Class 13: RNASeq with DESeq2

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Background

Today we will perform an RNASeq analysis of the effects of a common sertoid on airway cells.

In particular, dexamethasone (herafter just called "dex") on different airway smooth muscle cell lines (ASM cells).

Data Import

We need two different inputs:

-**countData** -**colData**: meta data that describes the columns in countData

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

Wee peek at counts and metadata.

```
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
```

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

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

```
[1] 4
```

Differential Gene Expression

We have 4 replicate drug treated and control (no drug) columns/experiments in our `counts` object.

We want one "mean" value for each gene (rows) in "treated" (drug) and one mean value for each gene in "control" cols.

Step 1. Find all "control" columns in `counts` Step 2. Extract these columns to a new object called `control.counts` Step 3. Then calculate the mean value for each gene

Step 1

```
control.inds <- metadata$dex == "control"
```

Step 2

```
control.counts <- counts[ , control.inds]
```

Step 3

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

> Q3. How would you make the above code in either approach more robust? Is there a function that could help here?

rowSums()

> Q4.Now do the same thing for the "treated" columns/experiments...

Step 1

```
treated.inds <- metadata$dex == "treated"
```

Step 2

```
treated.counts <- counts[ , treated.inds]
```

Step 3

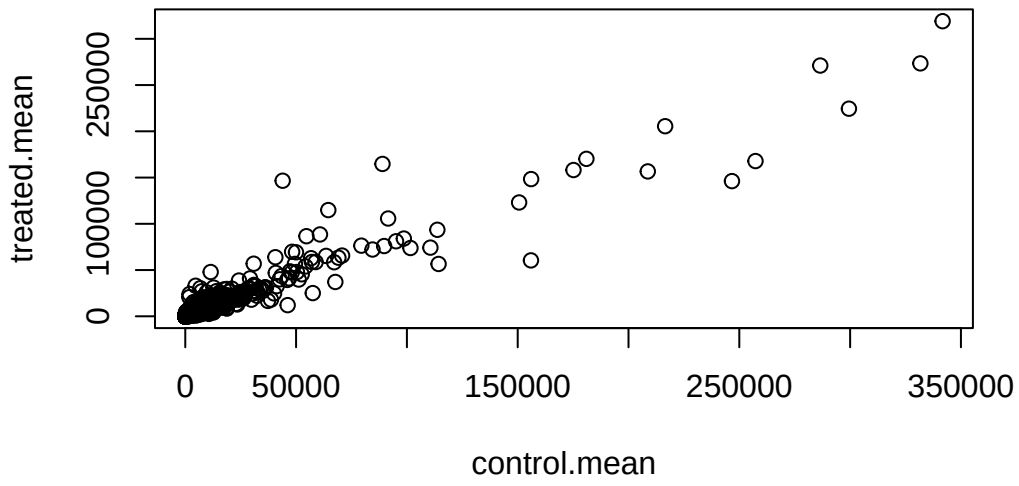
```
treated.mean <-rowMeans(treated.counts)
```

Put these together for easy book-keeping as `meancounts`

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

Q5. A quick plot

```
plot(meancounts)
```



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?

