RNA-Seq - differential expression using DESeq2

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Contents

The Snf2 dataset

The RNA-Seq dataset we will use in this practical has been produced by Gierliński *et al* ([@pmid26206307, @pmid27022035]). The dataset is composed of 48 WT yeast samples vs 48 Snf2 knock-out mutant cell line. The prepared RNA-Seq libraries (unstranded) were pooled and sequenced on seven lanes of a single flow-cell on an Illumina HiSeq 2000 resulting in a total of 1 billion 50-bp single-end reads across the 96 samples. RNA-Seq reads have been cleaned, mapped and counted to generated a count data matrix containing 7126 rows/genes. The primary objective of this study was to check whether the observed gene read counts distribution where consistent with theorical models (e.g. negative binomial). More information can be obtained in the original paper (pdf)

Loading the dataset

R enables to download data directly from the Web. The expression matrix and phenotypic information will be loaded into **R** using the **read.table** function. Both table will be converted into a data.frame object when loaded into R. The 'count.table' object will contains counts for each gene (row) and each sample (column).

```
# Download data files from the Web site (only if not done yet)
url.counts <- "http://jvanheld.github.io/stats_avec_RStudio_EBA/practicals/yeast_2x48_replicates/data/c

## Local paths
dir.snf2 <- ("~/ASG/practicals/rnaseq_snf2_Schurch_2015")
dir.counts <- file.path(dir.snf2, "data")
file.counts <- file.path(dir.counts, "counts.txt")
file.expDesign <- file.path(dir.counts, "expDesign.txt")

## Create a directory to download the dataset if it does not exist
dir.create(dir.counts, showWarnings = FALSE, recursive = TRUE)

## Download the data files if required
if (!file.exists(file.counts)) {
  message("Downloading count table from ", url.counts)
  download.file(url=file.path(url.counts, "counts.txt"), destfile = file.counts)
}
if (!file.exists(file.expDesign)) {
  message("Downloading design table from ", url.counts)
  download.file(url=file.path(url.counts, "expDesign.txt"), destfile = file.expDesign)
}

# Load the count table
count.table <- read.table(file=file.counts, sep="\t", header=TRUE, row.names=1)
# View(count.table)

# Load experimental design file
expDesign <- read.table(file=file.expDesign, sep="\t", header=TRUE)
#View(expDesign)
```

Phenotypic data

The dataset contains RNA-Seq count data for a wild type strain (**WT**) and a **Snf2** mutant, with 48 biological replicates for each genotype.

All phenotypic informations are enclosed in a dedicated file. Note that the produced data.frame encodes the 'strains' columns as a factor[1].

```
# Check the first and last line of the phenotypic data
head(expDesign)
```

```
  label strain
1   WT1     WT
2   WT2     WT
3   WT3     WT
4   WT4     WT
5   WT5     WT
6   WT6     WT
```

```
tail(expDesign)
```

```
   label strain
91 Snf43    Snf
92 Snf44    Snf
93 Snf45    Snf
94 Snf46    Snf
95 Snf47    Snf
96 Snf48    Snf
```

```
## Count the number of sample in each class
table(expDesign$strain)
```

```
Snf  WT
 48  48
```

```
## Define a strain-specific color for each sample,
## and add it as a supplementary column to the phenotypic data
col.strain <- c("WT"="green","Snf"="orange") # Choose one color per strain
expDesign$color <- col.strain[as.vector(expDesign$strain)]
```

- Draw a barplot showing the number of reads in each sample. Use either the `colSums()` or the `apply()` function (run `help(colSums()` if you don't know this function).
- What can you say from this diagram?

View solution| Hide solution

Solution

```
barplot(colSums(count.table)/1000000,
        main="Total number of reads per sample (million)",
        col=expDesign$color,
#        names.arg = "",
        las=1,  horiz=TRUE,
        ylab="Samples", cex.names=0.5,
        xlab="Million counts")
```

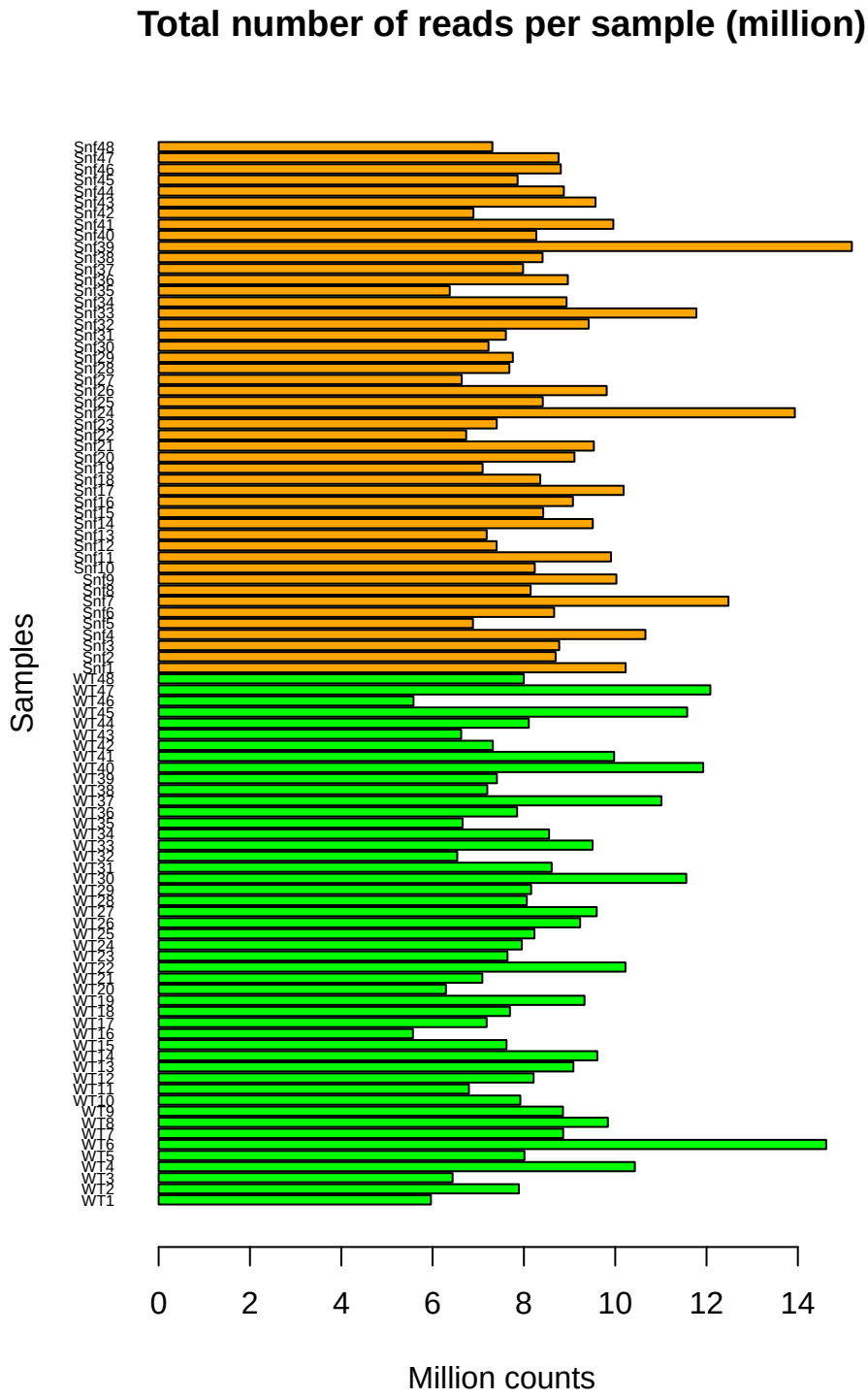


Figure 1: **Figure: Million counts per sample.**

The barplot indicates the library sizes (total number of reads) for each sample. We can see important differences, ranging from 5.57 to 15.2 million reads per sample.

This has important consequences: any read count should be interpreted relative to the sequencing depth of the corresponding sample. We will thus need to **normalise** the counts. Consequently, it makes not much sense to compute summary statistics per gene (mean counts, standard deviation, ...) before having normalized the data.

Descriptive statistics

Basic statistics

Before going further in the analysis, we will compute some descriptive statistics on the dataset. At this stage we only compute statistics per sample, since statistics per gene are meaningful only after library-wise normalization of the counts.

```
## Dimensions
nb_samples <- ncol(count.table)
print(nb_samples)
```

```
[1] 96
```

```
nb_genes <- nrow(count.table)
print(nb_genes)
```

```
[1] 7126
```

```
dim(count.table)
```

```
[1] 7126   96
```

```
## Min, Max, median (...).
## Here on the first 4 samples
head(summary(count.table[,1:4]))
```

```
      WT1              WT2              WT3              WT4        
 Min.   :     0   Min.   :     0   Min.   :     0   Min.   :     0  
 1st Qu.:    49   1st Qu.:    88   1st Qu.:    74   1st Qu.:   104  
 Median :   224   Median :   371   Median :   297   Median :   430  
 Mean   :   837   Mean   :  1107   Mean   :   903   Mean   :  1464  
 3rd Qu.:   561   3rd Qu.:   853   3rd Qu.:   670   3rd Qu.:  1019  
 Max.   :188825   Max.   :196804   Max.   :172119   Max.   :328674  
```

```
## A magic trick to convert column-wise summaries into a data.frame.
## The do.call() function produces a data frame with one col per sample,
## we transpose it to obtain one row per sample and one column per statistics.
stats.per.sample <- data.frame(t(do.call(cbind, lapply(count.table, summary))))
head(stats.per.sample)
```

[1] A factor is a vector with levels (categories), which permits an efficient storage and indexing, but can in some cases lead to misleading effects. To circumvent this, we will sometimes have to convert the factor to a vector, with the R command `as.vector()`.

```
    Min. X1st.Qu. Median Mean X3rd.Qu.   Max.
WT1    0     49.0    224  837      561 189000
WT2    0     88.0    371 1110      853 197000
WT3    0     74.2    297  903      670 172000
WT4    0    104.0    430 1460     1020 329000
WT5    0     84.0    353 1120      819 225000
WT6    0    153.0    667 2050     1600 357000
```

```
## We can now add some columns to the stats per sample
stats.per.sample$libsum <- apply(count.table, 2, sum) ## libsum
# Add some percentiles
stats.per.sample$perc05 <- apply(count.table, 2, quantile, 0.05)
stats.per.sample$perc10 <- apply(count.table, 2, quantile, 0.10)
stats.per.sample$perc90 <- apply(count.table, 2, quantile, 0.90)
stats.per.sample$perc95 <- apply(count.table, 2, quantile, 0.95)
stats.per.sample$zeros <- apply(count.table==0, 2, sum)
stats.per.sample$percent.zeros <- 100*stats.per.sample$zeros/nrow(count.table)

# View(stats.per.sample)
kable(stats.per.sample[sample(1:ncol(count.table), size = 10),],
      caption = "**Table: statistics per sample. ** We only display a random selection of 10 samples. "
```

