Class12

Yuntian Zhu (PID: A17816597)

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Backgroun

Today we will analyze some RNA-seq data on the effects of a common steroid on airway smooth muscle cells.

Data import

```r
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
```

There are 38694 genes in this dataset.

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

```
ncol(counts)
```

```
[1] 8
```

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

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

```
[1] 4
```

We have 4 control cell lines.

Toy differential gene experession

To start our analysis, let's calculate the mean counts of the 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 "control" columns

3 and 4. Do the same thing for "treated" groups

5. Compare these `control.mean` and `treated.mean` values.

Method 1:

```
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
```

> Q3. How would you make the above code in either approach more robust? Is there a function that could help here?

The method above finds the mean by first calculate the summation and divide it by 4 (the number of samples), which is not very robust. The primary reason is that there might be a different sample number and we need to change 4 to another number by ourselves. We can improve the code by using the function `rowMeans()`. By using `rowMeans()`, the mean is calculated automatically across however many samples are present, without requiring us to hard-code the sample count. This not only makes the code more flexible and maintainable, but also reduces the risk of errors when working with different datasets or experimental designs.

```
control.inds <- metadata$dex == "control"
control.count <- counts[, control.inds]
control.mean <- rowMeans(control.count)
head(control.mean)
```

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

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)

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

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

Store these all together for the ease of bookkeeping as `meancounts`

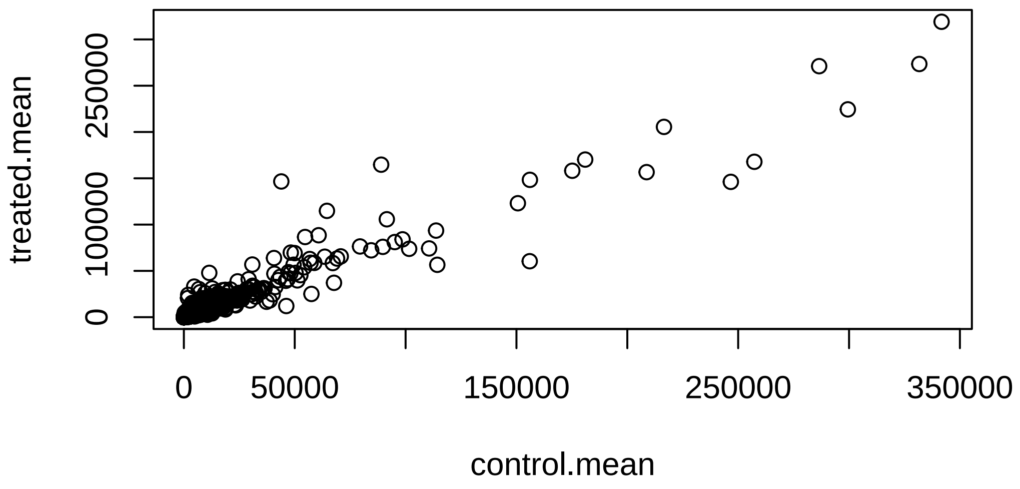
```r
meancounts <- data.frame(control.mean, treated.mean)
head(meancounts)
```

```
                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
```