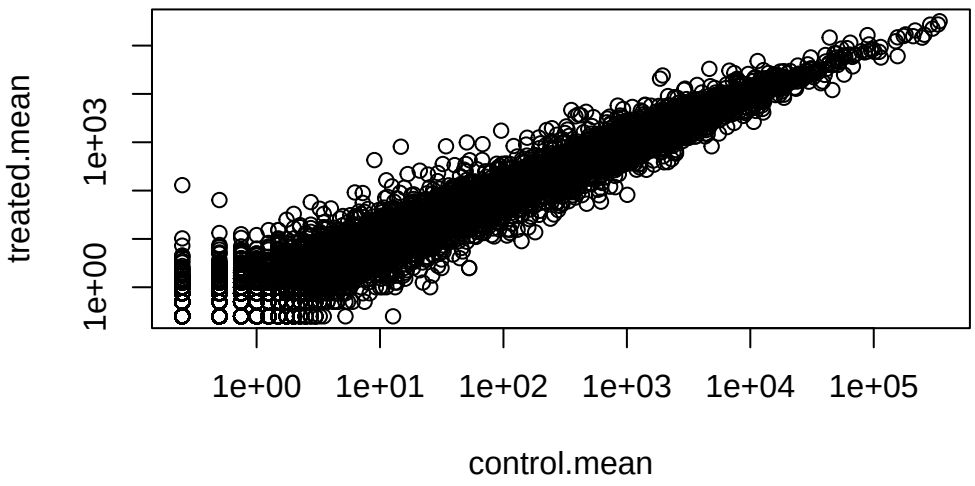
geom_point

Q6. Let's log transform this count 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
```



N.B. We most often use log2 for this type of data as it makes the interpretation much more straightforward.

If there was no change we would have a log2-fc of zero:

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

```
[1] 0
```

If we had double the amount of transcript around we would have a log2-fc of 1

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

```
[1] 1
```

If we had half as much transcript around we would have a log-fc of -1

```
log2(5/10)
```

```
[1] -1
```

Calculate a log2 fold change value for all our genes and add it as a new column to our `meancounts` object.

```
meancounts$log2F <- log2( meancounts$treated.mean / meancounts$control.mean )

head(meancounts)
```

```
                control.mean treated.mean       log2F
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
```

```
log2(40/10)
```

```
[1] 2
```

There are some funky log2fc values (NaN and -Inf) here that come about whenever we have 0 mean count values. Typically, we would remove these genes from any further analysis - as we can't say anything about them if we have no data for them.

```
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       log2F
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 purpose of the arr.ind argument in the which() function is so the which() give the row and column positions that meet the condition. We would take the first column because it has the row numbers that have zeros. The unique() function will remove duplicate row numbers just in case a gene has zeros in more than one column.

```
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?

250

> 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

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

Not fully because these results haven't proven to be statistically significant.

DeSEQ Analysis

Let's do this analysis with an estimate of statistical significance using the **DESeq2** package

```
library(DESeq2)
```

DESeq (like many bioconductor packages) want its input data in a very specific way.

```
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
```

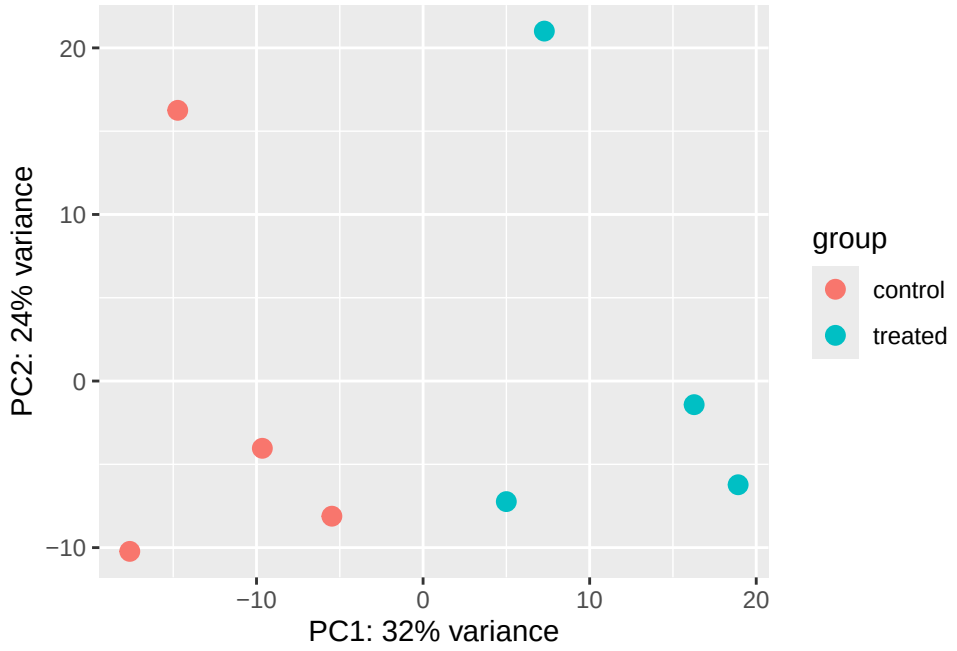
```
dds
```

