GTEx Hippocampus DEG

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Set working directory.

Load packages

```
##
## Attaching package: 'dplyr'

## The following objects are masked from 'package:stats':
##
##     filter, lag

## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union

## Loading required package: limma

##
## Attaching package: 'gplots'

## The following object is masked from 'package:stats':
##
##     lowess

##
## Attaching package: 'gridExtra'

## The following object is masked from 'package:dplyr':
##
##     combine

## Loading required package: foreach

## Loading required package: scales

## Loading required package: Biobase

## Loading required package: BiocGenerics

## Warning: package 'BiocGenerics' was built under R version 4.0.5

## Loading required package: parallel

##
## Attaching package: 'BiocGenerics'

## The following objects are masked from 'package:parallel':
##
##     clusterApply, clusterApplyLB, clusterCall, clusterEvalQ,
##     clusterExport, clusterMap, parApply, parCapply, parLapply,
##     parLapplyLB, parRapply, parSapply, parSapplyLB
```

```
## The following object is masked from 'package:gridExtra':
##
##     combine

## The following object is masked from 'package:limma':
##
##     plotMA

## The following objects are masked from 'package:dplyr':
##
##     combine, intersect, setdiff, union

## The following objects are masked from 'package:stats':
##
##     IQR, mad, sd, var, xtabs

## The following objects are masked from 'package:base':
##
##     anyDuplicated, 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

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

##
## Attaching package: 'variancePartition'

## The following object is masked from 'package:limma':
##
##     classifyTestsF
```

Read in files.

```
file1 <- read.delim("Brain_Hippocampus", header = TRUE, sep = "\t")
file2 <- read.delim("GTEx_Analysis_v8_Annotations_SubjectPhenotypesDS.txt",
                    header = TRUE, sep = "\t")
gtf.file <- "gencode.v26.GRCh38.genes.gtf"
gtf.gr <- rtracklayer::import(gtf.file)
gtf.df <- as.data.frame(gtf.gr)
gtf.df.condensed <-
  unique(gtf.df[,c("type","gene_type","gene_name","gene_id","seqnames")])
```

Reformat and rename files.

```
gtf.genes <- gtf.df.condensed[gtf.df.condensed$type == "gene",]

metadata <- file2
colnames(metadata) <- c("subject_id","sex","age","hardy_scale")

file1$X <- NULL
counts <- file1
colnames(counts)[1:2] <- c("gene_id","gene_name")
```

Merge to view info in one file.

```
counts.info <- left_join(counts, gtf.genes, by = "gene_id")
counts.info <- counts.info[,c(1,202:205,3:199)]
colnames(counts.info)[4] <- "gene_name"
```

Look to see if any NA values or replicated gene names.

```
table(is.na(counts.info$gene_name))
```

```
##
## FALSE
## 56200
```

```
table(duplicated(counts.info$gene_name))
```

```
##
## FALSE  TRUE
## 54592  1608
```

There are no NA values. However, 1,608 gene names are repetitive.

View the repetitive gene names.

```
table(counts.info$gene_name[duplicated(counts.info$gene_name)])
```

```
##
##       5_8S_rRNA         5S_rRNA             7SK           ACA59           ACA64
##               4              15               5               1               5
##        ACTR3BP2         AKAP17A         ALG1L9P         AMD1P2            ASMT
##               1               1               1               1               1
##           ASMTL       ASMTL-AS1         C2orf61            CD99         CD99P1
##               1               1               1               1               1
## Clostridiales-1           CRLF2         CSF2RA         CTSLP2        CYB561D2
##               2               1               1               1               1
##        DDX11L16           DHRSX       DHRSX-IT1      DNAJC9-AS1         DPH3P2
##               1               1               1               1               1
##           ELFN2         ELOCP24        FABP5P13        FAS-AS1         GOLGA8M
##               1               1               1               1               1
##          GTPBP6           IL3RA            IL9R       KRT18P53       LINC00102
##               1               1               1               1               1
##       LINC00106       LINC00484       LINC00685       LINC00901       LINC01115
##               1               1               1               1               1
##       LINC01238       LINC01297       LINC01347       LINC01422       LINC01481
##               1               1               1               1               1
##       LINC01598 LL0YNC03-29C1.1            LYNX1            MAL2     Metazoa_SRP
##               1               1               1               1             166
##       MIR3179-3       MIR3180-4         MIR3690         MIR6089         NBPF13P
##               1               1               1               1               1
##         OR7E47P           P2RY8          PLCXD1          PMS2P6         PPP2R3B
##               1               1               1               1               1
##            pRNA       PROX1-AS1      RAET1E-AS1            RGS5         RNA5-8S5
##               8               1               1               1               3
##       RNA5SP498   RP11-309M23.1   RP13-297E16.4   RP13-297E16.5   RP13-465B17.4
##               1               1               1               1               1
##   RP13-465B17.5         RPL14P5         RPS23P5        SCARNA11        SCARNA15
##               1               1               1               3               1
##        SCARNA16        SCARNA17        SCARNA18        SCARNA20        SCARNA21
```

```
##               4               3               1               6               3
##        SCARNA24         SCARNA6            SHOX         SLC25A6     snoMBII-202
##               1               1               1               1               1
## snoMe28S-Am2634          SNORA1         SNORA11         SNORA12         SNORA15
##               2               4               6               2               3
##         SNORA17         SNORA18         SNORA19          SNORA2         SNORA20
##               1               7               3               2               4
##         SNORA21         SNORA22         SNORA24         SNORA25         SNORA26
##               1               3               1              18              10
##         SNORA27          SNORA3         SNORA30         SNORA31         SNORA32
##               4               4               2              25               2
##         SNORA33         SNORA35         SNORA36         SNORA38          SNORA4
##               3               2               1               1               4
##         SNORA40         SNORA41         SNORA42         SNORA43         SNORA44
##              20               1               3               4               1
##         SNORA46         SNORA48         SNORA50         SNORA51         SNORA57
##               1              12               1              12               2
##         SNORA58         SNORA62         SNORA63         SNORA64         SNORA67
##               2               6              10               5               6
##         SNORA68         SNORA69          SNORA7         SNORA70         SNORA71
##               5               1               4              29               2
##         SNORA72         SNORA73         SNORA74         SNORA75         SNORA76
##               6               3               6               7               1
##         SNORA77         SNORA79          SNORA8         SNORA81          SNORA9
##               2               1               4               3               3
##         SNORD11        SNORD111        SNORD112        SNORD113        SNORD115
##               1               1               2               4               1
##        SNORD116         SNORD18         SNORD19        SNORD19B          SNORD2
##               2               1               1               1               1
##         SNORD22         SNORD23         SNORD27         SNORD28         SNORD30
##               1               1               1               1               1
##         SNORD33         SNORD37         SNORD38         SNORD39         SNORD41
##               1               2               3               3               1
##         SNORD42         SNORD45         SNORD46          SNORD5         SNORD51
##               1               3               2               2               1
##         SNORD56         SNORD59         SNORD60         SNORD63         SNORD65
##               6               1               2               3               3
##         SNORD66         SNORD67         SNORD70         SNORD74         SNORD75
##               1               1               1               6               1
##         SNORD77         SNORD78         SNORD81         snoU109          snoU13
##               4               1               2               5              31
##        snoU2_19           snoZ6         SPATA13           SPRY3          TRPC6P
##               2               3               1               1               1
##              U1              U2              U3              U4              U6
##              12              18              49               6              32
##              U7              U8          uc_338           VAMP7           Vault
##               5              21              31               1               1
##          WASH6P          WASIR1           Y_RNA           ZBED1
##               1               1             737               1
```

View the gene_type for the repetitive genes. This will help determine if we should remove repetitive gene names.

```
table(counts.info$gene_type[duplicated(counts.info$gene_name)])
```

```
##
##                          antisense                             lincRNA
##                                 12                                  15
##                             miRNA                            misc_RNA
##                                  3                                 949
##            processed_pseudogene              processed_transcript
##                                  9                                   1
##                  protein_coding                               rRNA
##                                 21                                  23
##                          scaRNA                       sense_intronic
##                                 22                                   2
##                          snoRNA                              snRNA
##                                468                                  73
##                           sRNA transcribed_unprocessed_pseudogene
##                                  2                                   3
##          unprocessed_pseudogene
##                                  5
```

----------CREATE DGE OBJECT----------

Remove duplicated names

```
keep <- !duplicated(counts.info$gene_name) # true when no duplicate
counts.info.unique <- counts.info[keep,]
dim(counts.info)
```

```
## [1] 56200   202
```

```
dim(counts.info.unique)
```

```
## [1] 54592   202
```

Went from 56,200 genes to 54,592.
RNAs were mainly lost.

