Statistical analysis of RNA-Seq data

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Introduction

Differential analysis

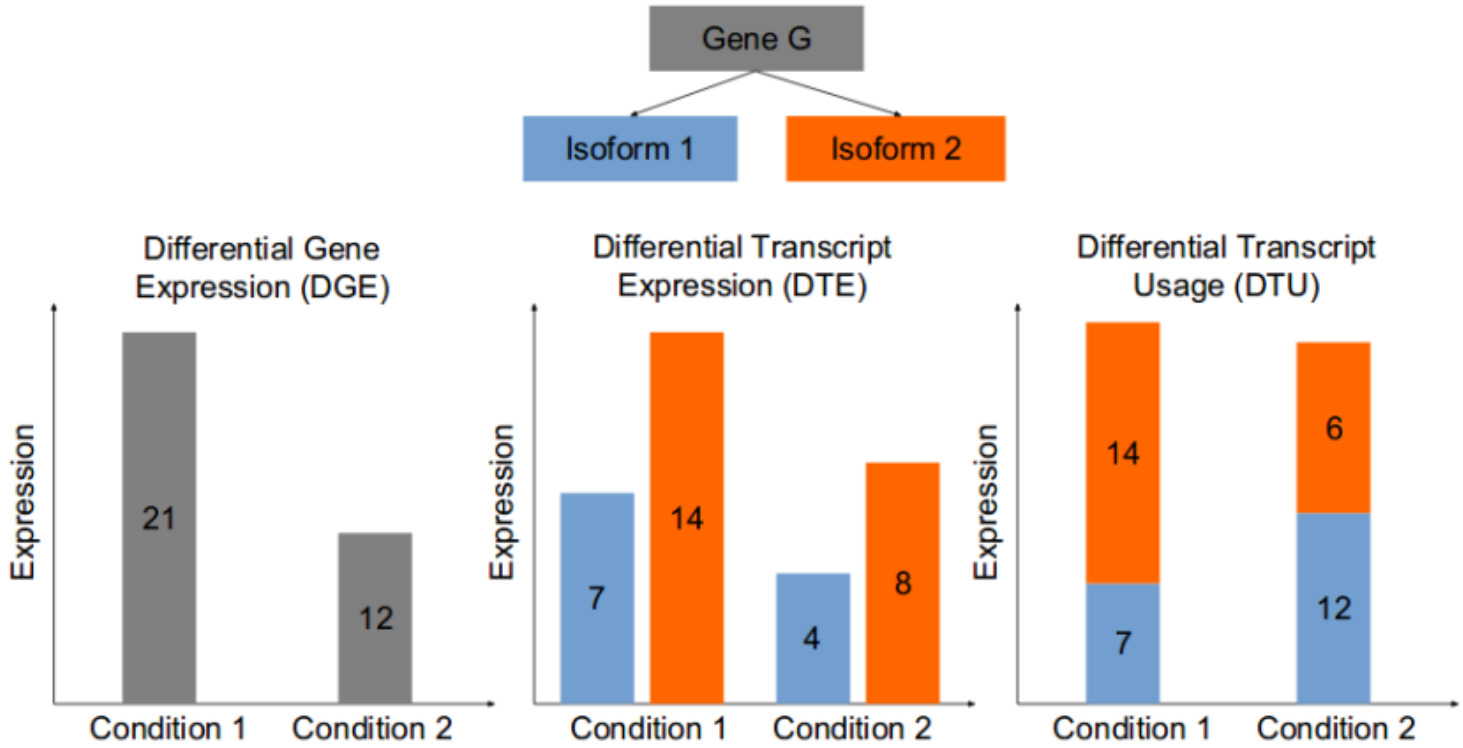
Comparison of treatments, states, conditions, ...
Example : ill vs healthy
$\Rightarrow$ statistical analysis based on tests

Particularities of NGS data :

- Very few individuals
- Many tests (one per variable)
- Count data (statistical distributions different from the ones used for continuous data from microarrays)

Introduction

DGE : differential gene expression, DTE : differential transcript expression, DTU : differential transcript usage



This course focuses on DGE

Differential analysis

A gene is declared differentially expressed if the observed difference between two conditions is statistically significant, that is to say higher than some natural random variation.

Key steps for statisticians :

- experimental design
- normalization
- differential analysis
- multiple testing

Plan

1. Experimental design
2. Exploratory data analysis
3. Normalization
4. Differential analysis
5. Presenting results

Not a recent idea !

To consult a statistician after an experiment is finished is often merely to ask him to conduct a post-mortem examination. He can perhaps say what the experiment died of (Ronald A. Fisher, Indian statistical congress, 1938, vol. 4, p 17).

While a good design does not guarantee a successful experiment, a suitably bad design guarantees a failed experiment (Kathleen Kerr, Inserm workshop 145, 2003)

Make an experimental design

Context of a RNA-seq experiment

Rule 0 : Share a common language in biology, bioinformatics and statistics.

Experimental design

All skills are needed to discussions right from project construction.

- Rule 1 : Well define the biological question, get together and collect a priori knowledge (e.g. reference genome, splicing),
- Rule 2 : Anticipate, identify all factors of variation and adapt Fisher's principles (1935), collect metadata from experiment and sequencing,
- Rule 3 : Choose a priori tools/methods for bioinformatics and statistical analyses,
- Rule 4 : Draw conclusions on results.

Experimental design

A good design is a list of experiments to conduct in order to answer to the asked question which maximize collected information and minimize experiments cost with respect to constraints.

Rule 1 : Well define the biological question : make a choice

- Identify differentially expressed genes,
- Detect and estimate isoforms,
- Construct a de novo transcriptome.

Rule 2 : adapt Fisher's principles : randomization and blocking AVOID CONFUSION between the biological variability of interest and a biological or technical source of variation

Experimental design

Biological vs technical replicate

Biological replicate : Repetition of the same experimental protocol but independent data acquisition (several samples).
Technical replicate : Same biological material but independent replications of the technical steps (several extracts from the same sample).

