Class 13

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```
library("DESeq2")
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```
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, saveRDS, setdiff,
    table, tapply, union, unique, unsplit, which.max, which.min
```

```
Attaching package: 'S4Vectors'

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

    findMatches

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
```

Import countData and ColData

There are two datasets I need to import/read

- `countData` the transcript counts per gene (rows) in the different experiments
- `colData` information about the columns(i.e experiments) in `countData`

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

We can have a peak at these with `head()`

```
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
```

38694

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

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

```
control treated
      4       4
```

4

We can find the average (mean) count values per gene for all "control" experiments and compare it to the mean values for "treated".

- Extract all "control" columns from the `counts` data
- Find the mean value for each gene

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

```
dim(control.counts)
```

```
[1] 38694     4
```

Now find the row wise mean

```
control.mean <- rowSums(control.counts)/ncol(control.counts)
head(control.mean)
```

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

```
treated.inds <- metadata$dex == "treated"
treated.counts <- counts [ ,treated.inds]
treated.mean <- rowSums(treated.counts)/ncol(treated.counts)
head(treated.mean)
```

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

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

The way to make this more robust is to just use `rowmeans` instead of `rowsums`

> 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 <- metadata[metadata[,"dex"] == "treated",]
treated.mean <- rowSums(counts[treated$id] )/4
names(treated.mean) <- counts$ensgene
head(treated.mean)
```

```
[1] 658.00   0.00 546.00 316.50  78.75   0.00
```

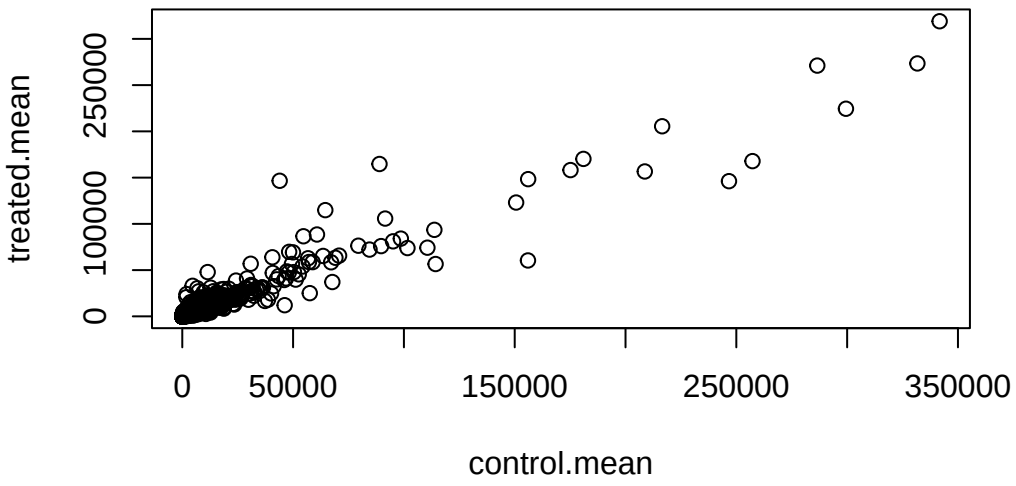
Lets put these two mean values together for easy book-keeping

```
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
```

Lets have a look at the plot of control.mean vs treated.mean > 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.

