Class 12: Transcriptomics & Analysis of RNA-Seq Data

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Bioconductor and DESeq2 setup

```
library(BiocManager)
library(DESeq2)
```

Import countData and colData

We need at least two things:

- count data (genes in rows and experiments in cols)
- metadata (aka. `colData`)

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

Check data structure

```
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
```

We can use the `==` to test for equality - is the right side equal to the left side (T/F). We can use the `all` function to check if all the inputs are T.

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

```
[1] TRUE
```

Q1. How many genes are in this dataset?

38694 genes.

```
nrow(counts)
```

```
[1] 38694
```

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

4 control cell lines.

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

```
control treated
      4       4
```

Toy differential gene expression

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

```
#We want a mean value across these rows (i.e. a mean count per gene)
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
```

```
control <- metadata[metadata[,"dex"]=="control",]
control.counts <- counts[ ,control$id]
control.mean <- rowSums(control.counts)/4
head(control.mean)
```

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

```
library(dplyr)
control <- metadata %>% filter(dex=="control")
control.counts <- counts %>% select(control$id)
control.mean <- rowSums(control.counts)/4
```

```
  head(control.mean)
```

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

```
  length(which("control"==metadata$dex))
```

```
[1] 4
```

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

Instead of dividing by 4 when calculating the control.mean, divide by the number of control samples (e.g., `length(which("control"==metadata$dex))`). Can also make the code into a function so that we can just input one variable.

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)

```
  treated <- metadata %>% filter(dex=="treated")
  treated.counts <- counts %>% select(treated$id)
  treated.mean <- rowSums(treated.counts)/4
  head(treated.mean)
```

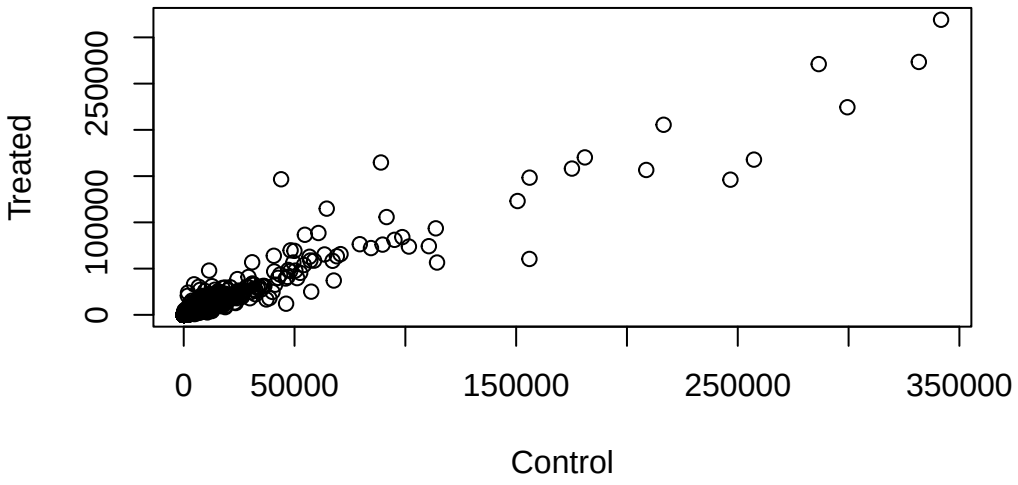
```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
         658.00             0.00           546.00           316.50            78.75
ENSG00000000938
           0.00
```

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

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

Q5 (a). Create a scatter plot showing the mean of the treated samples against the mean of the control samples.

