Class 12: RNA-Seq Analysis

AUTHOR
Hyeseung (Frankie) Son PID: A16025601

Quarto

Here we will be using DESeq package for RNA seq analysis. The data for today's class comes from a study of airway smooth muscle cells in a published RNA-seq experiment where airway smooth muscle cells were treated with dexamethasone, a synthetic glucocorticoid steroid with anti-inflammatory effects (Himes et al. 2014).

Import Data

We need two things for today's analysis:

- **countData** (counts for every transcript/gene in each experiment)
- **colData** (metadata that describes the environmental 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
```

There are 38,694 genes in this data set.

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

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

```
control treated
      4       4
```

another way:

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

```
[1] 4
```

There are 4 control cell lines in this data set.

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

- Step 1. Calculate the mean of control samples (i.e. columns in countData) Calculate the mean of treated samples.

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

- look in the metadata (a.k.a. colData), $dex column

```
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.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
```

> 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 ...

```
# We need to find which columns in countData are "treated" samples.
treated.inds <- metadata$dex == "treated"

# Extract all the control columns from `countData` and call it `treated.counts`.
treated.counts <- countData[ , treated.inds]

# Calculate the mean value across the rows of `treated.counts`.
treated.means <- rowMeans(treated.counts)
head(treated.means)
```

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

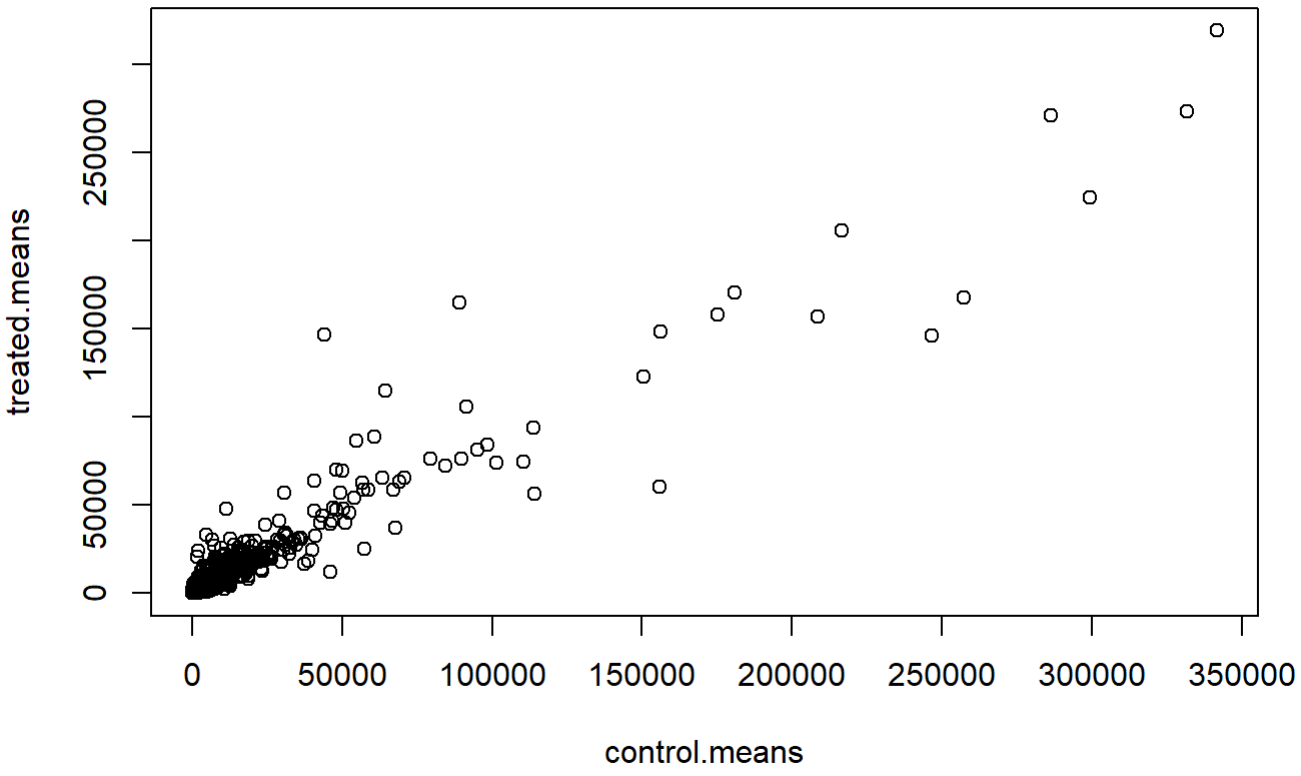
We now have control and treated mean count values. For ease of book-keeping I will combine these vectors into a new data.frame called `meancounts`

