by the adjusted p-value

```
ord <- order( res$padj )
#View(res[ord,])
head(res[ord,])
```

```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 6 rows and 10 columns
##                  baseMean log2FoldChange     lfcSE      stat      pvalue
##                 <numeric>      <numeric> <numeric> <numeric>   <numeric>
## ENSG00000152583   954.771        4.36836 0.2371268   18.4220 8.74490e-76
## ENSG00000179094   743.253        2.86389 0.1755693   16.3120 8.10784e-60
## ENSG00000116584  2277.913       -1.03470 0.0650984  -15.8944 6.92855e-57
## ENSG00000189221  2383.754        3.34154 0.2124058   15.7319 9.14433e-56
## ENSG00000120129  3440.704        2.96521 0.2036951   14.5571 5.26424e-48
## ENSG00000148175 13493.920        1.42717 0.1003890   14.2164 7.25128e-46
##                        padj      symbol      entrez     uniprot
##                   <numeric> <character> <character> <character>
## ENSG00000152583 1.32441e-71     SPARCL1        8404  A0A024RDE1
## ENSG00000179094 6.13966e-56        PER1        5187      O15534
## ENSG00000116584 3.49776e-53     ARHGEF2        9181      Q92974
## ENSG00000189221 3.46227e-52        MAOA        4128      P21397
## ENSG00000120129 1.59454e-44       DUSP1        1843      B4DU40
## ENSG00000148175 1.83034e-42        STOM        2040      F8VSL7
##                              genename
##                           <character>
## ENSG00000152583           SPARC like 1
## ENSG00000179094 period circadian reg..
## ENSG00000116584 Rho/Rac guanine nucl..
## ENSG00000189221    monoamine oxidase A
## ENSG00000120129 dual specificity pho..
## ENSG00000148175               stomatin
```

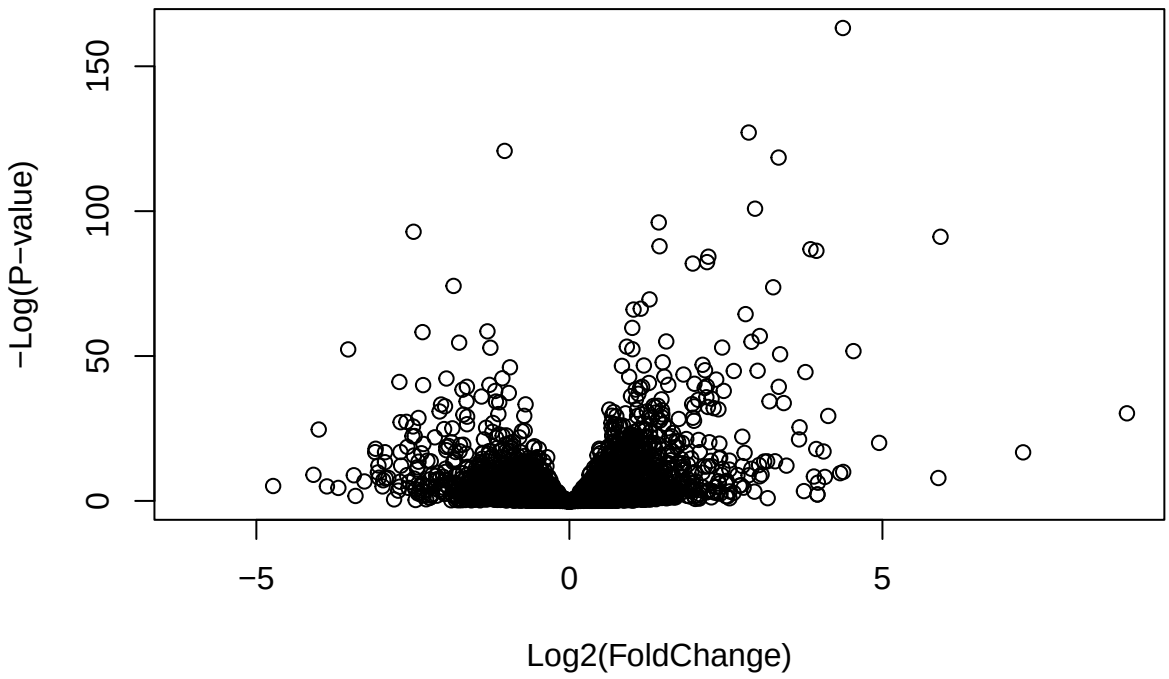
Finally, let's write out the ordered significant results with annotations. See the help for `?write.csv` if you are unsure here.

```
write.csv(res[ord,], "deseq_results.csv")
```

##6. Data Visualization Volcano plots Let's make a commonly produced visualization from this data, namely a so-called Volcano plot. These summary figures are frequently used to highlight the proportion of genes that are both significantly regulated and display a high fold change.

Typically these plots shows the log fold change on the X-axis, and the −log10 of the p-value on the Y-axis (the more significant the p-value, the larger the −log10 of that value will be). A very dull (i.e. non colored and labeled) version can be created with a quick call to plot() like so:

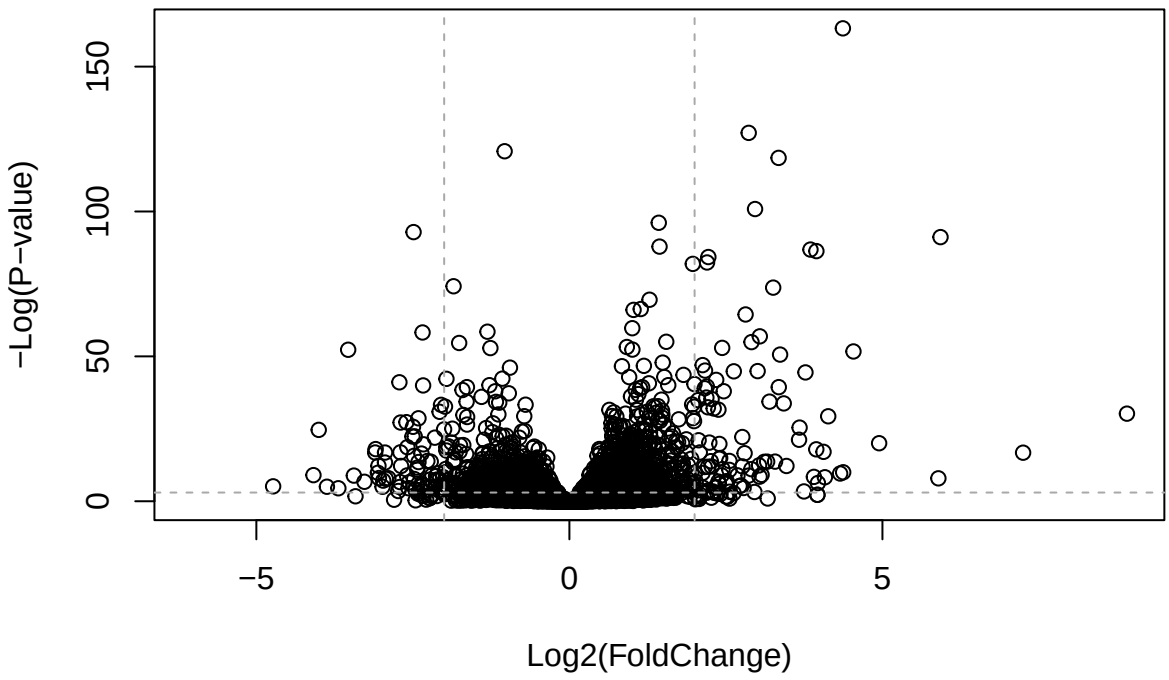
```
# log fold change on the X-axis, and the -log10 of the p-value on the Y-axis
plot( res$log2FoldChange,  -log(res$padj),
      xlab="Log2(FoldChange)",
      ylab="-Log(P-value)")
```



To make this more useful we can add some guidelines (with the abline() function) and color (with a custom color vector) highlighting genes that have padj<0.05 and the absolute log2FoldChange>2.

```
plot( res$log2FoldChange,  -log(res$padj),
 ylab="-Log(P-value)", xlab="Log2(FoldChange)")

# Add some cut-off lines
abline(v=c(-2,2), col="darkgray", lty=2)
abline(h=-log(0.05), col="darkgray", lty=2)
```



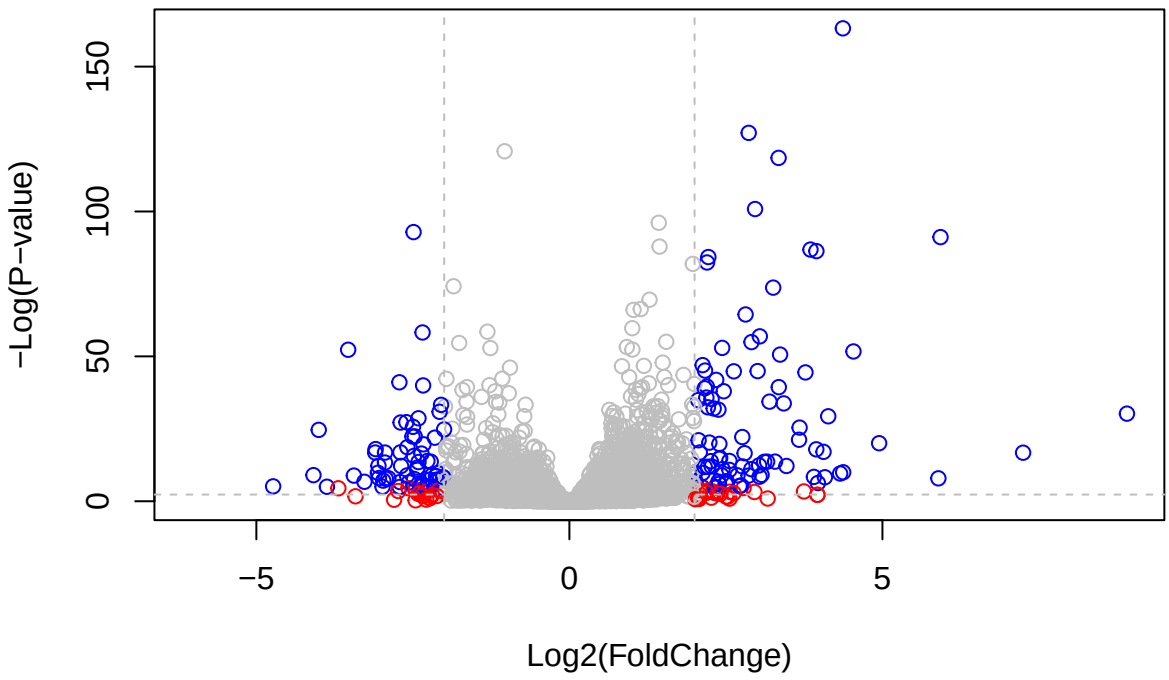
To color the points we will setup a custom color vector indicating transcripts with large fold change and significant differences between conditions:

```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res)) #replicate 'gray'
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red" #log2fc >2 assigned red

inds <- (res$padj < 0.01) & (abs(res$log2FoldChange) > 2 )
mycols[ inds ] <- "blue"

# Volcano plot with custom colors
plot( res$log2FoldChange,  -log(res$padj),
 col=mycols, ylab="-Log(P-value)", xlab="Log2(FoldChange)" )

# Cut-off lines
abline(v=c(-2,2), col="gray", lty=2)
abline(h=-log(0.1), col="gray", lty=2)
```



For even more customization you might find the EnhancedVolcano bioconductor package useful (Note. It uses ggplot under the hood):

First we will add the more understandable gene symbol names to our full results object res as we will use this to label the most interesting genes in our final plot.

