Class12

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```
#install.packages("BiocManager")
#BiocManager::install()
```

```
# For this class, you'll also need DESeq2:
#BiocManager::install("DESeq2")
```

```
library("BiocManager")
```

```
Bioconductor version '3.15' is out-of-date; the current release version '3.16'
  is available with R version '4.2'; see https://bioconductor.org/install
```

```
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
```

In today's class we will work with published RNA-seq experiment where airway smooth muscle cells were treated with dexamethasone, a synthetic glucocorticoid steroid with anti-inflammatory effects (Himes et al. 2014).

Data Import

We will use good old 'read.csv()' to read the two things we need for this analysis: - count data - col data (metadata)

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

Q1. How many genes are in this dataset?

How many transcripts do I have?

```
nrow(counts)
```

```
[1] 38694
```

There are 38694 genes in this dataset.

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

There are 4 'control' cell lines.

#'counts' data:

```
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
```

```
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
```

First, we should check the correspondence of the metadata and count data.

```
metadata$id
```

```
[1] "SRR1039508" "SRR1039509" "SRR1039512" "SRR1039513" "SRR1039516"
[6] "SRR1039517" "SRR1039520" "SRR1039521"
```

```
colnames(counts)
```

```
[1] "SRR1039508" "SRR1039509" "SRR1039512" "SRR1039513" "SRR1039516"
[6] "SRR1039517" "SRR1039520" "SRR1039521"
```

To check that these are all in the same order, we can use '==' test of equality.

```
all(metadata$id==colnames(counts))
```

```
[1] TRUE
```

```
all(c(T,T,T,T,T))
```

```
[1] TRUE
```

Analysis of CONTROL vs TREATED

The "treated" have the dex drug and the "control" do not. First, I need to be able to extract just the "control" columns in the 'counts' data set.

```
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
```

```
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.inds <- metadata$dex=="control"
control <- metadata[control.inds,]
control$id
```

```
[1] "SRR1039508" "SRR1039512" "SRR1039516" "SRR1039520"
```

Now I can use this to access just the "control" columns of my 'counts' data...

```
control.counts <- counts[,control$id]
head(control.counts)
```

```
                SRR1039508 SRR1039512 SRR1039516 SRR1039520
ENSG00000000003        723        904       1170        806
ENSG00000000005          0          0          0          0
ENSG00000000419        467        616        582        417
ENSG00000000457        347        364        318        330
ENSG00000000460         96         73        118        102
ENSG00000000938          0          1          2          0
```

Find the mean count value for each transcript/ gene by binding the 'rowMeans()'

```
control.mean <- rowMeans(control.counts)
head(control.mean)
```

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

Robust code:

```
control.inds <- metadata$dex=="control"
control <- metadata[control.inds,]
control$id
```

```
[1] "SRR1039508" "SRR1039512" "SRR1039516" "SRR1039520"
```

```
control.counts <- counts[,control$id]
head(control.counts)
```

```
                SRR1039508 SRR1039512 SRR1039516 SRR1039520
ENSG00000000003        723        904       1170        806
ENSG00000000005          0          0          0          0
ENSG00000000419        467        616        582        417
ENSG00000000457        347        364        318        330
ENSG00000000460         96         73        118        102
ENSG00000000938          0          1          2          0
```

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)

And now find a mean value for all the treated columns in the same way.

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

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

Now I have 'control.mean' and 'treated.mean'. Let's put them together for safe keeping and ease of use later.

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

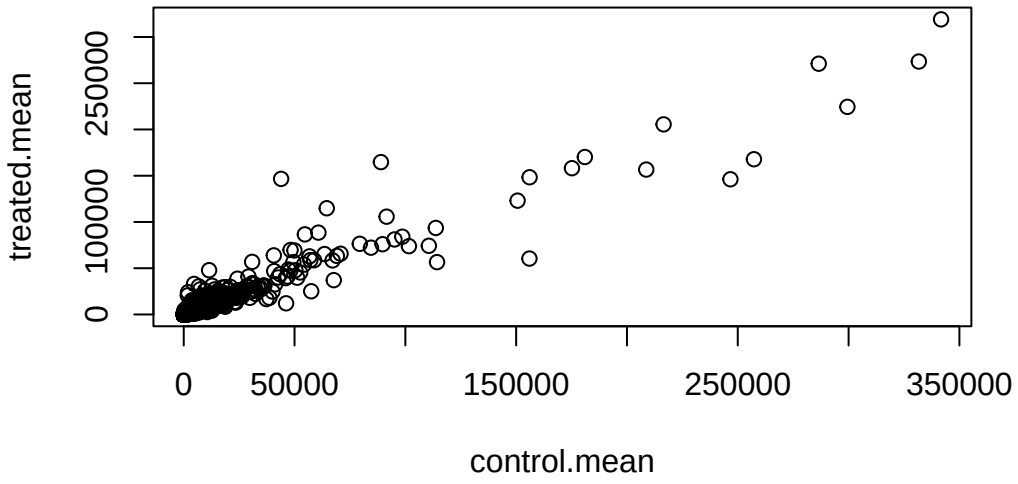
```
colSums(meancounts)
```