```
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.

```
plot(meancounts)
```



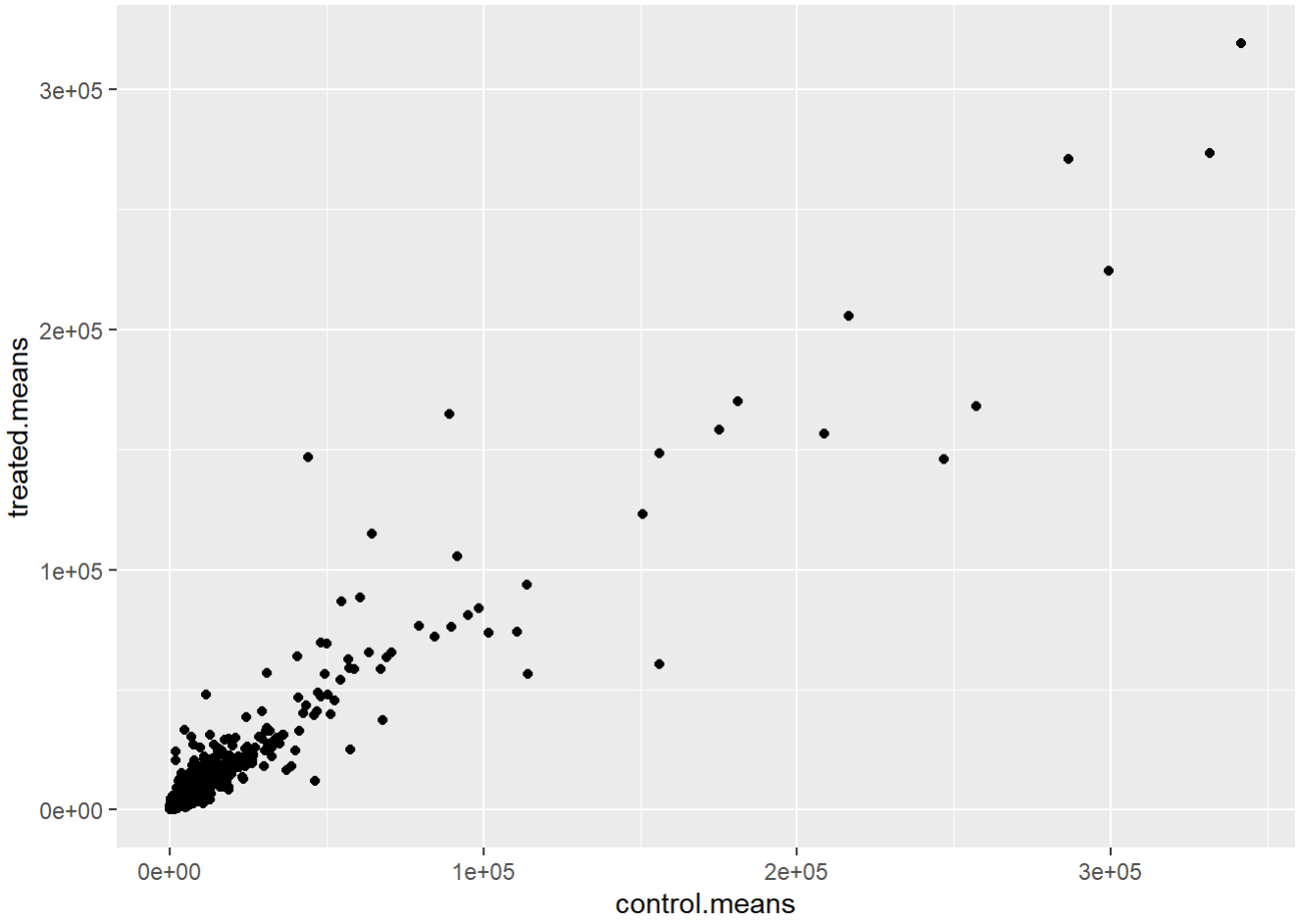
> Q5 (b).You could also use the ggplot2 package to make this figure producing the plot below. What geom_?() function would you use for this plot?

