Read counts to DGE, Part I

Contents

This script will show you how to:

- Read in `featureCounts` results into R.
- Use `DESeq2` to:
  - normalize read counts for differences in sequencing depth
  - transform reads to the log2 scale including variance reduction
- Accompany each step by exploratory plots.

You can generate an html document out of this entire script by clicking the `Knit HTML` button in RStudio.

```
options(stringsAsFactors = FALSE) # this will change a global setting, but just for this session

library(knitr)
opts_chunk$set(echo = TRUE, message = FALSE,cache=FALSE) # tuning knitr output
```

featureCounts

We aligned five samples for the WT and SNF2 condition, respectively. You can find those files here: `~/mat/precomputed/results_alignment`.

> How can you check which command was used to generate those `BAM` files?

```
## on the command line (!)
mkdir class/read_counts
cd class/read_counts
REF_DIR=~/mat/referenceGenomes/S_cerevisiae/

 # reads for yeast samples counted on the meta-feature level
~/mat/software/subread-1.6.0-Linux-x86_64/bin/featureCounts \
    -a ${REF_DIR}/Saccharomyces_cerevisiae.R64-1-1.81.gtf \
    -o featCounts_genes.txt \
     ~/mat/precomputed/results_alignment/*bam
```

Let's read the result file into R, i.e. download the table from our website or use the `scp` command to download the table that you generated on the server to your local machine.

Loading additional libraries:

```
library(ggplot2) # for making plots
```

```
## Warning: package 'ggplot2' was built under R version 3.5.2
```

```
library(magrittr) # for "pipe"-like coding in R
```

We will use the `DESeq2` package to normalize the samples for differences in their sequencing depths.

```
# not available via install.packages(), but through bioconductor
BiocManager::install("DESeq2")
```

```
library(DESeq2)
```

We will have to generate a `DESeqDataSet`; what is needed for this can be found out via `?DESeqDataSetFromMatrix`. The help indicates that we need two tables: `countData` and `colData`.

- `colData`: `data.frame` with all the variables you know about your samples, e.g., experimental condition, the type, and date of sequencing and so on. Its `row.names` should correspond to the unique sample names.
- `countData`: should contain a matrix of the actual values associated with the genes and samples. Is equivalent to `assay()`. Conveniently, this is almost exactly the format of the `featureCounts` output.

```
# reading in featureCounts output
readcounts <- read.table("~/Downloads/featCounts_genes.txt",
                          header=TRUE)
head(readcounts)
```

```
##      Geneid Chr Start   End Strand Length
## 1   YDL248W  IV  1802  2953      +   1152
## 2 YDL247W-A  IV  3762  3836      +     75
## 3   YDL247W  IV  5985  7814      +   1830
## 4   YDL246C  IV  8683  9756      -   1074
## 5   YDL245C  IV 11657 13360      -   1704
## 6   YDL244W  IV 16204 17226      +   1023
##   X.home.classadmin.mat.precomputed.results_alignment.SNF2_1_Aligned.sortedByCoord.out.bam
## 1                                                                                      109
## 2                                                                                        0
## 3                                                                                        6
## 4                                                                                        6
## 5                                                                                        1
## 6                                                                                       79
##   X.home.classadmin.mat.precomputed.results_alignment.SNF2_2_Aligned.sortedByCoord.out.bam
## 1                                                                                       84
## 2                                                                                        1
## 3                                                                                        6
## 4                                                                                        6
## 5                                                                                        6
## 6                                                                                       59
##   X.home.classadmin.mat.precomputed.results_alignment.SNF2_3_Aligned.sortedByCoord.out.bam
## 1                                                                                      100
## 2                                                                                        1
## 3                                                                                        1
## 4                                                                                        1
## 5                                                                                        9
## 6                                                                                       49
##   X.home.classadmin.mat.precomputed.results_alignment.SNF2_4_Aligned.sortedByCoord.out.bam
## 1                                                                                      112
## 2                                                                                        0
## 3                                                                                        3
## 4                                                                                        4
## 5                                                                                        5
## 6                                                                                       60
##   X.home.classadmin.mat.precomputed.results_alignment.SNF2_5_Aligned.sortedByCoord.out.bam
## 1                                                                                       62
## 2                                                                                        3
## 3                                                                                        4
## 4                                                                                        4
## 5                                                                                        3
## 6                                                                          37
##   X.home.classadmin.mat.precomputed.results_alignment.WT_1_Aligned.sortedByCoord.out.bam
## 1                                                                        47
## 2                                                                         0
## 3                                                                         2
## 4                                                                         1
## 5                                                                         6
## 6                                                                         9
##   X.home.classadmin.mat.precomputed.results_alignment.WT_2_Aligned.sortedByCoord.out.bam
## 1                                                                        65
## 2                                                                         0
## 3                                                                         3
## 4                                                                         3
## 5                                                                         2
## 6                                                                         8
##   X.home.classadmin.mat.precomputed.results_alignment.WT_3_Aligned.sortedByCoord.out.bam
## 1                                                                        60
## 2                                                                         1
## 3                                                                         4
## 4                                                                         2
## 5                                                                         5
## 6                                                                        12
##   X.home.classadmin.mat.precomputed.results_alignment.WT_4_Aligned.sortedByCoord.out.bam
## 1                                                                        95
## 2                                                                         0
## 3                                                                         7
## 4                                                                         4
## 5                                                                         5
## 6                                                                        30
##   X.home.classadmin.mat.precomputed.results_alignment.WT_5_Aligned.sortedByCoord.out.bam
## 1                                                                        43
## 2                                                                         0
## 3                                                                         9
## 4                                                                         0
## 5                                                                         6
## 6                                                                        14
```