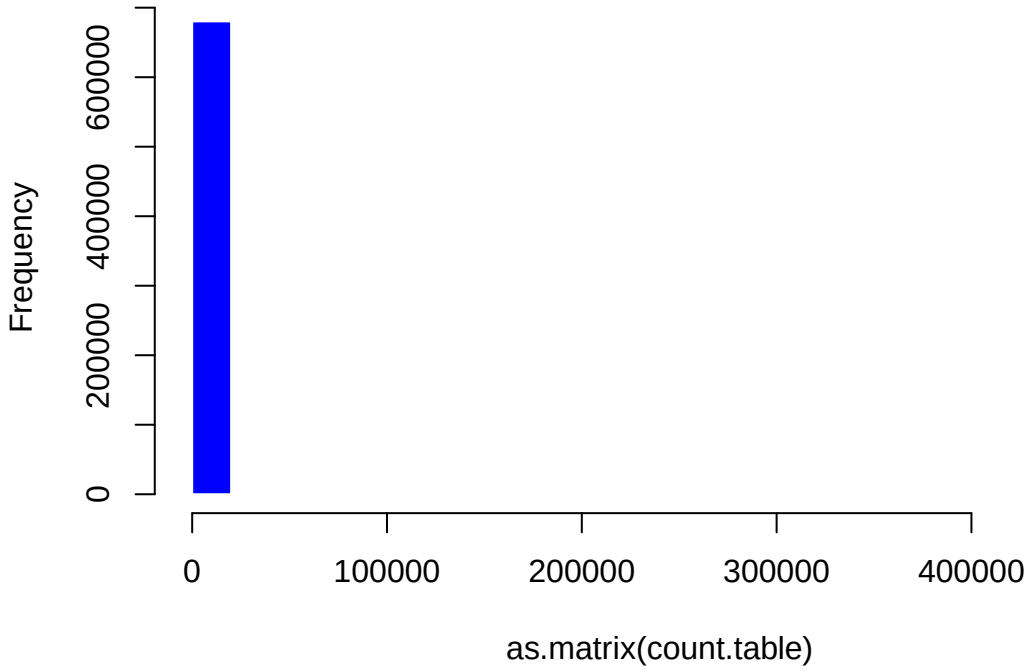
Table 1: **Table: statistics per sample.** We only display a random selection of 10 samples.

	Min.	X1st.Qu.	Median	Mean	X3rd.Qu.	Max.	libsum	perc05	perc10	perc90	perc95	zeros
Snf40	0	98.2	420	1160	942	181000	8271195	0	2	2146	3658	506
WT30	0	118.0	495	1620	1200	332000	11556151	0	2	2962	5308	501
Snf22	0	85.0	338	945	751	140000	6735821	0	2	1730	3066	522
WT5	0	84.0	353	1120	819	225000	8012039	0	2	1898	3437	525
WT11	0	55.0	244	954	611	256000	6795757	0	1	1600	2885	614
WT41	0	91.2	420	1400	1020	279000	9975448	0	2	2438	4340	517
WT29	0	83.0	353	1140	816	242000	8158307	0	2	1918	3487	514
WT33	0	91.0	390	1330	923	274000	9504114	0	2	2172	4132	531
Snf37	0	102.0	428	1120	954	152000	7982576	0	2	2106	3528	503
WT23	0	79.0	345	1070	822	185000	7640884	0	2	1934	3416	535

Distributions

The summary only displays a few milestone values (mean, median, quartiles). In order to get a better intuition of the data, we can draw an histogram of all values.

```
## Data distribution
hist(as.matrix(count.table), col="blue", border="white")
```



The histogram is not very informative so far, apparently due to the presence of a few very high count values, that impose a very large scale on the X axis. We can use a logarithmic transformation to improve the readability. Note that we will add pseudo count to avoid problems with "zero" counts observed for some genes in some samples.

```
## Data distribution in log scale.
epsilon <- 1 # pseudo-count to avoid problems with log(0)

## Logarithmic transformation
hist(as.matrix(log2(count.table + epsilon)), breaks=100, col="blue", border="white",
     main="", xlab="log2(counts)", ylab="Number of genes")
```

```
## Boxplots
boxplot(log2(count.table + epsilon), col=expDesign$color, pch=".",
        horizontal=TRUE, cex.axis=0.5,
        las=1, ylab="Samples", xlab="log2(Counts +1)")
```

Another way to get an intuition of the value distributions is to use the *plotDensity()* function, which draws one density curve for each sample.

```
## Density
## We will require one function from the affy package
if(!require("affy")){
  source("http://bioconductor.org/biocLite.R")
  biocLite("affy")
}
library(affy)
plotDensity(log2(count.table + epsilon), lty=1, col=expDesign$color, lwd=2)
```

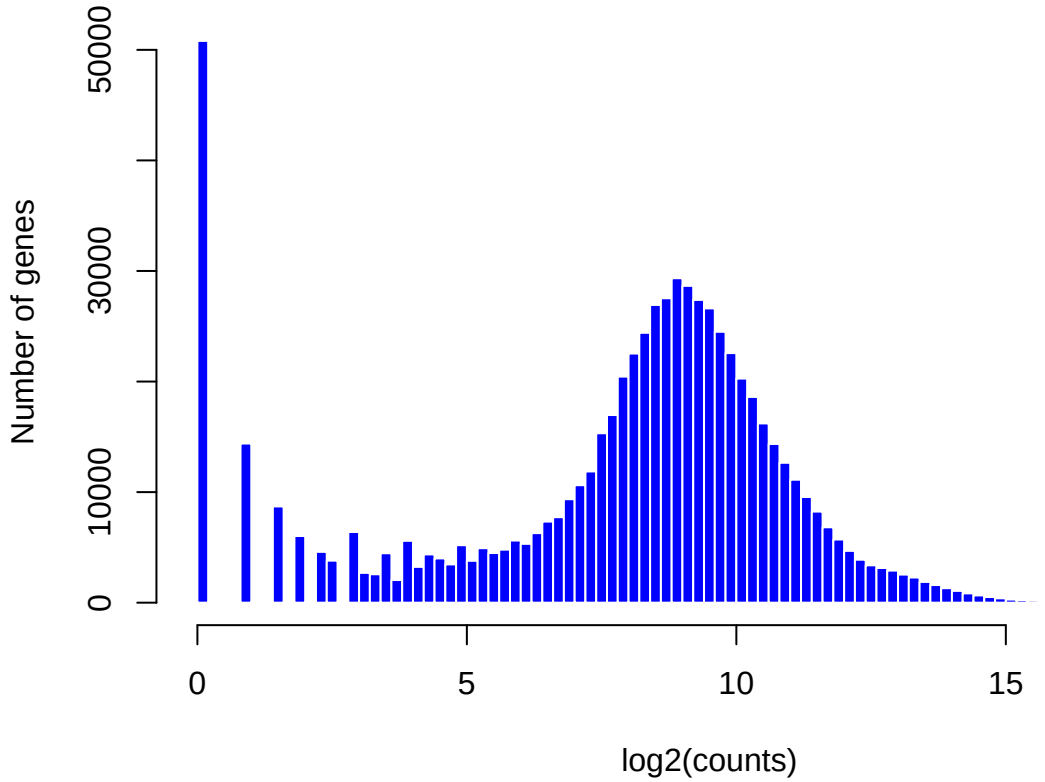
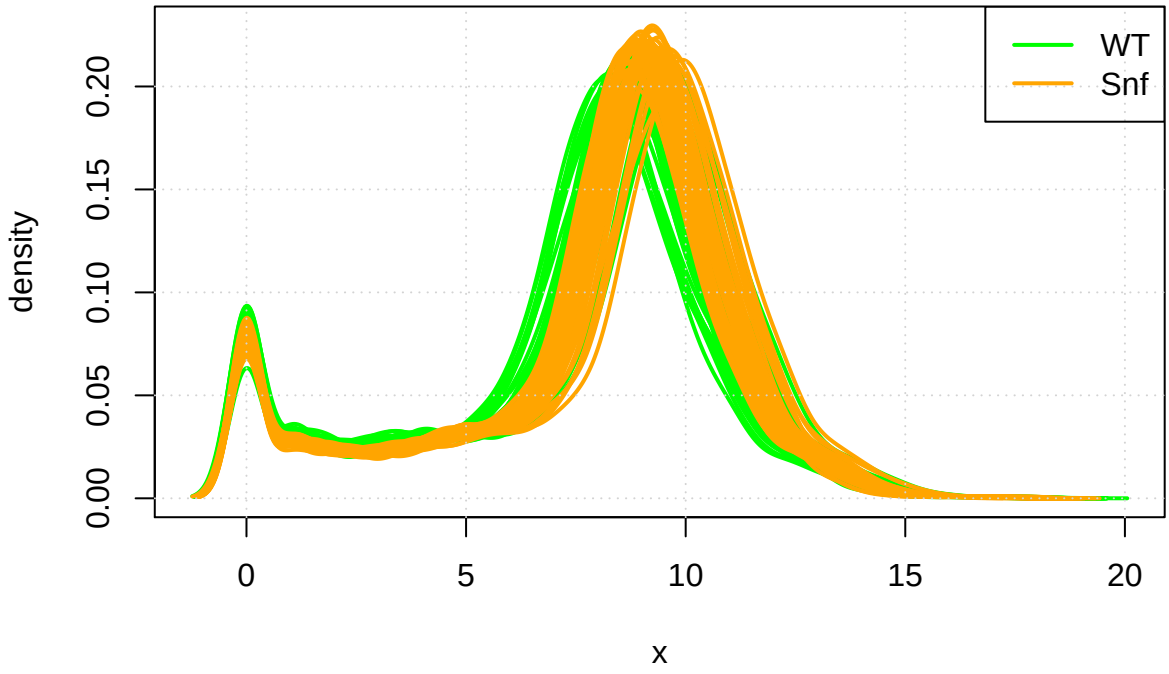


Figure 2: Histogram of log2-transformed counts per gene.

```
grid()
legend("topright", legend=names(col.strain), col=col.strain, lwd=2)
```



Beware: the R function *plotDensity()* does not display the actual distribution of your values, but a polynomial