If using ggplot2, we would use the `geom_point() layer.

```
library(ggplot2)
```

```
Warning: package 'ggplot2' was built under R version 4.2.3
```

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



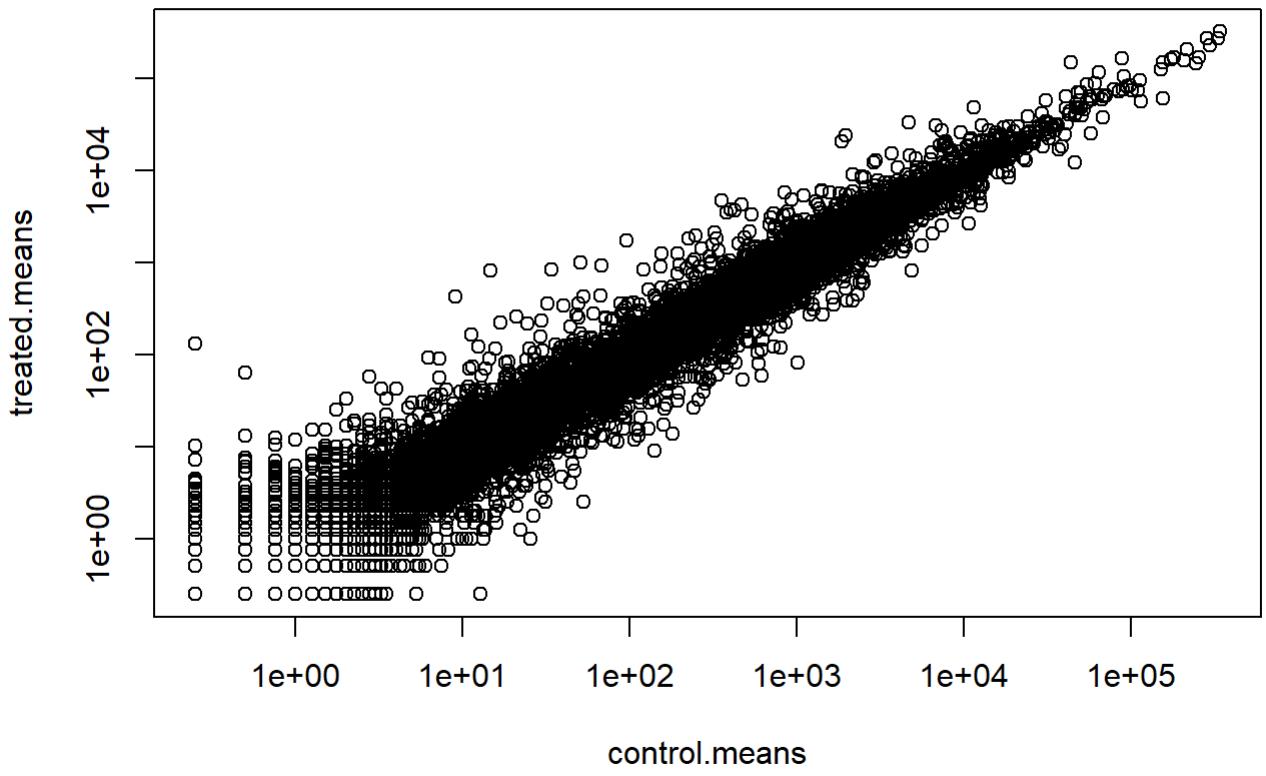
> Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

The plot argument `log = xy` allows us to plot both axes on a log10 scale.

```
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
```




```
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
ENSG00000000003        900.75        658.00
ENSG00000000419        520.50        546.00
ENSG00000000457        339.75        316.50
ENSG00000000460         97.25         78.75
ENSG00000000971       5219.00       6687.50
ENSG00000001036       2327.00       1785.75
```

> 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 lead which() to return both the row and column indices, where there are TRUE values. Here, this will tell us which rows and columns have zero counts, and ignore them. Calling unique() ensures we don't count any row twice if it has zero entries in both samples.

