Analysis of RNA-seq Data

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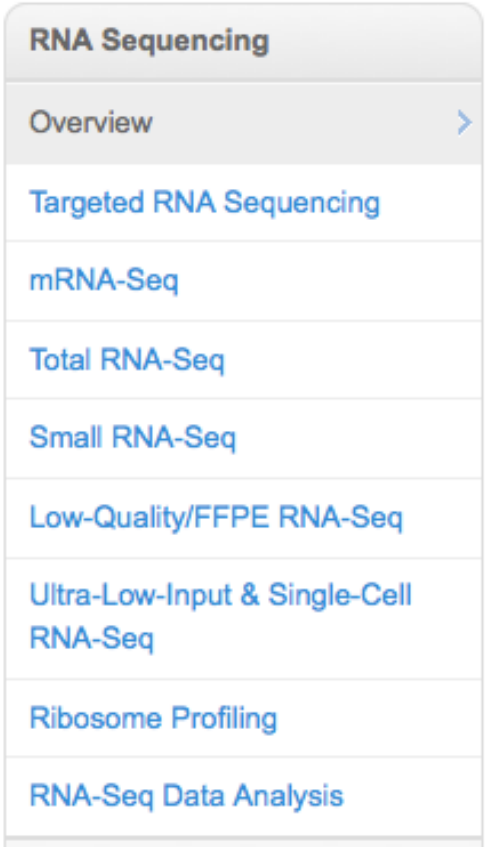
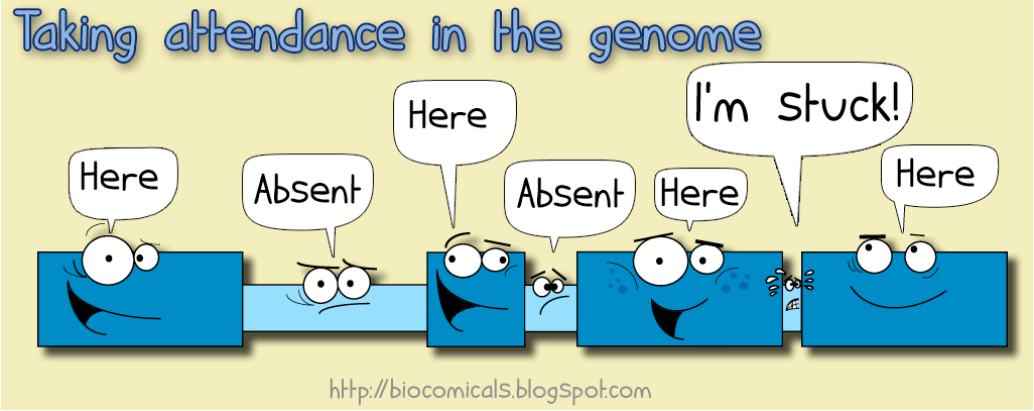
The many faces of RNA-seq



Applications

Discovery

- Find new transcripts
- Find transcript boundaries
- Find splice junctions



Comparison

Given samples from different experimental conditions, find effects of the treatment on

- Gene expression strengths
- Isoform abundance ratios, splice patterns, transcript boundaries

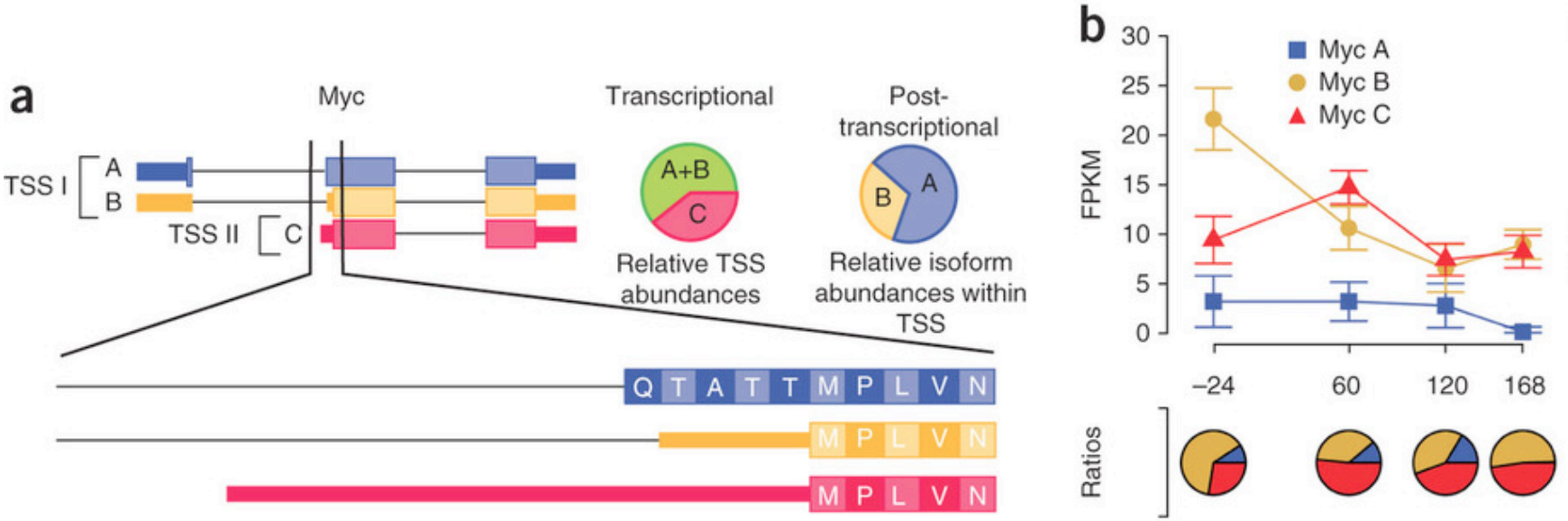
Applications

nature biotechnology

LETTERS

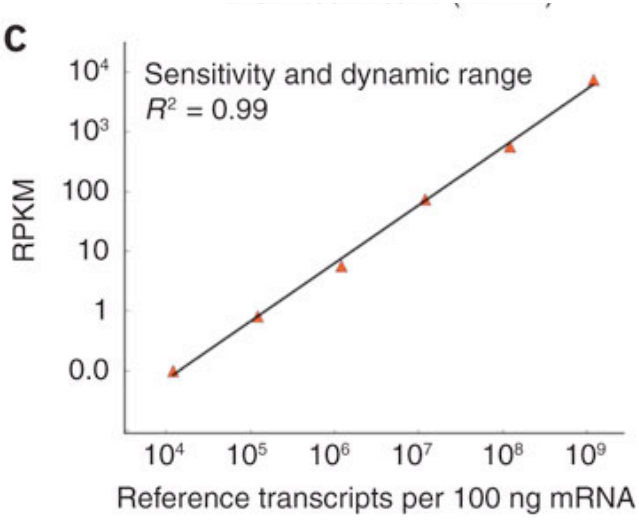
Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation

Cole Trapnell[1–3], Brian A Williams[4], Geo Pertea[2], Ali Mortazavi[4], Gordon Kwan[4], Marijke J van Baren[5], Steven L Salzberg[1,2], Barbara J Wold[4] & Lior Pachter[3,6,7]



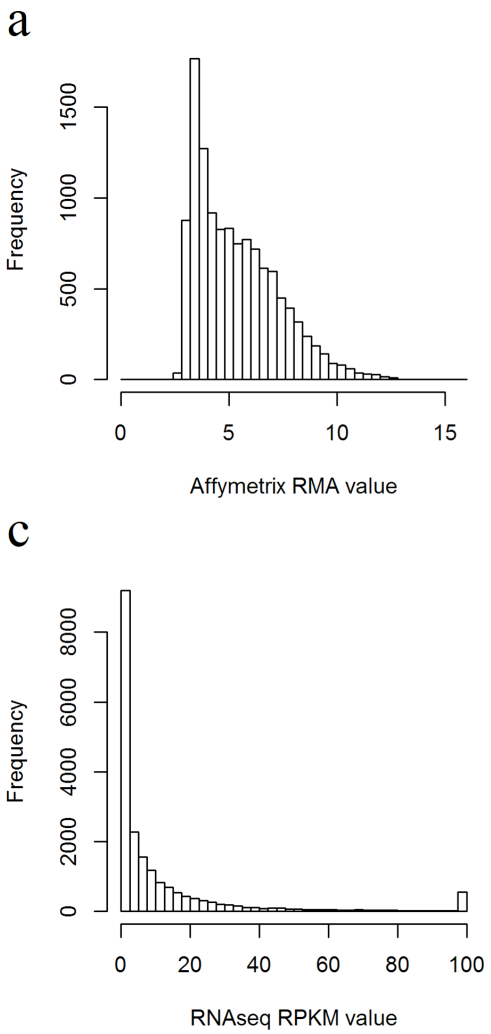
Differential Expression

- Comparing feature abundance under different conditions
- Assumes linearity of signal over a range of expression levels
- When *feature=gene*, well-established pre- and post-analysis strategies exist

