class12

Working on RNAseq today!

```
library(BiocManager)
library(DESeq2)
```

```
Loading required package: S4Vectors
```

```
Loading required package: stats4
```

```
Loading required package: BiocGenerics
```

```

Attaching package: 'BiocGenerics'
```

```
The following objects are masked from 'package:stats':

    IQR, mad, sd, var, xtabs
```

```
The following objects are masked from 'package:base':

    anyDuplicated, aperm, append, as.data.frame, basename, cbind,
    colnames, dirname, do.call, duplicated, eval, evalq, Filter, Find,
    get, grep, grepl, intersect, is.unsorted, lapply, Map, mapply,
    match, mget, order, paste, pmax, pmax.int, pmin, pmin.int,
    Position, rank, rbind, Reduce, rownames, sapply, 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


Attaching package: 'IRanges'

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

    windows

Loading required package: GenomicRanges

Loading required package: GenomeInfoDb

Loading required package: SummarizedExperiment

Loading required package: MatrixGenerics

Loading required package: matrixStats


Attaching package: 'MatrixGenerics'

The following objects are masked from 'package:matrixStats':

    colAlls, colAnyNAs, colAnys, colAvgsPerRowSet, colCollapse,
    colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
    colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
    colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
    colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
    colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
    colWeightedMeans, colWeightedMedians, colWeightedSds,
    colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAvgsPerColSet,
    rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
    rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
    rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
```

```
    rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
    rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs, rowVars,
    rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
    rowWeightedSds, rowWeightedVars

Loading required package: Biobase

Welcome to Bioconductor

    Vignettes contain introductory material; view with
    'browseVignettes()'. To cite Bioconductor, see
    'citation("Biobase")', and for packages 'citation("pkgname")'.


Attaching package: 'Biobase'

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

    rowMedians

The following objects are masked from 'package:matrixStats':

    anyMissing, rowMedians
```

```
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

```
nrow(counts)
```

```
[1] 38694
```

```
ncol(counts)
```

```
[1] 8
```

Let's make sure that the ID column of the metadata match the order of the columns in countData.

```
metadata$id == colnames(counts)
```

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

Can see if all the items in the vector are TRUE using `all()`.

```
all(metadata$id == colnames(counts))
```

```
[1] TRUE
```

you can also ask if all all the items are true.

```
#are there any items that are not true?
!all(metadata$id == colnames(counts))
```

```
[1] FALSE
```

#Analysis by hand

```
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
```

Let's first extract our counts for control samples as I want to compare this to the counts for treated (i.e. with drug) samples

```
control.inds <- metadata$dex == "control"
metadata$id[ control.inds]
```

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

```
control.counts <- counts[ control.inds]
head(control.counts)
```

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

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

Q3. How would you make the above code in either approach more robust? You would need to use rowMeans to find the average instead of manually dividing the sum by 4 samples.

I want a single summary counts value for each ene in the control experiment. I will start by taking the average.

```
#apply(control.counts,1, mean)
control.mean <-rowMeans(control.counts)
```

Q4. Follow the same procedure for the treated samples (i.e. calculate the mean per gene across drug treated samples and assign to a labeled vector called treated.mean)

```
treated.inds <- metadata$dex == "treated"
metadata$id[ treated.inds]
```

```
[1] "SRR1039509" "SRR1039513" "SRR1039517" "SRR1039521"
```

```
treated.counts <- counts[ treated.inds]
head(treated.counts)
```

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

