Class 13: RNASeq analysis with DESeq2

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> The data for this hands-on session comes from 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).

```
#install.packages("BiocManager")
#BiocManager::install("DESeq2")
```

```
library(DESeq2)
```

Data import

```
#Complete the missing code
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?

38694 genes.

```
nrow(counts)
```

```
[1] 38694
```

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

There are 4 controls.

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

```
[1] 4
```

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

```
control treated
      4       4
```

```
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
```

```
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
```

I want to compare the control to the treated columns. To do this I will

- Step 1. Identify and extract the "control" columns.
- Step 2. Calculate the mean value per gene fro all these "control" columns and save as `control.mean`.
- Step 3. Do the same for treated
- Step 4. Compare the `control.mean` and `treated.mean` values.

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

Step 1:

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

```
control.mean <- rowMeans(counts[,control.inds])
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`)

To calculate the treated group.

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

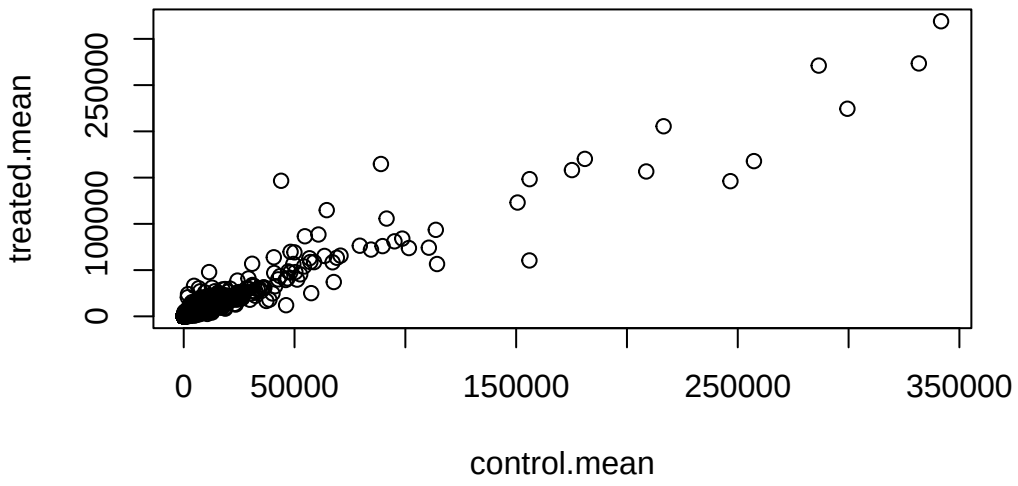
```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
         658.00            0.00          546.00          316.50            78.75
ENSG00000000938
           0.00
```

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

Let's see what these count values look like…

```
plot(meancounts)
```

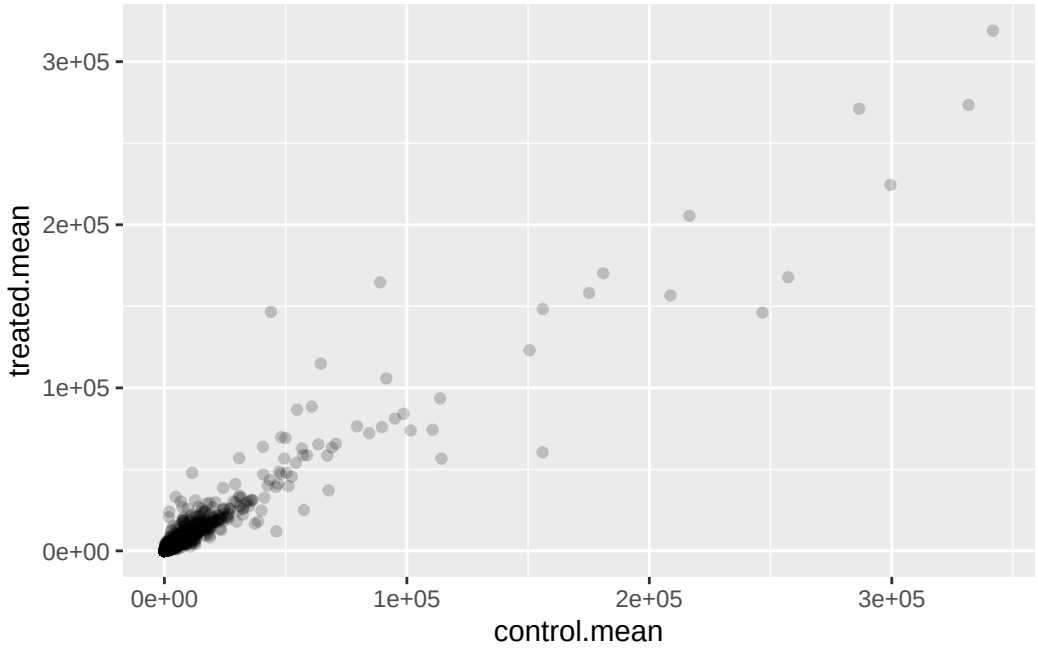


Q5 (b).You could also use the ggplot2 package to make this figure producing the plot below. What geom_?() function would you use for this plot?