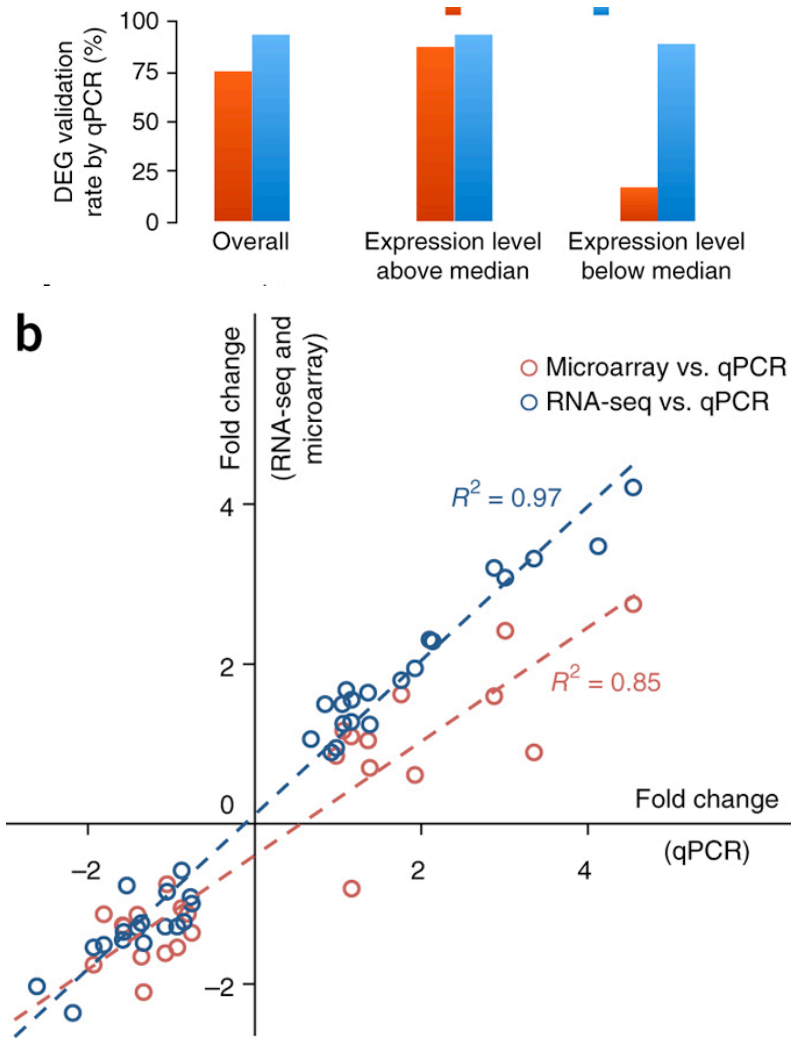


Mortazavi, A. et al (2008) *Nature Methods*

Range of detection

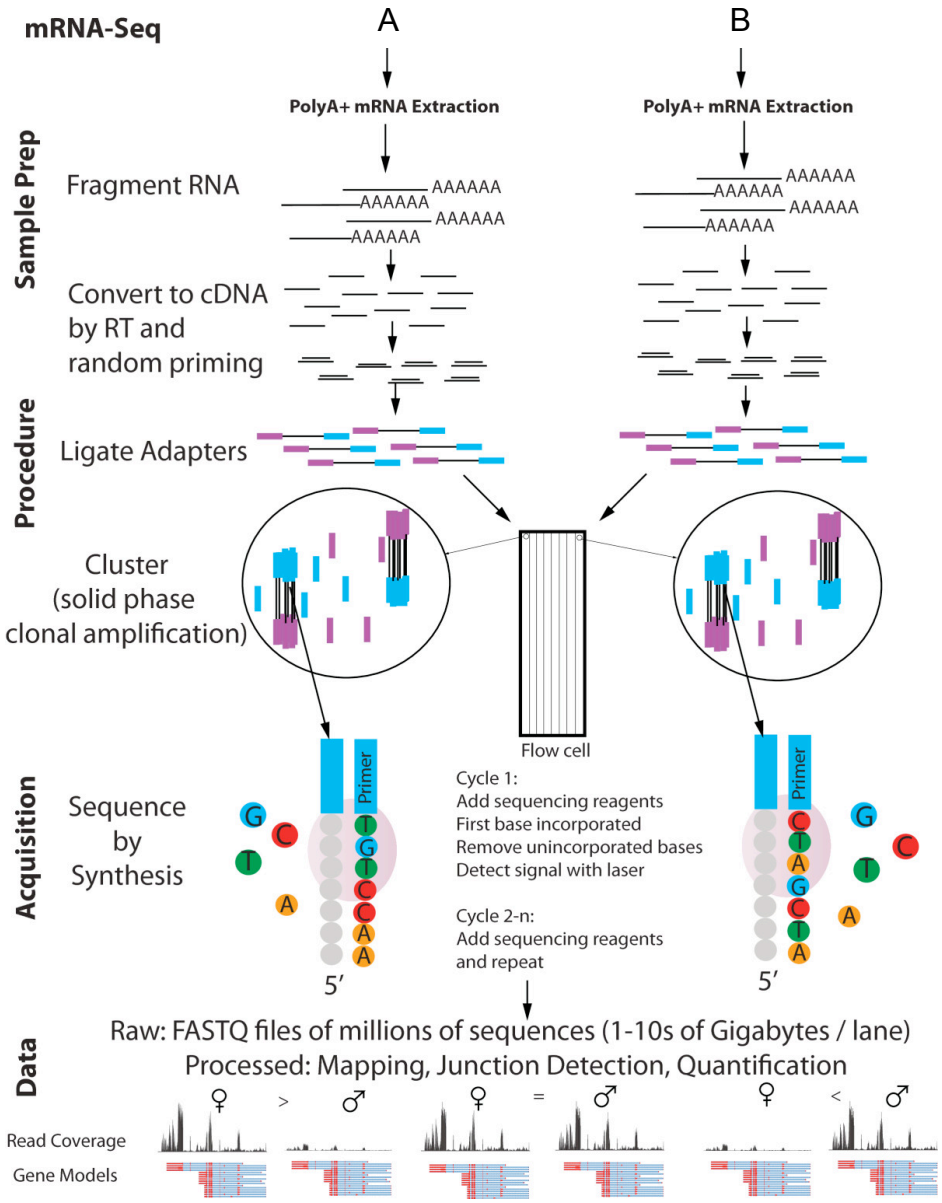


Guo et al. (2013) *Plos One*



Wang et al (2014) *Nature Biotech.*

Library Prep i



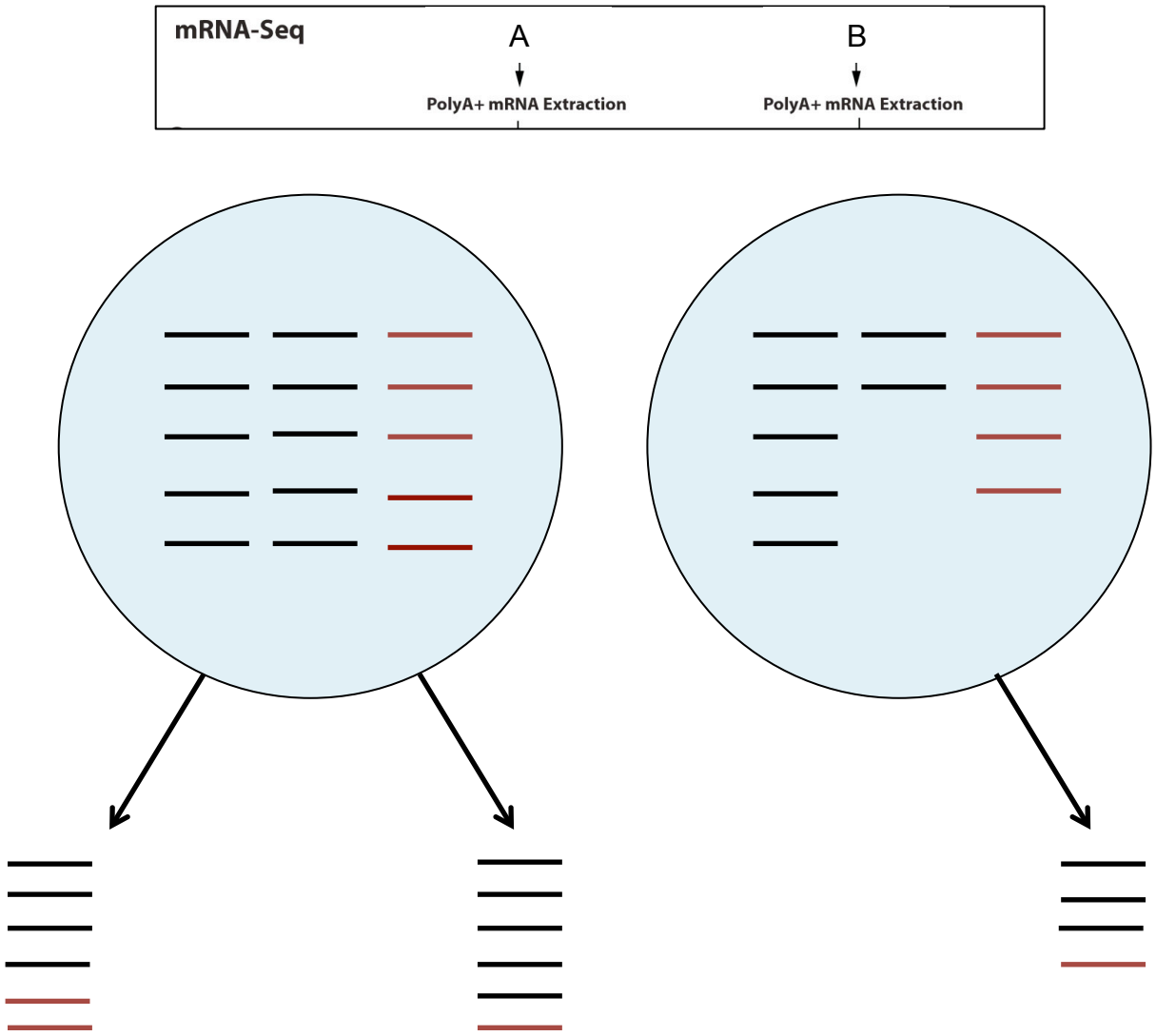
Malone, J.H. & Oliver, B.

