Class 13: RNASeq with DESeq2

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Today we will analyze some RNASeq data from Himes et. al. on the effects of dexamethasone (dex), a synthetic glucocorticoid steroid on airway smooth muscle cells (ASM).

Data import

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

A wee peak

```r
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
```

```r
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
```

> Q1. How many genes are in this dataset?

```r
nrow(counts)
```

```
[1] 38694
```

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

```r
sum(metadata$dex == "control")
```

```
[1] 4
```

```r
table(metadata$dex)
```

```

control treated 
      4       4 
```

Toy differential expression analysis

Calculate the mean per gene count values for all "control" samples (i.e. columns in counts) and do the same for "treated" and then compare them.

1. Find all "control" values/columns in counts

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

> Q3. How would you make the above code in either approach more robust? Is there a function that could help here?

To make the code more robust, you would use the apply function to apply the mean to the column above.

2. Find the mean per gene across all control columns.

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

> 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)

3. Do the same steps to find the `treated.mean` values.

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

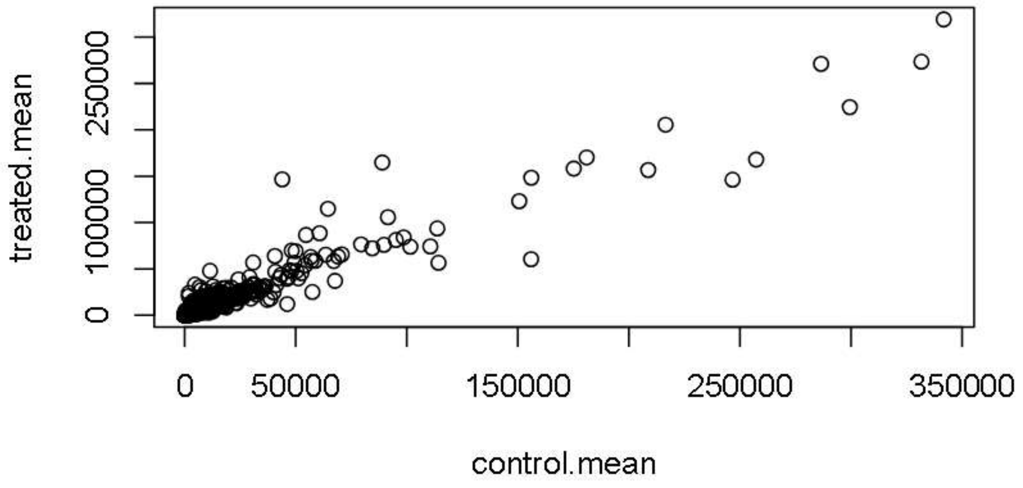
```
                SRR1039509 SRR1039513 SRR1039517 SRR1039521
ENSG00000000003        486        445       1097        604
ENSG00000000005          0          0          0          0
ENSG00000000419        523        371        781        509
ENSG00000000457        258        237        447        324
ENSG00000000460         81         66         94         74
ENSG00000000938          0          0          0          0
```

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

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

> 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)
```



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?

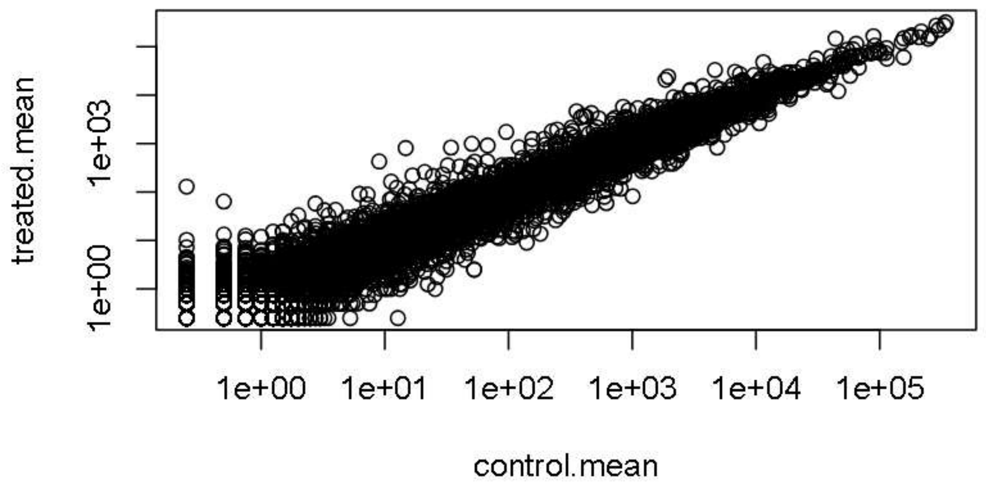
geom_(point)

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

```
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 frequently use log2 transformtions for this type of data.

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

```
[1] 0
```

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

```
[1] 1
```

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

```
[1] -1
```

These log 2 values make the interpretation of "fold-change" a little easier and a rule-of-thumb in the filed is a log2 fold-change of +2 or -2 is where we start to pay attention.

```
log2(40/10)
```

```
[1] 2
```

Let's calculate the log2(fold-change) and add it to our `meancounts` data.frame.

```
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
```

```
# meancounts[,1:2]==0
```

```
to.rm <- rowSums(meancounts[,1:2]==0) > 0
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 rgument in the which() function call above is to return both the row and column indices (i.e. positions) where there are TRUE values. Genes (rows) and samples (columns) with 0 counts will be displayed. Any genes that have 0 counts in any sample will be ignored (focus on the row answer). Calling unique() means no row will be counted twice if it has zero entries in both samples.

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

```r
nrow(mycounts)
```

```
[1] 21817
```

> Q. How many genes are "up" regulated upon drug treatment at a threshold of +2 log2-fold-change?

1. I need to extract the log2fc values
2. I need to find those that are above +2
3. Count them

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

```
[1] 250
```

> Q. How many genes are "down" regulated upon drug treatment?

