Lab 5 - Differential expression

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In this lab, we will be conducting a two-sample test for each gene/probe on the array to identify differentially expressed genes/probes between ketogenic rats and control diet rats. This small data set was run on the rat RAE230A Affymetrix array. The objective of the study was to determine differences in mRNA levels between brain hippocampi of animals fed a ketogenic diet (KD) and animals fed a control diet. "KD is an anticonvulsant treatment used to manage medically intractable epilepsies", so differences between the 2 groups of rats can provide biological insight into the genes that are regulated due to the treatment (source: GSE1155).

We are going to identify those genes/probes that are differentially expressed between the 2 rat diet groups and plot the results with a couple of different visual summaries.

1.) Download the GEO rat ketogenic brain data set and save as a text file.

```
# "rat_KD.zip" downloaded from Data sets section in course
# Decompress the zip files into a data directory
system("unzip -o ./data/rat_KD.zip -d ./data/")

# Check to make sure the unzip process went well
dir("data/")
```

```
## [1] "rat_KD.txt" "rat_KD.zip"
```

2.) Load into R, using `read.table()` function and `header=T`, `row.names=1` arguments.

```
# Read data into R as "dat"
dat <- read.table(file = "data/rat_KD.txt",
                  header = TRUE,
                  row.names = 1)

# Check out the data structure
str(dat)
```

```
## 'data.frame':    15923 obs. of  11 variables:
##  $ control.diet.19300  : num  76.4 94.7 27.9 174.3 87 ...
##  $ control.diet.19301  : num  86.1 73.4 44.5 151.8 94 ...
##  $ control.diet.19302  : num  80.6 88.7 33.9 167.4 120.3 ...
##  $ control.diet.19303  : num  93.8 111.6 60 200.5 114.6 ...
##  $ control.diet.19304  : num  73.1 92.1 39.2 170.7 100.2 ...
##  $ control.diet.19305  : num  97.7 96.4 37.6 196.8 88.4 ...
##  $ ketogenic.diet.19306: num  82.5 131.3 42.8 192.1 122.4 ...
##  $ ketogenic.diet.19307: num  77.2 114.9 50.1 206.3 131 ...
##  $ ketogenic.diet.19308: num  120.2 156.7 78.2 236 157.4 ...
##  $ ketogenic.diet.19309: num  99 117.2 47.9 202.8 110.4 ...
##  $ ketogenic.diet.19310: num  88.3 119.6 37 185.8 117.7 ...
```