Library Prep ii

Biological

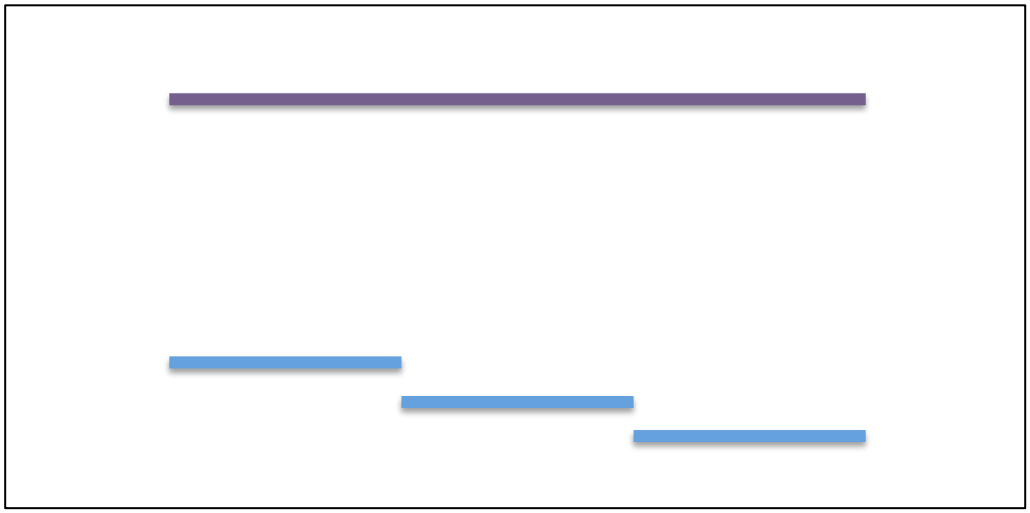
Technical

Library Prep iii

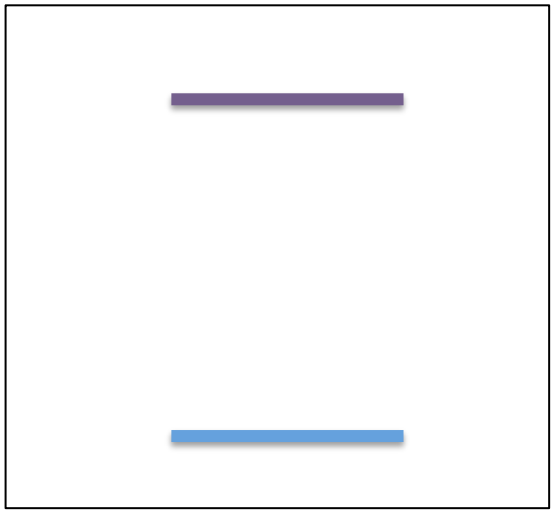


Library Prep iii

A



B



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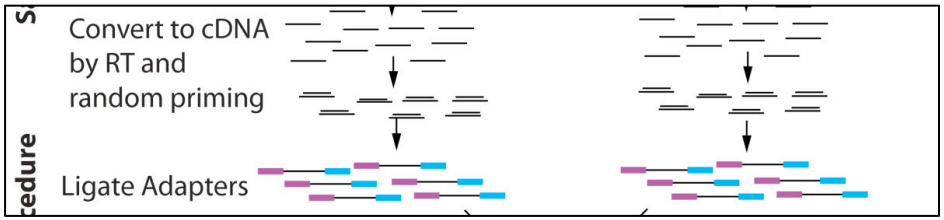
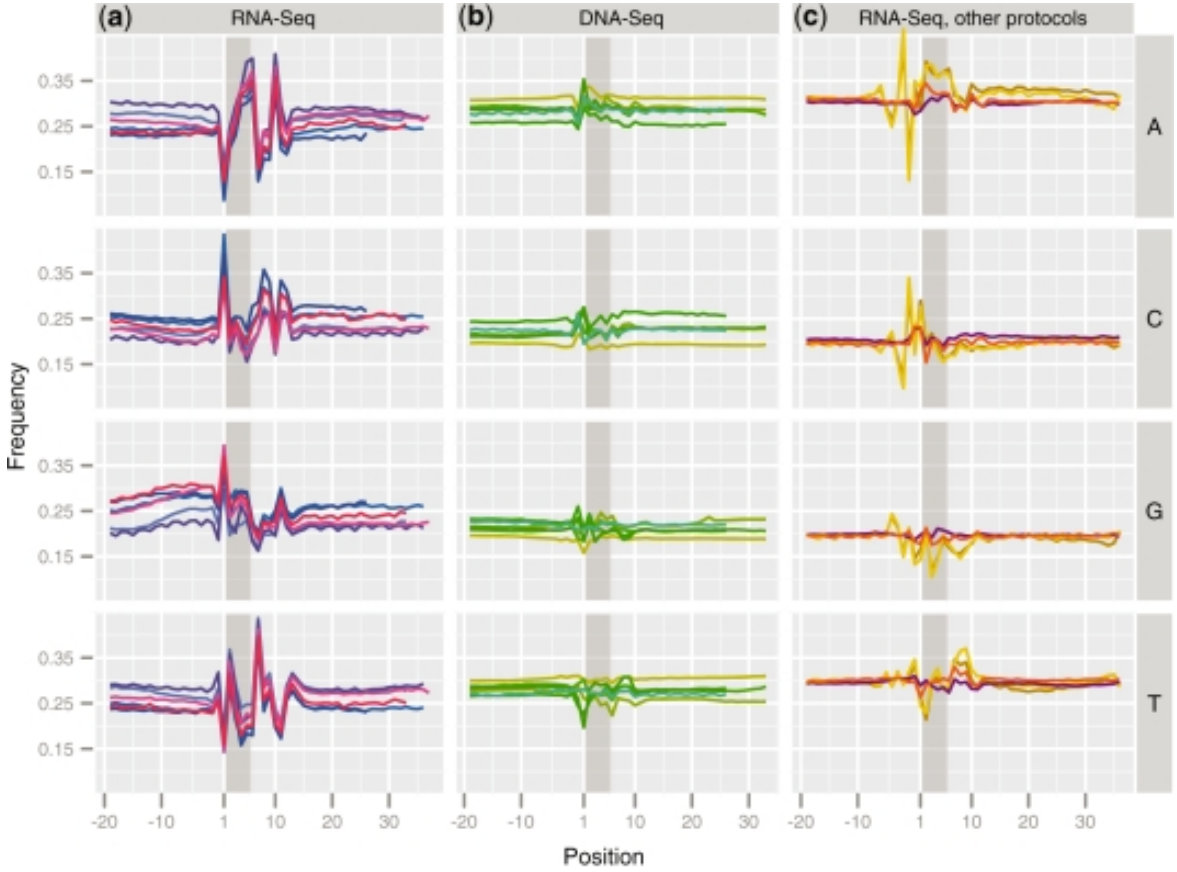
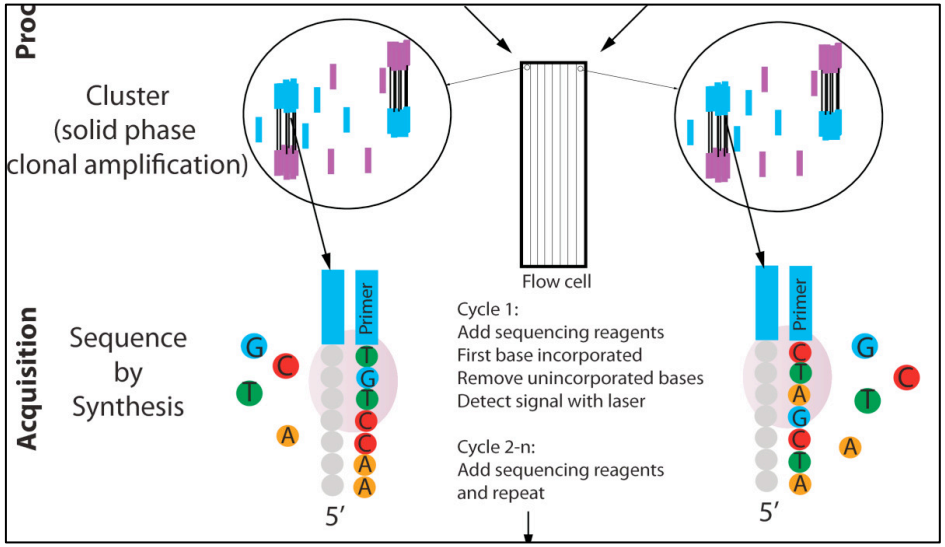




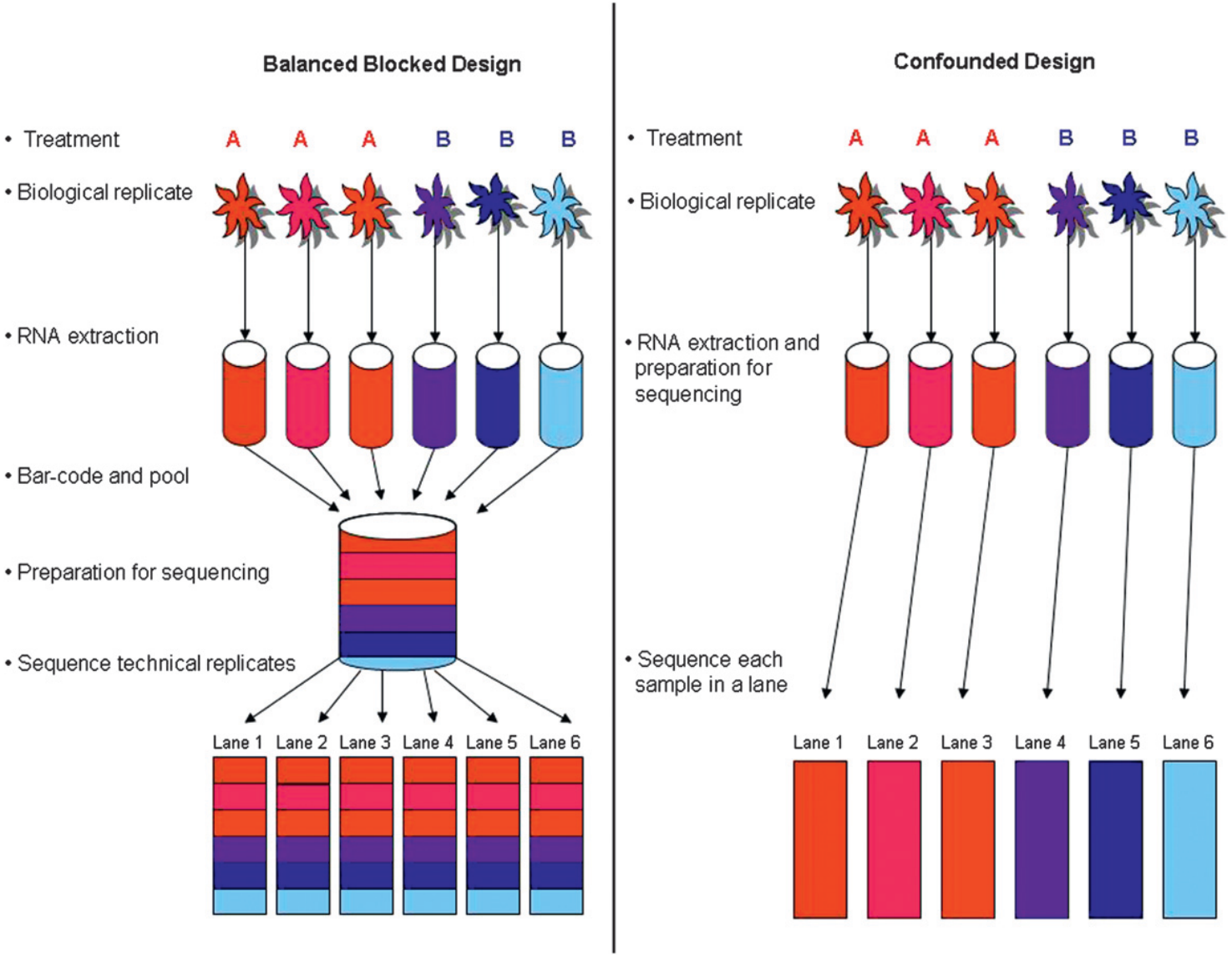
Hansen, K.D. et al. (2010) *Nuc. Acids Res.*

Library Prep v



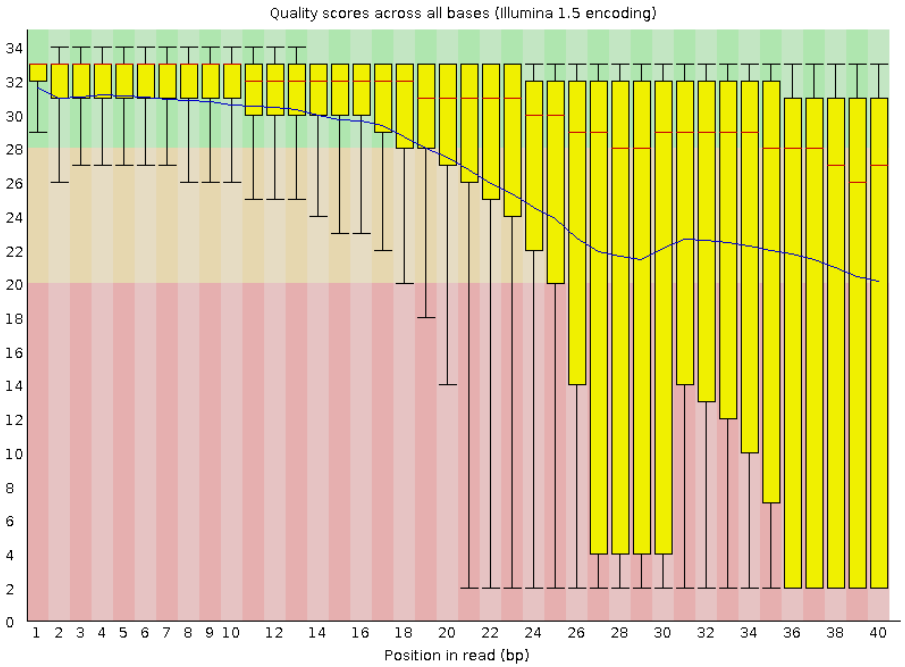
- Duplicates (optical & PCR)
- Sequence errors
- Indels
- Repetitive/problematic sequence

