Class 12

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Table of contents

Background

Today we will analyze some RNASeq data from Himes et al. on the effects of a common steroid (dexamethasone also called "dex") on airway smooth muscle cells (ASMs).

For this analysus we need two main inputs

- `countData`: a table of counts per gene (in rows) across experiments (in collumns)
- `colData`: **metadata** about the design of the experiments. The rows must match the columns in `countData`

Data Import

```
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
```

```
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 experiments (i.e columns in `counts` or rows in `metadata`) are there?

```
ncol(counts)
```

```
[1] 8
```

Q3 (Q2 in lab sheet). How many "control" experiments are there in this dataset?

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

```
[1] 4
```

Toy analysis example

1. Extract the "control" columns from `counts`
2. Calculate the mean value for each gene in these "control" columns

3-4. Do the same for the "treated" columns 5. Compare these mean values for each gene

Step 1.

```
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
```

Step 2.

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

Q4:

Step3.

```
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
```

Step 4.

```
treated.mean <- rowMeans(treated.counts)
```

For ease of book-keeping, we can store these together in one data frame called `meancounts`

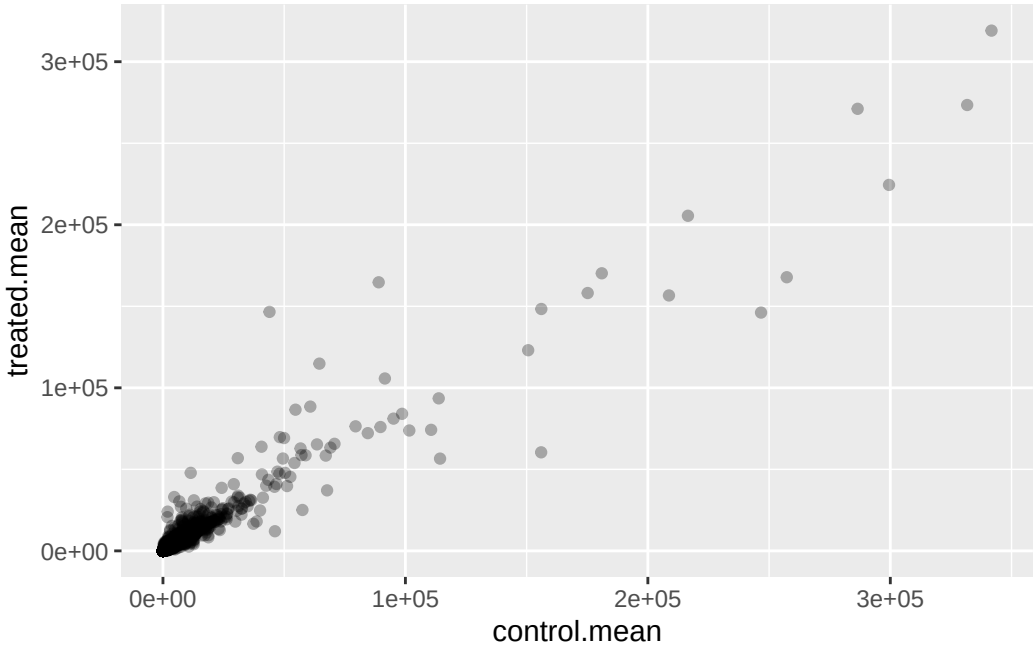
```
meancounts <- data.frame(control.mean, treated.mean)
head(meancounts)
```

```
                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
```

Q5: Step 5. Plot these against each other

```
library(ggplot2)