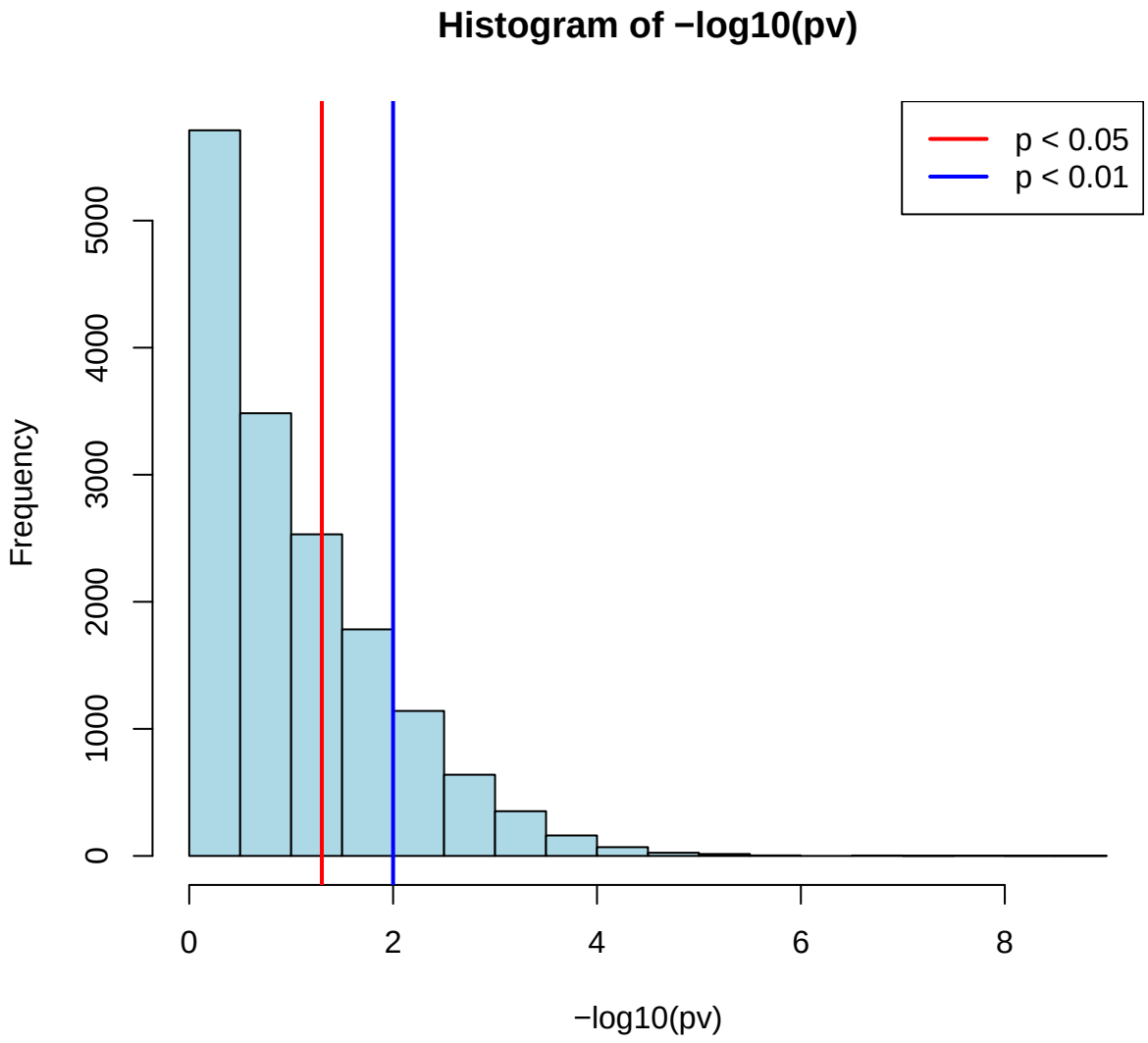
In the data, there appears to be **6 control diet** samples and **5 ketogenic diet** samples.

3.) First log_2 the data, then use the Student's t-test function in the notes to calculate the changing genes between the control diet and ketogenic diet classes. (Hint: use the `names()` function to determine where one class ends and the other begins).

```r
# Log transform the data
log2.dat <- log2(dat)

# Function from lecture notes
t.test.all.genes <- function(x,s1,s2) {
        x1 <- x[s1]; x2 <- x[s2]
        x1 <- as.numeric(x1); x2 <- as.numeric(x2)
        t.out <- t.test(x1,x2, alternative="two.sided",var.equal=T)
        out <- as.numeric(t.out$p.value)
        return(out)
}

# Gather indices of the groups
control <- grep("control", names(log2.dat))
keto <- grep("ketogenic", names(log2.dat))

# Get p-values
pv <- apply(log2.dat, 1, t.test.all.genes, s1 = control, s2 = keto)
```

4.) Plot a histogram of the p-values and report how many probesets have a p<0.05 and p<0.01. Then divide an alpha of 0.05 by the total number of probesets and report how many probesets have a p-value less than this value. This is a very conservative p-value thresholding method to account for multiple testing called the Bonferroni correction that we will discuss in upcoming lectures.

```
# Plot histogram of p-values
hist(-log10(pv), col = "lightblue")

# Vertical lines at p-value thresholds
abline(v = c(-log10(0.05), -log10(0.01)), col = c("red", "blue"), lwd = 2)

# Legend
legend(x = "topright", legend = c("p < 0.05", "p < 0.01"),
       col = c("red", "blue"), lty = 1, lwd = 2)
```



```r
# How many transcripts have p-values below alpha 0.05?
(lt0.05 <- sum(pv < 0.05))
```

```
## [1] 5160
```

```r
# How many transcripts have p-values below alpha 0.01?
(lt0.01 <- sum(pv < 0.01))
```

```
## [1] 2414
```

```r
# Bonferroni-corrected alpha
nProbesets <- length(names(pv))
bf.alpha <- 0.05/nProbesets

# How many are below Bonferroni-corrected alpha value?
(pv.sig <- sum(pv < bf.alpha))
```

```
## [1] 12
```