```r
sum(mycounts$log2fc < -2)
```

```
[1] 367
```

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

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

```
[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?

```r
sum(mycounts$log2fc < -2)
```

```
[1] 367
```

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

I do not trust these results because fold-chain can be large without being statistically significant as well as missing the stats of whether the differences we are seeing are significant. In order to obtain the stats, we need to use the DESeq2 package.

Wow hold on we are missing the stats here. Is the difference in the mean counts significant?

Let's do this analysis the right way with stats and use the **DESeq2** package

DESeq analysis

```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, saveRDS, setdiff,
    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


Attaching package: 'IRanges'

The following object is masked from 'package:grDevices':

    windows

Loading required package: GenomicRanges

Loading required package: GenomeInfoDb

Loading required package: SummarizedExperiment

Loading required package: MatrixGenerics

Loading required package: matrixStats

Warning: package 'matrixStats' was built under R version 4.4.2


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
```

The first function that we will use will setup the data in the way (format) DESeq wants it.

```
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
```

The function in the package is called `DESeq()` and we can run it on our `dds` object.

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

```
estimating size factors

estimating dispersions
```

```
gene-wise dispersion estimates

mean-dispersion relationship

final dispersion estimates

fitting model and testing
```

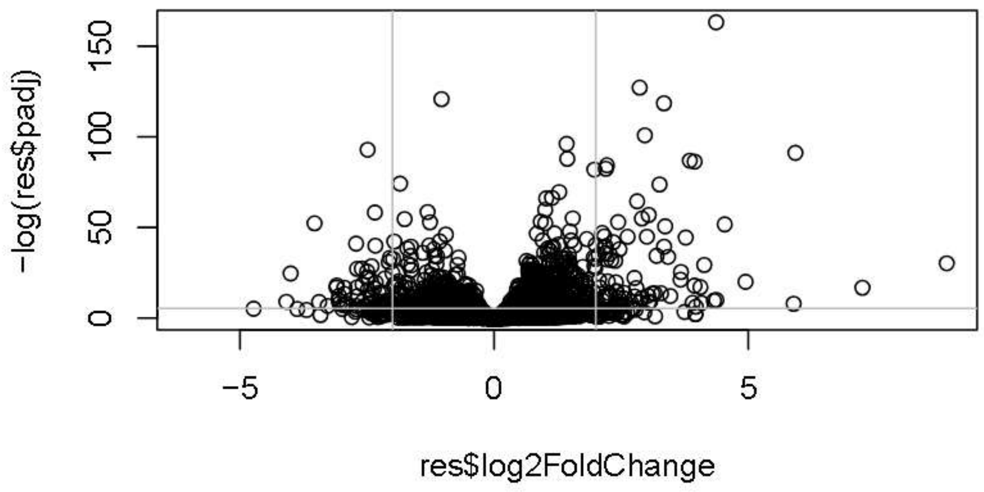
I will get the results from dds with the `results()` function:

```r
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
```

Make a common overall results figure from this analysis. This is designed to keep our inner biologist and inner stats nerd happy.

```r
plot(res$log2FoldChange, -log(res$padj))
abline(v=c(-2,2), col="gray")
abline(h= -log(0.005), col="gray")
```



```
log(0.0005)
```

```
[1] -7.600902
```

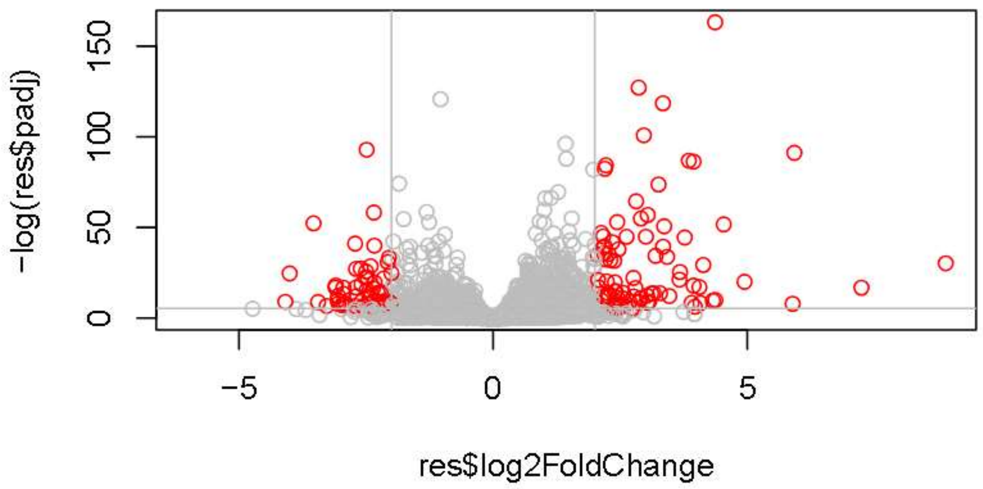
```
log(0.00000000005)
```

```
[1] -23.719
```

Add some color to this plot:

```
mycols <- rep("gray", nrow(res))
mycols[res$log2FoldChange > 2] <- "red"
mycols[res$log2FoldChange < -2] <- "red"
mycols[res$padj > 0.005] <- "gray"

plot(res$log2FoldChange, -log(res$padj), col=mycols )
abline(v=c(-2,2), col="gray")
abline(h= -log(0.005), col="gray")
```



I want to save my results to date out to disc

```
write.csv(res, file="myresults.csv")
```

We will pick this up next day and add annotation i.e. what are these genes of interest) and do pathway analysis (what biolog) are they known to be involved with.