```
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.

Let's do a quick plot to see how our data looks.

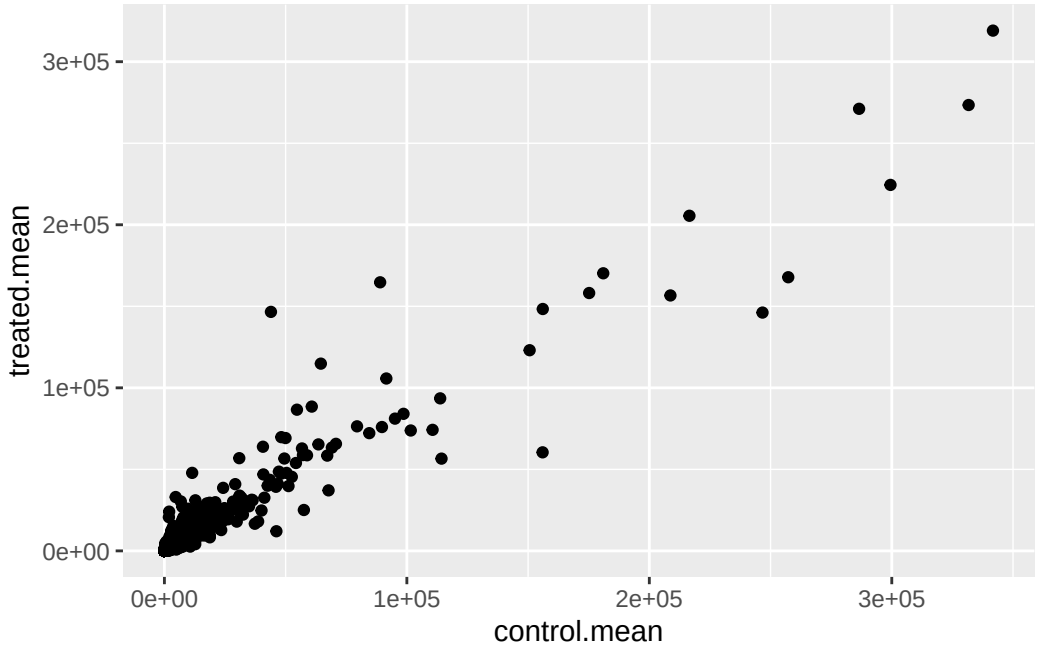
```
plot(meancounts)
```



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?

I would use geom_point for this plot.

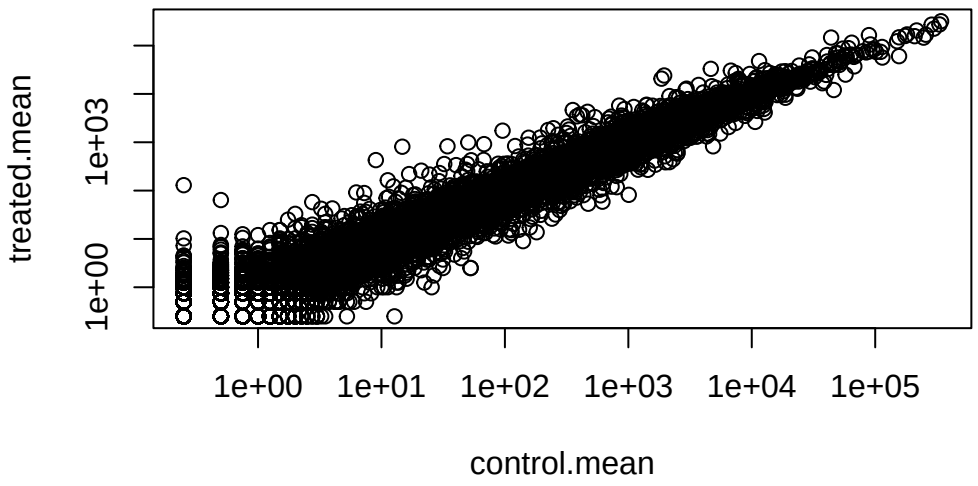
```
library(ggplot2)
ggplot(meancounts, aes(x=control.mean, y=treated.mean)) +
  geom_point()
```




```
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
```



