Class 12 : Transcriptomics and the analysis of RNA-Seq data

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Here we will use the DESeq2 package for RNASeq analysis.

Import Data:

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

We need two things for this analysis: - countData(called counts for me) (counts for every transcript/gene in each experiment) - colData (metadata that describes the experimental setup)

Take a Look at Each:

```
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
```

> Q1. How many genes are in this dataset?

```
nrow(counts)
```

```
[1] 38694
```

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

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

```
control treated
      4       4
```

Another way:

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

```
[1] 4
```

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

Look at code below.

- Step 1: Calculate the mean of the control samples (i.e.columns in countData) Calculate the mean of the treated samples

a. We need to find which columns are "control" samples.

- look in metadata (aka colData), $dex column

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

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

b. Extract all the control columns from `countData` and call it `control.counts`.

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

c. Calculate the mean value across the rows of `control.counts` i.e. calculate the mean count values for each gene in the control samples

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

```
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)

- Step 2: Calculate the mean of the **treated** samples.

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

```
ENSG00000000003 ENSG00000000005 ENSG00000000419
```

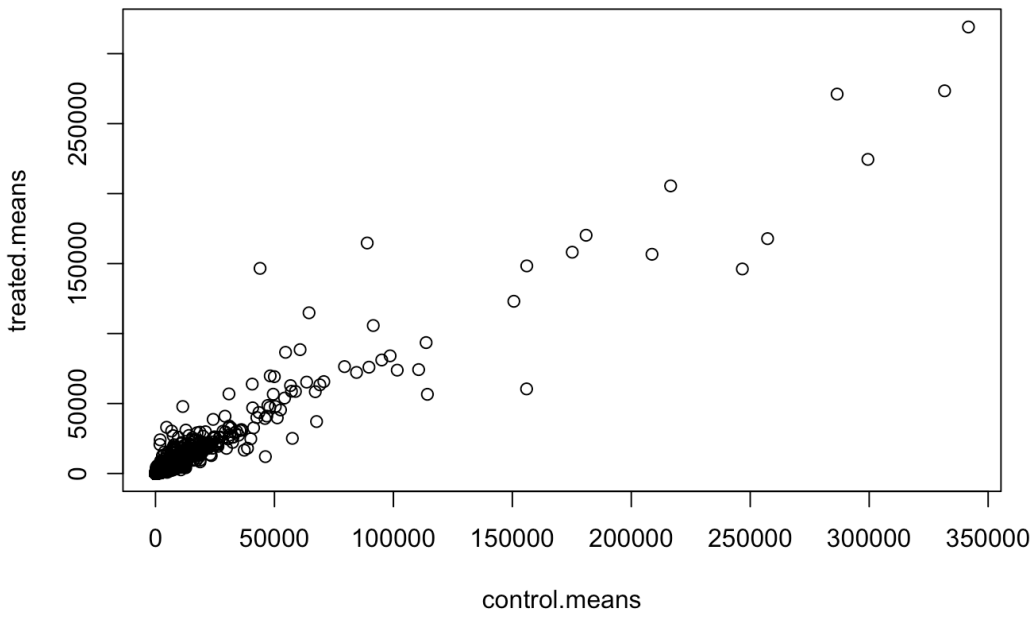
```
ENSG00000000457 ENSG00000000460
         658.00             0.00          546.00
316.50           78.75
ENSG00000000938
           0.00
```

We now have control and treated mean count values. For ease of book keeping, we will combine these vectors in to a new data.frame called `meancounts`.

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

> 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)
```



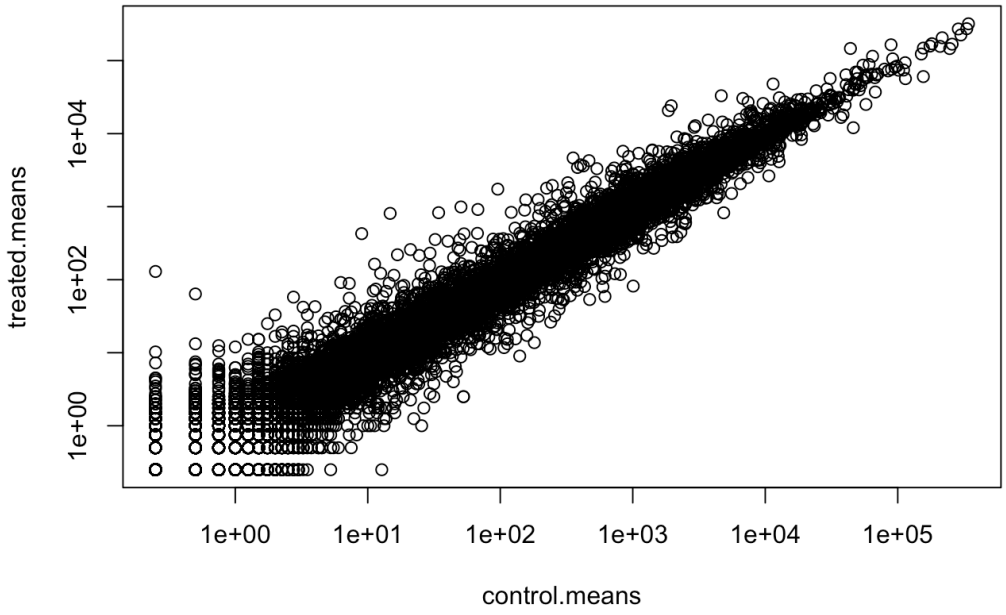
> Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

