

➤ Analyses biostatistiques de données RNA-seq

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INRAE



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➤ Outline

Exploratory analysis

- Introduction

- Experimental design

- Data exploration and quality assessment

Normalization

- Raw data filtering

- Interpreting read counts

Differential Expression analysis

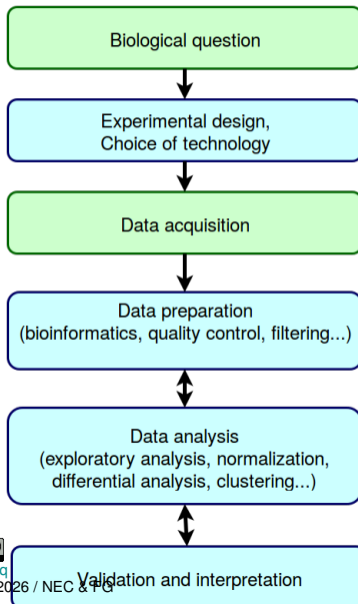
- Hypothesis testing and correction for multiple tests

- Differential expression analysis for RNAseq data

- Interpreting and improving the analysis



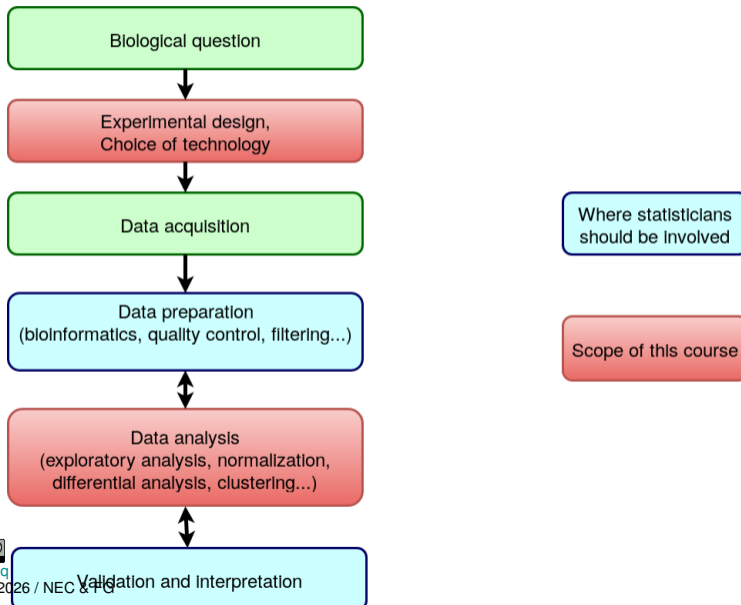
> A typical transcriptomic experiment



Where statisticians should be involved



> A typical transcriptomic experiment



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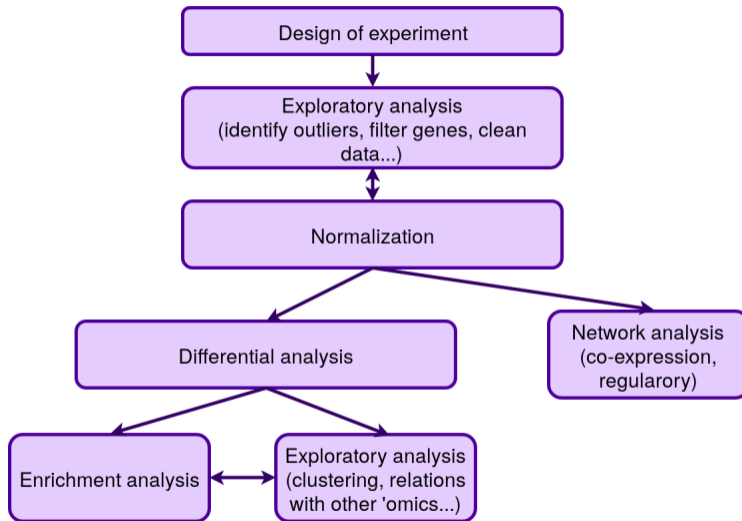
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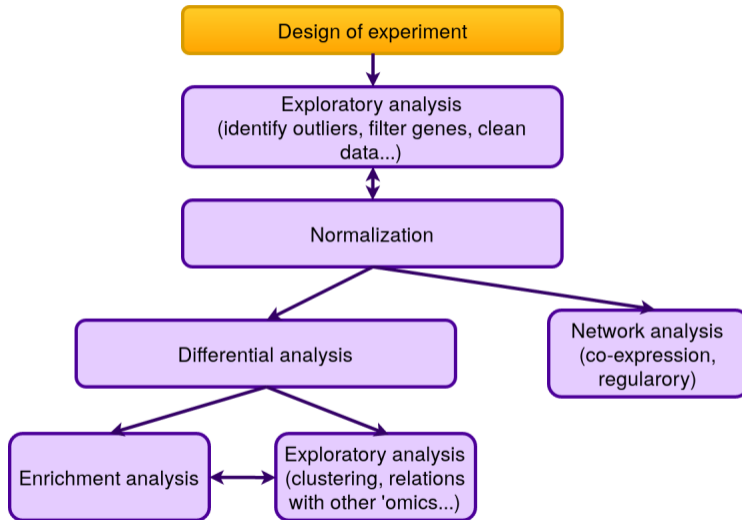
- Interpreting and improving the analysis



Steps in RNAseq data analysis



Part I: Experimental design



➤ Confounded effects: a simple example

Basic experiment: find differences between control/treated plants



control group plant



treated group plant

➤ Confounded effects: a simple example

Basic experiment: find differences between control/treated plants

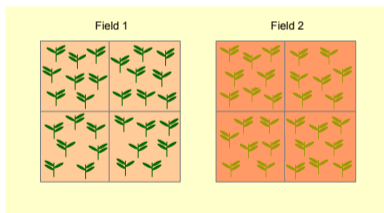


control group plant



treated group plant

A bad experimental design: grow all control group plants in one field and grow all treated group plants in another field



differences due to the field / the treatment can not be distinguished ⇒ **confounded**

effects

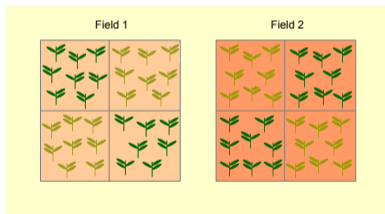
➤ Confounded effects: a simple example

Basic experiment: find differences between control/treated plants



control group plant treated group plant

A good experimental design: grow half control group plants (chosen at random) and half treated group plants in one field (and the rest in the other field)



differences due to the field / the treatment can be estimated separately

➤ Confounded effects: a simple example

Basic experiment: find differences between control/treated plants



control group plant



treated group plant

In summary, what is a good experimental design?

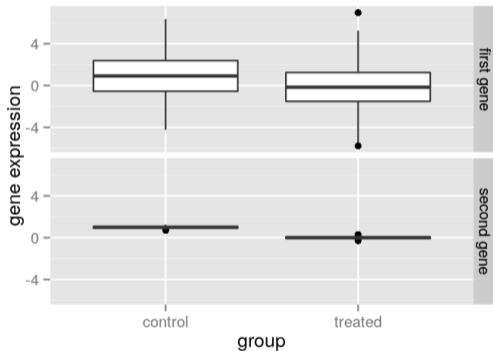
Experimental design are usually not as simple as this example: they can include **multiple experimental factors** (day of experiment, flow cell, ...) and **multiple covariates** (sex, parents, ...).

⇒ The experimental design must be **carefully** thought **before** starting the experiment and confounded effects must be searched for in a systematic manner.



➤ Effect & Variation

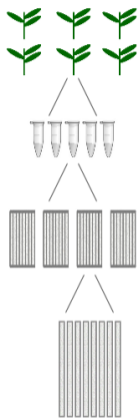
2 conditions, 2 genes whose expression distribution is:



- ▶ **first gene:** different median levels between the two groups but **large variance**: differences may be non significant
- ▶ **second gene:** different median levels between the two groups but **very small variance**: differences may be significant

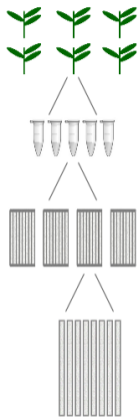


➤ Source of variation in RNA-seq experiments



1. at the top layer: **biological variations** (*i.e.*, individual differences due to *e.g.*, environmental or genetic factors)
2. at the middle layer: **technical variations** (library preparation effect)
3. at the bottom layer: **technical variations** (lane and cell flow effects)

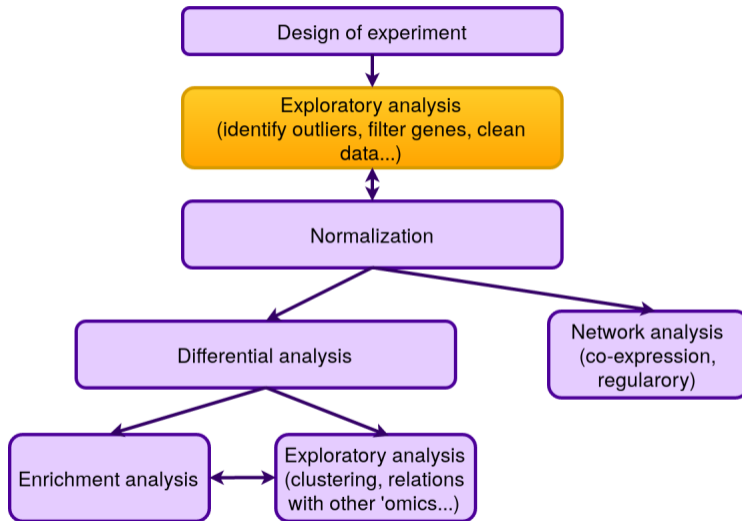
➤ Source of variation in RNA-seq experiments



1. at the top layer: **biological variations** (*i.e.*, individual differences due to *e.g.*, environmental or genetic factors)
2. at the middle layer: **technical variations** (library preparation effect)
3. at the bottom layer: **technical variations** (lane and cell flow effects)

lane effect < cell flow effect < library preparation effect \ll biological effect $\Rightarrow 2 \times 3$
biological replicates at least [[Liu et al., 2014](#)]

Part II: Exploratory analysis



➤ Some features of RNAseq data

What must be taken into account?