Preparing the count matrix for DESeq2DataSet class:

```r
# give meaningful and legible sample names
orig_names <- names(readcounts)
names(readcounts) <- gsub(".*alignment\\.", "" ,names(readcounts)) %>% gsub("_Aligned.*", "", .)

# gene IDs should be stored as row.names
row.names(readcounts) <- gsub("-", ".", readcounts$Geneid)

# exclude the columns without read counts (columns 1 to 6 contain additional
# info such as genomic coordinates)
readcounts <- readcounts[,-c(1:6)]
```

Always check your data set after you manipulated it!

```r
str(readcounts)
```

```
## 'data.frame':    7126 obs. of  10 variables:
##  $ SNF2_1: int  109 0 6 6 1 79 363 41 143 2 ...
##  $ SNF2_2: int  84 1 6 6 6 59 289 22 119 4 ...
##  $ SNF2_3: int  100 1 1 1 9 49 243 26 115 1 ...
##  $ SNF2_4: int  112 0 3 4 5 60 352 26 135 5 ...
##  $ SNF2_5: int  62 3 4 4 3 37 241 22 96 3 ...
##  $ WT_1  : int  47 0 2 1 6 9 192 25 239 2 ...
##  $ WT_2  : int  65 0 3 3 2 8 169 30 343 0 ...
##  $ WT_3  : int  60 1 4 2 5 12 190 18 251 1 ...
##  $ WT_4  : int  95 0 7 4 5 30 309 42 555 3 ...
##  $ WT_5  : int  43 0 9 0 6 14 171 27 323 1 ...
```

```
head(readcounts)
```

```
##           SNF2_1 SNF2_2 SNF2_3 SNF2_4 SNF2_5 WT_1 WT_2 WT_3 WT_4 WT_5
## YDL248W      109     84    100    112     62   47   65   60   95   43
## YDL247W.A      0      1      1      0      3    0    0    1    0    0
## YDL247W        6      6      1      3      4    2    3    4    7    9
## YDL246C        6      6      1      4      4    1    3    2    4    0
## YDL245C        1      6      9      5      3    6    2    5    5    6
## YDL244W       79     59     49     60     37    9    8   12   30   14
```

In addition to the read counts, we need some more information about the samples. According to `?colData`, this should be a `data.frame`, where the *rows* directly match the *columns* of the count data.