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

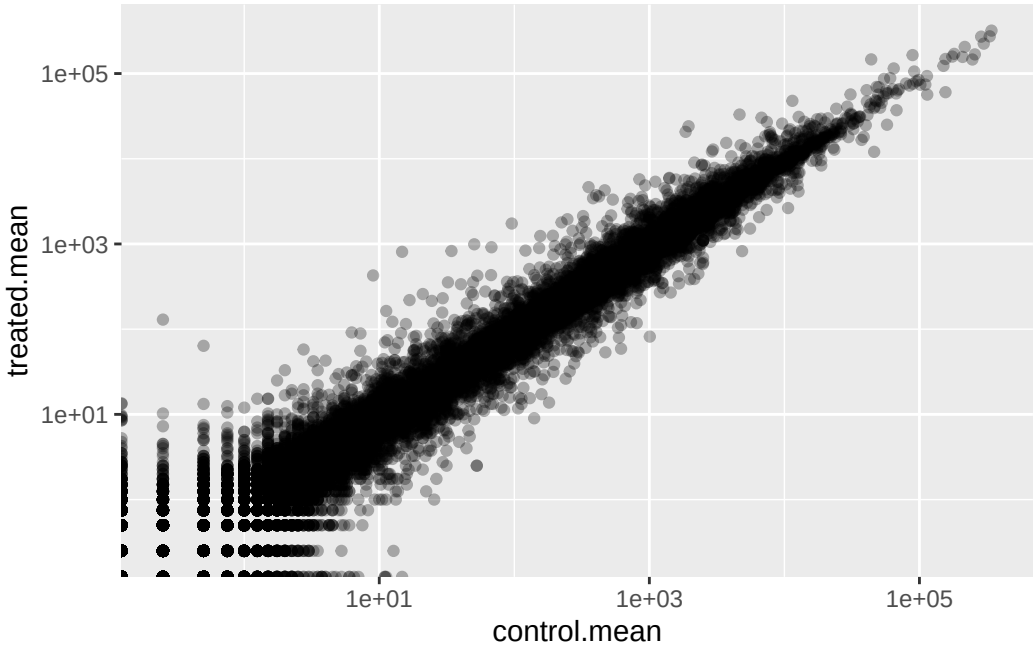


Q6.

```
#use log scale for both axes
ggplot(meancounts)+
  aes(control.mean, treated.mean)+
  geom_point(alpha=0.3)+
  scale_x_log10()+
  scale_y_log10()
```

```
Warning in scale_x_log10(): log-10 transformation introduced infinite values.
```

```
Warning in scale_y_log10(): log-10 transformation introduced infinite values.
```



We used "fold-change" as a way to compare - we usually use log2 units for fold change

```
#treated/control
log2(10/10)
```

```
[1] 0
```

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

```
[1] 1
```

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

```
[1] -1
```

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

```
[1] 2
```

```
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
```

A common "rule-of-thumb" threshold for calling something "up" regulated is a log2-fold change of +2 or greater. For "down" regulated the log2-fold change is -2 or less.

```
#filter out the NaNs and -inf values
nonzero.inds <- rowSums(counts) !=0
mycounts <-  meancounts[nonzero.inds,]
```

```
#alternate method
zero.inds <- which(meancounts[,1:2]==0, arr.ind=T)[,1]

mygenes <- meancounts[-zero.inds,]
```

> 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 arr.ind=TRUE allows us to get the row and column indeces where there are zero counts. We only need the row answer since that represents genes that have zero counts. The unique() function prevents rows from being counted twice if there is more than one zero within it.

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

```
sum(mygenes$log2fc >= 2)
```

```
[1] 314
```

> Q9. How many genes are "down" regulated at the -2 log2FC threshold?

```
sum(mygenes$log2fc <= -2)
```

```
[1] 485
```

Q10. Do you trust these results?

I do not trust these numbers until we have seen whether the difference is statistically significant.

DESeq analysis

Let's do this with DESeq2 and put some stats behind these numbers.

```
library(DESeq2)
```

```
Warning: package 'matrixStats' was built under R version 4.5.2
```

DESeq wants 3 things for analyis: 1. countData 2. colData 3. design

```
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 main function in the DESeq package to run analysis is called `DESeq()`.

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

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

```
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
```

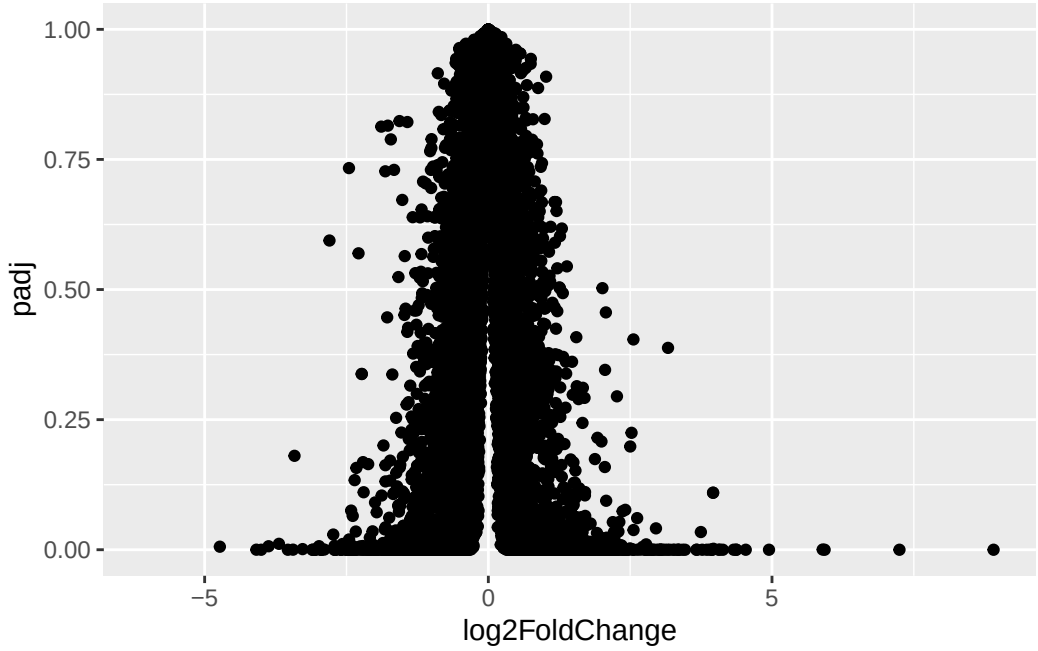
Note: the padj is corrected for multiple testing

Volcano plot

This is a plot of log2FC vs adjusted p-value

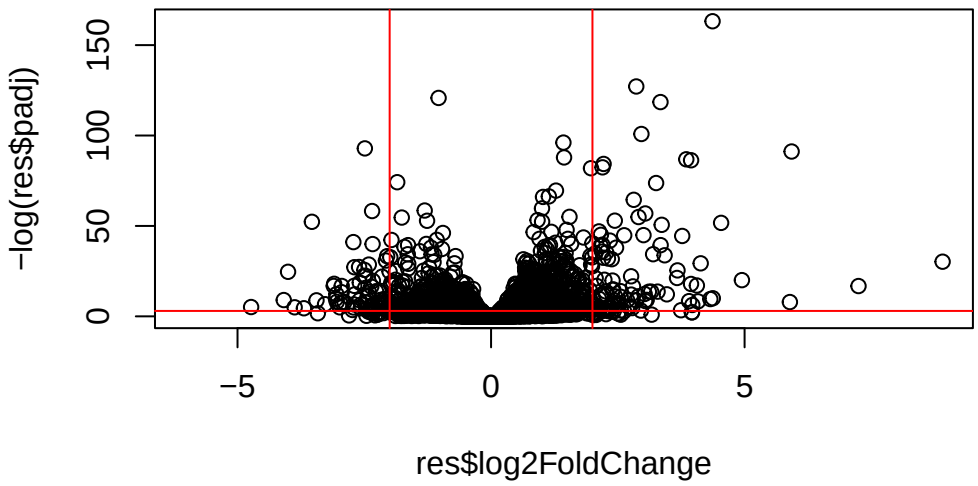
```
ggplot(res)+
  aes(log2FoldChange, padj)+
  geom_point()
```