Let's transform our data into something more useful. We use log transforms for skewed data such as this because we really care most about relative changes in magnitude.

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

If we have no change - i.e. same values in control and treated, we will have a log2 value of 0.

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

```
[1] 0
```

If I have double the amount, I will have a log2 fold change of +1.

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

```
[1] 1
```

If I have half the amount, I will have a log2 fold change of -1.

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

```
[1] -1
```

```
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
```

> Q8. How many genes are upregulated at the common threshold of +2 logFC values?

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

```
[1] 1910
```

> Q9. Can you determine how many down regulated genes we have at the greater than 2 fc level?

```
# for genes downregulated beyond the -2 threshold
sum (meancounts$log2fc < -2, na.rm = TRUE)
```

```
[1] 2212
```

```
# for genes down regulated at & beyond the -2 threshold
sum (meancounts$log2fc <= -2, na.rm = TRUE)
```

```
[1] 2330
```

Hold on, what about the stats! Yes these are big changes but are they significant changes?

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

We have yet to run a statistical analaysis to determine if the differences between the treated and control groups are statistically significant. To do this properly, we will turn to the DESeq2 package.

DESeq2 Analysis

```
library(DESeq2)
```

```
Warning: package 'DESeq2' was built under R version 4.2.2

Warning: package 'S4Vectors' was built under R version 4.2.2

Warning: package 'GenomicRanges' was built under R version 4.2.2

Warning: package 'GenomeInfoDb' was built under R version 4.2.2

Warning: package 'matrixStats' was built under R version 4.2.3
```

To use our DESeq we need our input countData and colData in a specific format 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 input.

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

```
estimating size factors

estimating dispersions

gene-wise dispersion estimates
```

```
mean-dispersion relationship

final dispersion estimates

fitting model and testing
```

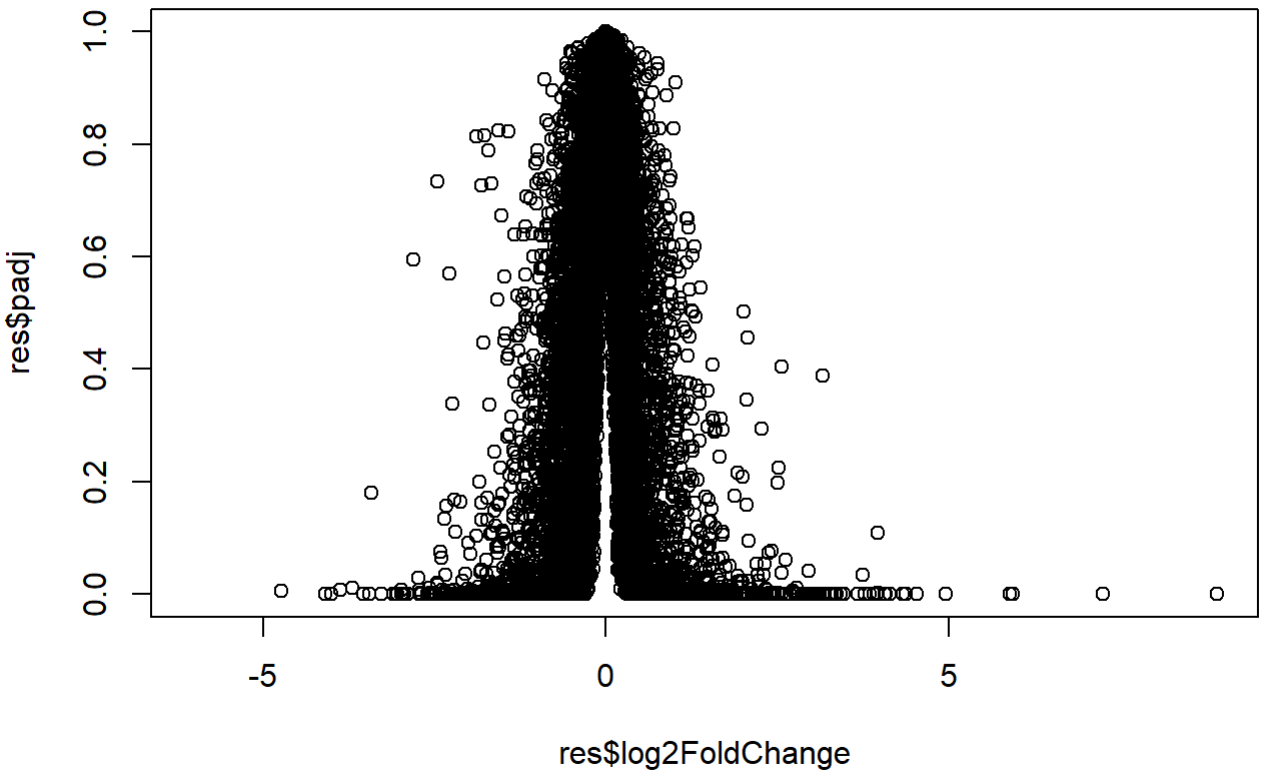
To get the results out of this `dds` object we can use the `results()` function from the package.

```
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
```

