# class 13: RNASeq Analysis

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The data for this hands-on session comes from a 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).

## 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 |

```
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
```

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

```
control treated
      4       4
```

### Check on the match of metadata with the counts data

```
colnames(counts)
```

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

```
metadata$id
```

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

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

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

If you want to know all the elements of a vector are TRUE we can use the `all()` function.

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

```
[1] FALSE
```

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

```
[1] TRUE
```

## Analysis

I want to start by comparing "control" and "treated" columns. To this I will first 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.

Lets extract the "control" columns first

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

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

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

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

Now do the same for the "treated" columns ie find the `treated.mean` values

```
treated.mean <- apply(counts[,metadata$dex == "treated"], 1, mean)
```

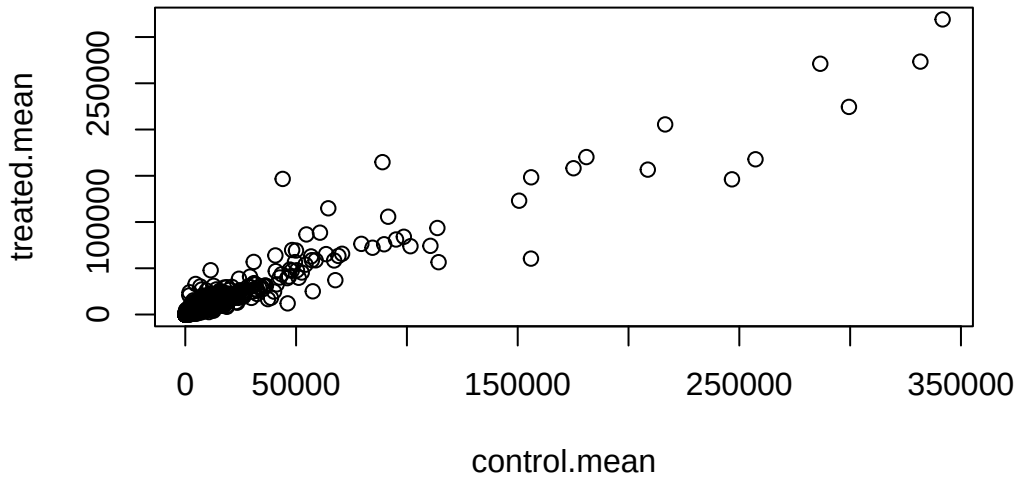
Put these two vectors together for ease of book-keeping

```
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
```

Lets have a wee look with a quick plot

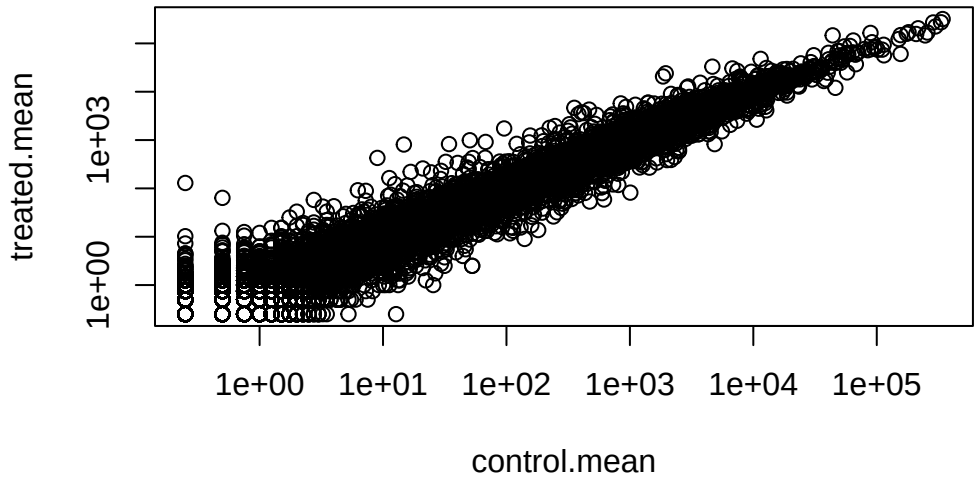
```
plot(meancounts)
```



```
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
```




```
log(10, base=2)
```

```
[1] 3.321928
```

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

```
[1] 0
```

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

```
[1] 1
```

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

```
[1] -1
```

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

```
[1] 2
```

We most often work in log2 units because they have a more simple interpretation.

Here we calculate the log2 fold change of treated/control values add it to our wee data frame of results.

```
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 funky answers in there 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.

```
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 genes filtering?

```
nrow(mycounts)
```

```
[1] 21817
```

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

Q. How many "up" regulated genes do we have?

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

```
[1] 314
```

Q. How many "down" regulated genes do we have?

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

```
[1] 485
```

## DESeq2 Analysis

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

```
library(DESeq2)
```

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

```
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

```
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

```
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
```

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="gray")
abline(v=+2, col="gray")
abline(h=-log(0.05), col="gray")
```