Sequencing technology does not eliminate biological variability.
(Nature Biotechnology Correspondence, 2011)

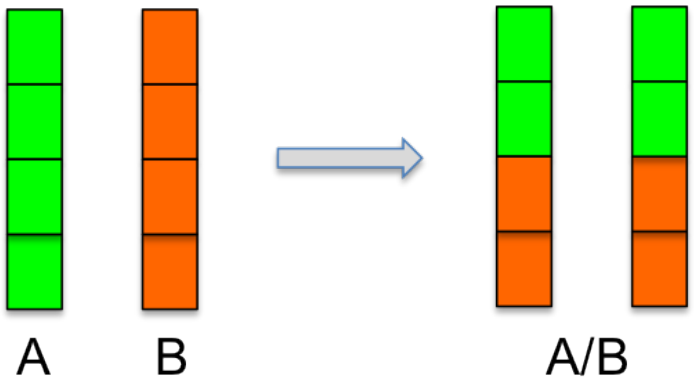
$$\text{lane effect} < \text{run effect} < \text{library prep effect} << \text{biological effect}$$

[Marioni et al., 2008],[Bullard et al., 2010]

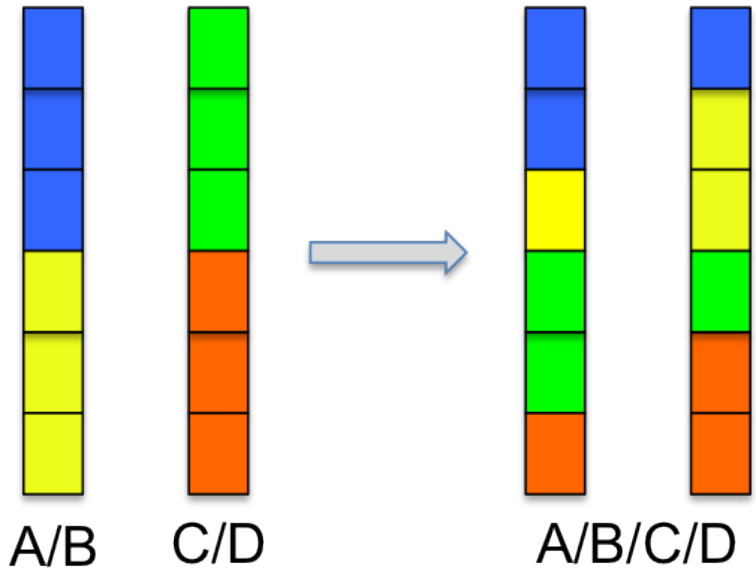
Include at least three biological replicates in your experiments !
Technical replicates are not necessary.

Experimental design

AVOID CONFUSION between the biological variability of interest and a biological or technical source of variation



Problem : Confusion between lane and condition
Solution : Distribute the conditions evenly on both lanes



Problem : Partial confusion between lane and condition
Solution : Distribute the conditions "evenly" on both lanes

Experimental design

Find genes that are differentially expressed between a normal skin and a damaged skin on mouse

Sample	Condition	RNA extraction date
S1	control	July 12th, 2016
S2	control	July 12th, 2016
S3	control	July 12th, 2016
S4	wound	July 20th, 2016
S5	wound	July 20th, 2016
S6	wound	July 20th, 2016

Confusion between skin status and RNA extraction date : comparing healthy and damaged skin is comparing RNAs extracted July 12th and 20th

Experimental design

Find genes that are differentially expressed between a normal skin and a damaged skin on mouse

Sample	Condition	RNA extraction date
S1	control	July 12th, 2016
S2	control	July 20th, 2016
S3	control	July 25th, 2016
S4	wound	July 12th, 2016
S5	wound	July 20th, 2016
S6	wound	July 25th, 2016

One solution : the day effect is evenly distributed across conditions.

Experimental design

Find genes that are differentially expressed between a normal skin and a damaged skin on mouse

Sample	Condition	RNA extraction date	mouse
S1	control	July 12th, 2016	m1
S2	control	July 20th, 2016	m2
S3	control	July 25th, 2016	m3
S4	wound	July 12th, 2016	m1
S5	wound	July 20th, 2016	m2
S6	wound	July 25th, 2016	m3

One solution : the day effect is evenly distributed across conditions.

In case of paired data the pairing may be confounded with the batch effect. These effects are NOT confounded with the biological effect of interest.

Experimental design

Why increasing the number of biological replicates ?

- To generalize to the population level
- To estimate with a higher degree of accuracy variation in individual transcript [Hart et al., 2013]
- To improve detection of DE transcripts and control of false positive rate [Soneson and Delorenzi, 2013]
- To focus on detection of low mRNAs, inconsistent detection of exons at low levels ($\leq$ 5 reads) of coverage [McIntyre et al., 2011]

More biological replicates or increasing sequencing depth ?

It depends ! [Haas et al., 2012], [Liu et al., 2014]

- DE transcript detection : (+) biological replicates
- Construction and annotation of transcriptome : (+) depth and (+) sampling conditions
- Transcriptomic variants search : (+) biological replicates and (+) depth

Support

- An experimental design using multiplexing,
- Tools for experimental design decisions : Scotty [Busby et al., 2013], RNAseqPower [Hart et al., 2013], PROPER [Wu et al., 2015]

And do not forget : budget also includes cost of biological data acquisition, sequencing data backup, bioinformatics and statistical analysis.

For a good (nice) experiment design ...

Before the experiment

- Ask a precise and well defined biological question
- List all possible biological confounding effects (sex, age, ...)
- Collect samples while taking care of the distribution of unwanted sources of variation across samples
- Include at least three biological replicates per condition. Technical replicates are not necessary
- Distribute samples on lanes and flow cells ...
  - according to the comparisons to be made
  - without introducing a confusion between technical effects and the biological effects of interest
  - applying the same multiplexing rate on all samples

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SARtools

SARTools : Statistical Analysis of RNA-Seq Tools [Varet et al., 2016]

- exports the results into easily readable **tab-delimited files**
- generates **a HTML report** which displays all the figures produced, explains the statistical methods and gives the results of the differential analysis.

- Exploratory data analysis
- Differential analysis including normalization and multiple testing

Available on R and Galaxy

Exploratory data analysis

Sample comparison for RNA-Seq [Schulze et al., 2012]