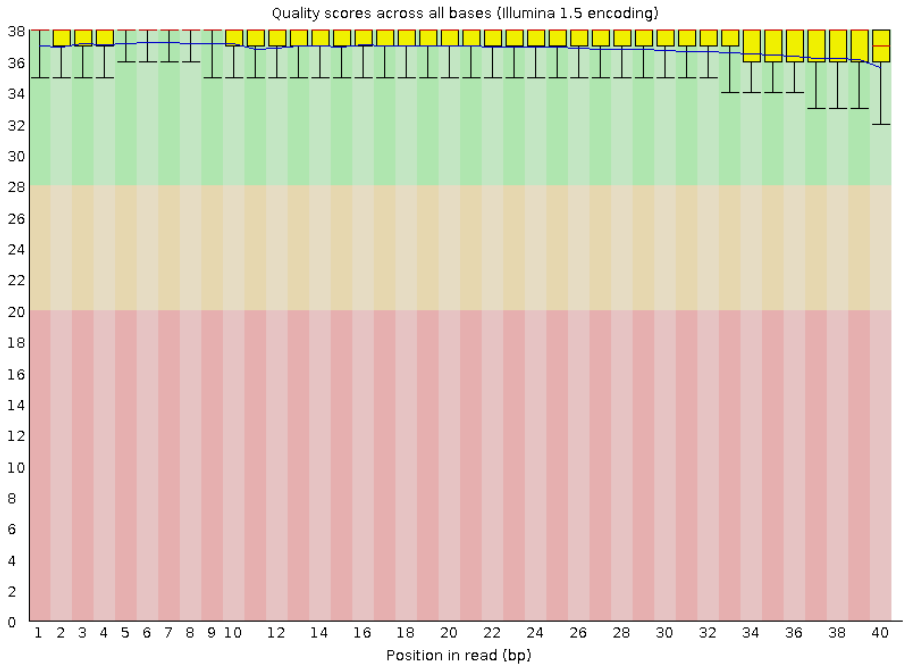
Hot off the sequencer…



Auer and Doerg (2010) *Genetics*

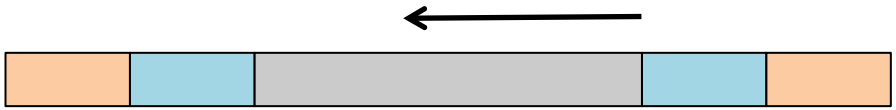
FASTQC

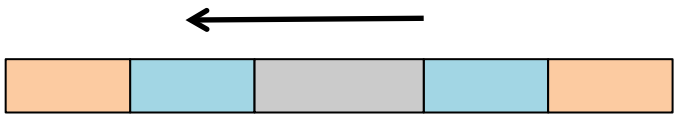

Quality scores across all bases (Illumina 1.5 encoding)
Position in read (bp)


Quality scores across all bases (Illumina 1.5 encoding)
Position in read (bp)

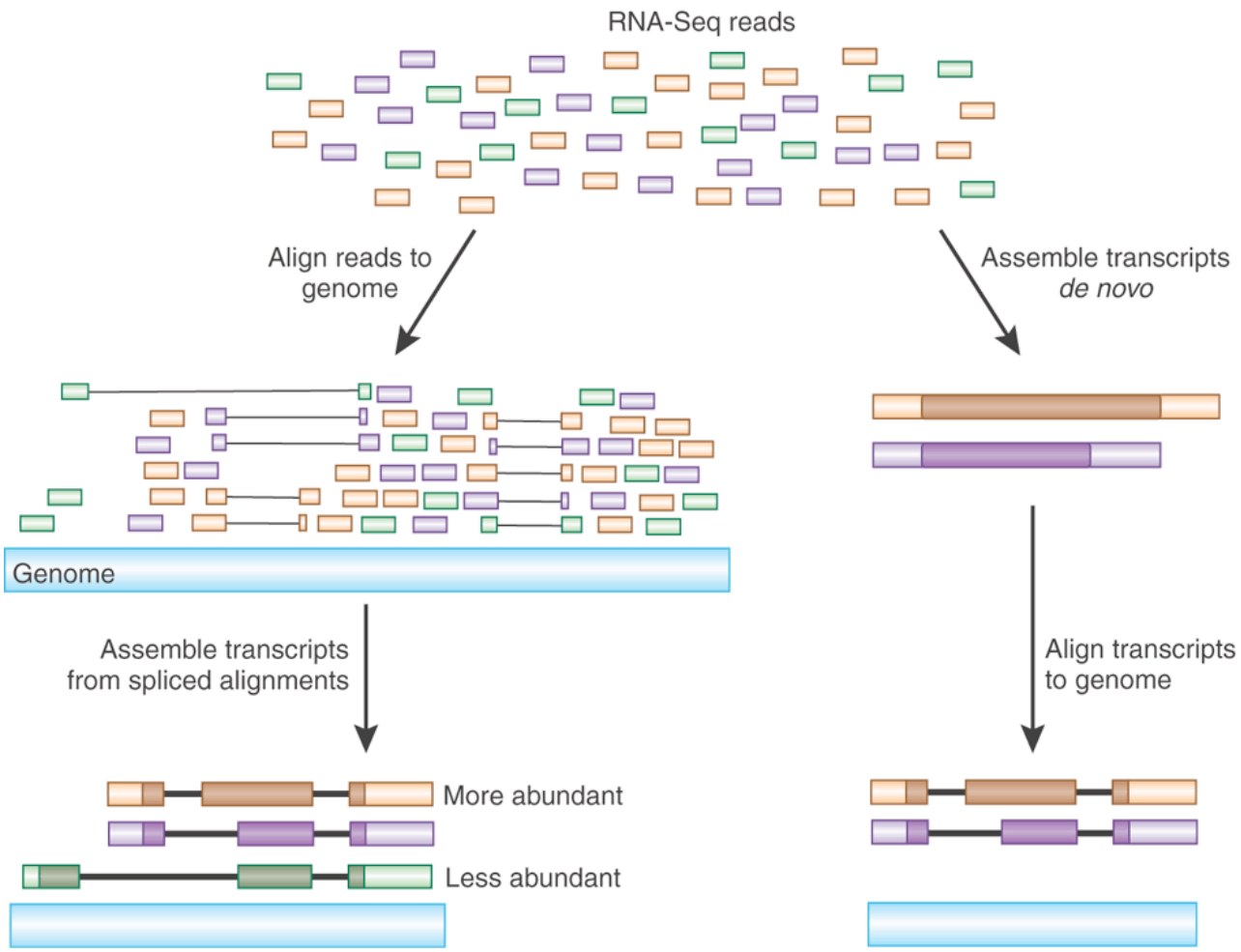
Trimming

- Quality-based trimming
- Adapter ‘contamination’





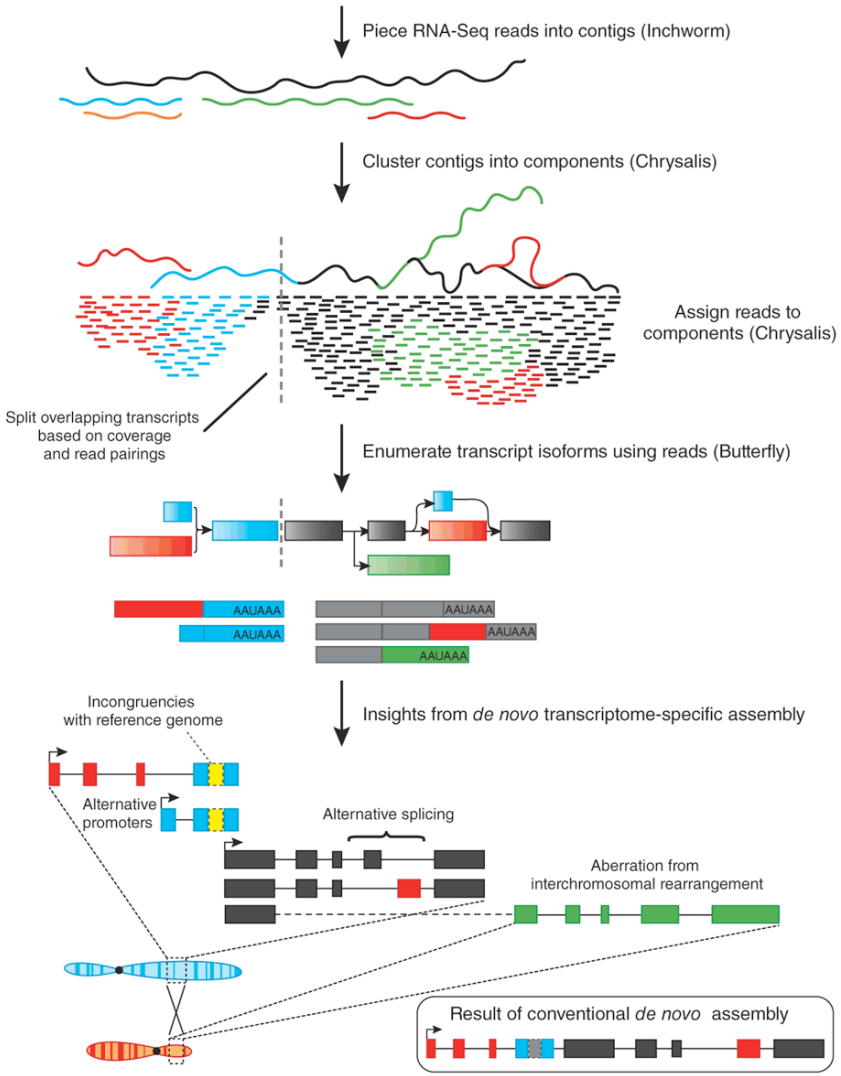
Sequence to sense



Haas, B.J. & Zody, M.C. (2010) *Nature Biotech.*

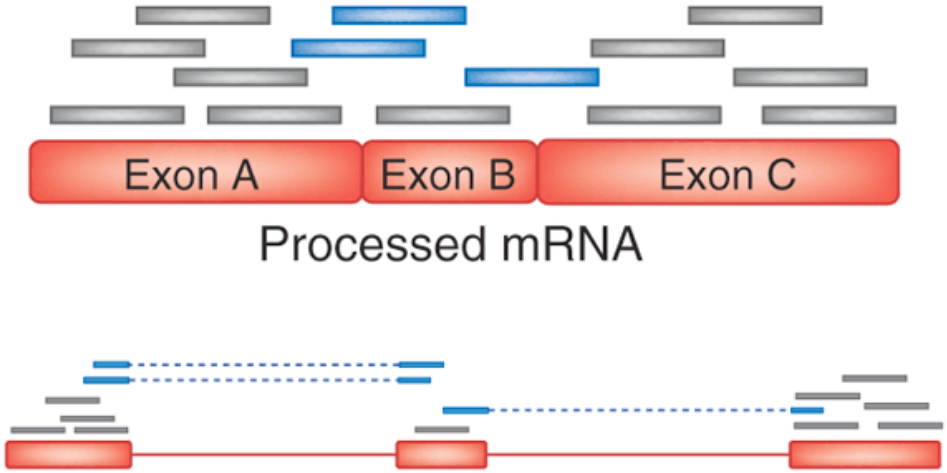
De novo assembly

- eg. Trinity



Haas, B.J.. et al (2013) *Nature Protocols*

Reference-based assembly



Genome mapping

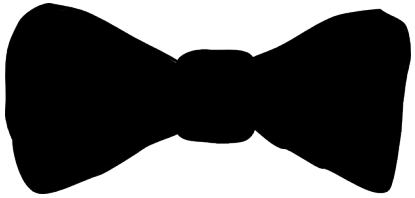
- Can identify novel features
- Spice aware?
- Can be difficult to reconstruct isoform and gene structures