- ▶ discrete, non-negative data (total number of aligned reads)

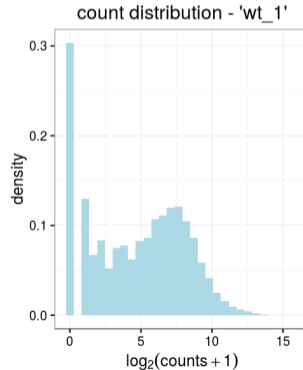
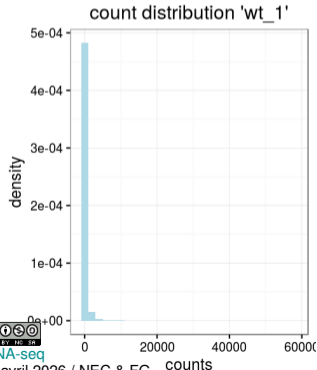


##	wt_1	wt_2	wt_3	mut1_1	mut1_2
## Medtr0001s0010.1	0	0	0	1	0
## Medtr0001s0070.1	0	0	0	0	0
## Medtr0001s0100.1	0	0	0	0	0
## Medtr0001s0120.1	0	0	0	0	0
## Medtr0001s0160.1	0	0	0	0	0
## Medtr0001s0190.1	0	0	0	0	0

➤ Some features of RNAseq data

What must be taken into account?

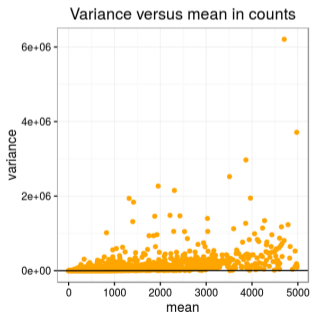
- ▶ discrete, non-negative data (total number of aligned reads)
- ▶ skewed data



➤ Some features of RNAseq data

What must be taken into account?

- ▶ discrete, non-negative data (total number of aligned reads)
- ▶ skewed data
- ▶ overdispersion (variance \gg mean)



black line is “variance = mean”



➤ Dataset used in the examples

Dataset provided by courtesy of the transcriptomic platform of IPS2

Three files:

- ▶ D1-counts.txt contains the raw counts of the experiment (13 columns: the first one contains the gene names, the others correspond to 12 different samples; gene names have been shuffled);
- ▶ D1-genesLength.txt contains information about gene lengths;
- ▶ D1-targets.txt contains information about the sample and the experimental design.

```
## labels group replicat
## 1 wt_1 wt replibio1
## 2 wt_2 wt replibio2
## 3 wt_3 wt replibio3
## 4 mut1_1 mut1 replibio1
## 5 mut1_2 mut1 replibio2
## 6 mut1_3 mut1 replibio3
## 7 mut2_1 mut2 replibio1
## 8 mut2_2 mut2 replibio2
## 9 mut2_3 mut2 replibio3
## 10 mut3_1 mut3 replibio1
## 11 mut3_2 mut3 replibio2
## 12 mut3_3 mut3 replibio3
```



➤ Dataset used in the examples

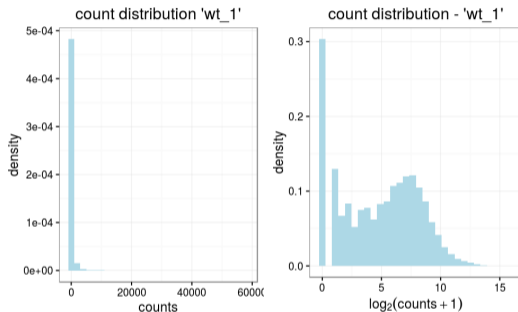
These text files are loaded with:

```
raw_counts <- read.table("D1-counts.txt", header = TRUE,  
                          row.names = 1)  
raw_counts <- as.matrix(raw_counts)  
design <- read.table("D1-targets.txt", header = TRUE)  
gene_lengths <- scan("D1-genesLength.txt")
```



➤ Count distribution

The **count distribution** (*i.e.*, the number of times a given count is obtained in the data) can be visualized with **histograms** (**boxplots** or **violin plots** can also be used):

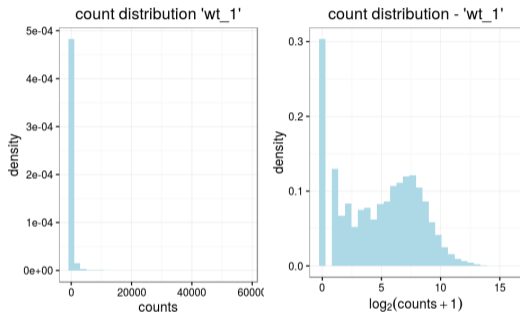


This distribution is highly skewed and it is better visualized using a **log₂ transformation** before it is displayed.



Count distribution

The **count distribution** (*i.e.*, the number of times a given count is obtained in the data) can be visualized with **histograms** (**boxplots** or **violin plots** can also be used):

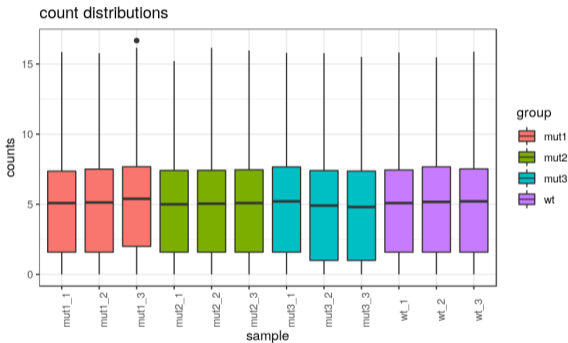


This distribution is highly skewed and it is better visualized using a **\log_2 transformation** before it is displayed.

The **library size** is the sum of all counts in a given sample.

➤ Count distribution between samples

The count distribution between different samples can be compared with **parallel boxplots** or **violin plots**:



It is expected that, within a given condition (group), the count distributions are similar. The same is often also expected between conditions.

➤ Check reproducibility between samples

MA plots can be used to visualize reproducibility between samples of an experiment (and thus check if normalization is needed). They plot the log-fold change (**M-values**) against the log-average (**A-values**):

M-values: log of ratio between counts between two samples:

$$M_g = \log_2(K_{gj}) - \log_2(K_{gj'})$$

A-values: average log counts between two samples:

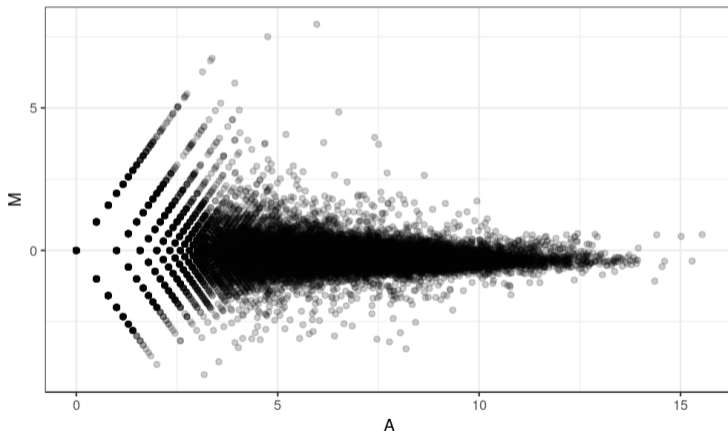
$$A_g = \frac{\log_2(K_{gj}) + \log_2(K_{gj'})}{2}$$

where K_{gj} stands for the counts for gene g in sample j .



➤ Check reproducibility between samples

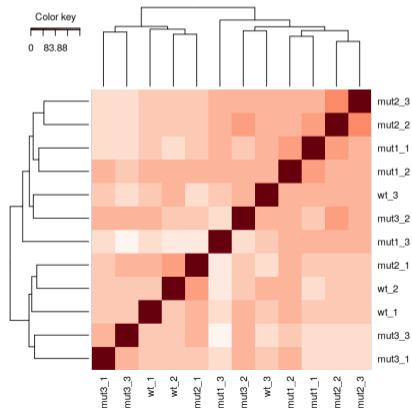
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Check similarity between samples

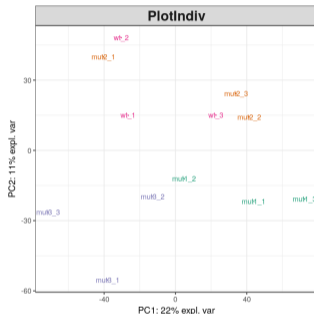
Similarities between samples can be visualized with a HAC and a heatmap:

- ▶ perform hierarchical ascending classification (HAC) using Euclidean distance between samples: $\delta(j, j') = \sum_g (\log_2(K_{gj}) - \log_2(K_{gj'}))^2$
- ▶ visualize the strength of the similarity with heatmap.