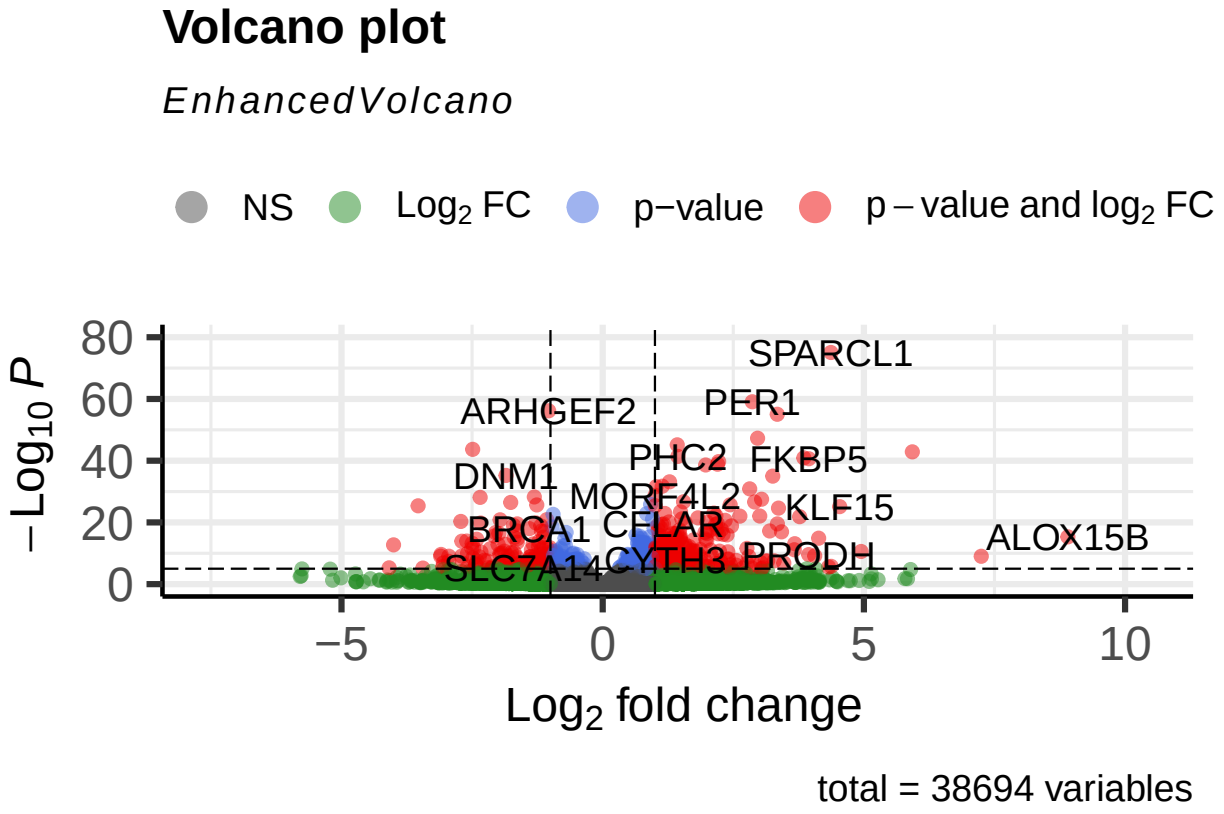
```
BiocManager::install("EnhancedVolcano")
```

```
library(EnhancedVolcano)
```

```
## Loading required package: ggrepel
```

```
## Registered S3 methods overwritten by 'ggalt':
##   method                  from
##   grid.draw.absoluteGrob  ggplot2
##   grobHeight.absoluteGrob ggplot2
##   grobWidth.absoluteGrob  ggplot2
##   grobX.absoluteGrob      ggplot2
##   grobY.absoluteGrob      ggplot2
```

```
x <- as.data.frame(res)
EnhancedVolcano(x,
    lab = x$symbol,
    x = 'log2FoldChange',
    y = 'pvalue')
```



##7. Pathway analysis Pathway analysis (also known as gene set analysis or over-representation analysis), aims to reduce the complexity of interpreting gene lists via mapping the listed genes to known (i.e. annotated) biological pathways, processes and functions.

#Patway analysis with R and Bioconductor There are many freely available tools for pathway or over-representation analysis. Ath the time of writting Bioconductor alone has over 80 packages categorized under gene set enrichment and over 120 packages categorized under pathways.

Here we play with just one, the GAGE package (which stands for Generally Applicable Gene set Enrichment), to do KEGG pathway enrichment analysis on our RNA-seq based differential expression results.

The KEGG pathway database, unlike GO for example, provides functional annotation as well as information about gene products that interact with each other in a given pathway, how they interact (e.g., activation, inhibition, etc.), and where they interact (e.g., cytoplasm, nucleus, etc.). Hence KEGG has the potential to provide extra insight beyond annotation lists of simple molecular function, process etc. from GO terms.

In this analysis, we check for coordinated differential expression over gene sets from KEGG pathways instead of changes of individual genes. The assumption here is that consistent perturbations over a given pathway (gene set) may suggest mechanistic changes.

Once we have a list of enriched pathways from gage we will use the pathview package to draw pathway diagrams, coloring the molecules in the pathway by their degree of up/down-regulation.

First we need to do our one time install of these required bioconductor packages:

```
# Run in your R console (i.e. not your Rmarkdown doc!)
BiocManager::install( c("pathview", "gage", "gageData") )
```

Now we can load the packages and setup the KEGG data-sets we need. The gageData package has pre-compiled databases mapping genes to KEGG pathways and GO terms for common organisms. kegg.sets.hs

is a named list of 229 elements. Each element is a character vector of member gene Entrez IDs for a single KEGG pathway.

```
library(pathview)
library(gage)
library(gageData)

data(kegg.sets.hs)

# Examine the first 2 pathways in this kegg set for humans
head(kegg.sets.hs, 2)
```

```
## $`hsa00232 Caffeine metabolism`
## [1] "10"   "1544" "1548" "1549" "1553" "7498" "9"
##
## $`hsa00983 Drug metabolism - other enzymes`
##  [1] "10"     "1066"   "10720"  "10941"  "151531" "1548"   "1549"   "1551"
##  [9] "1553"   "1576"   "1577"   "1806"   "1807"   "1890"   "221223" "2990"
## [17] "3251"   "3614"   "3615"   "3704"   "51733"  "54490"  "54575"  "54576"
## [25] "54577"  "54578"  "54579"  "54600"  "54657"  "54658"  "54659"  "54963"
## [33] "574537" "64816"  "7083"   "7084"   "7172"   "7363"   "7364"   "7365"
## [41] "7366"   "7367"   "7371"   "7372"   "7378"   "7498"   "79799"  "83549"
## [49] "8824"   "8833"   "9"      "978"
```

The main gage() function requires a named vector of fold changes, where the names of the values are the Entrez gene IDs.

