# Transcriptomics and the analysis of RNA-Seq data

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## 1. Bioconductor and DESeq2 setup

Install BioConductor and DESeq2

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

#BiocManager::install("DESeq2")
library(BiocManager)
library(DESeq2)
```

## 2. Import countData and colData

Read in our data

```
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?

```
nrow(counts)
```

```
## [1] 38694
```

ANSWER: There are 38694 genes in the dataset.

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

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

```
## [1] 4
```

ANSWER: There are 4 control cell lines

## 3. Toy differential gene expression

Let's calculate the mean counts per gene across these samples:

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

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

And do it again using the dplyr package

```
#remove.packages("rlang")
#install.packages("rlang")
library(rlang)
```

```
## Warning: package 'rlang' was built under R version 4.1.2
```

```
##
## Attaching package: 'rlang'
```

```
## The following object is masked from 'package:Biobase':
##
##     exprs
```

```
library(dplyr)
```

```
## Warning: package 'dplyr' was built under R version 4.1.2
```

```
##
## Attaching package: 'dplyr'
```

```
## The following object is masked from 'package:Biobase':
##
##     combine

## The following object is masked from 'package:matrixStats':
##
##     count

## The following objects are masked from 'package:GenomicRanges':
##
##     intersect, setdiff, union

## The following object is masked from 'package:GenomeInfoDb':
##
##     intersect

## The following objects are masked from 'package:IRanges':
##
##     collapse, desc, intersect, setdiff, slice, union

## The following objects are masked from 'package:S4Vectors':
##
##     first, intersect, rename, setdiff, setequal, union

## The following objects are masked from 'package:BiocGenerics':
##
##     combine, intersect, setdiff, union

## The following objects are masked from 'package:stats':
##
##     filter, lag

## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union
```

```
# install.packages("dplyr")
library(dplyr)
control <- metadata %>% filter(dex=="control")
control.counts <- counts %>% select(control$id)
control.mean <- rowSums(control.counts)/4
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?

To calculate the mean manually, the above codes use (control.counts)/4. However, a more robust way of writing this would be to use rowMeans(), as such:

```
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
```

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)

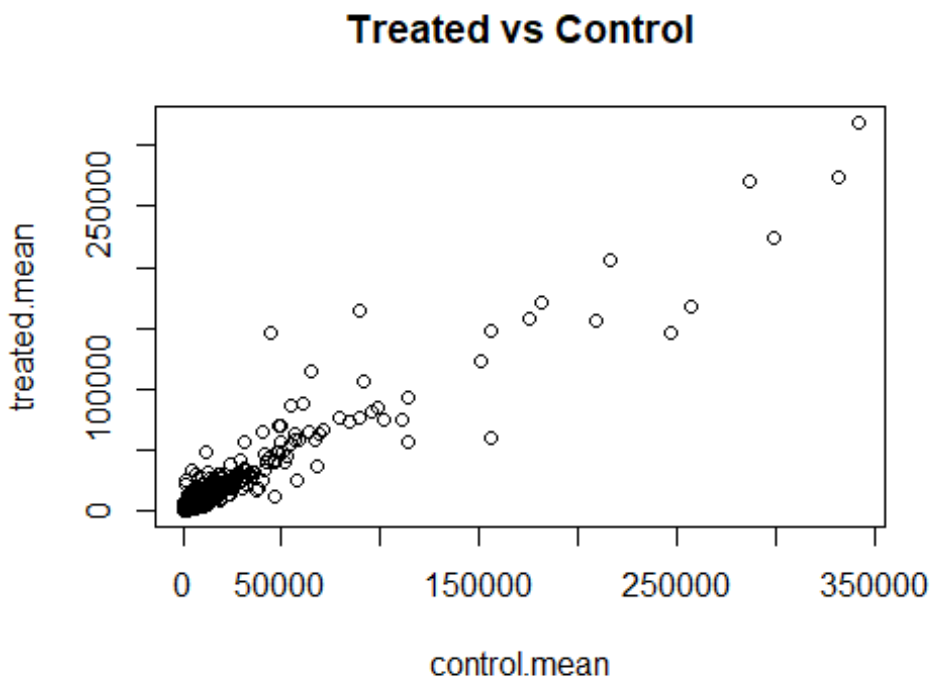
```
treated <- metadata[metadata[,"dex"]=="treated",]
treated.counts <- counts[ ,treated$id]
treated.mean <- rowMeans(treated.counts)
head(treated.mean)

