Project 07 - RNA-seq for DE

Author: Tianze (Vincent) Luo
Date: 2025-04-24

Background

- RNA-seq data on a single chromosome in multiple experiments
- Files: `/ix1/hugen2072-2025s/p7/`
- Interactive session command: `crc-interactive --teach -a hugen2072-2025s -t 4:00:00`

0. Setup JobArray Based on Experiments

```
######## 0. Setup ArrayJobs ########
exp=$(awk -v lineNumb=$(($SLURM_ARRAY_TASK_ID+1)) 'NR==lineNumb { print $0
}' ls_exp.txt)

## for output file names
exp_prefix=$(echo $exp | sed 's/_ERCC.*//')

## add read{1,2} as input
#       .read1.fastq.gz
#       .read2.fastq.gz
exp_read1="${exp}.read1.fastq.gz"
exp_read2="${exp}.read2.fastq.gz"


echo "Job for Experiment *** ${exp_prefix} *** started at: $(date)"

echo $exp_read1

echo $exp_read2
```

```
> ls_exp.txt
HBR_Rep1_ERCC-Mix2_Build37-ErccTranscripts-chr22
HBR_Rep2_ERCC-Mix2_Build37-ErccTranscripts-chr22
UHR_Rep1_ERCC-Mix1_Build37-ErccTranscripts-chr22
UHR_Rep2_ERCC-Mix1_Build37-ErccTranscripts-chr22
```

```
exp=$(awk -v lineNumb=$(($SLURM_ARRAY_TASK_ID+1)) 'NR==lineNumb { print $0
}' ls_exp.txt)
```

```
# $SLURM_ARRAY_TASK_ID = 0 --> line 1
# $SLURM_ARRAY_TASK_ID = 1 --> line 2
# $SLURM_ARRAY_TASK_ID = 2 --> ...
# $SLURM_ARRAY_TASK_ID = 3 --> ...

# Allows correspondance of jobarray ID to experiments

echo "Job for Experiment *** ${exp_prefix} *** started at: $(date)"
# Job for Experiment *** HBR_Rep1 *** started at: Thu Apr 24 23:01:35 EDT
2025

echo $exp_read1
# HBR_Rep1_ERCC-Mix2_Build37-ErccTranscripts-chr22.read1.fastq.gz

echo $exp_read2
# HBR_Rep1_ERCC-Mix2_Build37-ErccTranscripts-chr22.read2.fastq.gz
```

1. cutadapt: trim adapter sequence

```
mkdir -p ../output/1_cutadapt

# -a/-A: Universal Illumina adapters (3')
# -o/-p: OUTPUT (first/second)
cutadapt -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -A
AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT \
         -o ../output/1_cutadapt/${exp_prefix}.trimmed.R1.fastq.gz -p
../output/1_cutadapt/${exp_prefix}.trimmed.R2.fastq.gz \
         ../p7/${exp_read1} ../p7/${exp_read2}

         # Example
         # input: ../p7/HBR_Rep1_ERCC-Mix2_Build37-ErccTranscripts-
chr22.read{1,2}.fastq.gz

         # output: -o ../output/1_cutadapt/HBR_Rep1.trimmed.R1.fastq.gz
```

2. STAR: Aligning the reads

```
mkdir -p ../output/2_STAR

STAR --runThreadN 2 \
    --runMode alignReads \              # map reads
    --outSAMstrandField intronMotif \   # strand derived from the intron
motif. reads with inconsistent and/or non-canonical introns are filtered
out.
    --twopassMode Basic \
    --readFilesIn ../output/1_cutadapt/${exp_prefix}.trimmed.R1.fastq.gz \
                  ../output/1_cutadapt/${exp_prefix}.trimmed.R2.fastq.gz \
```

```
    --outFileNamePrefix ../output/2_STAR/${exp_prefix} \    #
../output/2_STAR/HBR_Rep1
    --quantMode GeneCounts \    # only count matrix; No output SAM/BAM
alignments
    --outStd Log \              # which output will be directed to stdout
(standard out) -- only log
    --outWigType bedGraph \
    --outWigReferencesPrefix ../output/2_STAR/${exp_prefix}_bedgraph \
    --genomeDir ../p7/STAR_reference/ \  # where genome indices where
generated (pre-generated)
    --readFilesCommand zcat \   # zcat - to uncompress .gz files
    --outSAMtype BAM SortedByCoordinate  # similar to samtools sort
command.
```