We like working with log transformed data as it can help make things more straightforward to interpret. If we have no change:

```
log2(20/20)
```

```
[1] 0
```

What about if we had a doubling

```
log2(40/20)
```

```
[1] 1
```

Half as much

```
log2(10/20)
```

```
[1] -1
```

```
log2(80/20)
```

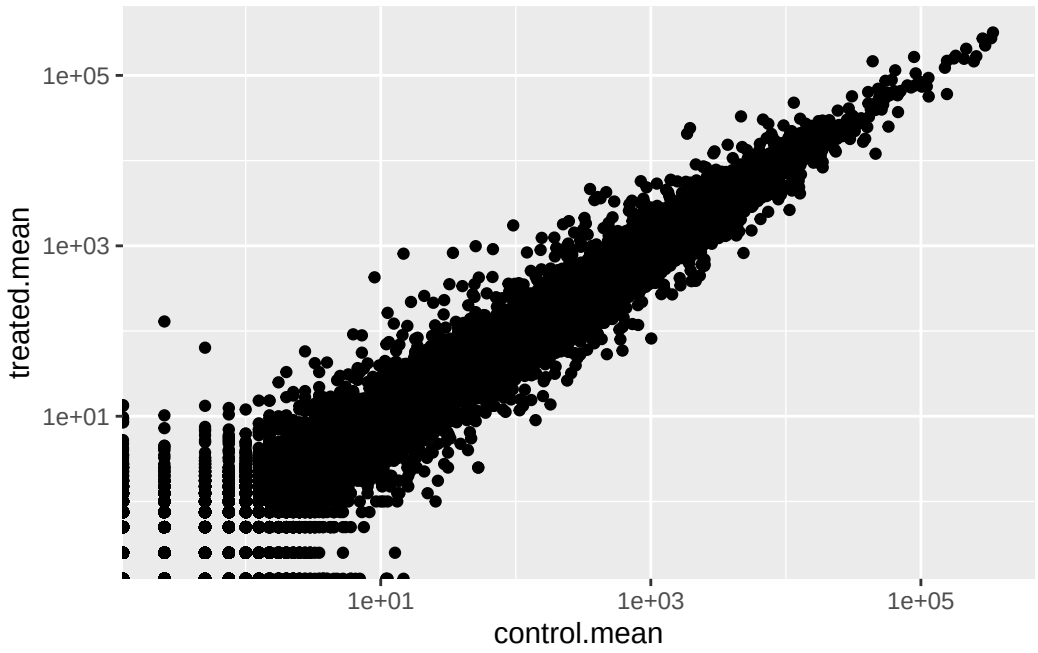
```
[1] 2
```

Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this? Thus is very heavly skewed and over a wide range- calling our for a log transform!

```
library(ggplot2)
ggplot(meancounts, aes(x=control.mean, y=treated.mean)) +
  geom_point()+
  scale_x_continuous(trans="log10")+
   scale_y_continuous(trans="log10")
```

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

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



We like working with log2 fold-change values. Let's calculate them for our data.

```
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
```

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

to.rm <- unique(zero.vals[,1])
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?

The purpose of the arr.ind argument in the which() function call above, is to display all values that are not equal to 0 . We take the first column of the output and need to call unique() function because we want to make sure that each row is included only once (and not twice).

We want to filter out any genees (that is the rows) where we have ZERO count data.

```
to.keep.inds <- rowSums(meancounts[,1:2]==0)==0
head(to.keep.inds)
```

```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
           TRUE           FALSE            TRUE            TRUE            TRUE
ENSG00000000938
          FALSE
```

```
mycounts <- meancounts[to.keep.inds,]
nrow(mycounts)
```

```
[1] 21817
```

A common threshold for calling genes as differentially expressed is a log2 fold-change of +2 or -2.

```
sum(mycounts$log2fc>= +2)
```

```
[1] 314
```

What fraction percent is this?

```
round((sum(mycounts$log2fc>= +2)/nrow(mycounts))*100,2)
```

```
[1] 1.44
```

```
round((sum(mycounts$log2fc<= -2)/nrow(mycounts))*100,2)
```

```
[1] 2.22
```

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

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

```
[1] 250
```

There are 250 up regulated genes we have at the greater than 2 fc level.

