Class 12: RNASeq Analysis

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Here we will use the DESeq2 package for RNASeq analysis. The data for today's class comes from a study of airway smooth muscle cells treated with dexamethasone, a synthetic glucocorticoid steroid with anti-inflammatory effects (Himes et al. 2014)

Import the data

We need two things for this analysis: - countData (counts for every transcript/gene in each experiment) - metdata (metadata that describes the experimental setup)

```
countData <- read.csv("airway_scaledcounts.csv", row.names = 1)
head(countData)
```

```
                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 <- read.csv("airway_metadata.csv")
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(countData)
```

```
[1] 38694
```

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

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

```
[1] 4
```

Step 1. - Calculate the mean of the control samples.

(a) We need to find which columns in countData are "control" samples.

```
control.inds <- metadata$dex == "control"
```

(b) Extract all the control columns from `countData` and call it `control.counts`.

```
control.counts <- countData[, control.inds]
```

(c) Calculate the mean value across the rows of `control.counts` i.e. calculate the mean count values for each gene in the control samples.

```
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?

You can use `rowMeans()` instead of `rowSum()`.

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)

Step 2. - Calculate the mean of the treated samples.

```
# (a)
treated.inds <- metadata$dex == "treated"

# (b)
treated.counts <- countData[, treated.inds]

# (c)
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
```

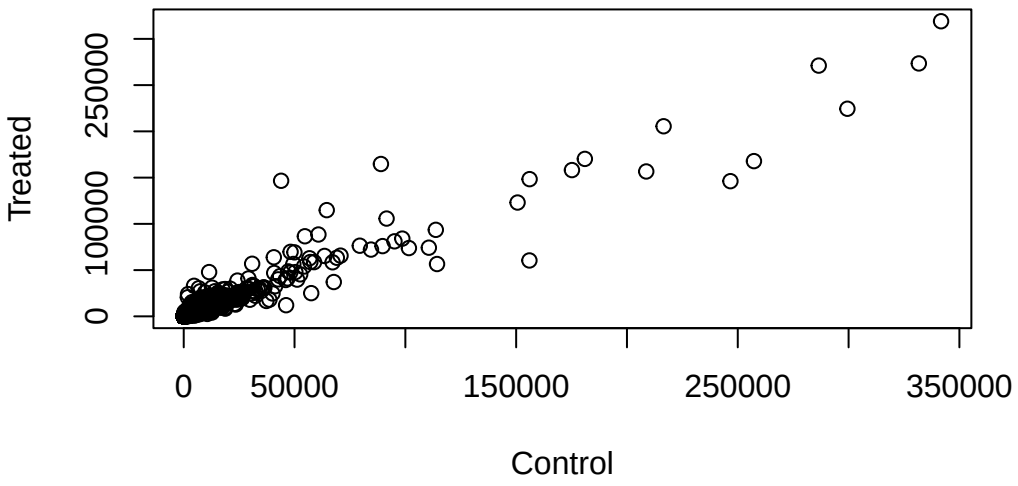
We now have control and treated mean count values. For ease of bookkeeping we can combine these vectors into a new data.frame called `meancounts`.