```
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
```

##Principal Component Analysis (PCA)

```
vsd <- vst(dds, blind = FALSE)
plotPCA(vsd, intgroup = c("dex"))
```

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




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

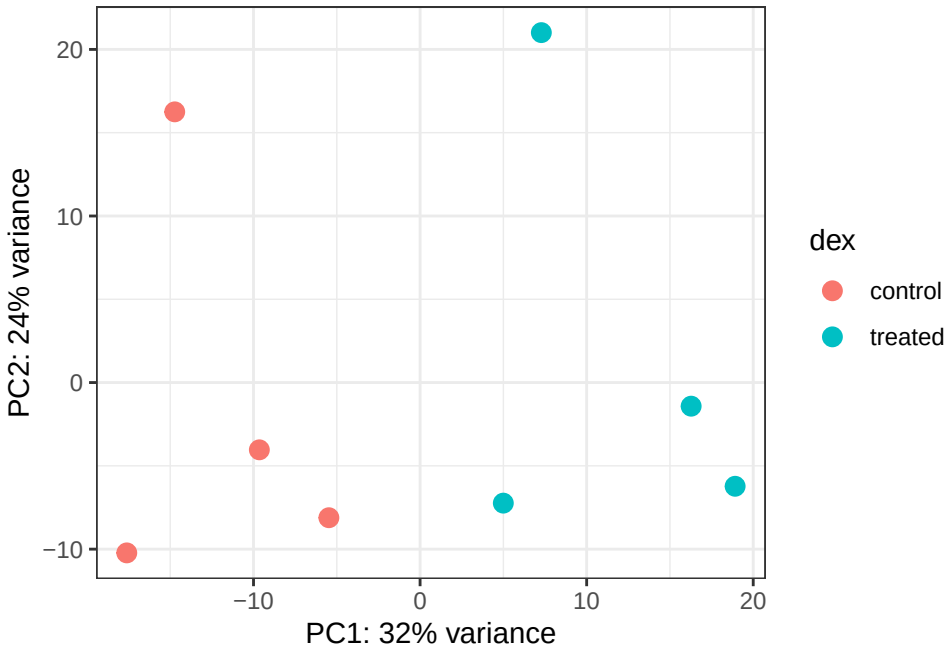
```
using ntop=500 top features by variance
```

```
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
```

```
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()
```



Run the DESeq analysis pipeline

The main function `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)
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
```

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

```
out of 25258 with nonzero total read count
adjusted p-value < 0.05
LFC > 0 (up)       : 1237, 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
```

Adding annotation data

We need to add missing annotation data to our main `res` results object. This includes the common gene "symbol"

```
library("AnnotationDbi")
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),
                     keytype="ENSEMBL",
                     column="SYMBOL",
                     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      -0.350703  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      symbol
                <numeric> <character>
ENSG00000000003  0.163017      TSPAN6
ENSG00000000005        NA        TNMD
```

```
ENSG00000000419  0.175937        DPM1
ENSG00000000457  0.961682       SCYL3
ENSG00000000460  0.815805       FIRRM
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
```

```
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      -0.350703  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      symbol      entrez     uniprot
                <numeric> <character> <character> <character>
ENSG00000000003  0.163017      TSPAN6        7105  A0A087WYV6
ENSG00000000005        NA        TNMD       64102      Q9H2S6
ENSG00000000419  0.175937        DPM1        8813      H0Y368
ENSG00000000457  0.961682       SCYL3       57147      X6RHX1
ENSG00000000460  0.815805       FIRRM       55732      A6NFP1
ENSG00000000938        NA         FGR        2268      B7Z6W7
                           genename
                        <character>
ENSG00000000003          tetraspanin 6
ENSG00000000005            tenomodulin
ENSG00000000419 dolichyl-phosphate m..
ENSG00000000457 SCY1 like pseudokina..
ENSG00000000460 FIGNL1 interacting r..
ENSG00000000938 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 0.2371306   18.4217 8.79214e-76
ENSG00000179094   743.253        2.86389 0.1755659   16.3123 8.06568e-60
ENSG00000116584  2277.913       -1.03470 0.0650826  -15.8983 6.51317e-57
ENSG00000189221  2383.754        3.34154 0.2124091   15.7316 9.17960e-56
ENSG00000120129  3440.704        2.96521 0.2036978   14.5569 5.27883e-48
ENSG00000148175 13493.920        1.42717 0.1003811   14.2175 7.13625e-46
                      padj      symbol      entrez     uniprot
                 <numeric> <character> <character> <character>
ENSG00000152583 1.33157e-71     SPARCL1        8404      B4E2Z0
ENSG00000179094 6.10774e-56        PER1        5187      A2I2P6
```