➤ Search for the main structures in the data: PCA

PCA (on \log_2 counts) can be used to project data into a small dimensional space and search for unexpected experimental effects in the data.



(MDS is equivalent to PCA when used with the standard Euclidean distance)

Remark: In **DESeq**, the function `plotPCA` performs PCA on the top genes with the highest variance.



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➤ Raw data filtering

Filtering consists in removing genes with low expression. Different strategies can be used:

- ▶ [Sultan et al., 2008]: filter out genes with a total read count smaller than a given threshold;
- ▶ [Bottomly et al., 2011]: filter out genes with zero count in an experimental condition;
- ▶ [Robinson and Oshlack, 2010]: filter out genes such that the number of samples with a CPM value (for this gene) smaller than a given threshold is larger than the smallest number of samples in a condition. With **CPM**: Count Per Million (*i.e.*, raw count divided by library size, this strategy takes into account differences in library sizes).



➤ Raw data filtering

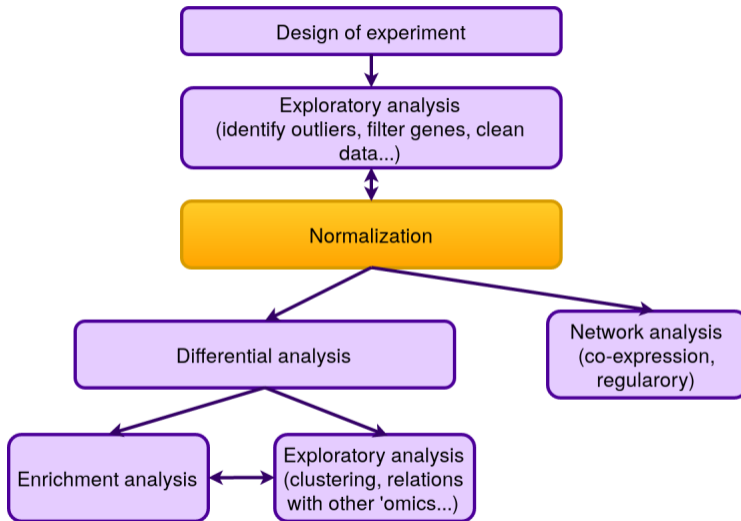
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More sophisticated filtering

To account for the fact that lowly expressed genes are almost never found differentially expressed, a more sophisticated filtering can be performed.

Part III: Normalization



➤ Purpose of normalization

- ▶ identify and correct technical biases (due to sequencing process) to **make counts comparable**
- ▶ **types of normalization**: within sample normalization and between sample normalization



➤ Within sample normalization

Example: (read counts)

	sample 1	sample 2	sample 3
gene A	752	615	1203
gene B	1507	1225	2455

counts for gene B are twice larger than counts for gene A because:



➤ Within sample normalization

Example: (read counts)

	sample 1	sample 2	sample 3
gene A	752	615	1203
gene B	1507	1225	2455

counts for gene B are twice larger than counts for gene A because:

- ▶ gene B is expressed with a number of transcripts twice larger than gene A



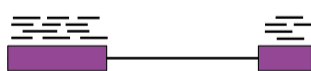
➤ Within sample normalization

Example: (read counts)

	sample 1	sample 2	sample 3
gene A	752	615	1203
gene B	1507	1225	2455

counts for gene B are twice larger than counts for gene A because:

- ▶ both genes are expressed with the same number of transcripts but gene B is twice longer than gene A



gene A



gene B

➤ Within sample normalization

- ▶ **Purpose of within sample normalization:** enabling comparisons of genes from a same sample
- ▶ **Sources of variability:** gene length, sequence composition (GC content)

These differences **need not to be corrected for a differential analysis** and are not really relevant for data interpretation.



➤ Between sample normalization

Example: (read counts)

	sample 1	sample 2	sample 3
gene A	752	615	1203
gene B	1507	1225	2455

counts in sample 3 are much larger than counts in sample 2 because:



➤ Between sample normalization

Example: (read counts)

	sample 1	sample 2	sample 3
gene A	752	615	1203
gene B	1507	1225	2455

counts in sample 3 are much larger than counts in sample 2 because:

- ▶ gene A is more expressed in sample 3 than in sample 2



gene A in sample 2



gene A in sample 3

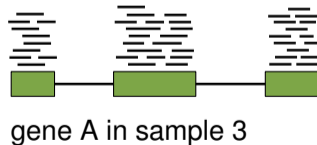
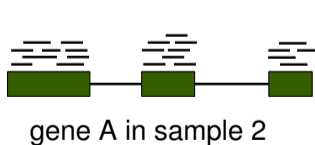
➤ Between sample normalization

Example: (read counts)

	sample 1	sample 2	sample 3
gene A	752	615	1203
gene B	1507	1225	2455

counts in sample 3 are much larger than counts in sample 2 because:

- ▶ gene A is expressed similarly in the two samples but sequencing depth is larger in sample 3 than in sample 2 (*i.e.*, differences in library sizes)



➤ Between sample normalization

- ▶ **Purpose of between sample normalization:** enabling comparisons of a gene in different samples
- ▶ **Sources of variability:** library size, ...

These differences **must be corrected for a differential analysis** and for data interpretation.



➤ Principles for sequencing depth normalization

Basics

1. choose an appropriate baseline for each sample
2. for a given gene, compare counts relative to the baseline rather than raw counts



Principles for sequencing depth normalization

Basics

1. choose an appropriate baseline for each sample
2. for a given gene, compare counts relative to the baseline rather than raw counts

In practice: Raw counts correspond to different sequencing depths

	control				treated		
Gene 1	5	1	0	0	4	0	0
Gene 2	0	2	1	2	1	0	0
Gene 3	92	161	76	70	140	88	70
:		:				:	
:		:				:	
:		:				:	
Gene G	15	25	9	5	20	14	17



Principles for sequencing depth normalization

Basics

1. choose an appropriate baseline for each sample
2. for a given gene, compare counts relative to the baseline rather than raw counts

In practice: A correction multiplicative factor is computed for every sample

	control				treated		
Gene 1	5	1	0	0	4	0	0
Gene 2	0	2	1	2	1	0	0
Gene 3	92	161	76	70	140	88	70
:							
:							
:							
Gene G	15	25	9	5	20	14	17
C_j	1.1	1.6	0.6	0.7	1.4	0.7	0.8

Principles for sequencing depth normalization

Basics

1. choose an appropriate baseline for each sample
2. for a given gene, compare counts relative to the baseline rather than raw counts

In practice: Every counts is multiplied by the correction factor corresponding to its sample

Gene 3	92	161	76	70	140	88	70
C_j	1.1	1.6	0.6	0.7	1.4	0.7	0.8
<hr/>							
Gene 3	101.2	257.6	45.6	49	196	61.6	56

x



Principles for sequencing depth normalization

Basics

1. choose an appropriate baseline for each sample
2. for a given gene, compare counts relative to the baseline rather than raw counts

Consequences: Library sizes for normalized counts are roughly equal.

	control				treated		
Gene 1	5.5	1.6	0	0	5.6	0	0
Gene 2	0	3.2	0.6	1.4	1.4	0	0
Gene 3	101.2	257.6	45.6	49	196	61.6	56
:							
:							
:							
Gene G	16.5	40	5.4	5.5	28	9.8	13.6
	+ size				+		
	13.1	13.0	13.2	13.1	13.2	13.0	13.1

$\times 10^5$

➤ Principles for sequencing depth normalization

Definition

If K_{gj} is the raw count for gene g in sample j then, the normalized counts is defined as:

$$\tilde{K}_{gj} = \frac{K_{gj}}{s_j \times D_j} \times 10^6$$

in which: $D_j = \sum_g K_{gj}$ is the library size of sample j , s_j is the correction factor of the library size for sample j and thus $C_j = \frac{10^6}{s_j D_j}$.

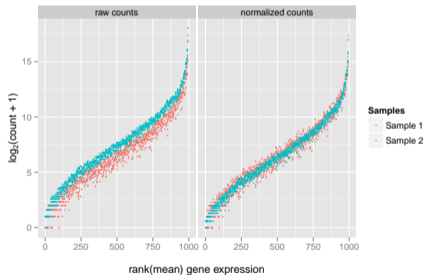


➤ Distribution adjustment

- ▶ Total read count adjustment [Mortazavi et al., 2008]

$$s_j = 1 \quad \text{and thus: } \tilde{K}_{gj} = \frac{K_{gj}}{D_j} \times 10^6$$

(Counts Per Million).



edgeR:

```
cpm(...,
      normalized.lib.sizes=FALSE)
```

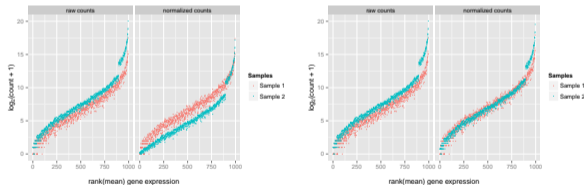


➤ Distribution adjustment

- ▶ Total read count adjustment [Mortazavi et al., 2008]
- ▶ (Upper) Quartile normalization [Bullard et al., 2010]

$$s_j = \frac{Q_j^{(p)}}{\frac{1}{N} \sum_{l=1}^N Q_l^{(p)}}$$

N : number of samples, $Q_j^{(p)}$: quantile in sample j



edgeR:

```
calcNormFactors(..., method = "upperquartile",  
p = 0.75)
```

➤ Method using gene lengths (intra & inter sample normalization)

RPKM: Reads Per Kilobase per Million mapped Reads

Assumptions: read counts are proportional to expression level, transcript length and sequencing depth

$$s_j = \frac{L_g}{10^3}$$

in which L_g is gene length (bp).

edgeR:

```
rpkm(..., gene.length = ...)
```

Unbiased estimation of number of reads but affect variability
[Oshlack and Wakefield, 2009].