```
treated.mean <-rowMeans(treated.counts)
```

To help us stay organized, let's make a new data.frame to stores these results together.

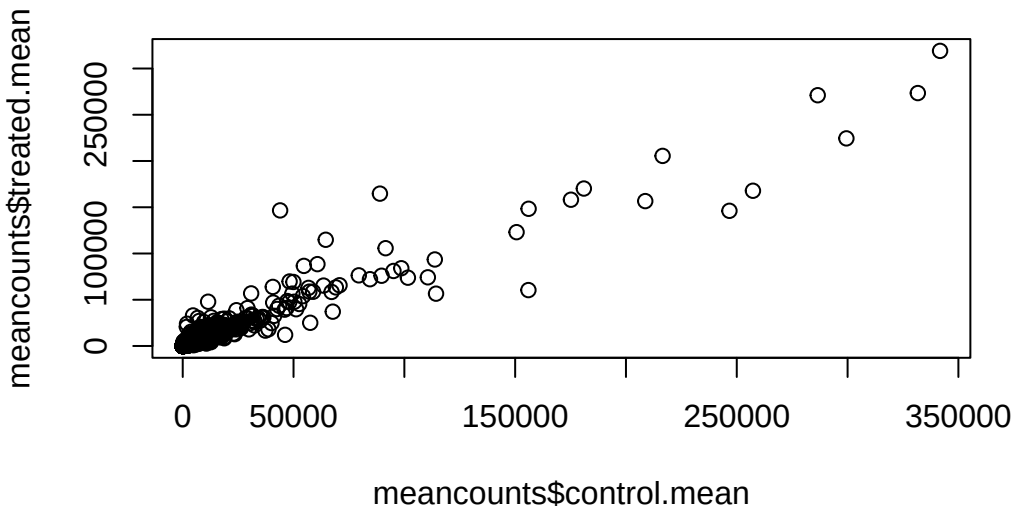
```
meancounts <- data.frame(control.mean, treated.mean)
head(meancounts)
```

```
                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$control.mean, meancounts$treated.mean)
```



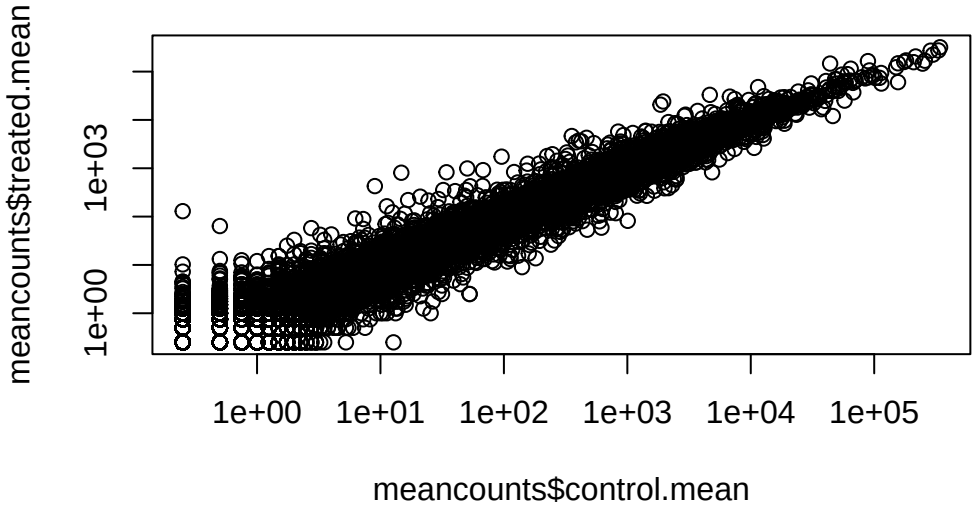
Q5 (b).You could also use the ggplot2 package to make this figure producing the plot below. What geom_?() function would you use for this plot? geom_point

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

```
plot(meancounts$control.mean, meancounts$treated.mean, 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
```



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()` will return all the rows that do not have zeros in it. The `unique()` function will make sure that rows with zeros in both control and treated will not be counted twice.

```
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
ENSG00000000003       900.75       658.00
ENSG00000000419       520.50       546.00
ENSG00000000457       339.75       316.50
ENSG00000000460        97.25        78.75
ENSG00000000971      5219.00      6687.50
ENSG00000001036      2327.00      1785.75
```

The most useful and straightforward to understand is log2 transform.