Here's how this could be generated in `R` matching the `readcounts` `data.frame` we already have:

```
sample_info <- DataFrame(condition = gsub("_[0-9]+", "", names(readcounts)),
                         row.names = names(readcounts) )
sample_info
```

```
## DataFrame with 10 rows and 1 column
##          condition
##        <character>
## SNF2_1        SNF2
## SNF2_2        SNF2
## SNF2_3        SNF2
## SNF2_4        SNF2
## SNF2_5        SNF2
## WT_1            WT
## WT_2            WT
## WT_3            WT
## WT_4            WT
## WT_5            WT
```

```
str(sample_info)
```

```
## Formal class 'DataFrame' [package "S4Vectors"] with 6 slots
##   ..@ rownames       : chr [1:10] "SNF2_1" "SNF2_2" "SNF2_3" "SNF2_4" ...
##   ..@ nrows          : int 10
##   ..@ listData       :List of 1
##   .. ..$ condition: chr [1:10] "SNF2" "SNF2" "SNF2" "SNF2" ...
##   ..@ elementType    : chr "ANY"
##   ..@ elementMetadata: NULL
##   ..@ metadata       : list()
```

Let's generate the `DESeqDataSet`:

```
DESeq.ds <- DESeqDataSetFromMatrix(countData = readcounts,
                                   colData = sample_info,
                              design = ~ condition)
DESeq.ds
```

```
## class: DESeqDataSet
## dim: 7126 10
## metadata(1): version
## assays(1): counts
## rownames(7126): YDL248W YDL247W.A ... RPM1 Q0297
## rowData names(0):
## colnames(10): SNF2_1 SNF2_2 ... WT_4 WT_5
## colData names(1): condition
```

```
head(counts(DESeq.ds))
```

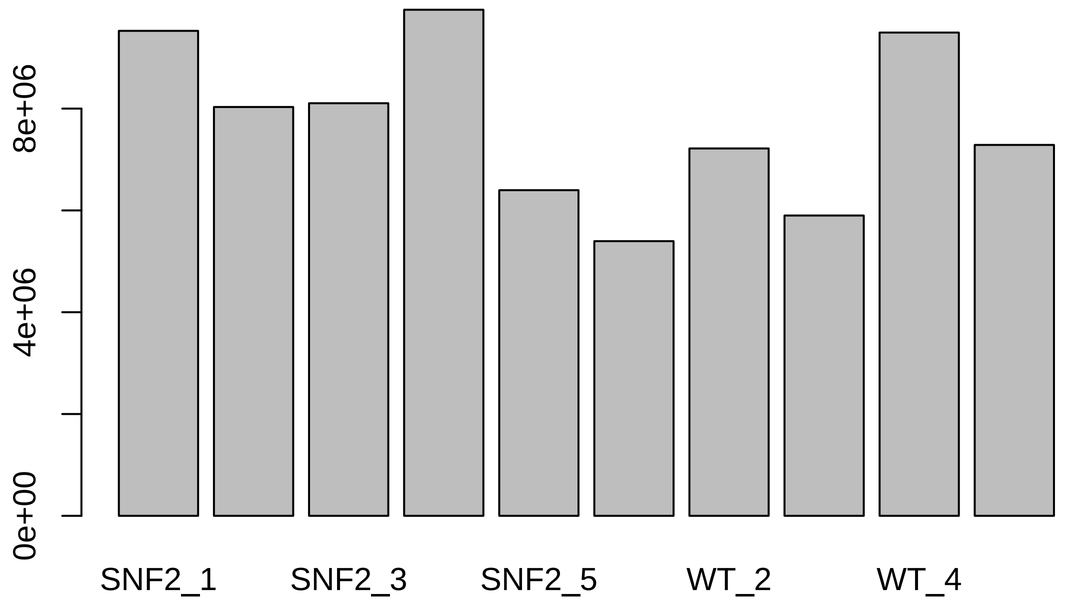
```
##             SNF2_1 SNF2_2 SNF2_3 SNF2_4 SNF2_5 WT_1 WT_2 WT_3 WT_4 WT_5
## YDL248W        109     84    100    112     62   47   65   60   95   43
## YDL247W.A        0      1      1      0      3    0    0    1    0    0
## YDL247W          6      6      1      3      4    2    3    4    7    9
## YDL246C          6      6      1      4      4    1    3    2    4    0
## YDL245C          1      6      9      5      3    6    2    5    5    6
## YDL244W         79     59     49     60     37    9    8   12   30   14
```

How many reads were counted for each sample (= library sizes)?

```
colSums(counts(DESeq.ds))
```

```
##  SNF2_1  SNF2_2  SNF2_3  SNF2_4  SNF2_5    WT_1    WT_2    WT_3    WT_4
## 9527395 8031694 8105366 9942250 6397208 5395963 7217177 5899432 9493974
##    WT_5
## 7286638
```

```
colSums(counts(DESeq.ds)) %>% barplot
```