Note that we used the mapIDs() function above to obtain Entrez gene IDs (stored in `res$entrez`) and we have the fold change results from DESeq2 analysis (stored in `res$log2FoldChange`).

```
foldchanges = res$log2FoldChange
names(foldchanges) = res$entrez
head(foldchanges)
```

```
##        7105       64102        8813       57147       55732        2268
## -0.35070302          NA  0.20610777  0.02452695 -0.14714205 -1.73228897
```

```
# Get the results
keggres = gage(foldchanges, gsets=kegg.sets.hs)
```

See help on the gage function with ?gage. Specifically, you might want to try changing the value of same.dir. This value determines whether to test for changes in a gene set toward a single direction (all genes up or down regulated) or changes towards both directions simultaneously (i.e. any genes in the pathway dysregulated). Here, we're using the default same.dir=TRUE, which will give us separate lists for pathways that are upregulated versus pathways that are down-regulated.

Now lets look at the object returned from gage().

```
attributes(keggres)
```

```
## $names
## [1] "greater" "less"    "stats"
```

It is a list with three elements, "greater", "less" and "stats".

You can also see this in your Environmnet panel/tab window of RStudio or use the R command str(keggres).

Like any list we can use the dollar syntax to access a named element, e.g. `head(keggres$greater)` and `head(keggres$less)`.

Lets look at the first few down (less) pathway results

```
# Look at the first three down (less) pathways
head(keggres$less, 3)
```

```
##                                       p.geomean stat.mean        p.val
## hsa05332 Graft-versus-host disease 0.0004250461 -3.473346 0.0004250461
## hsa04940 Type I diabetes mellitus  0.0017820293 -3.002352 0.0017820293
## hsa05310 Asthma                    0.0020045888 -3.009050 0.0020045888
##                                          q.val set.size         exp1
## hsa05332 Graft-versus-host disease 0.09053483       40 0.0004250461
## hsa04940 Type I diabetes mellitus  0.14232581       42 0.0017820293
## hsa05310 Asthma                    0.14232581       29 0.0020045888
```

Each `keggres$less` and `keggres$greater` object is data matrix with gene sets as rows sorted by p-value.

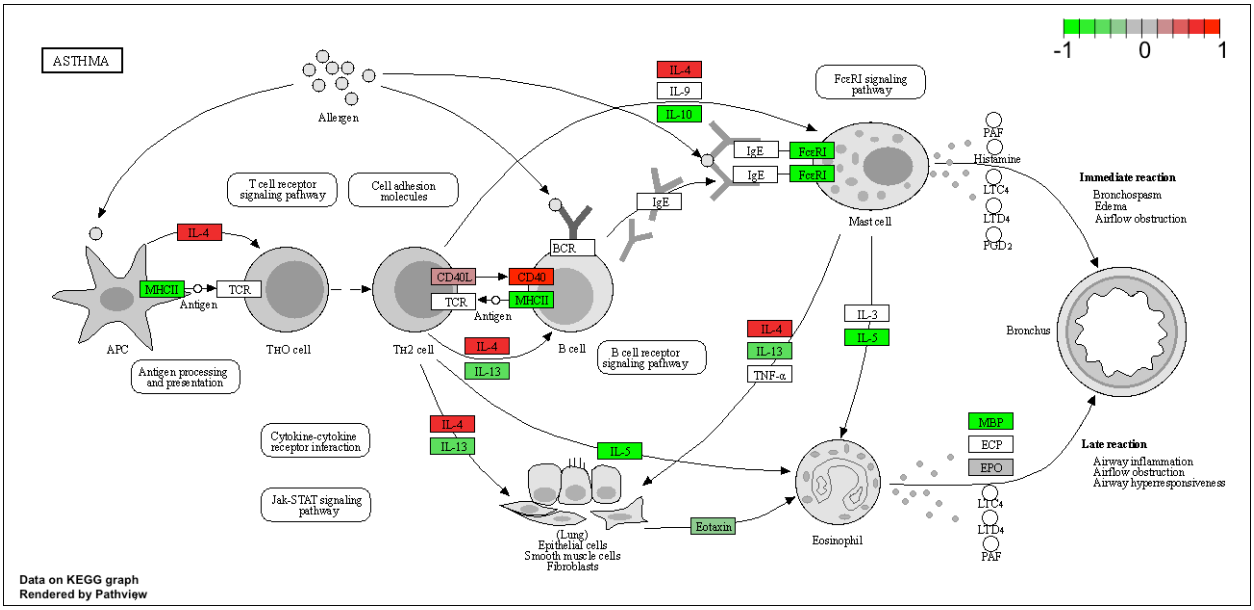
Now, let's try out the pathview() function from the pathview package to make a pathway plot with our RNA-Seq expression results shown in color. To begin with lets manually supply a pathway.id (namely the first part of the "hsa05310 Asthma") that we could see from the print out above

```
pathview(gene.data=foldchanges, pathway.id="hsa05310")
```

```
## 'select()' returned 1:1 mapping between keys and columns
```

```
## Info: Working in directory /Users/maywu/Desktop/bioinformatics/projects/bggn213_github/class15_RNAse
```

```
## Info: Writing image file hsa05310.pathview.png
```



Note how many of the genes in this pathway are perturbed (i.e. colored) in our results.

