

Class 12: Transcriptomics and the analysis of RNA-Seq data

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1. Bioconductor and DESeq2 setup

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

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Bioconductor version '3.16' is out-of-date; the current release version '3.17'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, rowCummmins, rowCumprods,
rowCumsums, rowDiffss, rowIQRDiffss, rowIQRs, rowLogSumExps,
rowMadDiffss, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
rowSdDiffss, rowSds, rowSums2, rowTabulates, rowVarDiffss, 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")

head(counts)
```

	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516
ENSG000000000003	723	486	904	445	1170
ENSG000000000005	0	0	0	0	0
ENSG000000000419	467	523	616	371	582
ENSG000000000457	347	258	364	237	318
ENSG000000000460	96	81	73	66	118
ENSG000000000938	0	0	1	0	2
	SRR1039517	SRR1039520	SRR1039521		
ENSG000000000003	1097	806	604		
ENSG000000000005	0	0	0		
ENSG000000000419	781	417	509		
ENSG000000000457	447	330	324		
ENSG000000000460	94	102	74		
ENSG000000000938	0	0	0		

```
nrow(counts)
```

[1] 38694

```
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

```
table(metadata$dex) ['control']
```

```
control
4
```

Q1. How many genes are in this dataset?

There are 38,694 genes (38694) in this dataset.

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

We have 4 'control' cell lines (using code `table(metadata$dex)`).

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

metadata[, 'dex']

[1] "control" "treated" "control" "treated" "control" "treated" "control"
[8] "treated"

metadata[, 'dex'] == 'control'

[1] TRUE FALSE  TRUE FALSE  TRUE FALSE  TRUE FALSE

metadata[metadata[, 'dex'] == 'control',]

      id      dex celltype      geo_id
1 SRR1039508 control    N61311 GSM1275862
3 SRR1039512 control    N052611 GSM1275866
5 SRR1039516 control    N080611 GSM1275870
7 SRR1039520 control    N061011 GSM1275874

control <- metadata[metadata[, "dex"]=="control",]
```

```
control$id

[1] "SRR1039508" "SRR1039512" "SRR1039516" "SRR1039520"

head(counts[,control$id])

      SRR1039508 SRR1039512 SRR1039516 SRR1039520
ENSG000000000003     723      904     1170      806
ENSG000000000005      0         0         0         0
ENSG00000000419     467      616      582      417
ENSG00000000457     347      364      318      330
ENSG00000000460      96       73      118      102
ENSG00000000938      0         1         2         0
```

```
control.counts <- counts[,control$id]

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?

```
control.counts <- counts[,control$id]
control.means <- rowMeans(control.counts)
```

Dividing by 4 makes the code non-reproducible if used in another context (e.g. more replicates/samples). As such, using rowMeans rather than dividing by an actual number makes the code more robust/usable outside of this dataset.

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 <- rowSums( treated.counts )/4  
head(treated.mean)
```

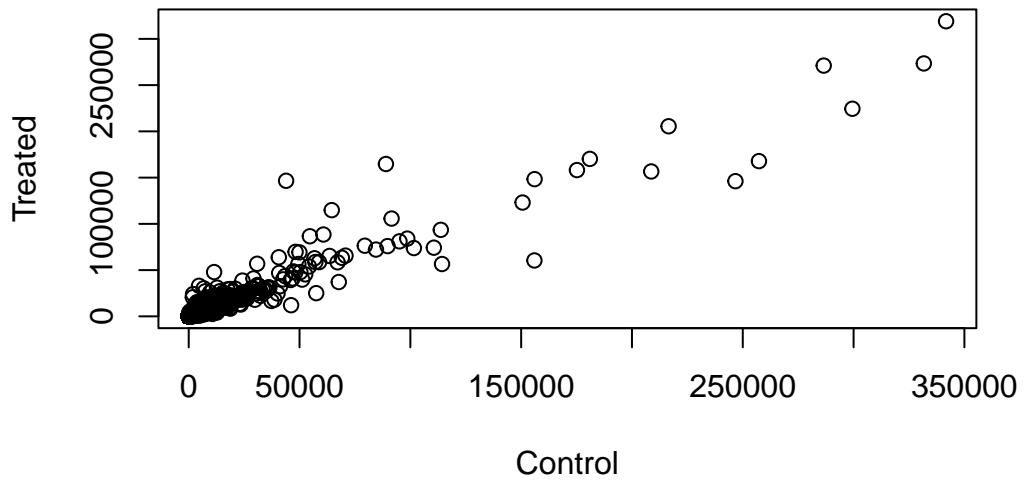
```
ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460  
658.00          0.00          546.00         316.50          78.75  
ENSG00000000938  
0.00
```

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

