Class 12: Transcriptomics & RNA-Seq

Kira

In this session we will read and explore the gene expression data from this experiment using base R functions and then perform a detailed analysis with the DESeq2 package from Bioconductor.

2. Import countData and colData

To begin our RNA-Seq analysis, we first need to import our data:

```
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

```
(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
7 SRR1039520 control  N061011 GSM1275874
8 SRR1039521 treated  N061011 GSM1275875
```

Q1. How many genes are in this dataset?

```
nrow(counts)
```

```
[1] 38694
```

There are 38694 genes in this dataset.

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

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

```
control treated
      4       4
```

There are 4 'control' cell lines.

We also want to ensure that the id column of the metadata matches the order of the columns in countData.

We can use the `all()` function to check if all the inputs are TRUE:

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

```
[1] TRUE
```

The column names of the count data matches the id column of the metadata, so we are all set to start our analysis.

3. Toy differential gene expression

Let's first extract our counts for the control samples so that we can compare this to the counts for the treated samples (i.e with drug).

```
# Extracting control information into a table
control.inds <- metadata$dex == "control"
control.ids <- metadata$id[control.inds]
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
```

I want a single summary counts value for each gene in the control experiments. To start, I will take the average:

```
# Finding an average count value for each 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
```

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

It is more robust to find the mean using either the `rowMeans()` function or the `apply()` function so that it can work for any amount of samples (i.e if the number of samples changed).

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)

```
# Extracting control information into a table
treated.inds <- metadata$dex == "treated"
treated.ids <- metadata$id[treated.inds]
```

```
treated.counts <- counts[, treated.ids]
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
```

```
# Finding an average count value for each gene
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
```

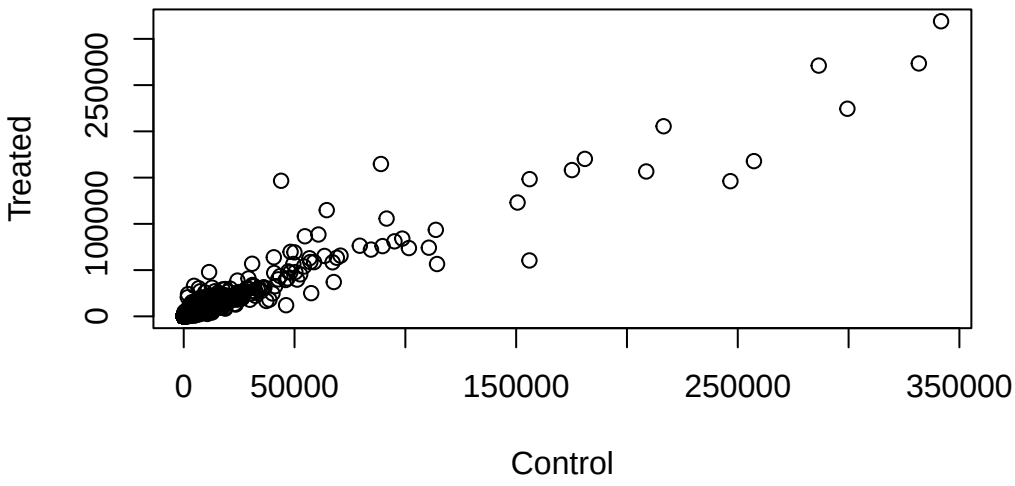
```
meancounts <- data.frame(control.mean, treated.mean)
```