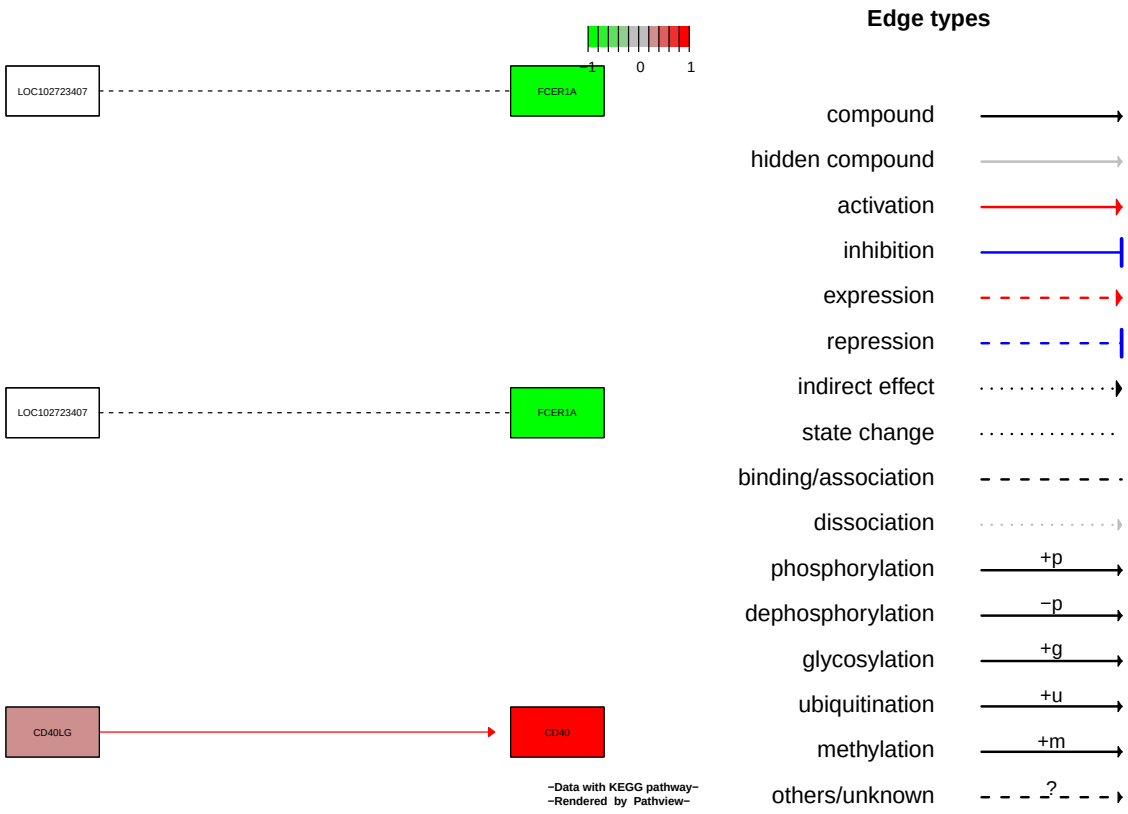
You can play with the other input arguments to pathview() to change the display in various ways including generating a PDF graph. For example:

```
pathview(gene.data=foldchanges, pathway.id="hsa05310", kegg.native=FALSE)
```

```
## 'select()' returned 1:1 mapping between keys and columns
```

```
## Info: Working in directory /Users/maywu/Desktop/bioinformatics/projects/bggn213_github/class15_RNAse
```

```
## Info: Writing image file hsa05310.pathview.pdf
```



Can you do the same procedure as above to plot the pathview figures for the top 2 down-reguled pathways?

```
pathview(gene.data=foldchanges, pathway.id="hsa04940")
```

```
## 'select()' returned 1:1 mapping between keys and columns
```

```
## Info: Working in directory /Users/maywu/Desktop/bioinformatics/projects/bggn213_github/class15_RNAse
```

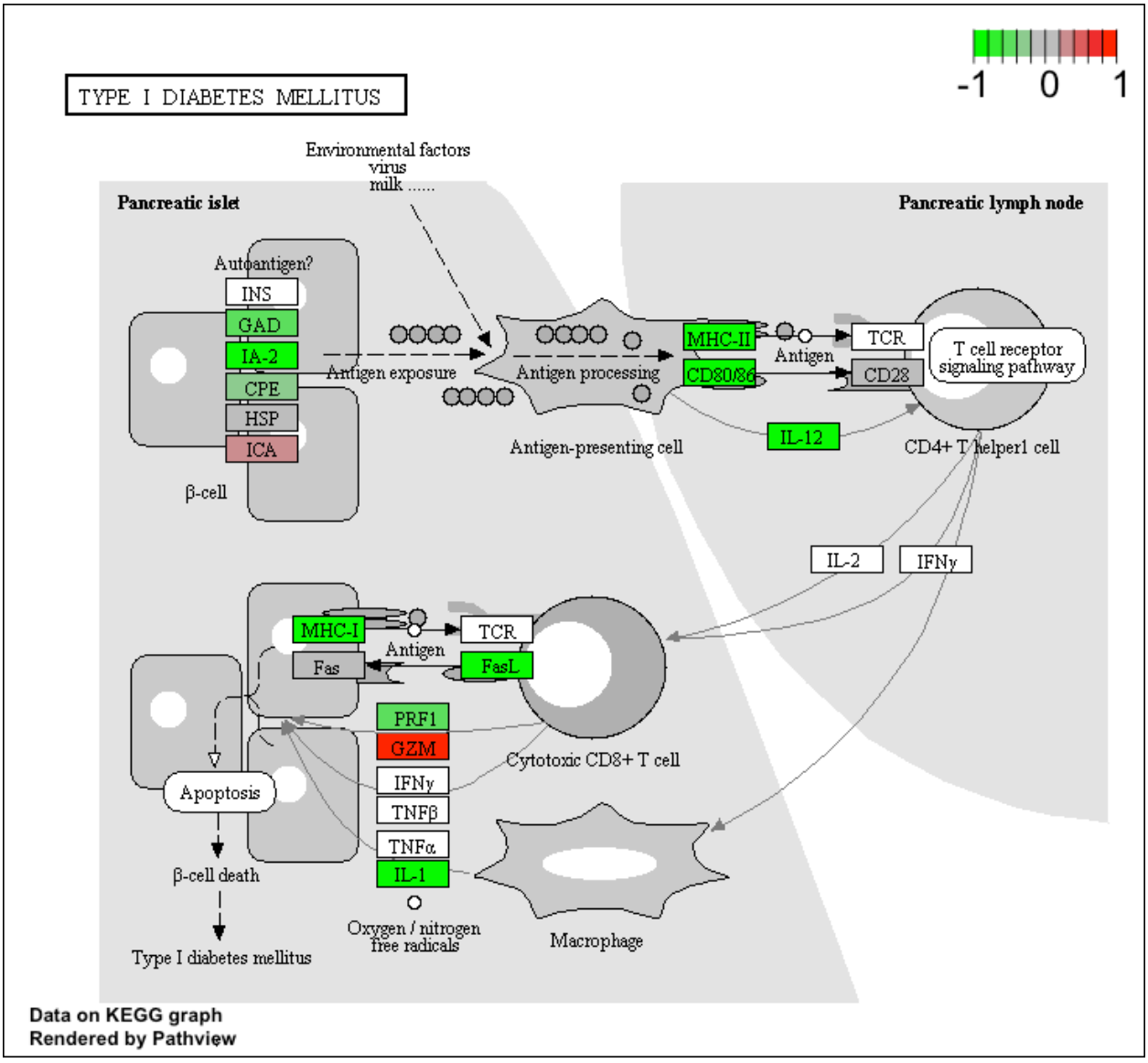
```
## Info: Writing image file hsa04940.pathview.png
```

