class_13

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Today we will analyze data from a published RNA-seq experiment where airway smooth muscle cells were treated with dexamethasone, a synthetic glucocorticoid steroid with anti-inflammatory effect.

```
library(BiocManager)
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, 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


Attaching package: 'IRanges'

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

    windows

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 databases I need to import/read

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

```
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? 38694 genes in this dataset.

```
nrow(counts)
```

```
[1] 38694
```

Q2. How many 'control' cell lines do we have? There are 4 'control' cell lines.

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

```
control treated
      4       4
```

Another method to get this data (shows how many are true for control):

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

```
[1] 4
```

We could 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 in these columns

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

```
                SRR1039508 SRR1039512 SRR1039516 SRR1039520
ENSG00000000003        723        904       1170        806
ENSG00000000005          0          0          0          0
ENSG00000000419        467        616        582        417
ENSG00000000457        347        364        318        330
ENSG00000000460         96         73        118        102
ENSG00000000938          0          1          2          0
```

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

Now, find the row wise mean. rowSums sums up all the rows in control.counts then divides by 4 because thats how many control there is.

```
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
```

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]
head(treated.counts)
```

```
                SRR1039509 SRR1039513 SRR1039517 SRR1039521
ENSG00000000003        486        445       1097        604
ENSG00000000005          0          0          0          0
ENSG00000000419        523        371        781        509
ENSG00000000457        258        237        447        324
ENSG00000000460         81         66         94         74
ENSG00000000938          0          0          0          0
```

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

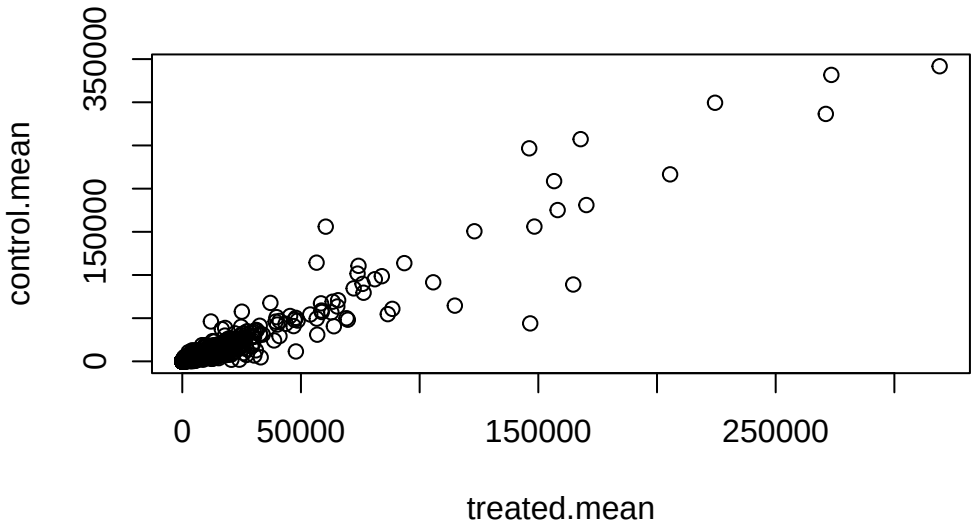
```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
         658.00            0.00          546.00          316.50           78.75
ENSG00000000938
           0.00
```

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.

First, lets put them together in a df:

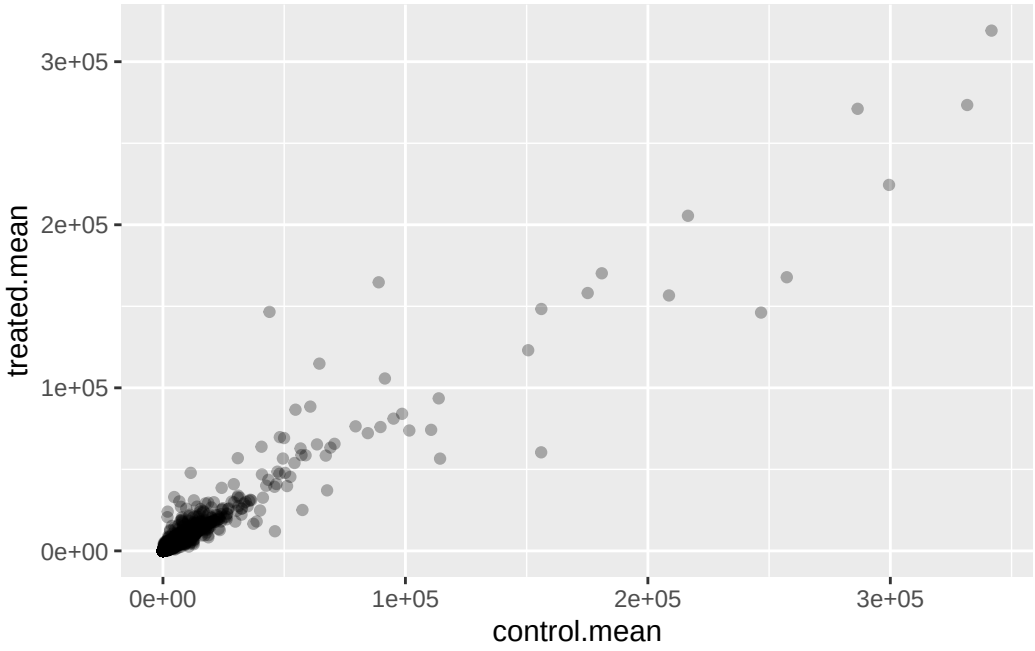
```
meancounts <- data.frame(treated.mean, control.mean)
```