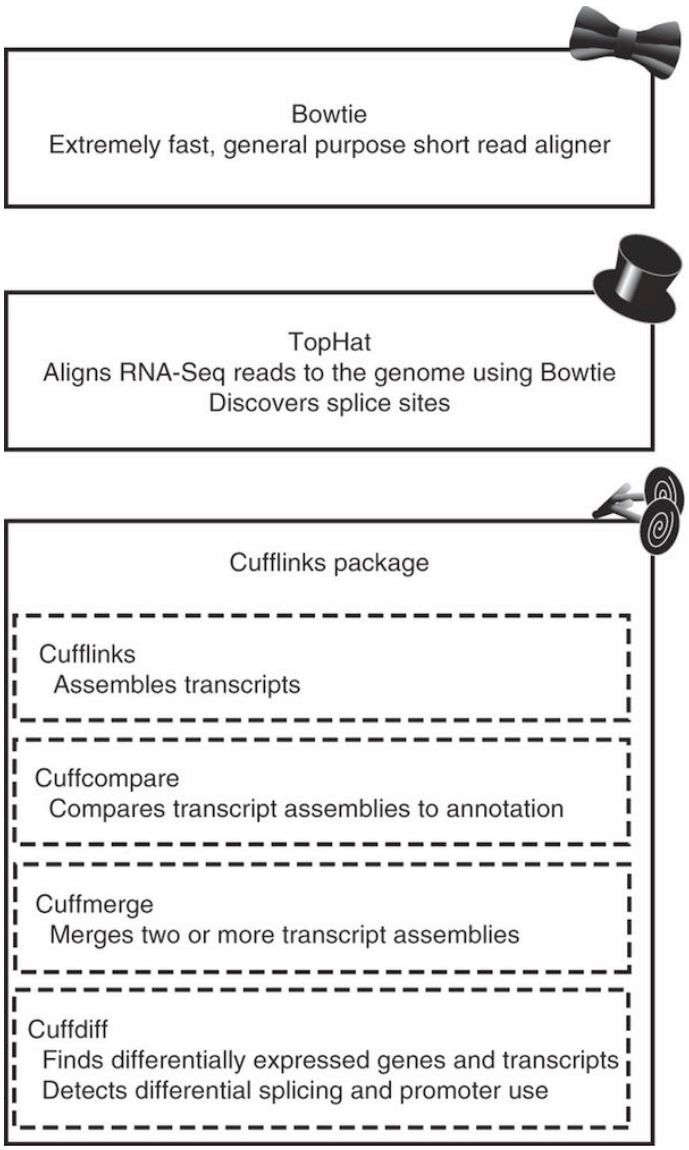
Transcriptome mapping

- No repetitive reference
- Overcomes issues of complex structures
- Novel features?
- How reliable is the transcriptome?

Trapnell & Salzberg (2009) *Nature Biotech*

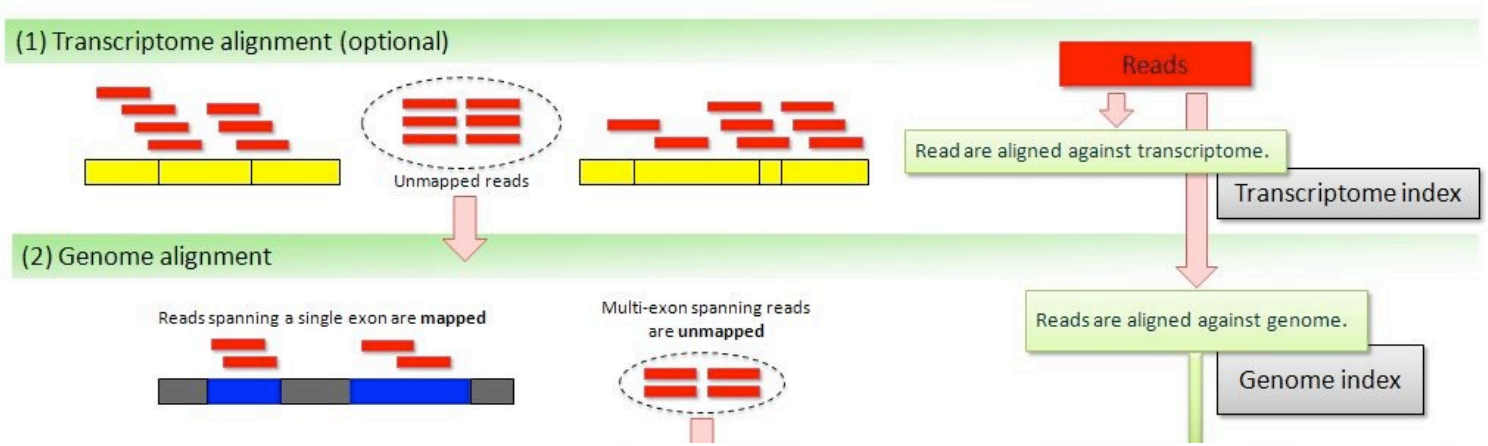
A smart suit(e)





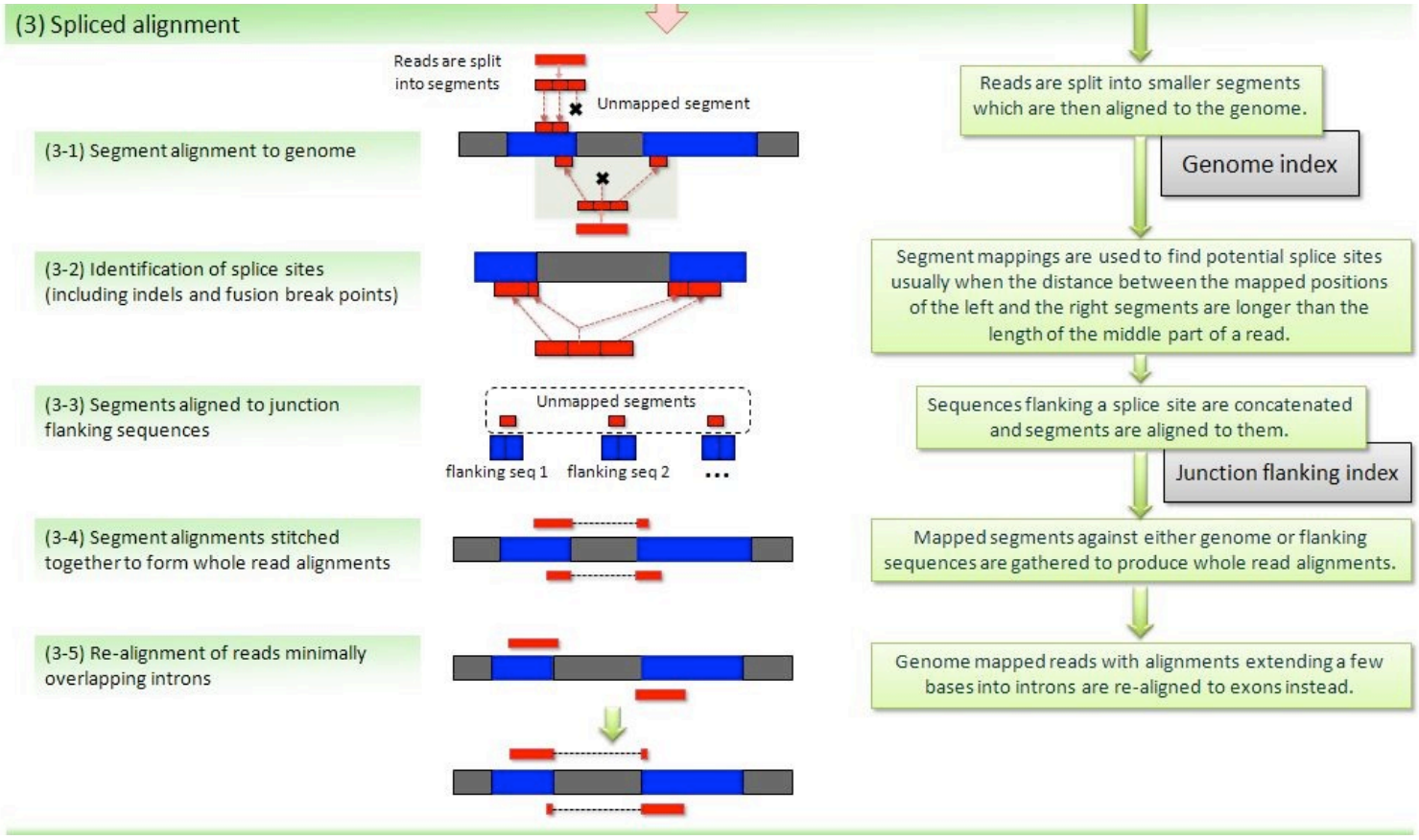
Trapnell, C. et al (2012) *Nature Protocols*