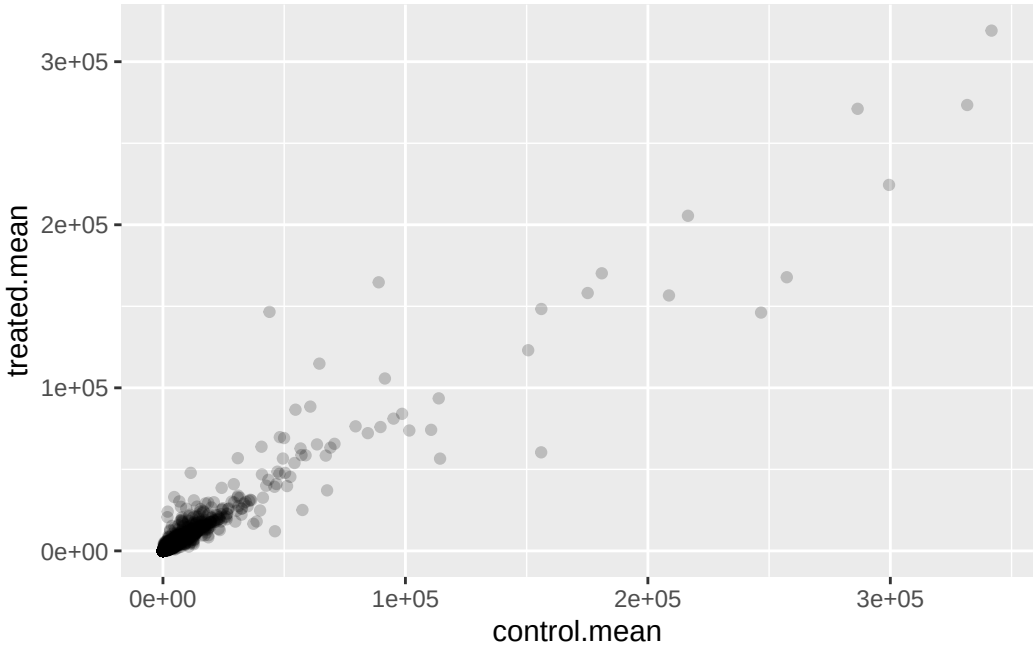
```
plot(meancounts)
```



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.mean, treated.mean) +
  geom_point(alpha=0.2)
```



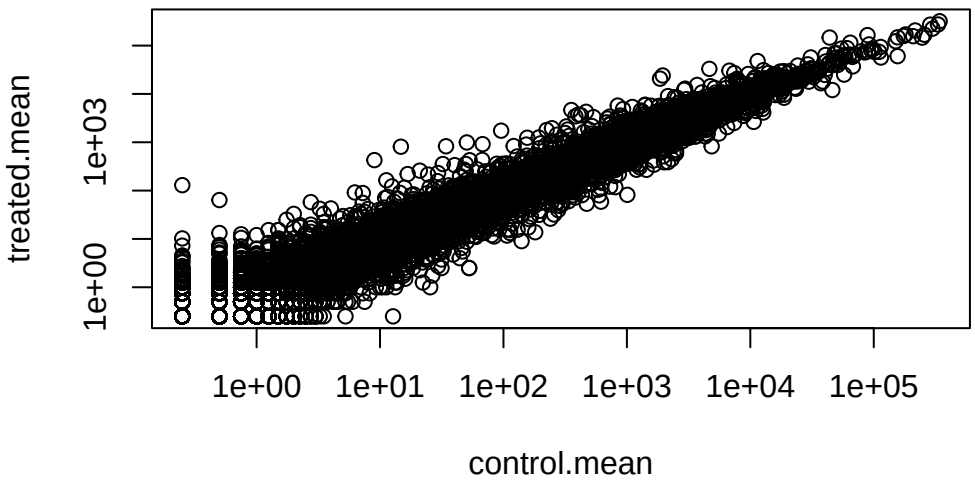
Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

Whenever we see the data that is so heavily skewed like this we often log transform the graph so we can see what is going on more easily.

```
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 most often work in log2 units as this makes the math easier. Lets have a play to see this

```
#treated/control
log2(20/20)
```

```
[1] 0
```

```
#treated/control
log2(20/40)
```

```
[1] -1
```

We can now add "log2 fold-change" values to our `meancounts` dataset.

```
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
```

We need to filter out zero cont genes -1.e remove the rows (genes) that have a 0 value in either control or treated means.

```
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 arr.ind=TRUE argument will clause which() to return both the row and column indices (i.e. positions) where there are TRUE values. In this case this will tell us which genes (rows) and samples (columns) have zero counts.

How many genes are "up" regulated at the common log2 fold-change threshold of +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 <- mycounts$log2fc > 2
sum(up.ind, na.rm = T)
```

```
[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, na.rm =T)
```

```
[1] 367
```

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

```
[1] 1910
```

How many genes are "down" regulated at the threshold of -2?

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

```
[1] 2330
```

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

No we do not trust these results due to not knowing which ones are statiscally significant due to the fold change altering.