Use geom_point.

```
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?

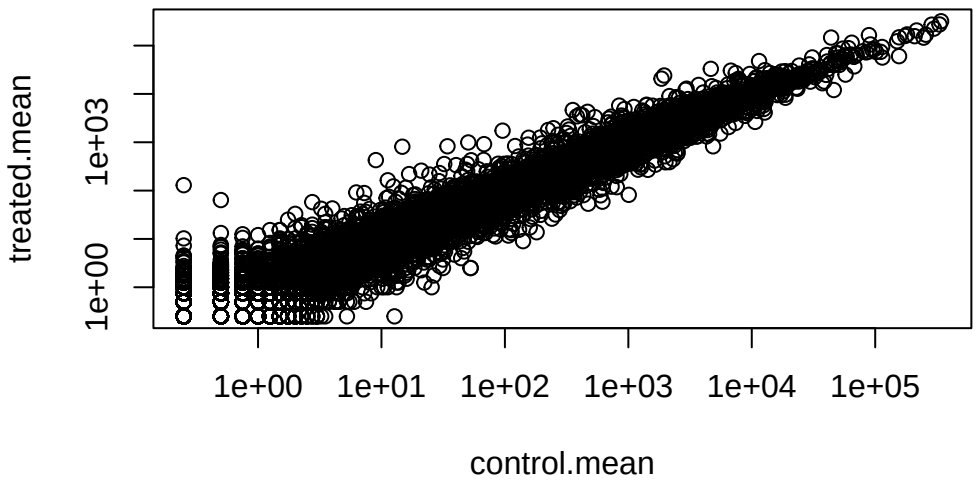
log .

a character string which contains "x" if the x axis is to be logarithmic, "y" if the y axis is to be logarithmic and "xy" or "yx" if both axes are to be logarithmic.

```
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
```



Logs are super useful when we have such skewed data

```
# Treated / control
# log2(10/10) = 0
# log2(5/10) = -1
```

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

```
[1] 1
```

Add log2(Fold-change) values to our wee results table.

```
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
```

I need to exclude any genes with zero counts as we can't say anything about them anyway form this experiment and it causes me math pain.

```
# What values in the first two cols are zero
# to remove
to.rm.inds <- rowSums(meancounts[,1:2] == 0) > 0
mycounts <- meancounts[!to.rm.inds,]
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
```

```
c(TRUE, FALSE, TRUE)
```

```
[1]  TRUE FALSE  TRUE
```

```
-c(TRUE, FALSE, TRUE)
```

```
[1] -1  0 -1
```

```
!c(TRUE, FALSE, TRUE)
```

```
[1] FALSE  TRUE FALSE
```

```
# Tell the elements that are TRUE
which(c(TRUE, FALSE, TRUE))
```

```
[1] 1 3
```

Another method:

```
#zero.vals <- which(meancounts[,1:2]==0, arr.ind=TRUE)
#to.rm <- unique(zero.vals[,1])
#mycounts <- meancounts[-to.rm,]
#head(mycounts)
```

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?

arr.ind logical; should array indices be returned when x is an array? Anything other than a single true value is treated as false.

unique() to prevent repetitive reports of both row and column == 0.

Q. How many genes do I have left?

```
nrow(mycounts)
```

```
[1] 21817
```

Q. How many genes are "up-regulated" i.e. have a log2(fold-change greater than +2?

```
sum(mycounts$log2fc > +2)
```

```
[1] 250
```

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$log2fc > 2
down.ind <- mycounts$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?

```
sum(down.ind)
```

```
[1] 367
```

367.

Q. How many genes are "down-regulated" i.e. have a log2(fold-change less than -2?

```
sum(mycounts$log2fc < -2)
```

```
[1] 367
```

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

We have to consider the statistical difference via p-value or p adjust.

Running DESeq

Like many bioconductor analysis packages DESeq wants it's inpit in a very particular way.

```
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
```

To run DESeq analysis we call the main function from the packages called `DESeq(dds)`.