Tophat/Bowtie



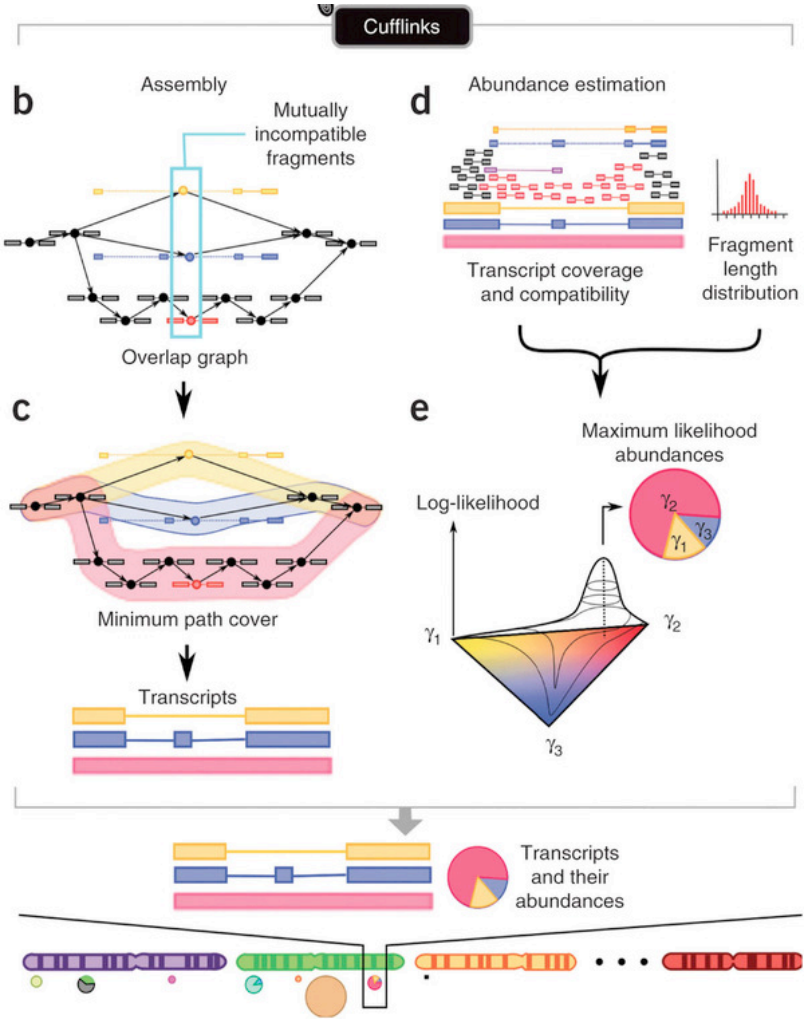
Kim, D. et al (2012) *Genome Biology*

Tophat/Bowtie



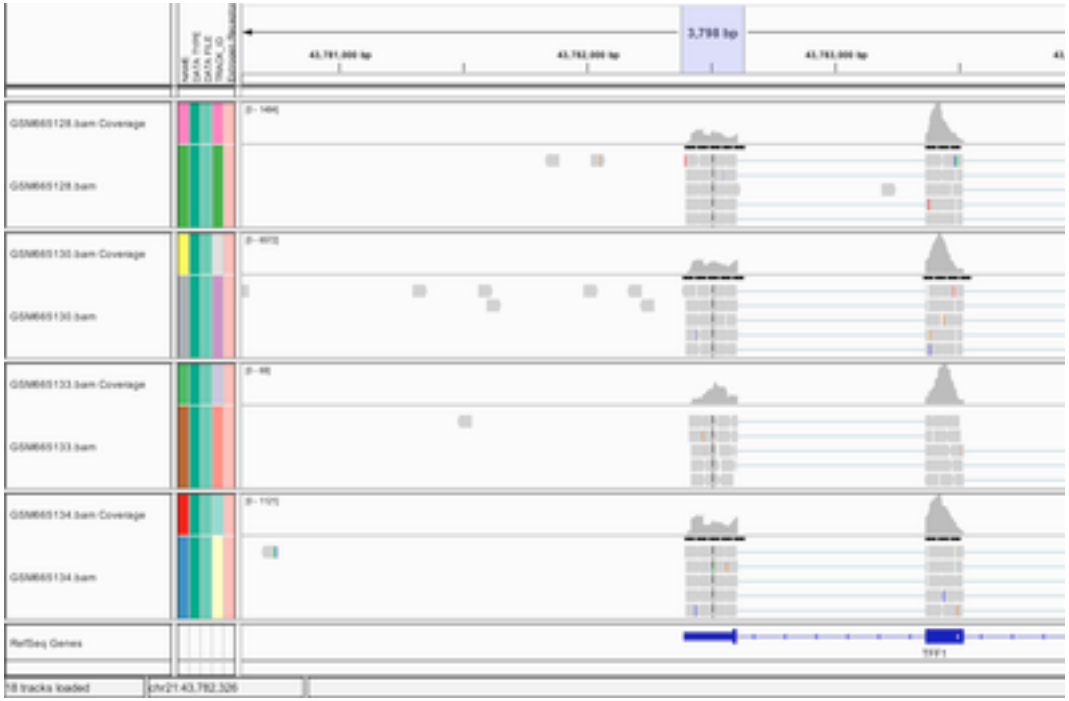
Kim, D. et al (2012) *Genome Biology*

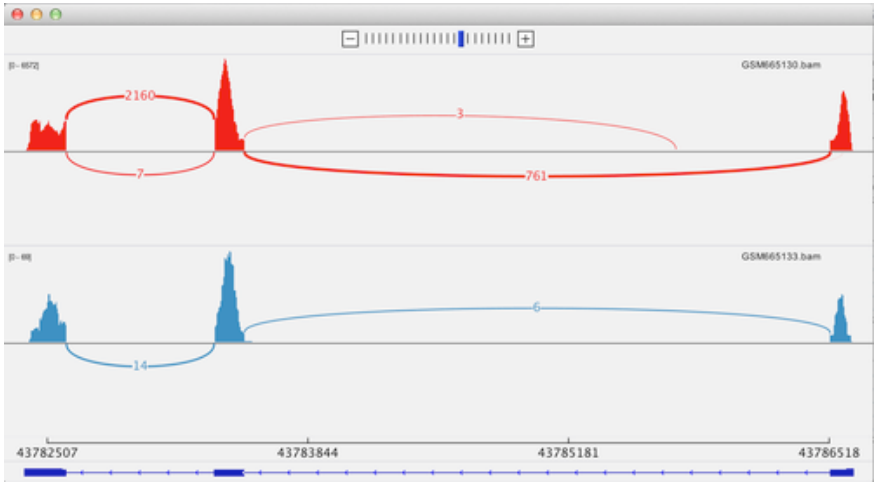
Cufflinks



Trapnell, C. et al. (2010) *Nature Biotech.*

How do we look?





Duplicates & RNA-seq

Intrinsically lower complexity

Highly expressed genes

Model as part of counting process

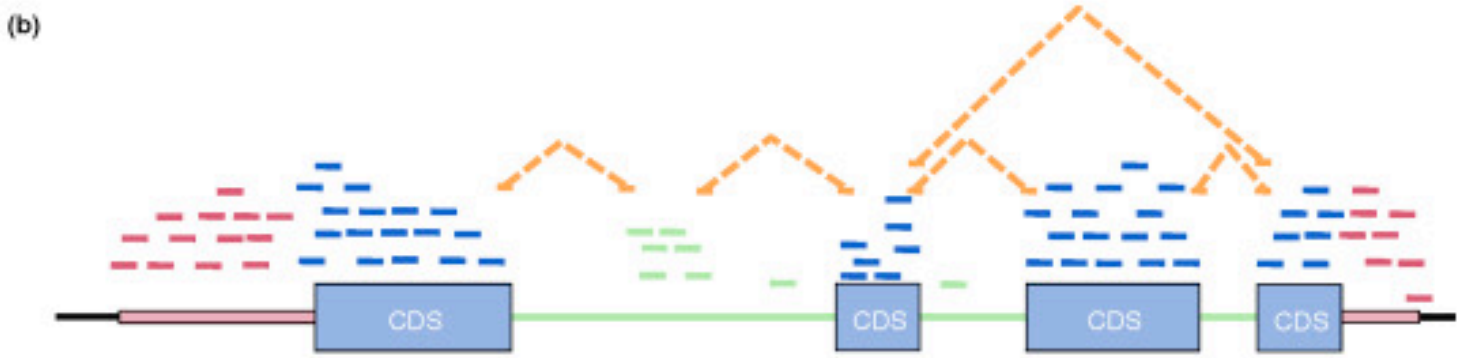
?

Variant calling vs DE analysis

Platform/pipeline

Single-end vs paired-end

Counting



Genome-based features

- Exon or gene boundaries?
- Isoform structures?
- Gene multireads?

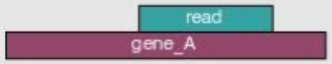
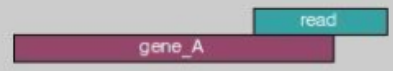
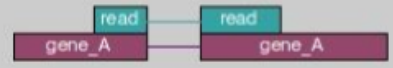
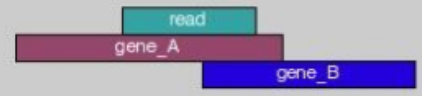
Transcript-based features

- Transcript assembly?
- Novel structures?
- Isoform multireads?

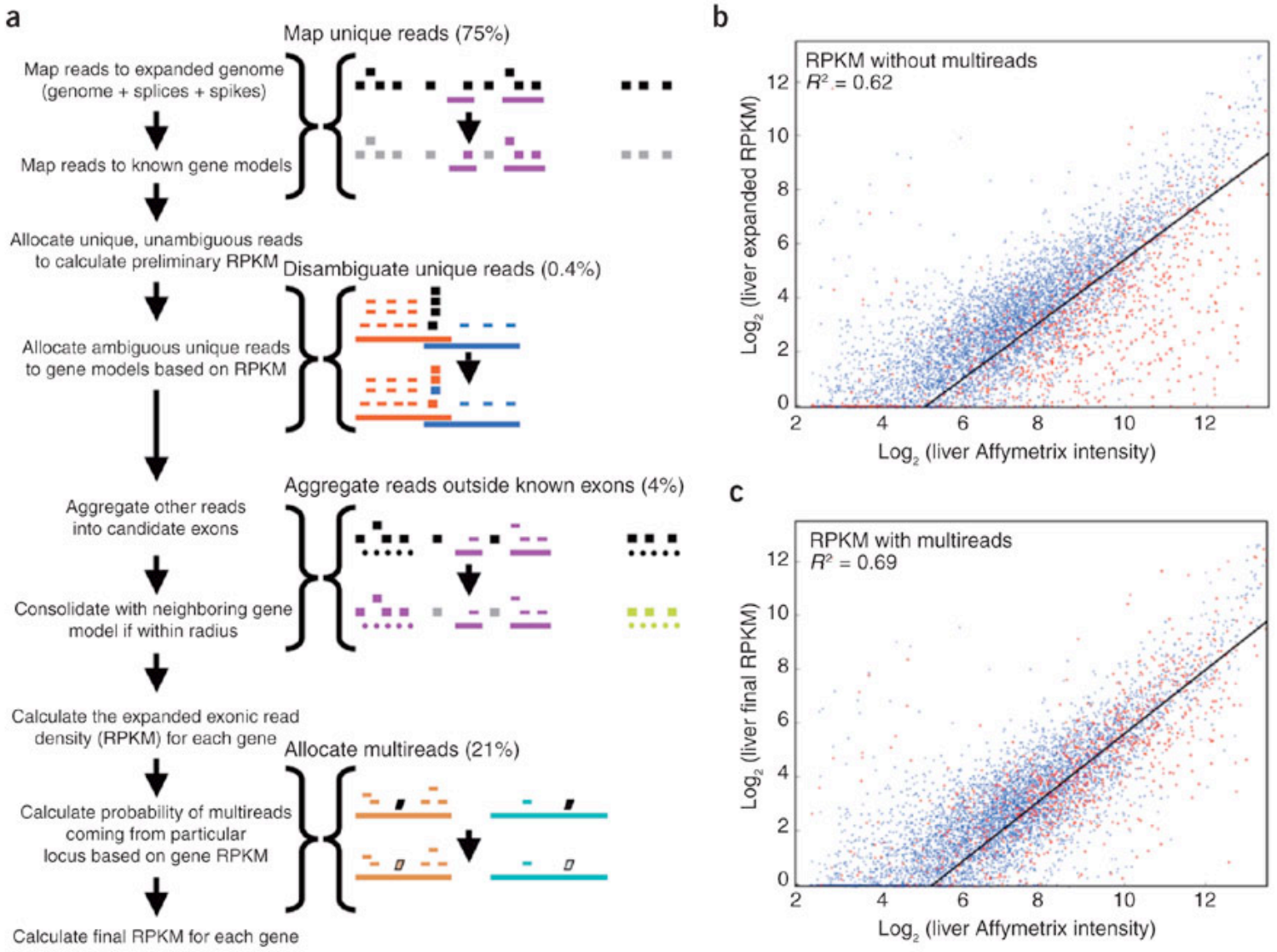
Oshlack, A. et al. (2010) *Genome Biology*

Counting

- eg. HTseq

	union	intersection _strict	intersection _nonempty
	gene_A	gene_A	gene_A
	gene_A	no_feature	gene_A
	gene_A	no_feature	gene_A
	gene_A	gene_A	gene_A
	gene_A	gene_A	gene_A
	ambiguous	gene_A	gene_A
	ambiguous	ambiguous	ambiguous