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

Combine our meancount data for bookkeeping purposes:

```
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
```

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) + title("Treated vs Control")
```

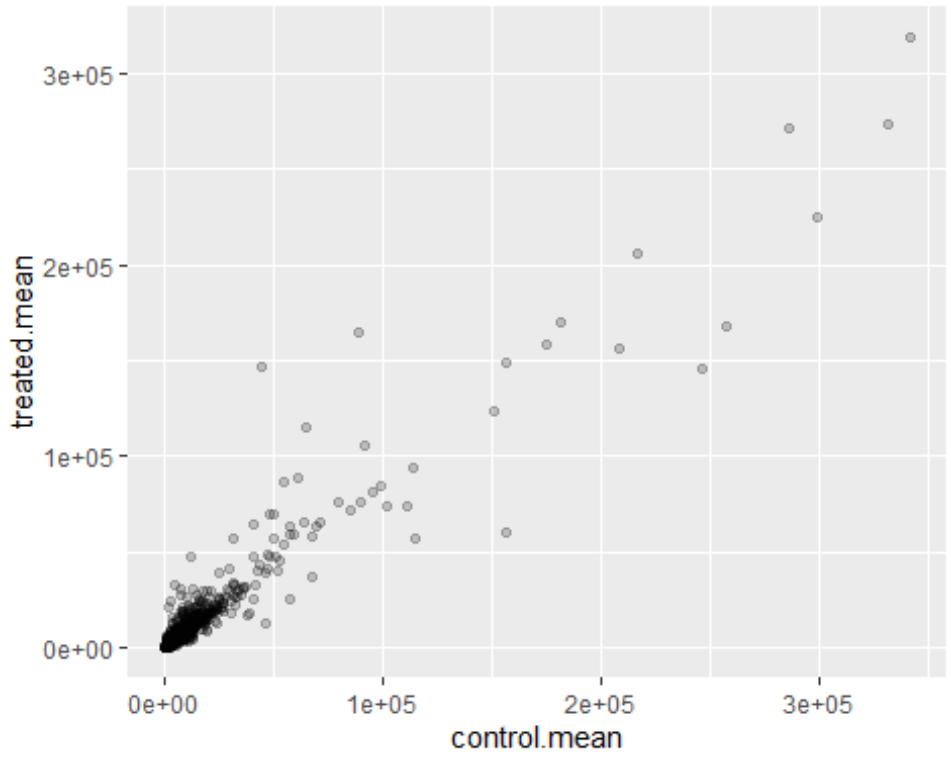



```
## integer(0)
```

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?

ANSWER: geom_point()

```
library(ggplot2)
ggplot(meancounts,aes(control.mean,treated.mean)) +
  geom_point(alpha=0.2)
```

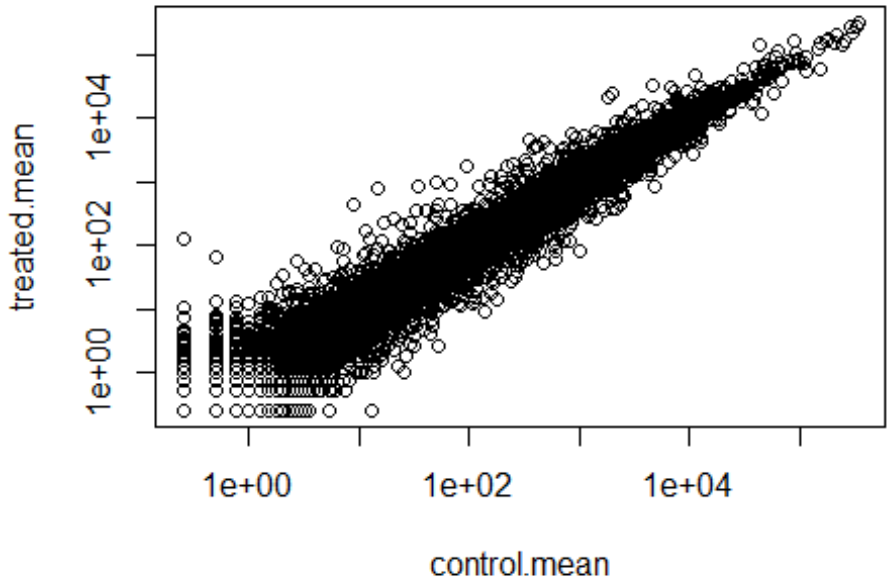



Q6. Try plotting both axes on a log scale. What is the argument to plot() that allows you to do this?

ANSWER: log