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

Let us make a plot of control vs. treated mean values

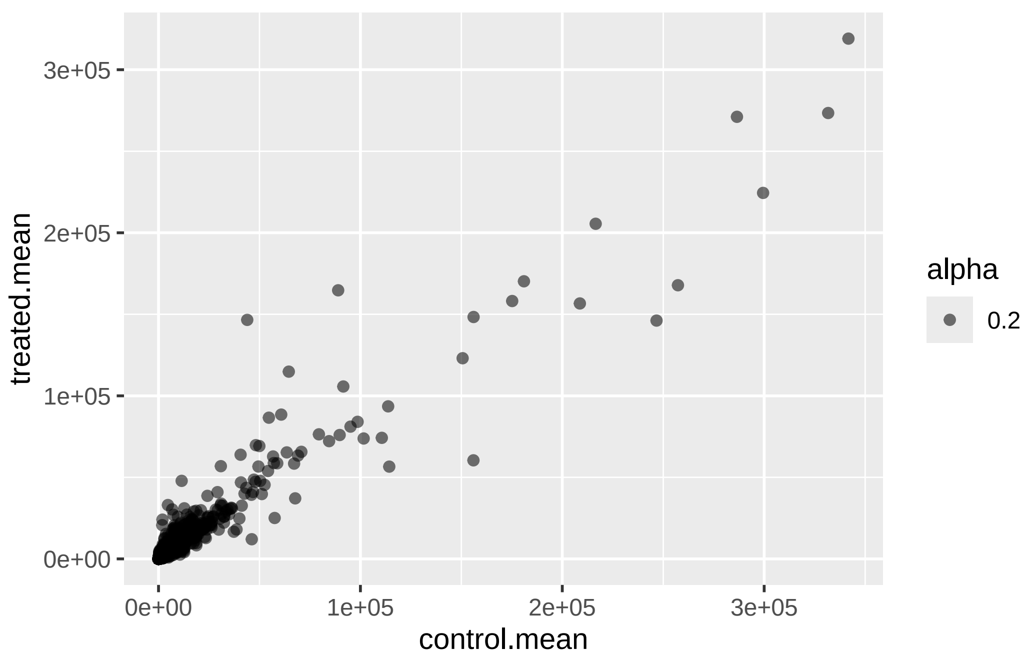
```r
plot(meancounts)
```



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?

Let us make another plot using ggplot2. We should use `geom_point()` here.

```
library(ggplot2)
  ggplot(meancounts) +
    aes(control.mean, treated.mean, alpha = 0.2) +
    geom_point()
```



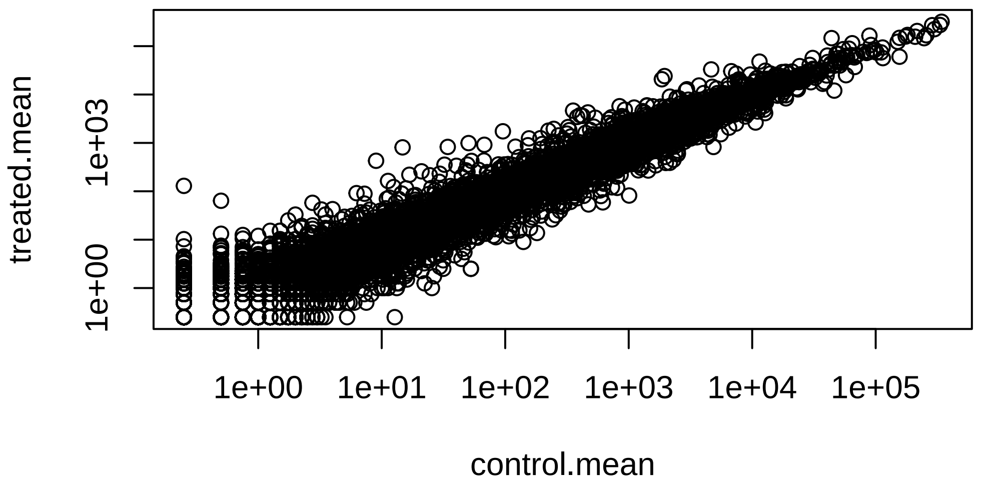
> Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

Make this a log log plot, so that the plot gets less crowded. The argument that we should use here is `log = "xy"`.

```
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 oftern talk about metrics like log2FC. Let's calculate the log2 fold change for our treated over control mean counts.

```
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
```

A common "rule of thumb" is a log2 fold change cutoff of +2 and -2 to callgenes "upregulated' or"downregulared".

First, we can find the number of upregulated genes

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

```
[1] 1910
```

Then, we can find the number of downregulated genes

```r
sum(meancounts$log2fc >= -2, na.rm = TRUE)
```

```
[1] 23046
```

We can make the data more polished by removing the genes that are not even expressed, so that we will not have things like NaN or -Inf.