```
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[, 1], meancounts[, 2], xlab = "Control", ylab = "Treated")
```



We us log transforms for skewed data such as this because we really care most about relative changes in magnitude.

We most often use log2 as the math is easier to interpret than log10 or others.

If we have no change - i.e. same values in control and treated - then we will have a log2 value of zero.

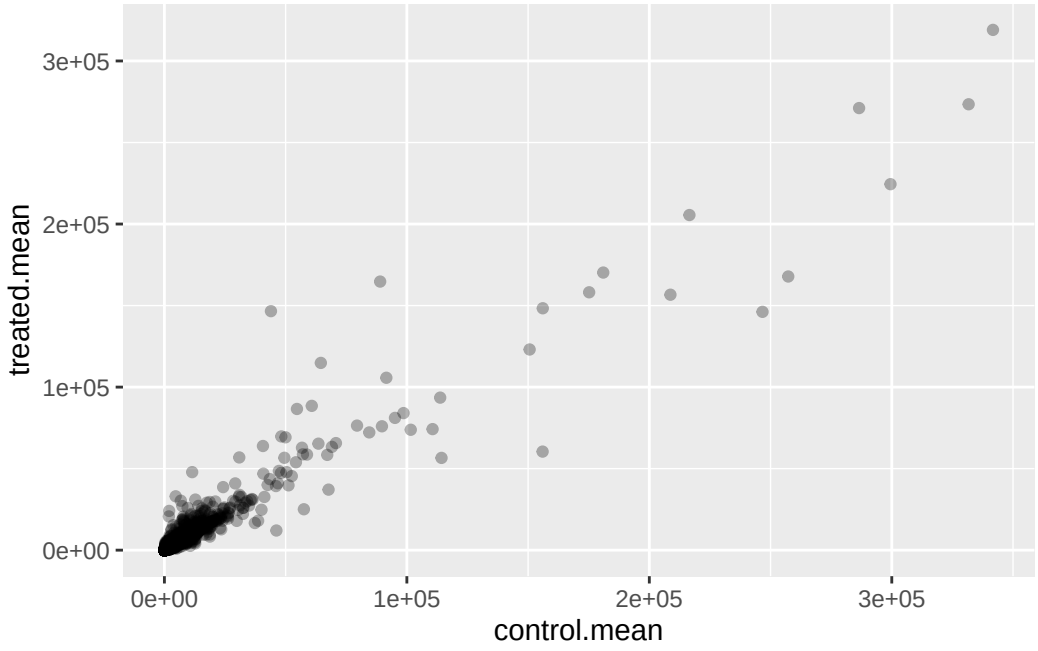
```
meancounts$log2fc <- log2(meancounts$treated.mean / meancounts$control.mean)
head(meancounts)
```

```
                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
```

Q5 (b).You could also use the ggplot2 package to make this figure. What geom_?() function would you use for this plot?

```
library(ggplot2)

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

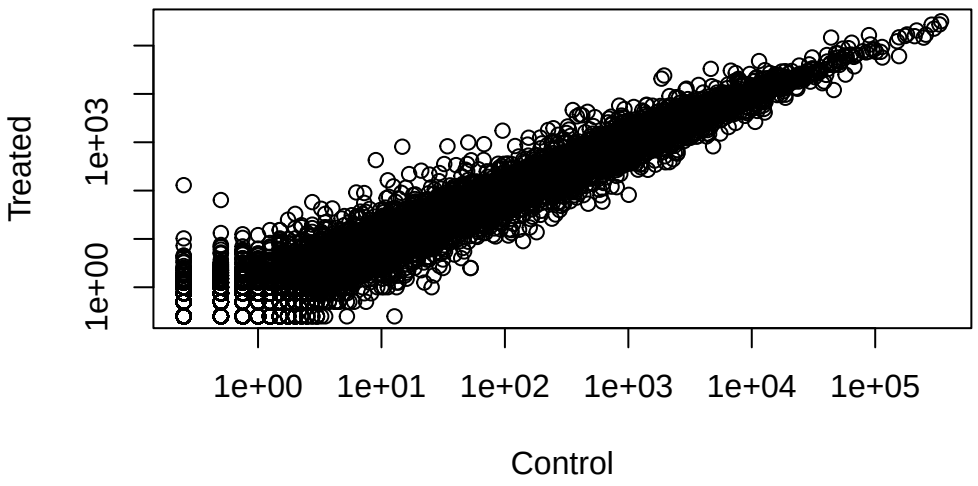


Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

```
plot(meancounts[, 1], meancounts[, 2], 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
```



Q. How many genes are up-regulated at the common threshold of +2 log2fc values?

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

```
[1] 1910
```

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 will clause which() to return both the row and column indices (i.e. positions) where there are TRUE values.

Calling unique() will ensure we dont count any row twice if it has zer entries in both samples.

```
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
```

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 we have not done anything to determine whether the differences we are seeing are significant so these results may be misleading.