Pearson's correlation coefficient

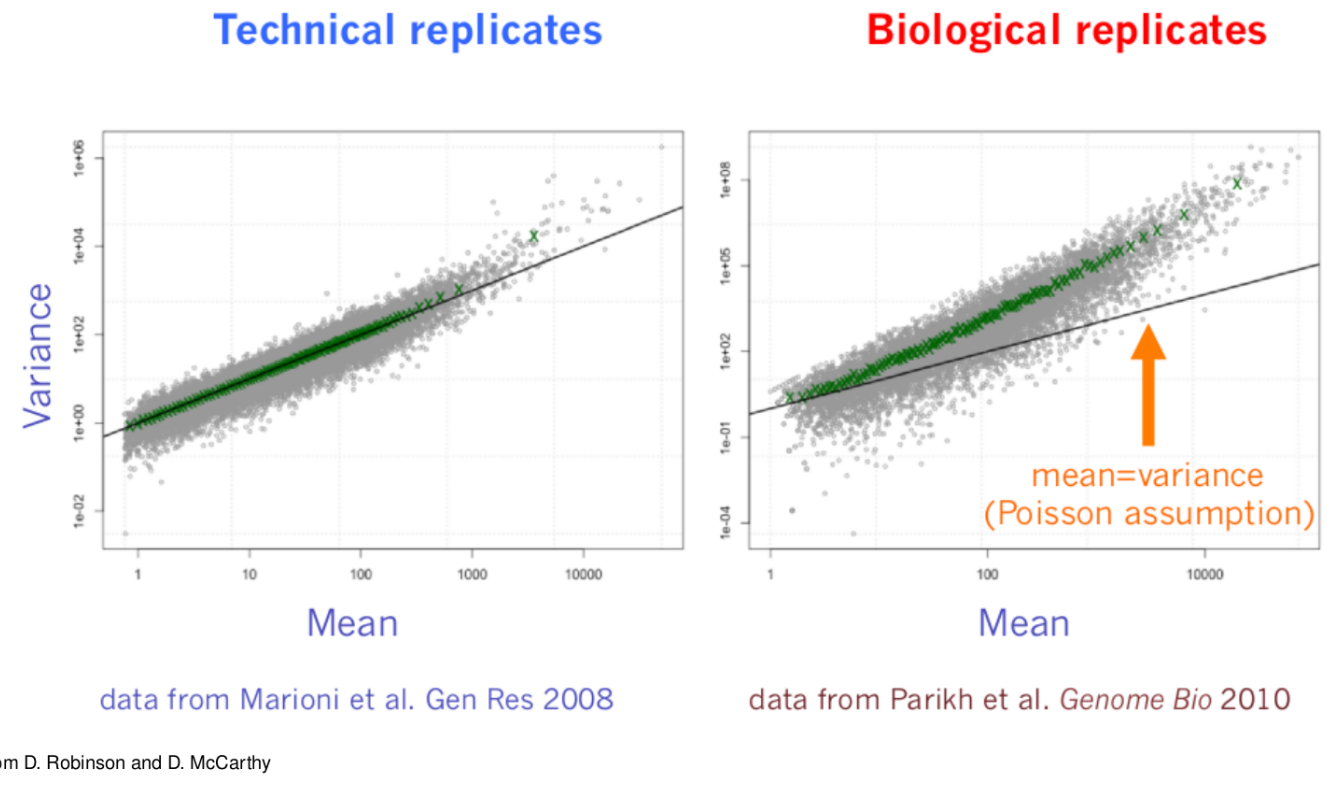
- widely used . . .
- . . .but highly dependent on sequencing depth and the range of expression samples inherent to the sample.

SERE : Simple Error Ratio Estimate

- ratio of observed variation to what would be expected from an ideal Poisson experiment
- interpretation unambiguous regardless of the total read count or the range of expression
- score of 1 : faithful replication
- score of 0 : data duplication
- scores > 1 true global differences between RNA-Seq libraries

Exploratory data analysis

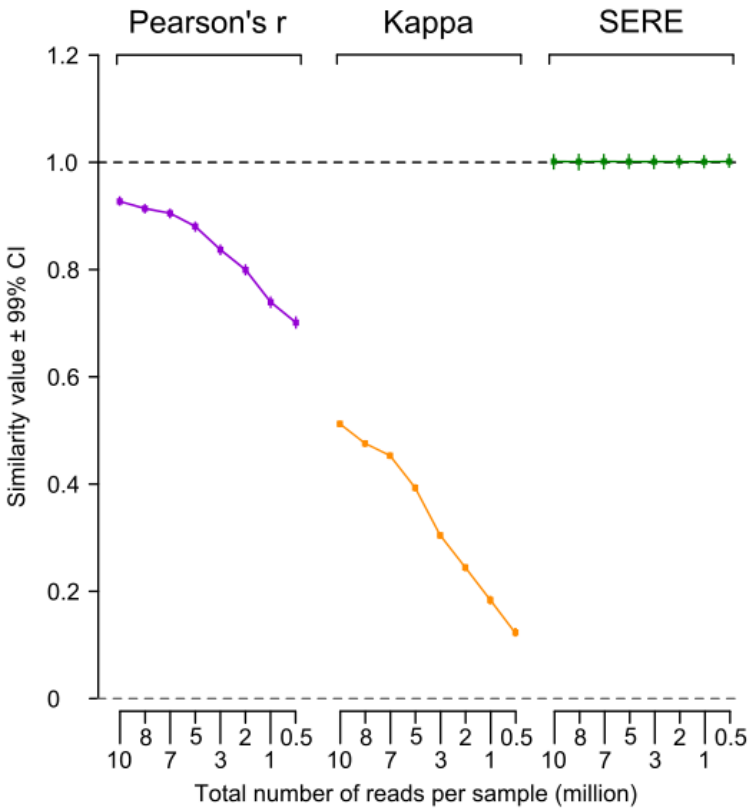
scores between 0 and 1 $\Rightarrow$ underdispersion (variance smaller than mean)



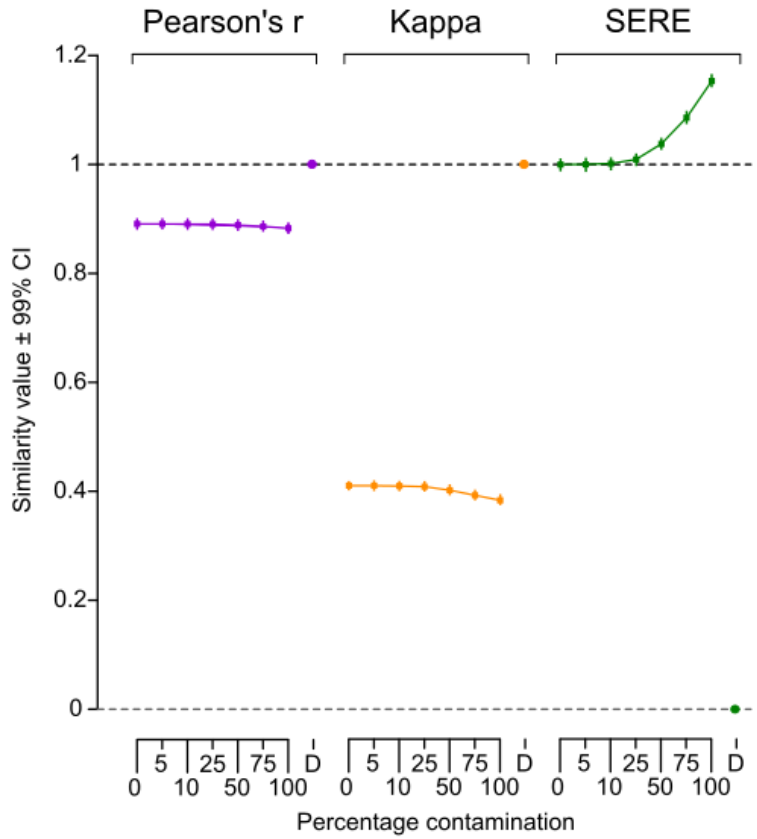
From D. Robinson and D. McCarthy

scores greater than 1 : overdispersion $\Rightarrow$ adapted to biological replicates

Sample comparison for RNA-Seq



total read count dependence



sensitivity to contamination

source : [Schulze et al., 2012]

Exploratory data analysis

Multivariate exploratory data analysis

Main goal : explore the structure of the dataset to better understand the proximity between samples and detect possible problems. **This is a quality control step**

Two main tools

- Principal Component Analysis (PCA) or MultiDimensional Scaling (MDS)
- Clustering

