class13

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Background

Today, we will analyze some RNA Sequencing data on the effects of a common steroid drug on airway cell lines.

There are two main inputs we need for this analysis:

- `countData`: counts for genes in rows with experiments in the columns
- `colData`: or metadata that tells us abut the design of the experiment (i.e. what is in the coolumns of `countData`)

```
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 'control' cell lines do we have?

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

```
control treated
      4       4
```

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

```
[1] 4
```

4 'control' cell lines.

Toy differential gene expression

Let's try finding the mean of the "control" and "treated" columns and see if they differ.

The `$dex` column tells me whether we have "control" or "treated"

```
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
```

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

```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
         900.75            0.00          520.50          339.75           97.25
ENSG00000000938
           0.75
```

Q3. Do the same for "treated" to get a `treated.mean`

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

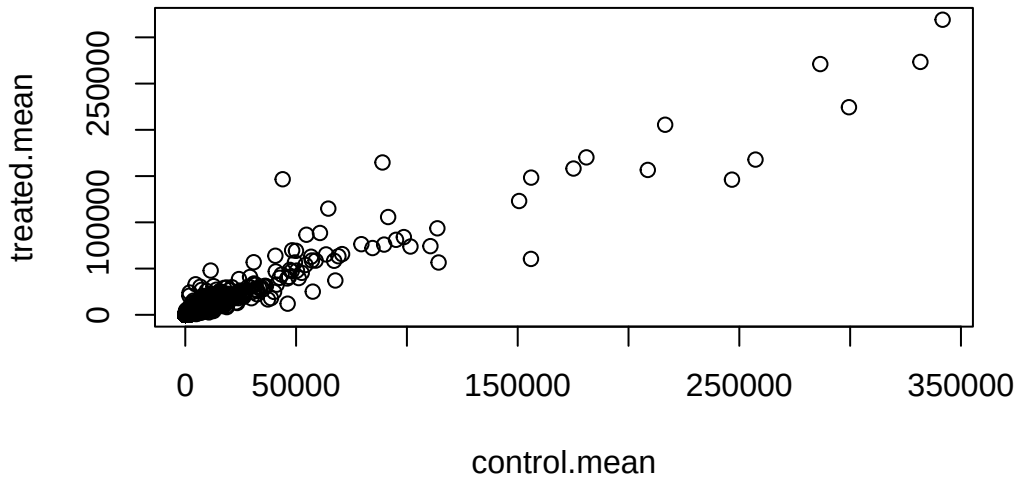
```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
         658.00            0.00          546.00          316.50           78.75
ENSG00000000938
           0.00
```

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

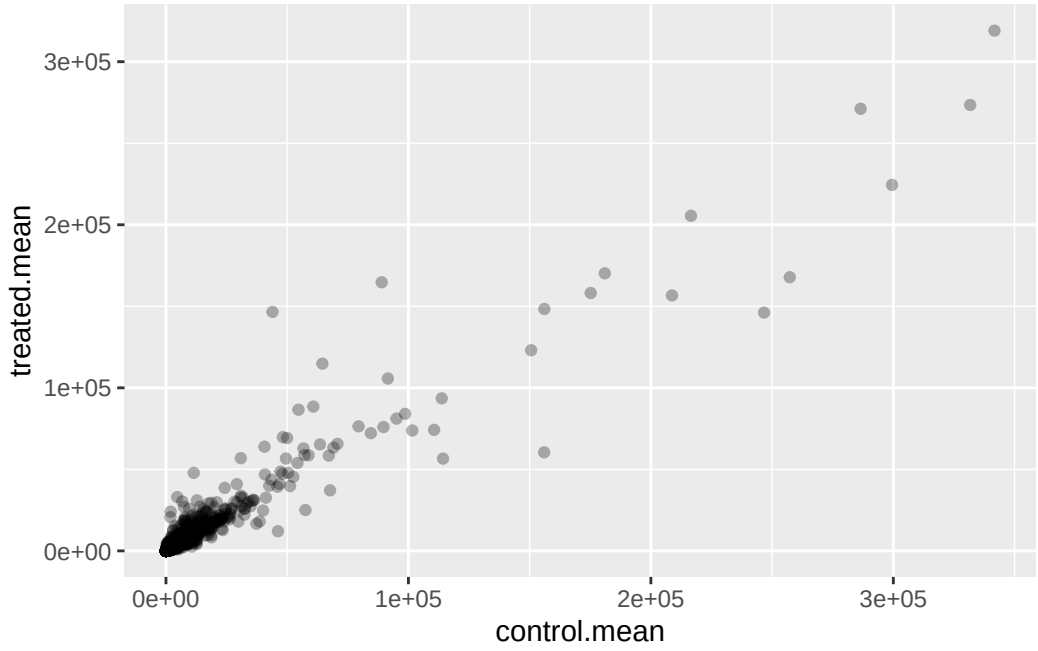
```
control.mean treated.mean
    23005324     22196524
```

Q4. Make a plot of `control.mean` vs `treated.mean`