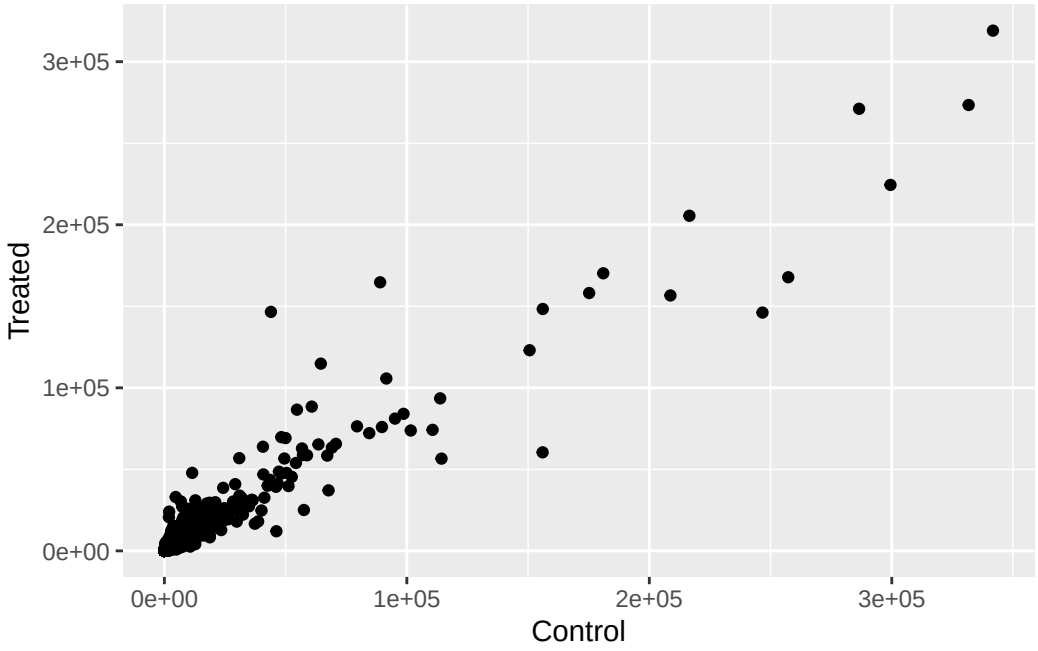
```
plot(meancounts, xlab="Control", ylab="Treated")
```



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()

```
library(ggplot2)
ggplot(meancounts) +
  aes(x=control.mean,y=treated.mean) +
  geom_point() +
  labs(x="Control", y="Treated")
```



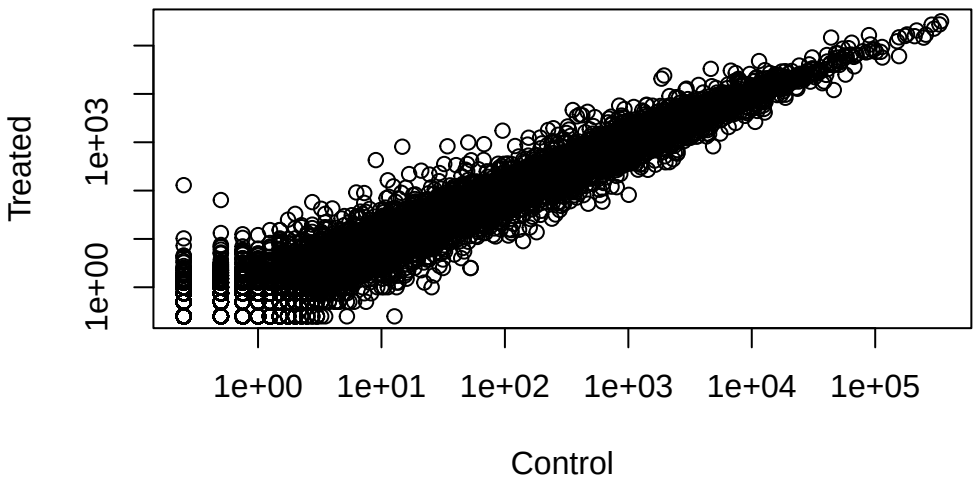
Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

For ggplot, `scale_x_log10()` and `scale_y_log10()`. For base R plot, `log="xy"`.

```
plot(meancounts, xlab="Control", ylab="Treated", log="xy")
```

```
Warning in xy.coords(x, y, xlabel, ylabel, log): 15032 x values <= 0 omitted
from logarithmic plot
```