Pre-requisite

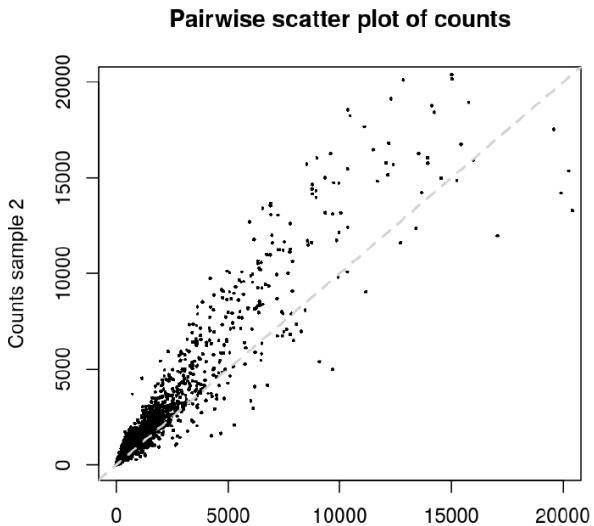
To apply these methods, make the data homoscedastic : **the variance must be independent of the intensity**

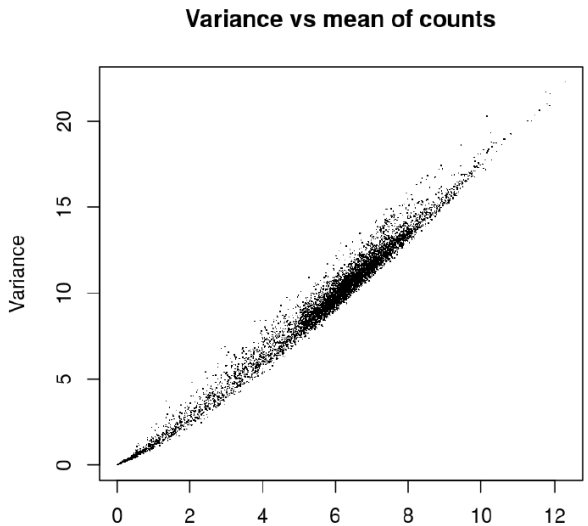
Exploratory data analysis

Transformations proposed :

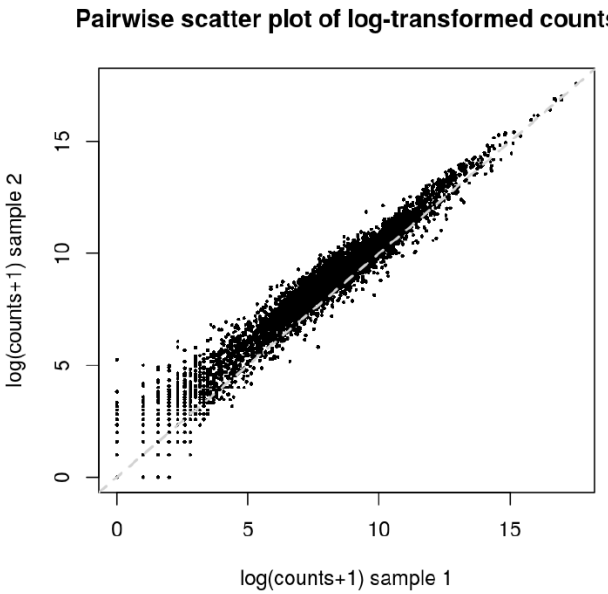
- DESeq2 : VST (Variance Stabilizing Transformation) or rlog (Regularized Log Transformation)
- edgeR : transformation of the count data as moderated log-counts-per-million

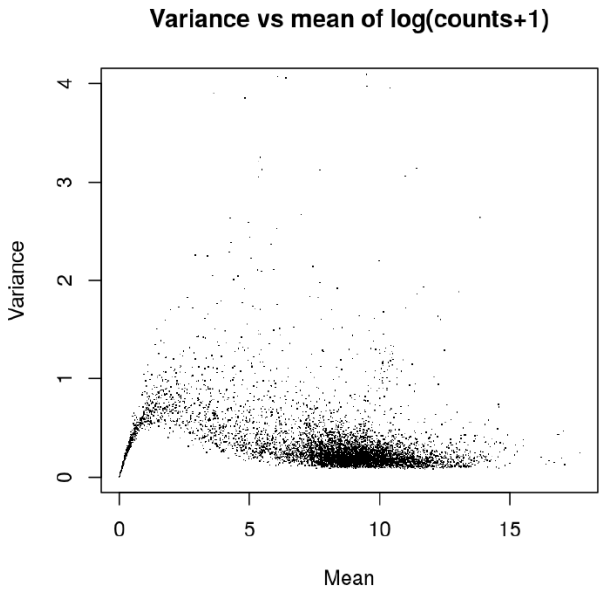
Illustration : Without transformation : variance increases with mean

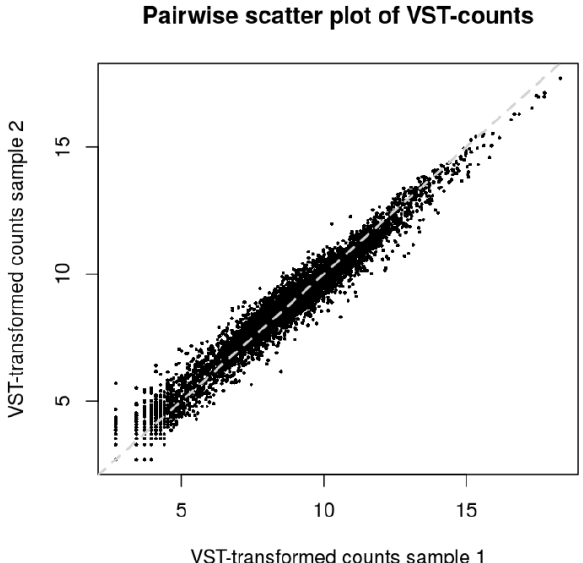


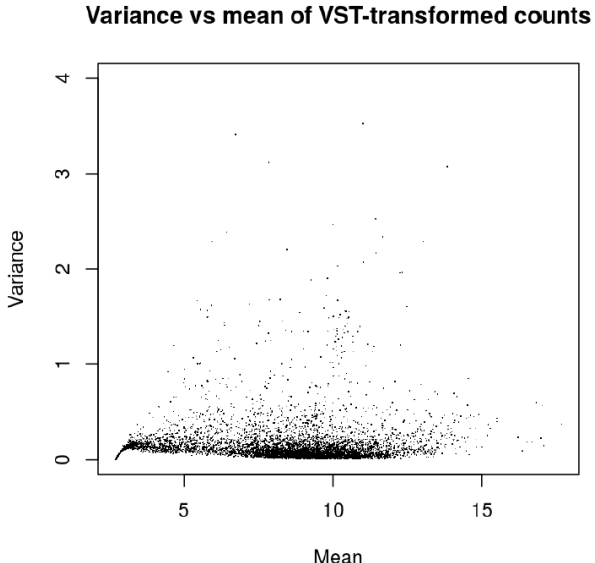


Exploratory data analysis - VST transformation









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Normalization

Definition

Normalization is a process designed to identify and correct **technical biases** removing the least possible biological signal. This step is technology and platform-dependant.