```
colSums(meancounts)
```

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

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.

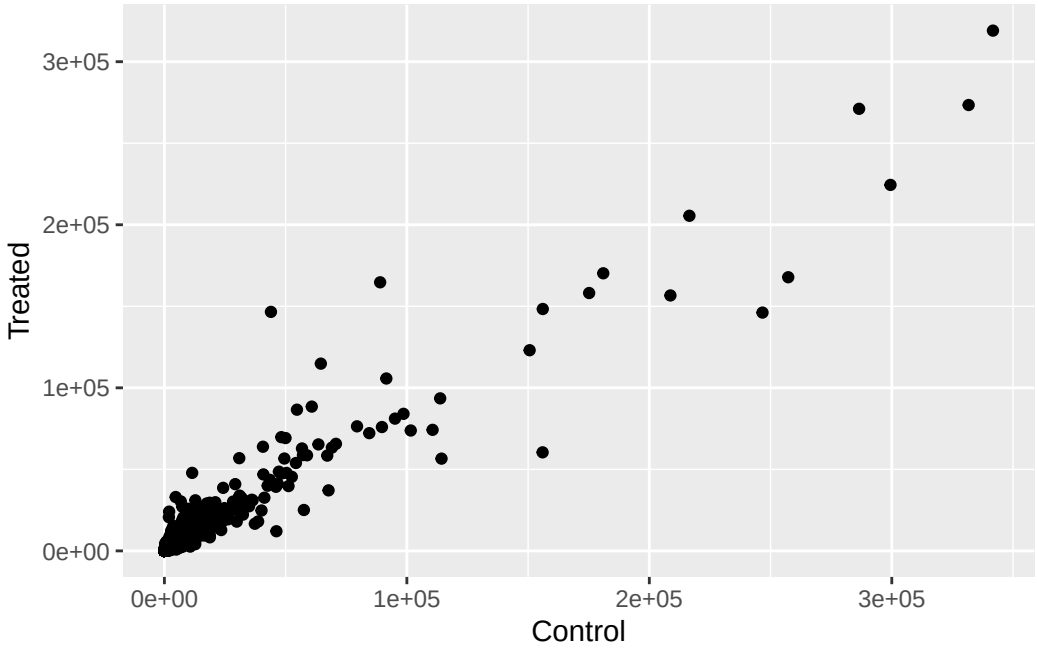
```
plot(meancounts$control.mean,meancounts$treated.mean, xlab="Control", ylab="Treated")
```



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?

The `geom_point()` function would be useful for this plot:

```
library(ggplot2)
ggplot(meancounts) + aes(control.mean,treated.mean) + geom_point() + xlab("Control") + yla
```



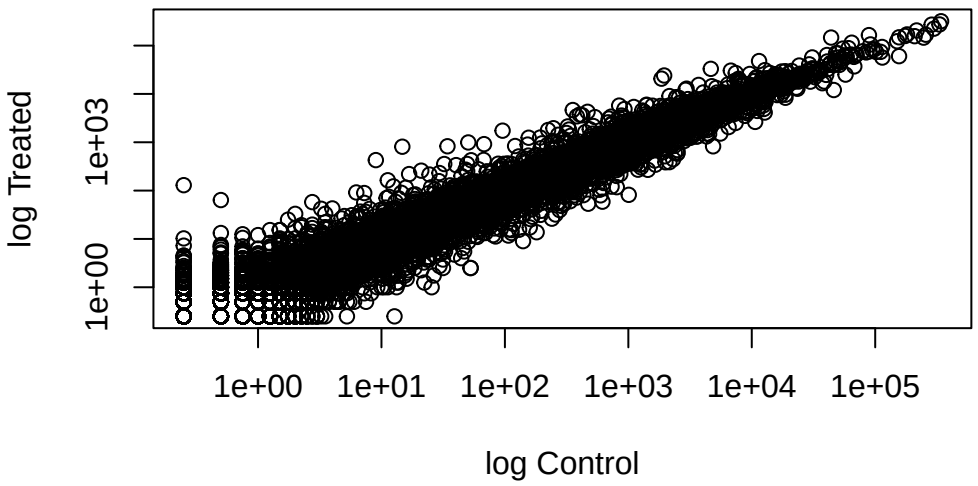
Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

We can use the log.scale argument to plot both axes on a log scale using base R plotting:

```
plot(meancounts$control.mean,meancounts$treated.mean, log="yx", xlab="log Control", ylab="
```

```
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
```



The most useful and most straightforward to understand is log2 transformation. We will add a "log2 fold-change" to our data frame by creating a new column:

```
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
```

Hmmm … we need to get rid of the genes where we have no count data as taking the log2 of these 0 counts does not tell us anything.

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

```
nrow(mycounts)
```

```
[1] 21817
```

```
# Another method to remove zero values for gene counts

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

#to.rm <- unique(zero.vals[,1])
#mycounts <- meancounts[-to.rm,]
#head(mycounts)
```

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 argument specifies that the array indices should be returned. We would use the `unique()` function to gather the zero values into a vector that we then can exclude from the counts dataset.

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

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

```
up.ind
```

```
[1] 314
```

There are 314 genes that are up regulated at the greater than 2 fc level.

Q9. Using the down.ind vector above can you determine how many down regulated genes we have at the greater than 2 fc level?