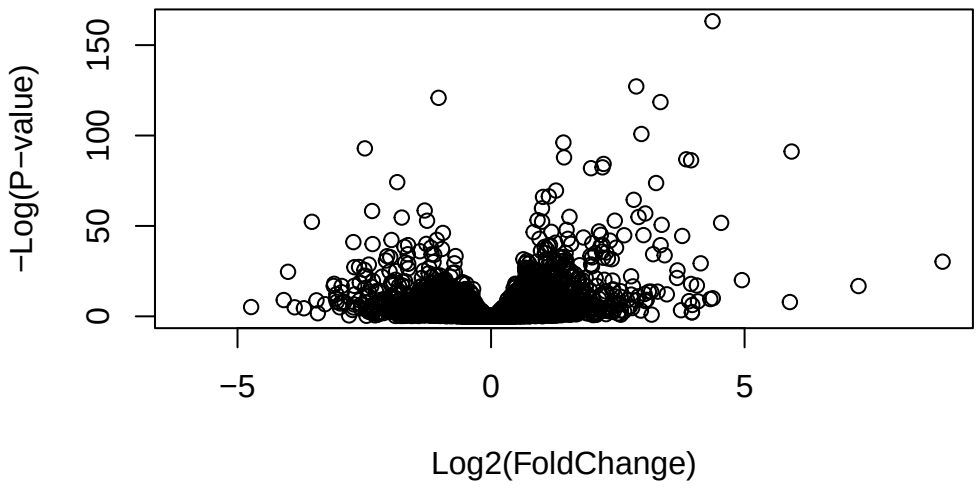
```
ENSG00000116584 3.28806e-53     ARHGEF2        9181  A0A8Q3SIN5
ENSG00000189221 3.47563e-52        MAOA        4128      B4DF46
ENSG00000120129 1.59896e-44       DUSP1        1843      B4DRR4
ENSG00000148175 1.80131e-42        STOM        2040      F8VSL7
                                genename
                              <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,], "results_annotated.csv")
```

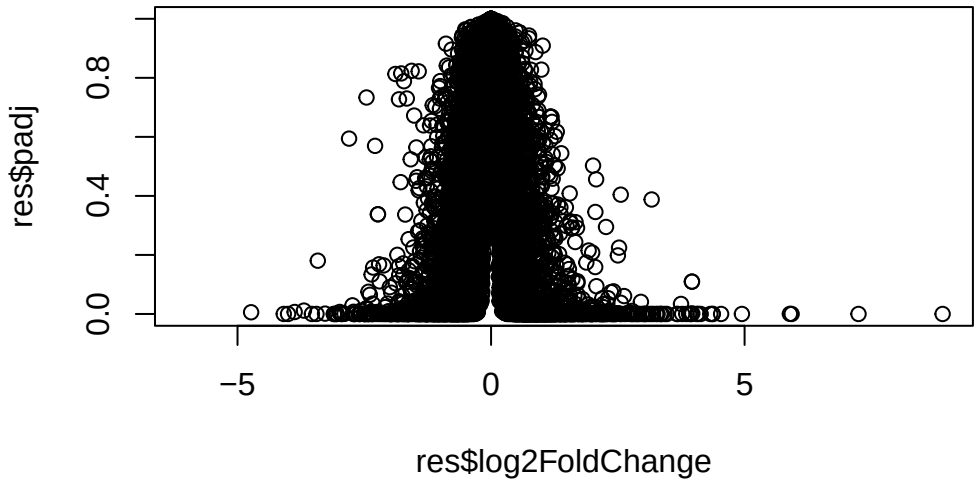
Volcano Plot

This is a main summary results figure from these kinds of studies. It is a plot of Log2 fold-change vs. P-value.