- Perform downstream analysis using `*ReadsPerGene.out.tab`

```
[til177@teach-cpu-n0 2_STAR]$ ll *ReadsPerGene.out.tab
# -rw-r--r-- 1 til177 mmarazita 1.5M Apr 24 23:08
HBR_Rep1ReadsPerGene.out.tab
# -rw-r--r-- 1 til177 mmarazita 1.5M Apr 24 23:08
HBR_Rep2ReadsPerGene.out.tab
# -rw-r--r-- 1 til177 mmarazita 1.5M Apr 24 23:08
UHR_Rep1ReadsPerGene.out.tab
# -rw-r--r-- 1 til177 mmarazita 1.5M Apr 24 23:08
UHR_Rep2ReadsPerGene.out.tab
```

3. DGE analysis in R

```
module load gcc/12.2.0 r/4.4.0
Rscript --vanilla p07_DE_tvl.R > ../output/DiffGeneExpr.log
```

Important Notes in the DGE analysis pipeline

1. When we read `*ReadsPerGene.out.tab` into R, each file has 3 samples

```
> head(hbr1)
#                   V1 V2 V3 V4
# 1 ENSG00000223972.5  0  0  0
# 2 ENSG00000227232.5  0  0  0
# 3 ENSG00000278267.1  0  0  0
# 4 ENSG00000243485.5  0  0  0
# 5 ENSG00000284332.1  0  0  0
# 6 ENSG00000237613.2  0  0  0
> head(hbr2)
#                   V1 V2 V3 V4
```

```
# 1 ENSG00000223972.5  0  0  0
# 2 ENSG00000227232.5  0  0  0
# 3 ENSG00000278267.1  0  0  0
# 4 ENSG00000243485.5  0  0  0
# 5 ENSG00000284332.1  0  0  0
# 6 ENSG00000237613.2  0  0  0
# ...
```

- The first column has the gene/transcript name
- Columns 2-4 of each data frame contain the expression levels
- **There are 6 HBR samples (stored in hbr1 and hbr2) & 6 UHR samples (stored in uhr1 and uhr2)**
- Each has the same number of rows (60,710 transcripts)

2. Combine the count data into one data.frame (same transcript for each row across experiments) -> matrix

```
## hbr first, uhr next
expr <- cbind(hbr1[,c(2:4)],
              hbr2[,c(2:4)],
              uhr1[,c(2:4)],
              uhr2[,c(2:4)])
expr <- as.matrix(expr)
class(expr)
# [1] "matrix" "array"
rownames(expr) <- hbr1$V1
colnames(expr) <- NULL

dim(expr)
# [1] 60710    12
> head(expr)
#                   [,1] [,2] [,3] [,4] [,5] [,6] [,7] [,8] [,9] [,10]
[,11]
# ENSG00000223972.5    0    0    0    0    0    0    0    0    0     0
0
# ENSG00000227232.5    0    0    0    0    0    0    0    0    0     0
0
# ENSG00000278267.1    0    0    0    0    0    0    0    0    0     0
0
# ENSG00000243485.5    0    0    0    0    0    0    0    0    0     0
0
# ENSG00000284332.1    0    0    0    0    0    0    0    0    0     0
0
# ENSG00000237613.2    0    0    0    0    0    0    0    0    0     0
0
#                   [,12]
# ENSG00000223972.5     0
# ENSG00000227232.5     0
# ENSG00000278267.1     0
# ENSG00000243485.5     0
```

```
# ENSG00000284332.1      0
# ENSG00000237613.2      0
```