Remove genes with no reads.

```
keep_genes <- rowSums(counts(DESeq.ds)) > 0
dim(DESeq.ds)
```

```
## [1] 7126   10
```

```
DESeq.ds <- DESeq.ds[ keep_genes, ]
dim(DESeq.ds)
```

```
## [1] 6680   10
```

```
counts(DESeq.ds) %>% str
```

```
##  int [1:6680, 1:10] 109 0 6 6 1 79 363 41 143 2 ...
##  - attr(*, "dimnames")=List of 2
##   ..$ : chr [1:6680] "YDL248W" "YDL247W.A" "YDL247W" "YDL246C" ...
##   ..$ : chr [1:10] "SNF2_1" "SNF2_2" "SNF2_3" "SNF2_4" ...
```

```
assay(DESeq.ds) %>% str
```

```
##  int [1:6680, 1:10] 109 0 6 6 1 79 363 41 143 2 ...
##  - attr(*, "dimnames")=List of 2
##   ..$ : chr [1:6680] "YDL248W" "YDL247W.A" "YDL247W" "YDL246C" ...
##   ..$ : chr [1:10] "SNF2_1" "SNF2_2" "SNF2_3" "SNF2_4" ...
```

Now that we have the data, we can start using `DESeq2`'s functions, e.g. `estimateSizeFactors()` for calculating a factor that will be used to correct for sequencing depth differences.

```
DESeq.ds <- estimateSizeFactors(DESeq.ds)
sizeFactors(DESeq.ds)
```

```
##    SNF2_1    SNF2_2    SNF2_3    SNF2_4    SNF2_5      WT_1      WT_2
## 1.4257668 1.1065006 1.0991960 1.4790643 0.8925667 0.5991287 0.9459106
##      WT_3      WT_4      WT_5
## 0.7697230 1.1385141 0.9026871
```

To see the details of how `DESeq2` calculates those size factors, you could look at the source code via `getMethod("estimateSizeFactors", "DESeqDataSet")`. A more verbose description can be found in the original paper by Anders and Huber:

> The purpose of the size factors is to render counts from different samples, which may have been sequenced to different depths, comparable. (...) The total number of reads (...) may seem to be a good measure of sequencing depth (...). Experience with real data, however, shows this not always to be the case, because a few highly and differentially expressed genes may have strong influence on the total read count, causing the ratio of total read counts not to be a good estimate for the ratio of expected counts. Hence, to estimate the size factors, we take the median of the ratios of observed counts (...) [where] each size factor is computed as the median of the ratios of the j-th sample's counts to those of the pseudo-reference, which is obtained by taking the geometric mean across *samples* [= columns].

In summary, the procedure is as follows:

1. for every gene (= row), determine the geometric mean of its read counts across all samples (yielding the "pseudo-reference", i.e. one value per gene);
2. divide every value of the count matrix by the corresponding pseudo-reference value;
3. for every sample (= column), determine the median of these ratios. This is the size factor.

```
## define a function to calculate the geometric mean
gm_mean <- function(x, na.rm=TRUE){
  exp(sum(log(x[x > 0]), na.rm=na.rm) / length(x))
}

## calculate the geometric means for each gene using that function
## note the use of apply(), which we instruct to apply the gm_mean()
```

```r
## function per row (this is what the second parameter, 1, indicates)
pseudo_refs <-  counts(DESeq.ds) %>% apply(., 1, gm_mean)

## divide each value by its corresponding pseudo-reference value
pseudo_ref_ratios <- counts(DESeq.ds) %>% apply(., 2, function(cts){ cts/pseudo_refs})

## if you want to see what that means at the single-gene level,
## compare the result of this:
counts(DESeq.ds)[1,]/pseudo_refs[1]
```

```
##    SNF2_1    SNF2_2    SNF2_3    SNF2_4    SNF2_5      WT_1      WT_2
## 1.4779492 1.1389700 1.3559167 1.5186267 0.8406683 0.6372808 0.8813458
##      WT_3      WT_4      WT_5
## 0.8135500 1.2881208 0.5830442
```

```r
## with
pseudo_ref_ratios[1,]
```