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

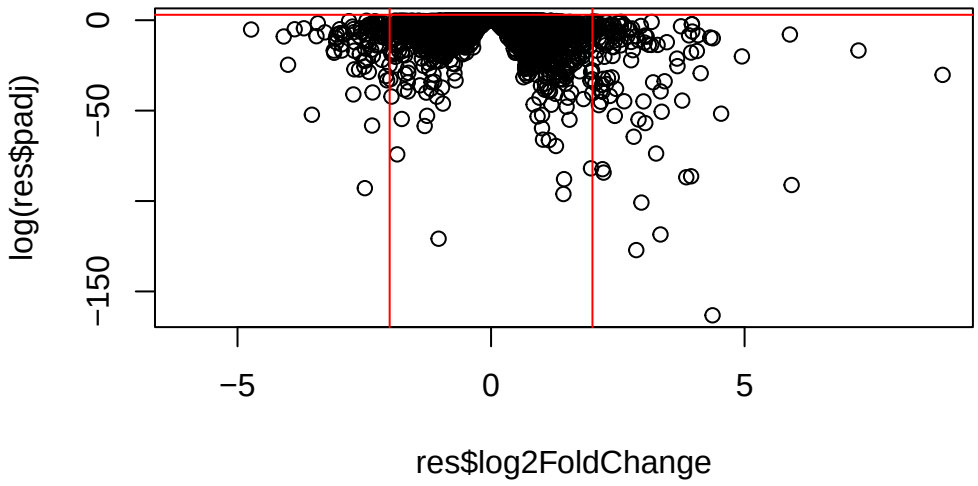



```
plot( res$log2FoldChange,
      res$padj)
```



Again this y-axis highly needs log transforming and we can flip the y-axis with a minus sign so it looks like every other volcano plot

```
plot(res$log2FoldChange,
     log(res$padj))
abline(v=-2, col="red")
abline(v=2, col="red")
abline(h=-log(0.05), col="red")
```



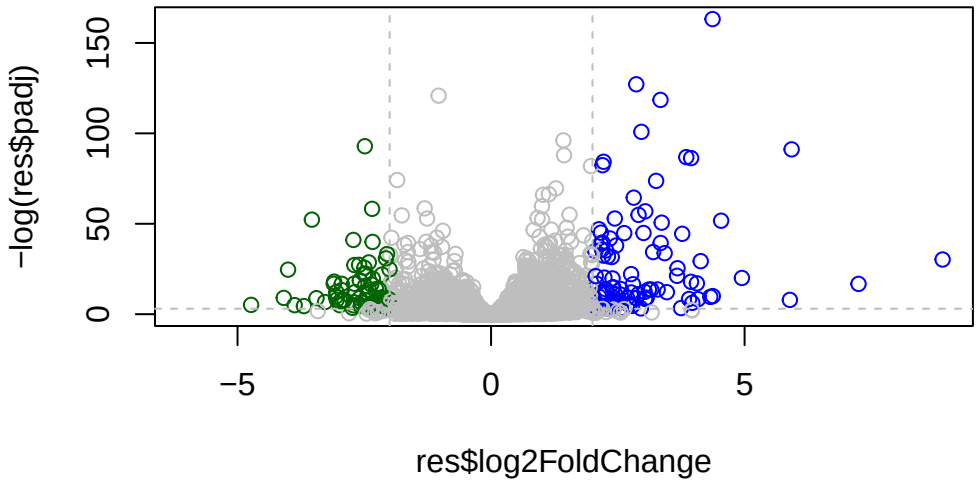
Adding some color annotation

Start with a default base color "gray"

