Class 13: RNASeq Analysis

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The data for today's lab comes from a published RNA-seq experiment where airway smooth muscle cells were treated with dexamethasone, a synthetic glucocorticoid steroid with anti-inflammatory effects.

Import Data

We need two things for this analysis: counts and metadata these are called "countData" and "colData" in the DESeq2 world

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

```
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
```

The counts are organized with a gene per row and experiment per column.

```
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
```

Examine Data

> 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
```

Check on match of metadata and coldata

```
colnames(counts)
```

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

```
metadata$id
```

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

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

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

If you want to know that all the elements are TRUE we can use the 'all()' function

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

```
[1] TRUE
```

Analysis

I want to start by comparing "control" and "treated" columns. To this I will find the average for each gene (row) in all "control" columns. Then I will find the average in the "treated" columns. Then I will compare them.

Let's extract all "control" columns first.

```r
control.inds <- metadata$dex == "control"
```

```r
control.counts <- counts[,control.inds]
```

Now find the mean value per gene using the 'apply()' function

```r
control.mean <- apply(control.counts, 1, mean)
```

> Q4. Extract all "treated" columns next.

```r
treated.inds <- metadata$dex == "treated"
```

```r
treated.counts <- counts[,treated.inds]
```

Now find the mean value per gene using the 'apply()' function

```r
treated.mean <- apply(treated.counts, 1, mean)
```

Put these two vectors together for ease of book-keeping

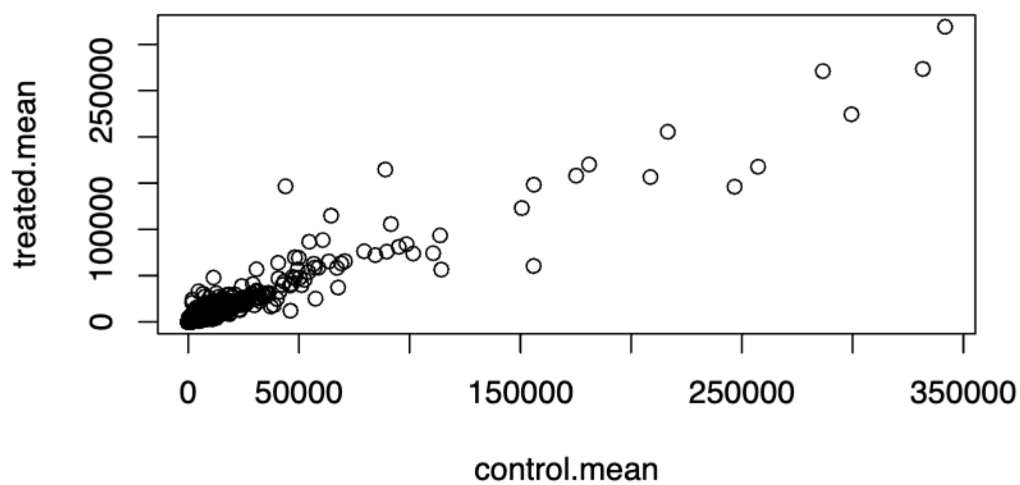
```r
meancounts <- data.frame(control.mean, treated.mean)
head(meancounts)
```

```
                control.mean treated.mean
ENSG00000000003       900.75       658.00
ENSG00000000005         0.00         0.00
ENSG00000000419       520.50       546.00
ENSG00000000457       339.75       316.50
ENSG00000000460        97.25        78.75
ENSG00000000938         0.75         0.00
```

Let's have a look with a quick plot

> Q5 (a). Create a scatter plot showing the mean of the treated samples against the mean of the control samples.

```r
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?