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

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

```
[1] 367
```

There are 367 down regulated genes we have at greater than 2 fc level.

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

In all honesty, I don't trust these results. The reason for this is because the results in their current form are likely to be misleading; Fold change can be large without being statistically significant.Also, we have not done anything yet to determine whether the differences we are seeing are significant. Hence, these results are not reliable.

We need some stats to check if the drug induced difference is sigificant

Turn to DESeq2

Let's turn to doing this the correct way with the DESeq2 package.

```
library(DESeq2)
```

The main function in the DESeq2 package is called 'deseq2'. It wants our count data and our colData (metadata) as input in a specific way.

```
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
```

Now I can run DESeq analysis

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

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

```
#results(dds)
```

Now what we have got so far is the log2 fold-change and the adjusted p-value for the significance.

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

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 6 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
                <numeric>
ENSG00000000003  0.163035
ENSG00000000005        NA
ENSG00000000419  0.176032
ENSG00000000457  0.961694
ENSG00000000460  0.815849
ENSG00000000938        NA
```

```
summary(res)
```

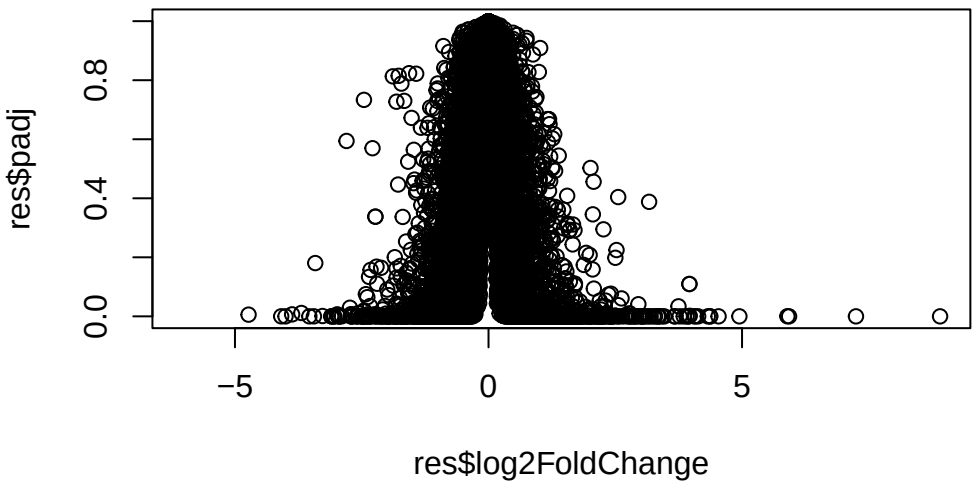
```
out of 25258 with nonzero total read count
adjusted p-value < 0.1
LFC > 0 (up)       : 1563, 6.2%
LFC < 0 (down)     : 1188, 4.7%
outliers [1]       : 142, 0.56%
low counts [2]     : 9971, 39%
(mean count < 10)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of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

```
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
```

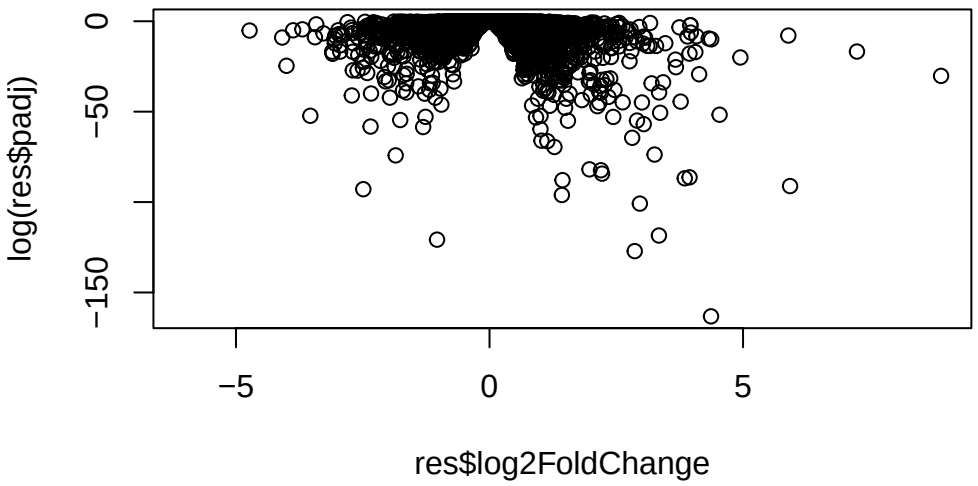
A first plot

```
plot(res$log2FoldChange, res$padj)
```