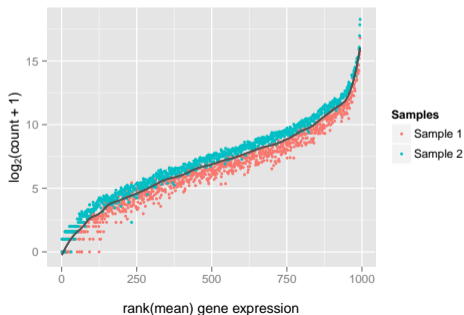
➤ Relative Log Expression (RLE)

Method:

1. compute a **pseudo-reference sample**: geometric mean across samples

$$R_g = \left(\prod_{j=1}^N K_{gj} \right)^{1/N}$$

(geometric mean is less sensitive to extreme values than standard mean)

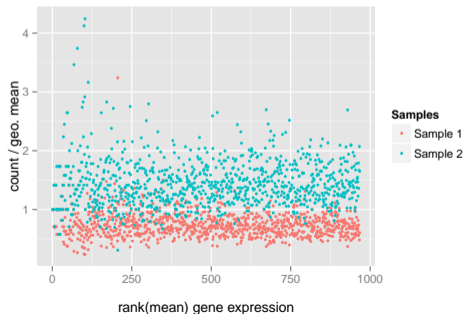


➤ Relative Log Expression (RLE)

Method:

1. compute a **pseudo-reference sample**
2. center samples compared to the reference

$$\tilde{K}_{gj} = \frac{K_{gj}}{R_g} \quad \text{with} \quad R_g = \left(\prod_{j=1}^N K_{gj} \right)^{1/N}$$

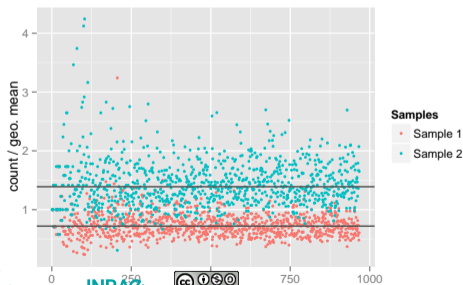


➤ Relative Log Expression (RLE)

Method:

1. compute a **pseudo-reference sample**
2. center samples compared to the reference
3. compute normalization factor: median of centered counts over the genes

$$\tilde{s}_j = \text{median}_g \{ \tilde{K}_{gj} \} \quad \text{factors multiply to 1:} \quad s_j = \frac{\tilde{s}_j}{\exp\left(\frac{1}{N} \sum_{l=1}^N \log(\tilde{s}_l)\right)}$$



with

$$\tilde{K}_{gj} = \frac{K_{gj}}{R_g}$$

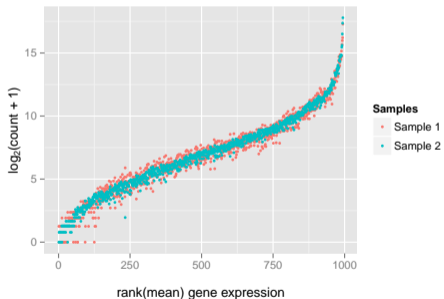
and

$$R_g = \left(\prod_{j=1}^N K_{gj} \right)^{1/N}$$

➤ Relative Log Expression (RLE)

Method:

1. compute a **pseudo-reference sample**
2. center samples compared to the reference
3. compute normalization factor: median of centered counts over the genes



```
## with edgeR
calcNormFactors(...,
  method="RLE")

## with DESeq
estimateSizeFactors(...)
```



➤ Trimmed Mean of M-values (TMM)

Assumptions behind the method

- ▶ the total read count strongly depends on a few highly expressed genes
- ▶ most genes are not differentially expressed



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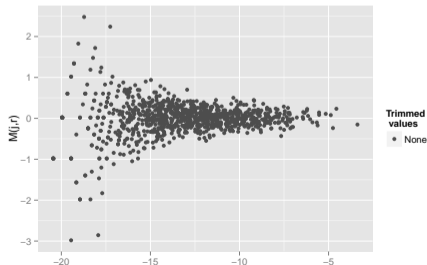
- ▶ the total read count strongly depends on a few highly expressed genes
- ▶ most genes are not differentially expressed

⇒ remove extreme data for fold-changed (M) and average intensity (A)

$$M_g(j, r) = \log_2 \left(\frac{K_{gj}}{D_j} \right) - \log_2 \left(\frac{K_{gr}}{D_r} \right) \quad A_g(j, r) = \frac{1}{2} \left[\log_2 \left(\frac{K_{gj}}{D_j} \right) + \log_2 \left(\frac{K_{gr}}{D_r} \right) \right]$$

select as a reference sample, the sample r with the upper quartile closest to the average upper quartile

M- vs A-values



➤ Trimmed Mean of M-values (TMM)

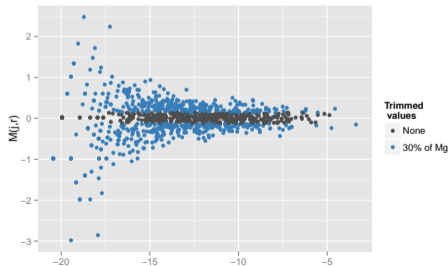
Assumptions behind the method

- ▶ the total read count strongly depends on a few highly expressed genes
- ▶ most genes are not differentially expressed

⇒ remove extreme data for fold-changed (M) and average intensity (A)

$$M_g(j, r) = \log_2 \left(\frac{K_{gj}}{D_j} \right) - \log_2 \left(\frac{K_{gr}}{D_r} \right) \quad A_g(j, r) = \frac{1}{2} \left[\log_2 \left(\frac{K_{gj}}{D_j} \right) + \log_2 \left(\frac{K_{gr}}{D_r} \right) \right]$$

Trim 30% on M-values



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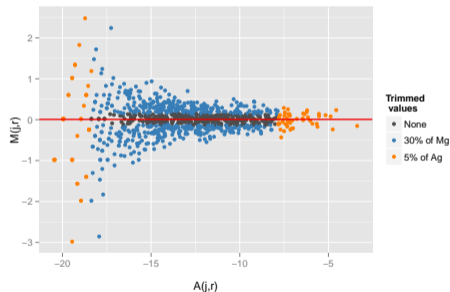
Trim 5% on A-values



➤ Trimmed Mean of M-values (TMM)

Assumptions behind the method

- ▶ the total read count strongly depends on a few highly expressed genes
- ▶ most genes are not differentially expressed



On remaining data, compute the **weighted mean of M-values**:

$$\text{TMM}(j, r) = \frac{\sum_{g:\text{not trimmed}} w_g(j, r) M_g(j, r)}{\sum_{g:\text{not trimmed}} w_g(j, r)}$$

$$\text{with } w_g(j, r) = \left(\frac{D_j - K_{gj}}{D_j K_{gj}} + \frac{D_r - K_{gr}}{D_r K_{gr}} \right).$$

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- ▶ the total read count strongly depends on a few highly expressed genes
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Correction factors:

$$\tilde{s}_j = 2^{\text{TMM}(j,r)} \quad \text{factors multiply to 1:} \quad s_j = \frac{\tilde{s}_j}{\exp\left(\frac{1}{N} \sum_{l=1}^N \log(\tilde{s}_l)\right)}$$

```
calcNormFactors(..., method="TMM")
```



➤ Comparison of the different approaches [Dillies et al., 2013]

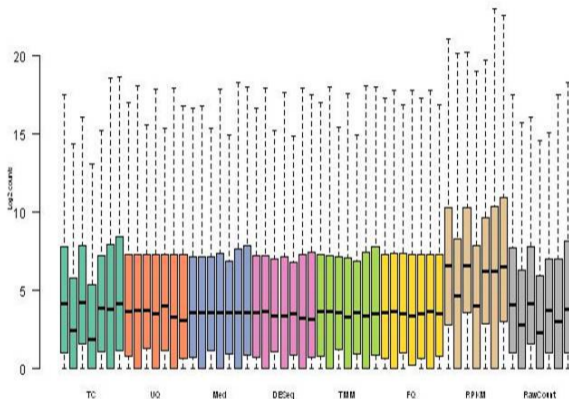
Purpose of the comparison:

- ▶ finding the “best” method for all cases is not a realistic purpose
- ▶ find an approach which is **robust enough** to provide relevant results in all cases
- ▶ **Method**: comparison based on several criteria to select a method which is valid for multiple objectives



➤ Comparison of the different approaches [Dillies et al., 2013]

Effect on count distribution:

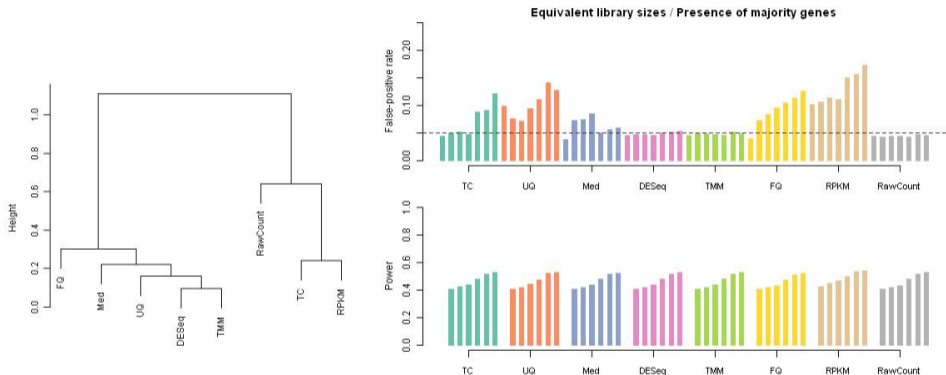


RPKM and TC are very similar to raw data.