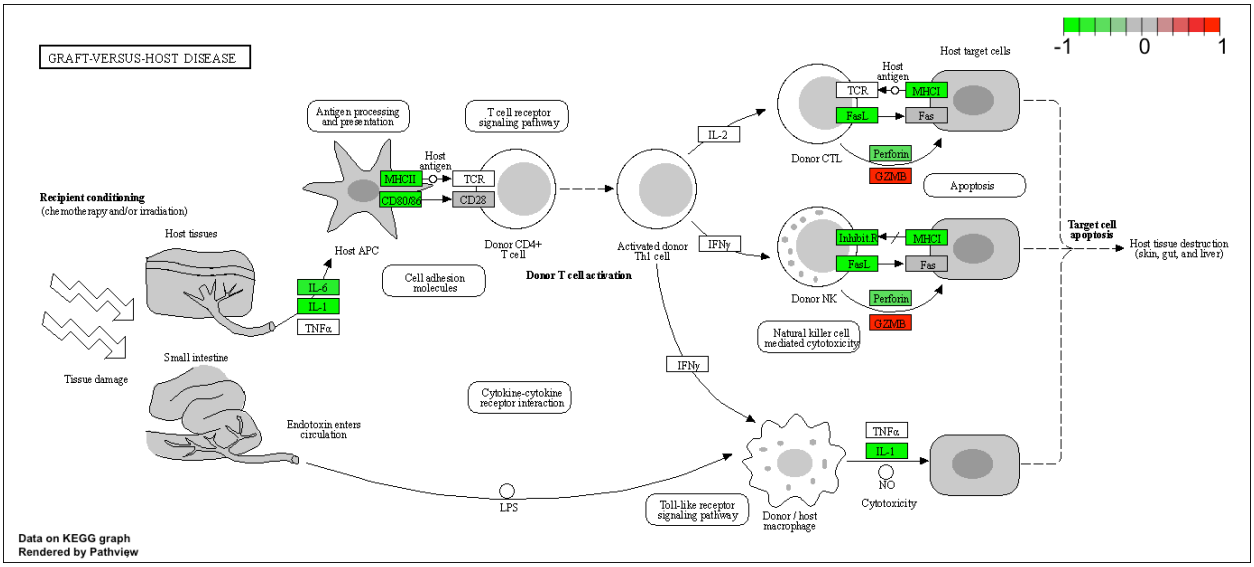
```
pathview(gene.data=foldchanges, pathway.id="hsa05332")
```

```
## 'select()' returned 1:1 mapping between keys and columns
```

```
## Info: Working in directory /Users/maywu/Desktop/bioinformatics/projects/bggn213_github/class15_RNAse
```

```
## Info: Writing image file hsa05332.pathview.png
```





OPTIONAL: Plotting counts for genes of interest

DESeq2 offers a function called plotCounts() that takes a DESeqDataSet that has been run through the pipeline, the name of a gene, and the name of the variable in the colData that you're interested in, and plots those values. See the help for ?plotCounts. Let's first see what the gene ID is for the CRISPLD2 gene using:

```
i <- grep("CRISPLD2", res$symbol)
res[i,]
```

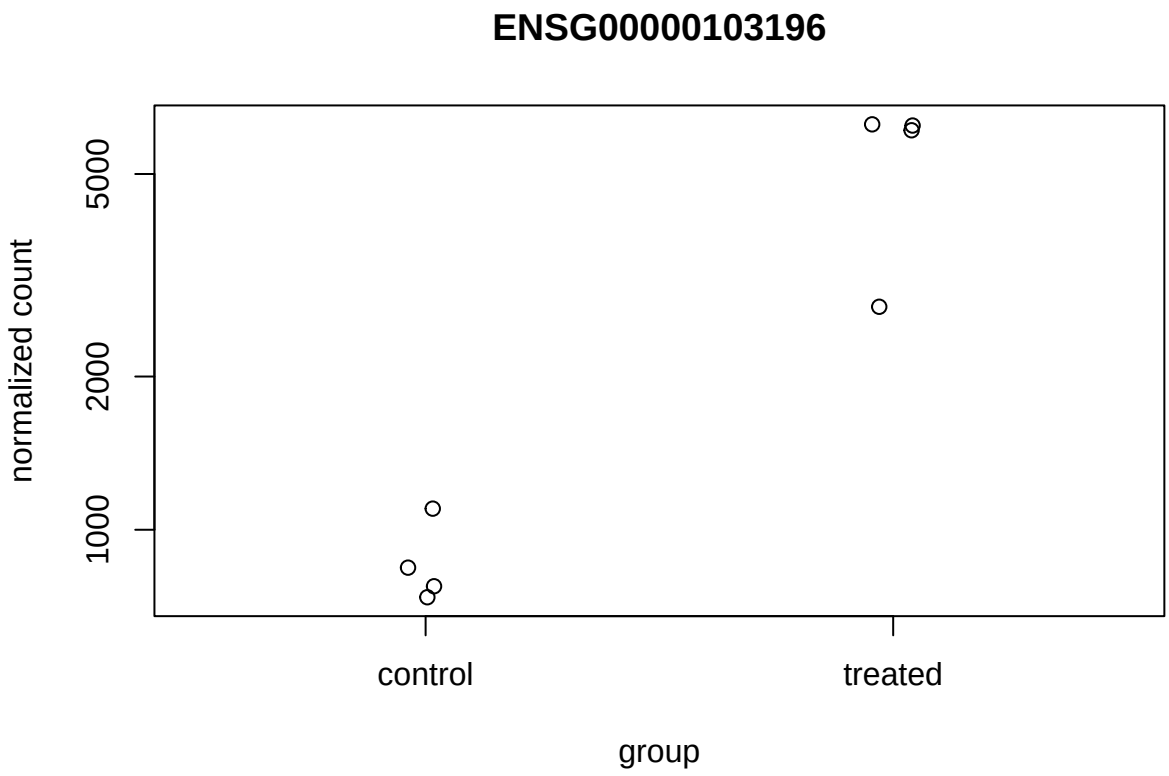
```
## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 1 row and 10 columns
##                  baseMean log2FoldChange     lfcSE      stat      pvalue
##                 <numeric>      <numeric> <numeric> <numeric>   <numeric>
## ENSG00000103196   3096.16        2.62603  0.267444   9.81899 9.32747e-23
##                      padj      symbol      entrez     uniprot
##                 <numeric> <character> <character> <character>
## ENSG00000103196 3.36344e-20    CRISPLD2       83716  A0A140VK80
##                            genename
##                         <character>
## ENSG00000103196 cysteine rich secret..
```

```
rownames(res[i,])
```

```
## [1] "ENSG00000103196"
```