Well that plot sucked. All the interesting P-values are down below zero. I am going to take the log of the p-value.

```
plot(res$log2FoldChange, log(res$padj))
```

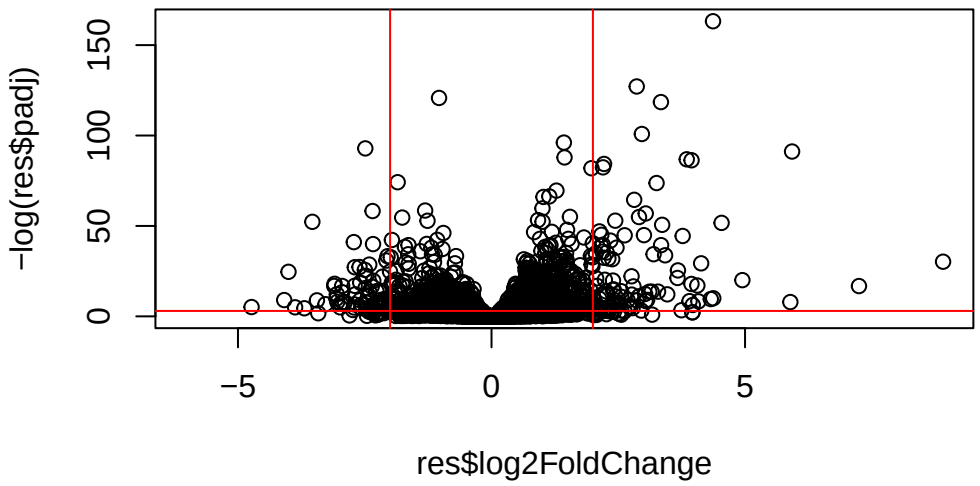



```
log(0.05)
```

```
[1] -2.995732
```

We can flip the y-axis so the plot does not look "upside down".

```
plot(res$log2FoldChange, -log(res$padj))
abline(v=c(-2,+2),col="red")
abline(h=-log(0.05),col="red")
```

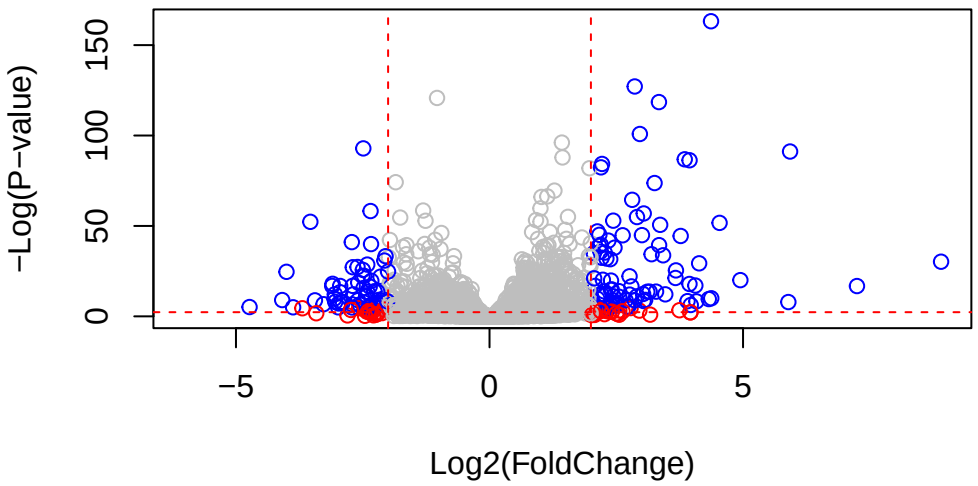



```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red"

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="red", lty=2)
abline(h=-log(0.1), col="red", lty=2)
```


−Log(P−value)
0
50
100
150
−5
0
5
Log2(FoldChange)