3. Record the group info (required when doing DE analysis), create `DGEList` object (for normalizations & input into DE analysis)

```
# The first 6 were from HBR, the second 6 from UHR
data_groups <- c(rep("hbr",6), rep("uhr",6))
data_groups
#  [1] "hbr" "hbr" "hbr" "hbr" "hbr" "hbr" "uhr" "uhr" "uhr" "uhr"
"uhr" "uhr"

d <- DGEList(counts=expr, group=factor(data_groups))
d
# An object of class "DGEList"
# $counts
#                   Sample1 Sample2 Sample3 Sample4 Sample5 Sample6
Sample7
# ENSG00000223972.5       0       0       0       0       0       0
0
# ENSG00000227232.5       0       0       0       0       0       0
0
# ENSG00000278267.1       0       0       0       0       0       0
0
# ENSG00000243485.5       0       0       0       0       0       0
0
# ENSG00000284332.1       0       0       0       0       0       0
0
#                   Sample8 Sample9 Sample10 Sample11 Sample12
# ENSG00000223972.5       0       0        0        0        0
# ENSG00000227232.5       0       0        0        0        0
# ENSG00000278267.1       0       0        0        0        0
# ENSG00000243485.5       0       0        0        0        0
# ENSG00000284332.1       0       0        0        0        0
# 60705 more rows ...

# $samples
#         group lib.size norm.factors
# Sample1   hbr    37357            1
# Sample2   hbr     3275            1
# Sample3   hbr    38242            1
# Sample4   hbr    45971            1
# Sample5   hbr     4047            1
# 7 more rows ...
```

4. Filter the data (filter OUT low-expressed transcripts across samples)

```
# cpm transformation -> T/F matrix -> rowSums (per transcript across
samples)
```

```
keep <- rowSums(cpm(d)>100) >= 2     # This picks out row numbers of
genes that are expressed (cpm > 100) in at least 2 samples
d <- d[keep,]                        # **Keep only the genes expressed
(cpm > 100) in at least 2 samples**
dim(d) # 654 genes are left
# [1] 654  12

d$samples$lib.size <- colSums(d$counts) # Update the "library size"
(the total number of transcripts) for each sample
d$samples
#          group lib.size norm.factors
# Sample1    hbr    37234            1
# Sample2    hbr     3262            1
# Sample3    hbr    38123            1
# Sample4    hbr    45780            1
# Sample5    hbr     4021            1
# Sample6    hbr    46862            1
# Sample7    uhr    63518            1
# Sample8    uhr     4339            1
# Sample9    uhr    64908            1
# Sample10   uhr    41848            1
# Sample11   uhr     3472            1
# Sample12   uhr    42082            1
```