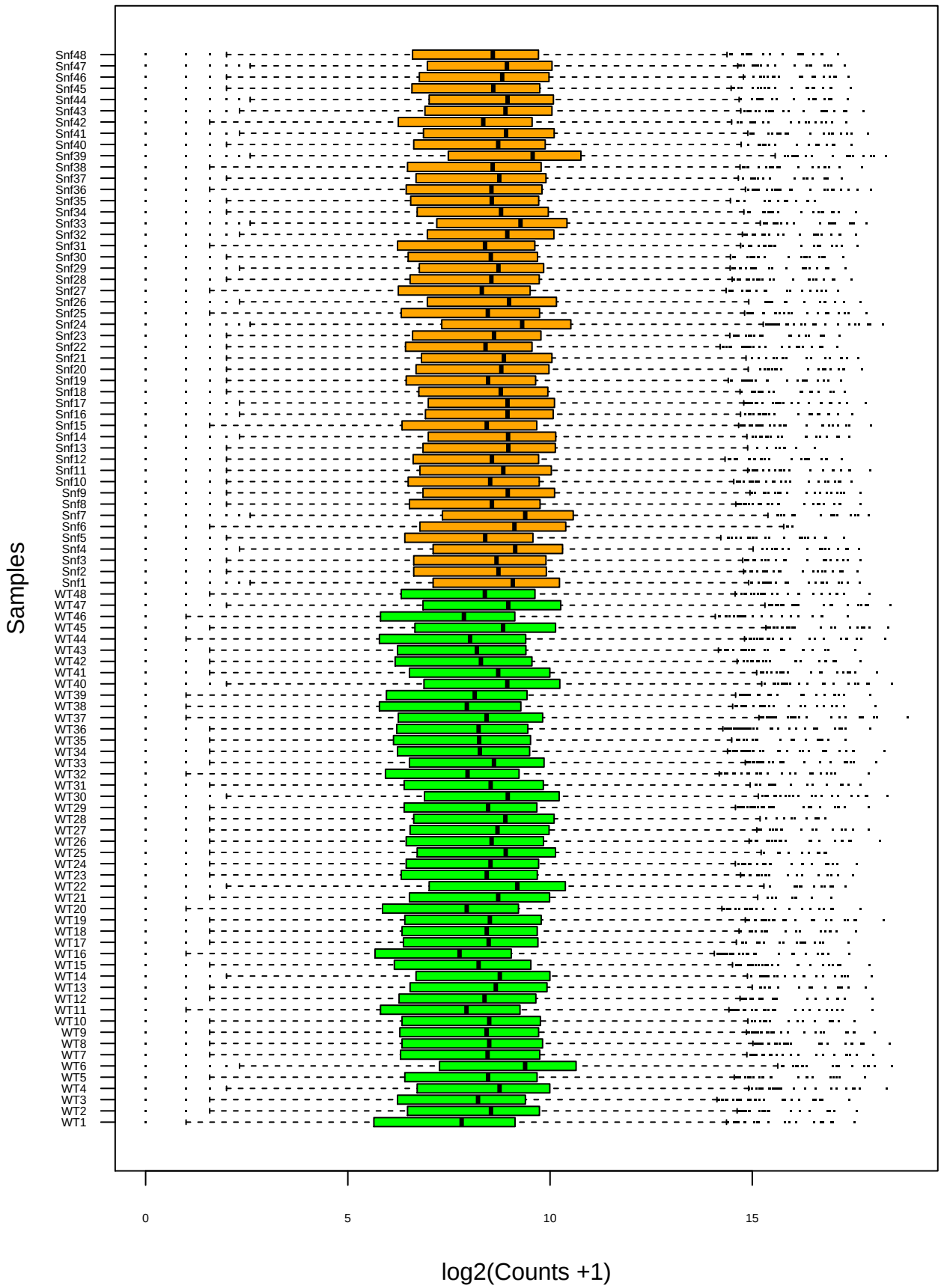


Figure 3: Box plots of non-normalized log2(counts) per sample.

fit. The representation thus generally looks smoother than the actual data. It is important to realize that, in some particular cases, the fit can lead to extrapolated values which can be misleading.

Scatter plots

Let's have a look at the scatter plots using the *pairs()* function. We will only represent 10 randomly selected samples.

```
## Define a function to draw a scatter plot for a pair of variables (samples) with density colors
plotFun <- function(x,y){
  dns <- densCols(x,y);
  points(x,y, col=dns, pch=".", panel.first=grid());
#  abline(a=0, b=1, col="brown")
  }

## Plot the scatter plot for a few pairs of variables selected at random
set.seed(123) # forces the random number generator to produce fixed results
pairs(log2(count.table[,sample(ncol(count.table),5)] + epsilon),
      panel=plotFun, lower.panel = NULL)
```

The command *pairs()* draws a scatter plot for each pair of columns of the input dataset. The plot shows a fairly good reproducibility between samples of the same type (WT or KO, respectively): all points are aligned along te diagonal, with a relatively wider dispersion at the bottom, corresponding to small number fluctuations.

In contrast, on all the plots comparing a WT and a KO sample, we can see some points (genes) discarding from the diagonal.

Eliminating undetected genes

All genes from genome the *S. cerevisiae* where quantified. However only a fraction of them where expressed and some of them where to weakly expressed to be detected in any of the sample. As a result the count table may contain rows with only zero values (null counts).

- What is the percentage of gene having null counts per sample. Draw a barplot.
- Some genes were not detected in any of the sample. Count their number, and delete them from the **count.table** data.frame.

View solution| Hide solution

Solution

```
prop.null <- apply(count.table, 2, function(x) 100*mean(x==0))
print(head(prop.null))
```

```
 WT1  WT2  WT3  WT4  WT5  WT6
9.22 7.27 7.37 7.07 7.37 6.05
```

```
barplot(prop.null, main="Percentage of null counts per sample",
        horiz=TRUE, cex.names=0.5, las=1,
        col=expDesign$color, ylab='Samples', xlab='% of null counts')
```

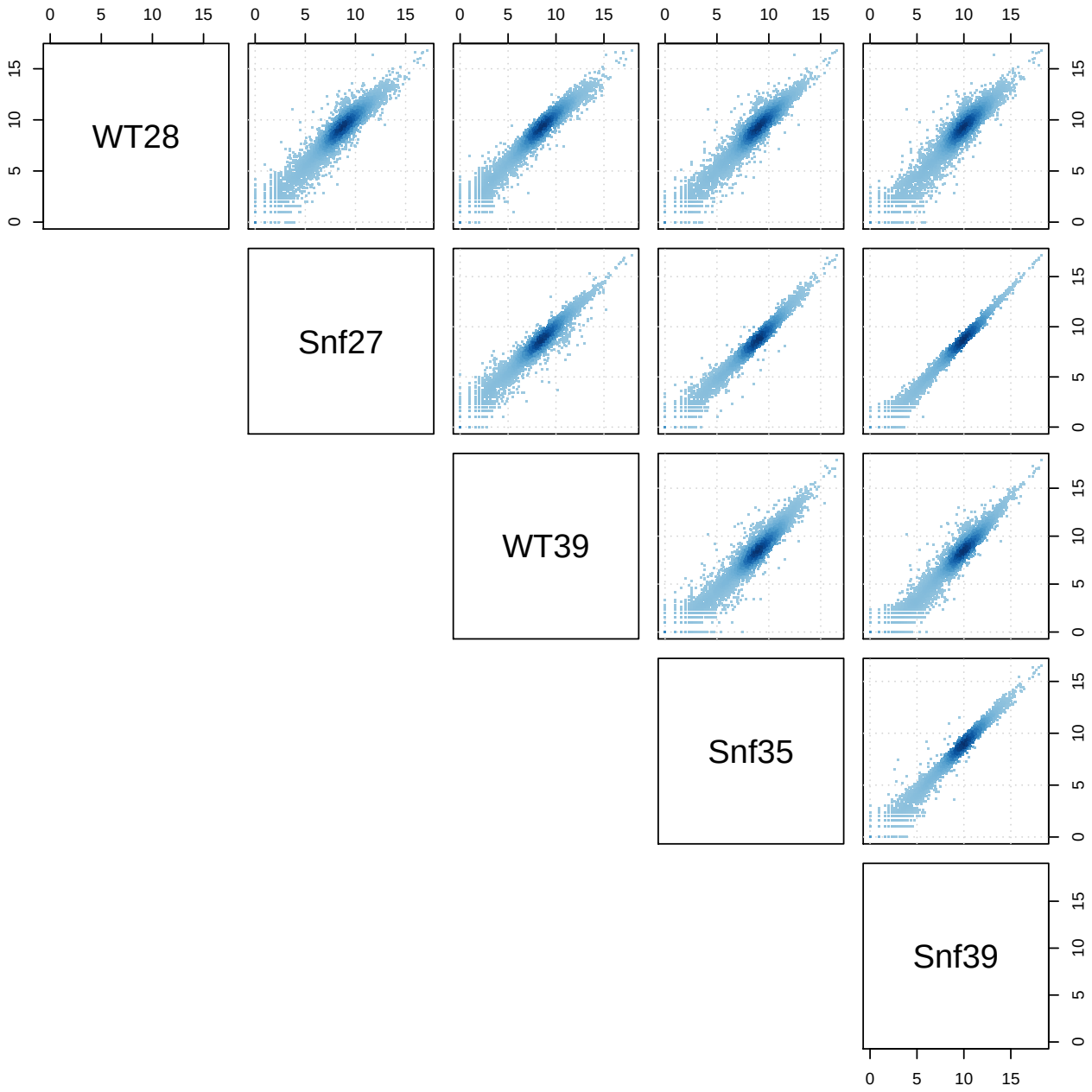


Figure 4: **Scatter plot of log2-counts for a random selection of samples.**

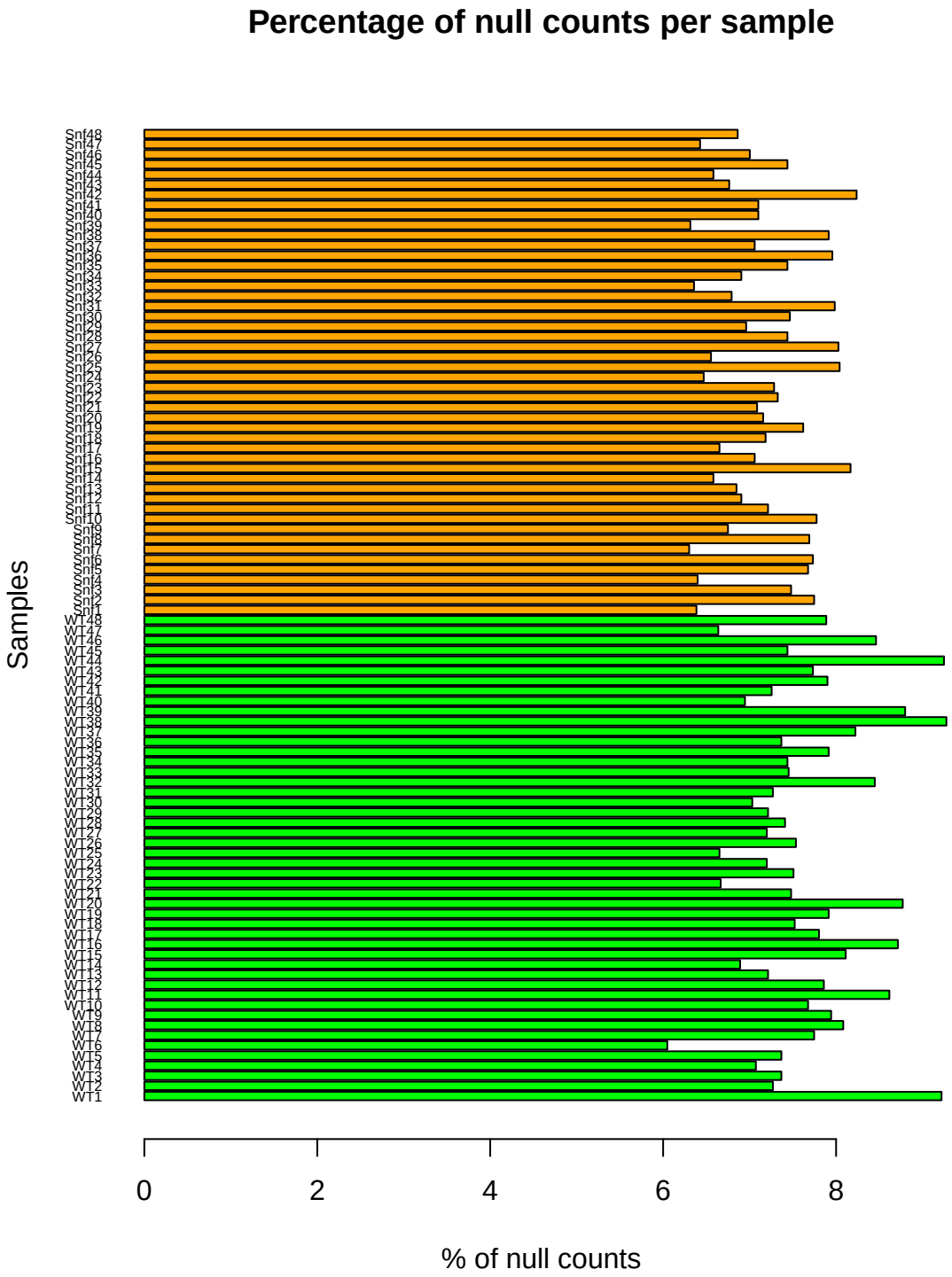


Figure 5: **Percentage of null counts per sample.**

```
## Some genes were not detected at all in these samples. We will discard them.
count.table <- count.table[rowSums(count.table) > 0,]
```