Counting



Mortazavi, A. et al (2008) *Nature Methods*

Counting & normalisation

- An estimate for the *relative* counts for each gene is obtained
- Assumed that this estimate is representative of the original population

Library size

- Sequencing depth varies between samples

Gene Properties

- GC content, length, sequence

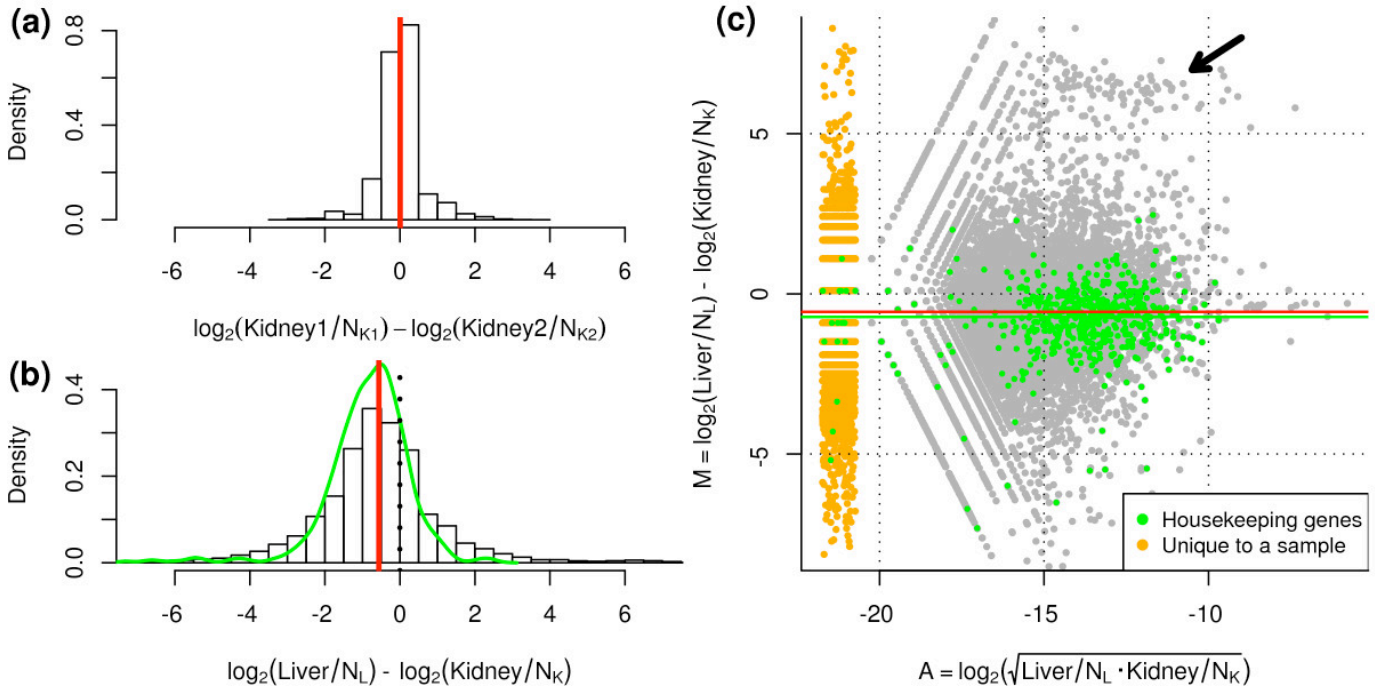
Library composition

- Highly expressed genes overrepresented at cost of lowly expressed genes

Normalisation i

Total Count

- Normalise each sample by total number of reads sequenced.
- Can also use another statistic similar tototal count; eg. median, upper quartile



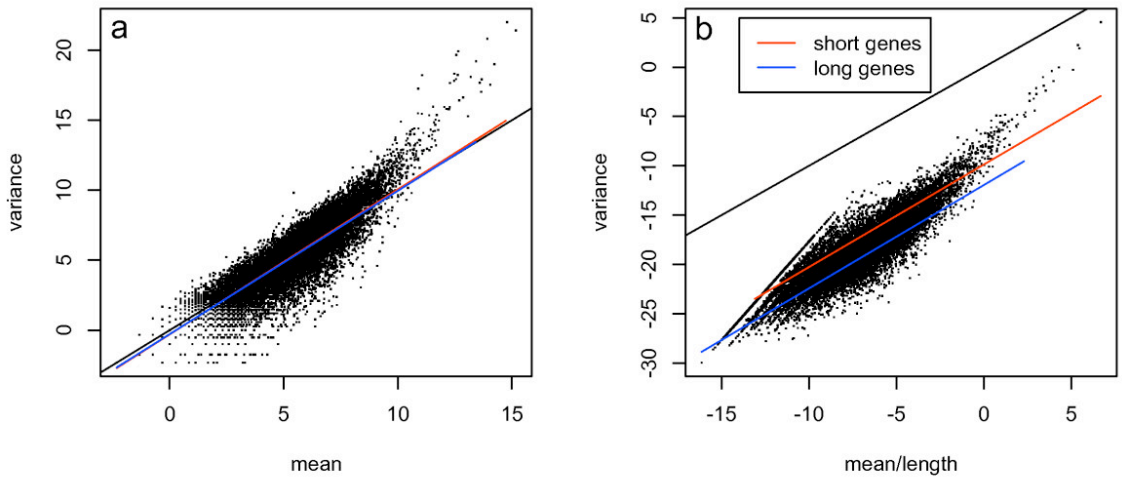
Robinson, M.D. & Oshlack, A. (2010) *Genome Biology*

Normalisation ii

RPKM

- Reads per kilobase per million =

$$\frac{\text{reads for gene } A}{\text{length of gene } A \text{ X Total number of reads}}$$

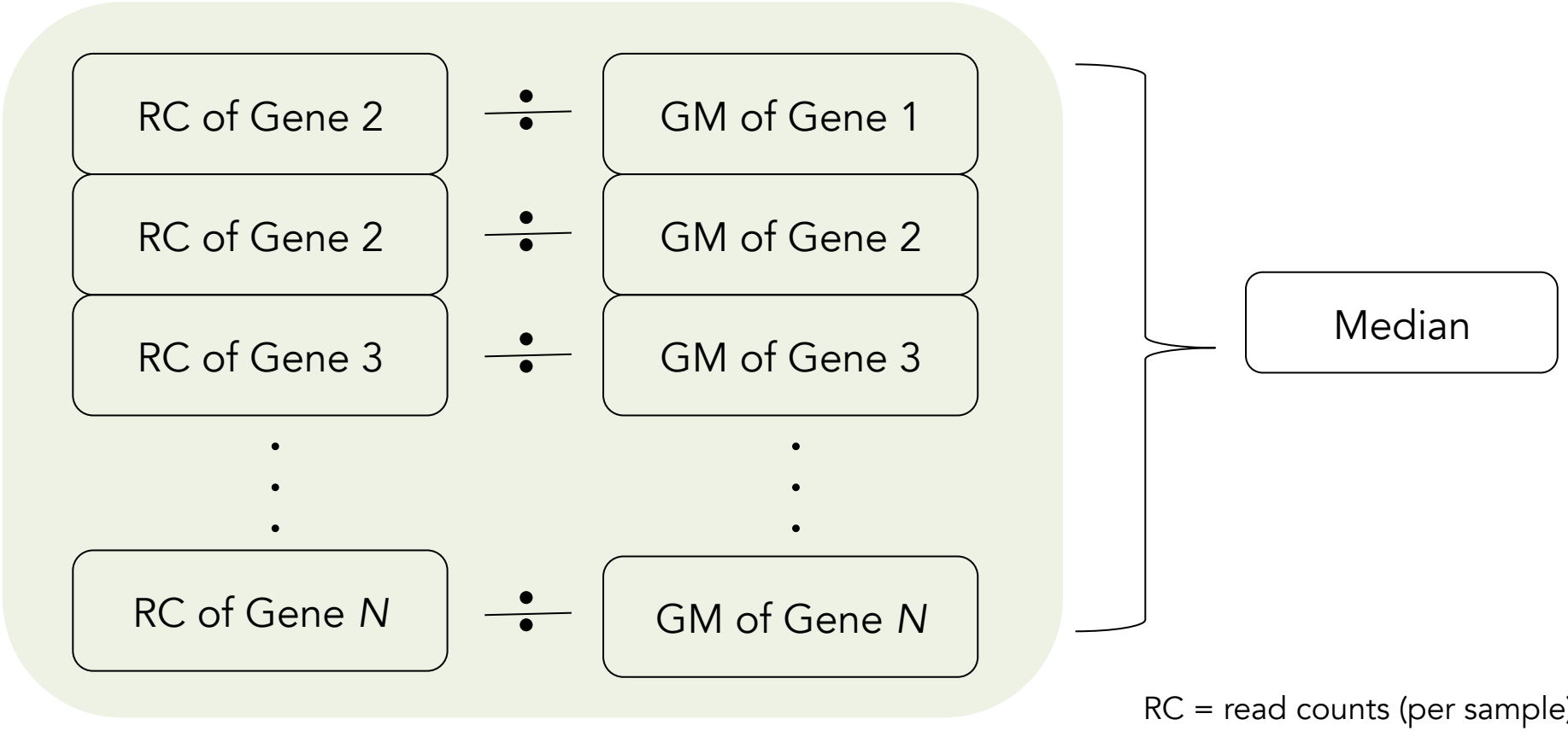


Oshlack, A. & Wakefield, M.J. (2009) *Biology Direct*

Normalisation iii

Geometric scaling factor

- Implemented in `DESeq`
- Assumes that most genes are not differentially expressed

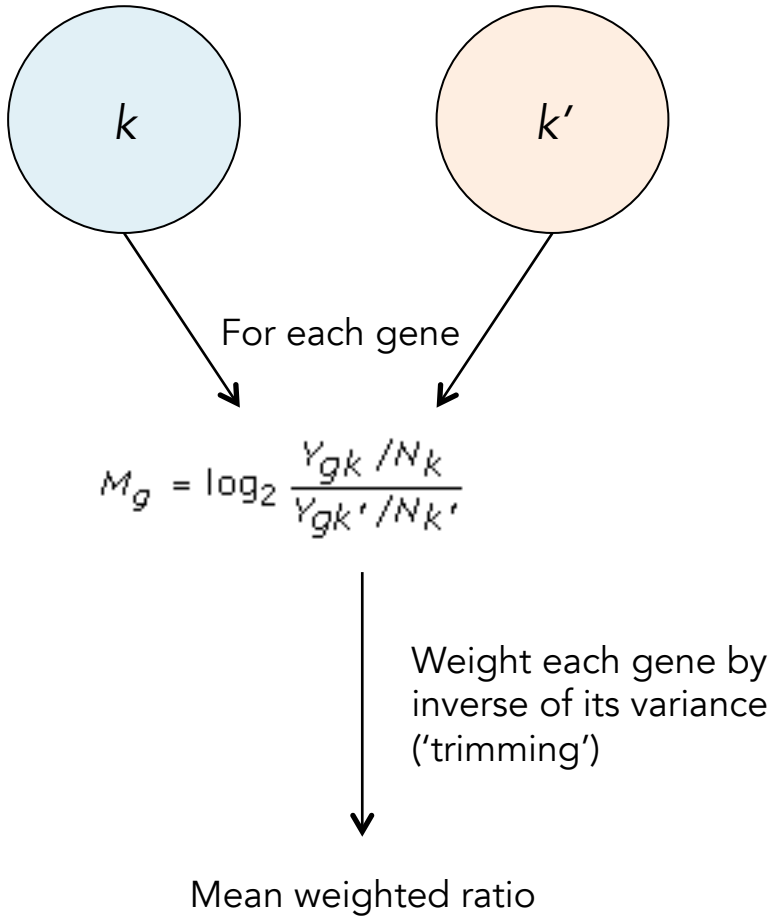


RC = read counts (per sample)
GM =geometric mean (all samples)