```
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 DESeq `results()` function.

```
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
```

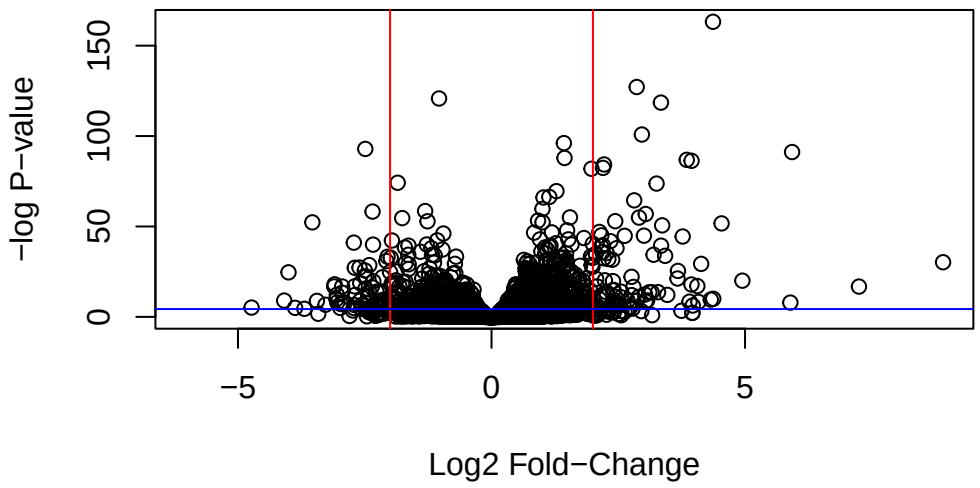
? To be more stringent. “padj”

```
-log2(0.05)
```

```
[1] 4.321928
```

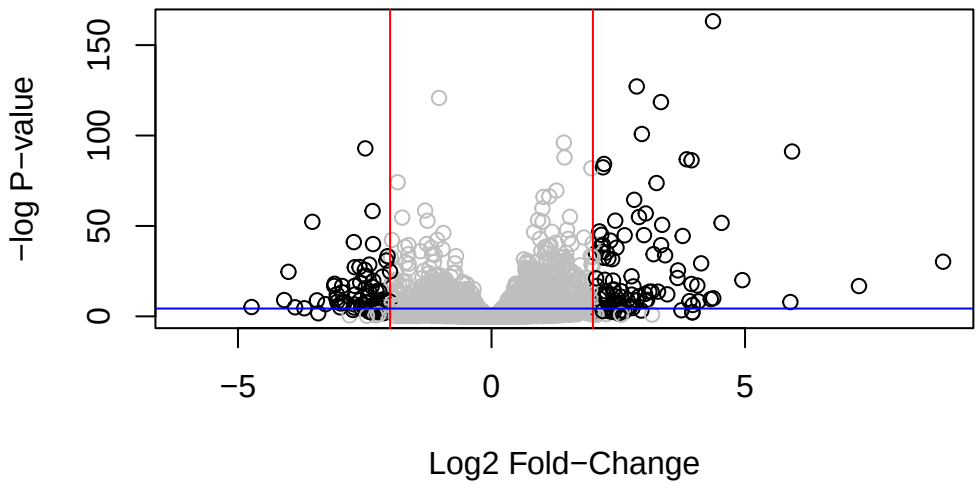
A common summary visualization is called a Volcano plot.

```
plot(res$log2FoldChange, -log(res$padj),
     xlab="Log2 Fold-Change",
     ylab="-log P-value")
#vertical
abline(v=c(-2,2), col="red")
#horizontal
abline(h=-log2(0.05), col="blue")
```




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

```
plot(res$log2FoldChange, -log(res$padj), col=mycols,
     xlab="Log2 Fold-Change",
     ylab="-log P-value")
#vertical
abline(v=c(-2,2), col="red")
#horizontal
abline(h=-log2(0.05), col="blue")
```