Selecting randomly samples

One of the question that will drive the analysis will be to define the impact of the number of sample on the analysis.

The original study contained 48 replicates per genotype, what happens if we select a smaller number?

Each attendee of this course select a given number (e.g. 3,4,5,10,15,20, 35, 40, 45...) and adapt the code below run the analysis with that number of replicates per genotype. We will at the end then compare the results (number of genes, significance, ...).

```
nb.replicates <- 10 ## Each attendee chooses a number (3,4,5,10,15 or 20)

samples.WT <- sample(1:48, size=nb.replicates, replace=FALSE)

## Random sampling of the Snf2 replicates (columns 49 to 96)
samples.Snf2 <- sample(49:96, size=nb.replicates, replace=FALSE)

selected.samples <- c(samples.WT, samples.Snf2)

# Don't forget to update colors
col.pheno.selected <- expDesign$color[selected.samples]
```

Differential analysis with DESeq2

In this section we will search for genes whose expression is affected by the genetic invalidation. You will first need to install the **DESeq2** bioconductor library then load it.

```
## Install the library if needed then load it
if(!require("DESeq2")){
  install.packages("lazyeval")
  install.packages("ggplot2")

  source("http://bioconductor.org/biocLite.R")
  biocLite("DESeq2")
}
library("DESeq2")
```

Creating a DESeqDataSet dataset

We will then create a **DESeqDataSet** using the **DESeqDataSetFromMatrix()** function. Get some help about the **DESeqDataSet** and have a look at some important accessor methods: **counts**, **conditions**, **estimateSizeFactors**, **sizeFactors**, **estimateDispersions** and **nbinomTest**.

```
## Use the DESeqDataSetFromMatrix to create a DESeqDataSet object
dds0 <- DESeqDataSetFromMatrix(countData = count.table[,selected.samples ], colData = expDesign[selecte
print(dds0)
```

```
class: DESeqDataSet
dim: 6887 20
metadata(1): version
assays(1): counts
rownames(6887): 15s_rrna 21s_rrna ... ty(gua)m2 ty(gua)o
rowData names(0):
colnames(20): WT3 WT25 ... Snf40 Snf26
colData names(3): label strain color
```

```
## What kind of object is it ?
is(dds0)
```

```
[1] "DESeqDataSet"                "RangedSummarizedExperiment"
[3] "SummarizedExperiment"        "Vector"
[5] "Annotated"
```

```
isS4(dds0)
```

```
[1] TRUE
```

```
## What does it contain ?
# The list of slot names
slotNames(dds0)
```

```
[1] "design"             "dispersionFunction" "rowRanges"
[4] "colData"            "assays"             "NAMES"
[7] "elementMetadata"    "metadata"
```

```
## Get some help about the "CountDataSet" class.
## NOT RUN
#?"DESeqDataSet-class"
```

Normalization

The normalization procedure (RLE) is implemented through the **estimateSizeFactors** function.

How is the scaling factor computed ?

Given a matrix with p columns (samples) and n rows (genes) this function estimates the size factors as follows: Each column element is divided by the **geometric means** of the rows. For each sample, the **median** (or, if requested, another location estimator) **of these ratios** (skipping the genes with a geometric mean of zero) is used as the size factor for this column.

The scaling factor for sample j is thus obtained as:

$$sf_j = median(\frac{K_{g,j}}{(\prod_{j=1}^{p} K_{g,j})^{1/p}})$$

```
### Let's implement such a function
### cds is a countDataset
estimSf <- function (cds){
    # Get the count matrix
    cts <- counts(cds)

    # Compute the geometric mean
    geomMean <- function(x) prod(x)^(1/length(x))

    # Compute the geometric mean over the line
    gm.mean  <-  apply(cts, 1, geomMean)

    # Zero values are set to NA (avoid subsequentcdsdivision by 0)
    gm.mean[gm.mean == 0] <- NA

    # Divide each line by its corresponding geometric mean
    # sweep(x, MARGIN, STATS, FUN = "-", check.margin = TRUE, ...)
    # MARGIN: 1 or 2 (line or columns)
    # STATS: a vector of length nrow(x) or ncol(x), depending on MARGIN
    # FUN: the function to be applied
    cts <- sweep(cts, 1, gm.mean, FUN="/")

    # Compute the median over the columns
    med <- apply(cts, 2, median, na.rm=TRUE)

    # Return the scaling factor
    return(med)
}
```

Now, check that the results obtained with our function are the same as those produced by DESeq. The method associated with normalization for the "CountDataSet" class is **estimateSizeFactors()**.

```
## Normalizing using the method for an object of class"CountDataSet"
dds.norm <-  estimateSizeFactors(dds0)
sizeFactors(dds.norm)
```

```
  WT3  WT25  WT42  WT47  WT21  WT46  WT20  WT28  WT23   WT5 Snf44 Snf12
0.771 1.218 0.820 1.296 1.075 0.619 0.644 1.197 0.889 0.903 1.283 0.994
 Snf2 Snf15 Snf42 Snf39 Snf30 Snf27 Snf40 Snf26
1.109 0.924 0.869 2.004 0.962 0.843 1.098 1.339
```

```
## Now get the scaling factor with our homemade function.cds.norm
head(estimSf(dds0))
```

```
  WT3  WT25  WT42  WT47  WT21  WT46
0.771 1.218 0.820 1.296 1.075 0.619
```

```
all(round(estimSf(dds0),6) == round(sizeFactors(dds.norm), 6))
```

```
[1] TRUE
```

```
## Checking the normalization
par(mfrow=c(2,2),cex.lab=0.7)
boxplot(log2(counts(dds.norm)+epsilon),  col=col.pheno.selected, cex.axis=0.7,
        las=1, xlab="log2(counts)", horizontal=TRUE, main="Raw counts")
boxplot(log2(counts(dds.norm, normalized=TRUE)+epsilon),  col=col.pheno.selected, cex.axis=0.7,
        las=1, xlab="log2(normalized counts)", horizontal=TRUE, main="Normalized counts")
plotDensity(log2(counts(dds.norm)+epsilon),  col=col.pheno.selected,
            xlab="log2(counts)", cex.lab=0.7, panel.first=grid())
plotDensity(log2(counts(dds.norm, normalized=TRUE)+epsilon), col=col.pheno.selected,
            xlab="log2(normalized counts)", cex.lab=0.7, panel.first=grid())
```