Within-sample normalization

Normalization enabling comparisons of fragments (genes) from a same sample.
No need in a differential analysis context.

Between-sample normalization

Normalization enabling comparisons of fragments (genes) from different samples.

Sources of variability

Read counts are proportional to expression level, gene length and sequencing depth (same RNAs in equal proportions).

Within-sample

- Gene length
- Sequence composition (GC content)

Between-sample

- Depth (total number of sequenced and mapped reads)
- Sampling bias in library construction ?
- Presence of majority fragments
- Sequence composition due to PCR-amplification step in library preparation [Pickrell et al., 2010], [Risso et al., 2011]

Comparison of normalization methods

A lot of different normalization methods...

- Some are part of models for DE, others are 'stand-alone'
- They do not rely on similar hypotheses
- But all of them claim to remove technical bias associated with RNA-seq data

Which one is the best ?

[Dillies et al., 2013], on behalf of StatOmique Group
Evaluation of normalization methods for RNA-Seq differential analysis at the gene level

Comparison of normalization methods

Focus on methods which aim at making read counts comparable across samples

Two main types

1. Methods that make read count distributions similar (if not equal)
2. Methods assuming that most genes are not differentially expressed

Note that :

- These methods apply on raw (integer) count data, to RNA-seq data (metagenomics), for differential expression analysis
- Other more complex methods have been proposed after the comparison [Risso et al., 2014]
- Library size : Number of reads that have been sequenced, mapped and counted for a given sample (sum on columns on the count table)

Which method should I use ? [Dillies et al., 2013]

In most cases

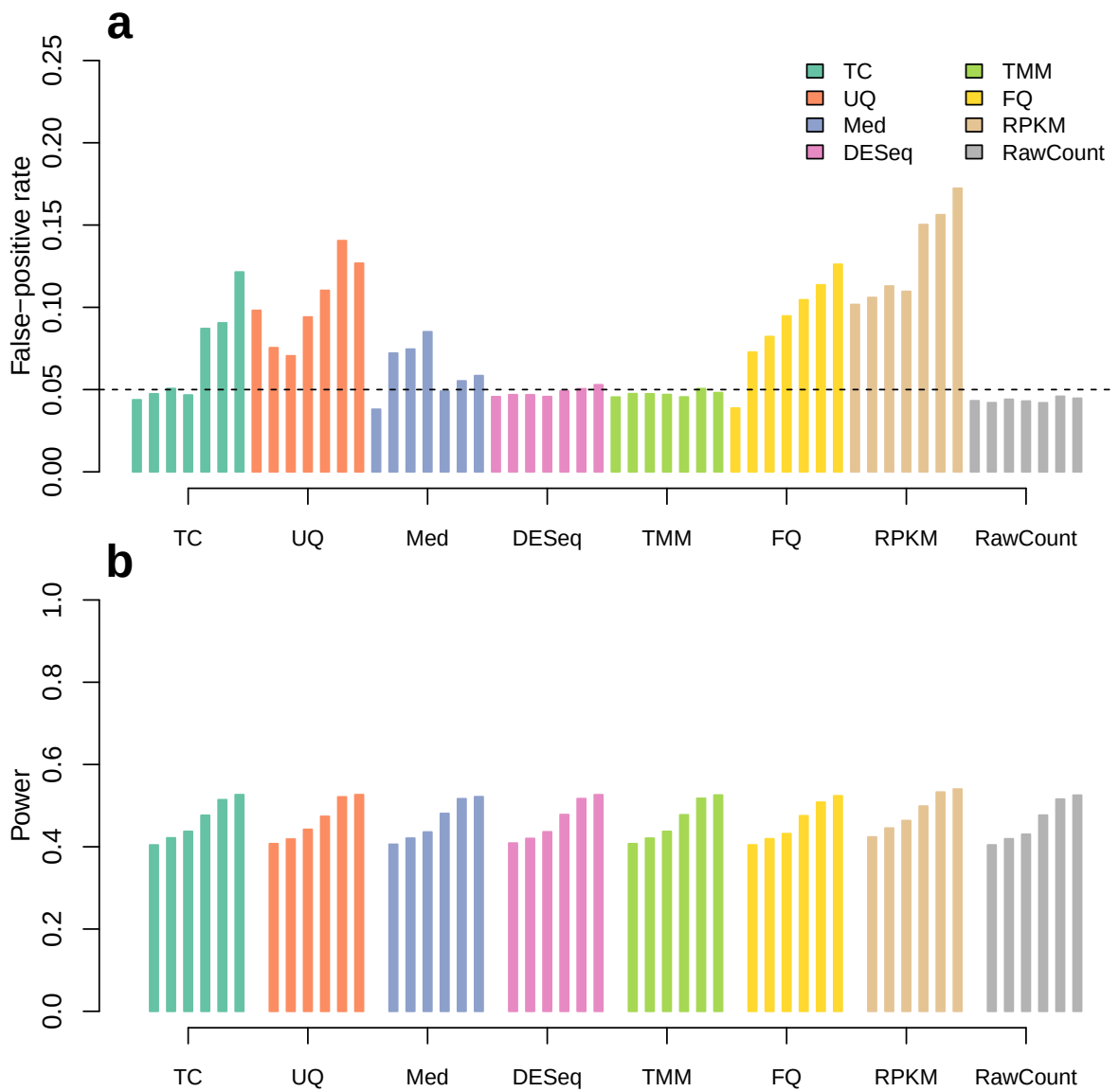
All methods provide comparable results

Anyway ...

Clear differences appear in the presence of high count genes or when the expressed RNA repertoire varies notably across samples

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

Which method should I use ? [Dillies et al., 2013]



Conclusions

- Hypothesis : the majority of genes is invariant between two samples.
- Differences between methods when presence of majority sequences, very different library depths.
- TMM and DESeq : performant and robust methods in a DE analysis context on the gene scale.
- Normalization is necessary and not trivial.
- Detection of differential expression in RNA-seq data is inherently biased (more power to detect DE of longer genes)
- Do not normalise by gene length in a context of differential analysis.

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Statistical significance and practical importance

Differential analysis :

Detect differentially expressed genes between two conditions

Fold change : measure describing how much a quantity changes. Various definitions (see Wikipedia, ipfs.io). In this course : ratio between measurements. If condition A measures 50 and condition B measures 100, fold change = 100/50 =2 and measure B is twice higher than measure A.

Log fold change : mean of normalised values in condition 1 - mean of normalised values in condition 2 (log B/A= log B - log A)

Question : Why not only using the fold change or log fold change to find differentially expressed genes ?

Statistical significance and practical importance

Fold change does not take the variance of the samples into account. Problematic since variability in omic data is partially marker-specific.

The difference between 102 and 100 is the same as between 4 and 2 but does not seem to have the same importance, regarding the baseline value.

Statistical significance and practical importance

Fold change does not take the variance of the samples into account. Problematic since variability in omic data is partially marker-specific.