```
treated.control_plot <- 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 = 0.3)
```



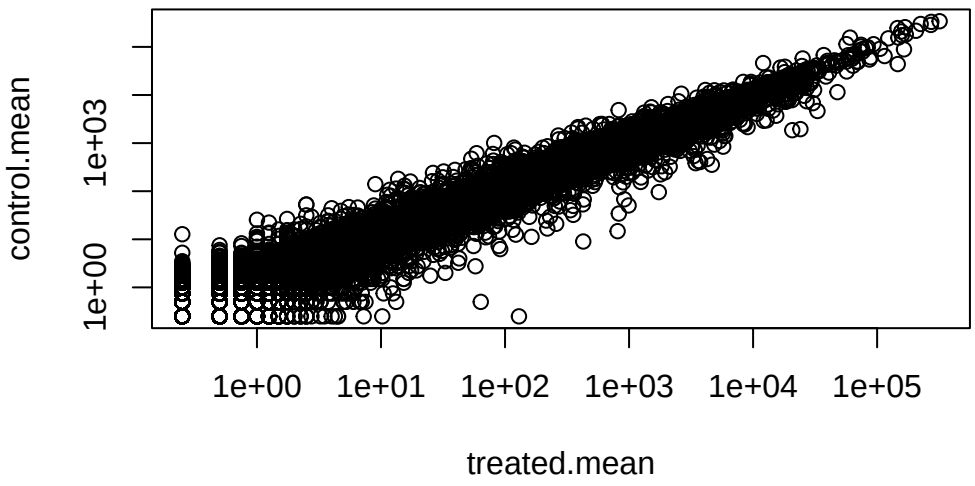
Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

Whenever we see data that's so heavily skewed like this we often log transform it so we can see what's going on more easily.

```
plot(meancounts, log = "xy")
```

```
Warning in xy.coords(x, y, xlabel, ylabel, log): 15281 x values <= 0 omitted
from logarithmic plot
```

```
Warning in xy.coords(x, y, xlabel, ylabel, log): 15032 y values <= 0 omitted
from logarithmic plot
```



We must often work in log2 units as this makes the math easier. Let's attempt this to see how it works.

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

```
[1] 0
```

```
log2(40/20)
```

```
[1] 1
```

```
log2(80/20)
```

```
[1] 2
```

The sign of the value tells you whether there was a decrease/increase in expression.

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

```
[1] -1
```

We can now add a "log2 fold-change" value to our 'meancounts' dataset.

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

head(meancounts) # it can be viewed on the table or you can call the column directly with hea
```

```
                treated.mean control.mean      log2fc
ENSG00000000003       658.00       900.75 -0.45303916
ENSG00000000005         0.00         0.00         NaN
ENSG00000000419       546.00       520.50  0.06900279
ENSG00000000457       316.50       339.75 -0.10226805
ENSG00000000460        78.75        97.25 -0.30441833
ENSG00000000938         0.00         0.75        -Inf
```

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

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

```
                treated.mean control.mean      log2fc
ENSG00000000003       658.00       900.75 -0.45303916
ENSG00000000419       546.00       520.50  0.06900279
ENSG00000000457       316.50       339.75 -0.10226805
ENSG00000000460        78.75        97.25 -0.30441833
ENSG00000000971      6687.50      5219.00  0.35769358
ENSG00000001036      1785.75      2327.00 -0.38194109
```

> Q7. What is the purpose of the arr.ind argument in the which() function call above? This argument allows the which() function to return the rows and columns with TRUE values, filtering out the ones with zero counts. Why would we then take the first column of the output and need to call the unique() function? The unique() function makes it so there arent redundancies, so rows won't be counted more than once for having zero counts.