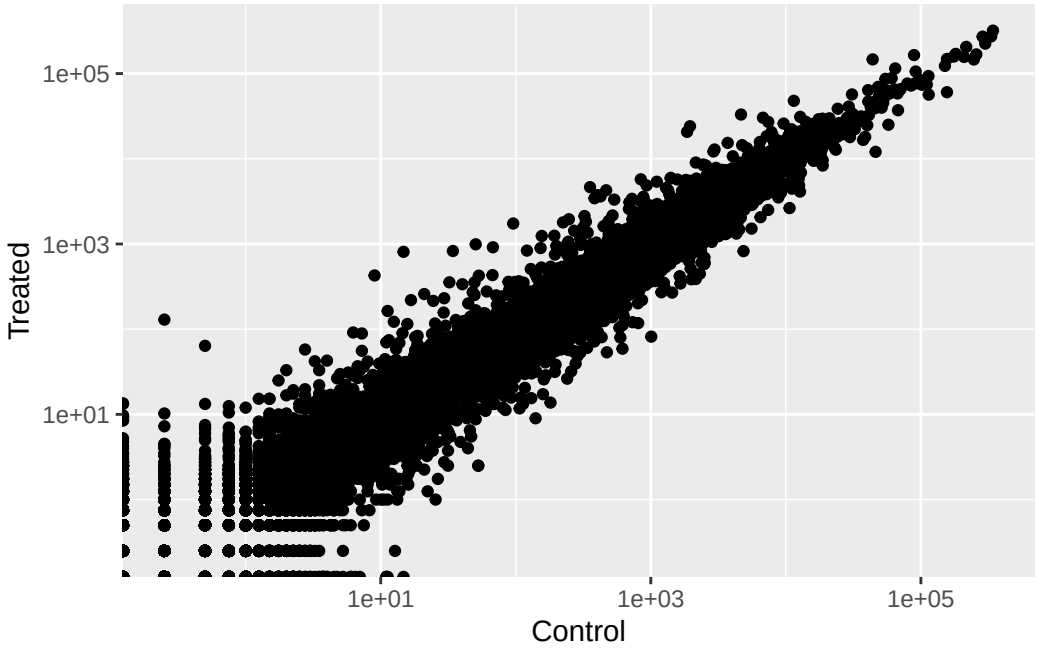
```
Warning in xy.coords(x, y, xlabel, ylabel, log): 15281 y values <= 0 omitted
from logarithmic plot
```




```
ggplot(meancounts) +
  aes(x=control.mean,y=treated.mean) +
  geom_point() +
  scale_x_log10() + scale_y_log10() +
  labs(x="Control", y="Treated")
```

```
Warning: Transformation introduced infinite values in continuous x-axis
```

```
Warning: Transformation introduced infinite values in continuous y-axis
```



Adding Log2(FoldChange)

```
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
```

```
zero.vals <- which(meancounts[,1:2]==0, arr.ind=TRUE)

to.rm <- unique(zero.vals[,1])
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
```

or...

```
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
```

```
nrow(mycounts)
```

```
[1] 21817
```

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` argument returns the row and column indices where the values are True (ie. rows/columns where there are zero counts). `unique()` ensures we don't count each row twice if it has two zero entries.

```
?which
```

```
Help on topic 'which' was found in the following packages:

  Package               Library
  base                  /Library/Frameworks/R.framework/Resources/library
```

```
  BiocGenerics          /Library/Frameworks/R.framework/Versions/4.2/Resources/library
```

```
Using the first match ...
```

A common threshold is 2/-2 FC.

```
#How many genes are upregulated at log2(FC) of +2 or greater?
sum(mycounts$log2fc>=2)
```

```
[1] 314
```

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

250.

```
up.ind <- mycounts$log2fc > 2
sum(up.ind)
```

```
[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?

367.

```
down.ind <- mycounts$log2fc < (-2)
sum(down.ind)
```

```
[1] 367
```

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

No. We would need the p value to see if the fold change is statistically significant.

DESeq2 analysis

It wants counts and colData and the "design" - aka. what to compare.

```
library(DESeq2)
```

```
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
```

```
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)
```

```
head(res)
```

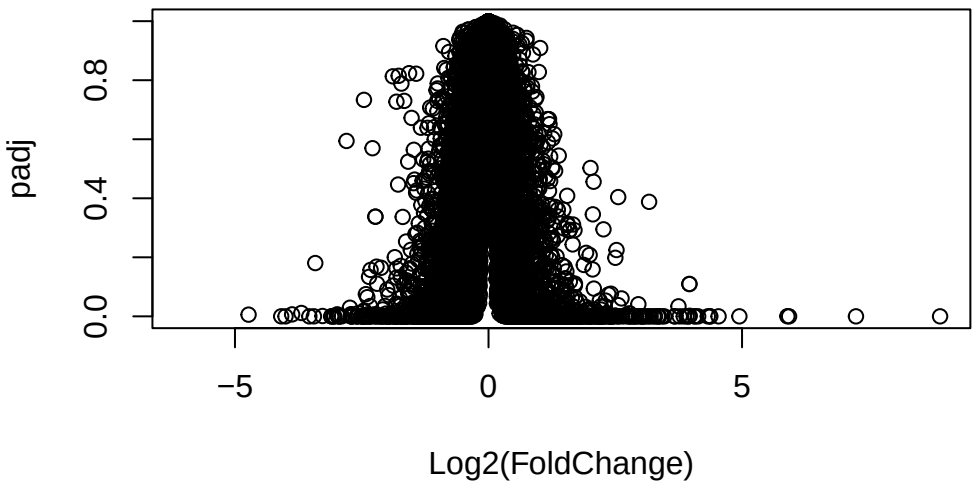
```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 6 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195     -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
```