The difference between 102 and 100 is the same as between 4 and 2 but does not seem to have the same importance, regarding the baseline value.

Practical importance and statistical significance have little to do with each other.

- An effect can be important, but undetectable (statistically insignificant) because the data are few, irrelevant, or of poor quality.
- An effect can be statistically significant (detectable) even if it is small and unimportant, if the data are many and of high quality.

Differential analysis

Aim : Detect differentially expressed genes between two conditions

- Discrete quantitative data
- Few replicates
- Overdispersion problem

Challenge : method which takes into account overdispersion and a small number of replicates

- Proposed methods : edgeR, DESeq for the most used and known [Anders et al., 2013]
- An abundant litterature
- Comparison of methods : [Pachter, 2011], [Kvam and Liu, 2012], [Soneson and Delorenzi, 2013], [Rapaport et al., 2013]

Statistical test

For each gene : is it differentially expressed between A and B ?

- Generalized linear framework
- Hypothesis to test : H_{0i} Equality of relative abundance of gene i in condition A and B vs H_{1i} non-equality
- Wald Test or Likelihood Ratio Test

p-value $p(t)$

For a realisation t of the T test statistic $p(t)$ is the probability (calculating under H_0) of obtaining a test statistic at least as extreme as the one that was actually observed.

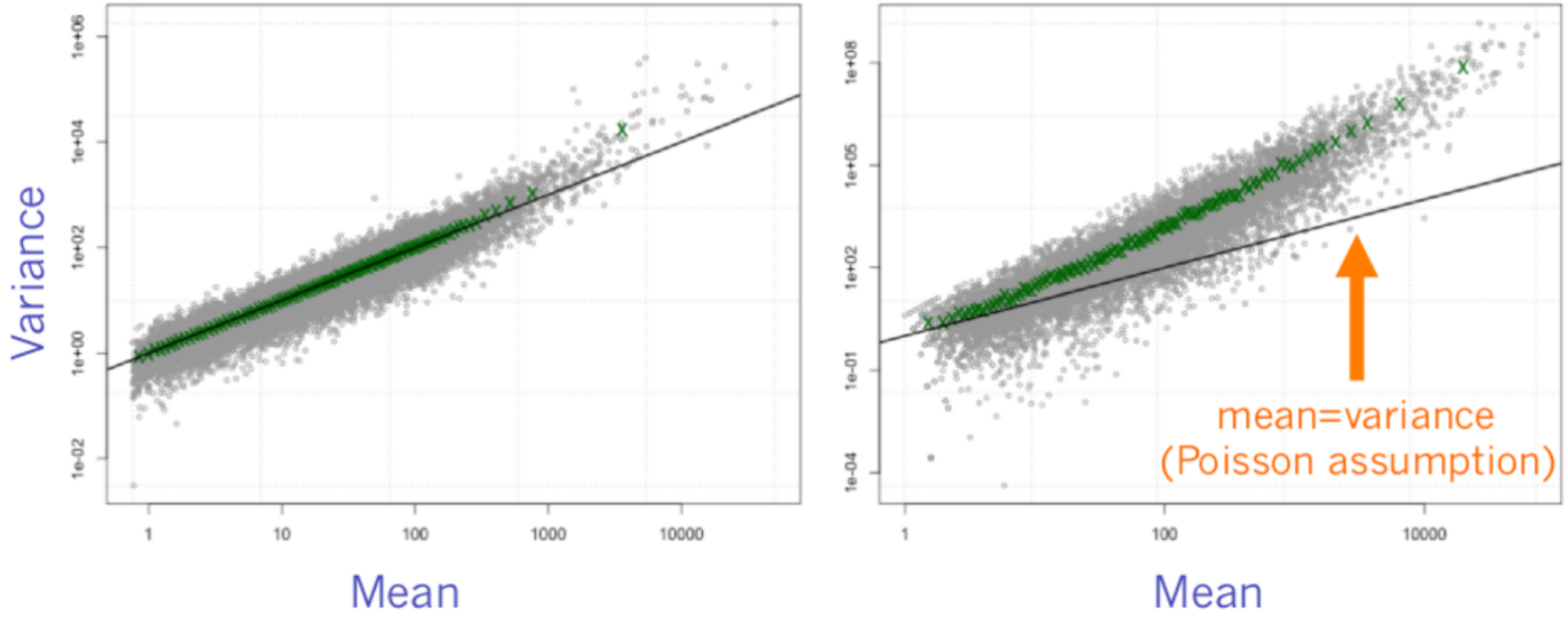
The p-value measures the agreement between H_0 and the obtained result.

Mean-Variance Relationship

The Poisson distribution to model counts

- Describes the number of occurences of rare events during a given time interval
- Property : Mean = Variance

Technical replicates | **Biological replicates**



data from Marioni et al. Gen Res 2008

data from Parikh et al. *Genome Bio* 2010

Overdispersion in RNA-seq data

Counts from biological replicates tend to have variance exceeding the mean (= overdispersion). Poisson describes only technical variation.

What causes this overdispersion ?

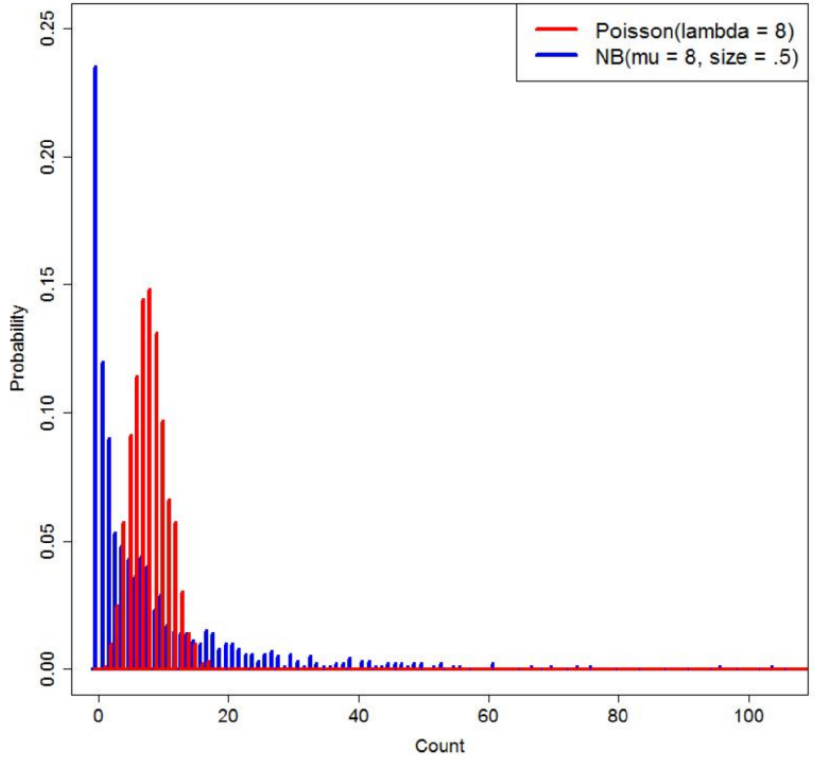
- Correlated gene counts
- Clustering of subjects
- Within-group heterogeneity
- Within-group variation in transcription levels
- Different types of noise present...