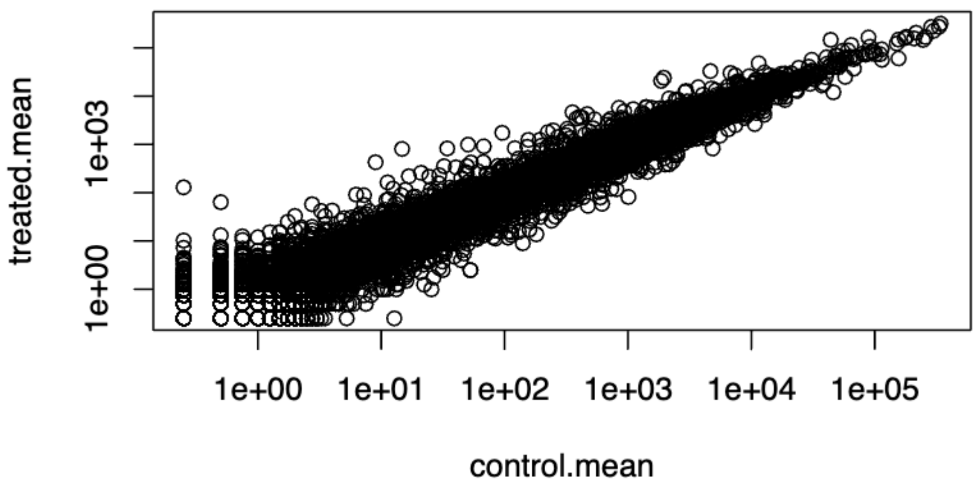
geom_point()

Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

```r
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 most often work with log2 units because they have a more simple interpretation.

```r
log2(10/10)
```

```
[1] 0
```

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

```
[1] 1
```

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

```
[1] -1
```

Here we calculate log2 Fold-change of treated/control values and add it to our data frame of results.

```r
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 some weird answers in here like NaN (not a number) and -Inf (minus infinity) that all come because I have zero count genes in my dataset.

It is common practice to filter these zero count genes out before we go too deep.

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

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

> Q. How many genes do we have left after zero count filtering?

```r
nrow(mycounts)
```

```
[1] 21817
```

A common threshold for calling a gene "up" or "down" is a log2 fold change of +2 or -2.

> Q8. How many "up" regulated genes do we have?

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

```
[1] 314
```

> Q9. How many "down" regulated genes do we have?

```r
sum(mycounts$log2fc >= -2)
```

```
[1] 21450
```

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

No, missing standard deviation of the results to determine if the difference is significant

DESeq Analysis

We need to do this analysis properly to keep our inner stats person happy.

```r
#/ message: false
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, aperm, 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 object is masked from 'package:utils':

    findMatches

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
```

To use DESeq we need to get our input data in a very particular format.

```r
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
```