Although **5160** probesets have p-values calculated to be below 0.05 and **2414** probesets appear to have p-values below 0.01, we need to account for the problem of multiple comparisons. Since we make 15923 separate comparisons to get all our p-values, we need to correct this potential source of error. After performing a conservative Bonferroni correction, we see that in actuality, only **12** probesets can be regarded as *likely* significant (below the corrected threshold p-value).

5.) Next calculate the mean for each gene, and calculate the fold change between the groups (control vs. ketogenic diet). Remember that you are on a log_2 scale.

We can find the fold change $FC_{ctrl.vs.keto} = (\frac{\bar{x}_{ctrl}}{\bar{x}_{keto}})$ by subtracting the logarithms:

$$log_2(\frac{\bar{x}_{ctrl}}{\bar{x}_{keto}}) = log_2(\bar{x}_{ctrl}) - log_2(\bar{x}_{keto})$$

```r
# Mean of each transcript in control sample
control.m <- apply(log2.dat[,control], 1, mean, na.rm = TRUE)

# Mean of each transcript in keto sample
keto.m <- apply(log2.dat[,keto], 1, mean, na.rm = TRUE)

# log2(FC) of all transcripts
log2fc <- control.m - keto.m
```

6.) What is the maximum and minimum fold change value, please report on the linear scale? Now report the probesets with a p-value less than the Bonferroni threshold you used in question 4 and |fold change| > 2. Remember that you are on a log_2 scale for your fold change and I am looking for a linear |fold| of 2.

To transpose the fold change, we will return it to the exponential with the following equation:

$$log_b(M) = N \implies M = b^N.$$

So, by raising 2 to the power of each log_2FC value, we obtain the non-transformed fold change.

```
# Linear scale FC
fc <- 2^(log2fc)

# Minimum and maximum values
min <- min(fc); max <- max(fc)

# Linear scale the subset of probesets with |log2fc| > 2
(filt.fc <- 2^log2fc[abs(log2fc) > 2])
```

```
## 1367553_x_at   1387011_at   1387408_at 1387696_a_at 1387827_x_at   1370239_at 1370240_x_at
##   9.00836617   5.25583892   0.19138963   0.24414227   7.06296231  12.99360895   8.76945002
## 1371102_x_at 1371245_a_at   1371272_at   1388358_at 1388608_x_at   1372087_at   1388804_at
##   7.28482038  55.15521320   5.09128353   0.10909850   4.88586193   0.23652737   0.24107415
##   1373938_at   1374132_at   1375213_at   1375608_at   1375758_at   1394198_at
##   0.24457633   4.56923312   0.14933507   4.11397625   0.08240443   4.73212583
```

```
# Get probesets whose p-value is less than Bonferroni alpha
(filt.pv <- pv[pv < bf.alpha])
```

```
## 1367553_x_at   1368071_at   1370239_at 1370240_x_at   1370355_at 1371102_x_at 1371245_a_at
## 1.224053e-08 1.108599e-06 5.280180e-08 1.622293e-09 1.909314e-06 2.583221e-08 6.370531e-09
## 1388608_x_at   1373040_at   1374641_at   1390092_at   1376005_at
## 1.743055e-07 2.773686e-06 2.217421e-07 2.450007e-06 2.919439e-07
```

```
# Find probeset names which appear in filt.fc and pv < bf.alpha sets
(less_than_bf.alpha  <- intersect(names(filt.fc), names(filt.pv)))
```

```
## [1] "1367553_x_at" "1370239_at"   "1370240_x_at" "1371102_x_at" "1371245_a_at" "1388608_x_at"
```

```
# Write file of probesets to upload to DAVID
write.table(
    less_than_bf.alpha,
    file = "data/probes.txt",
    quote = FALSE,
    row.names = FALSE,
    col.names = FALSE
)
```

After exponentiating the log-transformed fold changes into their linear values, the *minimum* fold change is **0.0824044** and the *maximum* fold change is **55.1552132**.