Remove MT genes.

```
keep <- counts.info.unique$seqnames != "chrM"
counts.info.uniqe.noMT <- counts.info.unique[keep,]
```

Make gene_name the row name.

```
rownames(counts.info.uniqe.noMT) <- counts.info.uniqe.noMT$gene_name
```

Change sex column from numbers to letters.

```
# male = 1, female = 2
sex_nums <- metadata$sex
sex_letters <- vector()
for (i in 1:length(sex_nums)) {
  if (sex_nums[i] == 1) {
    sex_letters <- c(sex_letters, "M")
  }
  else if (sex_nums[i] == 2) {
    sex_letters <- c(sex_letters, "F")
  }
```

```
}
head(cbind(sex_nums, sex_letters))
```

```
##      sex_nums sex_letters
## [1,] "2"      "F"
## [2,] "1"      "M"
## [3,] "1"      "M"
## [4,] "1"      "M"
## [5,] "1"      "M"
## [6,] "2"      "F"
```

```
metadata$sex <- sex_letters
metadata$subject_id <- make.names(metadata$subject_id)
```

Subset counts data.

```
counts <- counts.info.uniqe.noMT[,6:202]
```

Make counts columns same order as metadata rows.

```
metadata_all_samples <- metadata$subject_id
counts_hip_samples <- colnames(counts)

new_names_counts <- vector()
new_names_metadata <- vector()

for (i in 1:length(counts_hip_samples)) {
  for (j in 1:length(metadata_all_samples)) {
    checkpoint <- str_detect(counts_hip_samples[i], metadata_all_samples[j])
    if (checkpoint) {
      new_names_counts <- c(new_names_counts, counts_hip_samples[i])
      new_names_metadata <- c(new_names_metadata, metadata_all_samples[j])
    }
  }
}

head(cbind(new_names_counts, new_names_metadata)) #check
```

```
##      new_names_counts               new_names_metadata
## [1,] "GTEX.11DXW.0011.R1a.SM.DNZZD" "GTEX.11DXW"
## [2,] "GTEX.11EI6.0011.R1a.SM.DO93L" "GTEX.11EI6"
## [3,] "GTEX.11GS4.0011.R1a.SM.DO129" "GTEX.11GS4"
## [4,] "GTEX.11GSO.0011.R1b.SM.57WD3" "GTEX.11GSO"
## [5,] "GTEX.11GSP.0011.R1a.SM.9QEJ3" "GTEX.11GSP"
## [6,] "GTEX.11ONC.0011.R1a.SM.57WD4" "GTEX.11ONC"
```

Re-order dataframes.

```
counts <- counts[,new_names_counts]
tempdf <- as.data.frame(new_names_metadata)
colnames(tempdf) <- "subject_id"
metadata <- left_join(x = tempdf, y = metadata, by = "subject_id")
```

```
all.equal(colnames(counts), new_names_counts)
```

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

```
metadata$counts_id <- colnames(counts)
```

Create dge object

```
dge <- DGEList(counts,
               samples = metadata,
               genes = counts.info.uniqe.noMT[,1:5])
```

Reformat type of sample columns.

```
dge$samples[,c(5:7)] <-
  lapply(dge$samples[,c(5:7)], factor)
```

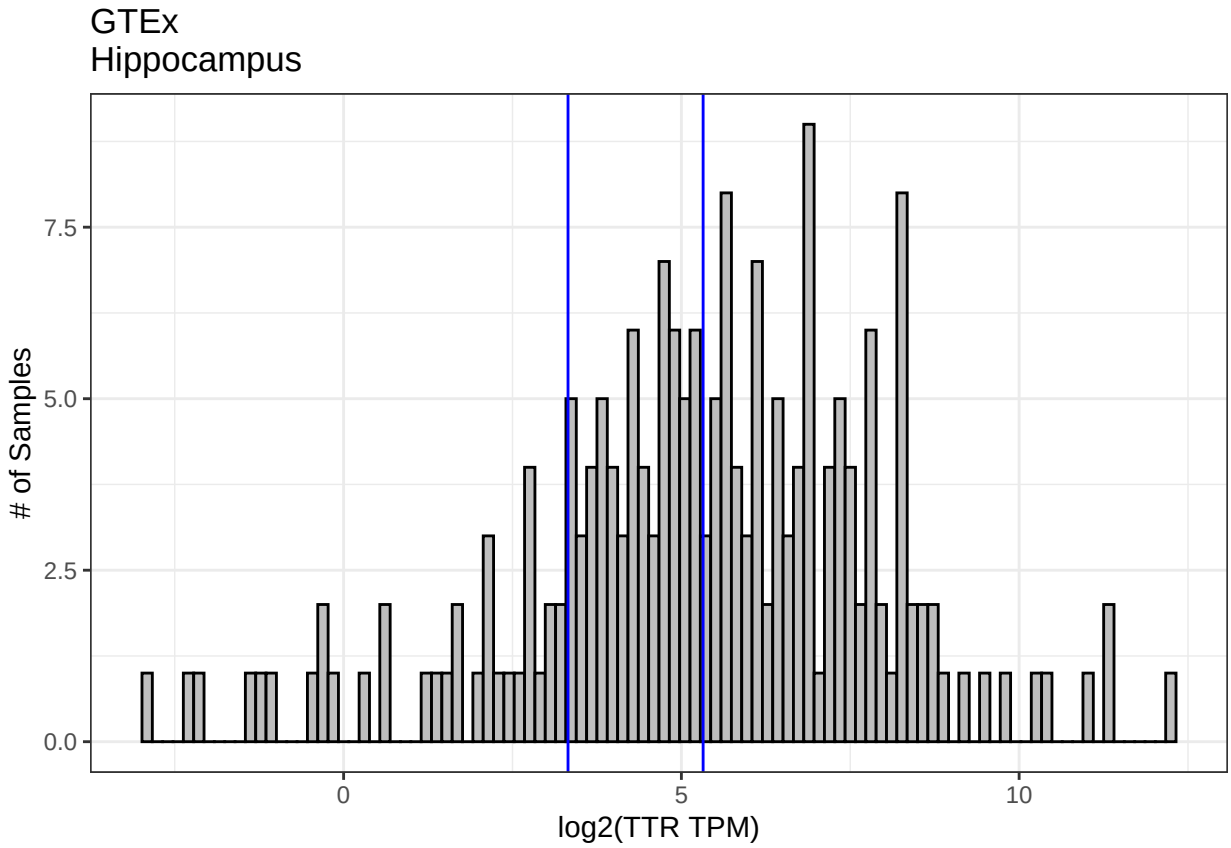
————————————TTR TPM HISTOGRAM————————————-