DESeq2 analysis

```
library(DESeq2)
```

```
Loading required package: S4Vectors
```

```
Loading required package: stats4
```

```
Loading required package: BiocGenerics
```

```
Attaching package: 'BiocGenerics'

The following objects are masked from 'package:stats':

    IQR, mad, sd, var, xtabs

The following objects are masked from 'package:base':

    anyDuplicated, aperm, append, as.data.frame, basename, cbind,
    colnames, dirname, do.call, duplicated, eval, evalq, Filter, Find,
    get, grep, grepl, intersect, is.unsorted, lapply, Map, mapply,
    match, mget, order, paste, pmax, pmax.int, pmin, pmin.int,
    Position, rank, rbind, Reduce, rownames, sapply, setdiff, sort,
    table, tapply, union, unique, unsplit, which.max, which.min


Attaching package: 'S4Vectors'

The following objects are masked from 'package:base':

    expand.grid, I, unname

Loading required package: IRanges

Loading required package: GenomicRanges

Loading required package: GenomeInfoDb

Loading required package: SummarizedExperiment

Loading required package: MatrixGenerics

Loading required package: matrixStats


Attaching package: 'MatrixGenerics'
```

```
The following objects are masked from 'package:matrixStats':

    colAlls, colAnyNAs, colAnys, colAvgsPerRowSet, colCollapse,
    colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
    colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
    colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
    colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
    colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
    colWeightedMeans, colWeightedMedians, colWeightedSds,
    colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAvgsPerColSet,
    rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
    rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
    rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
    rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
    rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs, rowVars,
    rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
    rowWeightedSds, rowWeightedVars

Loading required package: Biobase

Welcome to Bioconductor

    Vignettes contain introductory material; view with
    'browseVignettes()'. To cite Bioconductor, see
    'citation("Biobase")', and for packages 'citation("pkgname")'.


Attaching package: 'Biobase'

The following object is masked from 'package:MatrixGenerics':

    rowMedians

The following objects are masked from 'package:matrixStats':

    anyMissing, rowMedians
```

To use DESeq we need our input countData and colData in a specific formart that DESeq wants:

```
dds <- DESeqDataSetFromMatrix(countData = countData,
                              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
```

To run the analysis I can now use the main DESeq2 function called `DESeq()` with `dds` as an input.

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

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

To get the results out of this `dds` object we can use the `results()` function from the package.

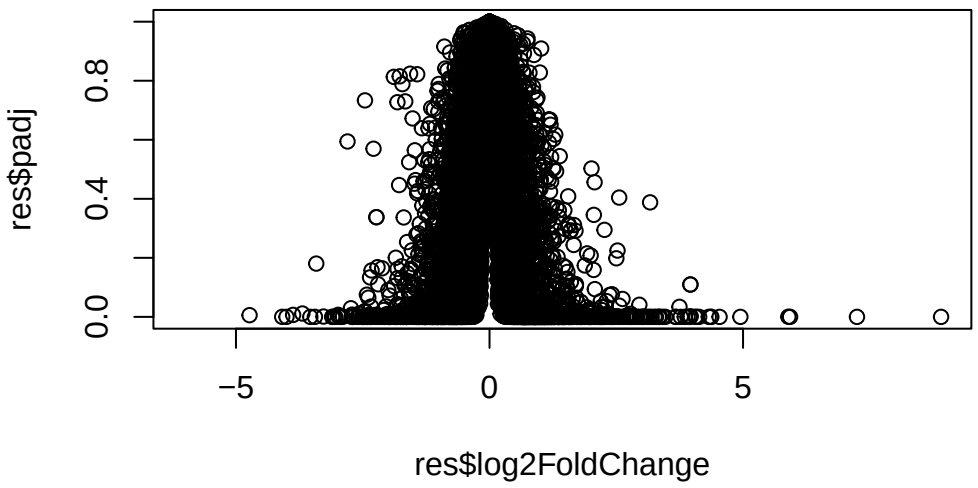
```
res <- results(dds)
head(res)
```

```
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
```