```
control.mean treated.mean  
23005324      22196524
```

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[,1],meancounts[,2], xlab="Control", ylab="Treated")
```

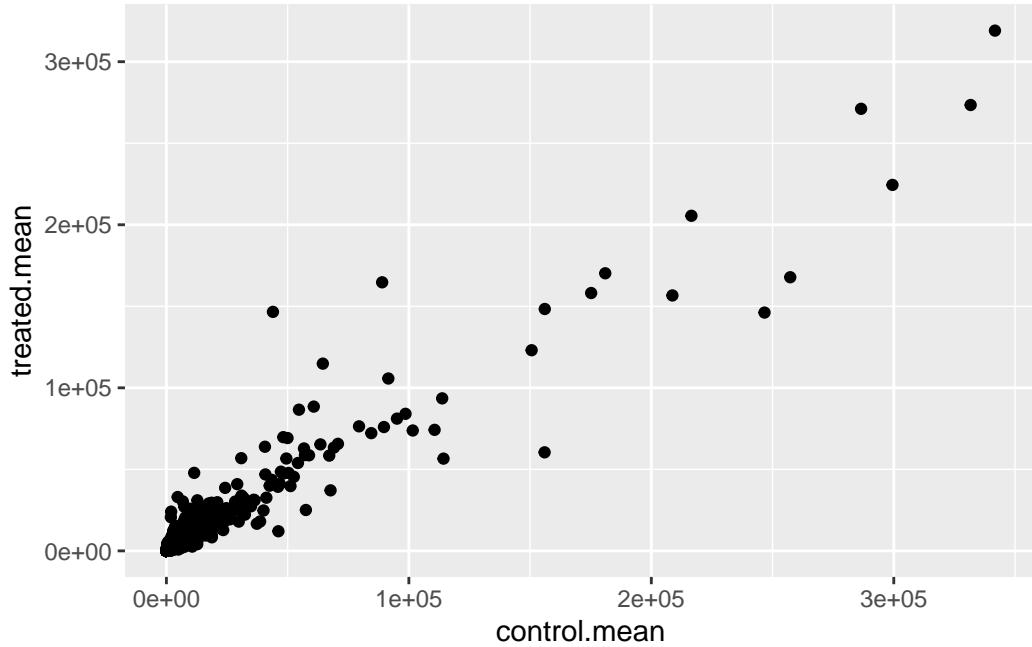


Q5 (b). You could also use the ggplot2 package to make this figure producing the plot below. What geom_?() function would you use for this plot?

```
library(ggplot2)

#meancounts

ggplot(data = meancounts) +
  aes(x = control.mean, y= treated.mean) +
  geom_point()
```