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

How many genes are "up" regulated at the common log2 fold-change threshold of +2.

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

```
[1] 1910
```

> Q8. Using the up.ind vector above can you determine how many up regulated genes we have at the greater than 2 fc level? 250.

```
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? 367.

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

```
[1] 367
```

> Q10. Do you trust these results? Why or why not? No, because we don't know if the results based on fold change are significant or not.

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

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

```
[1] 2330
```

DESeq2 analysis

```
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

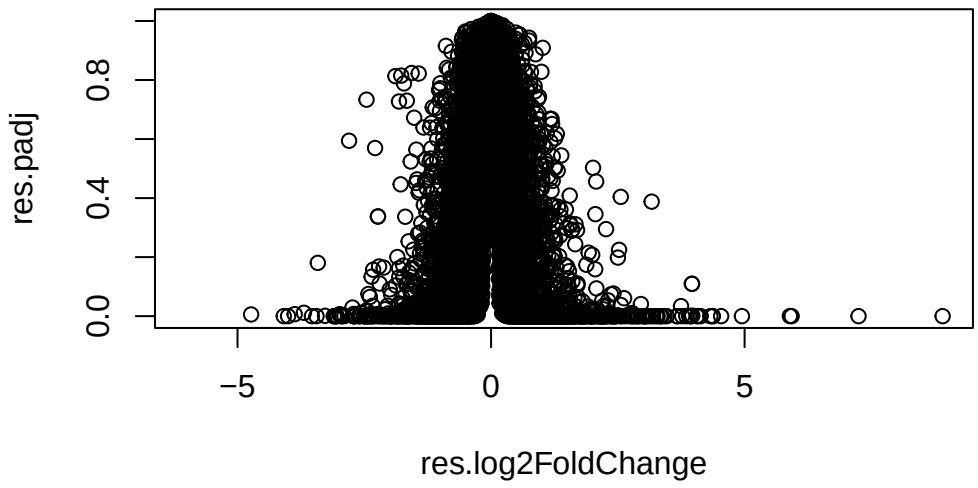
```
res <- results(dds)
head(res)
```

```
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 of fold-change vs P-value (adjusted for multiple testing)

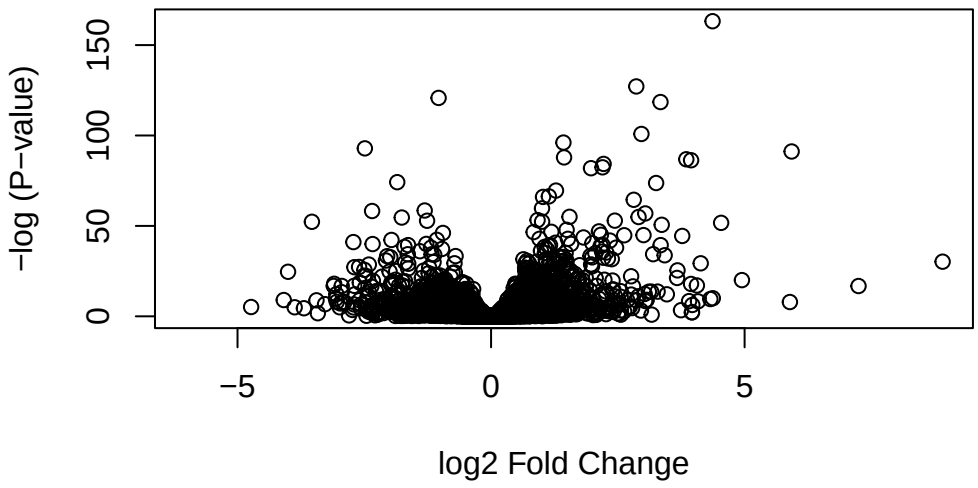
```
resPFC <- data.frame(res$log2FoldChange, res$padj)
plot(resPFC)
```



Take the log of the P-value, then it creates a plot where we notice that the higher the negative number, the lower the P-value. So, log of a P-value of 0.00001 would give a high negative number like -40.

We can flip the y axis by adding a negative number in front of padj to make it the y-axis instead of x-axis.

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



To finish off, let's make a nicer volcano plot.

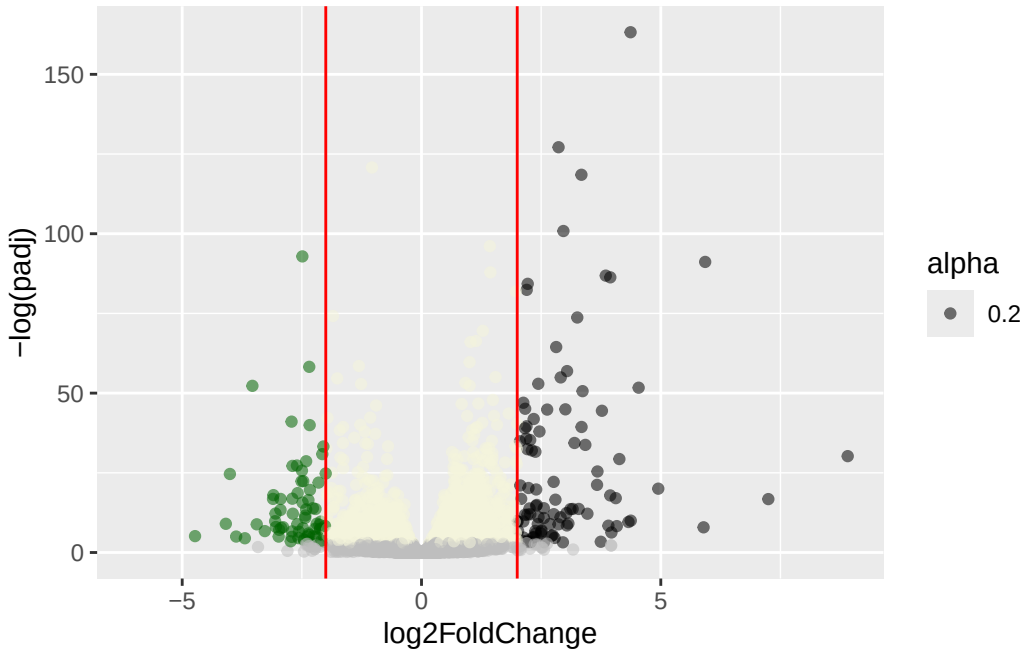
- Add the log2 threshold lines at +2/-2
- Add P-value threshold lines at 0.05
- Add color to highlight the subset of genes that meet both of the above thresholds.

Make it with ggplot

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