DESeq2 analysis

To do this the right way we need to consider the significance of the differences not just their magnitude

```
library(DESeq2)
```

To use this package it wants countData and colData in a 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
```

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

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

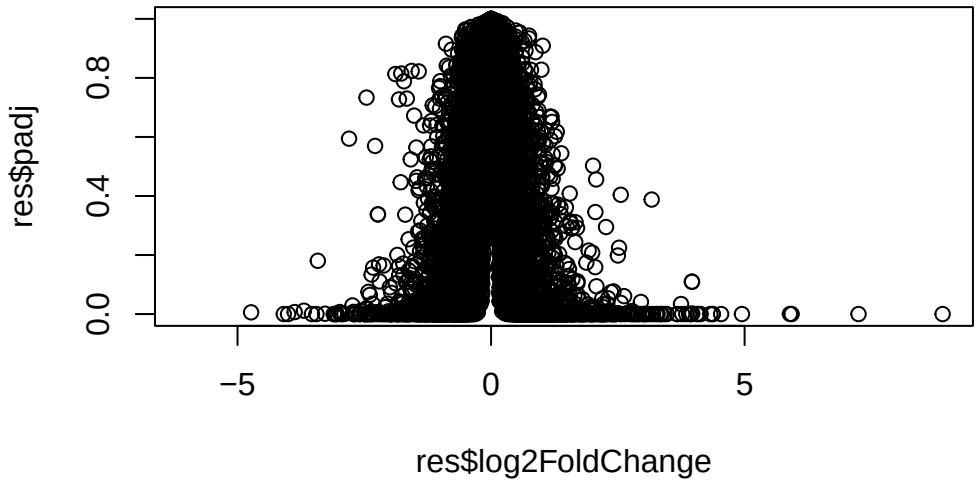
```
fitting model and testing
```

Extract my results

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

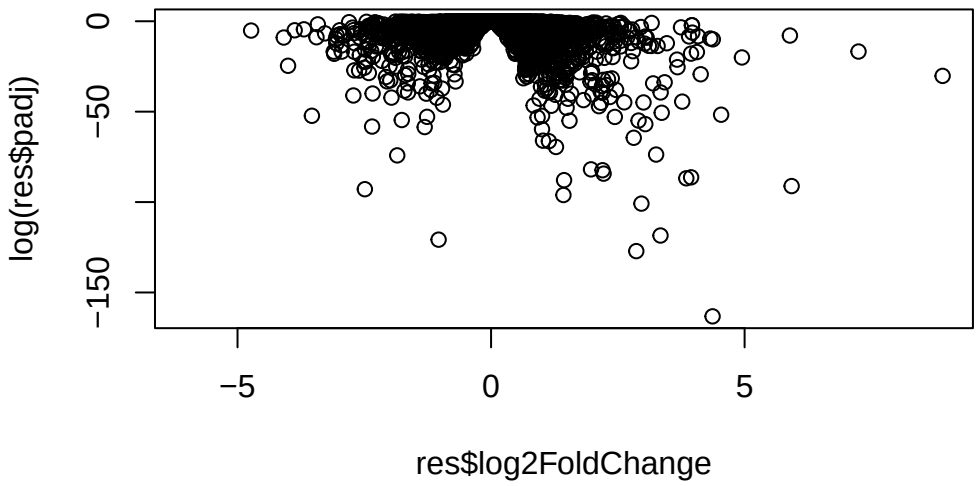
```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 6 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195 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