```
plot(meancounts)
```

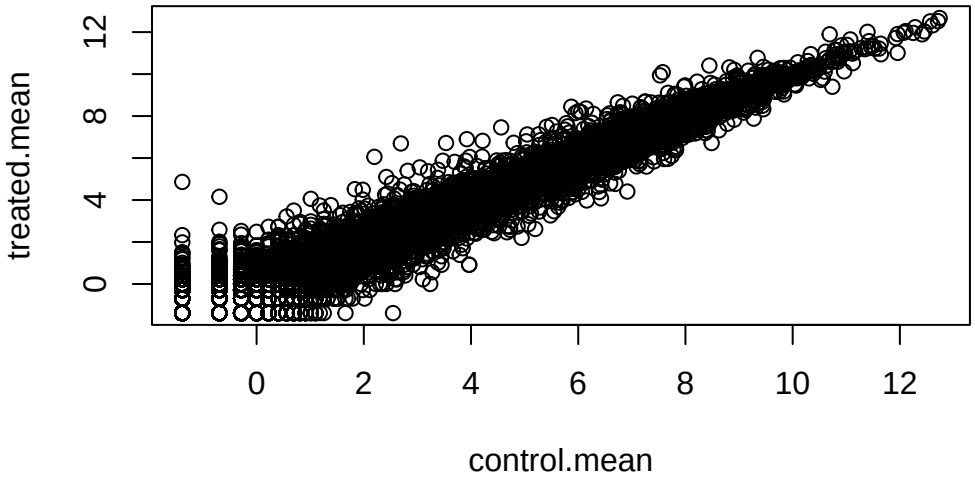



```
library(ggplot2)
ggplot(meancounts, aes(control.mean, treated.mean)) +
  geom_point(alpha=0.3)
```



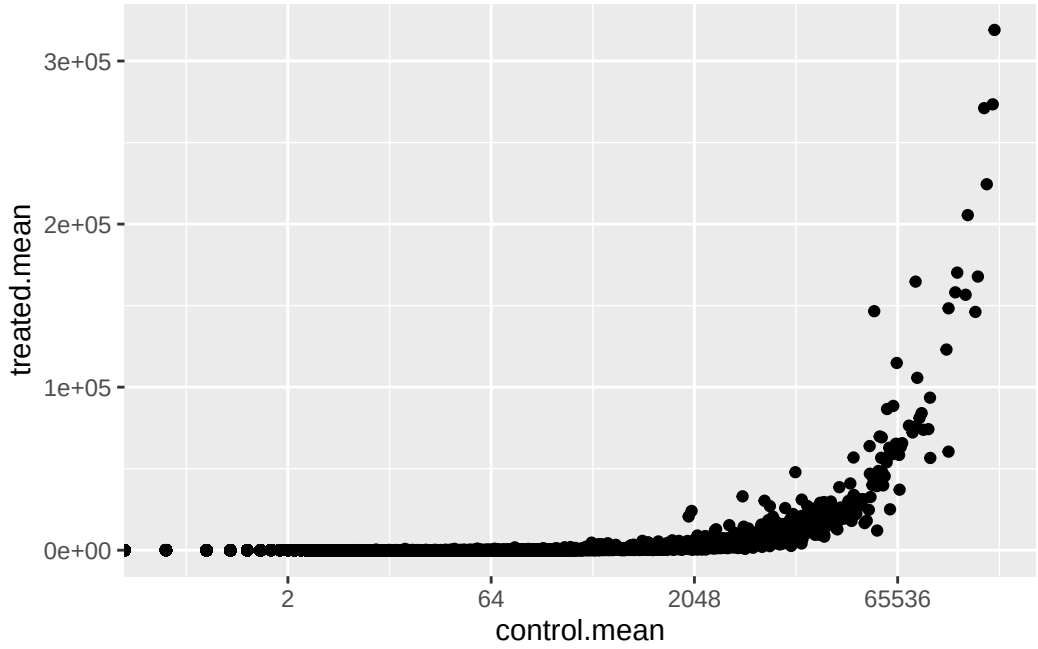
We totally need to log transform this data as it is so heavily skewed.

