Class12

Ebru Robinson

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Background

Today we will analyze some RNASeq data from Himes et al. on the effects of a common steroids (dexmethasone also called "dex") on airway smooth muscle cells (ASMs).

For this analysis we need two main inputs

- `countData`:a table of **counts** per gene(in rows) accross experiments (in columns).
- `-colData`: **metadata**about the design of the experiments. The rows match the columns in `coundtada`

Data Import

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

Let's have a peek at our `counts` data.

```
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
```

and the `metadata`

```
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
```

Q2. How many experiments (i.e. columns in `counts` or rows in `metadata`) do we have?

```
ncol(counts)
```

```
[1] 8
```

Q3. How many 'control' experiments do we have?

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

```
[1] 4
```

1. Extract the "control" columns from `counts`
2. Find the mean value for each gene in these "control" columns 3-4. Do the same for the "treated" columns
3. Compare these values for each gene

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

Step2.

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

Step3-4

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

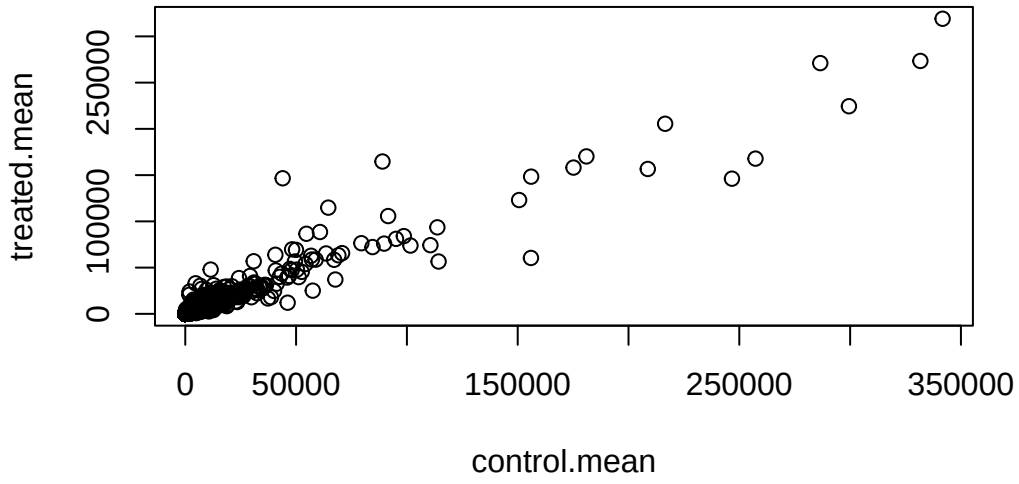
```
treated.mean <- rowMeans(treated.counts)
```

For ease of book-keeping we can store these together in one data frame called `meancounts`

```
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
```

```
plot(meancounts)
```

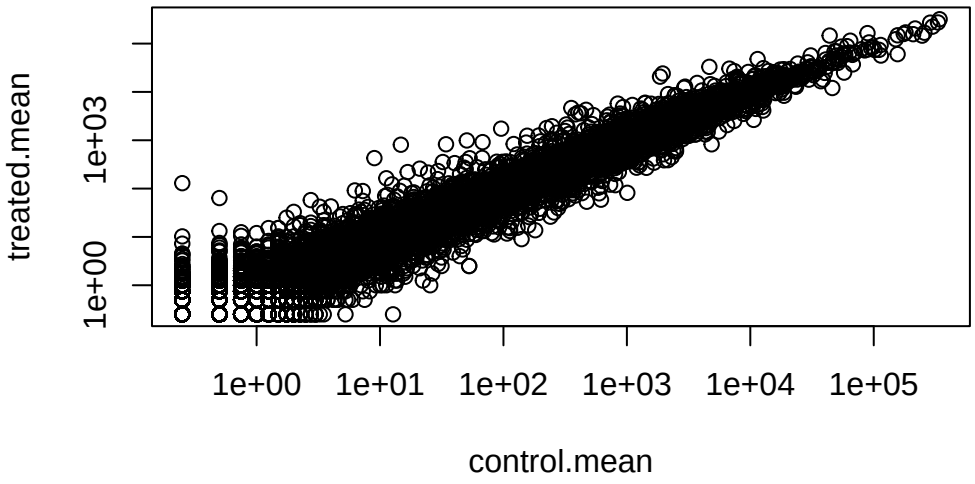


This is screaming at me to log transform this data!!

```
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
```



We use log2 "fold-change" as a way to compare

```
#treated/control
log2(10/10) #no change
```

```
[1] 0
```

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

```
[1] 1
```

```
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
```

2 folds means 4 times higher gene number A common "rule-of-thumb" threshold for calling something "up" regulated is a log2-fold-change of +2 or greater. For "down" regulated is -2 or less.