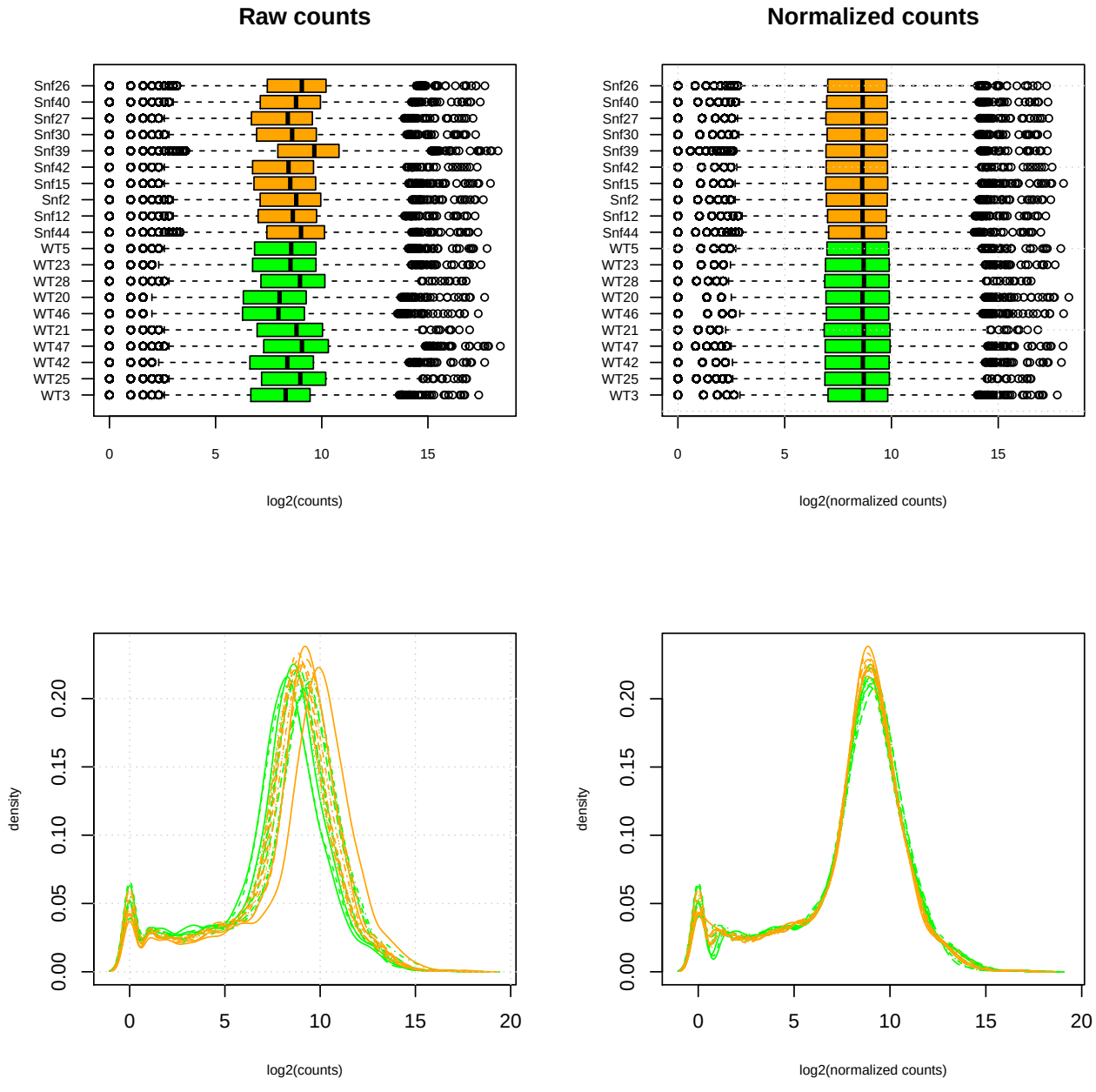


Figure 6: **Impact of the count normalization.**

Count variance is related to mean

As you can see from the following plot the relationship between variance and mean is not strictly linear. This can be shown by the poor fit that is obtained using a linear regression.

```
## Computing mean and variance
norm.counts <- counts(dds.norm, normalized=TRUE)
mean.counts <- rowMeans(norm.counts)
variance.counts <- apply(norm.counts, 1, var)

## sum(mean.counts==0) # Number of completely undetected genes

norm.counts.stats <- data.frame(
  min=apply(norm.counts, 2, min),
  mean=apply(norm.counts, 2, mean),
  median=apply(norm.counts, 2, median),
  max=apply(norm.counts, 2, max),
  zeros=apply(norm.counts==0, 2, sum),
  percent.zeros=100*apply(norm.counts==0, 2, sum)/nrow(norm.counts),
  perc05=apply(norm.counts, 2, quantile, 0.05),
  perc10=apply(norm.counts, 2, quantile, 0.10),
  perc90=apply(norm.counts, 2, quantile, 0.90),
  perc95=apply(norm.counts, 2, quantile, 0.95)
)

kable(norm.counts.stats)
```

	min	mean	median	max	zeros	percent.zeros	perc05	perc10	perc90	perc95
WT3	0	1213	407	223341	286	4.15	1.298	6.49	2162	3863
WT25	0	981	416	95177	235	3.41	0.821	5.75	2016	3376
WT42	0	1296	404	254884	324	4.71	1.219	6.10	2300	4071
WT47	0	1353	413	268933	234	3.40	0.771	6.17	2323	4179
WT21	0	957	417	118777	294	4.27	0.930	5.58	2097	3390
WT46	0	1309	400	273555	364	5.29	0.000	6.46	2280	4085
WT20	0	1419	399	324143	386	5.61	0.000	4.66	2431	4537
WT28	0	978	419	95311	289	4.20	0.835	4.18	2029	3347
WT23	0	1248	414	208093	296	4.30	1.125	5.62	2240	3980
WT5	0	1289	417	249736	286	4.15	1.108	6.65	2163	3903
Snf44	0	1005	405	130568	230	3.34	0.780	7.02	1848	3112
Snf12	0	1081	399	153508	253	3.67	1.006	7.04	1940	3435
Snf2	0	1138	400	180318	313	4.54	0.902	5.41	2066	3567
Snf15	0	1323	395	272840	343	4.98	1.082	5.41	2219	4094
Snf42	0	1152	397	188354	348	5.05	0.000	5.75	2103	3674
Snf39	0	1100	400	161407	211	3.06	0.998	5.99	2034	3519
Snf30	0	1091	404	161522	293	4.25	1.040	6.24	1985	3427
Snf27	0	1143	400	169171	333	4.83	1.186	5.93	2076	3692
Snf40	0	1094	402	164662	267	3.88	0.911	5.47	2016	3437
Snf26	0	1064	398	157066	228	3.31	0.747	5.97	1951	3329

```
## Mean and variance relationship
mean.var.col <- densCols(x=log2(mean.counts), y=log2(variance.counts))
plot(x=log2(mean.counts), y=log2(variance.counts), pch=16, cex=0.5,
```

```
     col=mean.var.col, main="Mean-variance relationship",
     xlab="Mean log2(normalized counts) per gene",
     ylab="Variance of log2(normalized counts)",
     panel.first = grid())
abline(a=0, b=1, col="brown")
```

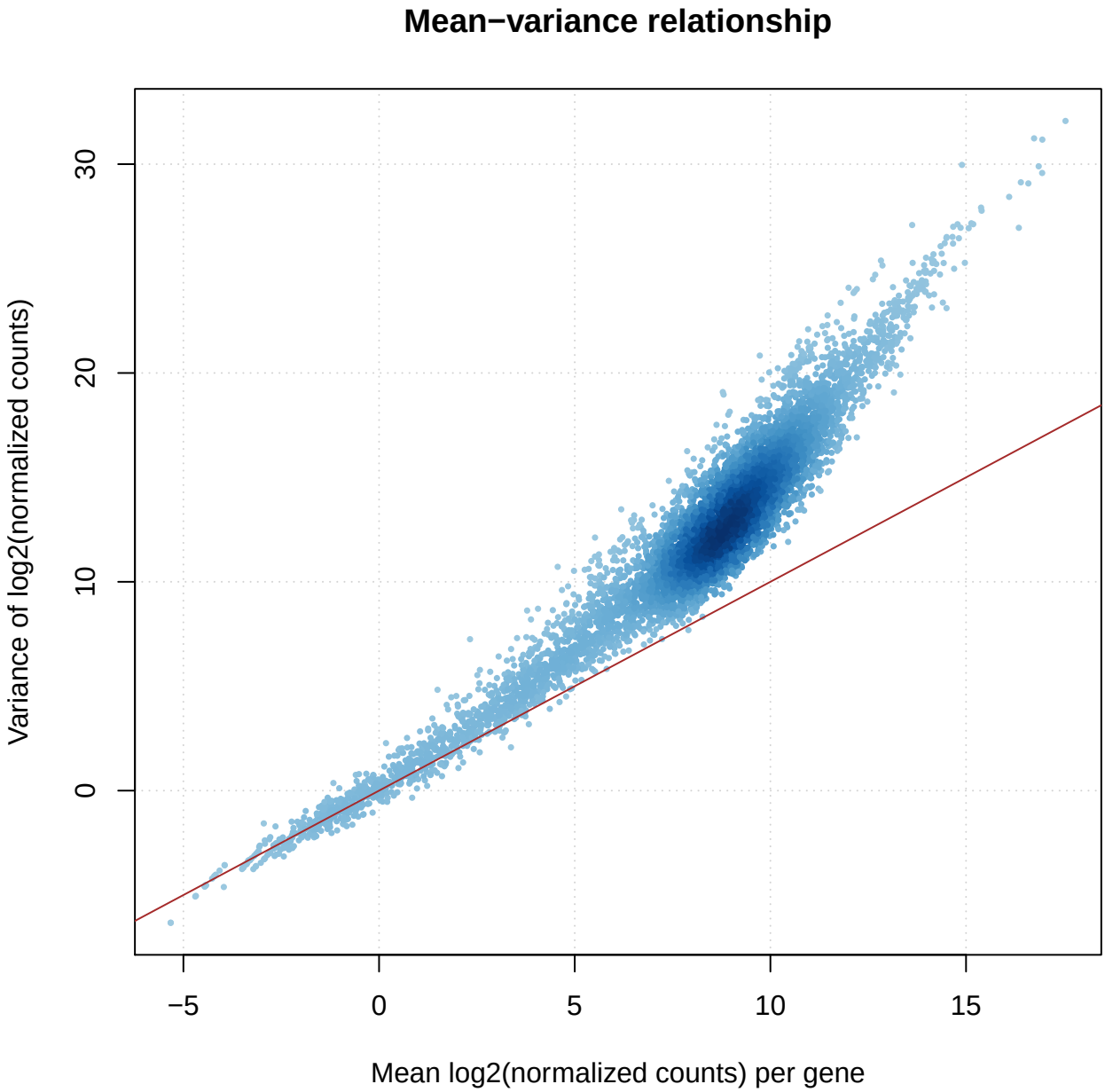


Figure 7: **Figure: variance/mean plot.** The brown line highlights $x = y$, which corresponds to the expected relationship between mean and variance for a Poisson distribution.

Modeling read counts

Let us imagine that we would produce a lot of RNA-Seq experiments from the same samples (technical replicates). For each gene g the measured read counts would be expected to vary rather slighlty around the

expected mean and would be probably well modeled using a Poisson distribution. However, when working with biological replicates more variations are intrinsically expected. Indeed, the measured expression values for each genes are expected to fluctuate more importantly, due to the combination of biological and technical factors: inter-individual variations in gene regulation, sample purity, cell-synchronization issues or reponses to environment (e.g. heat-shock).

The Poisson distribution has only one parameter indicating its expected mean : λ. The variance of the distribution equals its mean λ. Thus in most cases, the Poisson distribution is not expected to fit very well with the count distribution in biological replicates, since we expect some over-dispersion (greater variability) due to biological noise.

As a consequence, when working with RNA-Seq data, many of the current approaches for differential expression call rely on an alternative distribution: the *negative binomial* (note that this holds true also for other -Seq approaches, e.g. ChIP-Seq with replicates).

What is the negative binomial ?

The negative binomial distribution is a discrete distribution that give us the probability of observing x failures before a target number of succes n is obtained. As we will see later the negative binomial can also be used to model over-dispersed data (in this case this overdispersion is relative to the poisson model).

The probability of x failures before n success

First, given a Bernouilli trial with a probability p of success, the **negative binomial** distribution describes the probability of observing x failures before a target number of successes n is reached. In this case the parameters of the distribution will thus be p, n (in **dnbinom()** function of R, n and p are denoted by arguments **size** and **prob** respectively).

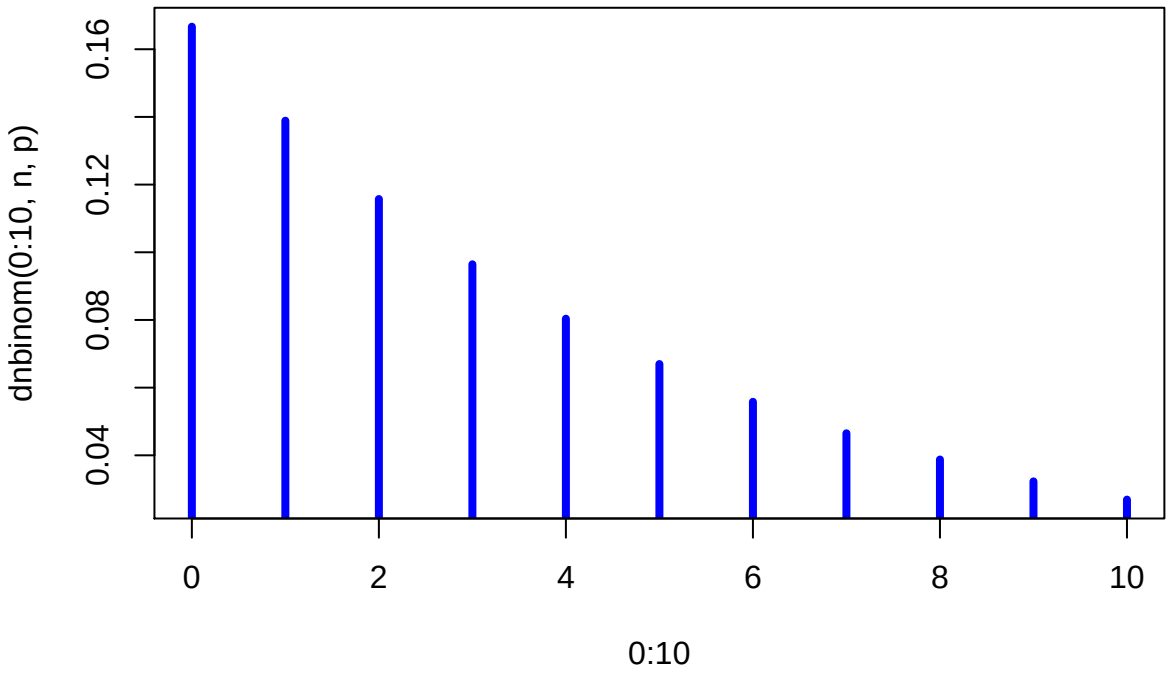
$$P_{NegBin}(x; n, p) = \binom{x+n-1}{x} \cdot p^n \cdot (1-p)^x = C^x_{x+n-1} \cdot p^n \cdot (1-p)^x$$

In this formula, p^n denotes the probability to observe n successes, $(1-p)^x$ the probability of x failures, and the binomial coefficient C^x_{x+n-1} indicates the number of possible ways to dispose x failures among the $x+n-1$ trials that precede the last one (the problem statement imposes for the last trial to be a success).