➤ Comparison of the different approaches [Dillies et al., 2013]

Effect on differential analysis (DESeq v. 1.6):



Inflated FPR for all methods except for TMM and DESeq (RLE).



➤ Comparison of the different approaches [Dillies et al., 2013]

Conclusion: Differences appear based on data characteristics

Method	Distribution	Intra-Variance	Housekeeping	Clustering	False-positive rate
TC	-	+	+	-	-
UQ	++	++	+	++	-
Med	++	++	-	++	-
DESeq	++	++	++	++	++
TMM	++	++	++	++	++
FQ	++	-	+	++	-
RPKM	-	+	+	-	-

TMM and DESeq (RLE) are performant in a differential analysis context.

➤ Outline

Exploratory analysis

- Introduction

- Experimental design

- Data exploration and quality assessment

Normalization

- Raw data filtering

- Interpreting read counts

Differential Expression analysis

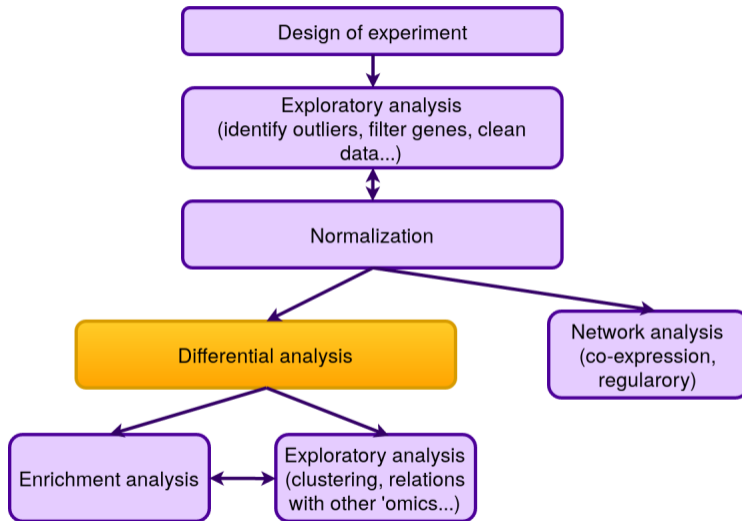
- Hypothesis testing and correction for multiple tests

- Differential expression analysis for RNAseq data

- Interpreting and improving the analysis



Part IV: Differential expression analysis

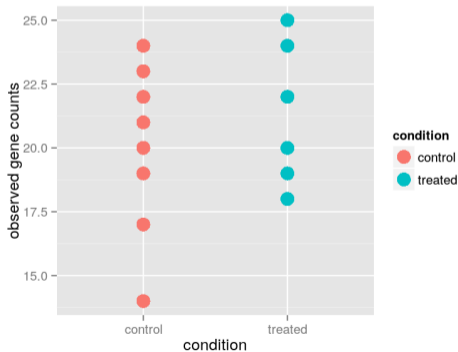


➤ Different steps in hypothesis testing

1. formulate an hypothesis H_0 :

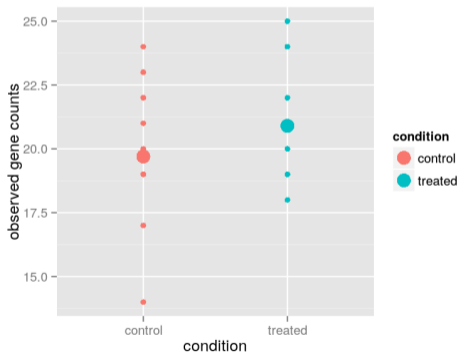
H_0 : the average count for gene g in the control samples is the same that the average count in the treated samples

which is tested against an alternative H_1 : the average count for gene g in the control samples is different from the average count in the treated samples



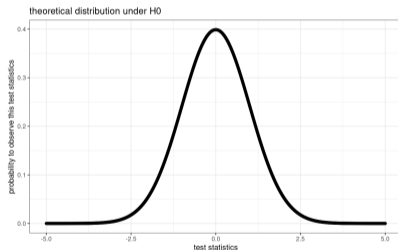
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2. from observations, compute a test statistics (e.g., the mean in the two samples)



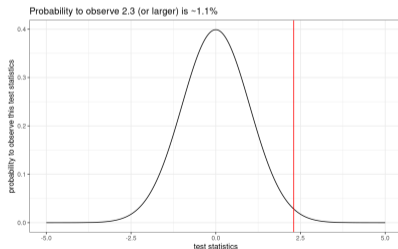
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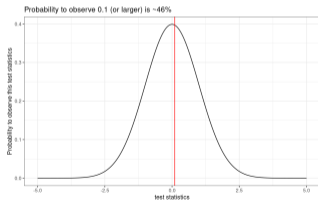
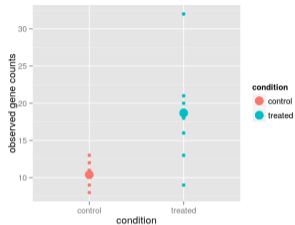
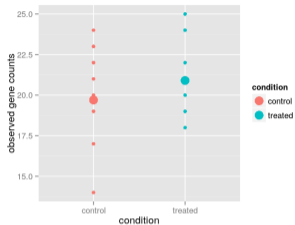


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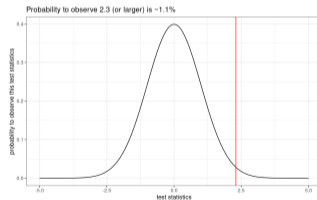
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4. deduce the probability that the observations occur under H_0 : this is called the p-value
5. conclude: if the p-value is low (usually below $\alpha = 5\%$ as a convention), H_0 is unlikely: we say that “ H_0 is rejected”.
We have that: $\alpha = \mathbb{P}_{H_0}(\text{H}_0 \text{ is rejected})$.
The smaller the p-value, the greater the confidence in rejecting H_0 .



Summary of the possible decisions



Do not reject H_0



Reject H_0



Types of errors in tests

		Reality	
		H_0 is true	H_0 is false
Decision	Do not reject H_0	Correct decision ☺ (True Negative)	Type II error ☹ (False Negative)
	Reject H_0	Type I error ☹ (False Positive)	Correct decision ☺ (True Positive)

$$P(\text{Type I error}) = \alpha \text{ (risk)}$$

$$P(\text{Type II error}) = 1 - \beta \text{ (\beta: power)}$$

➤ Why performing a large number of tests might be a problem?

Framework: Suppose you are performing G tests at level α .

$$\mathbb{P}(\text{at least one FP if } H_0 \text{ is always true}) = 1 - (1 - \alpha)^G$$

Ex: for $\alpha = 5\%$ and $G = 20$, $\mathbb{P}(\text{at least one FP if } H_0 \text{ is always true}) \simeq 64\%!!!$



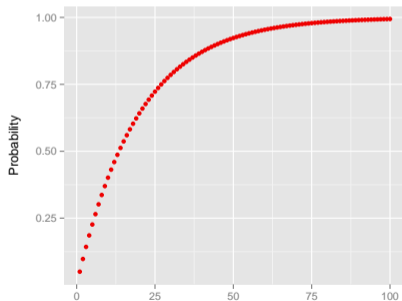
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Probability to have at least one false positive versus the number of tests performed when H_0 is true for all G tests



For more than 75 tests and if H_0 is always true, the probability to have at least one false positive is very close to 100%!