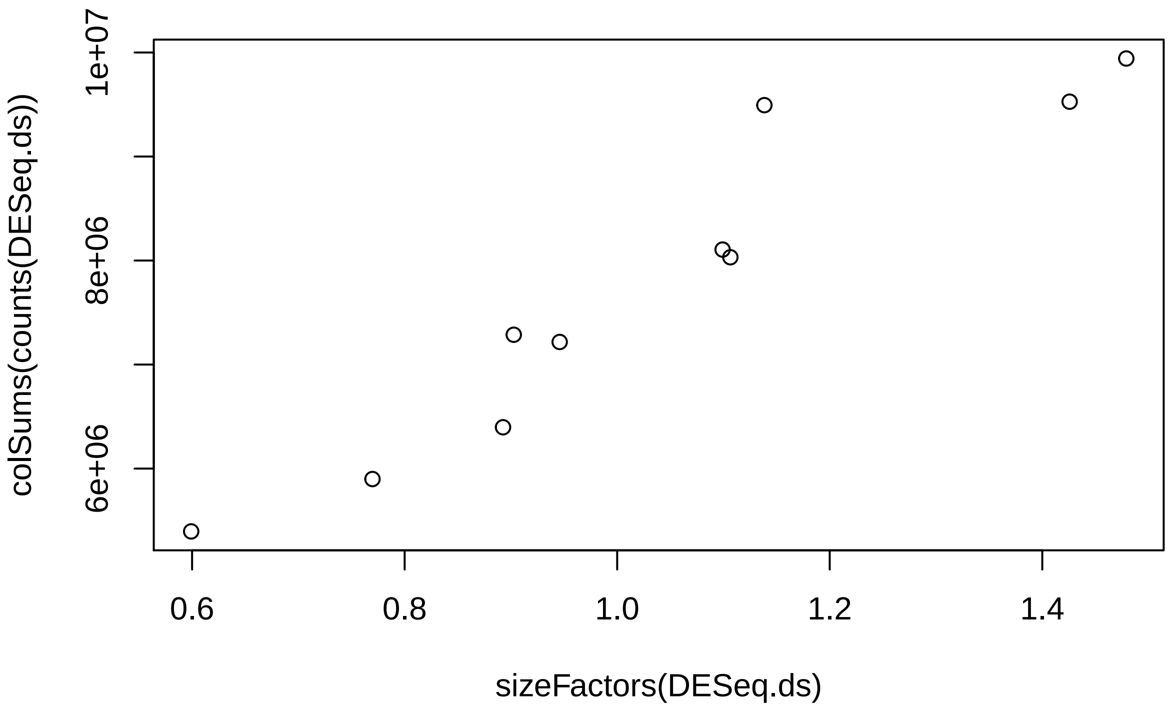
```
##    SNF2_1    SNF2_2    SNF2_3    SNF2_4    SNF2_5      WT_1      WT_2
## 1.4779492 1.1389700 1.3559167 1.5186267 0.8406683 0.6372808 0.8813458
##      WT_3      WT_4      WT_5
## 0.8135500 1.2881208 0.5830442
```

```r
## determine the median value per sample to get the size factor
apply(pseudo_ref_ratios, 2, median)
```

```
##    SNF2_1    SNF2_2    SNF2_3    SNF2_4    SNF2_5      WT_1      WT_2
## 1.4190635 1.0950712 1.0886771 1.4690623 0.8875478 0.5920460 0.9354326
##      WT_3      WT_4      WT_5
## 0.7662412 1.1306364 0.8959585
```

The result should be equivalent to 1.4257668, 1.1065006, 1.099196, 1.4790643, 0.8925667, 0.5991287, 0.9459106, 0.769723, 1.1385141, 0.9026871.