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

to.rm <- unique(zero.inds[,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
```

```
# ALTERNATE METHOD FIND ZERO VALUES
```

Q How many genes are "up" regulated at the +2 log2FC threshold?

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

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

```
[1] 250
```

Q How many genes are "down" regulated at the +2 log2FC threshold?

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

```
[1] 367
```

PCA

```
library(DESeq2)
```

```
Loading required package: S4Vectors
```

```
Loading required package: stats4
```

```
Loading required package: BiocGenerics
```

```
Loading required package: generics
```

```

Attaching package: 'generics'
```

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

    as.difftime, as.factor, as.ordered, intersect, is.element, setdiff,
    setequal, union
```

```

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, is.unsorted, lapply, Map, mapply, match, mget,
    order, paste, pmax, pmax.int, pmin, pmin.int, Position, rank,
    rbind, Reduce, rownames, sapply, saveRDS, table, tapply, unique,
    unsplit, which.max, which.min
```

```

Attaching package: 'S4Vectors'
```

```
The following object is masked from 'package:utils':

    findMatches

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

    expand.grid, I, unname

Loading required package: IRanges

Loading required package: GenomicRanges

Loading required package: Seqinfo

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
```

```r
# 1) Read data
counts   <- read.csv("airway_scaledcounts.csv", row.names = 1, check.names = FALSE)
metadata <- read.csv("airway_metadata.csv", stringsAsFactors = FALSE)

# 2) Clean whitespace/case everywhere
colnames(counts) <- trimws(colnames(counts))
names(metadata)  <- trimws(names(metadata))
metadata[] <- lapply(metadata, function(x) if (is.character(x)) trimws(x) else x)

# 3) Set sample IDs as rownames for metadata (adjust 'run' if your id column is named differe
id_col <- if ("run" %in% names(metadata)) "run" else names(metadata)[1]
rownames(metadata) <- metadata[[id_col]]

# 4) Align samples (keep shared samples, consistent order)
common <- intersect(colnames(counts), rownames(metadata))
counts   <- counts[, common, drop = FALSE]
metadata <- metadata[common, ,  drop = FALSE]

# 5) Normalize the 'dex' column values and inspect
metadata$dex <- tolower(trimws(metadata$dex))
table(metadata$dex, useNA = "ifany")  # should show only 'control' and 'treated' and no <NA>
```

```
control treated
      4       4
```

```
# If you still see NA, show the offending rows:
which(is.na(metadata$dex))            # indices with NA
```

```
integer(0)
```

```
metadata[is.na(metadata$dex), , drop = FALSE]
```

