Class 12

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1. Bioconductor and DESeq2 setup

```
library(BiocManager)
```

```
Bioconductor version '3.16' is out-of-date; the current release version '3.17'
  is available with R version '4.3'; see https://bioconductor.org/install
```

```
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, setdiff, sort,
    table, tapply, union, unique, unsplit, which.max, which.min
```

```
Attaching package: 'S4Vectors'

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
```

2. Import countData and colData

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

```
nrow(counts)
```

```
[1] 38694
```

```
View(metadata)
```

Q1. How many genes are in this dataset?

38694 genes

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

4 'control' cell lines

3. Toy differential gene expression

```
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
```

```
library(dplyr)
```

```
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
```

```
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?

```
# Subset metadata based on "dex" condition
control <- metadata[metadata$dex == "control", ]

# Validate if "id" column exists in control
if (!"id" %in% colnames(control)) stop("Control metadata does not contain 'id' column.")

# Subset counts based on control$id
control.counts <- counts[, control$id]

# Calculate control mean
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
```

This establishes a "validation check" to ensure that the "id" column exists in control.

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`)

```
# Subset metadata based on "dex" condition
treated <- metadata[metadata,"dex"] == "treated",]

# Validate if "id" column exists in treated
if (!"id" %in% colnames(treated)) stop("Treated metadata does not contain 'id' column.")

# Calculate treated mean
treated.mean <- rowSums(counts[, treated$id]) / 4
names(treated.mean) <- counts$ensgene
head(treated.mean)
```

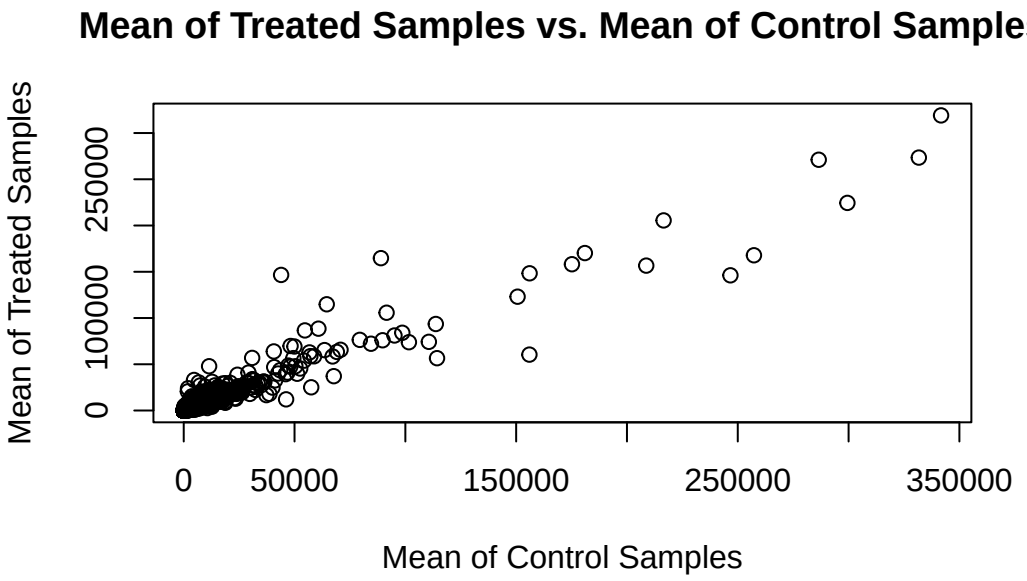
```
[1] 658.00   0.00 546.00 316.50  78.75   0.00
```

```
meancounts <- data.frame(Control = control.mean, Treated = treated.mean)
colSums(meancounts)
```