```
#log2 of 1 =0 --> a value of 1 would mean that the treated and control were the same, so l
log2(20/20)
```

```
[1] 0
```

doubling

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

```
[1] 1
```

half the amount

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

```
[1] -1
```

4x the amount

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

```
[1] 2
```

add a log2 fold-change”

```
#fc means fold-change
meancounts$log2fc <- log2(meancounts$treated.mean/meancounts$control.mean)
```

```
#NaN = "not a number"
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
```

Hmm.. we need to get rid of the genes where we have no count data as taking the log2 of these 0 counts does not tell us anything.

```
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
```

```
head(meancounts$control.mean ==0)
```

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

```
head(meancounts$treated.mean ==0)
```

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

```
to.keep <- rowSums(meancounts[,1:2]==0) ==0
mycounts <- meancounts[to.keep,]
```

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

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

```
[1] 250
```

How many genes are upregulated at the log2fc level of +2 or greater?

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

```
[1] 314
```

and down regulated...

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

```
[1] 485
```

Q9. Using the down.ind vector above can you determine how many down regulated genes we have at the greater than 2 fc level? 367 genes

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

```
[1] 367
```

Q10. Do you trust these results? Why or why not? No because they exclude those equal to +2 and -2. They also do not really tell us how significant these results are compared to others in the sample.

We are missing the stats. are these big changes significant?

#DESeq2 analysis

```
library(DESeq2)
```

Like most bioconductor packages, DESeq wants its input and output in a specific format

```
#design is where in the metadata it tells you the design of the experiment
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
```

The main DESeq function is called DESeq

```
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>
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
```

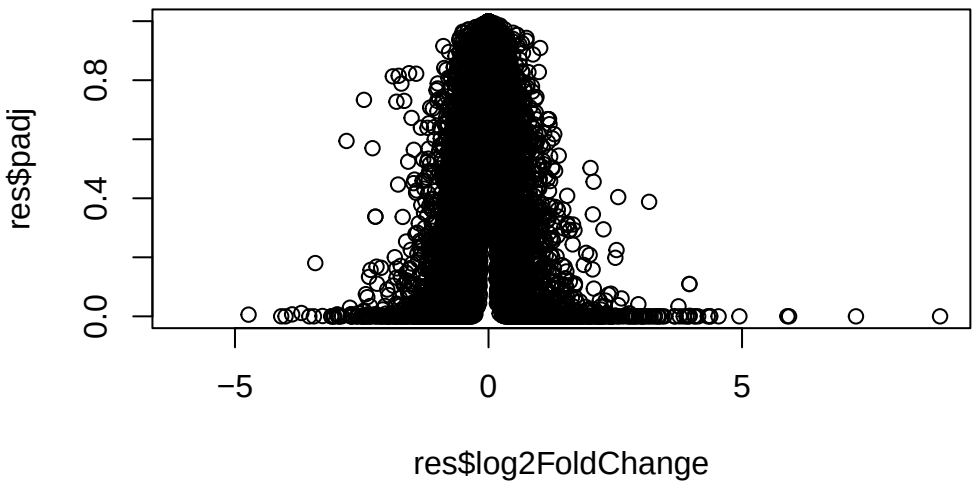
padj –> adjusted p-value -there are so many different samples -scale by the amount of tests you do -can change things from significant to not significant compared to the rest of the data

Volcano Plots

A major summary figure of this type of analysis is called a volcano plot. The idea here is to keep our inner biologist and inner statistician happy with one cool plot!

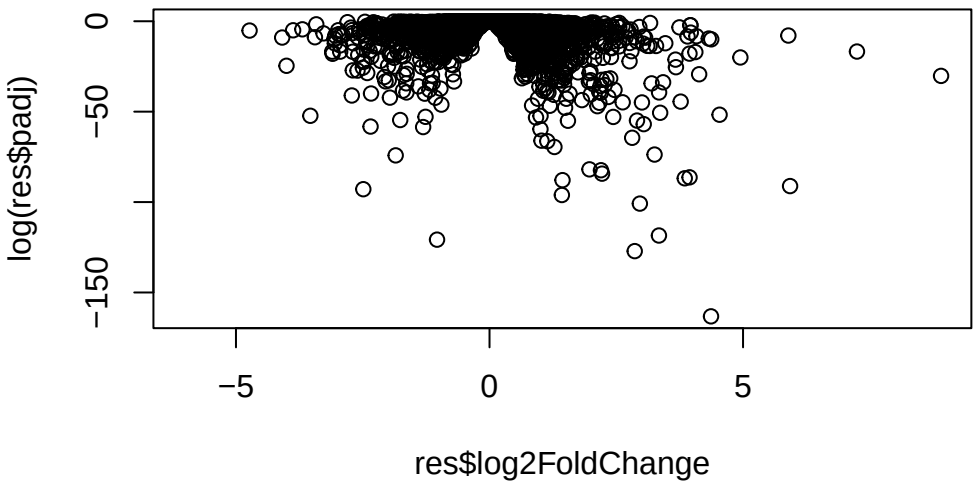
We want the more extreme values on the x axis (away from 0), and the lower p-values.