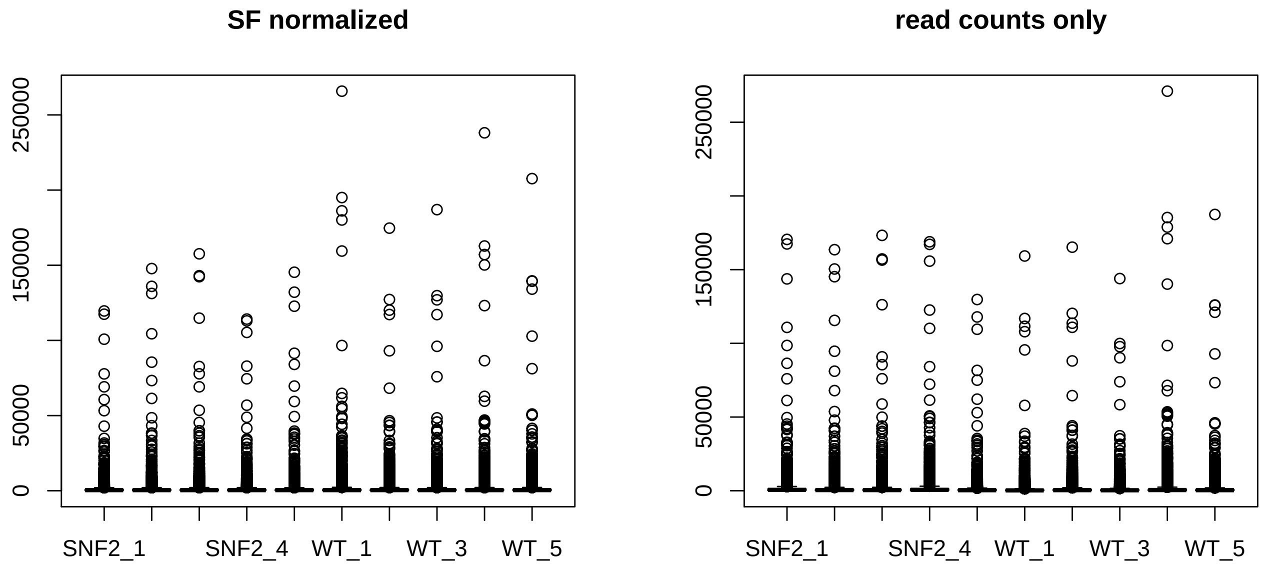
```r
plot(sizeFactors(DESeq.ds), colSums(counts(DESeq.ds)))
```



The read counts normalized for sequencing depth can be accessed via `counts(..., normalized = TRUE)`.

Let's check whether the normalization helped to adjust global differences between the samples.

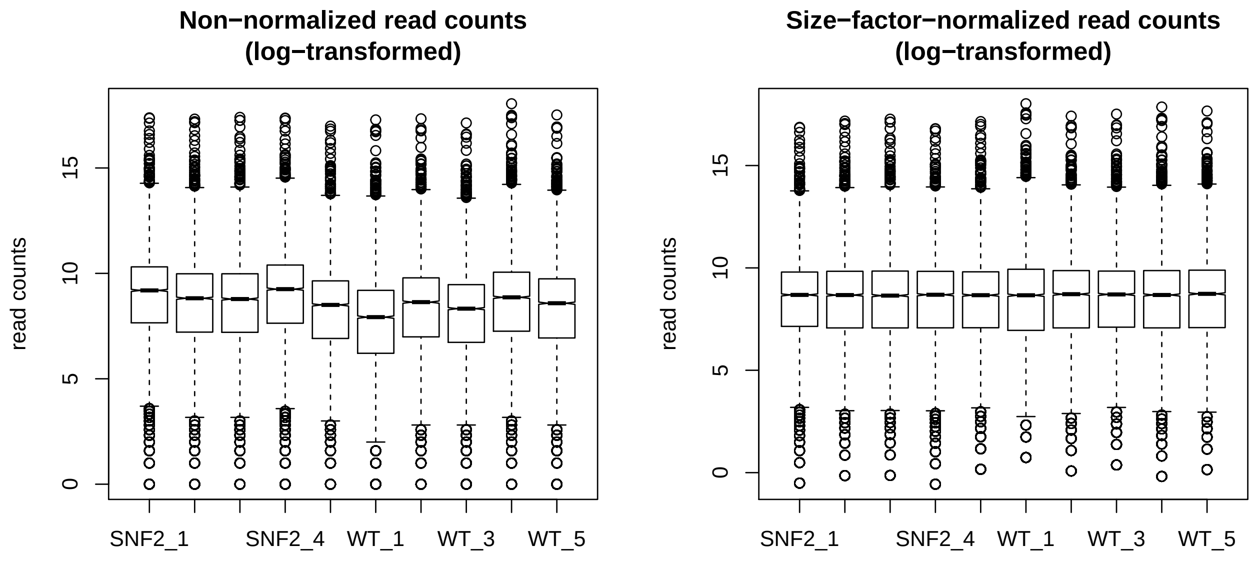
```r
# setting up the plotting layout
par(mfrow=c(1,2))
counts.sf_normalized <- counts(DESeq.ds, normalized=TRUE)

# adding the boxplots
boxplot(counts.sf_normalized, main = "SF normalized")
boxplot(counts(DESeq.ds), main = "read counts only")
```



We can't really see anything. It is usually helpful to *transform* the normalized read counts to bring them onto more similar scales.