The negative binomial distribution has expected value $n\frac{q}{p}$ and variance $n\frac{q}{p^2}$. Some examples of using this distribution in R are provided below.

Particular case: when $n = 1$ the negative binomial corresponds to the the **geometric distribution**, which models the probability distribution to observe the first success after x failures: $P_{NegBin}(x; 1, p) = P_{geom}(x; p) = p \cdot (1-p)^x$.

```
par(mfrow=c(1,1))

## Some intuition about the negative binomiale parametrized using n and p.
## The simple case, one success (see geometric distribution)
# Let's have a look at the density
p <- 1/6 # the probability of success
n <- 1   # target for number of successful trials

# The density function
plot(0:10, dnbinom(0:10, n, p), type="h", col="blue", lwd=4)
```




```
# the probability of zero failure before one success.
# i.e the probability of success
dnbinom(0, n , p)
```

```
[1] 0.167
```

```
## i.e the probability of at most 5 failure before one success.
sum(dnbinom(0:5, n , p)) # == pnbinom(5, 1, p)
```

```
[1] 0.665
```

```
## The probability of at most 10 failures before one sucess
sum(dnbinom(0:10, n , p)) # == pnbinom(10, 1, p)
```

```
[1] 0.865
```

```
## The probability to have more than 10 failures before one sucess
1-sum(dnbinom(0:10, n , p)) # == 1 - pnbinom(10, 1, p)
```

```
[1] 0.135
```

```
## With two successes
## The probability of x failure before two success (e.g. two six)
n <- 2
plot(0:30, dnbinom(0:30, n, p), type="h", col="blue", lwd=2,
     main="Negative binomial density",
     ylab="P(x; n,p)",
     xlab=paste("x = number of failures before", n, "successes"))
```

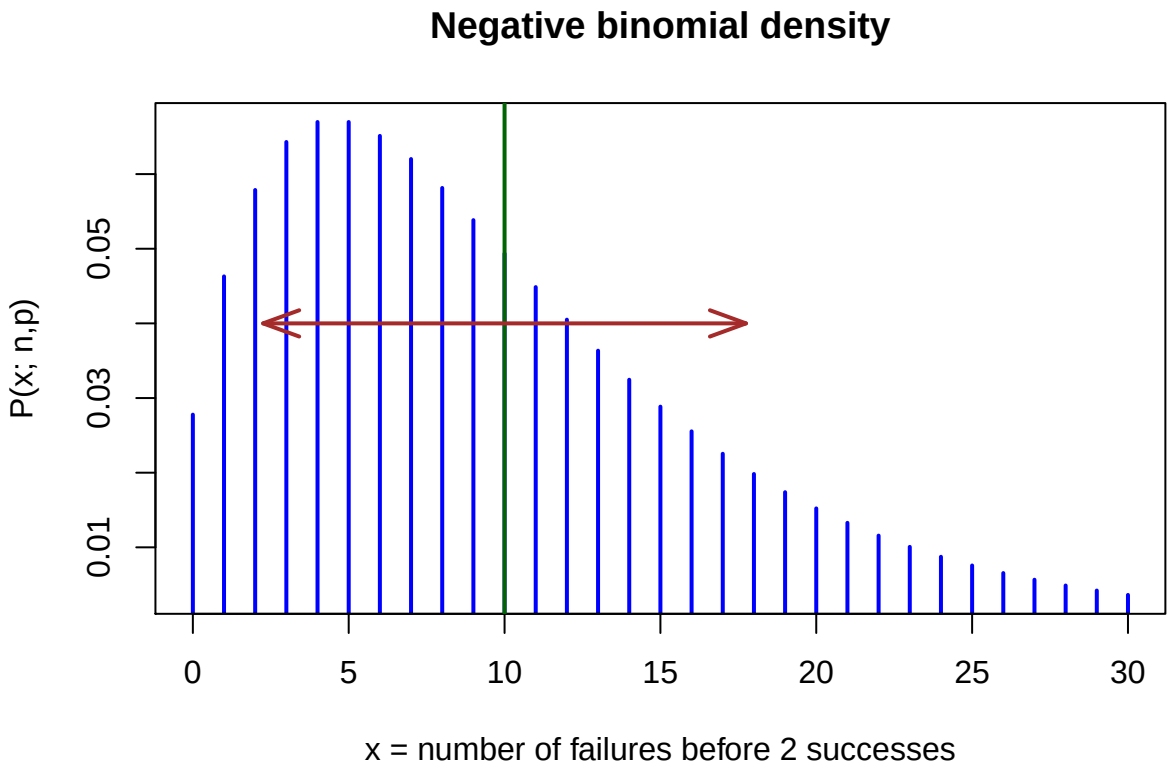
```r
# Expected value
q <- 1-p
(ev <- n*q/p)
```

```
[1] 10
```

```r
abline(v=ev, col="darkgreen", lwd=2)

# Variance
(v <- n*q/p^2)
```

```
[1] 60
```

```r
arrows(x0=ev-sqrt(v), y0 = 0.04, x1=ev+sqrt(v), y1=0.04, col="brown",lwd=2, code=3, , length=0.2, angle=
```



Using mean and dispersion

The second way of parametrizing the distribution is using the mean value m and the dispersion parameter r (in **dnbinom()** function of R, m and r are denoted by arguments **mu** and **size** respectively). The variance of the distribution can then be computed as $m + m^2/r$.

Note that m can be deduced from n and p.

```r
n <- 10
p <- 1/6
q <- 1-p
mu <- n*q/p

all(dnbinom(0:100, mu=mu, size=n) == dnbinom(0:100, size=n, prob=p))
```

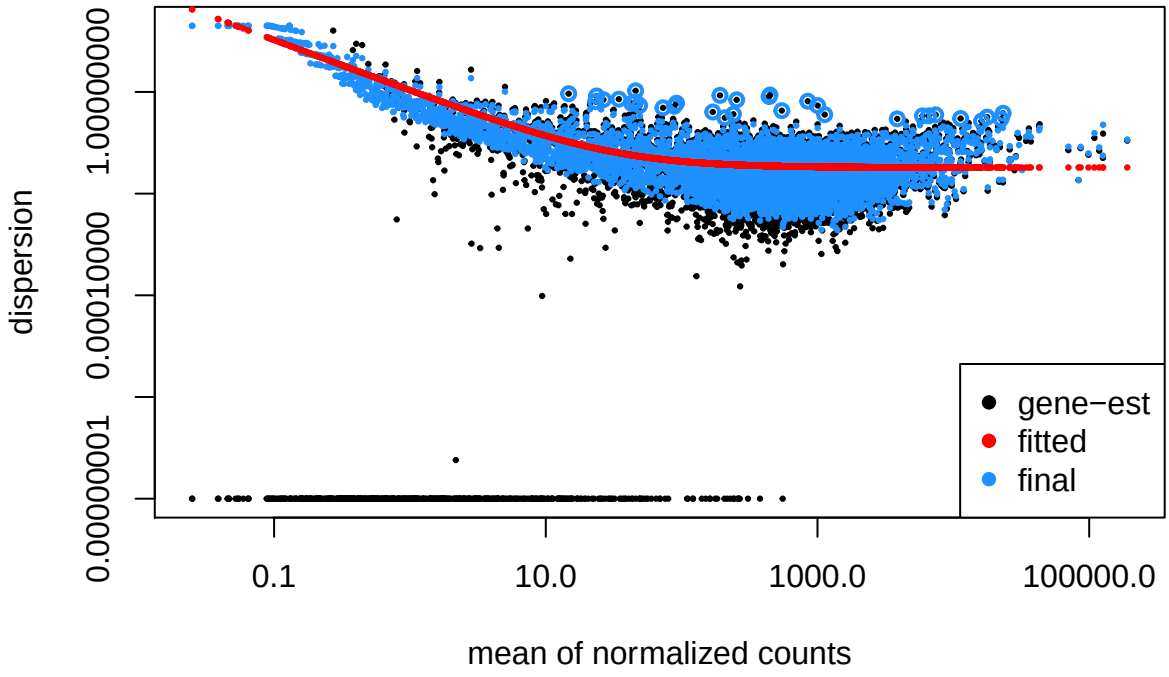
```
[1] TRUE
```

Modelling read counts through a negative binomial

To perform diffential expression call DESeq will assume that, for each gene, the read counts are generated by a negative binomial distribution. One problem here will be to estimate, for each gene, the two parameters of the negative binomial distribution: mean and dispersion.