```
 Control  Treated
23005324 22196524
```

Q5(a). Create a scatter plot showing the mean of the treated samples against the mean of the control samples.

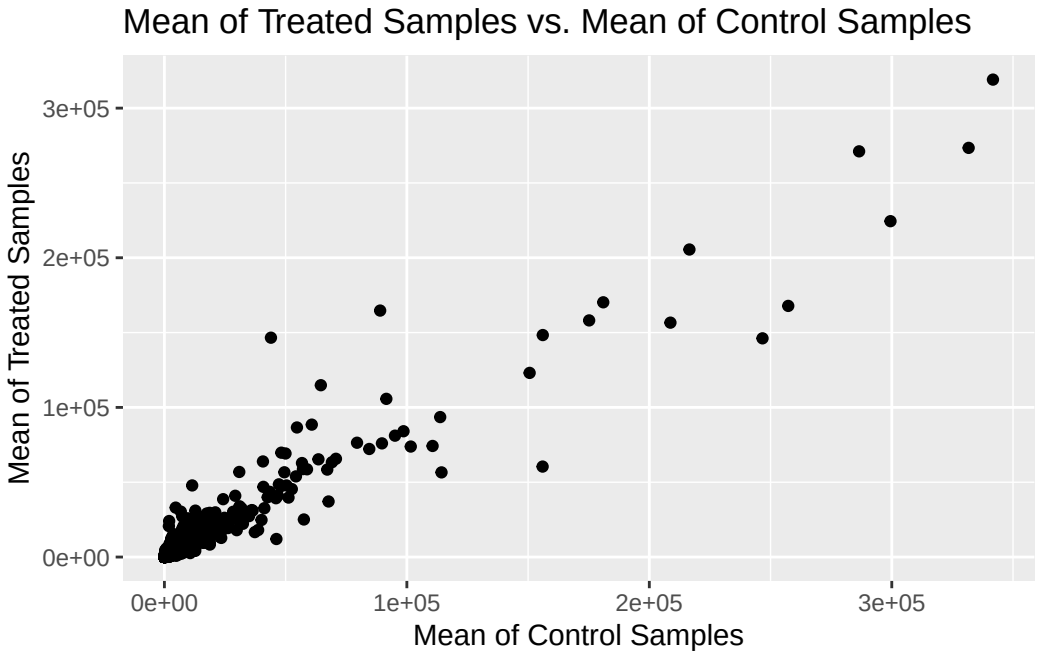
```
# Create a scatter plot using base R
plot(control.mean, treated.mean,
     xlab = "Mean of Control Samples",
     ylab = "Mean of Treated Samples",
     main = "Mean of Treated Samples vs. Mean of Control Samples")
```



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)

# Create the scatter plot using ggplot2
ggplot(meancounts, aes(x = Control, y = Treated)) +
  geom_point() +
  labs(x = "Mean of Control Samples", y = "Mean of Treated Samples") +
  ggtitle("Mean of Treated Samples vs. Mean of Control Samples")
```



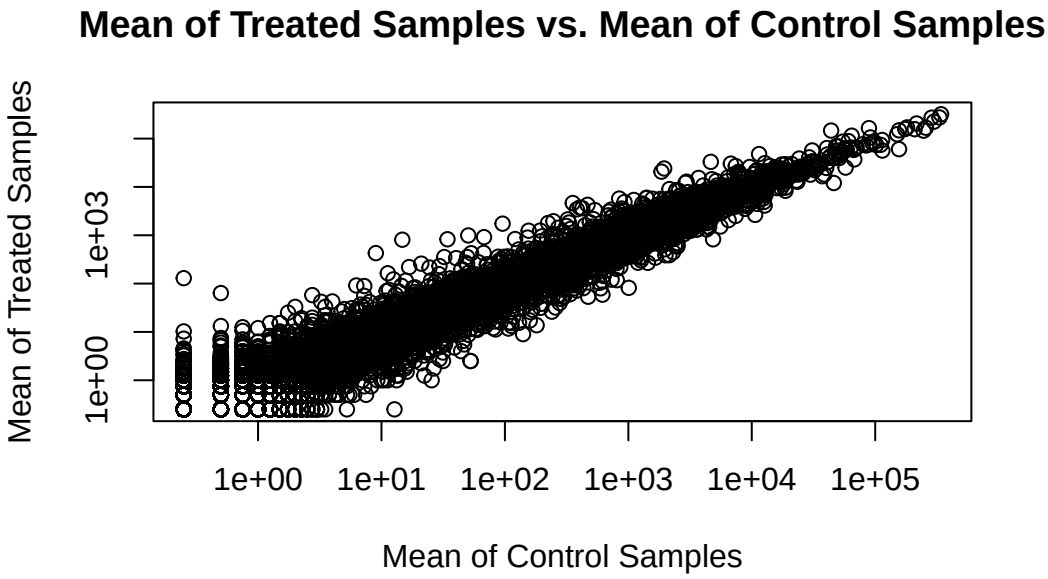
geom_point()

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

```
plot(control.mean, treated.mean,
     xlab = "Mean of Control Samples",
     ylab = "Mean of Treated Samples",
     main = "Mean of Treated Samples vs. Mean of Control Samples",
     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
```



log = "xy"

```
meancounts$log2fc <- log2(meancounts[,"Treated"]/meancounts[,"Control"])
head(meancounts)
```

```
                Control Treated      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
```

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

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

```
                Control Treated      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 tells **`which()`** to return the results as indices in array/matrix form, allowing us to extract the row indices of the elements with zero values.

The first column of the output is extracted because it contains the row indices of elements with zero values. **`unique()`** is used to obtain only the unique row indices since there might be cases where multiple elements have a value of zero in the same row.