```
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
```




Here we calculate log2foldchange, add it to our meancounts data.frame and inspect the results either with the head() or the View() function for example.

```
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
```

There are a couple of wird NaN or Inf points. Lets filter these out.

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

ANSWER: The purpose of arr.ind argument in the which() function is to return the true row and column indices as a matrix. If arr.ind=FALSE (default), it would return the true results as as integers. We then take the first column of the output and call the unique() function because although the first line gives us rows containing zerors, some rows may have zeroes in both columns annd may be repeated. So, we'd want to focus on the unique row numbers. We ultimately want non-zero genes, so we use negative (-)to.rm to select the rows that do not have zeros.

Let's filter to see how many genes are up or down regulated

```
up.ind <- mycounts$log2fc > 2
down.ind <- mycounts$log2fc < (-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?

```
sum(up.ind)
```

```
## [1] 250
```

ANSWER: There are 250 genes that are up-regulated at the greater than 2 fc level.

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
```

ANSWER: There are 267 genes that are down-regulated greater than the 2 fc level.

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

ANSWER: Ultimately, one would have to look at another statistical test to gain greater confidence in these results. For example, a large log2 fc may occur yet be statistically insignificant. Therefore, some p-value test would be required to see if the cahnges in expression are significant.

## 4. DESeq2 Analysis

```
library(DESeq2)
citation("DESeq2")
```

```
##
##   Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change
```

```
##   and dispersion for RNA-seq data with DESeq2 Genome Biology 15(12):550
##   (2014)
##
## A BibTeX entry for LaTeX users is
##
##   @Article{,
##     title = {Moderated estimation of fold change and dispersion for RNA-
seq data with DESeq2},
##     author = {Michael I. Love and Wolfgang Huber and Simon Anders},
##     year = {2014},
##     journal = {Genome Biology},
##     doi = {10.1186/s13059-014-0550-8},
##     volume = {15},
##     issue = {12},
##     pages = {550},
##   }
```

Import the data

```
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

## class: DESeqDataSet
## dim: 38694 8
## metadata(1): version
## assays(1): counts
## rownames(38694): ENSG00000000003 ENSG00000000005 ... ENSG00000283120
##   ENSG00000283123
## rowData names(0):
## colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
## colData names(4): id dex celltype geo_id
```

DESeq Analysis

```
dds <- DESeq(dds)