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



Take the log of the P-value

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




```
log(0.01)
```

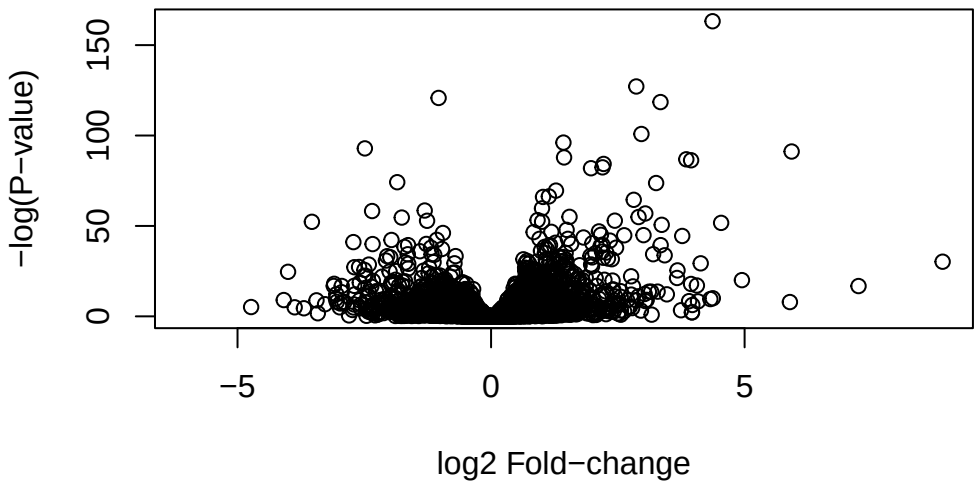
```
[1] -4.60517
```

```
log(0.0000000001)
```

```
[1] -23.02585
```

We can just flip the Y-axis by putting a minus sign on it

```
plot(res$log2FoldChange, -log(res$padj), xlab= "log2 Fold-change",
     ylab="-log(P-value)")
```



Lets save our work to date

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

To finish off lets make a nicer volcano plot

- Add the log2 threshold lines at +2 / -2
- Add P-value threshold lines at 0.05
- Add color to highlight the subset of genes that meet both of the above thresholds.

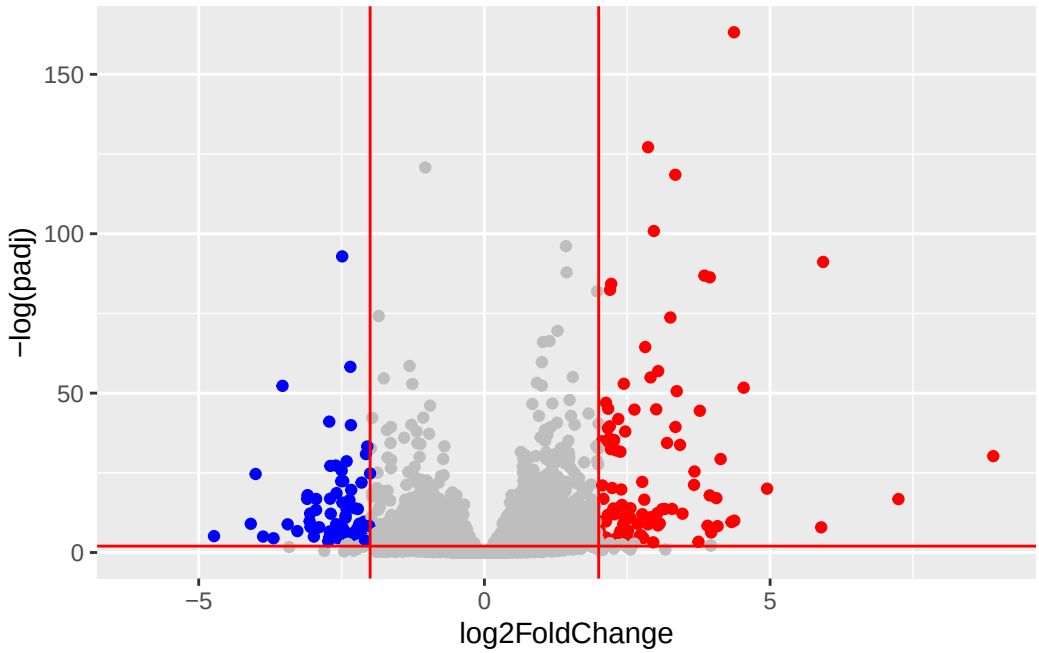
Make it with ggplot.

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

```
library(ggplot2)

ggplot(res) +
  aes(log2FoldChange, -log(padj))+
  geom_point(col = mycols) +
  geom_vline(xintercept = c(-2,2), col= "red") +
  geom_hline(yintercept = 2, col= "red")
```

```
Warning: Removed 23549 rows containing missing values or values outside the scale range
(`geom_point()`).
```



Add gene annotation data

Now the question is

We will use some BioConductor packages to "map" the ENSEMBLE ids to more useful gene SYMBOL name/ids.