```
plot(log(meancounts))
```



```
ggplot(meancounts, aes(control.mean, treated.mean)) +
  geom_point() +
  scale_x_continuous(trans="log2")
```

```
Warning in scale_x_continuous(trans = "log2"): log-2 transformation introduced
infinite values.
```



A common "rule-of-thumb" is to focus on genes with a log2 "fold-change" of +2 as so-called UP REGULATED and -2 as DOWN REGULATED

Let's add a log2 fold-change value to our `meancounts` data.

```
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
```

Q. Remove and "zero count" genes from our dataset for further analysis.

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

```
[1] 21817
```

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

Q8. How many genes are "up" regulated at a log2fc threshold of +2?

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

```
[1] 314
```

Q9. How many genes are "down" regulated at a log2fc threshold of -2?

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

```
[1] 485
```

We're missing stats.

DESeq2 Analysis

Let's do this properly and consider the stats - are the differences in the means significant? We will use DESeq2 to do this.

```
library(DESeq2)
citation("DESeq2")
```

```
To cite package 'DESeq2' in publications use:

  Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change
  and dispersion for RNA-seq data with DESeq2 Genome Biology 15(12):550
  (2014)

A BibTeX entry for LaTeX users is

  @Article{,
    title = {Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2
    author = {Michael I. Love and Wolfgang Huber and Simon Anders},
    year = {2014},
    journal = {Genome Biology},
    doi = {10.1186/s13059-014-0550-8},
    volume = {15},
    issue = {12},
    pages = {550},
  }
```

The first function we will use from this package sets up the input in the particular 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
```

We can now run our DESeq analysis

```
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)
```

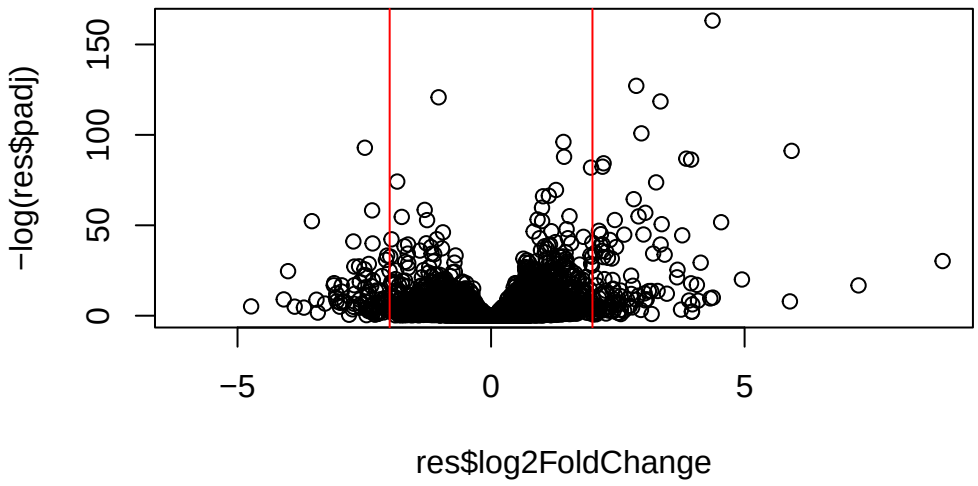
Peak at results

```
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
```

We can flip the y-axis by adding a minus sign. This will make it easier to interpret.