## estimating size factors

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship
```

```
## final dispersion estimates

## fitting model and testing
```

get results

```
res <- results(dds)
res

## log2 fold change (MLE): dex treated vs control
## Wald test p-value: dex treated vs control
## DataFrame with 38694 rows and 6 columns
##                   baseMean log2FoldChange     lfcSE      stat    pvalue
##                  <numeric>      <numeric> <numeric> <numeric> <numeric>
## ENSG00000000003  747.1942     -0.3507030  0.168246 -2.084470 0.0371175
## ENSG00000000005    0.0000             NA        NA        NA        NA
## ENSG00000000419  520.1342      0.2061078  0.101059  2.039475 0.0414026
## ENSG00000000457  322.6648      0.0245269  0.145145  0.168982 0.8658106
## ENSG00000000460   87.6826     -0.1471420  0.257007 -0.572521 0.5669691
## ...                    ...            ...       ...       ...       ...
## ENSG00000283115  0.000000             NA        NA        NA        NA
## ENSG00000283116  0.000000             NA        NA        NA        NA
## ENSG00000283119  0.000000             NA        NA        NA        NA
## ENSG00000283120  0.974916      -0.668258   1.69456 -0.394354  0.693319
## ENSG00000283123  0.000000             NA        NA        NA        NA
##                      padj
##                 <numeric>
## ENSG00000000003  0.163035
## ENSG00000000005        NA
## ENSG00000000419  0.176032
## ENSG00000000457  0.961694
## ENSG00000000460  0.815849
## ...                   ...
## ENSG00000283115        NA
## ENSG00000283116        NA
## ENSG00000283119        NA
## ENSG00000283120        NA
## ENSG00000283123        NA
```

## 5. Adding annotation data

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

##
## Attaching package: 'AnnotationDbi'

## The following object is masked from 'package:dplyr':
##
##     select
```

```
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    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),
```

```
                    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

ord <- order( res$padj )
#View(res[ord,])
head(res[ord,])

## 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>
## ENSG00000152583   954.771        4.36836 0.2371268   18.4220 8.74490e-76
## ENSG00000179094   743.253        2.86389 0.1755693   16.3120 8.10784e-60
## ENSG00000116584  2277.913       -1.03470 0.0650984  -15.8944 6.92855e-57
## ENSG00000189221  2383.754        3.34154 0.2124058   15.7319 9.14433e-56
## ENSG00000120129  3440.704        2.96521 0.2036951   14.5571 5.26424e-48
## ENSG00000148175 13493.920        1.42717 0.1003890   14.2164 7.25128e-46
##                       padj      symbol      entrez     uniprot
##                  <numeric> <character> <character> <character>
## ENSG00000152583 1.32441e-71     SPARCL1        8404  A0A024RDE1
## ENSG00000179094 6.13966e-56        PER1        5187      O15534
## ENSG00000116584 3.49776e-53     ARHGEF2        9181      Q92974
## ENSG00000189221 3.46227e-52        MAOA        4128      P21397
## ENSG00000120129 1.59454e-44       DUSP1        1843      B4DU40
## ENSG00000148175 1.83034e-42        STOM        2040      F8VSL7
##                              genename
##                           <character>
## ENSG00000152583           SPARC like 1
## ENSG00000179094 period circadian reg..
## ENSG00000116584 Rho/Rac guanine nucl..
## ENSG00000189221    monoamine oxidase A
```

```
## ENSG00000120129 dual specificity pho..
## ENSG00000148175                stomatin
```

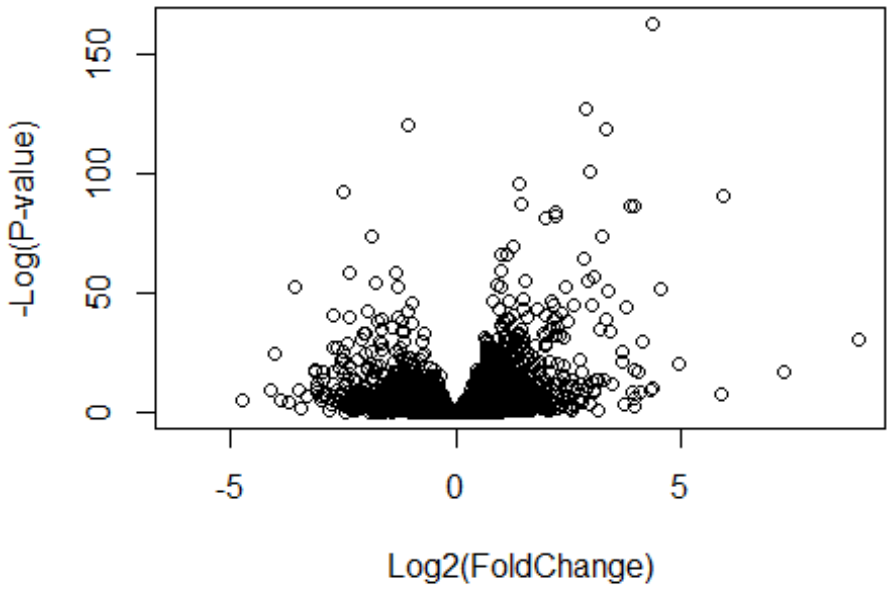
Let's write out the ordered significant results with annotations:

```
write.csv(res[ord,], "deseq_results.csv")
```