➤ Notations for multiple tests

Number of decisions for G independent tests:

	True null hypotheses	False null hypotheses	Total
Not rejected	$G_0 - U$	$G_1 - V$	$G - R$
Rejected	U	V	R
Total	G_0	G_1	G



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Total	G_0	G_1	G

Instead of the risk α , control:

- ▶ **familywise error rate (FWER)**: $\text{FWER} = \mathbb{P}(U > 0)$ (i.e., probability to have at least one false positive decision)
- ▶ **false discovery rate (FDR)**: $\text{FDR} = \mathbb{E}(Q)$ with

$$Q = \begin{cases} U/R & \text{if } R > 0 \\ 0 & \text{otherwise} \end{cases}$$

➤ Adjusted p-values

Settings: p-values p_1, \dots, p_G (e.g., corresponding to G tests on G different genes)

Adjusted p-values

adjusted p-values are $\tilde{p}_1, \dots, \tilde{p}_G$ such that

$$\text{Rejecting tests such that } \tilde{p}_g < \alpha \iff \mathbb{P}(U > 0) \leq \alpha \text{ or } \mathbb{E}(Q) \leq \alpha$$



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2. compute $\tilde{p}_{(g)} = a_g p_{(g)}$
 - ▶ with **Bonferroni** method: $a_g = G$ (FWER)
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 - ▶ with **Benjamini & Hochberg** method: $a_g = G/g$ (FDR)
3. if adjusted p-values $\tilde{p}_{(g)}$ are larger than 1, correct $\tilde{p}_{(g)} \leftarrow \min\{\tilde{p}_{(g)}, 1\}$



Adjusting p-values in practice

- ▶ compute adjusted p-values (Bonferroni or BH procedures for instance)
- ▶ select all genes for which this adjusted p-values is below 5% (for instance)
- ▶ this is equivalent to controlling either the probability to have at least one FP (FWER) or the average proportion of FP (FDR)

```
> head(res_et$table)
```

	logFC	logCPM	risk < 10% PValue	FDR < 10% padj_BH	FWER < 10% padj_Bonferonni
genes1	-1.25598945	17.35035	2.216483e-05	0.0002216483	0.0002216483
genes2	-0.62262459	16.85998	8.691765e-02	0.2172941253	0.8691765014
genes3	-0.06069361	16.94473	8.954297e-01	0.9949218968	1.0000000000
genes4	0.85148982	16.67618	1.458169e-02	0.0729084286	0.1458168571
genes5	0.56274616	16.76017	1.086896e-01	0.2173792897	1.0000000000
genes6	0.25631998	16.55039	4.992014e-01	0.7131449114	1.0000000000

➤ Fisher's exact test for contingency tables

After normalization, one may build a contingency table like this one:

	treated	control	Total
gene g	n_{gA}	n_{gB}	n_g
other genes	$N_A - n_{gA}$	$N_B - n_{gB}$	$N - n_g$
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Question: is the number of reads of gene g in the treated sample significantly different than in the control sample?



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Method

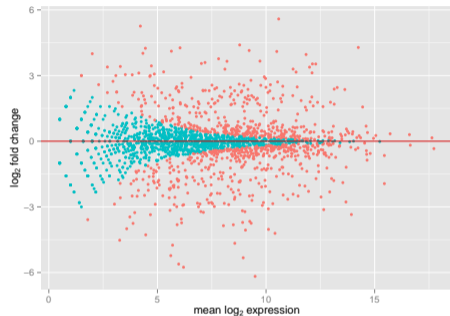
Direct computation of the probability to obtain such a contingency table (or a “more extreme” contingency table) with:

- ▶ independency between the two columns of the contingency tables;
- ▶ the same marginals (“Total”).



➤ Example of results obtained with the Fisher test

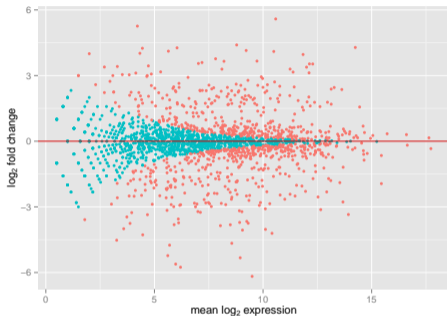
Genes declared significantly differentially expressed are in pink:



Main remark: more conservative for genes with a low expression

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Limitation of Fisher test

Highly expressed genes have a very large variance! As Fisher test does not estimate variance, it **tends to detect false positives among highly expressed genes** ⇒ do not use it!



➤ Basic principles of tests for count data: 2 conditions and replicates

Notations: for gene g , $K_{g1}^1, \dots, K_{gn_1}^1$ (condition 1) and $K_{g1}^2, \dots, K_{gn_2}^2$ (condition 2)

- ▶ choose an appropriate distribution to model count data (discrete data, overdispersion)

- ▶ estimate its parameters for both conditions

- ▶ conclude by computing p-value



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$$K_{gj}^k \sim \text{NB}(s_j^k \lambda_{gk}, \phi_g)$$

in which:

- ▶ s_j^k is library correction factor of sample j in condition k
- ▶ λ_{gk} is the proportion of counts for gene g in condition k
- ▶ ϕ_g is the (over)dispersion (parameter) of gene g (supposed to be identical for all samples)
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- $\lambda_{g1} \quad \lambda_{g2} \quad \phi_g$
- ▶ conclude by computing p-value \Rightarrow Test

$$H_0 : \{\lambda_{g1} = \lambda_{g2}\}$$

➤ First method: Exact Negative Binomial test

2 conditions only



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Normalization is performed to get equal size libraries \Rightarrow s



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2 conditions only

Normalization is performed to get equal size libraries $\Rightarrow s$

The test is performed similarly as for Fisher test (exact probability is computed according to NB distribution after parameters have been estimated)

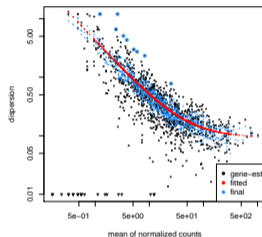


Estimating the dispersion parameter ϕ_g

Some methods:

- ▶ **DESeq, DESeq2:** ϕ_g is a smooth function of $\lambda_g = \lambda_{g1} = \lambda_{g2}$

```
dge <- estimateDispersion(dge)
```



- ▶ **edgeR:** estimate a common dispersion parameter for all genes and use it as a prior in a Bayesian approach to estimate a gene specific dispersion parameter by log-likelihood maximization

```
dge <- estimateDisp(dge)
```



➤ Perform the test

Some methods:

- ▶ **DESeq, DESeq2**: exact (**DESeq**) or approximate (Wald and LR in **DESeq2**) tests

```
res <- nbinomWaldTest(dge)
results(res)
```

```
res <- nbinomLR(dge)
results(res)
```

- ▶ **edgeR**: exact tests

```
res <- exactTest(dge)
topTags(res)
```

(comparison between methods in [[Zhang et al., 2014](#)])



➤ More complex experiments: GLM

Framework:

$$K_{gj} \sim \text{NB}(\mu_{gj}, \phi_g) \quad \text{with} \quad \log(\mu_{gj}) = \log(s_j) + \log(\lambda_{gj})$$

in which:

- ▶ s_j is the library size correction for sample j ;



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- ▶ $\log(\lambda_{gj})$ is estimated (for instance) by a **Generalized Linear Model (GLM)**:

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in which \mathbf{x}_j is a vector of covariates.



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GLM allows to decompose the effects on the mean of

- ▶ different factors
- ▶ their interactions



➤ More complex experiments: GLM in practice

edgeR

```
dge <- estimateDisp(dge, design) # estimation of dispersion
fit <- glmFit(dge, design) # estimation of parameters
res <- glmLRT(fit, ...) # tests (likelihood ratio)
topTags(res)
```

DESeq, DESeq2

```
dge <- estimateDispersions(dge)
fit <- fitNbinomGLMs(dge, count ~ ...)
fit0 <- fitNbinomGLMs(dge, count ~ 1)
res <- nbinomGLMTest(fit, fit0)
p.adjust(res, method = "BH")
```



Example

In an experiment, gene expression is influenced by:

- ▶ diets: A (reference diet) and B (another diet)
- ▶ genotypes: G (reference genotype), H (mutant 1), K (mutant 2)



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The model with two additional effects writes:

$$\log(\lambda) = \underbrace{\beta_0}_{\text{basal level for reference}} + \underbrace{\beta_1 \mathbf{1}_{\text{diet B}}}_{\text{additional expression for diet B}} + \underbrace{\beta_2 \mathbf{1}_{\text{genotype H}}}_{\text{additional expression for mutant 1}} + \underbrace{\beta_3 \mathbf{1}_{\text{genotype K}}}_{\text{additional expression for mutant 2}}$$

Tests:

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Tests:

- ▶ Testing if the diet as an effect is equivalent to testing “ $\beta_1 = 0$ ” coef = 2 in glmLRT of **edgeR**



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Tests:

- ▶ Testing if genotype K has an expression different to genotype H is equivalent to testing “ $\beta_2 = \beta_3$ ” contrast = $c(0, 0, 1, -1)$ in `glmLRT` of **edgeR**



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- ▶ genotypes: G (reference genotype), H (mutant 1), K (mutant 2)

The model with two additional effects writes:

$$\log(\lambda) = \underbrace{\beta_0}_{\text{basal level for reference}} + \underbrace{\beta_1 \mathbf{1}_{\text{diet B}}}_{\text{additional expression for diet B}} + \underbrace{\beta_2 \mathbf{1}_{\text{genotype H}}}_{\text{additional expression for mutant 1}} + \underbrace{\beta_3 \mathbf{1}_{\text{genotype K}}}_{\text{additional expression for mutant 2}}$$

Tests:

- ▶ Testing if the genotype has an effect is equivalent to testing the full model above against the model $\log(\lambda) = \beta_0 + \beta_1 \mathbf{1}_{\text{diet B}}$ or testing $\beta_2 = \beta_3 = 0$ (coef = 3:4 glmLRT of **edgeR**)