```
ggplot(res) +
  aes(log2FoldChange, -log(padj), alpha = 0.2) +
  geom_point(col = mycols) +
  geom_vline(xintercept = c(-2,2), col = "red")
```

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



Let's save our up to date work

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

We will use some BioConductor packages to "map" the ENSEMBL ids to more useful gene SYMBOL names/

We can install these packages with "BiocManager::install("AnnotationDbi")

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

What database 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

Let's add SYMBOL, and ENTREZID

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

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

```
res$uniprot <- mapIds(org.Hs.eg.db,
                     keys=row.names(res),
                     column="UNIPROT",
                     keytype="ENSEMBL",
                     multiVals="first")
```

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

```
res$genename <- mapIds(org.Hs.eg.db,
                     keys=row.names(res),
                     column="GENENAME",
                     keytype="ENSEMBL",
                     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 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      entrez     uniprot               genename
                <numeric> <character> <character>            <character>
ENSG00000000003  0.163035        7105  A0A087WYV6          tetraspanin 6
ENSG00000000005        NA       64102      Q9H2S6            tenomodulin
ENSG00000000419  0.176032        8813      H0Y368 dolichyl-phosphate m..
ENSG00000000457  0.961694       57147      X6RHX1 SCY1 like pseudokina..
ENSG00000000460  0.815849       55732      A6NFP1 FIGNL1 interacting r..
ENSG00000000938        NA        2268      B7Z6W7 FGR proto-oncogene, ..
```

##Pathway Analysis

Now, I know the gene names 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 tons of different BioConductor packages for pathway and we use just one of them called **gage** and **pathway**. I will install these packages with 'BiocManager::install(c("gage", "pathview", "gageData"))'

```
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)
```

Load up the KEGG gene

```
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 'gage' function.

For input

```
foldchanges = res$log2FoldChange
```

Vectors "names" that are useful for bookkeeping what a given value corresponds to, e.g.

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

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

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

Now we can run "pathway analysis"

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

```
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
```

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 C:/Users/profe/OneDrive/Desktop/bimm143/class_13
```

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

Insert this figure in my report