log

```
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?

geom_point

We use log transforms for skewed data such as this and because we really care most about relative changes in magnitude.

We must often use log2 as our transform as the math is easier to interpret than log10 or others.

If we have no change i.e. some values in control and treated we will have...

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

```
[1] 0
```

If I have double the amount i.e. 20 compared to 10 for example I will have a log2 fold-change of +1:

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

```
[1] 1
```

If I have half the amount I will have a log2 fold-change of -1:

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

```
[1] -1
```

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

```
[1] 2
```

```
meancounts$log2fc <- log2(meancounts$treated.means / meancounts
head(meancounts)
```

```
                control.means treated.means      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
```

> Q8. How many genes are up regulated at the common threshold of +2 log2FC values?

```
sum(meancounts$log2fc >= 2, na.rm=TRUE)
```

```
[1] 1910
```

> Q9.

```
sum(meancounts$log2fc <= 2, na.rm=TRUE)
```

```
[1] 23412
```

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

No because we have not done anything yet to determine whether the differences we are seeing are significant.

Hold on what about the stats?! Yes these are big changes but are these changes significant!?

To do this properly we will turn to **DESeq2** package.

##DESeq2 analysis:

```
library(DESeq2)
```

To use DESeq we need our input countData and colData in a specific format that DESeq wants:

```
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
```

To run the analysis, I can now use the main DESeq2 function called `DESeq()` with `dds` as input.

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

```
estimating size factors
```

```
estimating dispersions

gene-wise dispersion estimates

mean-dispersion relationship

final dispersion estimates

fitting model and testing
```

To get the results out of this `dds` object, we cna use the `results()` function from the package.

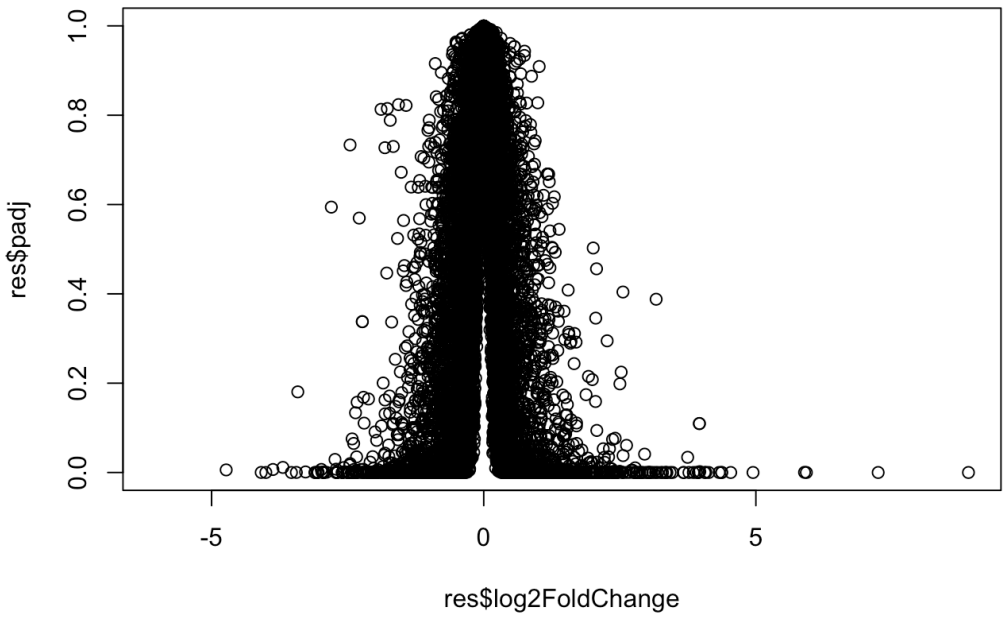
```
res <- results(dds)
head(res)
```