```
plot(res$log2FoldChange, res$padj)
```



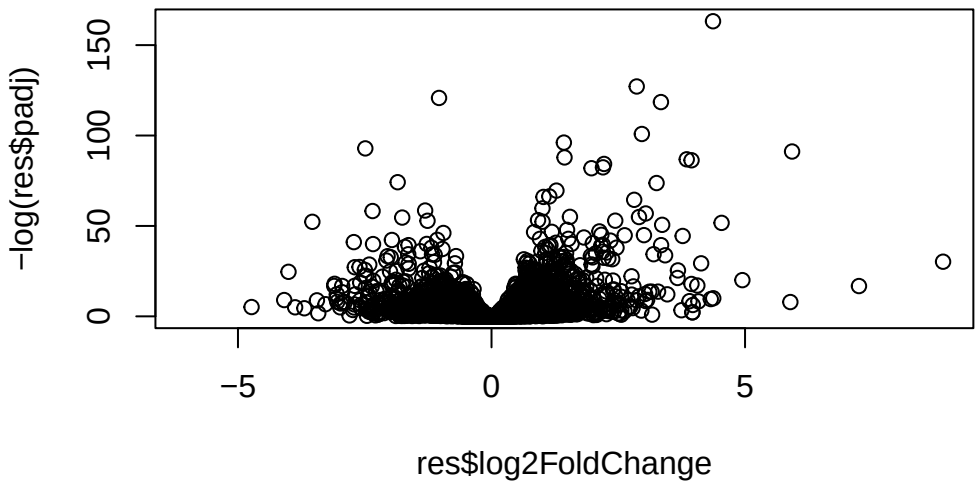
Improve this plot by taking the log of the p-value axis.

```
plot(res$log2FoldChange, log(res$padj))
```



I want to flip this y-axis so the values I care about (i.e. the low p-values or high log(p-value) are at the top).