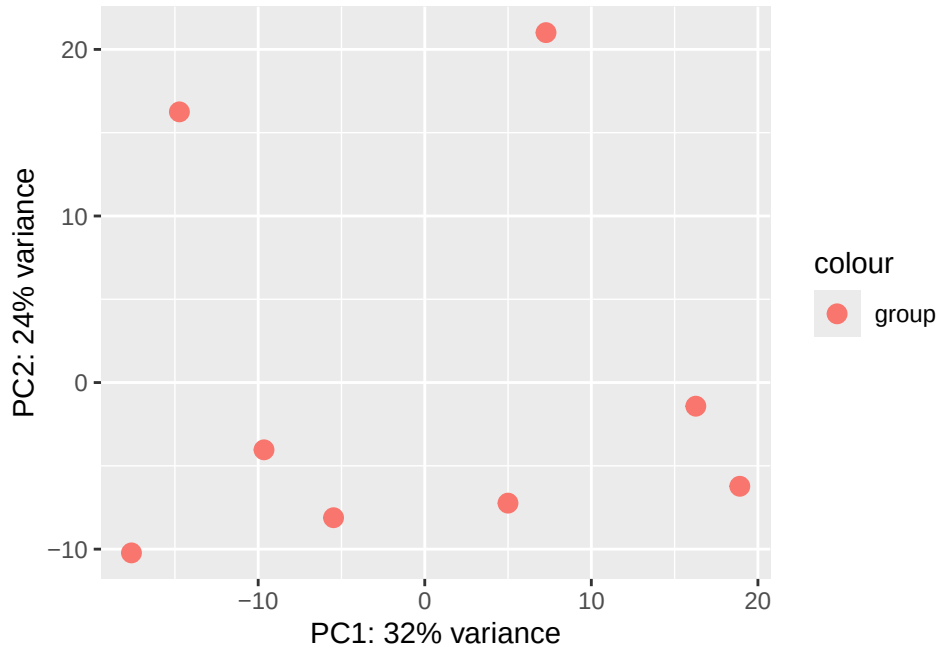
```
[1] id       dex      celltype geo_id
<0 rows> (or 0-length row.names)
```

```
# 6) Drop samples with missing dex OR fix the values
keep <- !is.na(metadata$dex) & metadata$dex %in% c("control","treated")
counts   <- counts[, keep, drop = FALSE]
metadata <- metadata[keep, ,  drop = FALSE]

# 7) Make factor with correct levels (control as reference)
metadata$dex <- factor(metadata$dex, levels = c("control","treated"))

# 8) Build DESeq2 object (ensure integer counts)
counts_mat <- as.matrix(round(counts))
dds <- DESeqDataSetFromMatrix(countData = counts_mat,
                              colData   = metadata,
                              design    = ~ dex)
```

```
converting counts to integer mode
```

```
# Optional: filter zero-count genes
dds <- dds[rowSums(counts(dds)) > 0, ]

# 9) PCA prep and plot
vsd <- vst(dds, blind = FALSE)
plotPCA(vsd, intgroup = "dex")
```

```
using ntop=500 top features by variance
```




```
pcaData <- plotPCA(vsd, intgroup=c("dex"), returnData=TRUE)
```

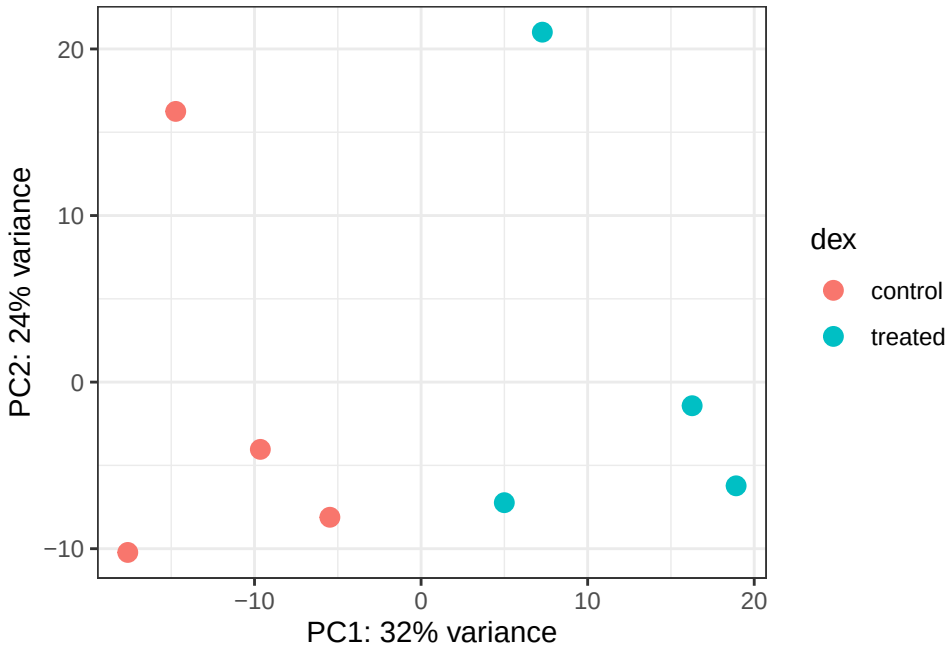
```
using ntop=500 top features by variance
```

```
head(pcaData)
```

```
                 PC1        PC2   group       name         id     dex celltype
SRR1039508 -17.607922 -10.225252 control SRR1039508 SRR1039508 control   N61311
SRR1039509   4.996738  -7.238117 treated SRR1039509 SRR1039509 treated   N61311
SRR1039512  -5.474456  -8.113993 control SRR1039512 SRR1039512 control  N052611
SRR1039513  18.912974  -6.226041 treated SRR1039513 SRR1039513 treated  N052611
SRR1039516 -14.729173  16.252000 control SRR1039516 SRR1039516 control  N080611
SRR1039517   7.279863  21.008034 treated SRR1039517 SRR1039517 treated  N080611
               geo_id sizeFactor
SRR1039508 GSM1275862  1.0193796
SRR1039509 GSM1275863  0.9005653
SRR1039512 GSM1275866  1.1784239
SRR1039513 GSM1275867  0.6709854
SRR1039516 GSM1275870  1.1731984
SRR1039517 GSM1275871  1.3929361
```

```
# Calculate percent variance per PC for the plot axis labels
percentVar <- round(100 * attr(pcaData, "percentVar"))
```

```
library(ggplot2)
ggplot(pcaData) +
  aes(x = PC1, y = PC2, color = dex) +
  geom_point(size =3) +
  xlab(paste0("PC1: ", percentVar[1], "% variance")) +
  ylab(paste0("PC2: ", percentVar[2], "% variance")) +
  coord_fixed() +
  theme_bw()
```