Data is already in TPM (transcripts per million). Create histogram of TPM TTR values.

```
ttr.counts <- as.data.frame(dge$counts["TTR",])
colnames(ttr.counts) <- "ttr.counts"

log2.ttr.counts <- log2(ttr.counts + 0.01)
colnames(log2.ttr.counts) <- "log2.ttr.counts"
```

```
h1 <- ggplot(ttr.counts, aes(x = ttr.counts)) +
  geom_histogram(bins = 100, fill = "gray", color = "black") +
  labs(title = "A.", x=NULL, y=NULL) +
  xlab("TTR TPM") + ylab("# of Samples") +
  geom_vline(xintercept = 20, col = "red") +
  theme_bw()

h2 <- ggplot(ttr.counts, aes(x = ttr.counts)) +
  geom_histogram(bins = 100, fill = "gray", color = "black") +
  labs(title = "B.", x=NULL, y=NULL) +
  xlab("TTR TPM") + ylab("# of Samples") +
  xlim(0, 500) + geom_vline(xintercept = 20, col = "red") +
  theme_bw()

h3 <- ggplot(log2.ttr.counts, aes(x = log2.ttr.counts)) +
  geom_histogram(bins = 100, fill = "gray", color = "black") +
  labs(title = "GTEx\nHippocampus", x=NULL, y=NULL) +
  xlab("log2(TTR TPM)") + ylab("# of Samples") +
  theme_bw() +
  geom_vline(xintercept = log2(20) + 1, col = "blue") +
  geom_vline(xintercept = log2(20) - 1, col = "blue")
```

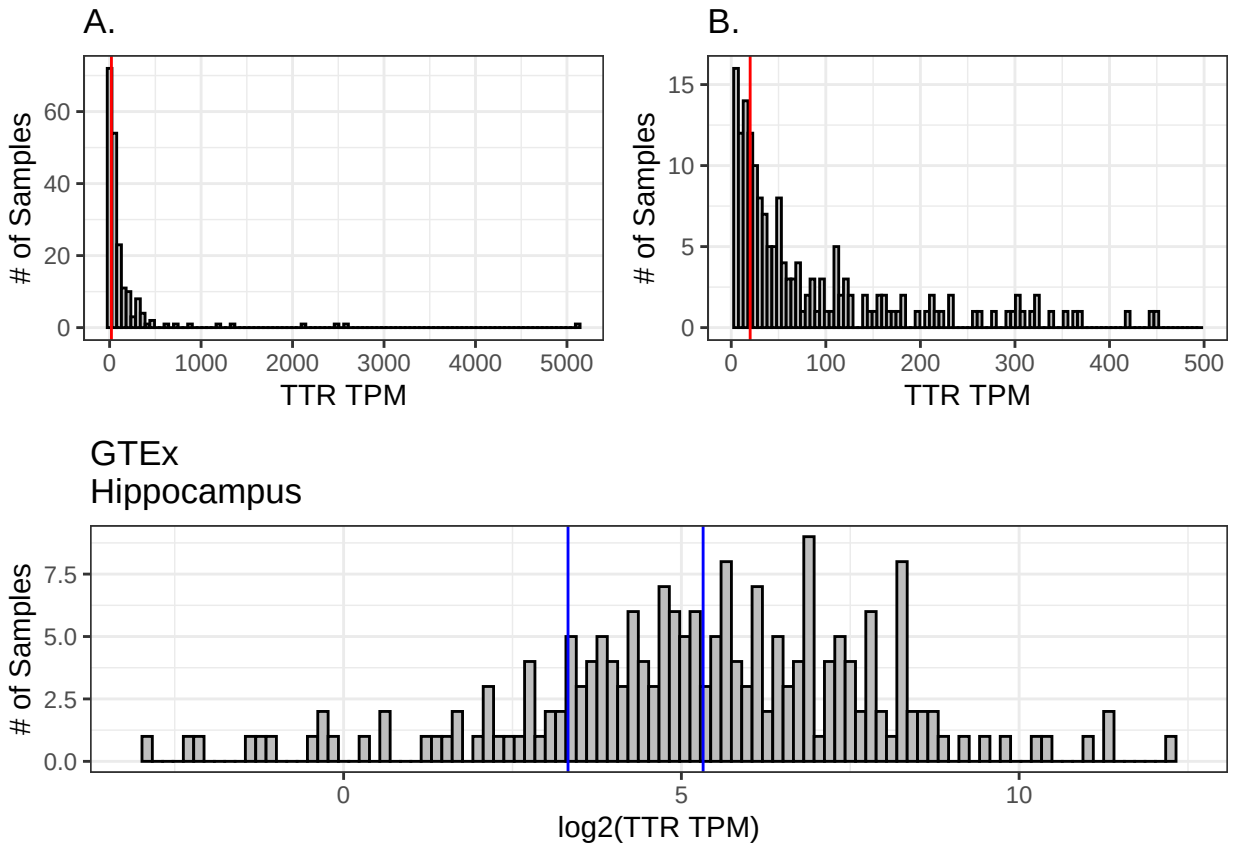
```
h3
```



Arrange graphs in grid.

```
plots1 <- list(h1,h2,h3)

layout1 <- rbind(c(1,2),c(3))

grid1 <- grid.arrange(grobs = plots1, layout_matrix = layout1)
```

```
## Warning: Removed 9 rows containing non-finite values (stat_bin).
## Warning: Removed 2 rows containing missing values (geom_bar).
```



ASSIGN DIRTY/CLEAN/NEITHER

Lets looks at groups +/- 1 of the cutoff

```
cutoff <- log2(20)
clean_threshold <- cutoff - 1 # values <= this are clean
clean_threshold
```

```
## [1] 3.321928
```

```
cutoff
```

```
## [1] 4.321928
```

```
dirty_threshold <- cutoff + 1 # values >= this are dirty
dirty_threshold
```

```
## [1] 5.321928
```

Assign clean or dirty

```
clean_or_dirty <- vector()
vector.log2.ttr.counts <- as.vector(log2.ttr.counts$log2.ttr.counts)

for (i in 1:length(vector.log2.ttr.counts)){
  if (vector.log2.ttr.counts[i] <= clean_threshold){
    clean_or_dirty <- c(clean_or_dirty, "clean")
  }
  else if (vector.log2.ttr.counts[i] >= dirty_threshold){
    clean_or_dirty <- c(clean_or_dirty, "dirty")
  }
```

```
  else {
    clean_or_dirty <- c(clean_or_dirty, "neither")
  }
}
table(clean_or_dirty)
```

```
## clean_or_dirty
##   clean   dirty neither
##      34     100      63
```