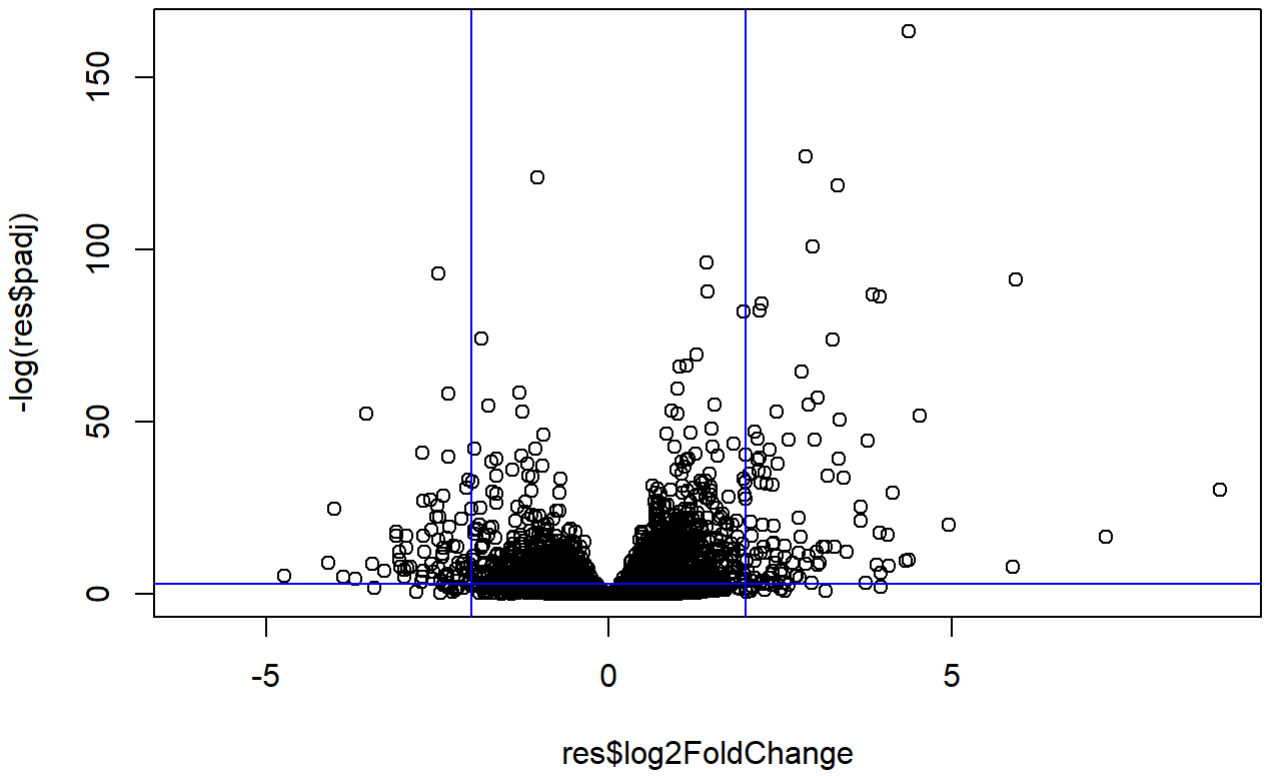
Let's make a final (for today) plot of log2 fold-change vs the adjusted p-value.

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



It is the low P-values that we care about and these are lost in the skewed plot above.