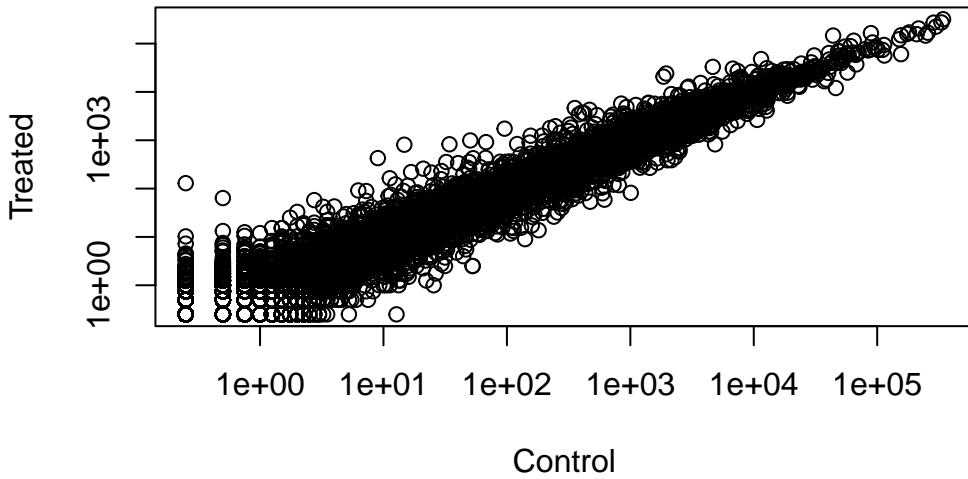


Q6. Try plotting both axes on a log scale. What is the argument to `plot()` that allows you to do this?

```
plot(meancounts[,1],meancounts[,2], xlab="Control", ylab="Treated", 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



The argument is `log`.

To calculate the `log2` of the fold change between treated and control...

```
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

```
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
ENSG000000000003	900.75	658.00	-0.45303916
ENSG000000000419	520.50	546.00	0.06900279
ENSG000000000457	339.75	316.50	-0.10226805
ENSG000000000460	97.25	78.75	-0.30441833
ENSG000000000971	5219.00	6687.50	0.35769358
ENSG000000001036	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 argument returns the row and column positions where there are TRUE values, which will tell us which genes and samples have a count of zero (want to ignore any genes that have zero counts in order to avoid linearizing the matrix). Unique() will make sure that none of the rows are counted twice if there are zero entries in both samples.

```
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

There are 250 up regulated genes 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

There are 367 downregulated genes greater than 2 fc level.

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

Not entirely, mainly because we are missing the statistics. The actual numbers/code makes sense, but we don't know if the differences are significant or generated by chance. We don't know if the number of replicates we have used is enough to say whether or not the differences we see are real, and as such more analysis is needed to confirm the results.

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
```

DESeq analysis

```
#results(dds)  
dds <- DESeq(dds)
```

```
estimating size factors
```

```
estimating dispersions
```

```
gene-wise dispersion estimates
```

```
mean-dispersion relationship
```

```
final dispersion estimates
```

```
fitting model and testing
```

```
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>
ENSG000000000003  747.1942 -0.3507030  0.168246 -2.084470 0.0371175
ENSG000000000005  0.0000      NA       NA       NA       NA
ENSG000000000419  520.1342  0.2061078  0.101059  2.039475 0.0414026
ENSG000000000457  322.6648  0.0245269  0.145145  0.168982 0.8658106
ENSG000000000460  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>
ENSG000000000003  0.163035
ENSG000000000005  NA
ENSG000000000419  0.176032
ENSG000000000457  0.961694
ENSG000000000460  0.815849
...
...
ENSG00000283115  NA
ENSG00000283116  NA
ENSG00000283119  NA
ENSG00000283120  NA
ENSG00000283123  NA

```

```
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

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

columns(org.Hs.eg.db)

[1] "ACNUM"          "ALIAS"           "ENSEMBL"         "ENSEMLPROT"      "ENSEMLTRANS"
[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>
ENSG000000000003 747.194195 -0.3507030 0.168246 -2.084470 0.0371175
ENSG000000000005 0.000000      NA       NA       NA       NA
ENSG000000000419 520.134160  0.2061078 0.101059  2.039475 0.0414026
ENSG000000000457 322.664844  0.0245269 0.145145  0.168982 0.8658106
ENSG000000000460 87.682625  -0.1471420 0.257007 -0.572521 0.5669691
ENSG000000000938 0.319167  -1.7322890 3.493601 -0.495846 0.6200029
  padj      symbol
  <numeric> <character>
ENSG000000000003 0.163035    TSPAN6
ENSG000000000005  NA          TNMD
ENSG000000000419 0.176032    DPM1
ENSG000000000457 0.961694    SCYL3
ENSG000000000460 0.815849    C1orf112
ENSG000000000938  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
```