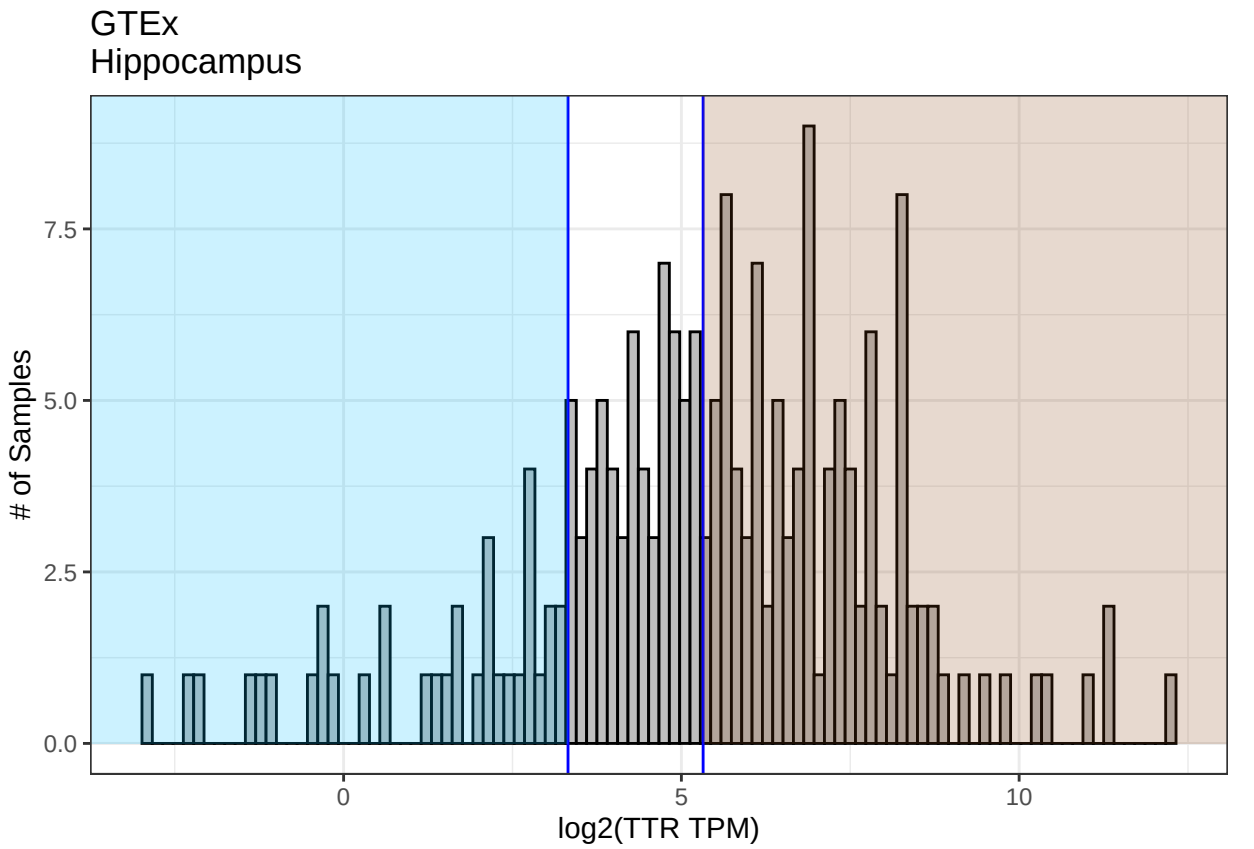
```
dge$samples$group <- factor(clean_or_dirty)
head(dge$samples)
```

```
##                               group lib.size norm.factors subject_id sex   age
## GTEX.11DXW.0011.R1a.SM.DNZZD neither 295069.0            1 GTEX.11DXW   M 40-49
## GTEX.11EI6.0011.R1a.SM.DO93L   dirty 255731.1            1 GTEX.11EI6   M 60-69
## GTEX.11GS4.0011.R1a.SM.DO129   dirty 286639.2            1 GTEX.11GS4   M 60-69
## GTEX.11GSO.0011.R1b.SM.57WD3 neither 290899.0            1 GTEX.11GSO   M 60-69
## GTEX.11GSP.0011.R1a.SM.9QEJ3   clean 288836.8            1 GTEX.11GSP   F 60-69
## GTEX.11ONC.0011.R1a.SM.57WD4   clean 214828.4            1 GTEX.11ONC   M 60-69
##                              hardy_scale                    counts_id
## GTEX.11DXW.0011.R1a.SM.DNZZD           2 GTEX.11DXW.0011.R1a.SM.DNZZD
## GTEX.11EI6.0011.R1a.SM.DO93L           4 GTEX.11EI6.0011.R1a.SM.DO93L
## GTEX.11GS4.0011.R1a.SM.DO129           2 GTEX.11GS4.0011.R1a.SM.DO129
## GTEX.11GSO.0011.R1b.SM.57WD3           2 GTEX.11GSO.0011.R1b.SM.57WD3
## GTEX.11GSP.0011.R1a.SM.9QEJ3           2 GTEX.11GSP.0011.R1a.SM.9QEJ3
## GTEX.11ONC.0011.R1a.SM.57WD4           2 GTEX.11ONC.0011.R1a.SM.57WD4
```

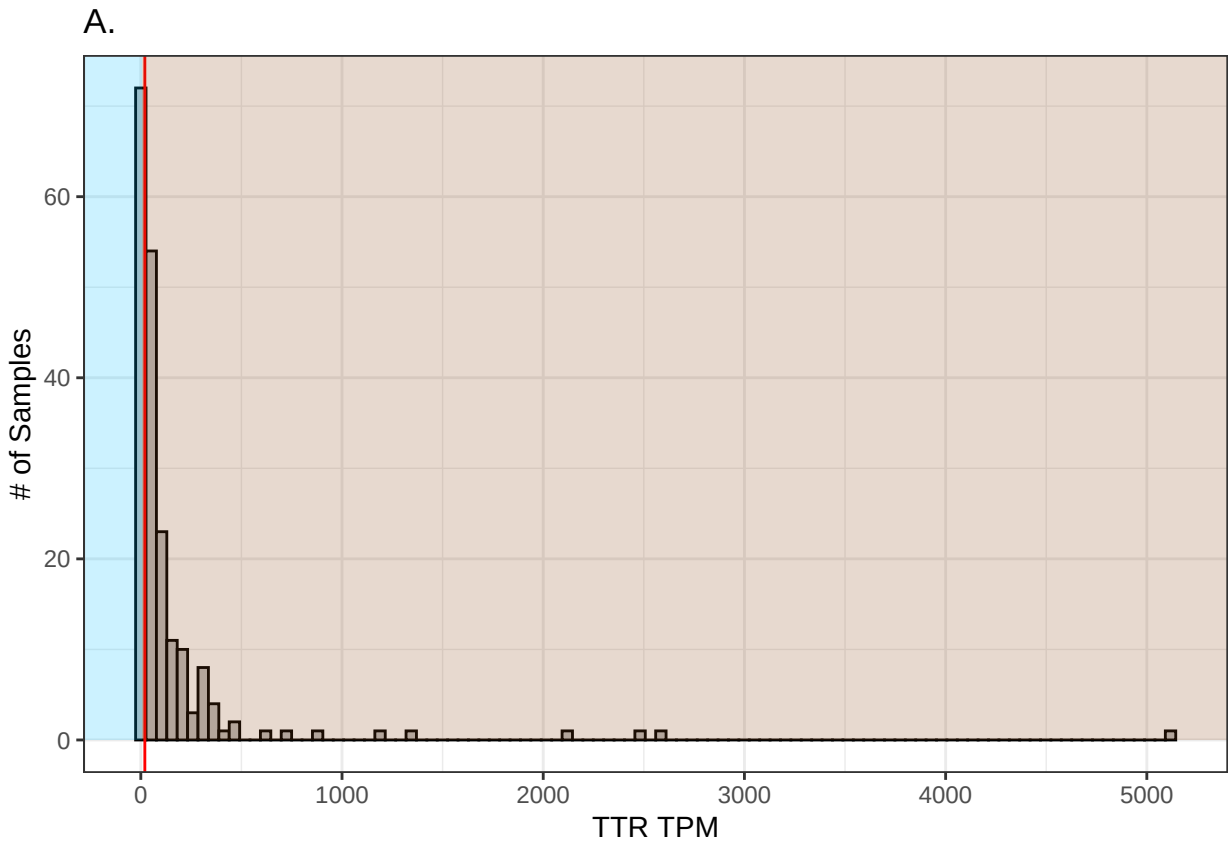
```
h4 <- h3 +
  annotate("rect",
              xmin = -Inf,
              xmax = clean_threshold,
              ymin = 0,
              ymax=Inf,
              alpha=0.2,
              fill="deepskyblue") +
  annotate("rect",
              xmin = dirty_threshold,
              xmax = Inf,
              ymin = 0,
              ymax=Inf,
              alpha=0.2,
              fill="chocolate4")
h5 <- h1 +
  annotate("rect",
              xmin = -Inf,
              xmax = clean_threshold,
              ymin = 0,
              ymax=Inf,
              alpha=0.2,
              fill="deepskyblue") +
  annotate("rect",
              xmin = dirty_threshold,
              xmax = Inf,
              ymin = 0,
```

```
              ymax=Inf,
              alpha=0.2,
              fill="chocolate4")
```

```
h4
```




```
h5
```



FILTER LOWLY EXPRESSED GENES

TPM to CPM

```
cpm <- cpm(dge)
```

Filter

```
# produces a logical matrix
thresh <- cpm > 2 # cpm greater than 2

# keep genes that have at least 3 TRUES in each row of thresh
keep <- rowSums(thresh) >= 3
summary(keep)
```

```
##    Mode   FALSE    TRUE
## logical   30225   24330
```

```
# redefine object, filter by logical
dge.filtered <- dge[keep, keep.lib.sizes = FALSE]

dim(dge)
```

```
## [1] 54555   197
```

```
dim(dge.filtered)
```

```
## [1] 24330   197
```

Went from 54,592 genes to 18,883 genes.