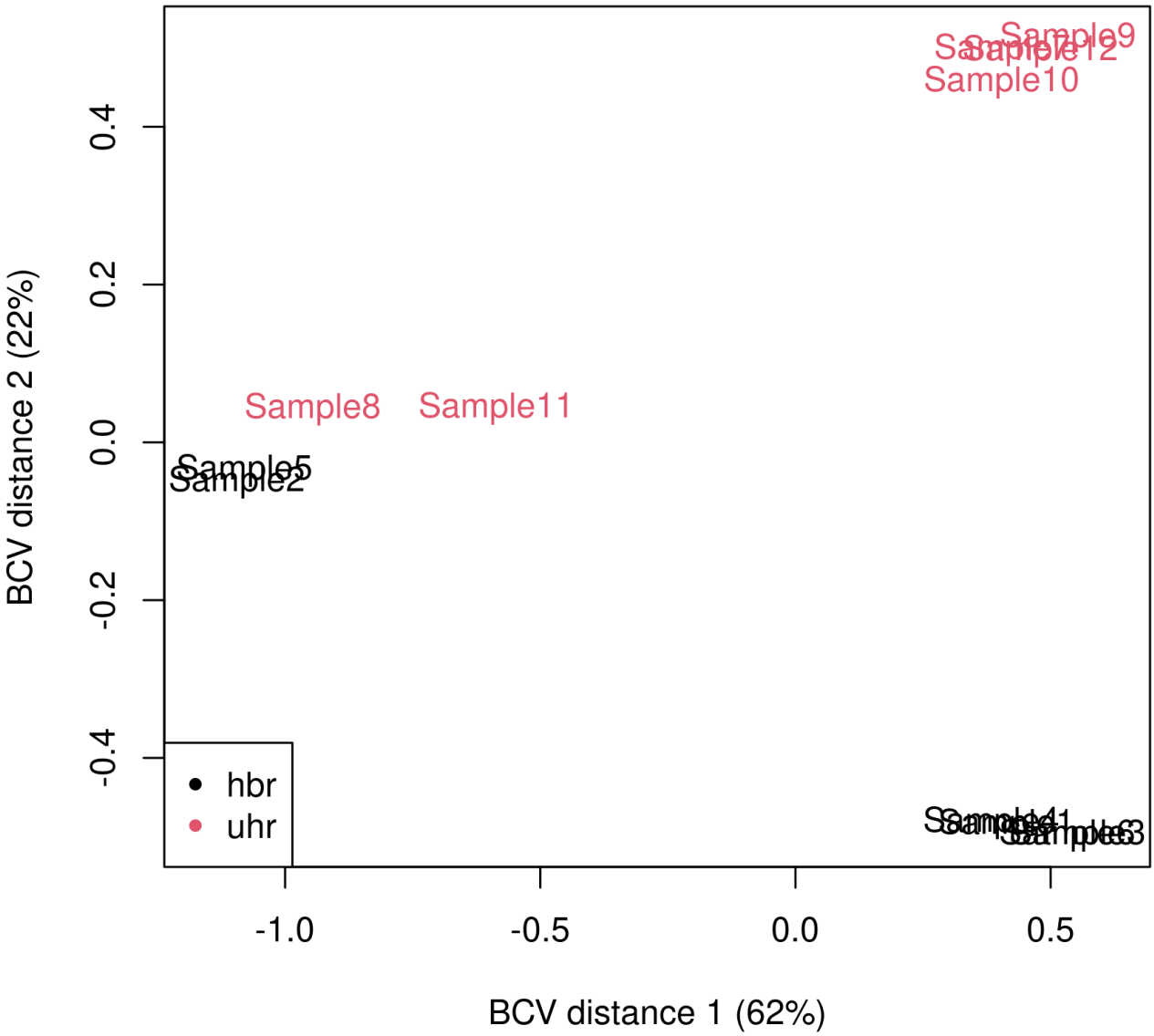
5. Normalize the reads and plot them

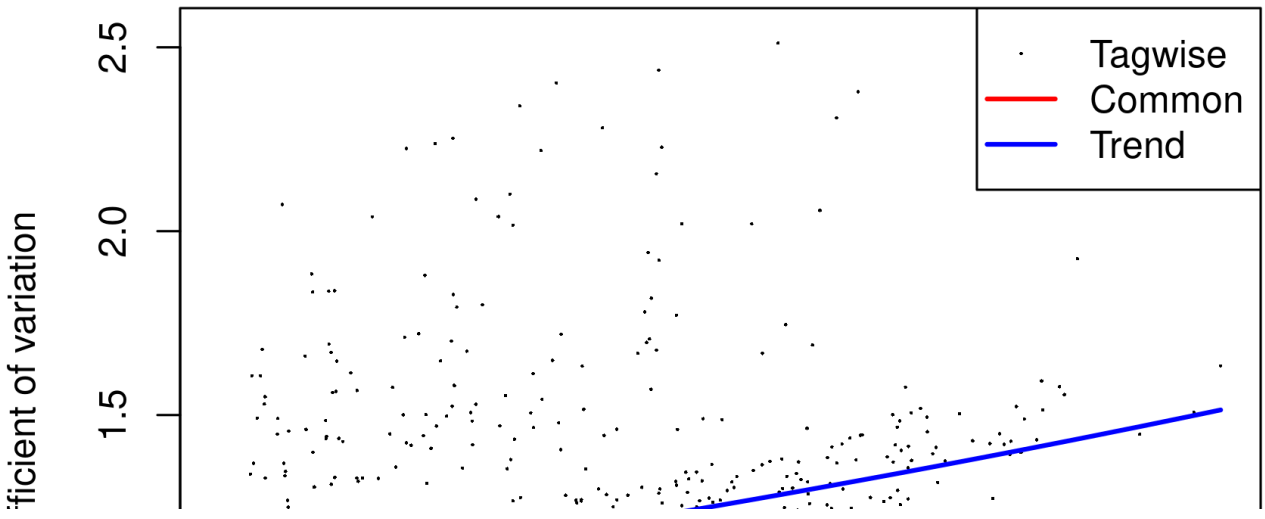
```
d <- calcNormFactors(d) # This stores the scaling factors for
normalization in d$samples$norm.factors
d
# An object of class "DGEList"
# $counts
#                    Sample1 Sample2 Sample3 Sample4 Sample5 Sample6
Sample7
# ENSG00000198062.15       0       0       0       0       0       0
3
# ENSG00000206195.11       3       0       3       1       0       1
107
# ENSG00000271127.2        0       0       0       0       0       0
11
# ENSG00000232775.6        0       0       0       1       1       0
2
# ENSG00000272872.1        1       0       1       1       0       1
22