```
down.ind
```

```
[1] 485
```

There are 485 genes that are down regulated at the greater than 2 fc level.

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

We can't really trust these results yet because we haven't determined if the difference in mean and fold change values are statistically significant or not.

4. DESeq2 analysis

```
library(DESeq2)
```

Like most bioconductor packages, DESeq2 wants its input and output in a very specific 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
```

The main DESeq function 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)
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
```

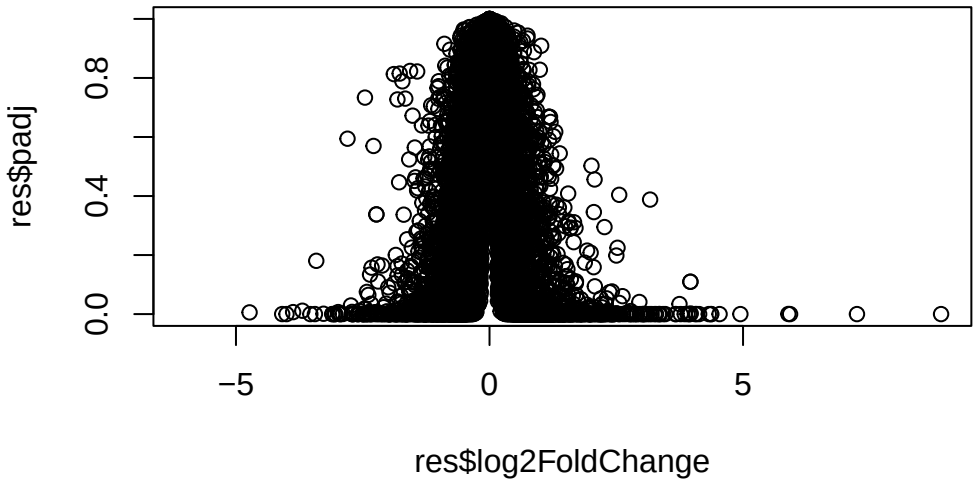
5. Adding annotation data

We skipped this section due to time

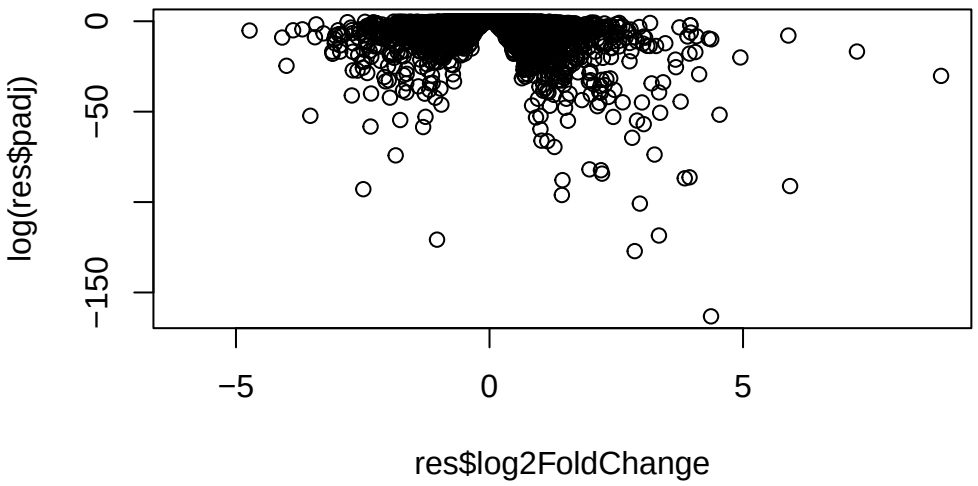
6. Data Visualization: Volcano Plot

These summary figures are frequently used to highlight the proportion of genes that are both significantly regulated and display a high fold change (this will keep both our inner biologist and statistician happy).

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

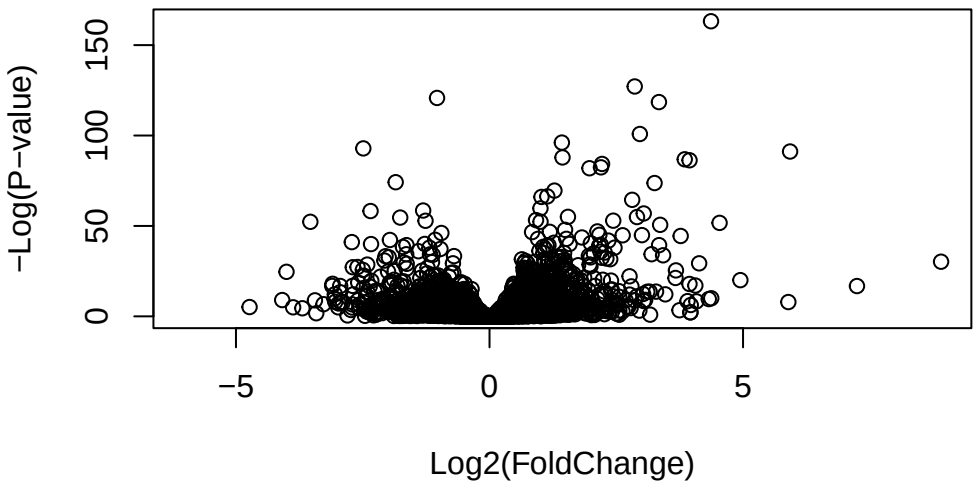