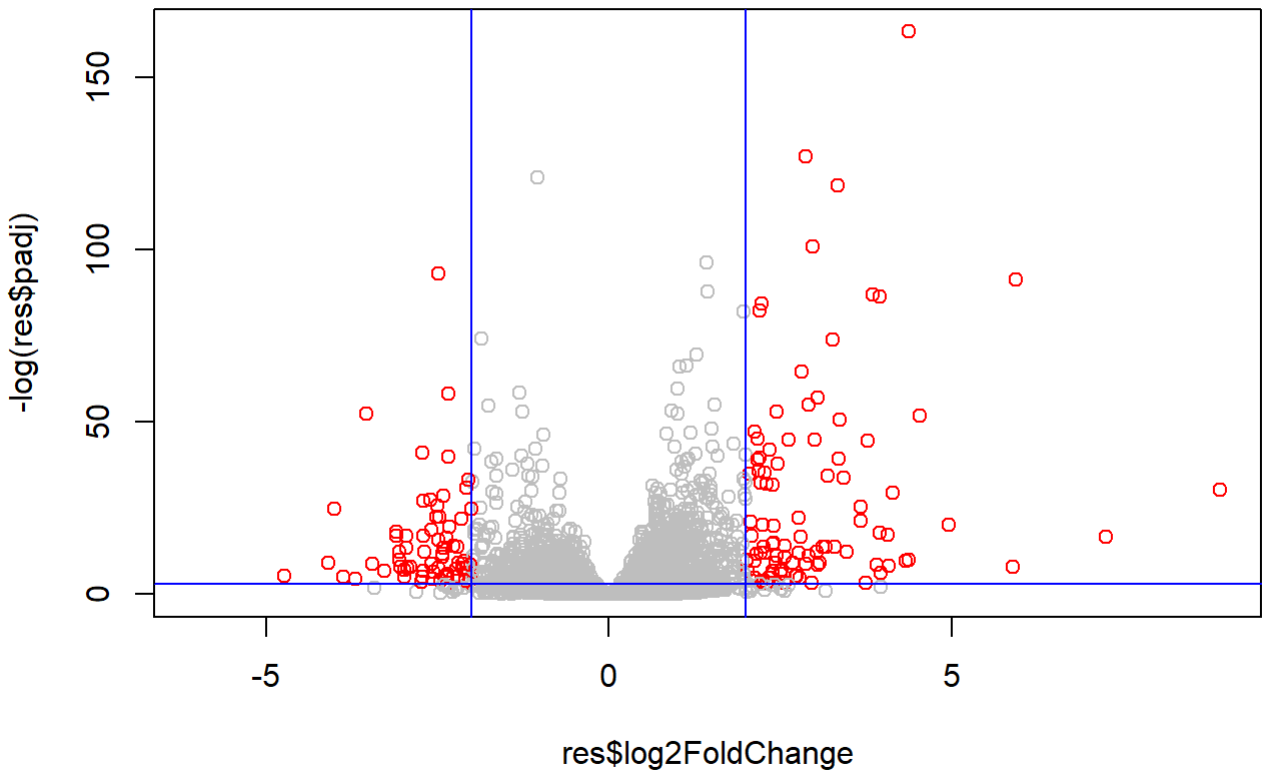
```
plot(res$log2FoldChange, -log(res$padj))
abline(v=c(+2, -2), col = "blue")
abline(h=-log(0.05), col = "blue")
```



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")
```



We're done for the day :).

Adding Annotation data

We will use one of Bioconductor's main annotation packages to help with mapping between various ID schemes. We'll use the AnnotationDbi package and the annotation data package for humans org.Hs.eg.db.

```
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
```

We can translate (a.k.a. "map") between all these database id formats

```
library("AnnotationDbi")
```

```
Warning: package 'AnnotationDbi' was built under R version 4.2.2
```

```
library("org.Hs.eg.db")
```

```
columns(org.Hs.eg.db)
```

```
 [1] "ACCNUM"       "ALIAS"        "ENSEMBL"      "ENSEMBLPROT"  "ENSEMBLTRANS"
 [6] "ENTREZID"     "ENZYME"       "EVIDENCE"     "EVIDENCEALL"  "GENENAME"
[11] "GENETYPE"     "GO"           "GOALL"        "IPI"          "MAP"
[16] "OMIM"         "ONTOLOGY"     "ONTOLOGYALL"  "PATH"         "PFAM"
[21] "PMID"         "PROSITE"      "REFSEQ"       "SYMBOL"       "UCSCKG"
[26] "UNIPROT"
```

My ids are stored as the rownames of `res`.