> Contrasts

$$\log(\lambda) = \underbrace{\beta_0}_{\text{basal level for reference}} + \underbrace{\beta_1 \mathbf{1}_{\text{diet B}}}_{\text{additional expression for diet B}} + \underbrace{\beta_2 \mathbf{1}_{\text{genotype H}}}_{\text{additional expression for mutant 1}} + \underbrace{\beta_3 \mathbf{1}_{\text{genotype K}}}_{\text{additional expression for mutant 2}}$$

testing if genotype K has an expression different to genotype H:

	β_0	β_1	β_2	β_3
genotype K	1	0	0	1
- genotype H	1	0	1	0

> Contrasts

$$\log(\lambda) = \underbrace{\beta_0}_{\text{basal level for reference}} + \underbrace{\beta_1 \mathbf{1}_{\text{diet B}}}_{\text{additional expression for diet B}} + \underbrace{\beta_2 \mathbf{1}_{\text{genotype H}}}_{\text{additional expression for mutant 1}} + \underbrace{\beta_3 \mathbf{1}_{\text{genotype K}}}_{\text{additional expression for mutant 2}}$$

testing if genotype K has an expression different to genotype H:

	β_0	β_1	β_2	β_3
genotype K	1	0	0	1
- genotype H	1	0	1	0
⇒ contrast:	0	0	-1	1

➤ Example

In an experiment, gene expression is influenced by:

- ▶ leg: L1, L2, L3, L4
- ▶ type: pull, push



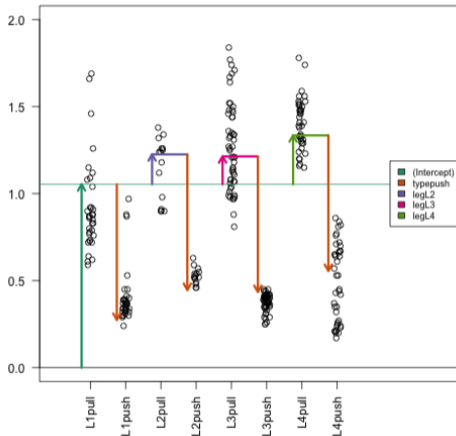
➤ Example

In an experiment, gene expression is influenced by:

- ▶ leg: L1, L2, L3, L4
- ▶ type: pull, push

```
model.matrix(~ type + leg)
```

$$\beta_0 + \beta_1 \mathbf{1}_{L2} + \beta_2 \mathbf{1}_{L3} + \beta_3 \mathbf{1}_{L4} + \gamma \mathbf{1}_{\text{push}}$$



➤ Example

In an experiment, gene expression is influenced by:

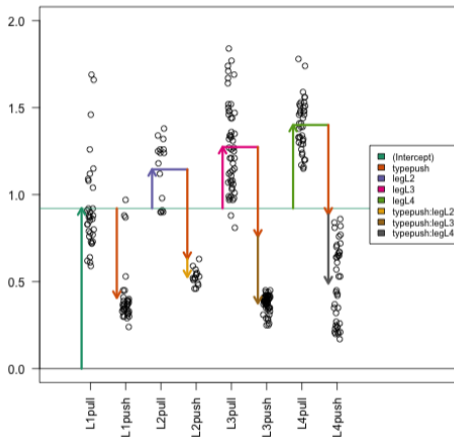
- ▶ leg: L1, L2, L3, L4
- ▶ type: pull, push

with the interaction term

`model.matrix(~ type + leg + type:leg)`

$$\beta_0 + \beta_1 \mathbf{1}_{\text{push}} + \beta_2 \mathbf{1}_{L2} + \beta_3 \mathbf{1}_{L3} + \beta_4 \mathbf{1}_{L4} + \gamma_1 \mathbf{1}_{\text{push} \& L2} + \gamma_2 \mathbf{1}_{\text{push} \& L3} + \gamma_3 \mathbf{1}_{\text{push} \& L4}$$

Testing interaction: `coef = 6:8`



➤ Example

In an experiment, gene expression is influenced by:

- ▶ leg: L1, L2, L3, L4
- ▶ type: pull, push

equivalently, with group = leg × type

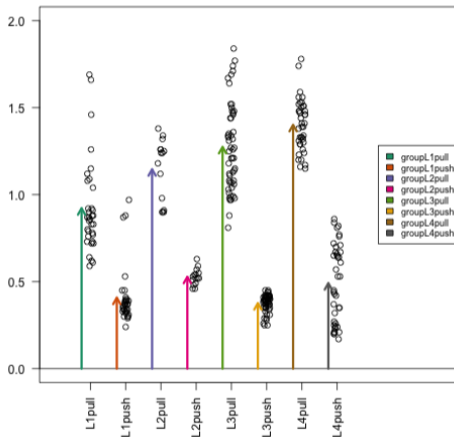
`model.matrix(~ 0 + group)`

$\beta_1 \mathbf{1}_{L1 \& \text{pull}} + \beta_2 \mathbf{1}_{L1 \& \text{push}} +$

$\beta_3 \mathbf{1}_{L2 \& \text{pull}} + \beta_4 \mathbf{1}_{L2 \& \text{push}} +$

$\beta_5 \mathbf{1}_{L3 \& \text{pull}} + \beta_6 \mathbf{1}_{L3 \& \text{push}} +$

$\beta_7 \mathbf{1}_{L4 \& \text{pull}} + \beta_8 \mathbf{1}_{L4 \& \text{push}}$



➤ Alternative approach: linear model for count data

Basic idea:

1. data are transformed so that they are approximately normally distributed

```
tcount <- voom(counts, design)
```

2. a linear (Gaussian) model is fitted (with a Bayesian approach to improve FDR [McCarthy and Smyth, 2009]):

$$\tilde{K}_{gj} \sim \mathcal{N}(\mu_{gj}, \sigma_g^2)$$

with

$$\mathbb{E}(\tilde{K}_{gj}) = \beta_0 + \mathbf{x}_j^T \beta_g$$

```
fit <- lmFit(tcount, design)
fit <- eBayes(fit)
topTables(fit, ...)
```



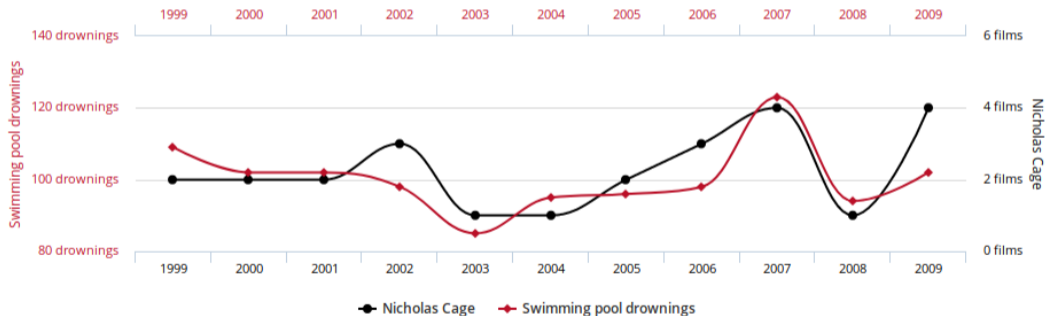
➤ But never forget: correlation is not causality!

Number of people who drowned by falling into a pool

correlates with

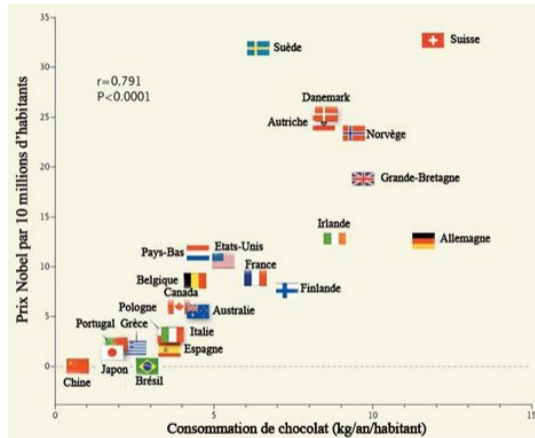
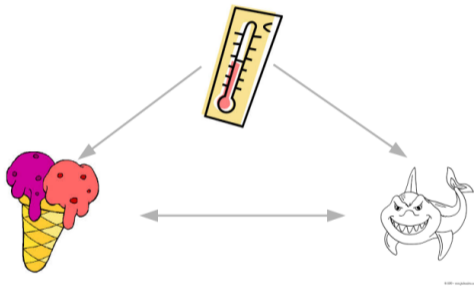
Films Nicolas Cage appeared in

Correlation: 66.6% ($r=0.666004$)

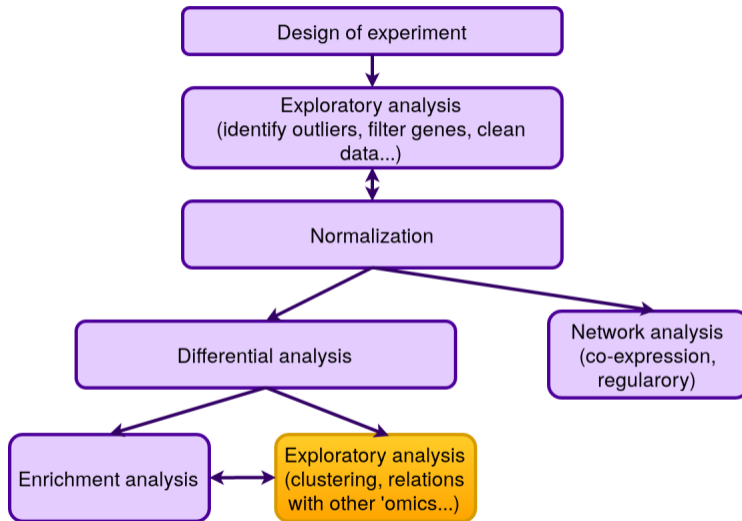


Spurious correlations: <http://www.tylervigen.com/spurious-correlations>

➤ ... and be aware of the Simpson's effect!