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

plot(res$log2FoldChange,
     -log(res$padj),
     col=mycols)
```

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

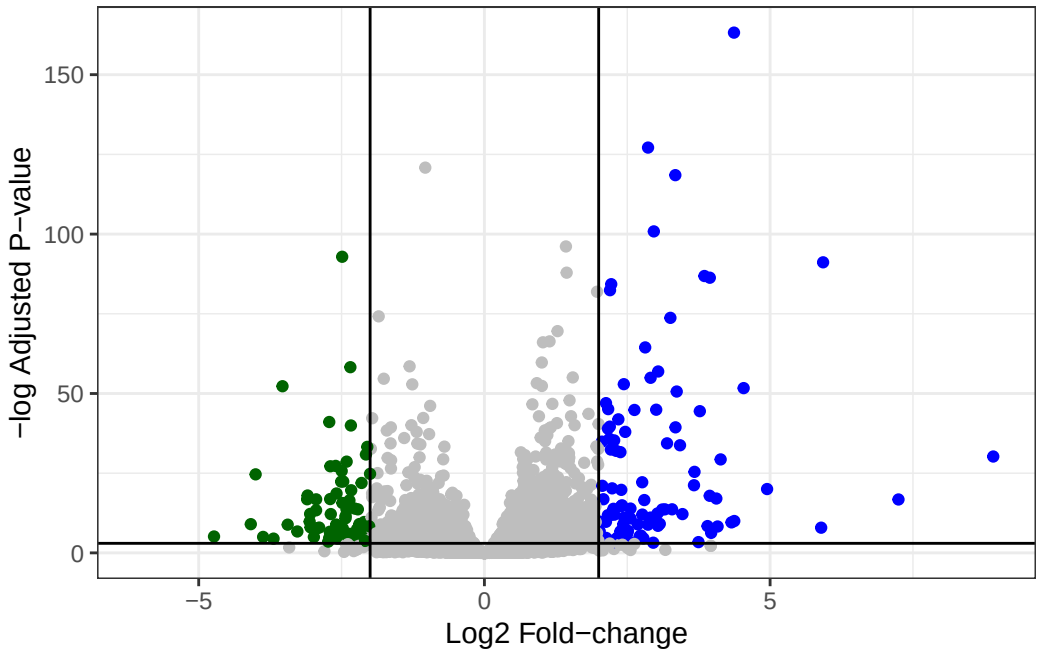


Q. Make a presentation quality ggplot version of this plot. Include clear axis labels, a clean theme, your custom colors, cut-offlines and a plot title.

```
library(ggplot2)

ggplot(res)+
  aes(log2FoldChange,
      -log(padj))+
  geom_point(color=mycols)+
  labs(x="Log2 Fold-change",
       y="-log Adjusted P-value")+
  geom_vline(xintercept=c(-2,2))+
  geom_hline(yintercept =-log(0.05))+
  theme_bw()
```

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




```
library(EnhancedVolcano)
```

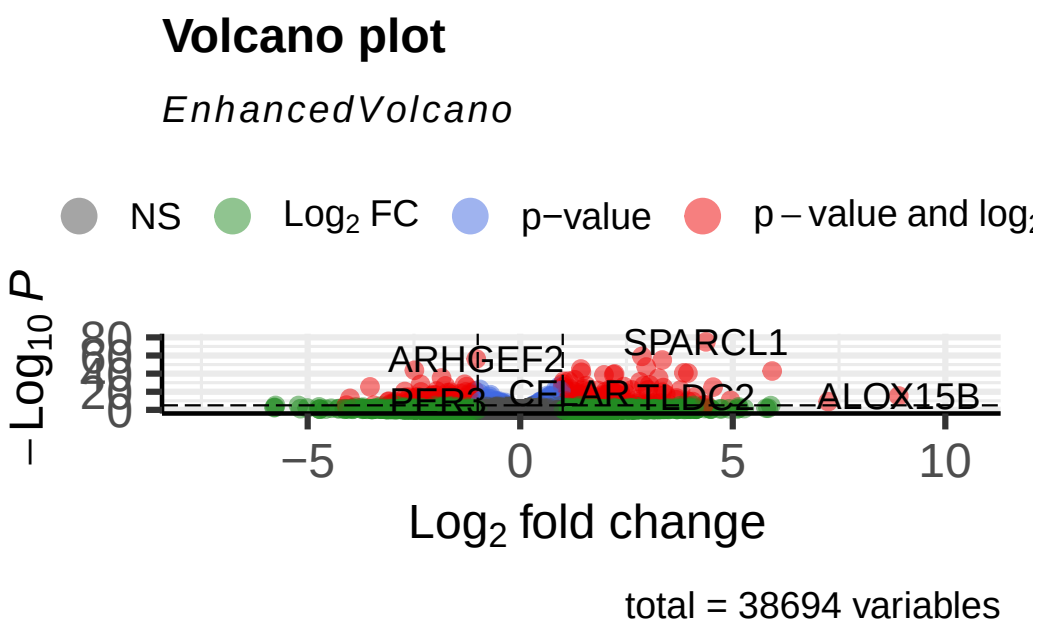
```
Loading required package: ggrepel
```

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

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