In case of overdispersion, increase of the type I error rate (probability to declare incorrectly a gene DE).

Negative Binomial Models

A supplementary dispersion parameter ϕ to model the variance

Poisson vs Negative Binomial models



Technical variability is the main source of variability in low counts, whereas biological variability is dominant in high counts

Available tests

Models of count data

- Data transformation and gaussian-based model : limma - voom
- Poisson : TSPM
- Negative Binomial : edgeR, DESeq(2), NBPSeq, baySeq, ShrinkSeq, ...

Statistical approaches

- Frequentist Approach : edgeR, DESeq(2), NBPSeq, TSPM, ...
- Bayesian Approach : baySeq, ShrinkSeq, EBSeq, ...
- Non-parametric approach : SAMSeq, NOISeq, ...

Comparison of two conditions [Soneson and Delorenzi, 2013]

A comparison of methods for differential analysis of RNA-Seq data [Soneson and Delorenzi, 2013]

- 11 statistical tests included in the study
- R packages
- input data are raw counts (gene-level analysis)
- TMM or DESeq normalization

Main results

- **With only two biological replicates, all the methods show low performances.** They either lack power or poorly control the false positive rate.
- No method outperforms the others in all circumstances : **the method should be chosen according to the dataset**

How to choose ?

- Number of replicates of the experiment
- Presence / absence of outliers
- Constant / variable within-group dispersion
- Balanced / unbalanced differential expression
  (results are more accurate and less variable between methods if DE genes are regulated in both directions)
- Simple / complex experiment design

edgeR and DESeq(2)

DESeq2 et edgeR : similarities . . .

- Easy to use and well documented R packages
- A 3-step analysis process : normalization, dispersion estimation, statistical test
- Negative Binomial distribution of counts and Generalized Linear Models (GLM) : allows analysis of simple and complex designs

. . . and differences

- outlier detection and processing
- low counts filtering
- **dispersion estimation**

In both cases, the version matters

Estimating the dispersion : the key question

Problem

Estimate a reliable dispersion from a very small number of replicates (sometimes less than 5)

Why using sophisticated approaches ?

- gene-specific tests $\Rightarrow$ lack of sensitivity (proportion of true positives among positives) due to the lack of information
- common dispersion parameter for all tests $\Rightarrow$ many false positives

Example : empirical bayesian approaches = compromise between gene-specific and common dispersion parameter estimation

Empirical bayesian approaches

Principles

- Bayes theorem : $P(A/B) = P(B/A)P(A)$
- "empirical" $\Rightarrow$ priors from the observed data

$$\tilde{\theta_g} = \widehat{\theta_c} + b(\widehat{\theta_g} - \widehat{\theta_c})$$

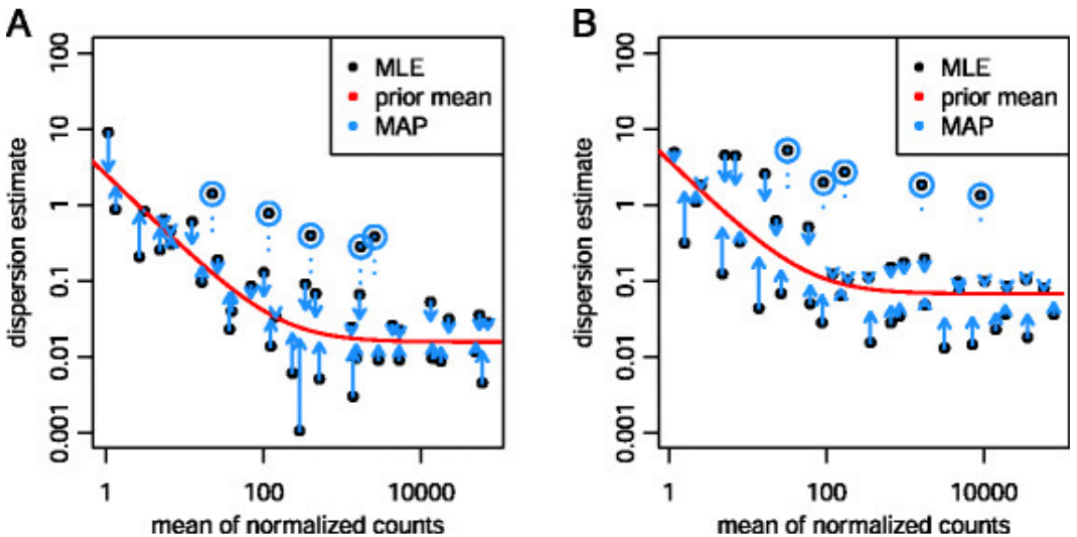
with $\tilde{\theta_g}$ = shrinkage estimator
$\widehat{\theta_c}$ = estimator of the mean population
$\widehat{\theta_g}$ = usual empirical estimator gene by gene
b =shrinkage factor

$b = 1 \Rightarrow \tilde{\theta_g} = \widehat{\theta_g}$
$b = 0 \Rightarrow \tilde{\theta_g} = \widehat{\theta_c}$

Dispersion estimation with DESeq2

Hypothesis : genes of similar average expression strength have similar dispersion

1. Estimate gene-wise dispersion estimates using maximum likelihood (ML) (black dots)
2. Fit a smooth curve (red line)
3. Shrink the gene-wise dispersion estimates (empirical Bayes approach) toward the values predicted by the curve to obtain final dispersion values (blue arrow heads).



Dispersion estimation with edgeR

1. Estimate gene-wise dispersion estimates using ML
2. Estimate a common dispersion parameter by ML
3. Moderate gene-wise dispersion estimates toward a common estimate or toward a local estimate from genes with similar expression strength using a weighted conditional likelihood.

Differences :

- DESeq2 estimates the width of the prior distribution from the data and therefore automatically controls the amount of shrinkage based on the observed properties of the data.
- edgeR requires a user-adjustable parameter, the prior degrees of freedom, which weights the contribution of the individual gene estimate and edgeR's dispersion fit.

