Class 12: RNASeq

Le, Sarah (A18518276)

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Background

Today, we will analyze some RNASeq data from Himes et al. on the effects of a common steroid on airway smooth muscle cells (ASM cells).

At starting point is the "counts" data and "metadata" that contain the count values for each gene in their different experiments (i.e. cell lines with or without the drug).

Data import

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

Let's have a wee peak at these objects:

```
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
```

Q. How many different experiments (columns in counts or rows in metadata) are there?

```
ncol(counts)
```

```
[1] 8
```

```
nrow(metadata)
```

```
[1] 8
```

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

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

```
[1] 4
```

Toy differential gene expression

To start our analysis, let's calculate the mean counts for all genes in the "control" experiments.

1. Extract all "control" columns from the `counts` object.
2. Calculate the mean for all rows (i.e. genes) of these "contol" columns. 3-4. Do the same for "treated".
3. Compare these `control.mean` and `treated.mean` values.

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

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

```
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? Is there a function that could help here?

Instead of using rowSums() by 4, we can use rowMeans() function instead as it will automatically calculate the mean accross rows regardless of the amount of samples so it is more flexible if we need to add more samples.

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.inds <- metadata$dex == "treated"
treated.counts <- counts[, treated.inds]
```

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

Store these together for ease of book keeping as `meancounts`.

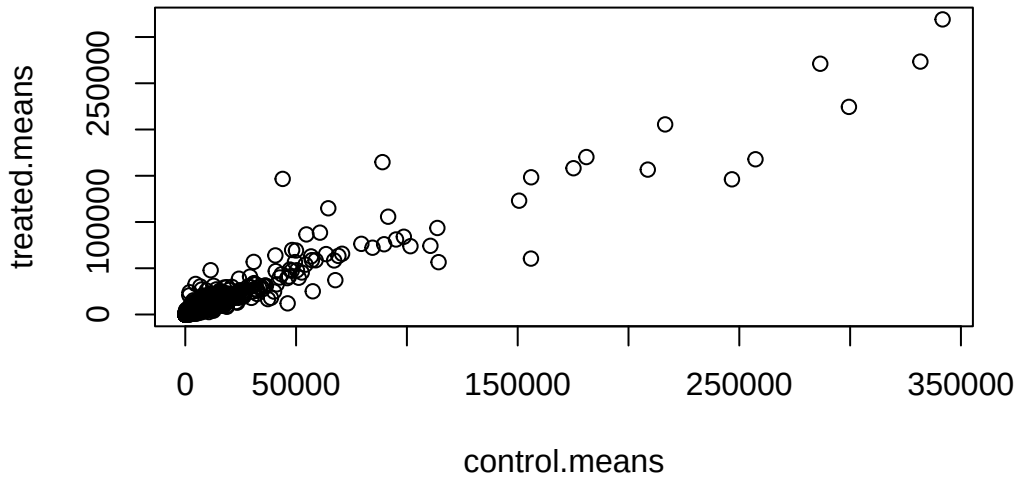
```
meancounts <- data.frame(control.means, treated.means)
head(meancounts)
```

```
                control.means treated.means
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 (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.

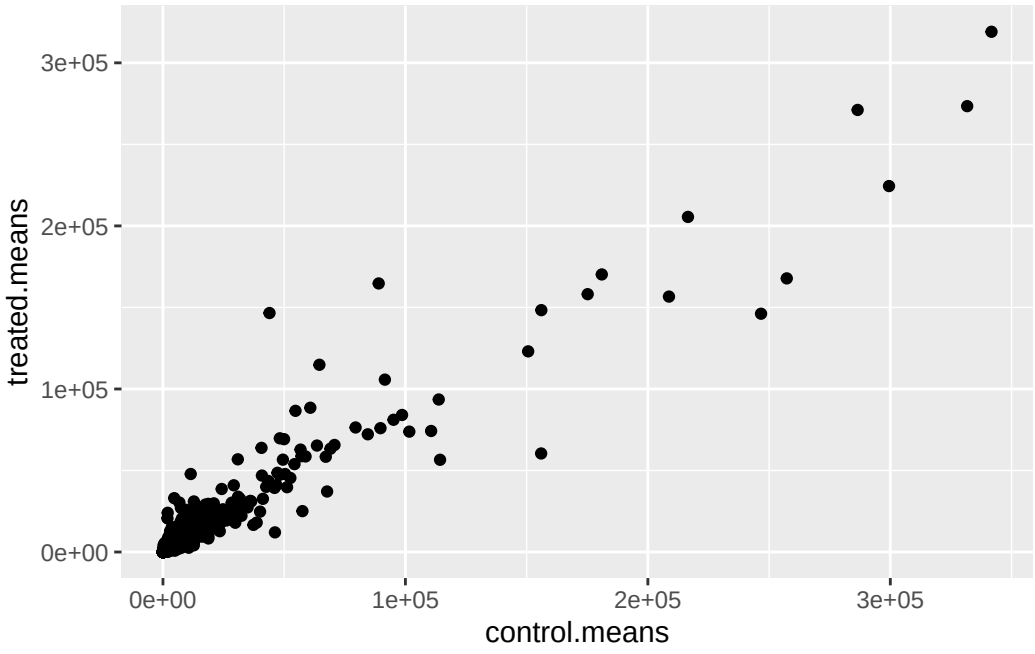
Make a plot of control vs treated mean values for all genes.

```
plot(meancounts)
```



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?