```
Warning: Using `size` aesthetic for lines was deprecated in ggplot2 3.4.0.
i Please use `linewidth` instead.
i The deprecated feature was likely used in the EnhancedVolcano package.
  Please report the issue to the authors.
```

```
Warning: The `size` argument of `element_line()` is deprecated as of ggplot2 3.4.0.
i Please use the `linewidth` argument instead.
i The deprecated feature was likely used in the EnhancedVolcano package.
  Please report the issue to the authors.
```



Save our results

Write a CSV file

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

Pathway Analysis

What known biological pathways do our differntially expressed genes overlap with (i.e. play a role in)?

There is lot's of bioconductor packages to do this type of analysis.

We will use one othe oldest called **gage** along with **pathview** to render nice pics of the pathways we find.

We can install these with the command `BiocManager::install( c("pathview", "gage", "gageData") )`

```
library(pathview)
library(gage)
library(gageData)
```

Have a wee peek what is in `gageData`

```
# Examine the first 2 pathways in this kegg set for humans
data(kegg.sets.hs)
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 that does the actual work wants a simple vector as input.

```
foldchanges = res$log2FoldChange
names(foldchanges) = res$entrez
head(foldchanges)
```

```
       7105       64102        8813       57147       55732        2268
-0.35070296          NA  0.20610728  0.02452701 -0.14714263 -1.73228897
```

The KEG database uses ENTREZ ids so we need to provide these in our input vector for **gage**

```
names(foldchanges) <- res$entrez
```

Now we can run `gage()`

```
keggress = gage(foldchanges, gsets=kegg.sets.hs)
```

What is in the output object `keggress`

```
attributes(keggress)
```

```
$names
[1] "greater" "less"    "stats"
```

```
head(keggress$less, 3)
```

```
                                    p.geomean stat.mean        p.val
hsa05332 Graft-versus-host disease 0.0004250607 -3.473335 0.0004250607
hsa04940 Type I diabetes mellitus  0.0017820379 -3.002350 0.0017820379
hsa05310 Asthma                    0.0020046180 -3.009045 0.0020046180
                                         q.val set.size         exp1
hsa05332 Graft-versus-host disease 0.09053792       40 0.0004250607
hsa04940 Type I diabetes mellitus  0.14232788       42 0.0017820379
hsa05310 Asthma                    0.14232788       29 0.0020046180
```

We can use the **pathview** function to render a figure of any of these pathways along with annotation for our DEGs.