Differences between edgeR and DESeq(2)

- **edgeR** : borrow information across genes for stable estimates of ϕ ; 3 ways to estimate ϕ (common, trend, moderated)
- **DESeq2** : relationship of variance and mean + dispersion and fold change shrinkage (for PCA and Gene Set Enrichissment Analysis) + detection of outliers

Robustness

- **edgeR :** one option : moderate dispersion less towards trend
  Allows dispersions to be driven more by the data
- **DESeq2 :** calculate Cook's distance and filter genes with outliers
  Can inadvertently filter interesting genes

Robustness - edgeR and DESeq(2)

- Robust edgeR (not by default in R) suffers a tiny bit in power with no outliers, but has good capacity to dampen their effect if present (be careful with reviews which take the value by default of edgeR) resulting in (sometimes drastic) drop in power
- DESeq2 is very powerful in the absence of outliers, but policy to filter outliers results in loss of power
- edgeR and edgeR robust are a bit liberal (5% FDR might mean 6% or 7%)

Comparaison of differential analysis methods

[Soneson and Delorenzi, 2013]

- Small number of replicates (2-3) or low expression $\rightarrow$ be careful ! !
- Large number of replicates (10 or so) or very high expression $\rightarrow$ method choice does not matter much.
- Outlier counts affect different methods in different ways. Removing genes with outlier counts or using non-parametric methods reduce the sensitivity to outliers
- Allow tagwise dispersion values
- Normalization methods have problems when all DE genes are regulated in one direction. Results are more accurate and less variable between methods if DE genes are regulated in both directions.

Comparaison of differential analysis methods

[Rapaport et al., 2013]

Evaluation on methods using SEQC benchmark dataset and ENCODE data.

- Significant differences between methods.
- Array-based methods adapted perform comparably to specific methods.
- Increasing the number of replicates samples significantly improves sensitivity over increased sequencing depth.

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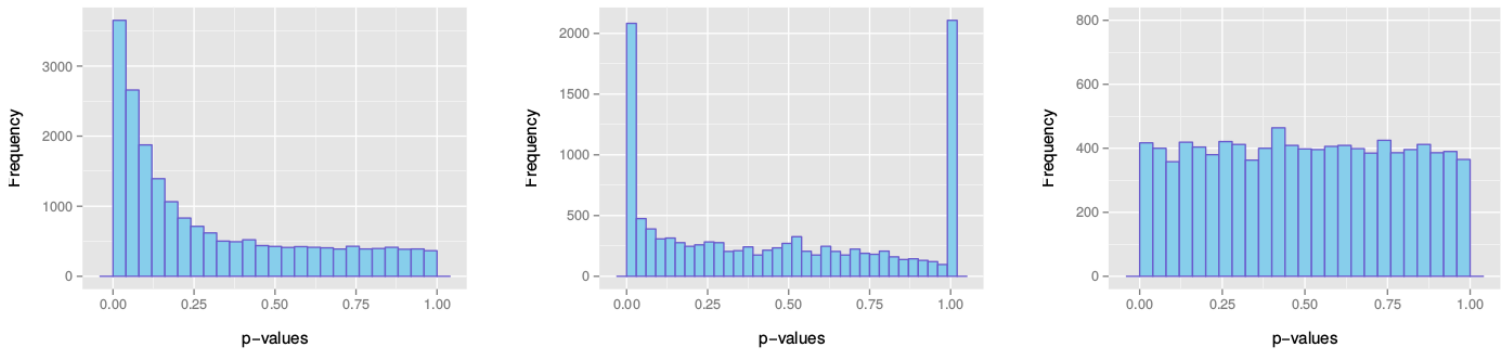
p-values histograms for diagnosis

Examples of expected overall distribution



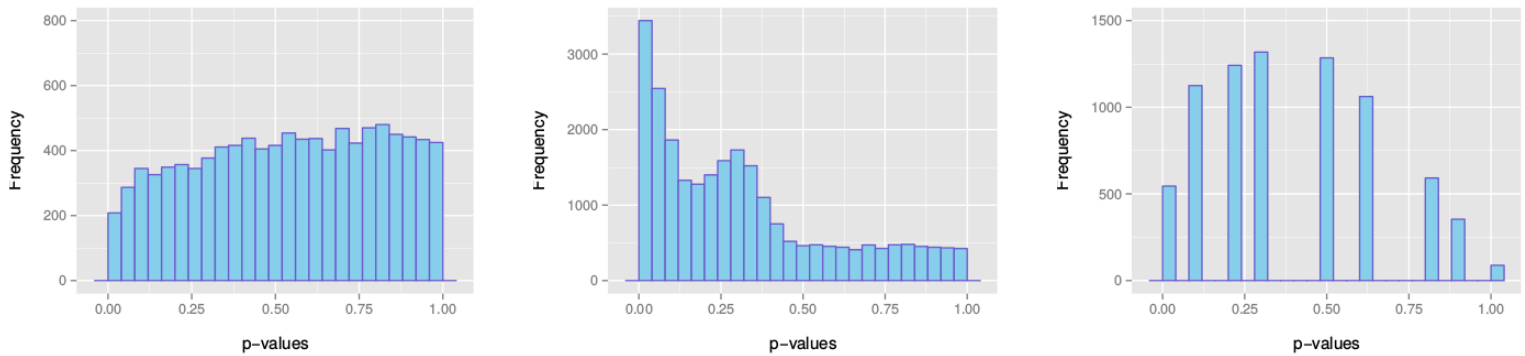
(a) : the most desirable shape

(b) : very low counts genes usually have large p-values

(c) : do not expect positive tests after correction

p-values histograms for diagnosis

Examples of not expected overall distribution



(a) : indicates a batch effect (confounding hidden variables)

(b) : the test statistics may be inappropriate (due to strong correlation structure for instance)

(c) : discrete distribution of p-values : unexpected

Multiple testing : key points

- Important to control for multiple tests
- FDR or FWER depends on the cost associated to FN and FP

Controlling the FWER :

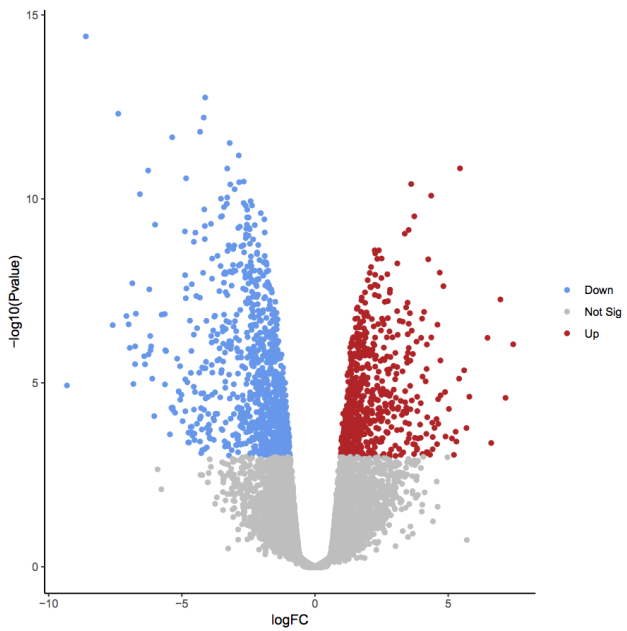
Having a great confidence on the DE elements (strong control). Accepting to not detect some elements (lack of sensitivity $\Leftrightarrow$ a few DE elements)

Controlling the FDR :

Accepting a proportion of FP among DE elements. Very interesting in exploratory study.

Volcano plot

Compromise between statistical significance and importance.
One can adapt the definition of differentially expressed by saying for exemple "A gene is declared differentially expressed (DE) if the observed difference between two conditions is statistically significant at 5% and the fold change is higher than 2"

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