To see the influence of the sequencing depth normalization, make two box plots of log2(read counts) - one for unnormalized counts, the other one for normalized counts (exclude genes with zero reads in all samples).

```
par(mfrow=c(1,2)) # to plot the two box plots next to each other
boxplot(log2(counts(DESeq.ds)), notch=TRUE,
        main = "Non-normalized read counts\n(log-transformed)",
        ylab="read counts")
boxplot(log2(counts(DESeq.ds, normalize= TRUE)), notch=TRUE,
        main = "Size-factor-normalized read counts\n(log-transformed)",
        ylab="read counts")
```



Understanding more properties of read count data

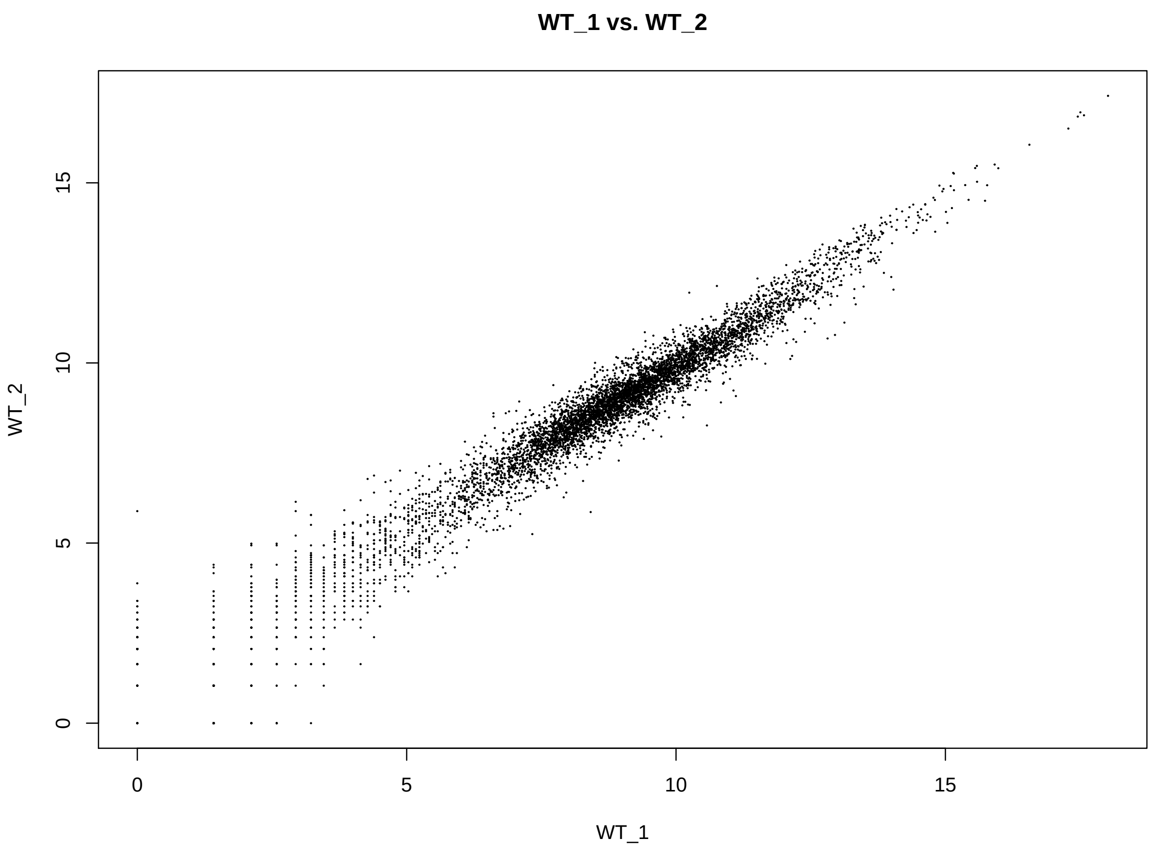
Characteristics we've touched upon so far:

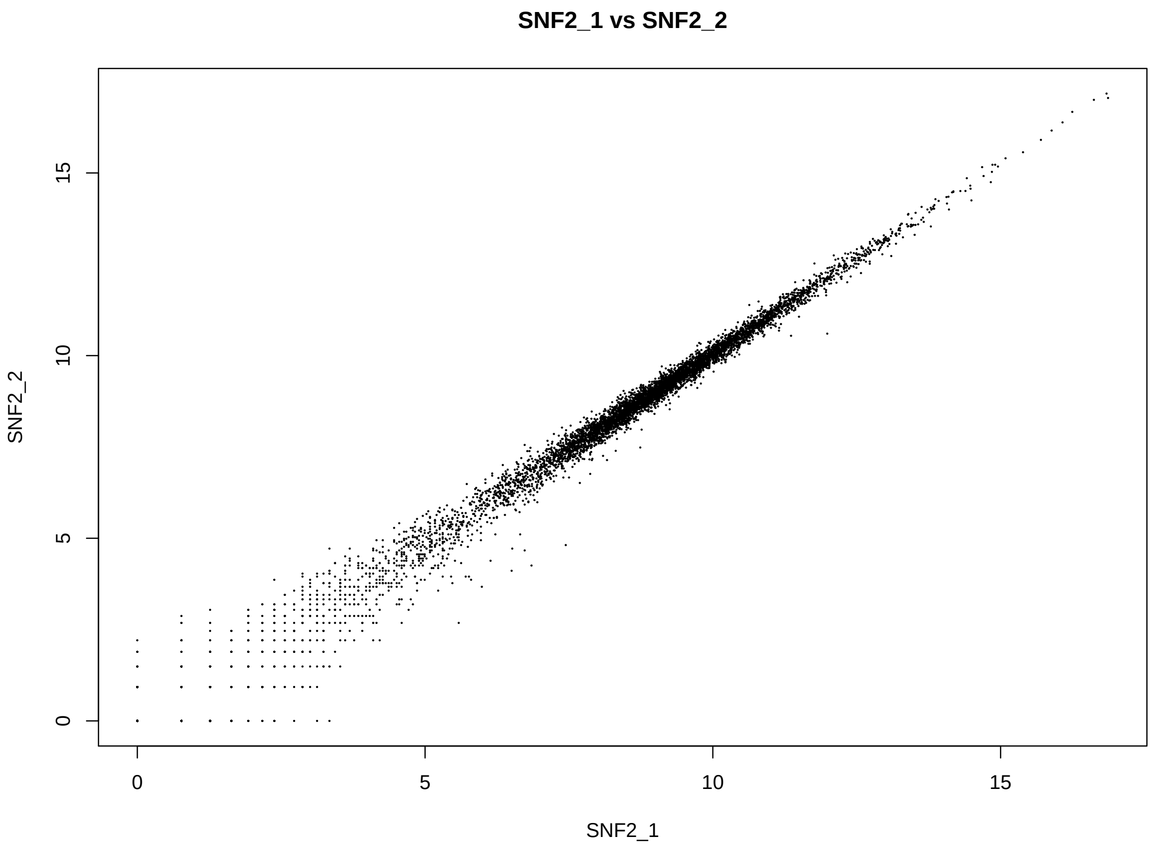
- zeros can mean two things: no expression or no detection
- fairly large dynamic range

  Make a scatterplot of log normalized counts against each other to see how well the actual values correlate which each other per sample and gene.

```
# non-normalized read counts plus pseudocount
log.counts <- log2(counts(DESeq.ds, normalized = FALSE) + 1)
# instead of creating a new object, we could assign the values to a distinct matrix
# normalized and log2-transformed read counts
assay(DESeq.ds, "log.norm.counts") <- log2(counts(DESeq.ds, normalized=TRUE) + 1)
```

```
par(mfrow=c(2,1))
DESeq.ds[, c("WT_1","WT_2")] %>% assay(.,  "log.norm.counts") %>% plot(., cex=.1, main = "WT_1 vs. WT_2
DESeq.ds[, c("SNF2_1","SNF2_2")] %>% assay(.,  "log.norm.counts") %>% plot(., cex=.1, main = "SNF2_1 vs
```


WT_1 vs. WT_2
WT_2
WT_1
0
5
10
15
0
5
10
15


SNF2_1 vs SNF2_2
SNF2_2
SNF2_1
0
5
10
15
0
5
10
15