```
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>
ENSG000000000003	747.194195	-0.3507030	0.168246	-2.084470	0.0371175
ENSG000000000005	0.000000	NA	NA	NA	NA
ENSG000000000419	520.134160	0.2061078	0.101059	2.039475	0.0414026
ENSG000000000457	322.664844	0.0245269	0.145145	0.168982	0.8658106
ENSG000000000460	87.682625	-0.1471420	0.257007	-0.572521	0.5669691
ENSG000000000938	0.319167	-1.7322890	3.493601	-0.495846	0.6200029
	padj	symbol	entrez	uniprot	
	<numeric>	<character>	<character>	<character>	
ENSG000000000003	0.163035	TSPAN6	7105	AOA024RCI0	
ENSG000000000005	NA	TNMD	64102	Q9H2S6	
ENSG000000000419	0.176032	DPM1	8813	060762	
ENSG000000000457	0.961694	SCYL3	57147	Q8IZE3	
ENSG000000000460	0.815849	C1orf112	55732	AOA024R922	

ENSG000000000938	NA	FGR	2268	P09769
		genename		
		<character>		
ENSG000000000003		tetraspanin 6		
ENSG000000000005		tenomodulin		
ENSG000000000419		dolichyl-phosphate m..		
ENSG000000000457		SCY1 like pseudokina..		
ENSG000000000460		chromosome 1 open re..		
ENSG000000000938		FGR proto-oncogene, ..		

```

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

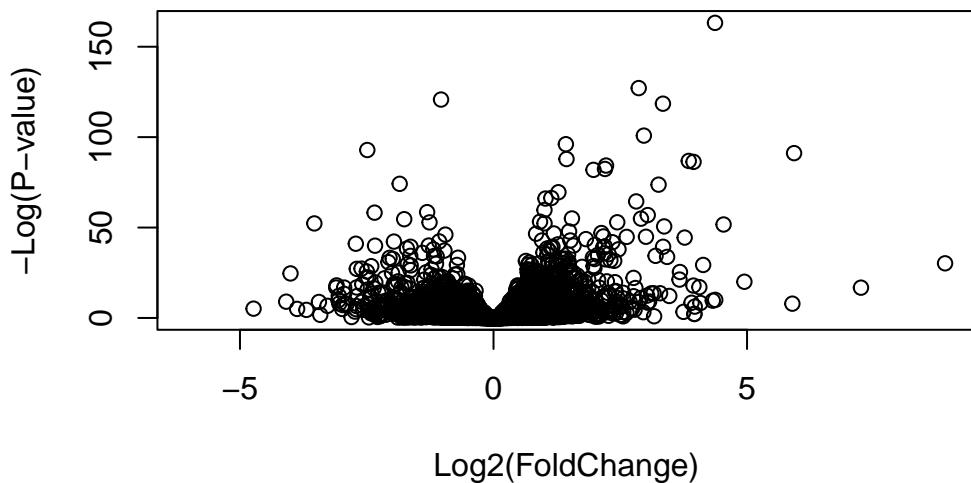
	padj	symbol	entrez	uniprot
	<numeric>	<character>	<character>	<character>
ENSG00000152583	1.32441e-71	SPARCL1	8404	A0A024RDE1
ENSG00000179094	6.13966e-56	PER1	5187	Q15534
ENSG00000116584	3.49776e-53	ARHGEF2	9181	Q92974
ENSG00000189221	3.46227e-52	MAOA	4128	P21397
ENSG00000120129	1.59454e-44	DUSP1	1843	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			

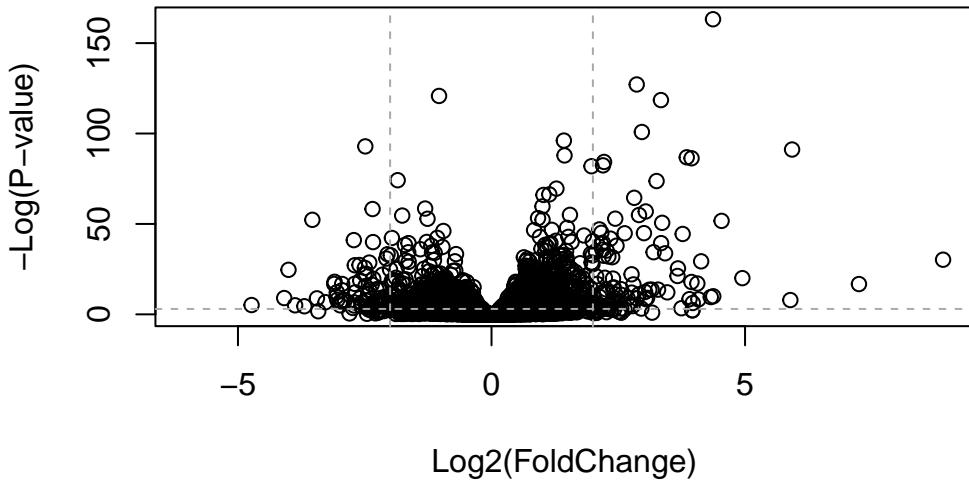
```
write.csv(res[ord,], "deseq_results.csv")
```

6. Data Visualization