Now, with that gene ID in hand let's plot the counts, where our intgroup, or "interesting group" variable is the "dex" column.

```
plotCounts(dds, gene="ENSG00000103196", intgroup="dex")
```

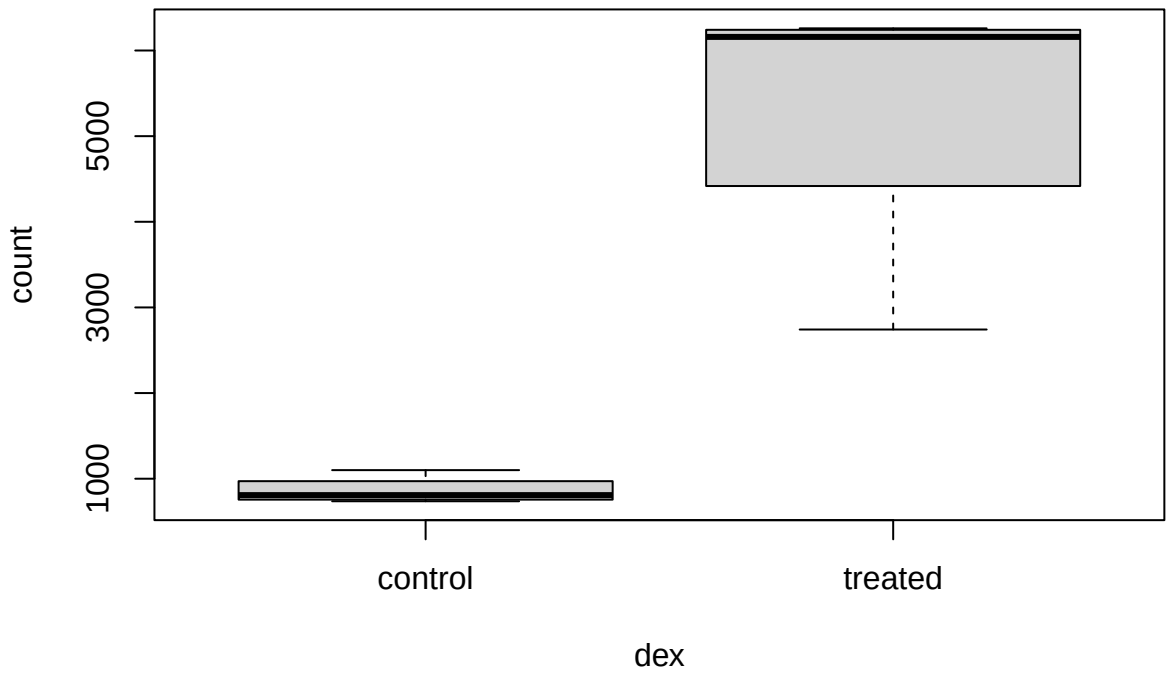


That's just okay. Keep looking at the help for ?plotCounts. Notice that we could have actually returned the data instead of plotting. We could then pipe this to ggplot and make our own figure. Let's make a boxplot.

```
d <- plotCounts(dds, gene="ENSG00000103196", intgroup="dex", returnData=TRUE)
head(d)
```

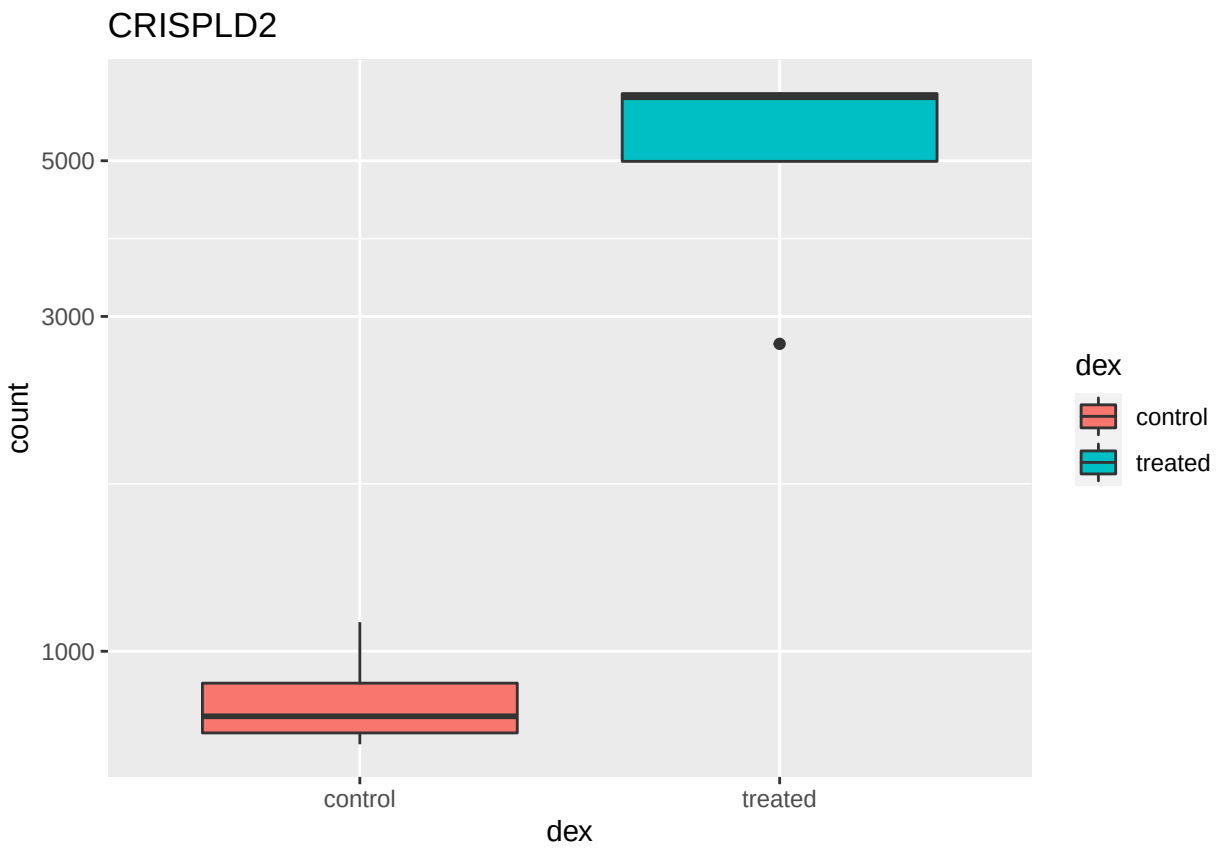
```
##                count     dex
## SRR1039508  774.5002 control
## SRR1039509 6258.7915 treated
## SRR1039512 1100.2741 control
## SRR1039513 6093.0324 treated
## SRR1039516  736.9483 control
## SRR1039517 2742.1908 treated
```

```
# a formula, such as y ~ grp, where y is a numeric vector of data values to be split into groups accord
boxplot(count ~ dex , data=d)
```