- The mean will be estimated from the observed normalized counts in both conditions.
- The first step will be to compute a gene-wise dispersion. When the number of available samples is insufficient to obtain a reliable estimator of the variance for each gene, DESeq will apply a **shrinkage** strategy, which assumes that counts produced by genes with similar expression level (counts) have similar variance (note that this is a strong assumption). DESeq will regress the gene-wise dispersion onto the means of the normalized counts to obtain an estimate of the dispersion that will be subsequently used to build the binomial model for each gene.

```
## Performing estimation of dispersion parameter
dds.disp <- estimateDispersions(dds.norm)

## A diagnostic plot which
## shows the mean of normalized counts (x axis)
## and dispersion estimate for each genes
plotDispEsts(dds.disp)
```



Performing differential expression call

Now that a negative binomial model has been fitted for each gene, the **nbinomWaldTest** can be used to test for differential expression. The output is a data.frame which contains nominal p-values, as well as FDR values (correction for multiple tests computed with the Benjamini–Hochberg procedure).

```
alpha <- 0.0001
wald.test <- nbinomWaldTest(dds.disp)
res.DESeq2 <- results(wald.test, alpha=alpha, pAdjustMethod="BH")

## What is the object returned by nbinomTest()
class(res.DESeq2)
```

```
[1] "DESeqResults"
attr(,"package")
[1] "DESeq2"
```

```
is(res.DESeq2) # a data.frame
```

```
[1] "DESeqResults"    "DataFrame"       "DataTable"       "SimpleList"
[5] "DataTableORNULL" "List"            "Vector"          "Annotated"
```

```
slotNames(res.DESeq2)
```

```
[1] "rownames"        "nrows"           "listData"        "elementType"
[5] "elementMetadata" "metadata"
```

```
head(res.DESeq2)
```

```
log2 fold change (MAP): strain WT vs Snf
Wald test p-value: strain WT vs Snf
DataFrame with 6 rows and 6 columns
          baseMean log2FoldChange     lfcSE      stat    pvalue      padj
         <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
15s_rrna     14.74          0.896    0.4289      2.09  0.036655    0.0705
21s_rrna     91.73          0.979    0.3858      2.54  0.011192    0.0249
hra1          2.75          0.681    0.3860      1.77  0.077515    0.1340
icr1        142.71         -0.189    0.0911     -2.07  0.038038    0.0728
lsr1        202.49          0.851    0.2522      3.37  0.000743    0.0022
nme1         24.92          0.298    0.2207      1.35  0.176897    0.2688
```

```
## The column names of the data.frame
## Note the column padj
## contains FDR values (computed Benjamini-Hochberg procedure)
colnames(res.DESeq2)
```

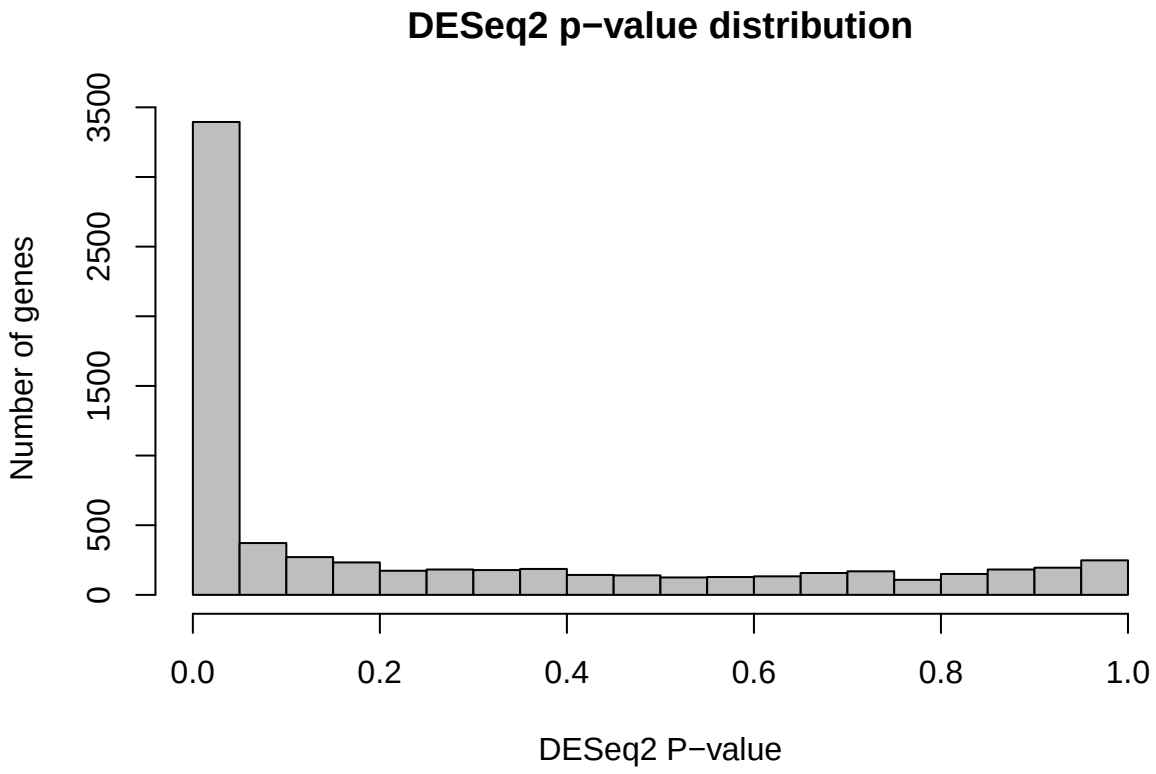
```
[1] "baseMean"       "log2FoldChange" "lfcSE"          "stat"
[5] "pvalue"         "padj"
```

```
## Order the table by decreasing p-valuer
res.DESeq2 <- res.DESeq2[order(res.DESeq2$padj),]
head(res.DESeq2)
```

```
log2 fold change (MAP): strain WT vs Snf
Wald test p-value: strain WT vs Snf
```

```
DataFrame with 6 rows and 6 columns
         baseMean log2FoldChange     lfcSE      stat    pvalue      padj
        <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
yar071w      1483           4.00    0.0973      41.1         0         0
yer001w       670           2.35    0.0620      37.9         0         0
ygr234w      2823           4.10    0.0984      41.6         0         0
yhr215w      1169           4.16    0.1090      38.1         0         0
yil121w       860           2.79    0.0570      49.0         0         0
yor290c       878           7.16    0.1806      39.7         0         0
```

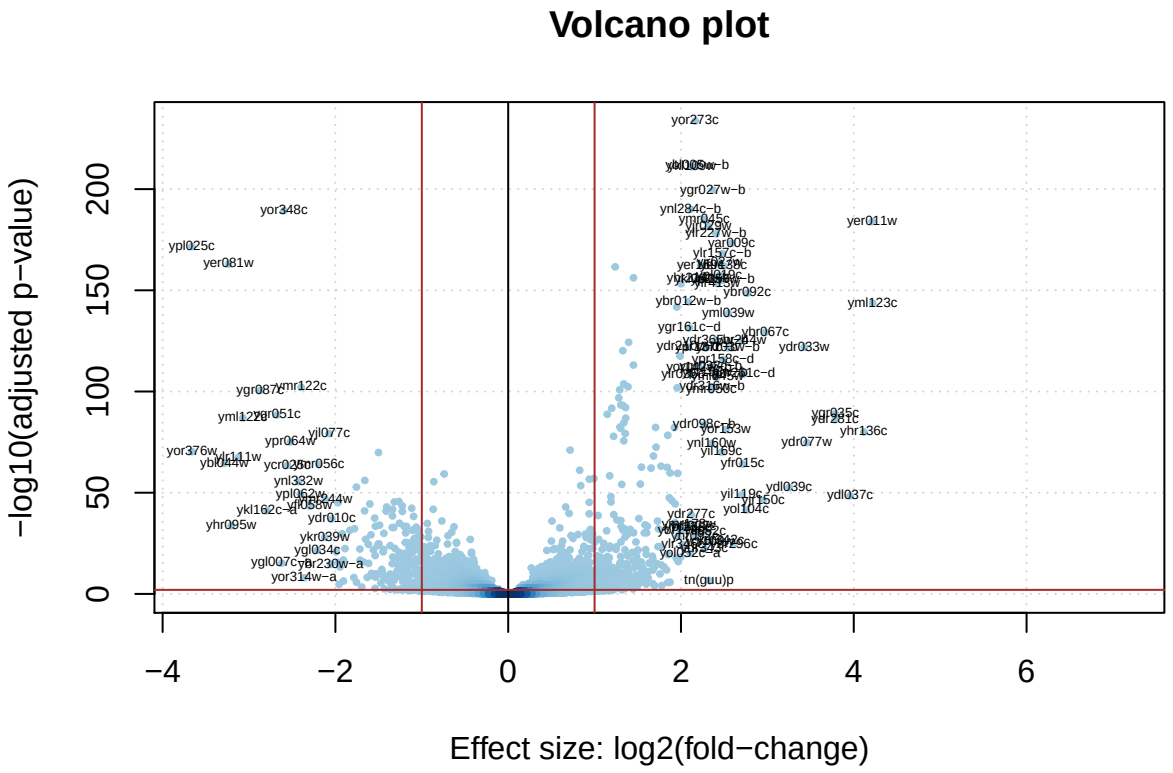
```
## Draw an histogram of the p-values
hist(res.DESeq2$padj, breaks=20, col="grey", main="DESeq2 p-value distribution", xlab="DESeq2 P-value",
```



Volcano plot

```
alpha <- 0.01 # Threshold on the adjusted p-value
cols <- densCols(res.DESeq2$log2FoldChange, -log10(res.DESeq2$pvalue))
plot(res.DESeq2$log2FoldChange, -log10(res.DESeq2$padj), col=cols, panel.first=grid(),
     main="Volcano plot", xlab="Effect size: log2(fold-change)", ylab="-log10(adjusted p-value)",
     pch=20, cex=0.6)
abline(v=0)
abline(v=c(-1,1), col="brown")
abline(h=-log10(alpha), col="brown")

gn.selected <- abs(res.DESeq2$log2FoldChange) > 2 & res.DESeq2$padj < alpha
text(res.DESeq2$log2FoldChange[gn.selected],
```

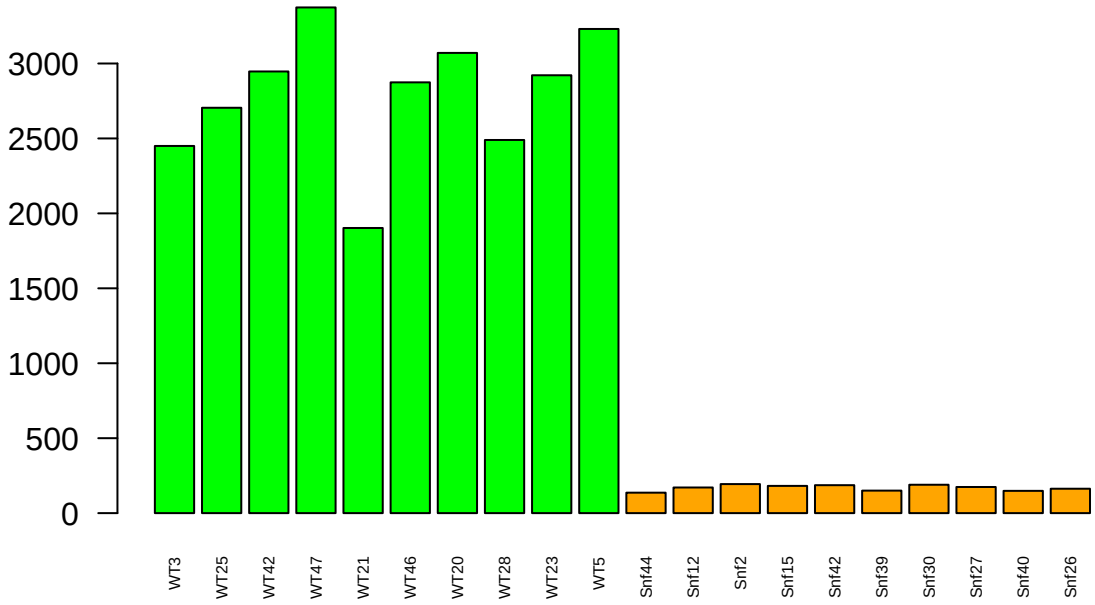
```
    -log10(res.DESeq2$padj)[gn.selected],
    lab=rownames(res.DESeq2)[gn.selected ], cex=0.4)
```



