RNA-Seq Analysis

Estudios in silico en Biomedicina

(Máster en Bioinformática, Universidad de Valencia)

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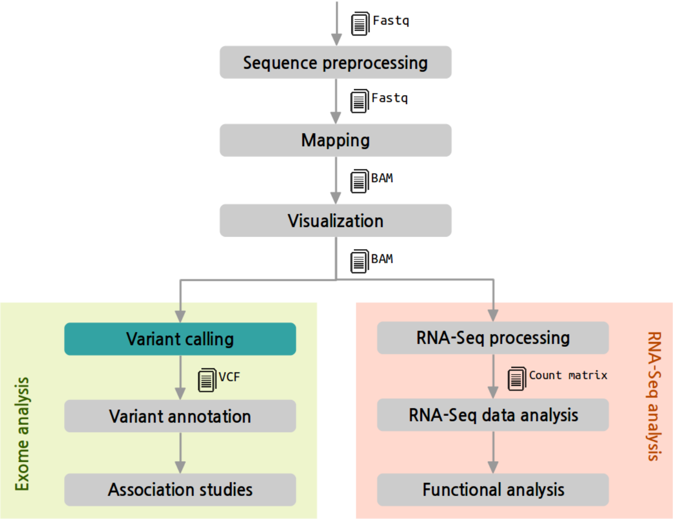
NGS pipeline



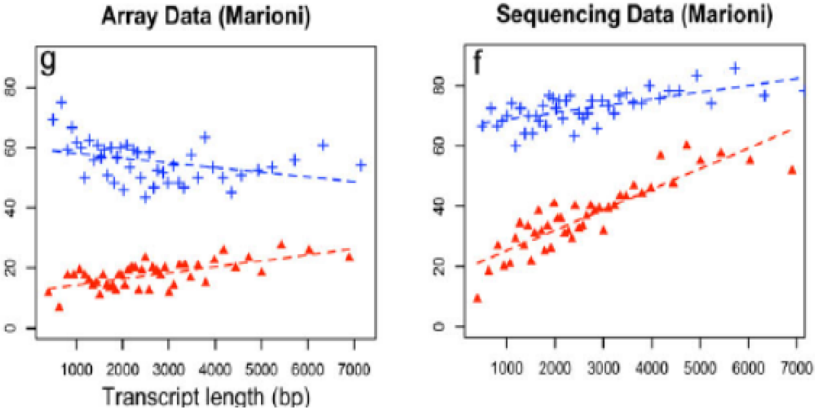
Experiment considerations

- Reference genome
  - Yes: map against it
  - No: try to assemble transcripts an then map
- Reference transcriptome
  - Yes: estimate known transcripts abundance & discover new transcripts
  - No: find expressed regions & their transcript combination

Gene transcript length dependence

Counts are proportional to . . .

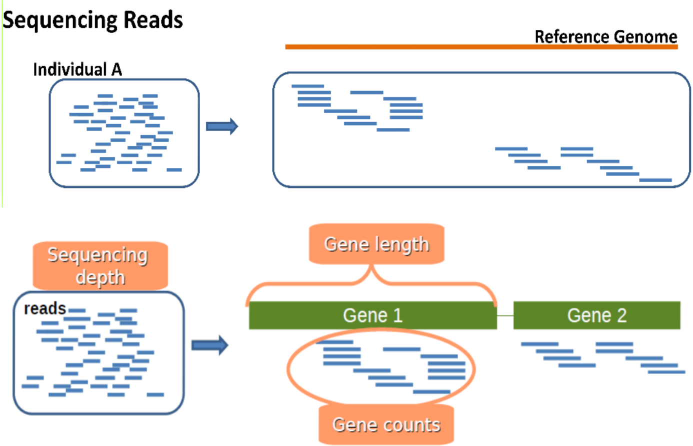
- the mRNA expression level
- the *transcript length*



Array Data (Marioni)

Sequencing Data (Marioni)

Gene transcript length dependence



Library size dependence

More reads in the library -> more reads per transcript
Library size matters.
Correction is needed for some analysis:

- Descriptive statistics: PCA, clustering, . . .

May not be so important for other analyses:

- Differential expression at gene level . . .

Count Normalization

- Transcript length: within library
- Library size: between libraries
- Many other biases . . .
  - Differences on the read count distribution among samples.
  - GC content of the gene affects the detection of that gene (Illumina)
  - sequence-specific bias is introduced during the library preparation

RPKM

RPKM: Reads Per Kilobase of the transcript per Million mapped reads

$$RPKM = 10^9 \frac{C}{NL}$$

- **C**: number of reads mapped to the exons of the gene.
- **N**: total number of mappable reads in the experiment.
- **L**: total length of the exons of the gene.

Fragments Per Kilobase of exon per Million fragments mapped (FPKM)

Many other count corrections

- RPKM (Mortazavi et al., 2008)
- TC: Gene counts are divided by the sequencing depth associated to that sample and multiplied by the average of the total counts across all the samples. Gene counts are divided by the gene length (kb) times the total number of millions of mapped reads.
- Upper-quartile (Bullard et al., 2010): Gene counts are divided by the upper quartile of counts for genes with at least one read.
- Median (Bullard et al., 2010): Gene counts are divided by the median of counts for genes with at least one read.
- Quantile (Irizarry et al., 2003): This method matches the gene count distributions across samples.
- edgeR (Robinson et al., 2009): overdispersed Poisson model. Bioconductor package

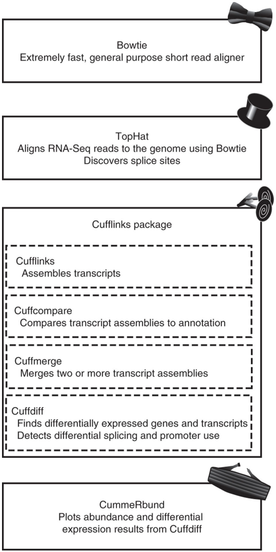
Many other count corrections

- TMM (Robinson & Oshlack, 2010): Trimmed Mean of M-values. Based on the hypothesis that most of the genes are not DE. A sample is taken as the reference. For each sample, the scaling factor is the weighted mean of log ratios between this sample and the reference after removing the most expressed genes and those with the largest log ratios. Gene counts are divided by this factor re-scaled by the mean of the normalized library sizes.
- DESeq (Anders & Huber, 2010): Based on the hypothesis that most of the genes are not DE.
- The scaling factor for a given sample is the median of the ratio, for each gene, of its counts over its geometric mean across all the samples. Gene counts are divided by this scaling factor.
- FPKM (Trapnell et al., 2010): implemented in Cufflinks, similar to RPKM .

Software

- TopHat / Bowtie: alignment
- Cufflinks:
  - assembly
  - compare with the known transcripts
- Cuffdiff: differential expression
  - estimate fragment length distribution
  - calculate transcript abundances FPKM
  - sequence bias correction
- CummeRbund:
  - an R package to handle results

Software



Bowtie
Extremely fast, general purpose short read aligner

TopHat
Aligns RNA-Seq reads to the genome using Bowtie
Discovers splice sites

Cufflinks package

Cufflinks
Assembles transcripts

Cuffcompare
Compares transcript assemblies to annotation

Cuffmerge
Merges two or more transcript assemblies

Cuffdiff
Finds differentially expressed genes and transcripts
Detects differential splicing and promoter use

CummeRbund
Plots abundance and differential
expression results from Cuffdiff