```
library(ggplot2)
ggplot(meancounts, aes(control.means, treated.means))+
  geom_point()
```

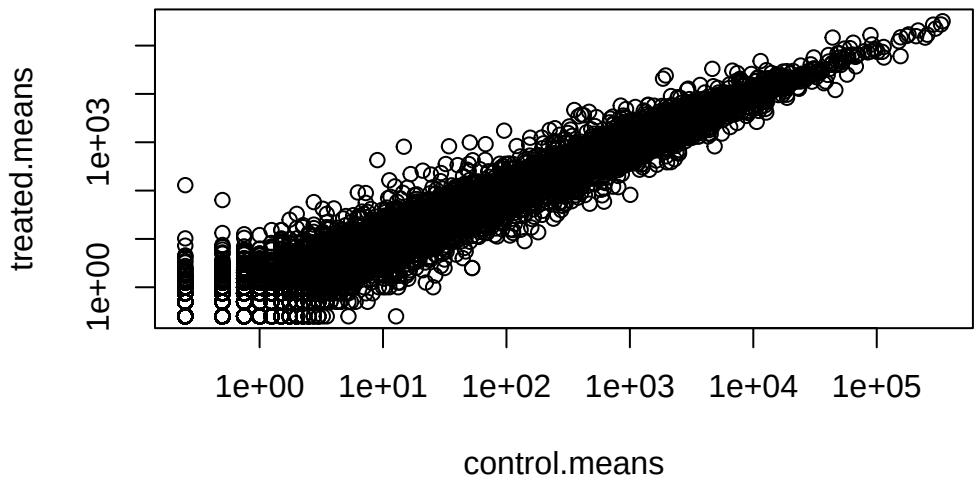


Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

```
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
```



We often talk metrics like "log2 fold-change".

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

```
[1] 0
```

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

```
[1] -1
```

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

```
[1] 1
```

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

```
[1] 2
```

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

```
[1] -2
```

Let's calculate the log2 fold chain for our treated over control mean counts.

```
meancounts$log2fc <-
log2(meancounts$treated.means/
  meancounts$control.means)
```

```
head(meancounts)
```

```
                control.means treated.means      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
```

Let's filter our data to remove these genes.

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

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

```
                control.means treated.means      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
```

> 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?

arr.ind = TRUE will returns row or column locations. [,1] will keep only the row indices (genes) and then unique() will remove duplicates so we only filter each gene once.

A common "rule of thumb" is a log2 fold chain cutoff of +2 and -2 to call genes "Up regulated" or "Down regulated".

Number of "Up regulated" genes.

```
sum(meancounts$log2fc >= +2, na.rm= T)
```

```
[1] 1910
```

Number of "Down regulated" genes at -2 threshold.

```
sum(meancounts$log2fc <= -2, na.rm = T)
```

```
[1] 2330
```

> 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 <- 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?

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

```
[1] 367
```

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

No because these results are only at fold-change values without testing for statistical significance (p-values) so large fold-changes can occure due to noise or by change. We need to use DESeq2 to confirm which genes are truly differentially expressed.

DESeq2

Let's do this analysis properly and keep our inner stats nerd happy - i.e. are the difference we see between drug and no drug significant given the replicate experiments.

```
library(DESeq2)
```

For DESeq analysis, we need three things.

- count values (`countData`).
- metadata telling us about the columns in `countData` (`colData`).
- design of the experiment (i.e. what do you want to compare?).

Our first function from DESeq2 will setup the input required for analysis by storing all these 3 things together.

```
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 DESeq2 that runs the 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)
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
```

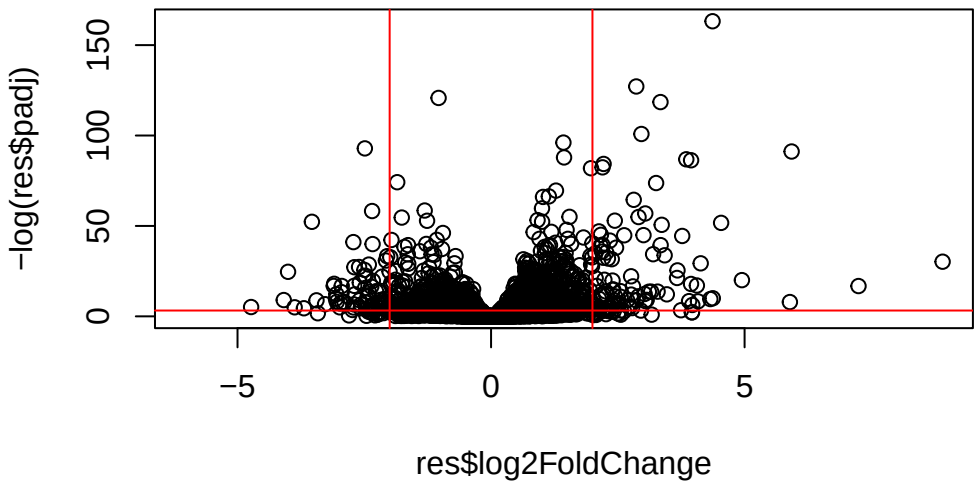
```
36000*0.05
```

```
[1] 1800
```

Volcano Plot

This is common summary result figure from these types of experiments and plot the log2 fold-change vs the adjusted p-value.

```
plot(res$log2FoldChange, -log(res$padj))
abline(v=c(-2,2), col="red")
abline(h=-log(0.04), col= "red")
```




```
log(0.1)
```

```
[1] -2.302585
```

```
log(0.00001)
```

```
[1] -11.51293
```

Save our results

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