```
head( rownames(res) )
```

```
[1] "ENSG00000000003" "ENSG00000000005" "ENSG00000000419" "ENSG00000000457"
[5] "ENSG00000000460" "ENSG00000000938"
```

```
res$symbol <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",   # The format of our genenames
                     column="SYMBOL",     # The new format we want to add
                     multiVals="first")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 7 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      symbol
                <numeric> <character>
ENSG00000000003  0.163035      TSPAN6
ENSG00000000005        NA        TNMD
ENSG00000000419  0.176032        DPM1
ENSG00000000457  0.961694       SCYL3
ENSG00000000460  0.815849    C1orf112
ENSG00000000938        NA         FGR
```

> Q11. Run the mapIds() function two more times to add the Entrez ID and UniProt accession and GENENAME as new columns called res$entrez, res$uniprot and res$genename.

```
res$entrez <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",   # The format of our genenames
                     column="ENTREZID",     # The new format we want to add
                     multiVals="first")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
res$uniprot <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",   # The format of our genenames
                     column="UNIPROT",   # The new format we want to add
                     multiVals="first")
```

```
'select()' returned 1:many mapping between keys and columns
```

```
res$genename <- mapIds(org.Hs.eg.db,
                     keys=row.names(res), # Our genenames
                     keytype="ENSEMBL",   # The format of our genenames
                     column="GENENAME",   # The new format we want to add
                     multiVals="first")
```

```
'select()' returned 1:many mapping between keys and columns
```

Save our results to date

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

Pathway analysis

We can use the KEGG database of biological pathways to get some more insight into our differentially expressed genes and the kinds of biology they are involved in.

```
# results = hide
library(pathview)
```

```
##############################################################################
Pathview is an open source software package distributed under GNU General
Public License version 3 (GPLv3). Details of GPLv3 is available at
http://www.gnu.org/licenses/gpl-3.0.html. Particullary, users are required to
formally cite the original Pathview paper (not just mention it) in publications
or products. For details, do citation("pathview") within R.

The pathview downloads and uses KEGG data. Non-academic uses may require a KEGG
license agreement (details at http://www.kegg.jp/kegg/legal.html).
##############################################################################
```

```
library(gage)
```

```
library(gageData)
```

Look at the first two KEGG pathways.

```
data(kegg.sets.hs)

# Examine the first 2 pathways in this kegg set for humans
head(kegg.sets.hs, 2)
```

```
$`hsa00232 Caffeine metabolism`
[1] "10"   "1544" "1548" "1549" "1553" "7498" "9"

$`hsa00983 Drug metabolism - other enzymes`
 [1] "10"     "1066"   "10720"  "10941"  "151531" "1548"   "1549"   "1551"
 [9] "1553"   "1576"   "1577"   "1806"   "1807"   "1890"   "221223" "2990"
[17] "3251"   "3614"   "3615"   "3704"   "51733"  "54490"  "54575"  "54576"
[25] "54577"  "54578"  "54579"  "54600"  "54657"  "54658"  "54659"  "54963"
[33] "574537" "64816"  "7083"   "7084"   "7172"   "7363"   "7364"   "7365"
[41] "7366"   "7367"   "7371"   "7372"   "7378"   "7498"   "79799"  "83549"
[49] "8824"   "8833"   "9"      "978"
```

Make a new vector of fold-change values that I will use as input for `gage` this will have the ENTREZ IDs as names.