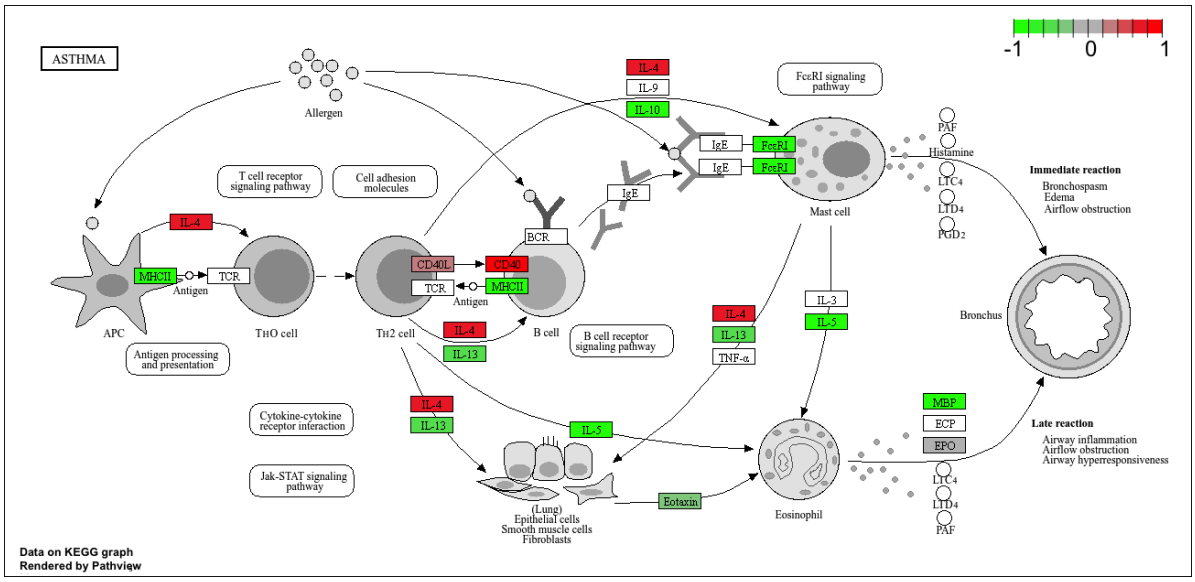
Let's see the hsa05310 Asthma pathway with our DEGs colored up:

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

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

```
Info: Working in directory /Users/rachellamm/bimm143- Rachel Lamm/class13
```

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



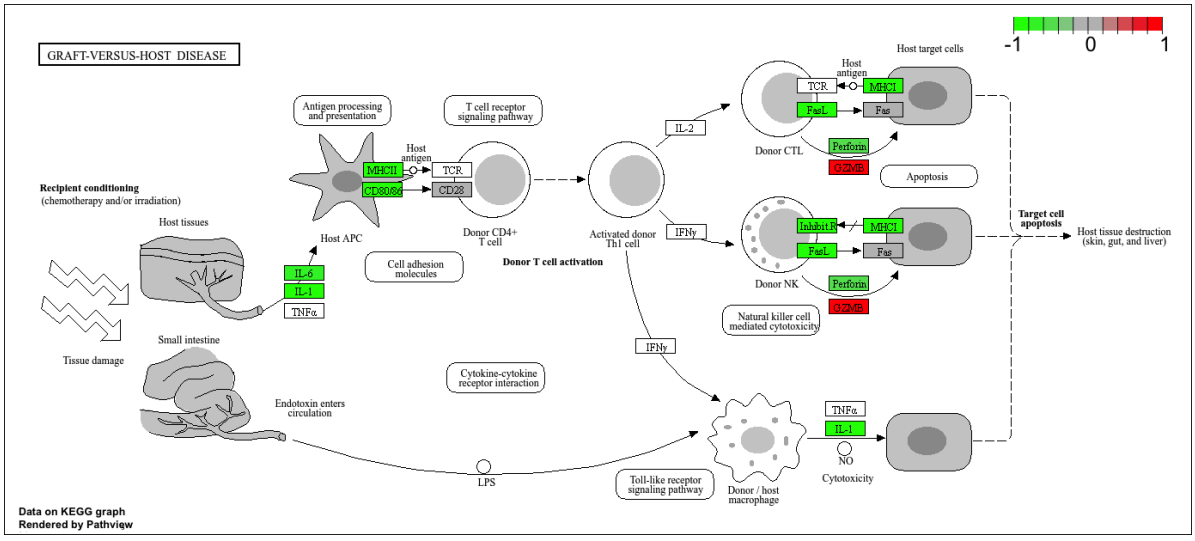
Q. Can you render and insert here the pathway figure for "Graft-versus-host disease" and "Type I diabetes"?

```
pathview(gene.data=foldchanges, pathway.id="hsa05332")
```

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

```
Info: Working in directory /Users/rachellamm/bimm143- Rachel Lamm/class13
```

```
Info: Writing image file hsa05332.pathview.png
```



```
pathview(gene.data=foldchanges, pathway.id="hsa04940")
```

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

```
Info: Working in directory /Users/rachellamm/bimm143- Rachel Lamm/class13
```

```
Info: Writing image file hsa04940.pathview.png
```