Additionally, there are 6 probesets with **both** a linear $|FC| > 2$ and $p < 3.1401118 \times 10^{-6}$ (Bonferroni-corrected alpha).

7.) Go to NetAffx or another database source if you like and identify gene information for the probesets that came up in #6. What is the general biological function that associates with these probesets?

Functional Annotation Table

Help and Manual

Current Gene List: probes
Current Background: Rattus norvegicus
3 DAVID IDs

3 record(s) Download File

1371102_x_at, 1371245_a_at	beta globin minor gene(LOC100134871)	Related Genes	Rattus norvegicus
GOTERM_BP_DIRECT	oxygen transport,		
GOTERM_CC_DIRECT	hemoglobin complex,		
GOTERM_MF_DIRECT	oxygen transporter activity, iron ion binding, oxygen binding, heme binding,		
INTERPRO	Globin, Haemoglobin, beta, Globin-like, Globin, structural domain,		
KEGG_PATHWAY	African trypanosomiasis, Malaria,		
UP_KEYWORDS	Acetylation, Complete proteome, Direct protein sequencing, Heme, Iron, Metal-binding, Methylation, Oxygen transport, Phosphoprotein, Reference proteome, S-nitrosylation, Transport,		
UP_SEQ_FEATURE	chain:Hemoglobin subunit beta-2, metal ion-binding site:Iron (heme distal ligand), metal ion-binding site:Iron (heme proximal ligand), modified residue, sequence variant,		
1367553_x_at	**hemoglobin subunit beta(Hbb)**	**Related Genes**	**Rattus norvegicus**
GOTERM_BP_DIRECT	glutathione metabolic process, positive regulation of cell death, regulation of eIF2 alpha phosphorylation by heme, oxygen transport, hemopoiesis, response to hydrogen peroxide, hydrogen peroxide catabolic process, erythrocyte development, protein heterooligomerization, renal absorption, platelet aggregation,		
GOTERM_CC_DIRECT	hemoglobin complex, haptoglobin-hemoglobin complex, myelin sheath, extracellular exosome, blood microparticle,		
GOTERM_MF_DIRECT	peroxidase activity, oxygen transporter activity, iron ion binding, oxygen binding, heme binding, hemoglobin binding, haptoglobin binding, hemoglobin alpha binding, hemoglobin beta binding,		
INTERPRO	Globin, Haemoglobin, beta, Globin-like, Globin, structural domain,		
KEGG_PATHWAY	African trypanosomiasis, Malaria,		
UP_KEYWORDS	3D-structure, Acetylation, Complete proteome, Direct protein sequencing, Heme, Iron, Metal-binding, Methylation, Oxygen transport, Phosphoprotein, Polymorphism, Proteomics identification, Reference proteome, S-nitrosylation, Transport,		
UP_SEQ_FEATURE	chain:Hemoglobin subunit beta-1, helix, metal ion-binding site:Iron (heme distal ligand), metal ion-binding site:Iron (heme proximal ligand), modified residue, sequence conflict, sequence variant, turn,		
1370239_at, 1388608_x_at, 1370240_x_at	**hemoglobin, alpha 1(Hba1)**	**Related Genes**	**Rattus norvegicus**
GOTERM_BP_DIRECT	in utero embryonic development, positive regulation of cell death, oxygen transport, response to stilbenoid, response to hydrogen peroxide, hydrogen peroxide catabolic process, negative regulation of blood pressure, erythrocyte development, protein heterooligomerization, regulation of sensory perception of pain,		
GOTERM_CC_DIRECT	hemoglobin complex, membrane, cytosolic small ribosomal subunit, haptoglobin-hemoglobin complex, myelin sheath, synapse, extracellular exosome, blood microparticle,		
GOTERM_MF_DIRECT	beta-amyloid binding, peroxidase activity, oxygen transporter activity, iron ion binding, protein binding, oxygen binding, heme binding, haptoglobin binding,		
INTERPRO	Globin, Haemoglobin, alpha, Haemoglobin, pi, Globin-like, Globin, structural domain,		
KEGG_PATHWAY	African trypanosomiasis, Malaria,		
UP_KEYWORDS	3D-structure, Acetylation, Complete proteome, Direct protein sequencing, Heme, Iron, Metal-binding, Oxygen transport, Phosphoprotein, Polymorphism, Reference proteome, Transport,		
UP_SEQ_FEATURE	chain:Hemoglobin subunit alpha-1/2, helix, metal ion-binding site:Iron (heme distal ligand), metal ion-binding site:Iron (heme proximal ligand), modified residue, sequence conflict, sequence variant, turn,		