Check the expression levels of the most differentially expressed gene

It may be important to check the validity of our analysis by simply assessing the expression level of the most highly differential gene.

```
gn.most.sign <- rownames(res.DESeq2)[1]
gn.most.diff.val <- counts(dds.norm, normalized=T)[gn.most.sign,]
barplot(gn.most.diff.val, col=col.pheno.selected, main=gn.most.sign, las=2, cex.names=0.5)
```



Looking at the results with a MA plot

One popular diagram in dna chip analysis is the M versus A plot (MA plot) between two conditions a and b. In this representation :

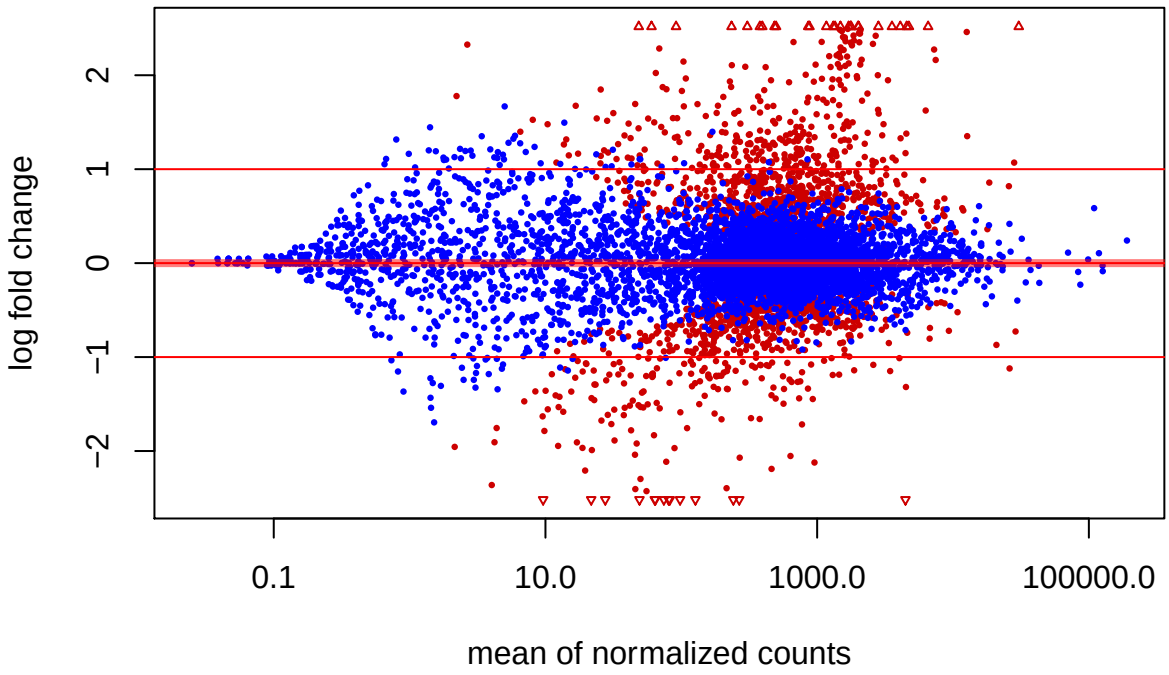
- M (Minus) is the log ratio of counts calculated for any gene.

$$M_g = log2(\bar{x}_{g,a}) - log2(\bar{x}_{g,b})$$

- A (add) is the average log counts which corresponds to an estimate of the gene expression level.

$$A_g = \frac{1}{2}(log2(\bar{x}_g, a) + log2(\bar{x}_g, b))$$

```
## Draw a MA plot.
## Genes with adjusted p-values below 1% are shown
plotMA(res.DESeq2, colNonSig = "blue")
abline(h=c(-1:1), col="red")
```



Hierarchical clustering

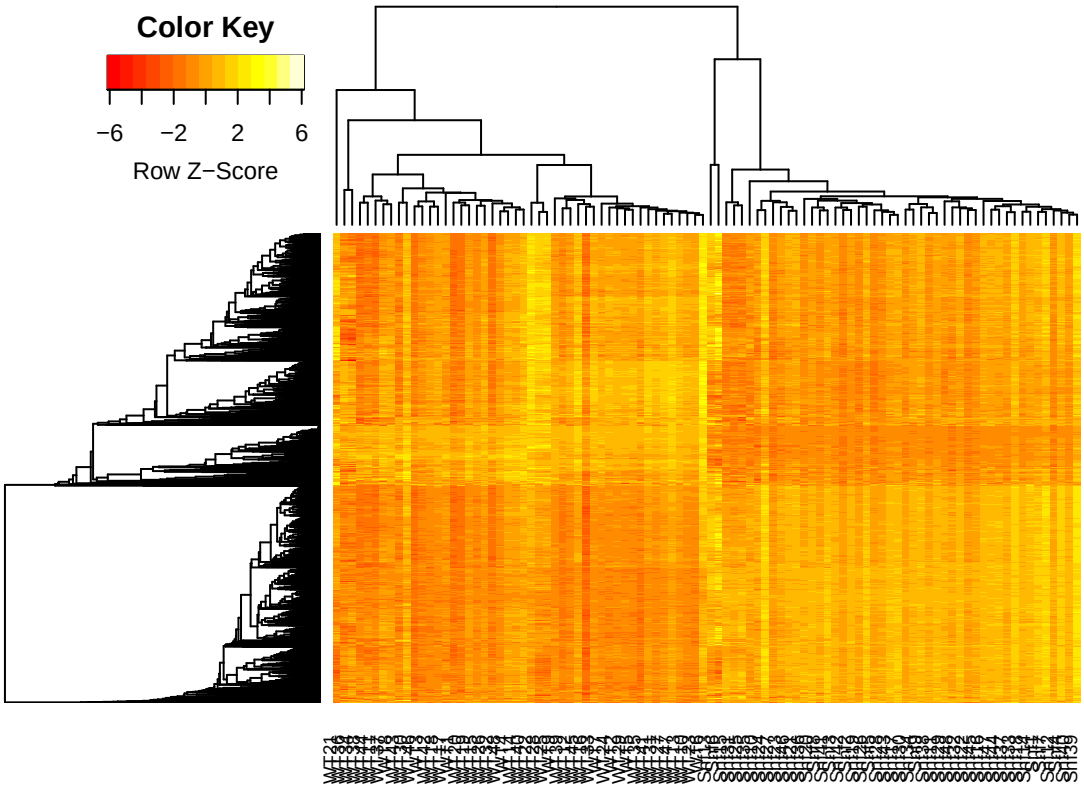
To ensure that the selected genes distinguish well between "treated"" and "untreated" condition we will perform a hierachical clustering using the **heatmap.2()** function from the gplots library.

```
## We select gene names based on FDR (1%)
gene.kept <- rownames(res.DESeq2)[res.DESeq2$padj <= alpha & !is.na(res.DESeq2$padj)]

## We retrieve the normalized counts for gene of interest
count.table.kept <- log2(count.table + epsilon)[gene.kept, ]
dim(count.table.kept)
```

```
[1] 2753   96
```

```
## Install the gplots library if needed then load it
if(!require("gplots")){
  install.packages("gplots")
}
library("gplots")

## Perform the hierarchical clustering with
## A distance based on Pearson-correlation coefficient
## and average linkage clustering as agglomeration criteria
heatmap.2(as.matrix(count.table.kept),
          scale="row",
          hclust=function(x) hclust(x,method="average"),
          distfun=function(x) as.dist((1-cor(t(x)))/2),
          trace="none",
```

```
          density="none",
          labRow="",
          cexCol=0.7)
```



Functional enrichment

We will now perform functional enrichment using the list of induced genes. This step will be performed using the gProfileR R library.

```
library(gProfileR)

res.DESeq2.df <- na.omit(data.frame(res.DESeq2))
induced.sign <- rownames(res.DESeq2.df)[res.DESeq2.df$log2FoldChange >= 2 &  res.DESeq2.df$padj < alpha
# head(induced.sign)
# names(term.induced)

term.induced <- gprofiler(query=induced.sign, organism="scerevisiae")
term.induced <- term.induced[order(term.induced$p.value),]
# term.induced$p.value
kable(term.induced[1:10,c("term.name",
                      "term.size",
                      "query.size",
                      "overlap.size",
                      "recall",
                      "precision",
                      "p.value",
```

```
                      "intersection")],
      format.args=c(engeneer=TRUE, digits=3), caption="**Table: functional analysis wit gProfileR. ** "
```

	term.name	term.si
32	RNA-DNA hybrid ribonuclease activity	
3	DNA integration	
22	RNA-directed DNA polymerase activity	
35	aspartic-type peptidase activity	
37	aspartic-type endopeptidase activity	
23	DNA-directed DNA polymerase activity	
31	endoribonuclease activity, producing 5'-phosphomonoesters	
21	DNA polymerase activity	
28	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters	
30	endoribonuclease activity	

And now using the list of repressed genes.

```
res.DESeq2.df <- na.omit(data.frame(res.DESeq2))
repressed.sign <- rownames(res.DESeq2.df)[res.DESeq2.df$log2FoldChange <= -2 &  res.DESeq2.df$padj < alp
head(repressed.sign)
```

```
[1] "yor348c" "ypl025c" "yer081w" "ymr122c" "ygr087c" "ygr051c"
```

```
term.repressed <- gprofiler(query=repressed.sign, organism="scerevisiae")
term.repressed <- term.repressed[order(term.repressed$p.value),]
kable(head(term.induced[,c("p.value", "term.name","intersection")], 10))
```

	p.value	term.name
32	0	RNA-DNA hybrid ribonuclease activity
3	0	DNA integration
22	0	RNA-directed DNA polymerase activity
35	0	aspartic-type peptidase activity
37	0	aspartic-type endopeptidase activity
23	0	DNA-directed DNA polymerase activity
31	0	endoribonuclease activity, producing 5'-phosphomonoesters
21	0	DNA polymerase activity
28	0	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters
30	0	endoribonuclease activity

Assess the effect of sample number on differential expression call

Using a loop, randomly select 10 times 2,5,10,15..45 samples from WT and Snf2 KO. Perform differential expression calls and draw a diagram showing the number of differential expressed genes.

```r
## Create a directory to store the results that will be obtained below
dir.results <- file.path(dir.snf2, "results")
dir.create(dir.results, showWarnings = FALSE, recursive = TRUE)

## Export the table with statistics per sample.
write.table(stats.per.sample, file=file.path(dir.results, "stats_per_sample.tsv"),
            quote=FALSE, sep="\t", col.names =NA, row.names = TRUE)

# Export the DESeq2 result table
DESeq2.table <- file.path(dir.results, "yeast_Snf2_vs_WT_DESeq2_diff.tsv")
write.table(res.DESeq2, file=DESeq2.table, col.names = NA, row.names = TRUE, sep="\t", quote = FALSE)
```