DESeq analysis

Let's do this with DESeq2 and put some stats behind these numbers

```
library(DESeq2)
```

DESeq wants 3 things for analysis,countData, colData and desing.

```
dds <- DESeqDataSetFromMatrix(countData=counts, colData = metadata, design= ~dex)
```

```
converting counts to integer mode
```

The main function in the DESeq package to run analysis 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
```

get the results out of this DESeq object with the function `results()`

```
res <- results(dds)
head(res)
```

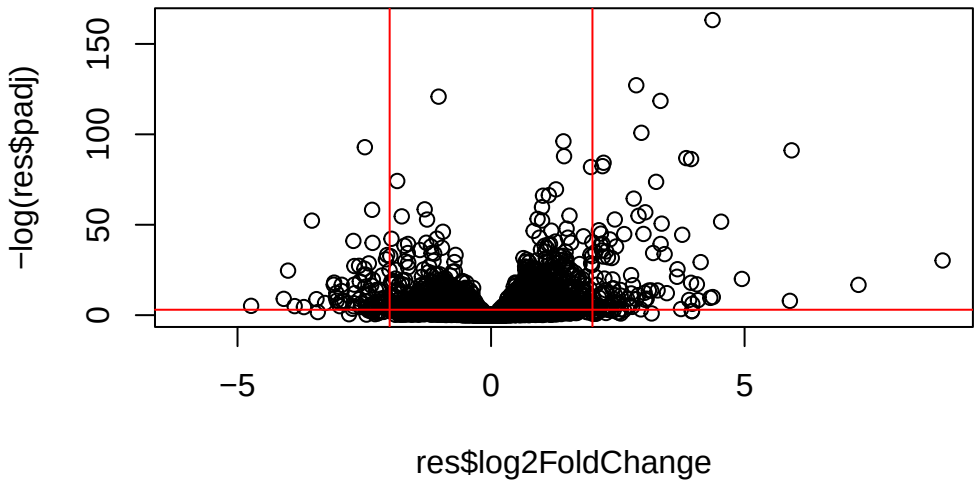
```
log2 fold change (MLE): dex treated vs control
Wald test p-value: dex treated vs control
DataFrame with 6 rows and 6 columns
                  baseMean log2FoldChange     lfcSE      stat    pvalue
                 <numeric>      <numeric> <numeric> <numeric> <numeric>
ENSG00000000003 747.194195      -0.350703  0.168242 -2.084514 0.0371134
ENSG00000000005   0.000000             NA        NA        NA        NA
ENSG00000000419 520.134160       0.206107  0.101042  2.039828 0.0413675
ENSG00000000457 322.664844       0.024527  0.145134  0.168996 0.8658000
ENSG00000000460  87.682625      -0.147143  0.256995 -0.572550 0.5669497
ENSG00000000938   0.319167      -1.732289  3.493601 -0.495846 0.6200029
                     padj
                <numeric>
```