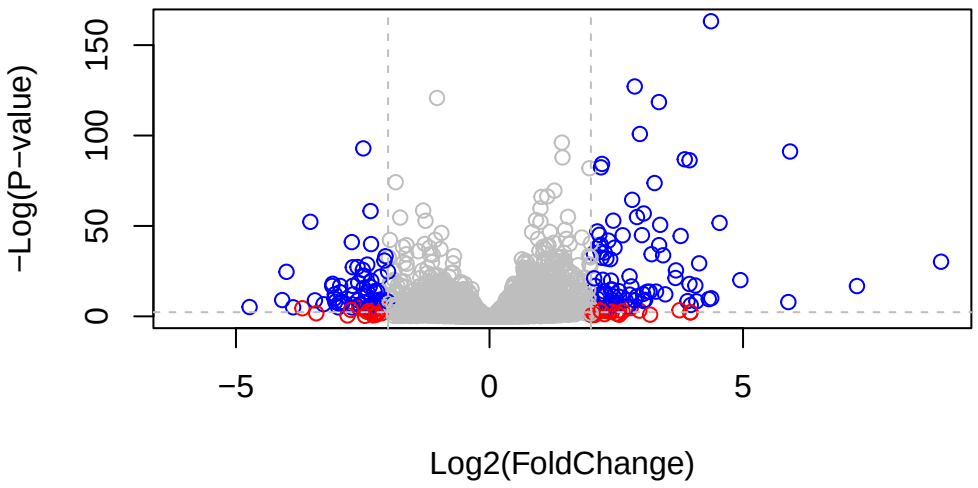



```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red"

inds <- (res$padj < 0.01) & (abs(res$log2FoldChange) > 2 )
mycols[ inds ] <- "blue"

# Volcano plot with custom colors
plot( res$log2FoldChange,  -log(res$padj),
 col=mycols, ylab="-Log(P-value)", xlab="Log2(FoldChange)" )

# Cut-off lines
abline(v=c(-2,2), col="gray", lty=2)
abline(h=-log(0.1), col="gray", lty=2)
```




```
BiocManager::install("EnhancedVolcano")
```

```
Bioconductor version 3.18 (BiocManager 1.30.22), R 4.3.1 (2023-06-16 ucrt)
```

```
Warning: package(s) not installed when version(s) same as or greater than current; use
  `force = TRUE` to re-install: 'EnhancedVolcano'
```

```
Installation paths not writeable, unable to update packages
  path: C:/Program Files/R/R-4.3.1/library
  packages:
    foreign, KernSmooth, lattice, mgcv, nlme, rpart, spatial, survival
```

```
Old packages: 'BiocVersion', 'dplyr', 'GenomeInfoDb', 'httr2', 'Matrix',
  'shiny', 'stringi', 'stringr', 'xfun'
```

```
library(EnhancedVolcano)
```

```
Loading required package: ggrepel
```

```
library(ggrepel)
```

```
#x <- as.data.frame(res)

#EnhancedVolcano(x,lab = x$symbol,x = 'log2FoldChange',y = 'pvalue')
```

Save our results to date

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

Adding annotation data

We need to translate or "map" our ensemble IDs into more understandable gene names and the identifiers that other useful databases use.

```
#BiocManager::install("AnnotationDbi")
```

```
#BiocManager::install("org.Hs.eg.db")
```

```
library(AnnotationDbi)
```

```
Warning: package 'AnnotationDbi' was built under R version 4.3.2
```

```
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"
```

```
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       FIRRM
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
```

```
head(res)
```

```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 10 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      entrez     uniprot
                <numeric> <character> <character> <character>
ENSG00000000003  0.163035      TSPAN6        7105  A0A024RCI0
ENSG00000000005        NA        TNMD       64102      Q9H2S6
ENSG00000000419  0.176032        DPM1        8813      O60762
ENSG00000000457  0.961694       SCYL3       57147      Q8IZE3
ENSG00000000460  0.815849       FIRRM       55732  A0A024R922
```

```
ENSG00000000938        NA         FGR        2268      P09769
                                genename
                              <character>
ENSG00000000003          tetraspanin 6
ENSG00000000005            tenomodulin
ENSG00000000419 dolichyl-phosphate m..
ENSG00000000457 SCY1 like pseudokina..
ENSG00000000460 FIGNL1 interacting r..
ENSG00000000938 FGR proto-oncogene, ..
```

```
# Run in your R console (i.e. not your Rmarkdown doc!)
#BiocManager::install( c("pathview", "gage", "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)
```

```
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"
```

$hsa00232 Caffeine metabolism #ENTREZID [1] “10” “1544” “1548” “1549” “1553” “7498” “9”

More easily to hit $hsa00983 Drug metabolism - other enzymes Because there are more genes in it Calibration

```
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
```

ENTREZID

Run gage:

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

```
attributes(keggres)
```

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

```
# Look at the first three down (less) pathways
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
```

To look at the pathway of hsa05310 Asthma

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

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

```
Info: Working in directory D:/UCSD BioSci/Courses/Year 1/Fall_BGGN 213 Bioinformatics/Previou
```

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