Run DESeq analysis

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

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

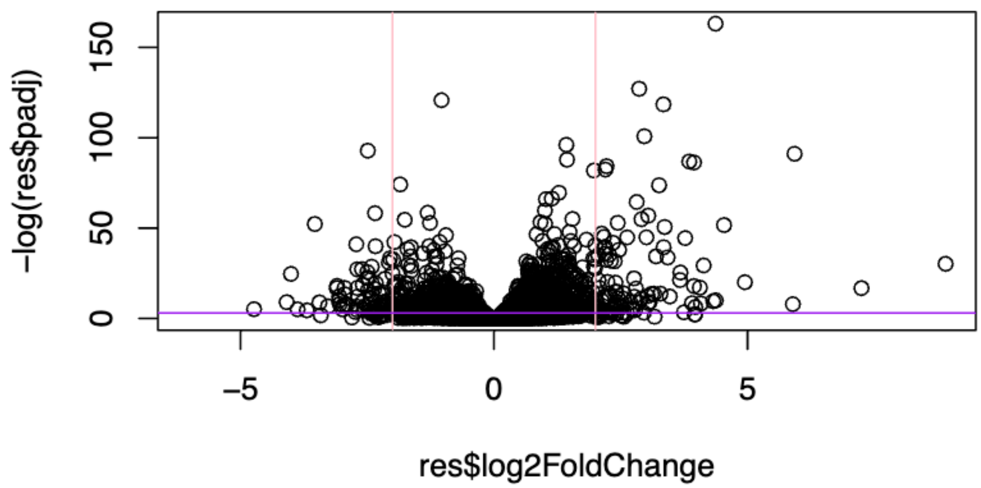
Get the results

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

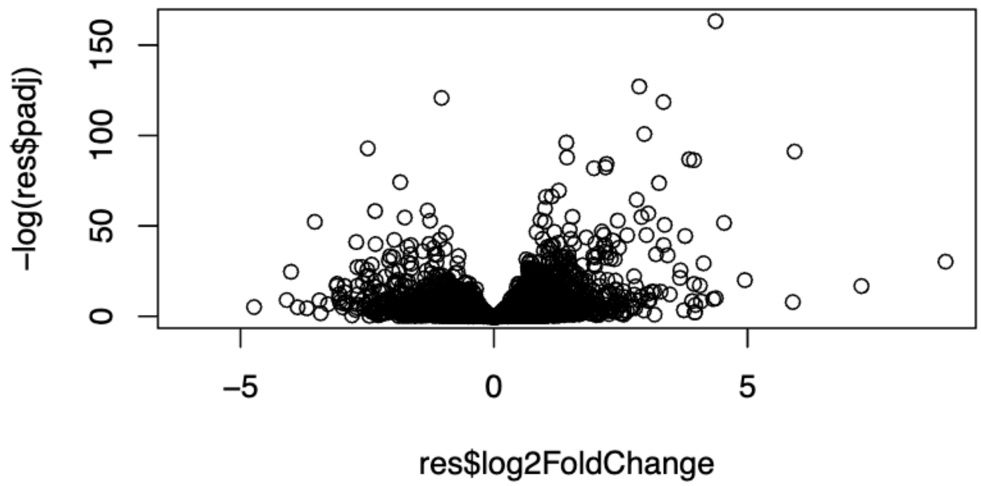
```
class: DESeqDataSet
dim: 6 8
metadata(1): version
assays(4): counts mu H cooks
rownames(6): ENSG00000000003 ENSG00000000005 ... ENSG00000000460
  ENSG00000000938
rowData names(22): baseMean baseVar ... deviance maxCooks
colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
colData names(5): id dex celltype geo_id sizeFactor
```

I want to make a figure showing an overview of all my results to date. A plot of **log2 fold change** vs the **p-value** (adjusted p-value)

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




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

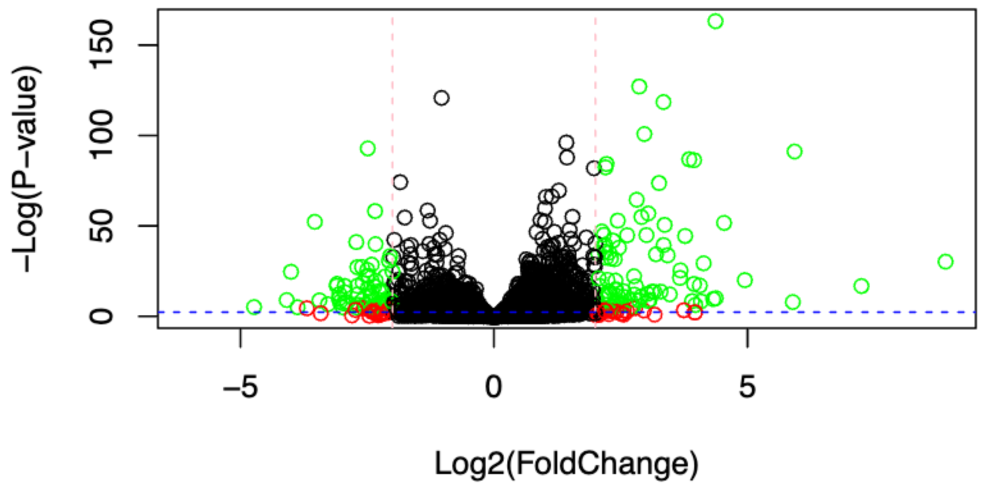



```
# Setup our custom point color vector
mycols <- rep("black", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red"

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

# 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="pink", lty=2)
abline(h=-log(0.1), col="blue", lty=2)
```



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