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

What databse identifiers can I translate between here

```
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"
```

We can now use the `mapIDs()` function to translate/map between these different identifier formats.

Lets add SYMBOL, GENENAME and ENTREZID

```
res$symbol <- mapIds(org.Hs.eg.db,
              keys=rownames(res),
               keytype="ENSEMBL",
                column = "SYMBOL")
```

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

```
res$genome <- mapIds(org.Hs.eg.db,
              keys=rownames(res),
               keytype="ENSEMBL",
                column = "GENENAME")
```

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

```
  res$entrez <- mapIds(org.Hs.eg.db,
              keys=rownames(res),
               keytype="ENSEMBL",
                column = "ENTREZID")
```

```
'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 9 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                 genome      entrez
                <numeric> <character>            <character> <character>
ENSG00000000003  0.163035      TSPAN6          tetraspanin 6        7105
ENSG00000000005        NA        TNMD            tenomodulin       64102
ENSG00000000419  0.176032        DPM1 dolichyl-phosphate m..        8813
ENSG00000000457  0.961694       SCYL3 SCY1 like pseudokina..       57147
ENSG00000000460  0.815849       FIRRM FIGNL1 interacting r..       55732
ENSG00000000938        NA         FGR FGR proto-oncogene, ..        2268
```

Now I know the gene name and their IDs in different databases I want to know what type of biology they are involved in …

This is the job of "pathway analysis" (a.k.a "gene set enrichment")

there are tones of different bioconductor packages for pathway analysis here we use juse on of them called **gage**, and **pathview**. I will installl these packages with `BiocManager::install(c("gage", "pathview", "gageData"))`

```
library("gage")
```

```
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("gageData")
```

Load up the KEGG genesets

```
data(kegg.sets.hs)
```

```
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"
```

We will use these KEGG genesets (a.k.a pathways) and our `res` results to see what overlaps. To do this we will use the `gage()` function.

For input`gage()` wnats just a vector of importance - in our case FoldChange values.

```
foldchanges <- res$log2FoldChange
```

Vectors in R can have "names that are useful for bookkeepingso we know what a given value corresponds to e.g

```
x<- c(10, 100, 20)
names(x) <- c("barry", "alice", "chandra")
x
```

```
  barry   alice chandra
     10     100      20
```

Let's put names on our `foldchanges` vector - here we will use `res$entrez`

```
names(foldchanges) <- res$entrez
head(foldchanges)
```

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

Now we can run "pathway analysis"

```
keggres = gage(foldchanges, gsets=kegg.sets.hs)
```

```
head(keggres$less)
```

```
                                                      p.geomean stat.mean
hsa05332 Graft-versus-host disease                   0.0004250461 -3.473346
hsa04940 Type I diabetes mellitus                    0.0017820293 -3.002352
hsa05310 Asthma                                      0.0020045888 -3.009050
hsa04672 Intestinal immune network for IgA production 0.0060434515 -2.560547
hsa05330 Allograft rejection                         0.0073678825 -2.501419
hsa04340 Hedgehog signaling pathway                  0.0133239547 -2.248547
                                                             p.val      q.val
hsa05332 Graft-versus-host disease                   0.0004250461 0.09053483
hsa04940 Type I diabetes mellitus                    0.0017820293 0.14232581
hsa05310 Asthma                                      0.0020045888 0.14232581
hsa04672 Intestinal immune network for IgA production 0.0060434515 0.31387180
hsa05330 Allograft rejection                         0.0073678825 0.31387180
hsa04340 Hedgehog signaling pathway                  0.0133239547 0.47300039
                                                     set.size         exp1
hsa05332 Graft-versus-host disease                         40 0.0004250461
hsa04940 Type I diabetes mellitus                          42 0.0017820293
hsa05310 Asthma                                            29 0.0020045888
hsa04672 Intestinal immune network for IgA production      47 0.0060434515
hsa05330 Allograft rejection                               36 0.0073678825
hsa04340 Hedgehog signaling pathway                        56 0.0133239547
```

We can get a pathway image file with our genesets highlighted via the `pathview()` function.

```
pathview(foldchanges, pathway.id = "hsa05310")
```

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

```
Info: Working in directory /Users/yaniviny/Desktop/BIMM143/Class13

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

Insert this figure in my report by