```
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
```

250 up regulated genes.

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 down regulated genes.

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

The log2 fold change threshold of greater than 2 or less than -2 is a common cutoff used in differential expression analysis. Because of this, I think we are on the right path but more statistical analysis (specifically significance) is needed to ensure that these results are not misleading.

4. DESeq2 analysis

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

```
To cite package 'DESeq2' in publications use:

  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},
  }
```

```
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
```

```
# Run the DESeq pipeline on the dds object, and reassign the whole thing back to dds
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
```

```
res.df <- as.data.frame(res)
View(res)
```

```
summary(res)
```

```
out of 25258 with nonzero total read count
adjusted p-value < 0.1
LFC > 0 (up)       : 1563, 6.2%
LFC < 0 (down)     : 1188, 4.7%
outliers [1]       : 142, 0.56%
```

```
low counts [2]     : 9971, 39%
(mean count < 10)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of ?results
```

```
res05 <- results(dds, alpha=0.05)
summary(res05)
```

```
out of 25258 with nonzero total read count
adjusted p-value < 0.05
LFC > 0 (up)       : 1236, 4.9%
LFC < 0 (down)     : 933, 3.7%
outliers [1]       : 142, 0.56%
low counts [2]     : 9033, 36%
(mean count < 6)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of ?results
```

5. Adding annotation data

```
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`.

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

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

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

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

```
# Add gene name column
res$genename <- mapIds(org.Hs.eg.db,
                       keys = row.names(res),
                       keytype = "ENSEMBL",
                       column = "GENENAME",
                       multiVals = "first")
```

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

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

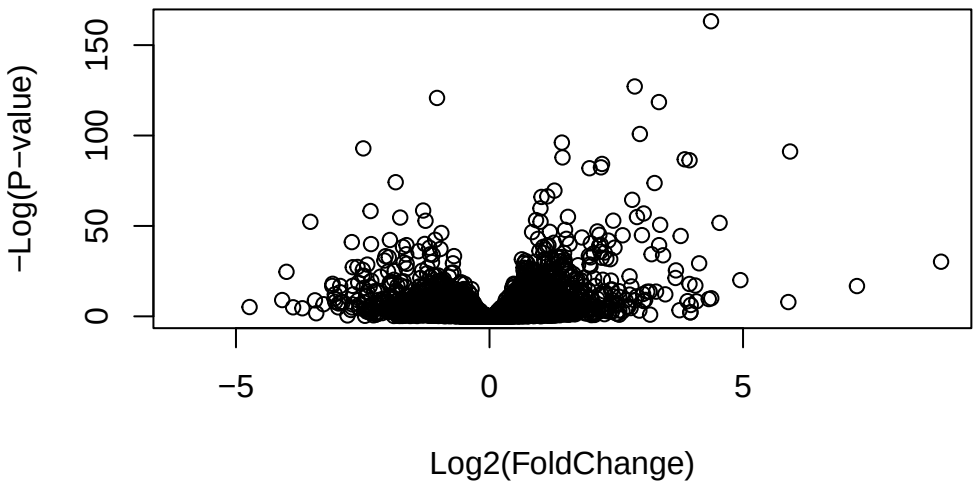
```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
```

```
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
```