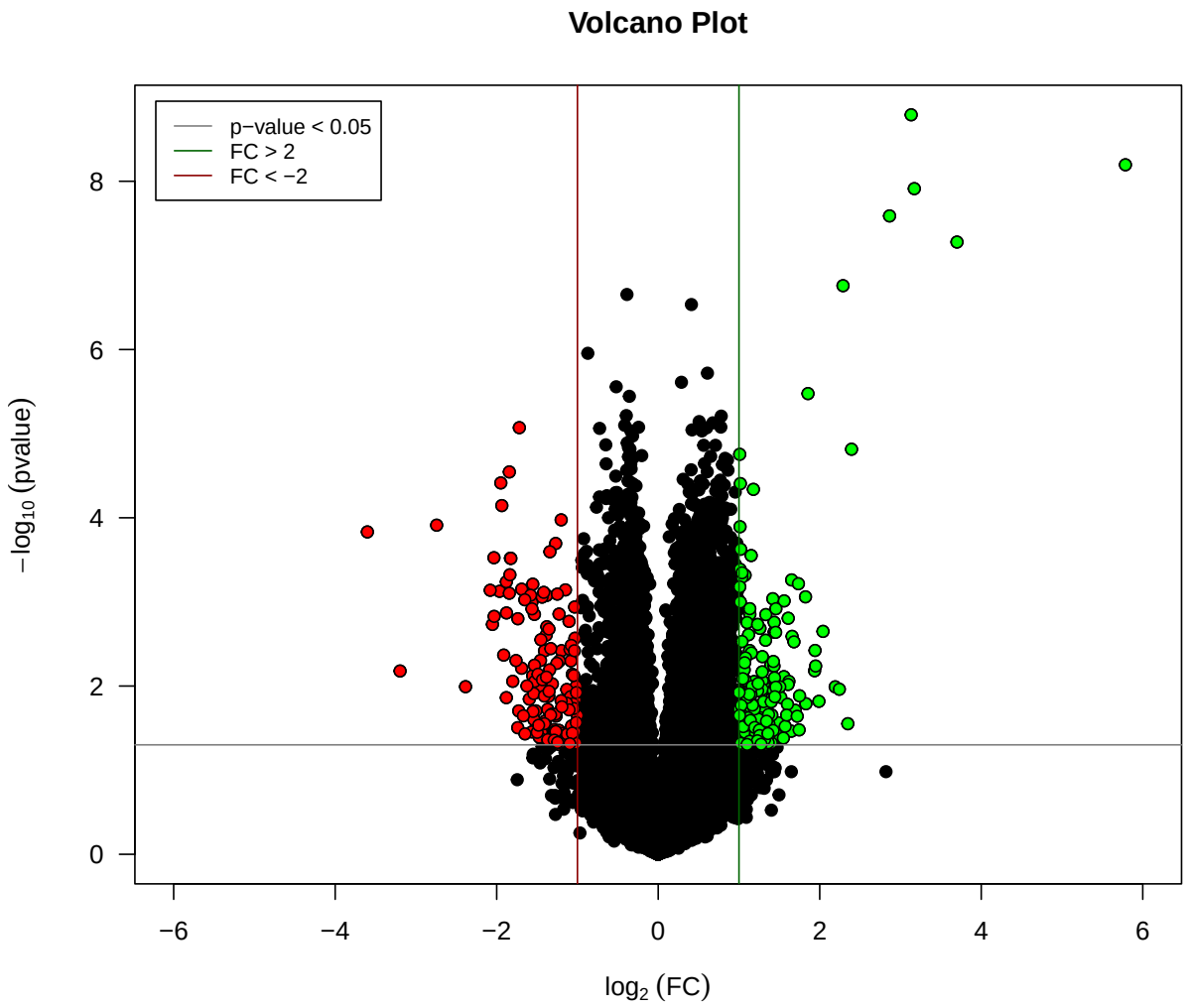
As seen in the results above all of the probe-sets are involved in:

- Cellular Component: **hemoglobin complex** (GO:0005833)
- Biological Process: **oxygen transport** (GO:0015671)
- Molecular Functions: **oxygen transporter activity** (GO:0005344), **iron ion binding** (GO:0005506), **oxygen binding** (GO:0019825), **heme binding** (GO:0020037)

Essentially, they are all associated with the biological functionality of red blood cells. To re-create, upload `probes.txt` to the DAVID tool, select "AFFYMETRIX_3PRIME_IVT_ID" as the identifier, and check "Gene List" as the list type. Then, click the "Functional Annotation Table" on the bottom of the page to retrieve the table pictured above (as of 08 July 2021).

8.) Transform the p-value ($-log_{10}(p.value)$) and create a volcano plot with the p-value and fold change vectors (see the lecture notes). Make sure to use a log_{10} transformation for the p-value and a log_2 (R function `log2()`) transformation for the fold change. Draw the horizontal lines at fold values of 2 and -2 ($log_2(p) = 1$) and the vertical p-value threshold line at p = 0.05 (remember that it is transformed in the plot).

```
# Transform p-value
p.trans <- -log10(pv)

# Volcano plot
plot(
    range(log2fc),
    range(p.trans),
    type = "n", las = 1,
    main = "Volcano Plot",
    xlab = expression(log[2] ~ (FC)),
    ylab = expression(-log[10] ~ (pvalue)),
    xlim = c(-6, 6)
)
points(log2fc,
       p.trans,
       pch = 21,
       col = "black",
       bg = "black")

# Up-regulated genes
points(log2fc[(p.trans > -log10(.05) &
                   log2fc > log2(2))],
       p.trans[(p.trans > -log10(.05) &
                    log2fc > log2(2))],
       pch = 21, col = "black", bg = "green")

# Down-regulated genes
points(log2fc[(p.trans > -log10(.05) &
                   log2fc < -log2(2))],
       p.trans[(p.trans > -log10(.05) &
                    log2fc < -log2(2))],
       pch = 21, col = "black", bg = "red")

# Plot markers
abline(h = -log10(0.05), col = "grey50")
abline(v = log2(2), col = "darkgreen")
abline(v = -log2(2), col = "darkred")

# Legend
legend(
    "topleft",
    legend = c("p-value < 0.05", "FC > 2", "FC < -2"),
    col = c("grey50", "darkgreen", "darkred"),
    lty = 1, cex = 0.8, inset = 0.02
)
```


Volcano Plot
p−value < 0.05
FC > 2
FC < −2
−log10 (pvalue)
log2 (FC)
8
6
4
2
0
−6
−4
−2
0
2
4
6

Session Info

```
sessionInfo()
```

```
## R version 4.1.0 (2021-05-18)
## Platform: x86_64-apple-darwin17.0 (64-bit)
## Running under: macOS Big Sur 11.4
##
## Matrix products: default
## LAPACK: /Library/Frameworks/R.framework/Versions/4.1/Resources/lib/libRlapack.dylib
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## loaded via a namespace (and not attached):
##  [1] compiler_4.1.0    magrittr_2.0.1    htmltools_0.5.1.1 tools_4.1.0       yaml_2.2.1
##  [6] stringi_1.6.2     rmarkdown_2.9     knitr_1.33        stringr_1.4.0     xfun_0.24
## [11] digest_0.6.27     rlang_0.4.11      evaluate_0.14
```