```
plot(res$log2FoldChange, -log(res$padj))
```



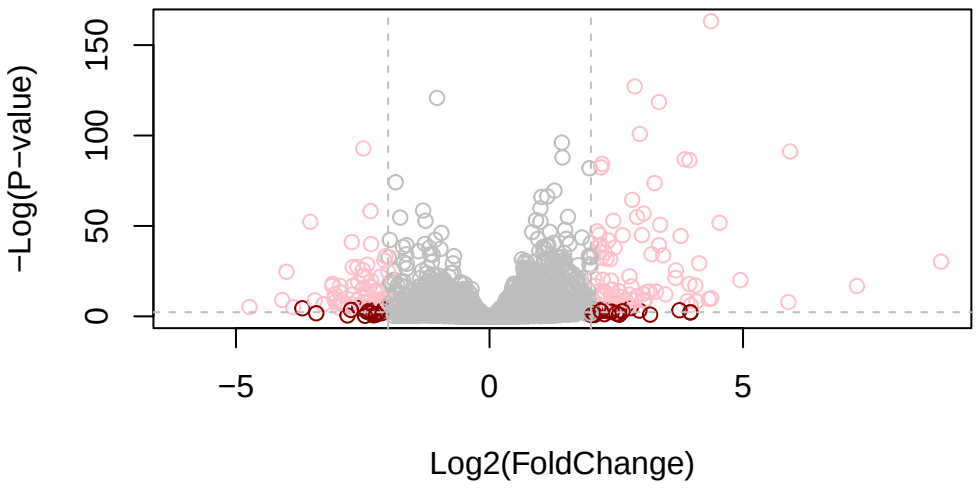
Let's finish up for today by adding some color to better highlight the suset of genes that we will fous on next day (i.e. those with big log2fc values at +2/-2 threshold) and significant p-values (less than 0.05 for example).

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

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

# 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)
```



Adding annotation data

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

We will use one of Bioconductor's main annotation packages to help with mapping between various ID schemes. Here we load the `AnnotationDbl` package and the annotation data packages for humans `org.Hs.eg.db`.

Look at what types of IFs I can translate between from the `org.Hs.eg.db` package with the `columns()` function.

```
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(x = org.Hs.eg.db,
                     column= "SYMBOL",
                     keys = rownames(res),
                     keytype = "ENSEMBL")
```

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

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>
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    C1orf112       55732  A0A024R922
ENSG00000000938        NA         FGR        2268      P09769
                           genename
                        <character>
ENSG00000000003          tetraspanin 6
ENSG00000000005            tenomodulin
ENSG00000000419 dolichyl-phosphate m..
ENSG00000000457 SCY1 like pseudokina..
ENSG00000000460 chromosome 1 open re..
ENSG00000000938 FGR proto-oncogene, ..
```

Pathway analysis

We will finish this lab with a quick pathway analysis. Here we play with just the **GAGE package**

```
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"
```

The main `gage()` function requires a named vector of fold changes where the names of the values are the Entrez gene IDs.

```
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
```

Now„ let's run the pathway analysis

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

Now let's look at the object returned from `gage()`

```
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
```

Let's pull up the highlgihted pathways and show our diffferentially epressed genes on the pathway. I will use the 'hss KEGG, I would get the pathway from KEGG and foldchange vector to show my genes

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

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

Info: Working in directory C:/Users/MacCh/OneDrive/Documents/BIMM 143/class12

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

Put this into my document

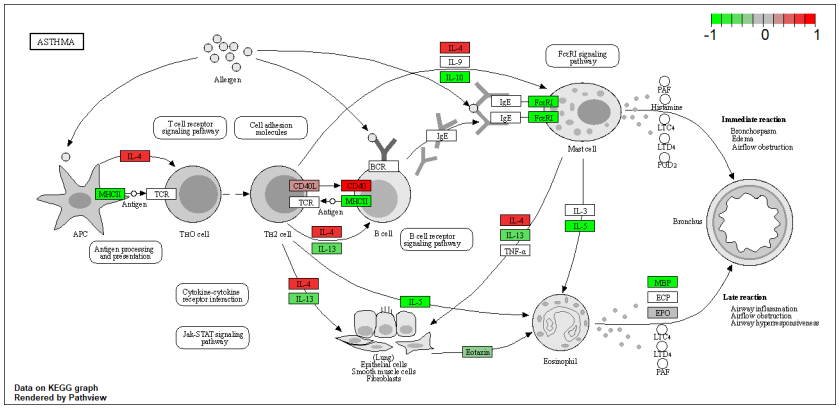


Figure 1: the Asthma pathway with my highlighted differentially epressed genes in order