TRIMMED MEAN OF M-VALUES

Now, we want to normalize gene expression distributions. We do this by normalizing library size differences in each sample. The method Trimmed Mean of M-values (TMM) is used with the calcNormFactors in the edgeR package. The normalization factors calculated here are used as a scaling factor for the library size.

Trimmed mean of M values (TMM) normalization estimates sequencing depth after excluding genes for which the ratio of counts between a pair of experiments is too extreme or for which the average expression is too extreme. The edgeR software implements a TMM normalization (Biostar Handbook 2020).

```
# creates another large DGEList
dge.filtered.norm <- calcNormFactors(dge.filtered, method = "TMM")

# view norm factors
dge.filtered.norm$samples$norm.factors
```

```
##   [1] 0.9208014 0.9397341 1.0664196 1.1657822 1.1034621 1.0757837 0.9692125
##   [8] 1.0345089 1.1286779 0.9074237 1.0777276 1.1876111 1.0649142 0.9585218
##  [15] 0.9699986 0.9005879 0.9866129 0.9987741 0.9415839 0.9032043 1.0152109
##  [22] 1.0121925 1.0371544 0.8992394 1.0453845 0.9016306 1.0659189 1.1260111
##  [29] 0.9450652 0.9096229 0.8623565 0.8448163 1.1555415 0.9492569 0.9825655
##  [36] 1.0605614 1.0104486 0.8361482 0.8753509 0.9654817 1.0096480 0.8608656
##  [43] 0.8320688 0.9922814 1.1346967 0.9275243 1.0780612 1.0901378 1.0437759
##  [50] 0.8624394 1.0029232 1.0540529 1.1874402 0.8926142 1.0670313 1.0547220
##  [57] 1.0384668 0.7318076 0.9500912 1.1119956 0.8796994 0.9476418 0.9584379
##  [64] 1.0863173 1.0988247 1.0302707 1.0276728 1.1872606 0.9843724 0.7001994
##  [71] 1.0412301 1.1075697 1.1262024 0.8636979 1.0865561 1.1177207 1.0470575
##  [78] 1.0812113 1.0651687 1.0803114 0.8892324 1.0766973 0.9631251 1.0482810
##  [85] 1.1339810 1.0978243 0.9991410 0.9488282 1.0089296 0.9492502 1.0222190
##  [92] 0.9941390 1.0646432 0.9794406 0.9119222 0.9667743 0.9246916 0.9639162
##  [99] 1.0380671 0.9249168 0.9808264 1.0000709 1.1134358 0.9578064 0.9663822
## [106] 0.9866986 0.6440762 0.8832843 1.0252993 0.9320145 1.1172923 0.9903614
## [113] 1.0588432 0.9264131 1.0554269 0.9254882 0.9490689 0.8831684 1.1087182
## [120] 0.8840364 1.0230063 1.0438604 0.9622401 0.8130978 1.0115068 1.0412311
## [127] 0.9575043 0.9875283 0.9423856 1.0974353 0.9971180 1.0841743 1.0317499
## [134] 0.9764619 1.0516356 1.0403124 0.8842430 0.8214545 1.1038727 0.9825239
## [141] 0.8676495 1.0425724 0.8893092 0.8093808 1.1624619 1.0098526 1.0763740
## [148] 1.1643346 0.9752810 1.0302402 1.0366424 0.8129844 1.0329518 1.0349895
## [155] 0.9732349 1.0351201 0.9280301 0.9094309 1.0713761 0.9071833 1.1432864
## [162] 1.1622661 1.0131992 0.9996608 1.0791143 1.0572388 1.0152764 0.9515026
## [169] 1.1424870 0.9718733 1.1850443 1.2132065 1.0591215 0.9334512 1.1576479
## [176] 1.0268450 1.0141231 1.0059438 1.1929826 0.9018986 0.9574237 1.0124440
## [183] 1.0337595 1.0564354 1.0587401 1.1084535 1.0184384 1.0201374 1.0178207
## [190] 0.9576280 1.0526098 1.0964601 1.0651772 0.9379478 0.9691619 1.1172171
## [197] 1.0581417
```

```
summary(dge.filtered.norm$samples$norm.factors)
```

```
##    Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
##  0.6441  0.9488  1.0124  1.0047  1.0652  1.2132
```

Looking to be around ~1.

-Variance Partition-

See what options we have to choose from.

```
colnames(dge.filtered.norm$samples)
```

```
## [1] "group"        "lib.size"     "norm.factors" "subject_id"   "sex"
## [6] "age"          "hardy_scale"  "counts_id"
```

Specify variables to consider

```
#register(SnowParam(4)) # work in parallel

# geneExpr: matrix of gene expression values
# info: information/metadata about each sample
#geneExpr <- as.matrix(dge.filtered.norm$counts)
#info <- as.data.frame(dge.filtered.norm$samples)
#info$hardy_scale <- as.factor(info$hardy_scale)

# Age is USUALLY continuous so model it as a fixed effect "age"
# However, this metadata categorizes it into bins so it's categorical
# Sex is categorical, so model them as random effects "(1|sex)"
# Note the syntax
#form <- ~ (1|group) +
#  (1|age) +
#  (1|sex) +
#  (1|hardy_scale)

#varPart <- fitExtractVarPartModel(geneExpr, form, info)
#vp <- sortCols(varPart)
```

Violin plots

```
#plotVarPart(vp)
```

Percent bars

```
#plotPercentBars(vp[1:10,])
```

```
# sort genes based on variance explained by group
#varPart.df <- as.data.frame(varPart)
#order.varPart.df <- varPart.df[order(varPart.df$group, #decreasing = TRUE),]
#order.varPart.df["group"]
```

DESIGN MATRIX

```
sex <- dge.filtered.norm$samples$sex
age <- dge.filtered.norm$samples$age
```

Create a design matrix and specify the samples we want to compare to each other.