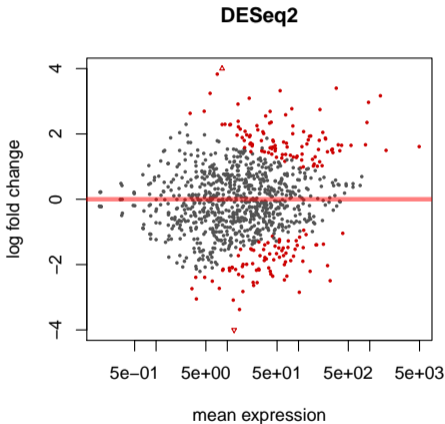
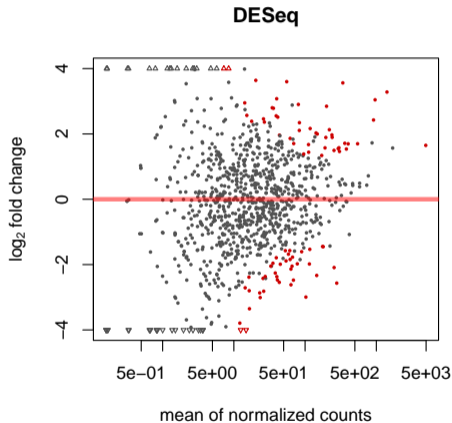


➤ Part V: Interpreting differential analysis results



➤ Overview of the results: MA-plot

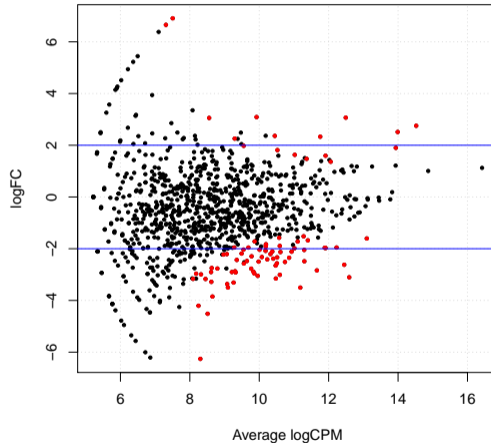
```
plotMA(..., main="DESeq", ylim=c(-4,4))  
plotMA(..., main="DESeq2", ylim=c(-4,4))
```



(the last one includes a prior on log₂ fold change which results in more moderated estimates for low count genes)

➤ Overview of the results: MA-plot

```
plotSmear(..., de.tags = ...)
```

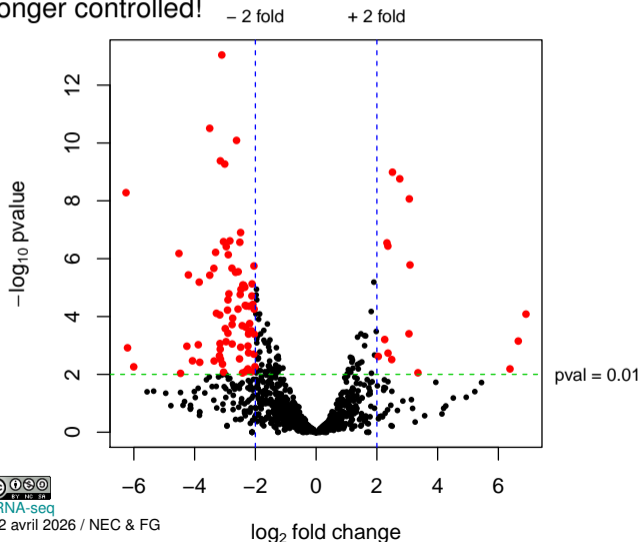


➤ Fold change and p-value: the Volcano plot

p-value versus fold change (both log scaled) scatterplot. Significant genes are in red ?



FDR is no longer controlled!



➤ Gene clustering

Prior clustering: transform data to obtain counts with similar variance

- ▶ **DESeq, DESeq2**

```
varianceStabilizingTransformation(...)
```

- ▶ **DESeq2**

```
rlog(...)
```

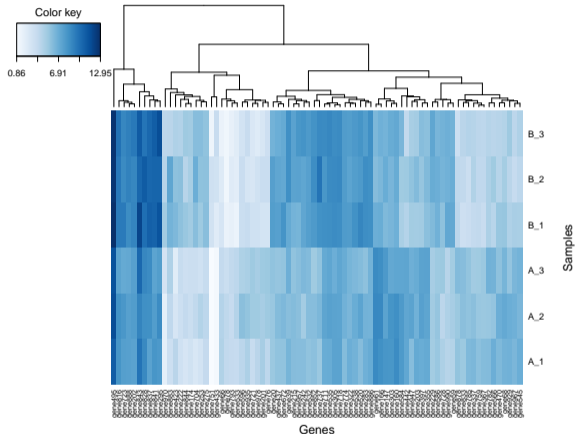
- ▶ **edgeR**

```
cpm(..., prior.count=2, log=TRUE)
```



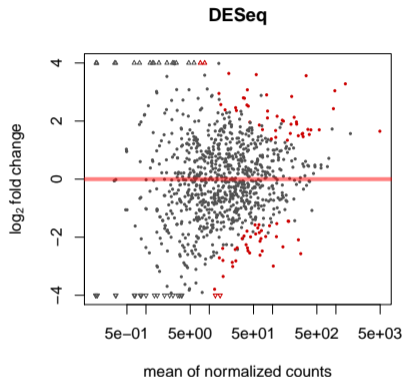
➤ Gene clustering

On transformed data, use *e.g.*, heatmap:



which is useful to visualize which genes are over/under-expressed in one condition.

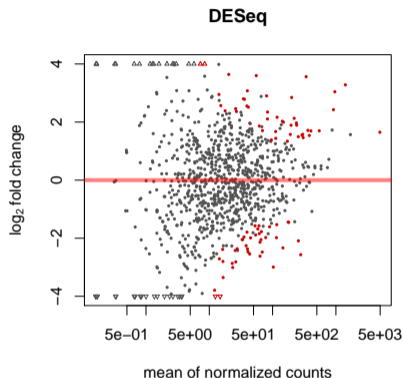
➤ Standard property of usual DE analyses



Remark: low read counts have a too large variance to be found differentially expressed.



➤ Standard property of usual DE analyses

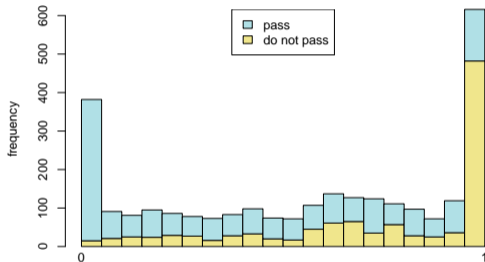


Remark: low read counts have a too large variance to be found differentially expressed.

Consequence: filtering out these genes before the DE analysis because it improves the power of the test because of multiple test correction.

➤ Example

Filtering out the 40% genes that have the lowest overall counts does not affect much low p-values:

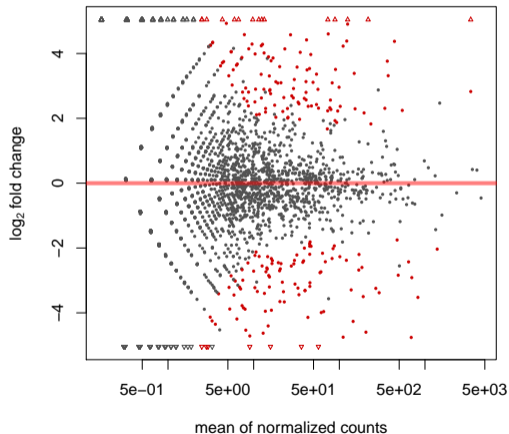


but leads to find new DE genes that were previously discarded by multiple test correction.



Filtering in practice

```
cdsFilt <- HTSFilter(..., plot=FALSE)$filteredData  
res <- exactTest(cdsFilt)
```



> In summary... (with **edgeR**)

preparation of the **design of the experiment**

| **sequencing**

count data

exploratory analysis (hist, boxplot...)

| creating an R object with count data (**DGEList**)

a **DGEList** object

| normalization (**calcNormFactors**)

a **DGEList** object with normalization factors

| fitting the model (**estimateDisp**)

a **DGEList** object with dispersion estimates

| filtering low count genes (**HTSFilter**)

a **DGEList** object without filtered genes

| test (**exactTest** or **glmFit/glmLRT**)

a **DGEEexact** or **DGELRT** object




exploratory analysis (**topTags**, **plotSmear**...)

Thank you for giving us feedback!






<https://sondages.inrae.fr/index.php/84236?lang=fr>



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