```
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
```

##VOLCANO PLOT

Let's make a final (for today) plot of log2 fold-change vs the adjusted P-value.

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

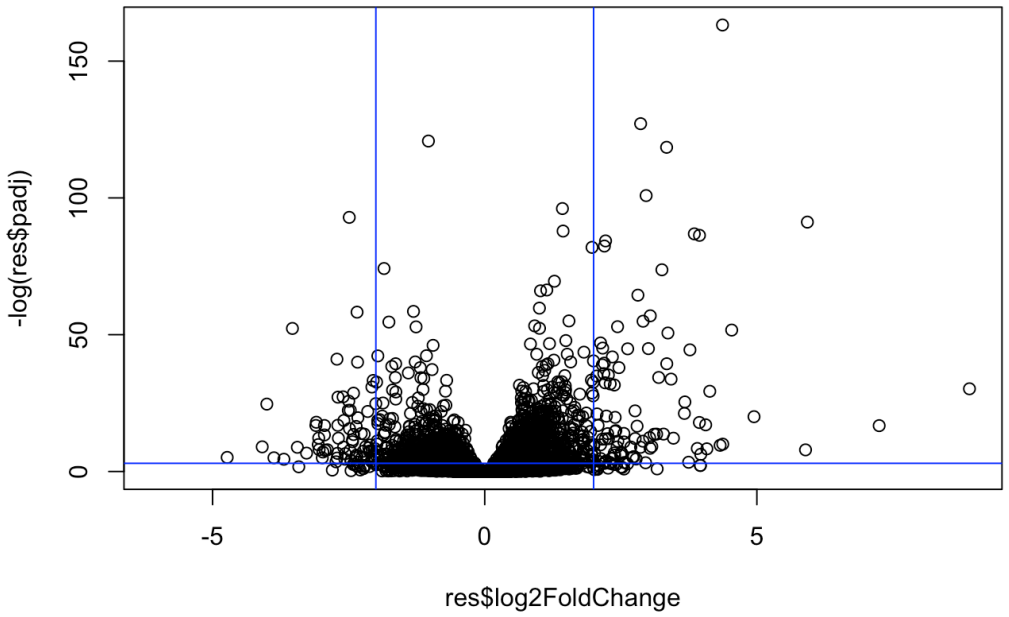


It is the low P-values that we care about and these are lost in the skewed plot above. Lets take the log of our `res$padj` values for our plot.

Add negative sign.

Volcano Plot!:

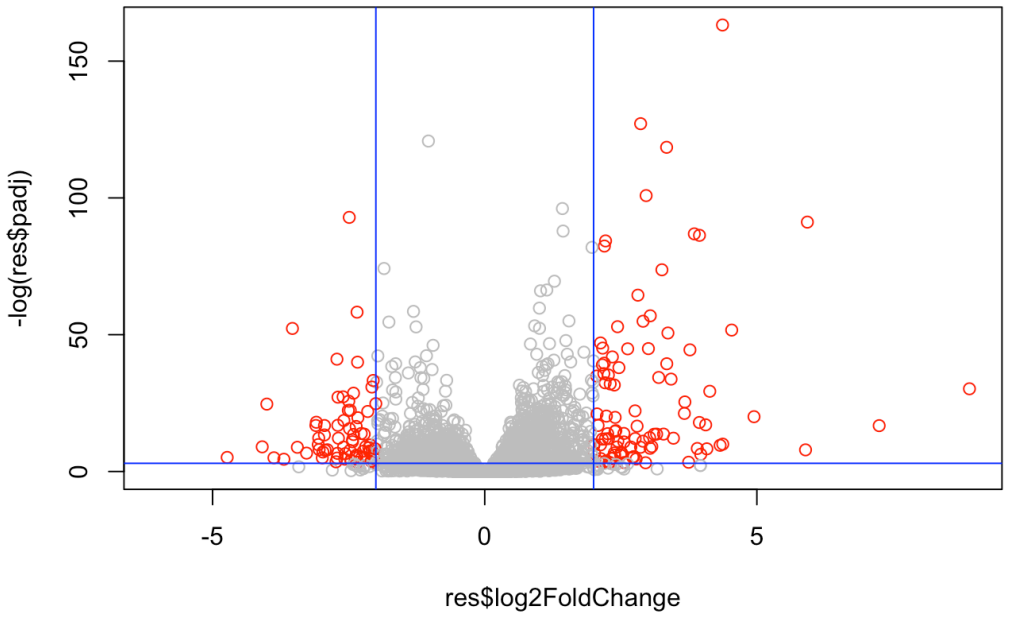
```
plot(res$log2FoldChange, -log(res$padj))
abline(v=c(+2,-2), col="blue")
abline(h=(-log(0.05)), col="blue")
```



Finally we can make a color vector to use in the plot to better highlight the genes we care about.

```
mycols <- rep("gray", nrow(res))
# Or use abs for one of these:
mycols[res$log2FoldChange >= 2] <- "red"
mycols[res$log2FoldChange <= -2] <- "red"
mycols[res$padj > 0.05] <- "gray"

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


-log(res$padj)
150
100
50
0
-5
0
5
res$log2FoldChange