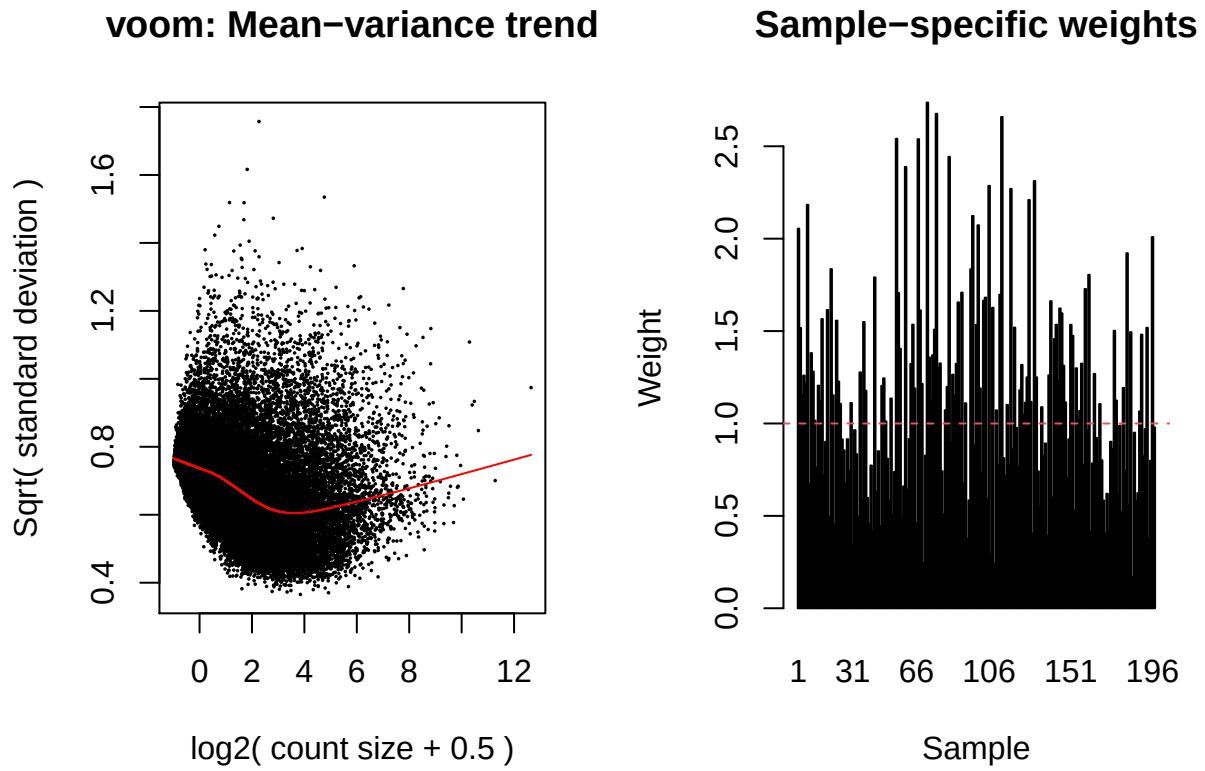
```
# interaction computes a factor which represents the interaction of the given factors
group <- interaction(dge.filtered$samples$group)

design <- model.matrix(~ 0 + group + sex + age)
colnames(design) <- make.names(colnames(design))
colnames(design)[1:2] <- c("clean","dirty")
```

Run voom with quality weights. This combines observational-level weights with sample-specific quality weights in an experiment. Normalizes expression intensities so that the log-ratios have similar distributions across a set of samples. To quantile normalize, add normalize.method = "quantile".

Quality weights in RNA-seq data improves data quality when samples with high variance are present. This

variation is modeled by taking into account global intensity-dependent trends using voom.
Sample-specific variability is taken into account with a log-linear model that shares parameters between genes.
https://doi.org/10.1093/nar/gkv412

```
v <- voomWithQualityWeights(dge.filtered.norm, design, plot = TRUE)
```



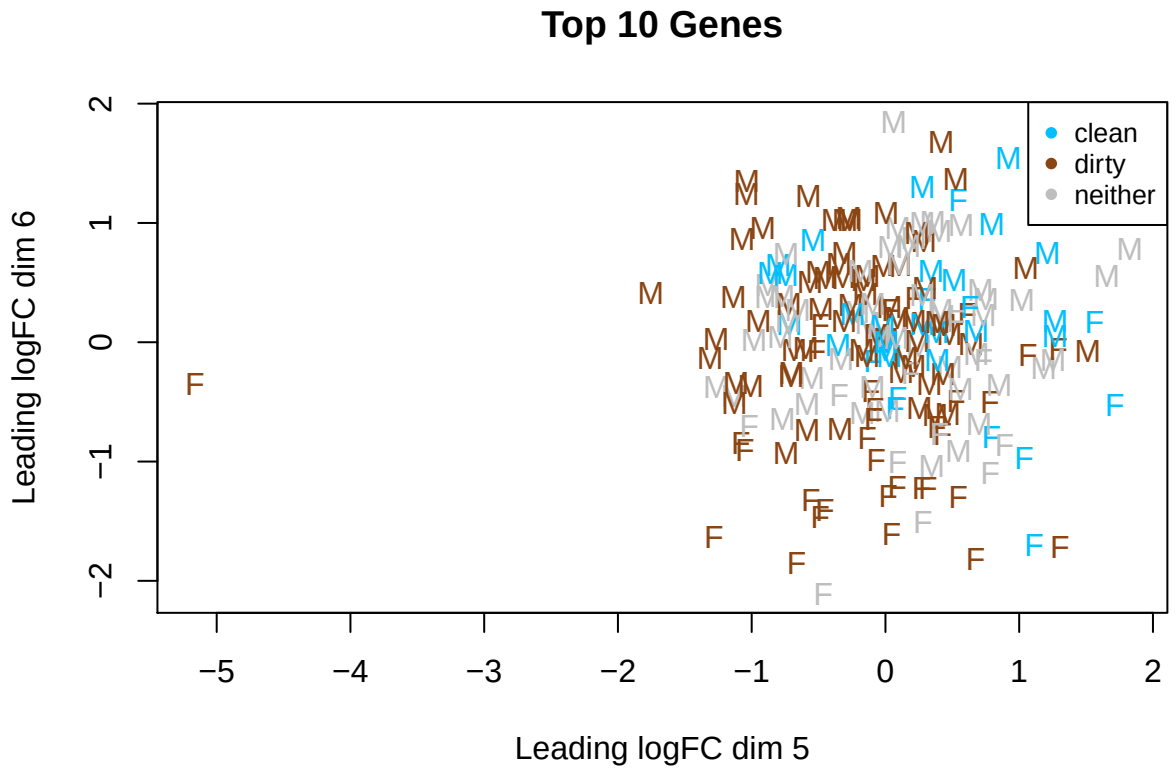
MDS PLOT

```
group_colors <- c("deepskyblue","chocolate4","gray")[v$targets$group] # blue is clean, brown is dirty
v$targets$sex <- as.factor(v$targets$sex)
point_shapes <- c("F","M")[v$targets$sex] # will say F or M for points
head(data.frame(v$targets$group, group_colors, v$targets$sex,point_shapes)) # Check everything matches
```

```
##   v.targets.group group_colors v.targets.sex point_shapes
## 1         neither         gray             M            M
## 2           dirty   chocolate4             M            M
## 3           dirty   chocolate4             M            M
## 4         neither         gray             M            M
## 5           clean  deepskyblue             F            F
## 6           clean  deepskyblue             M            M
```

```
#create a png file# png(filename = "mds_1and2.png", width = 400, height = 400)
#default height and width are 480, default units are "px" (pixels)

mds <- plotMDS(
  v, # our data object
  top = 10, # only looking at top 10 genes
  pch = point_shapes,
  cex = 1, # point size
  dim.plot = c(5,6), # specifying principal components to be plotted
  plot = TRUE, # if TRUE then plot is created on current graphics device
```

```
  col = group_colors # assigning our colors for each genotype
)

legend(
  "topright",
  pch = 16,
  legend = c("clean","dirty","neither"),
  col = c("deepskyblue","chocolate4","gray"),
  cex = 0.8
)

title("Top 10 Genes")
```



———————-HIERARCHIAL HEAT CLUSTERING———————-

ds[order(factor(Month, levels = c(“Apr”, “May”, “Jan”, “Feb”, “Mar”))), .SD,ID]

```
current_order <- dge.filtered.norm$samples$counts_id

# reorder levels
group_order <- dge.filtered.norm$samples
group_order <- group_order[order(factor(group, levels = c("dirty","neither","clean"))),]
sample_group_order <- group_order$counts_id
dge.group <- dge.filtered.norm[,sample_group_order]

# sample group colors
sample_group_color <- c("deepskyblue", "chocolate4", "gray")[dge.group$samples$group]
as.data.frame(dge.group$samples$group, sample_group_color)
```

```
##             dge.group$samples$group
## chocolate4                    dirty
```

```
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```