#                    Sample8 Sample9 Sample10 Sample11 Sample12
# ENSG00000198062.15       0       3        5        1        4
# ENSG00000206195.11       0     107       72        1       71
# ENSG00000271127.2        0      11        1        0        1
# ENSG00000232775.6        2       0        5        5        0
# ENSG00000272872.1        0      22       12        0       12
# 649 more rows ...

# $samples
```

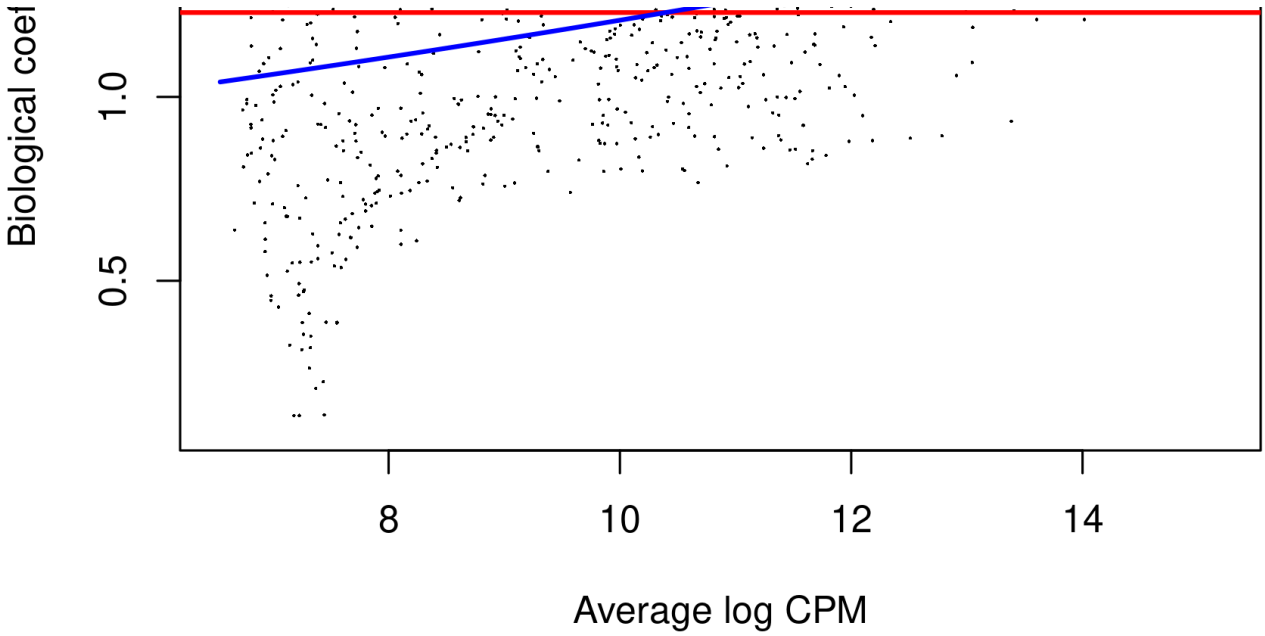
```
#         group lib.size norm.factors
# Sample1   hbr    37234    0.7271597
# Sample2   hbr     3262    2.4586720
# Sample3   hbr    38123    0.7191907
# Sample4   hbr    45780    0.7275449
# Sample5   hbr     4021    2.2131558
# 7 more rows ...

plotMDS(d, method="bcv", col=as.numeric(d$samples$group))
legend("bottomleft", as.character(unique(d$samples$group)), col=1:2,
pch=20)
```