## 6. Data Visualization

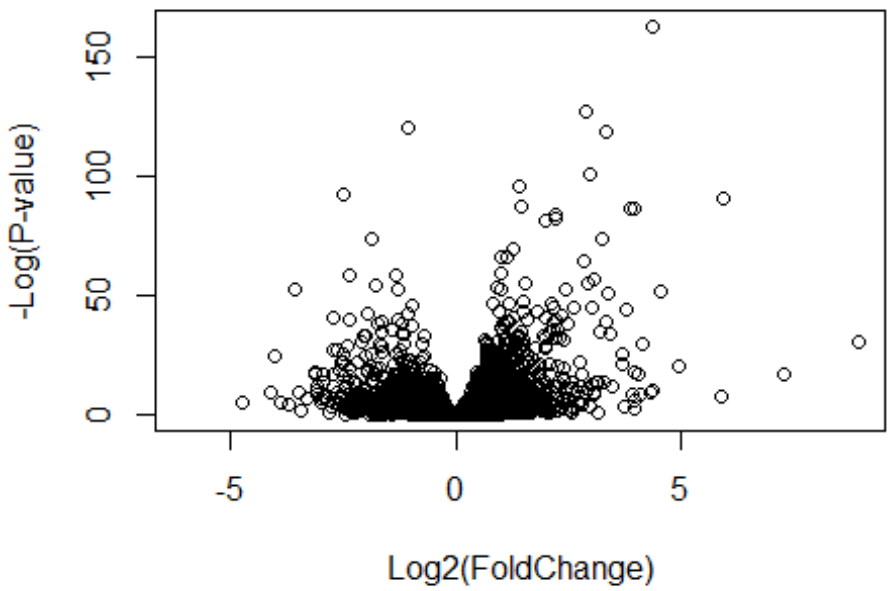
Volcano plot!

```
plot( res$log2FoldChange,  -log(res$padj),
      xlab="Log2(FoldChange)",
      ylab="-Log(P-value)")
```

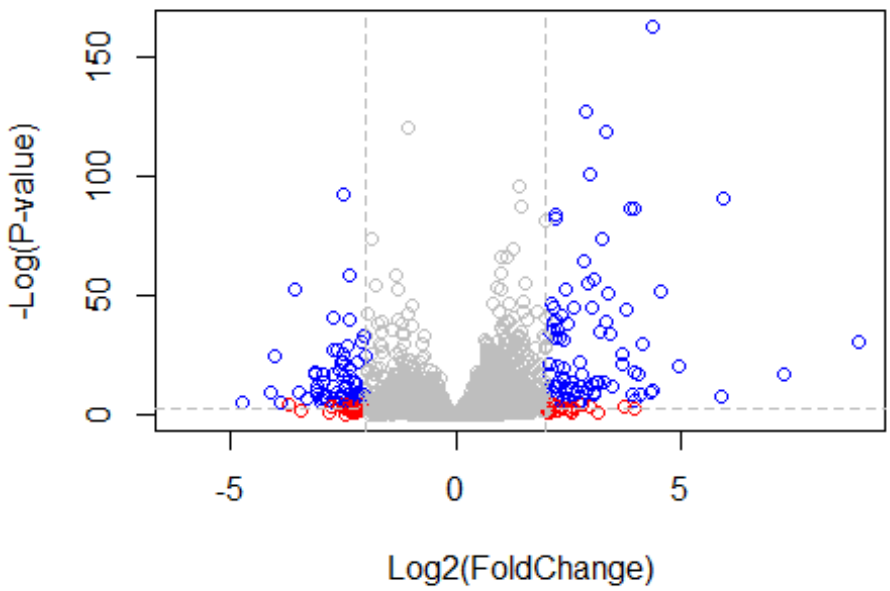




```
plot( res$log2FoldChange,  -log(res$padj),
      xlab="Log2(FoldChange)",
      ylab="-Log(P-value)")
```