As the returned object is a data.frame it is also all setup for ggplot2 based plotting. For example:

```
library(ggplot2)
ggplot(d, aes(dex, count, fill=dex)) +
  geom_boxplot() +
  scale_y_log10() +
  ggtitle("CRISPLD2")
```



Session Information The sessionInfo() prints version information about R and any attached packages. It's a good practice to always run this command at the end of your R session and record it for the sake of reproducibility in the future.

```
sessionInfo()
```

```
## R version 4.1.1 (2021-08-10)
## Platform: x86_64-apple-darwin17.0 (64-bit)
## Running under: macOS Big Sur 10.16
##
## Matrix products: default
## BLAS:   /Library/Frameworks/R.framework/Versions/4.1/Resources/lib/libRblas.0.dylib
## LAPACK: /Library/Frameworks/R.framework/Versions/4.1/Resources/lib/libRlapack.dylib
##
## 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] stats4    stats     graphics  grDevices utils     datasets  methods
## [8] base
##
## other attached packages:
##  [1] gageData_2.32.0             gage_2.44.0
##  [3] pathview_1.34.0             EnhancedVolcano_1.12.0
##  [5] ggrepel_0.9.1               org.Hs.eg.db_3.14.0
##  [7] AnnotationDbi_1.56.2        ggplot2_3.3.5
```

```
##  [9] dplyr_1.0.7                 DESeq2_1.34.0
## [11] SummarizedExperiment_1.24.0 Biobase_2.54.0
## [13] MatrixGenerics_1.6.0        matrixStats_0.61.0
## [15] GenomicRanges_1.46.0        GenomeInfoDb_1.30.0
## [17] IRanges_2.28.0              S4Vectors_0.32.2
## [19] BiocGenerics_0.40.0         BiocManager_1.30.16
##
## loaded via a namespace (and not attached):
##  [1] bitops_1.0-7           bit64_4.0.5            ash_1.0-15
##  [4] RColorBrewer_1.1-2     httr_1.4.2             Rgraphviz_2.38.0
##  [7] tools_4.1.1            utf8_1.2.2             R6_2.5.1
## [10] vipor_0.4.5            KernSmooth_2.23-20     DBI_1.1.1
## [13] colorspace_2.0-2       withr_2.4.2            tidyselect_1.1.1
## [16] ggrastr_1.0.0          ggalt_0.4.0            bit_4.0.4
## [19] compiler_4.1.1         extrafontdb_1.0        graph_1.72.0
## [22] DelayedArray_0.20.0    labeling_0.4.2         KEGGgraph_1.54.0
## [25] scales_1.1.1           proj4_1.0-10.1         genefilter_1.76.0
## [28] stringr_1.4.0          digest_0.6.28          rmarkdown_2.11
## [31] XVector_0.34.0         pkgconfig_2.0.3        htmltools_0.5.2
## [34] extrafont_0.17         fastmap_1.1.0          highr_0.9
## [37] maps_3.4.0             rlang_0.4.12           RSQLite_2.2.8
## [40] generics_0.1.1         farver_2.1.0           BiocParallel_1.28.0
## [43] RCurl_1.98-1.5         magrittr_2.0.1         GO.db_3.14.0
## [46] GenomeInfoDbData_1.2.7 Matrix_1.3-4           Rcpp_1.0.7
## [49] ggbeeswarm_0.6.0       munsell_0.5.0          fansi_0.5.0
## [52] lifecycle_1.0.1        stringi_1.7.5          yaml_2.2.1
## [55] MASS_7.3-54            zlibbioc_1.40.0        grid_4.1.1
## [58] blob_1.2.2             parallel_4.1.1         crayon_1.4.2
## [61] lattice_0.20-45        Biostrings_2.62.0      splines_4.1.1
## [64] annotate_1.72.0        KEGGREST_1.34.0        locfit_1.5-9.4
## [67] knitr_1.36             pillar_1.6.4           geneplotter_1.72.0
## [70] XML_3.99-0.8           glue_1.5.0             evaluate_0.14
## [73] png_0.1-7              vctrs_0.3.8            Rttf2pt1_1.3.9
## [76] gtable_0.3.0           purrr_0.3.4            cachem_1.0.6
## [79] xfun_0.28              xtable_1.8-4           survival_3.2-13
## [82] tibble_3.1.6           beeswarm_0.4.0         memoise_2.0.0
## [85] ellipsis_0.3.2
```