6. Fit a trend in CV vs. mean (single dispersion & GLM)

```
d1 <- estimateCommonDisp(d, verbose=T)
# Disp = 1.44585 , BCV = 1.2024
d1 <- estimateTagwiseDisp(d1)
plotBCV(d1)

# Estimate and plot again, using a generalize linear model to get
better fit
design.mat <- model.matrix(~ 0 + d$samples$group)
colnames(design.mat) <- levels(d$samples$group)
d2 <- estimateGLMCommonDisp(d,design.mat)
d2 <- estimateGLMTrendedDisp(d2,design.mat, method="power")
d2 <- estimateGLMTagwiseDisp(d2,design.mat)
plotBCV(d2)
```


Sample9
Sample12
Sample10
Sample8
Sample11
Sample5
Sample2
hbr
uhr
BCV distance 2 (22%)
BCV distance 1 (62%)
0.4
0.2
0.0
-0.2
-0.4
-1.0
-0.5
0.0
0.5


Tagwise
Common
Trend
2.5
2.0
1.5

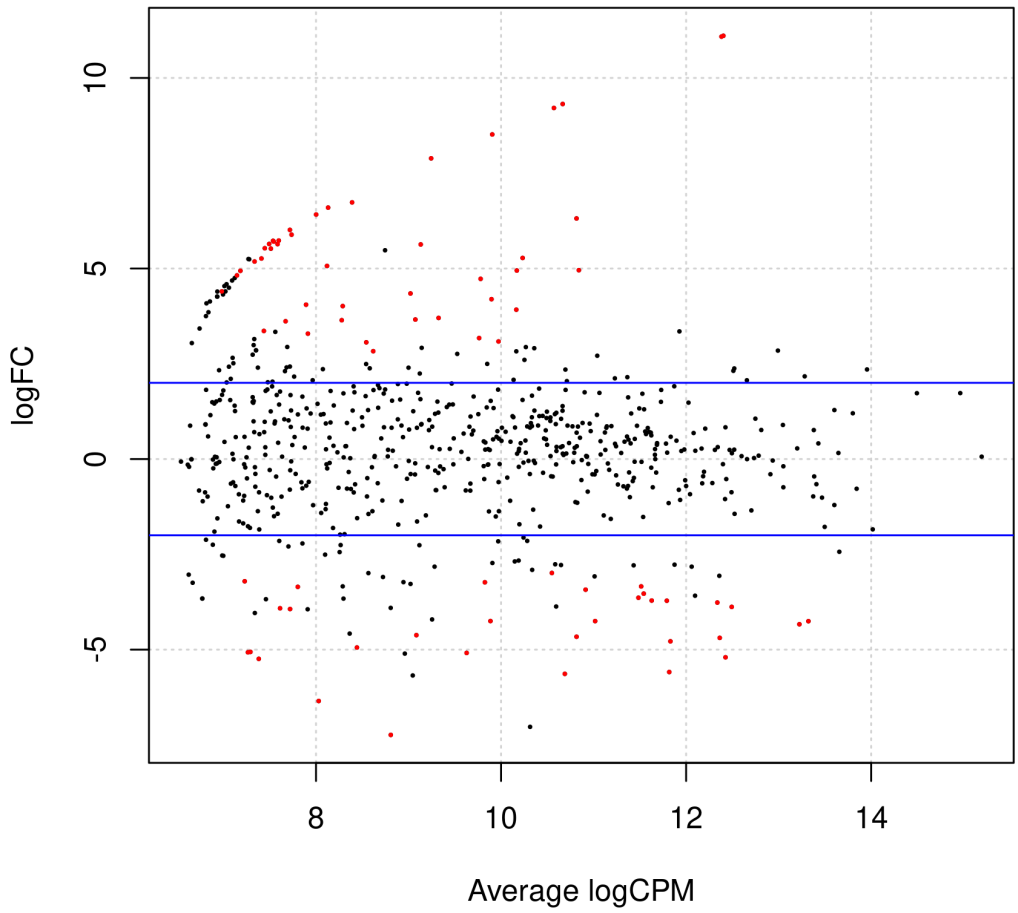


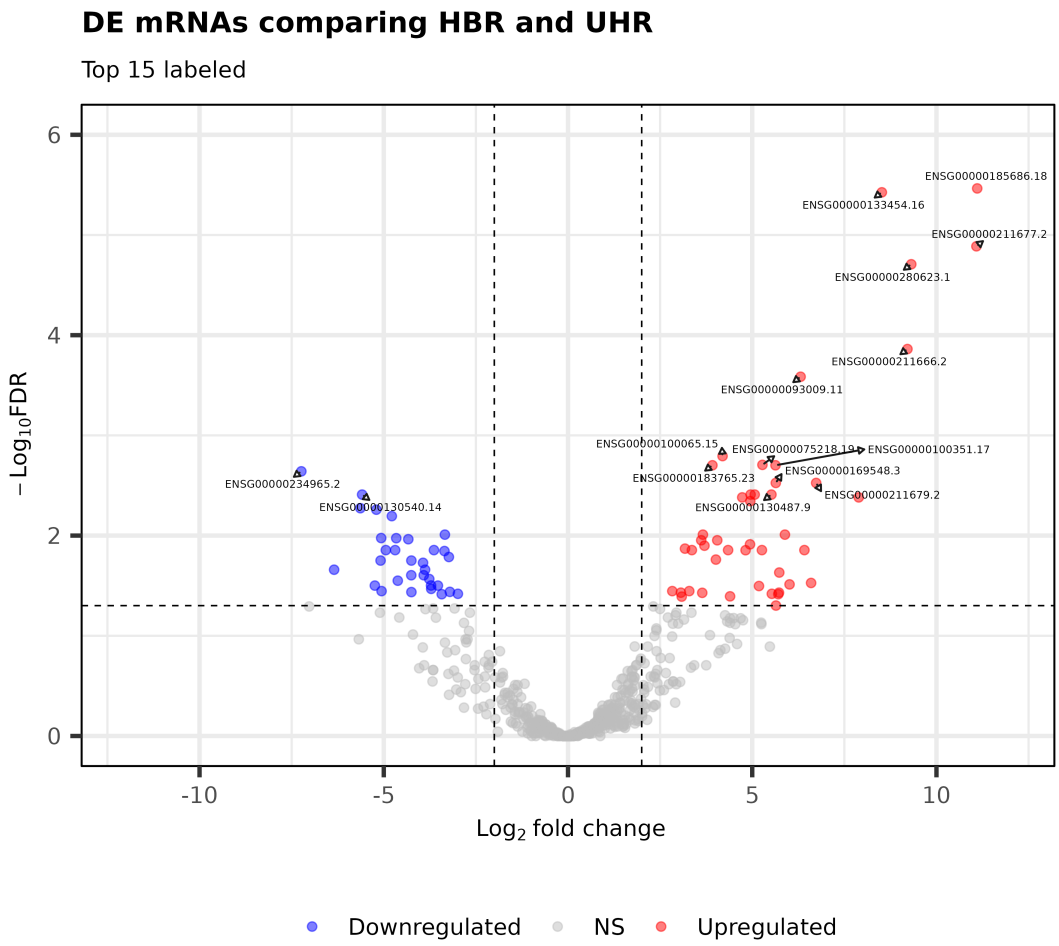
7. DE analysis **between the UHR - HBR**