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

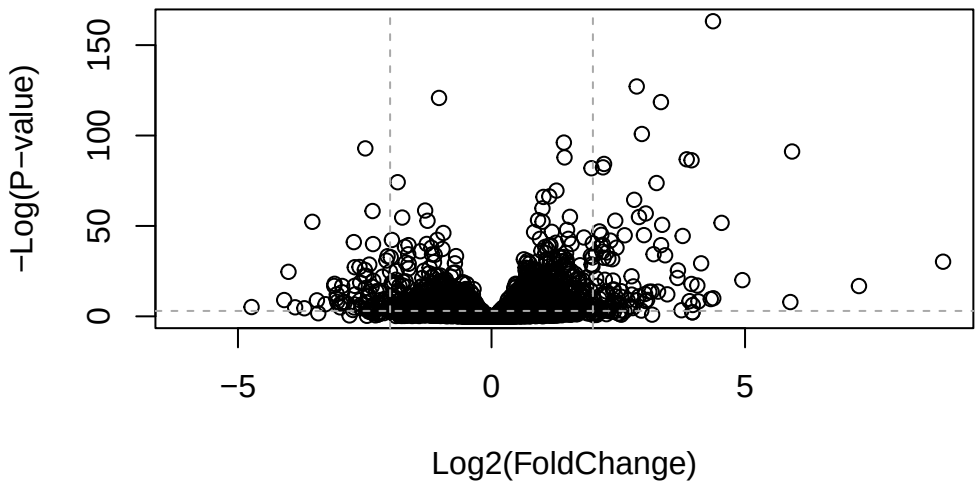
6. Data Visualization

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




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

# Add some cut-off lines
abline(v=c(-2,2), col="darkgray", lty=2)
abline(h=-log(0.05), col="darkgray", lty=2)
```

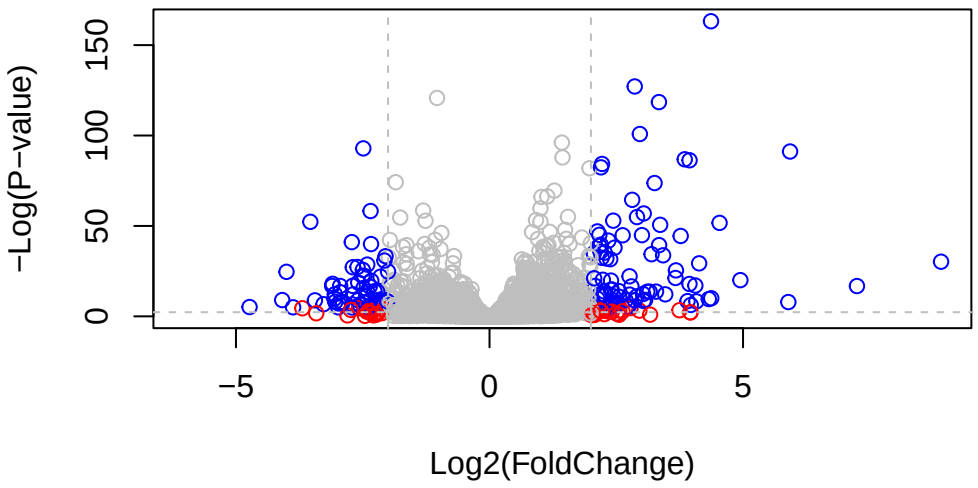



```
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ]  <- "red"

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)
```

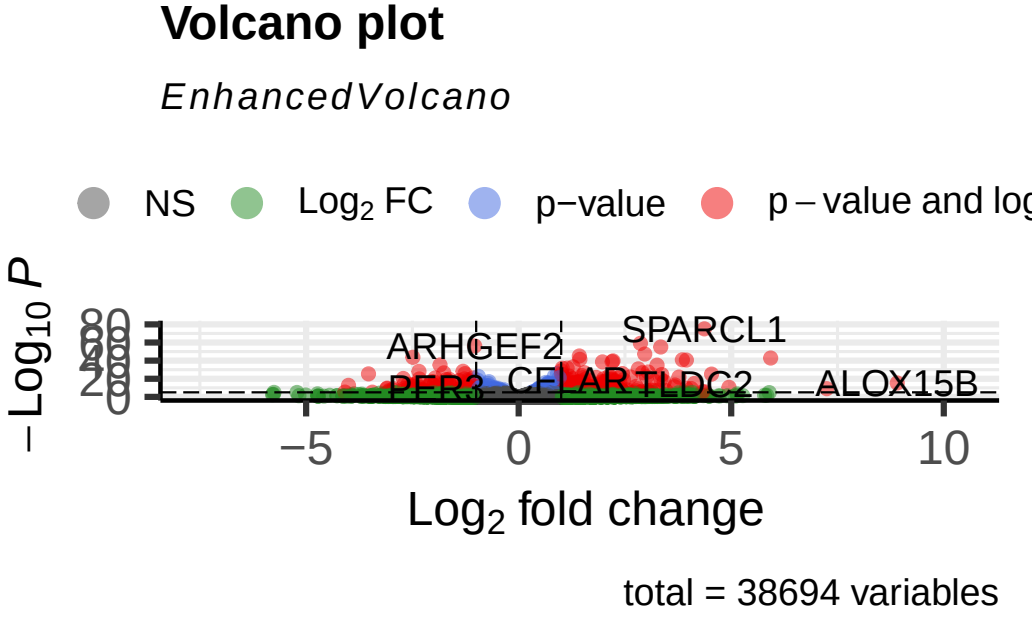



```
library(EnhancedVolcano)
```

```
Loading required package: ggrepel
```

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

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


Volcano plot
EnhancedVolcano
NS
Log2 FC
p−value
p – value and log
−Log10 P
80
60
40
20
0
ARHGEF2
SPARCL1
PFKP
CFL1
ARTLDC2
ALOX15B
−5
0
5
10
Log2 fold change
total = 38694 variables