```
foldchanges = res$log2FoldChange
names(foldchanges) = res$entrez
```

```
head(foldchanges)
```

```
      7105       64102        8813       57147       55732        2268
-0.35070302          NA  0.20610777  0.02452695 -0.14714205 -1.73228897
```

```
# Get the results
keggres = gage(foldchanges, gsets=kegg.sets.hs)
```

Let's look at the attributes of keggres

```
attributes(keggres)
```

```
$names
[1] "greater" "less"    "stats"
```

Look at the top 3 "LESS"

```
head(keggres$less, 3)
```

```
                                   p.geomean stat.mean        p.val
hsa05332 Graft-versus-host disease 0.0004250461 -3.473346 0.0004250461
hsa04940 Type I diabetes mellitus  0.0017820293 -3.002352 0.0017820293
hsa05310 Asthma                    0.0020045888 -3.009050 0.0020045888
                                        q.val set.size         exp1
hsa05332 Graft-versus-host disease 0.09053483       40 0.0004250461
hsa04940 Type I diabetes mellitus  0.14232581       42 0.0017820293
hsa05310 Asthma                    0.14232581       29 0.0020045888
```

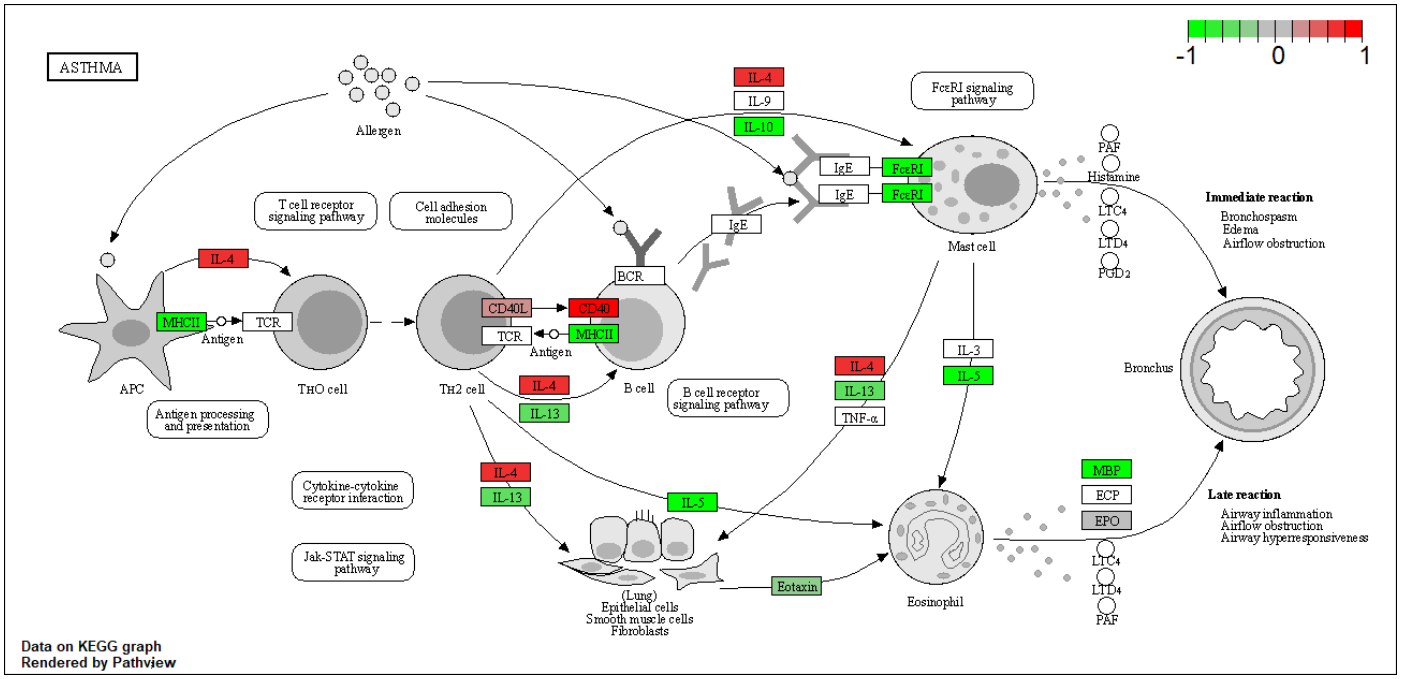
Now I can use the KEGG IDs of these pathways from gage to view our genes mapped to these pathways.

```
# message = false
pathview(gene.data=foldchanges, pathway.id="hsa05310")
```

```
'select()' returned 1:1 mapping between keys and columns

Info: Working in directory C:/Users/frank/Downloads/class12

Info: Writing image file hsa05310.pathview.png
```



Asthma pathway from KEGG with our genes shown in color