```
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)
```



```

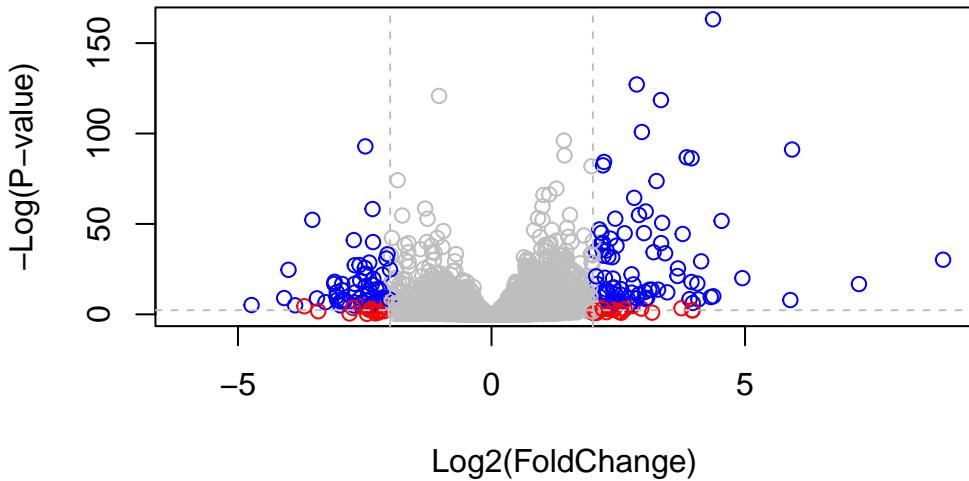
# Setup our custom point color vector
mycols <- rep("gray", nrow(res))
mycols[ abs(res$log2FoldChange) > 2 ] <- "red"

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)

```



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

```
library(EnhancedVolcano)
```

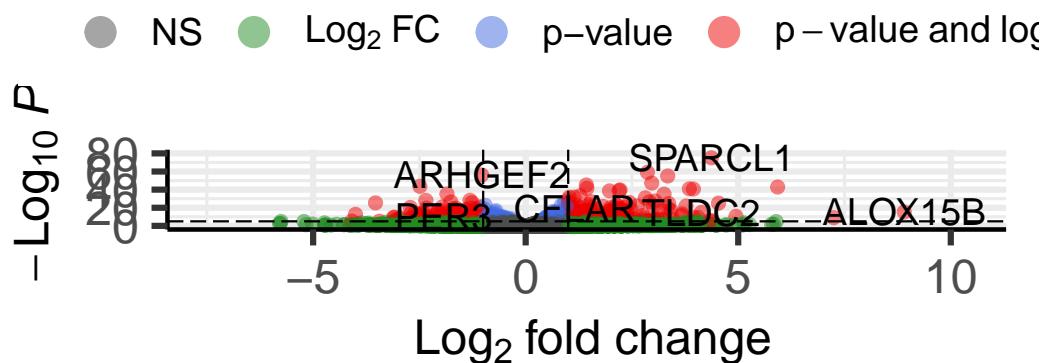
Loading required package: ggrepel

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

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

Volcano plot

Enhanced Volcano



total = 38694 variables