```
ENSG00000000003  0.163017
ENSG00000000005        NA
ENSG00000000419  0.175937
ENSG00000000457  0.961682
ENSG00000000460  0.815805
ENSG00000000938        NA
```

Volcano Plot

This is plot of log2FC vs adjusted p-value

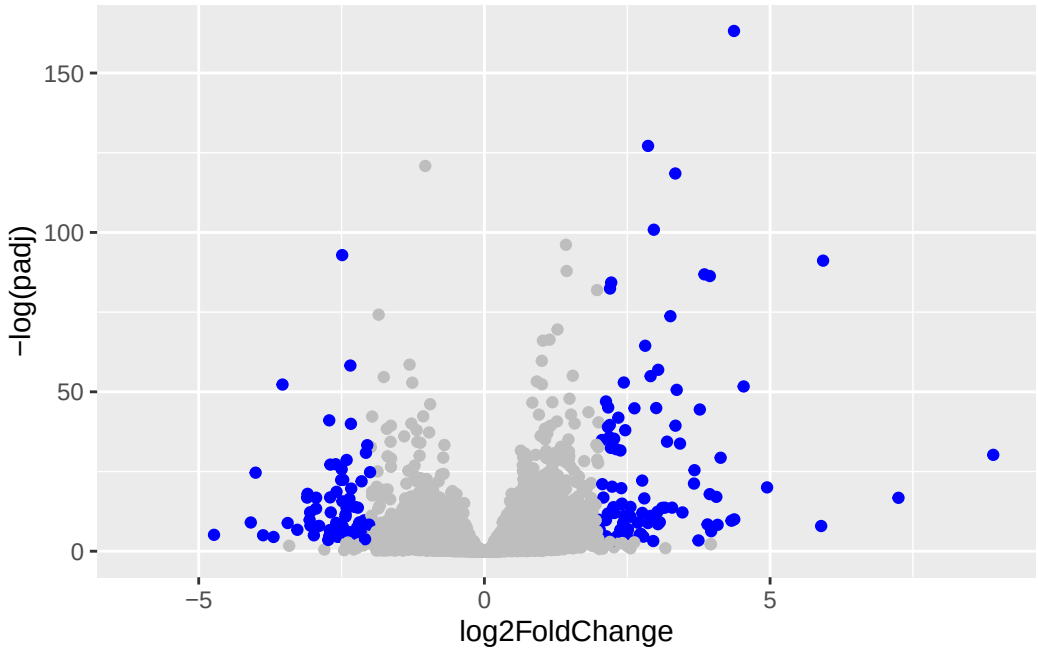
```
plot(res$log2FoldChange, -log(res$padj))

abline(v=c(-2,2), col="red")
abline(h=-log(0.05),col="red")
```



A nicer ggplot volcano plot

```
library(ggplot2)

mycols <- rep("gray", nrow(res))
mycols[abs(res$log2FoldChange)>2] <- "blue"
mycols[res$padj>=0.05] <- "gray"

ggplot(res)+ aes(log2FoldChange, -log(padj))+ geom_point(col=mycols)
```

```
Warning: Removed 23549 rows containing missing values or values outside the scale range
(`geom_point()`).
```



Save our results

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

```
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)
```

```
[1] -0.35070296          NA  0.20610728  0.02452701 -0.14714263 -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 q.val
hsa00232 Caffeine metabolism                 NA       NaN    NA    NA
hsa00983 Drug metabolism - other enzymes     NA       NaN    NA    NA
hsa01100 Metabolic pathways                  NA       NaN    NA    NA
                                      set.size exp1
hsa00232 Caffeine metabolism                 0   NA
hsa00983 Drug metabolism - other enzymes     0   NA
hsa01100 Metabolic pathways                  0   NA
```

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

```
Warning: None of the genes or compounds mapped to the pathway!
Argument gene.idtype or cpd.idtype may be wrong.
```

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

```
Info: Working in directory /Users/ebruy/Desktop/UCSD/Bioinformatics 213/class12.
```

```
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)
```

```
Warning: None of the genes or compounds mapped to the pathway!
Argument gene.idtype or cpd.idtype may be wrong.
```

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

```
Info: Working in directory /Users/ebruy/Desktop/UCSD/Bioinformatics 213/class12.

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