```
res05 <- results(dds, alpha=0.05)
summary(res05)
```

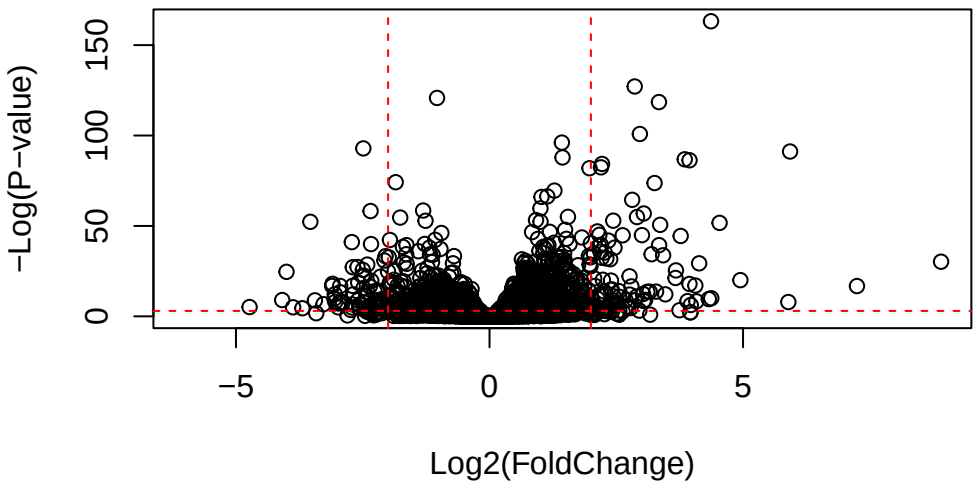
```
out of 25258 with nonzero total read count
adjusted p-value < 0.05
LFC > 0 (up)       : 1236, 4.9%
LFC < 0 (down)     : 933, 3.7%
outliers [1]       : 142, 0.56%
low counts [2]     : 9033, 36%
(mean count < 6)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of ?results
```

Data Visualization

```
plot( res$log2FoldChange,  res$padj,
      xlab="Log2(FoldChange)",
      ylab="padj")
```




```
#Take log of p-value
plot( res$log2FoldChange,  -log(res$padj),
      xlab="Log2(FoldChange)",
      ylab="-Log(P-value)")
abline(v=c(-2,2), col="red", lty=2)
abline(h=-log(0.05), col="red", lty=2)
```

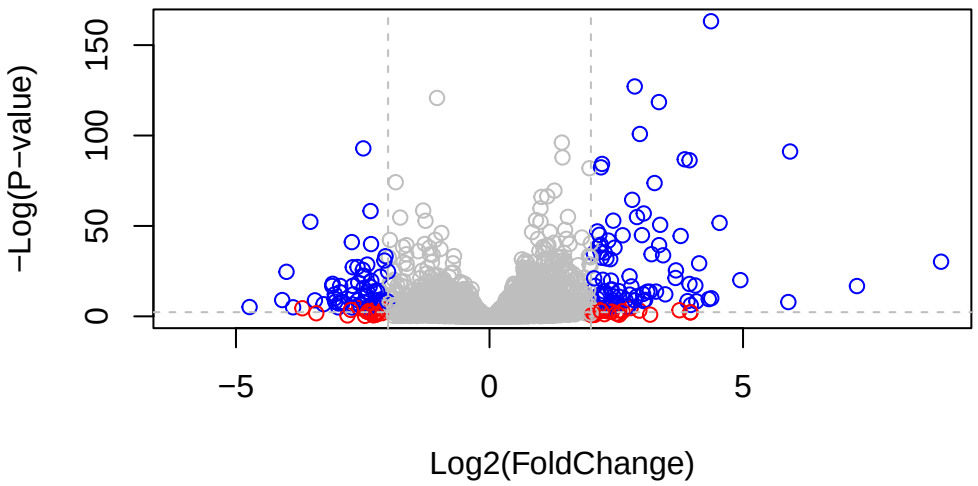



```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red"

inds <- (res$padj < 0.01) & (abs(res$log2FoldChange) > 2 )
mycols[ inds ] <- "blue"

# Volcano plot with custom colors
plot( res$log2FoldChange,  -log(res$padj),
 col=mycols, ylab="-Log(P-value)", xlab="Log2(FoldChange)" )

# Cut-off lines
abline(v=c(-2,2), col="gray", lty=2)
abline(h=-log(0.1), col="gray", lty=2)
```


−Log(P−value)
0
50
100
150
−5
0
5
Log2(FoldChange)