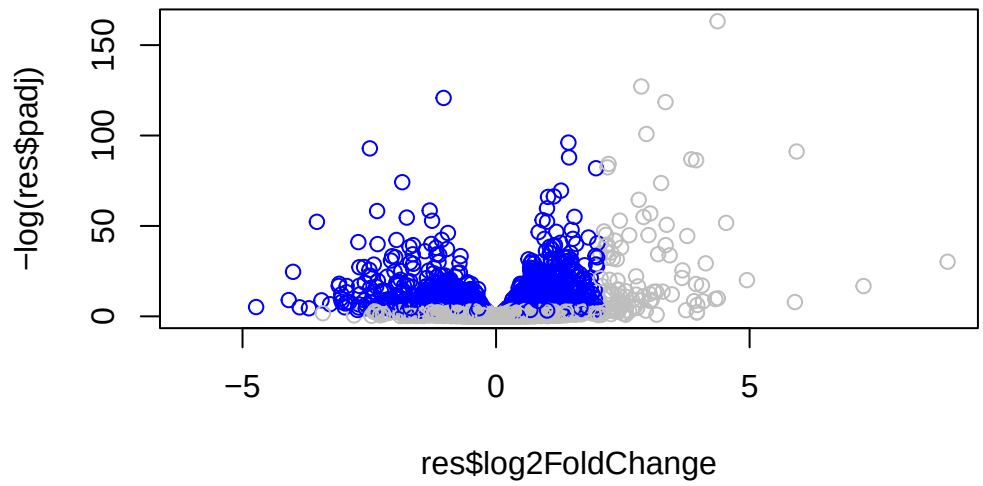
```
plot(res$log2FoldChange, -log(res$padj))
abline(v=-2, col="red")
abline(v=+2, col="red")
```



Let's add some color.

```
mycols <- rep("gray", nrow(res))
mycols[ res$log2FoldChange <= -2 ] <- "blue"
mycols[ res$log2FoldChange <= 2 ] <- "blue"

mycols[ res$padj >= 0.05] <- "gray"
plot(res$log2FoldChange, -log(res$padj), col=mycols)
```



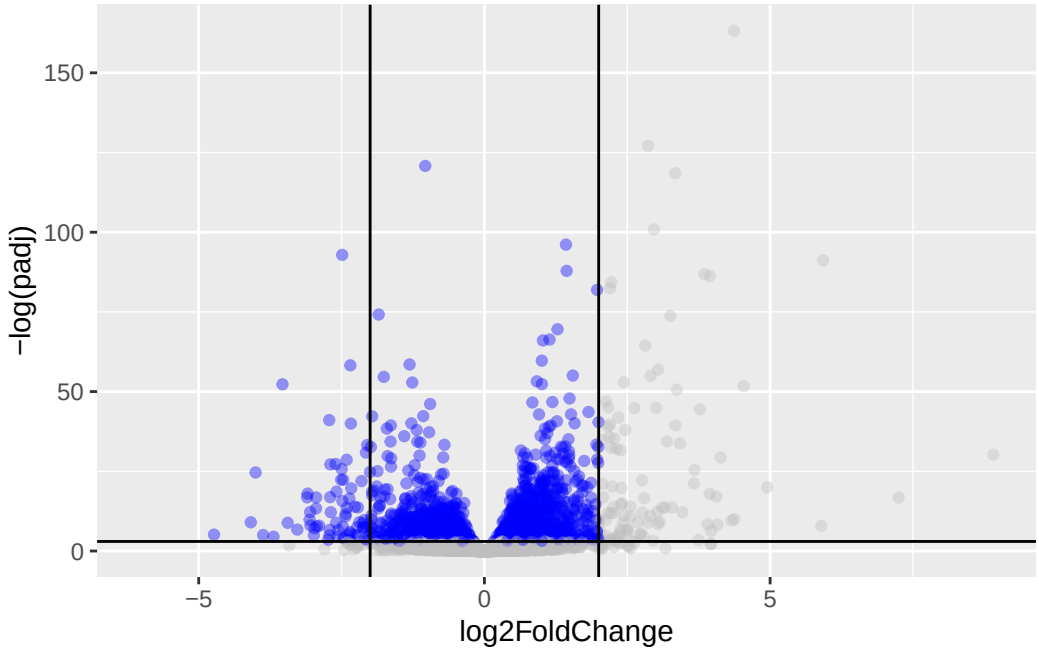

```
head(res)
```

```
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
```

Q. Make a ggplot volcano plot with colors and lines as annotation along with nice axis labels.

```
ggplot(res, aes(log2FoldChange, -log(padj))) +
  geom_point(alpha=0.4, color=mycols) +
  geom_vline(xintercept=c(-2, 2)) +
  geom_hline(yintercept= -log(0.05))
```

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




```
  labs(
    title = "Volcano ggplot",
    x = "log 2 fold-change",
    y = "-log(Adjusted P-Value)"
  ) +
  theme_bw()
```

```
NULL
```