```
# We estimate the variance for each row in the logcounts matrix
#logcounts <- cpm(dge.group, log = TRUE) # log2 values
#var_genes <- apply(logcounts, 1, var)

# Get the gene names for the top 100 most variable genes
#select_var <- names(sort(var_genes, decreasing=TRUE))[1:50]
#select_var

# Subset logcounts matrix
#highly_variable_lcpm <- logcounts[select_var,]
```

```
#my_palette <- colorRampPalette(c("red", "black", "green"))

# Plot the heatmap
#map <- heatmap.2(highly_variable_lcpm,
 #          main = "Top 50 most variable genes across samples",
 #          col = my_palette, # heatmap color scale
 #          ColSideColors = sample_group_color,
  #         trace = "none",
#           dendrogram = "row",
```

```
  #       Colv = sample_group_order,
  #       colCol = sample_group_color,
  #       srtCol = 45,
  #       xlab = "Samples"
  #       )
```

LINEAR MODELS

Fit linear models for comparisons of interest. Linear modeling in limma is carried out using the lmFit and contrasts.fit functions.

What do lm.fit and contrasts.fit do? 1) Fit a separate model to the expression values for each gene. 2) An empirical Bayes moderation is carried out by borrowing info across all the gene to obtain more precise estimates of gene-wise variability. 3) The model's residual variances are plotted against average expression values in the next figure.

It can be seen from this plot that the variance is no longer dependent on the mean expression level.

```
# Fits linear model for each gene given a series of arrays
fit <- lmFit(
  v,  # object containing log-expression values for a series of arrays
  design  # design matrix
  )
```

Contrast design for differential expression.

```
contrasts <-
  makeContrasts(
    dirty_vs_clean = dirty - clean,
    levels = colnames(design)
  )

head(contrasts)
```

```
##               Contrasts
## Levels         dirty_vs_clean
##   clean                    -1
##   dirty                     1
##   groupneither              0
##   sexM                      0
##   age30.39                  0
##   age40.49                  0
```

Assign allComparisons to a vector for later use.

```
allComparisons <- colnames(contrasts)
allComparisons # check
```

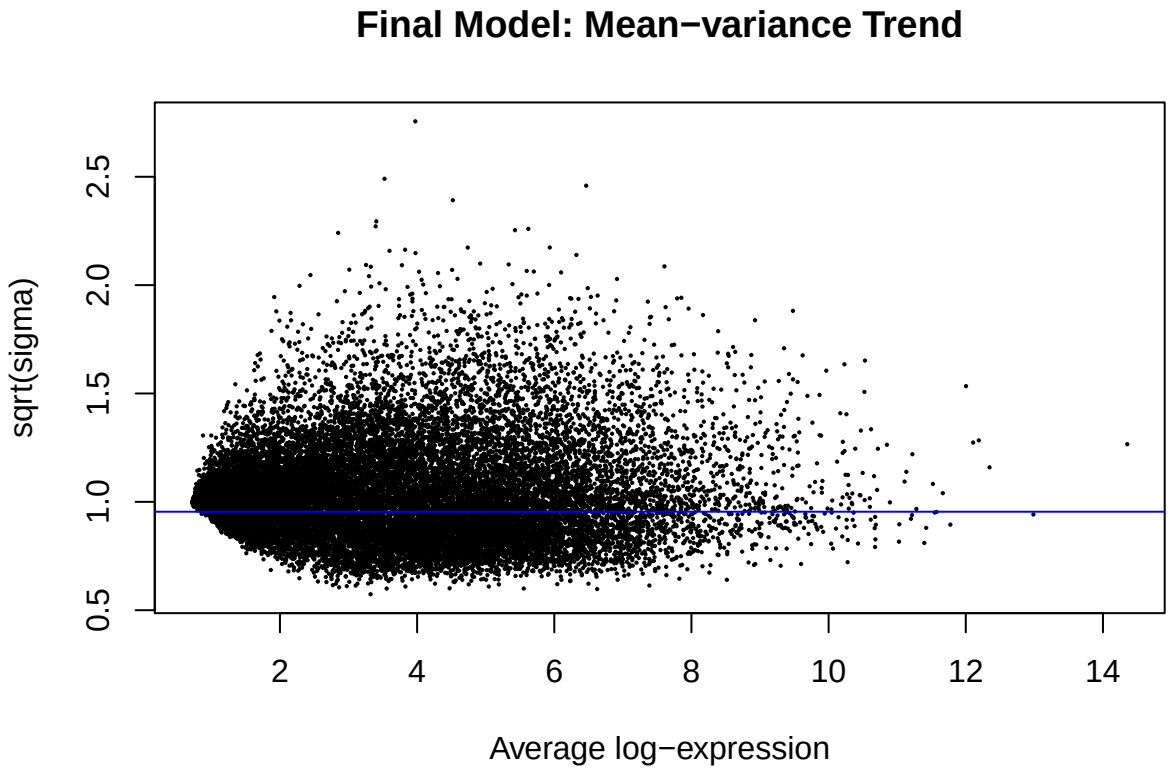
```
## [1] "dirty_vs_clean"
```

Run contrast analysis. Given a linear model fit, compute estimated coefficients and standard errors for a given set of contrasts.

```
vfit <- contrasts.fit(fit, contrasts = contrasts)
```

Compute differential expression based on the empirical Bayes moderation of the standard errors towards a common value.

```
veBayesFit <- eBayes(vfit)
plotSA(veBayesFit, main = "Final Model: Mean-variance Trend")
```



Looking at N of differentially expressed genes (DEGs) with adjusted p < 0.01 and log2FC > 2.

decideTests() identifies which genes are significantly differentially expressed for each contrast from a fit object containing p-values and test statistics.

```
sumTable <-
  summary(decideTests(
    vfit,  # object
    # by default the method = "separate"
    adjust.method = "BH",
    p.value = 0.05,
    lfc = 1  # numeric, minimum absolute log2-fold change required
  ))

head(sumTable)
```

```
##        dirty_vs_clean
## Down               25
## NotSig          24236
## Up                 69
```

DEG TABLES

For each comparison. . . extract table of the top-ranked genes (DEGs) from a linear model fit AND output a table.

NOTE: Log2FC of 1 is equivalent to linear fold change of 2.

```
coef = 1
for (i in allComparisons) {

  # p < 1, log2fc > 0 ------------------------------------------------------------
  vTopTableAll <-
```

```
    topTable(
      veBayesFit,
      coef = coef,
      n = Inf,
      p.value = 1,
      lfc = 0
    )
  #output as txt file
  path <- paste("DEG_", i, "_FDRq1_Log2FC0.txt", sep = "")
  write.table(
    vTopTableAll,
    path,
    sep = "\t",
    row.names = TRUE,
    quote = FALSE
  )

  # p < 0.05, log2fc > 1 ------------------------------------------------------
  vTopTable1 <-
    topTable(
      veBayesFit,
      coef = coef,
      n = Inf,
      p.value = 0.05,
      lfc = 1
    )
  path <- paste("DEG_", i, "_FDRq0.05_Log2FC1.txt", sep = "")
  write.table(
    vTopTable1,
    path,
    sep = "\t",
    row.names = TRUE,
    quote = FALSE
  )

  # increment the coefficient -------------------------------------------------
  coef <- coef + 1
}
```

-VOLCANO PLOTS

Using the DEG tables we made, graph a volcano plot. Read in file.