```
plot( res$log2FoldChange, log(res$padj))
```



We want to flip the y-axis so that the values we're interested in (i.e the low p-value or high log(p-value)) are at the top of the axis. We can improve this plot by taking the negative log of the y-axis (padj):

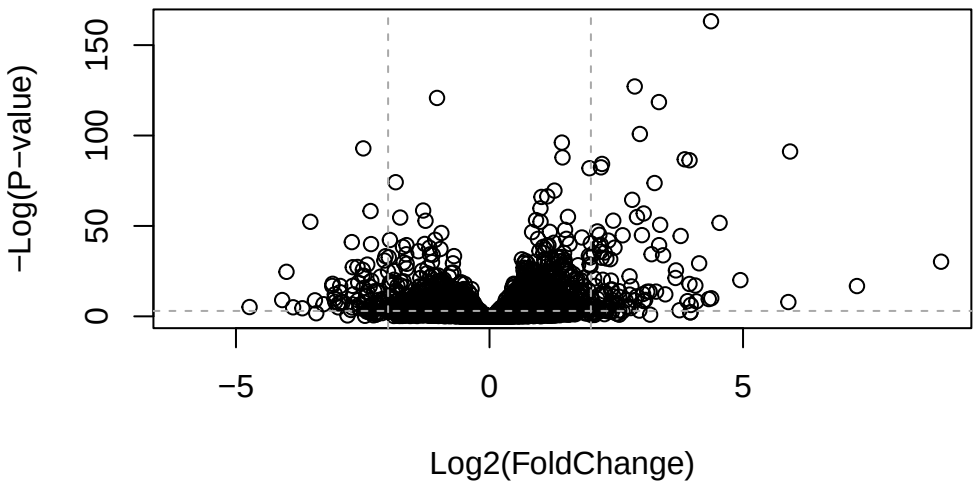
```
plot( res$log2FoldChange,  -log(res$padj),
      xlab="Log2(FoldChange)",
      ylab="-Log(P-value)")
```



To make this more useful we can add some guidelines and color highlighting genes that have padj <0.05 and the absolute log2FoldChange >2.

```
plot( res$log2FoldChange,  -log(res$padj),
 ylab="-Log(P-value)", xlab="Log2(FoldChange)")

# Add some cut-off lines
abline(v=c(-2,2), col="darkgray", lty=2)
abline(h=-log(0.05), col="darkgray", lty=2)
```

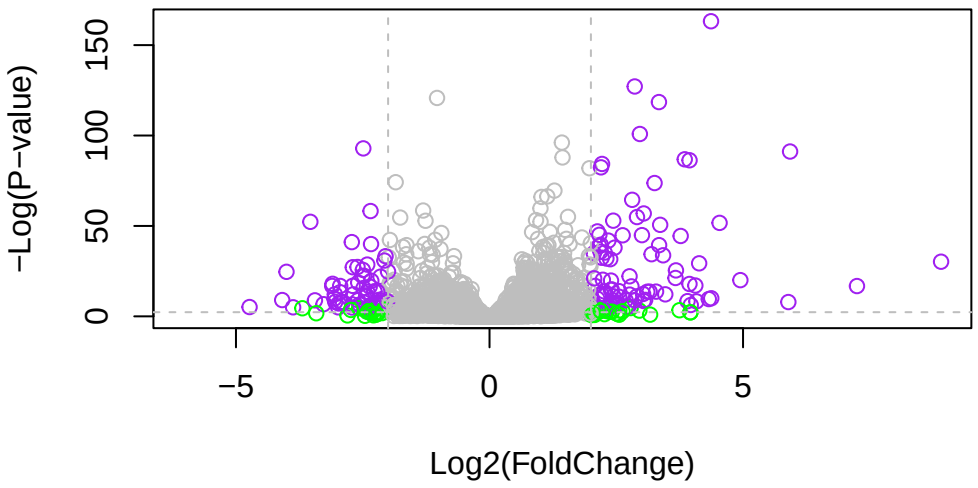



```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "green"

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

# 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)
```



These colored transcripts have both a large fold change and a significant difference between conditions.