```
Warning: Removed 23549 rows containing missing values or values outside the scale range
(`geom_point()`).
```



We care about the small p-values, so let's take the log

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



A nicer ggplot volcano plot

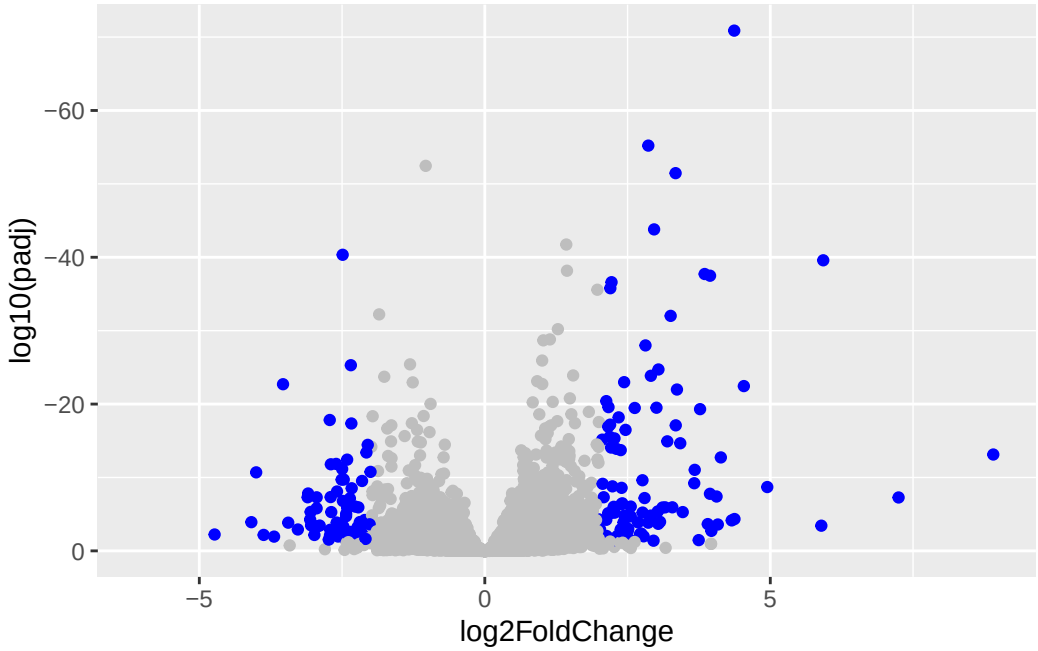
```
mycols <-  rep("gray", nrow(res))

#makes points that have logFC above or below 2 blue
mycols[abs(res$log2FoldChange) > 2] <- "blue"

#make the points that are not significant gray
mycols[res$padj >= 0.05] <- "gray"

ggplot(res)+
  aes(log2FoldChange, log10(padj))+
  geom_point(col=mycols)+
  scale_y_reverse()
```

```
Warning: Removed 23549 rows containing missing values or values outside the scale range
(`geom_point()`).
```



Save our results

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