- **Genewise Exact Test for NB distributed counts between UHR - HBR**
  - > "so if the pair is c("A","B") then the comparison is B - A, so genes with positive log-fold change are up-regulated in group B compared with group A (and vice versa for genes with negative log-fold change)."
  - `pair=c(1,2)`: 1 = HBR, 2 = UHR
  - UHR - HBR
- We are finding DE genes for UHR/cancer
  - 77 DEGs (32 down + 45 up)

```
# compare groups 1 and 2 (HBR, UHR)
et12 <- exactTest(d1, pair=c(1,2))

# Look at the top 10 DE genes
topTags(et12, n=10)
# Comparison of groups:  uhr-hbr
#                         logFC    logCPM       PValue          FDR
# ENSG00000185686.18 11.105786 12.402565 5.243387e-09 3.429175e-06
# ENSG00000133454.16  8.517490  9.904674 1.146932e-08 3.750466e-06
# ENSG00000211677.2  11.084370 12.382343 5.950079e-08 1.297117e-05
# ENSG00000280623.1   9.316181 10.666070 1.198342e-07 1.959289e-05
# ENSG00000211666.2   9.212263 10.571262 1.051225e-06 1.375002e-04
# ...

# How many are up- or down-regulated?
de1 <- decideTestsDGE(et12, adjust.method="BH", p.value=0.05)
summary(de1)
#        uhr-hbr
# Down        32
```

```
# NotSig     577
# Up          45


#### Plot log(fold change) vs. log(cpm) ####
#   Significantly differently-expressed genes are shown in red
de1tags12 <- rownames(d1)[as.logical(de1)]
plotSmear(et12, de.tags=de1tags12)
abline(h = c(-2, 2), col = "blue")


#### Report the list of the differentially expressed genes (DEGs) ####
# Get the summary table for: ***ALL DE genes with FDR < 0.05***
DEG_fdr_tab = topTags(et12, n = Inf, adjust.method = "BH", p.value =
0.05) #adjusted p-value cutoff

# 77 DEGs
nrow(DEG_fdr_tab)
# [1] 77
# 32 down + 45 up
summary(de1)
#        uhr-hbr
# Down        32
# NotSig     577
# Up          45

#### Output ####
library(tidyverse)
df_DEG_fdr_tab = rownames_to_column(as.data.frame(DEG_fdr_tab), var =
"ENSG")

# 1. summary table
write_csv(df_DEG_fdr_tab,
file="../output/3_DiffGeneExpr_result/table_DEGs_fdr.csv")
# 2. list of DEGs
write.table( rownames(DEG_fdr_tab),
file="../output/3_DiffGeneExpr_result/ls_DEGs_fdr.txt",
            quote = FALSE, sep=" ", row.names = FALSE, col.names =
FALSE)
```


logFC
10
5
0
-5
8
10
12
14
Average logCPM



UHR - HBR

- see `output/DiffGeneExpr.log` and `code/Rplots.pdf` for details.

4. Interpretation of DGE analysis

Based on the description of the experiments, describe the biological function you think the differentially expressed genes should be associated with.

- *Universal Human Reference (UHR)* is total RNA isolated from a diverse set of 10 *cancer cell lines*.
- *Human Brain Reference (HBR)* is total RNA isolated from the *brains* of 23 Caucasians, male and female, of varying age but *mostly 60-80 years old*.

My hypothesis (UHR - HBR)

The mRNAs DE in UHR compared to HBR should be enriched in biological pathways involving:

- Positive regulation in metabolism and cell proliferation (or cell cycle);
- Negatively associated with degeneration, cell death, apoptosis, neuronal functions.

Overrepresentation Test in DAVID (future direction, post conversion to gene symbol)