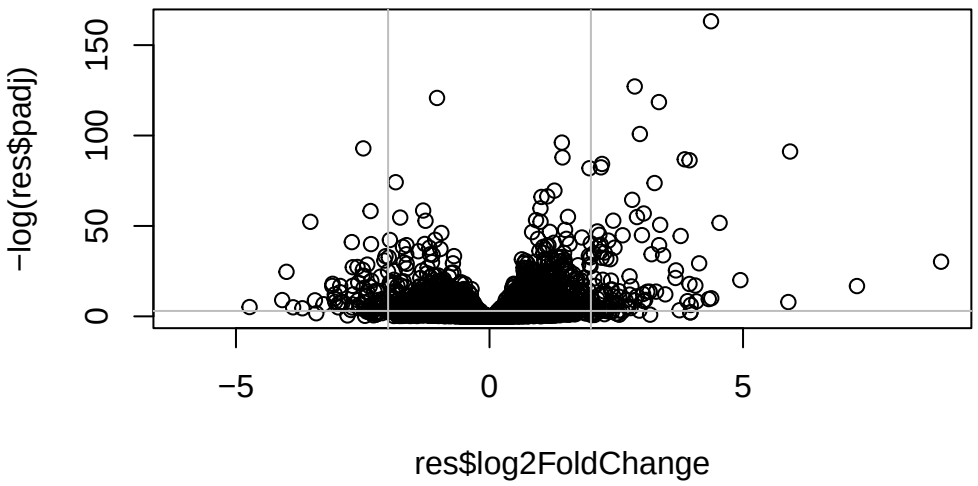
Lrt's make a final plot of log2 fold-change vs. the adjusted P-value.

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



It is the low P-values that we care about and these are lost in the skewed plot above. Let's take the log of the `$padj` values for our plot.

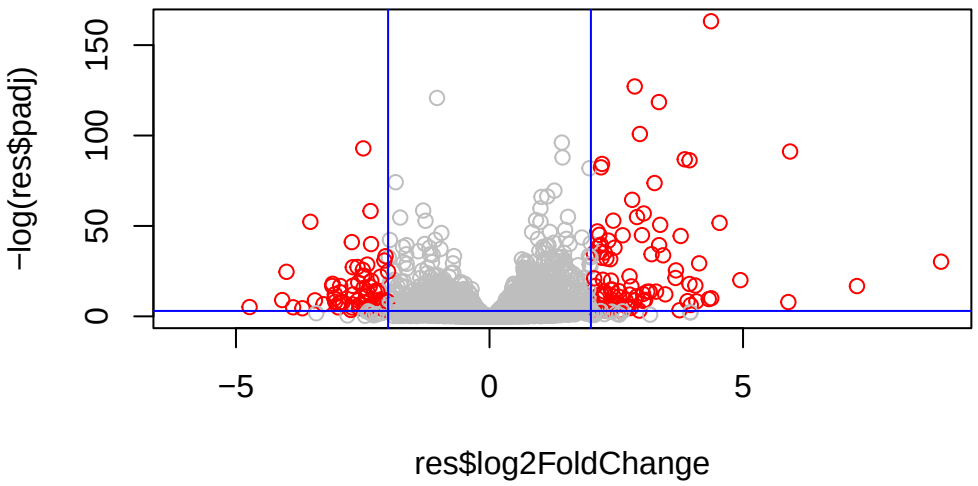
```
plot(res$log2FoldChange, -log(res$padj))
abline(v = c(+2, -2), col = "gray")
abline(h = -log(0.05), col = "gray")
```



Finally we can make a color vector to use in the plot to better highlight the genes we care about.

```
mycols <- rep("gray", nrow(res))
mycols[abs(res$log2FoldChange) >= 2] <- "red"
mycols[res$padj > 0.05] <-"gray"

plot(res$log2FoldChange, -log(res$padj), col = mycols)
abline(v = c(+2, -2), col = "blue")
abline(h = -log(0.05), col = "blue")
```


−log(res$padj)
0
50
100
150
−5
0
5
res$log2FoldChange