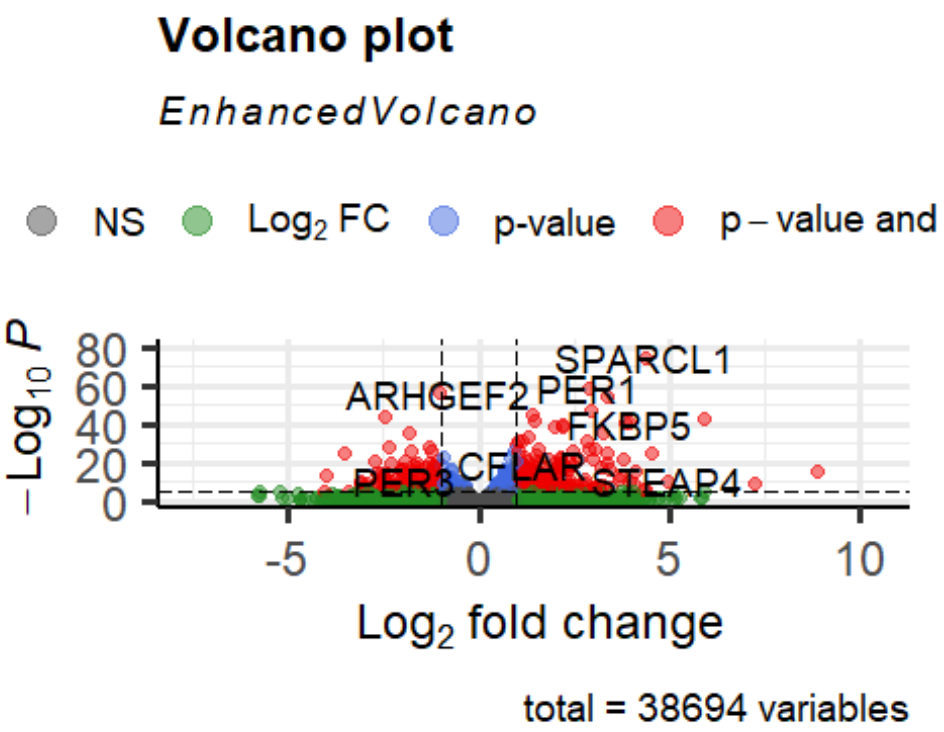



```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red"

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 3.13 (BiocManager 1.30.16), R 4.1.1 (2021-08-10)

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

## Installation paths not writeable, unable to update packages
##   path: C:/Program Files/R/R-4.1.1/library
##   packages:
##     class, foreign, lattice, MASS, Matrix, mgcv, nlme, nnet, rpart,
spatial,
##     survival

## Old packages: 'digest'

library(EnhancedVolcano)

## Loading required package: ggrepel

## Warning: package 'ggrepel' was built under R version 4.1.2

## Registered S3 methods overwritten by 'ggalt':
##   method                  from
##   grid.draw.absoluteGrob  ggplot2
##   grobHeight.absoluteGrob ggplot2
##   grobWidth.absoluteGrob  ggplot2
```

```
##   grobX.absoluteGrob      ggplot2
##   grobY.absoluteGrob      ggplot2

x <- as.data.frame(res)

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

## Warning: Ignoring unknown parameters: xlim, ylim
```




## 7. Pathway Analysis

```
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"

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)

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
```

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

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

```
## Info: Working in directory C:/Users/meggg/Documents/bioinfo/class11
```

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

```
# A different PDF based output of the same data
pathview(gene.data=foldchanges, pathway.id="hsa05310", kegg.native=FALSE)
```

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

```
## Info: Working in directory C:/Users/meggg/Documents/bioinfo/class11
```

```
## Info: Writing image file hsa05310.pathview.pdf
```