```
dirty_vs_clean <-
  read.table(
    "DEG_dirty_vs_clean_FDRq1_Log2FC0.txt",
    header = TRUE,
    sep = "\t",
    stringsAsFactors = F
  )
```

```
dirty_vs_clean$gene_symbol <- rownames(dirty_vs_clean)
```

```
summary(decideTests(
    vfit,  # object
```

```
    adjust.method = "BH", # by default the method = "separate"
    p.value = 0.05,
    lfc = 1
  ))
```

```
##        dirty_vs_clean
## Down               25
## NotSig          24236
## Up                 69
```

```
color_values <- vector()
max <- nrow(dirty_vs_clean)

for(i in 1:max){
  if (dirty_vs_clean$adj.P.Val[i] < 0.05){
    if (dirty_vs_clean$logFC[i] > 1){
      color_values <- c(color_values, 1) # 1 when logFC > 1 and pval < 0.05
    }
    else if (dirty_vs_clean$logFC[i] < -1){
      color_values <- c(color_values, 2) # 2 when logFC < -1 and pval < 0.05
    }
    else{
      color_values <- c(color_values, 3) # 3 when -1 =< logFC <= 1 pval < 0.05
    }
  }
  else{
    color_values <- c(color_values, 3) # 3 when pval >= 0.05
  }
}

dirty_vs_clean$color_p0.05_lfc1 <- factor(color_values)
```

```
hadjpval <- (-log10(max(dirty_vs_clean$adj.P.Val[dirty_vs_clean$adj.P.Val < 0.05], na.rm=TRUE)))
hadjpval
```

```
## [1] 1.302149
```

Subset 10 lowest FDRq for up and down regulated genes.

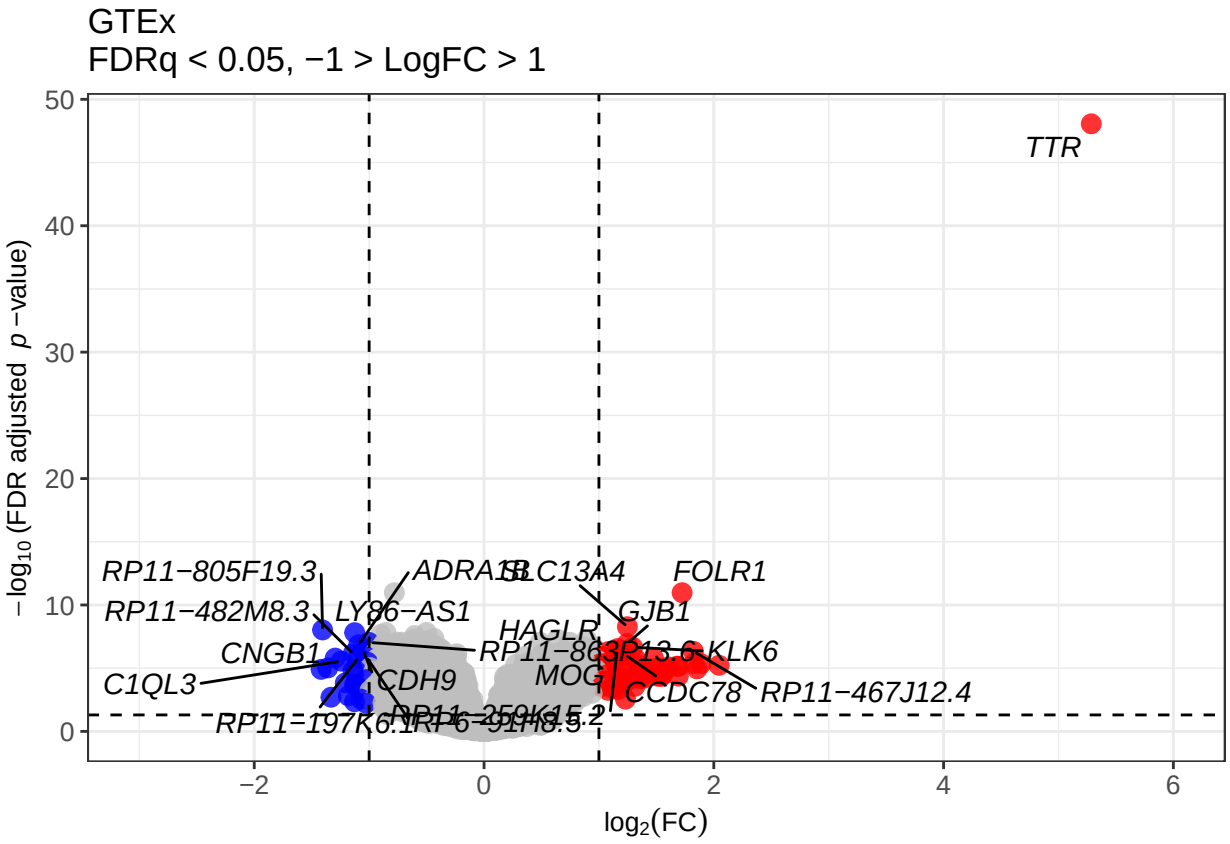
```
keep <- dirty_vs_clean$color_p0.05_lfc1 == 1
up10 <- dirty_vs_clean[keep,]
up10 <- up10[order(up10$adj.P.Val),]
up10 <- up10[c(1:10),c(4,6,10)]

keep <- dirty_vs_clean$color_p0.05_lfc1 == 2
down10 <- dirty_vs_clean[keep,]
down10 <- down10[order(down10$adj.P.Val),]
down10 <- down10[1:10, c(4,6,10)]
```

```
p <-
  ggplot(data = dirty_vs_clean,
         aes(x = logFC, # x-axis is logFC
             y = -log10(adj.P.Val), # y-axis will be log10 of adj.P.Val
             color = color_p0.05_lfc1)) + # color is based on factored Color column
  geom_point(alpha = 0.8, size = 3) + # create scatterplot, alpha makes points transparent
  theme_bw() +
```

```
  theme(legend.position = "none") + # no legend
  xlim(c(-3, 6)) + #ylim(c(0, 25)) + # x and y axis limits
  scale_color_manual(values = c("red", "blue", "grey")) + # set factor colors
  labs(
    title = "", # no main title
    x = expression(log[2](FC)), # x-axis title
    y = expression(-log[10] ~ "(FDR adjusted " ~ italic("p") ~ "-value)") # y-axis title
  ) +
  theme(axis.title.x = element_text(size = 10),
        axis.text.x = element_text(size = 10)) +
  theme(axis.title.y = element_text(size = 10),
        axis.text.y = element_text(size = 10)) +
  geom_hline(yintercept = hadjpval,
                     colour = "#000000",
                     linetype = "dashed") +
    geom_vline(xintercept = 1,
              colour = "#000000",
              linetype = "dashed") +
  geom_vline(xintercept = -1,
              colour = "#000000",
              linetype = "dashed") +
  ggtitle("GTEx\nFDRq < 0.05, -1 > LogFC > 1") +
  geom_text_repel(data = up10,
                  aes(x = logFC, y = -log10(adj.P.Val), label = gene_name),
                  color = "black",
                  fontface="italic",
                  max.overlaps = getOption("ggrepel.max.overlaps", default = 80)
                  ) +
  geom_text_repel(data = down10,
                  aes(x = logFC, y = -log10(adj.P.Val), label = gene_name),
                  color = "black",
                  fontface = "italic",
                  max.overlaps = getOption("ggrepel.max.overlaps", default = 80)
  )

p
```


GTEx
FDRq < 0.05, −1 > LogFC > 1
50
40
30
20
10
0
−2
0
2
4
6
− log10 (FDR adjusted p −value)
log2(FC)
TTR
FOLR1
GJB1
HAGLR
MOG
CCDC78
RP11−467J12.4
RP11−805F19.3
RP11−482M8.3
LY96−AS1
CNGB1
C1QL3
CDH9
RP11−197K6.1
RP11−91H8.3