```r
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
```

> 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 purpose of `arr.ind` is to get row and column coordinates instead of just linear positions, because normally, `which()` returns just the indices of TRUE values in a vector. The purpose of taking the first column is to find which rows (genes) have a zero in either control.mean or treated.mean. The first column of `zero.vals` corresponds to the row index of each zero entry, so that we can remove any genes that have zero counts in any samples. The purpose of calling the `unique()` function is that A single row (gene) might appear multiple times if both control.mean and treated.mean are zero, or if repeated zeros are found. To avoid removing the same row multiple times, we take only unique row indices.

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

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

```
[1] 250
```

We have 250 upregulated genes 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?

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

```
[1] 367
```

We have 367 downregulated genes at the greater than 2 fc level.

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

Not really. The results only reflect whether the log2FC of a certain gene is greater than the 2 cutoff or less than the -2 cutoff. In order to make the result more trustable, we should focus on the genes that show statistically significant change. In other words, we should also consider the p-values in addition to log2FC. Then, we can focus on the genes that not only have log2FC > 2 or log2FC < -2 but also have a small p-value showing the statistical significance.

DESeq2 analysis

Let;s do this analysis properly and find genes that have a statistically significant change.

```r
library(DESeq2)
```

For DESeq analysis, we need three things:

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

Our first function from DESeq2 will set up the input for our analysis by storing all these three things together.

```r
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 differential expression analysis is called `DESeq()`

```r
dds <- DESeq(dds)
```

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

```r
res <- results(dds)
head(res)
```

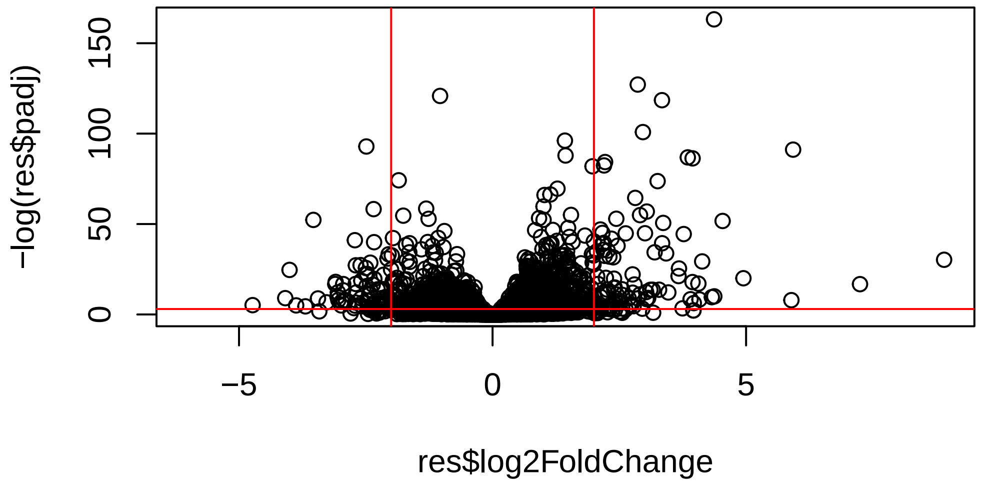
```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 6 columns
                 baseMean log2FoldChange     lfcSE      stat    pvalue
                <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195      -0.350703  0.168242 -2.084514 0.0371134
ENSG00000000005   0.000000             NA        NA        NA        NA
```

```
ENSG00000000419 520.134160        0.206107  0.101042  2.039828 0.0413675
ENSG00000000457 322.664844        0.024527  0.145134  0.168996 0.8658000
ENSG00000000460  87.682625       -0.147143  0.256995 -0.572550 0.5669497
ENSG00000000938   0.319167       -1.732289  3.493601 -0.495846 0.6200029
                     padj
                <numeric>
ENSG00000000003  0.163017
ENSG00000000005        NA
ENSG00000000419  0.175937
ENSG00000000457  0.961682
ENSG00000000460  0.815805
ENSG00000000938        NA
```

Volcano Plot

This is a common summery result figure from these types of experiments an 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(0.05), col = "red")
```



Save our results

```r
write.csv(res, file = "myresults.csv")
```