Normalisation iv

Trimmed mean of *M*

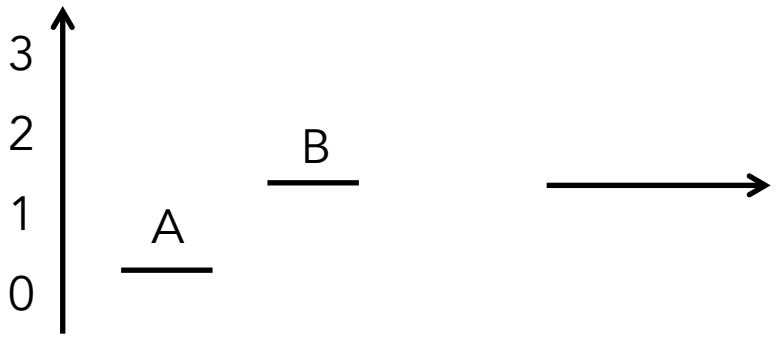
- Implemented in edgeR
- Assumes most genes are not differentially expressed



g = each gene

Differential expression

- Simple



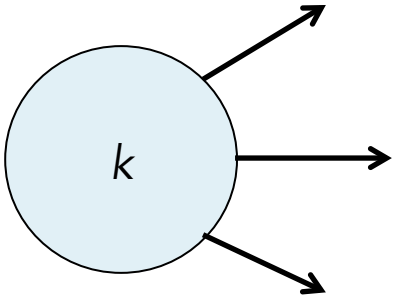
	Cond A	Cond B
Gene X		
Other		

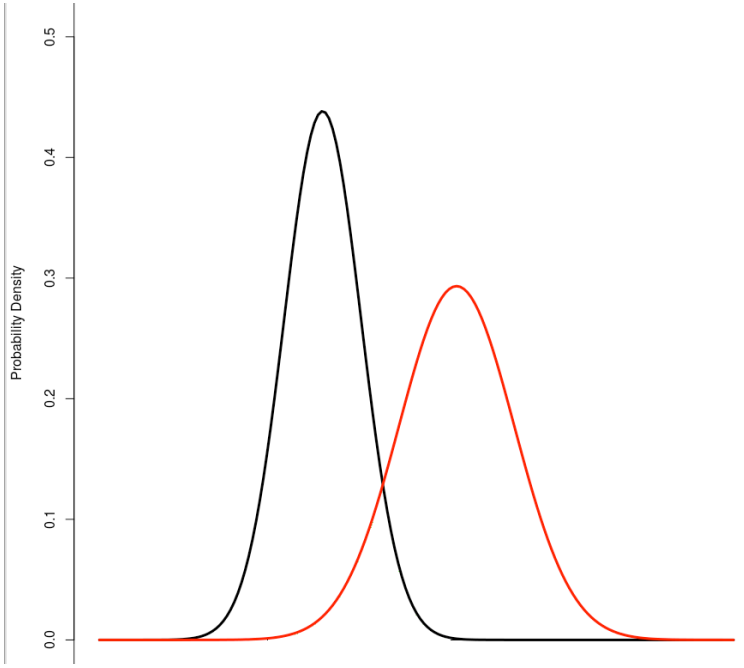
All we need

- Know what the data looks like
- Some measure of difference

Modelling – old trends

- Technical replicates introduce some variance

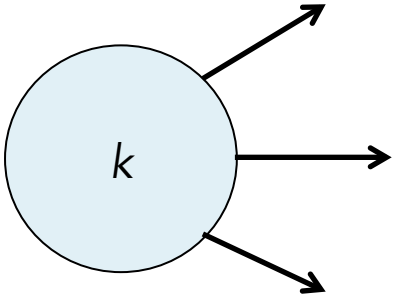


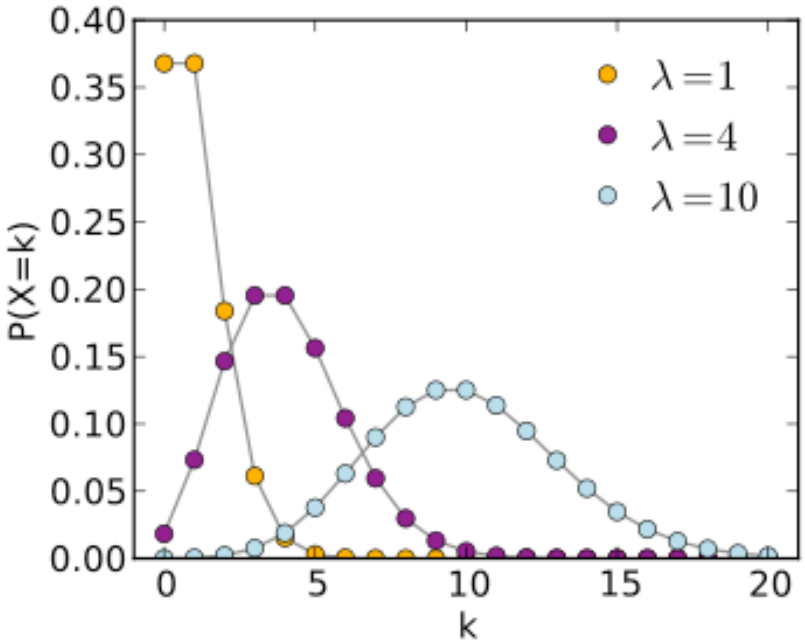


- What the data looks like: ***normal distribution***
- Some measure of difference: ***t-test etc***

Modelling – in fashion

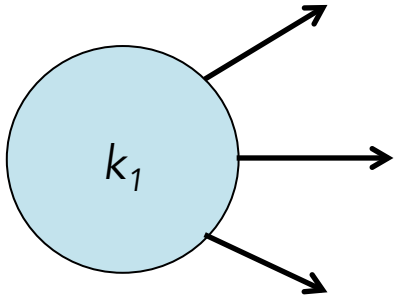
- Use the Poisson distribution for count data from technical replicates
- Just one parameter required – the mean

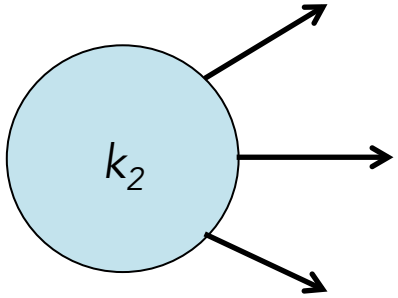


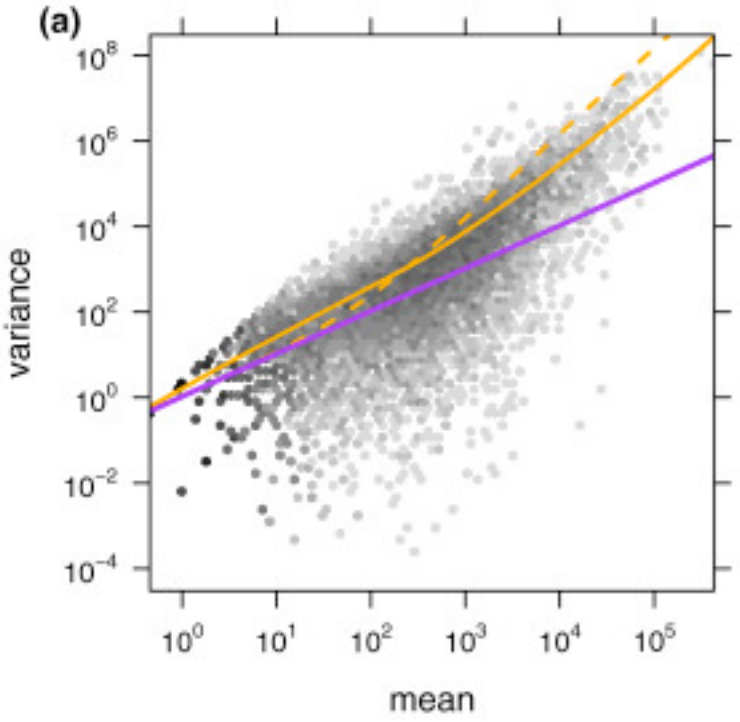


Modelling – in fashion

- Biology is never that simple…





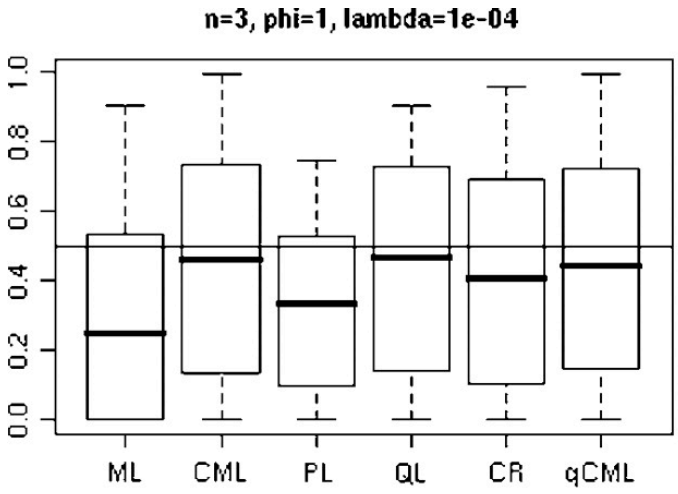


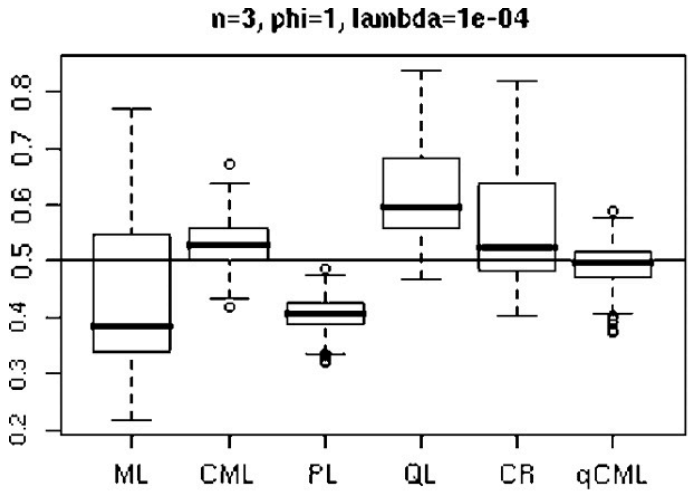
- The negative binomial distribution represents an *overdispersed* Poisson distribution, and has parameters for both the mean and the overdispersion.

Anders, S. & Huber, W. (2010) *Genome Biology*

Modelling – in fashion

- Estimating the dispersion parameter can be difficult with a small number of samples
- 'Share' information from all genes to obtain global estimate



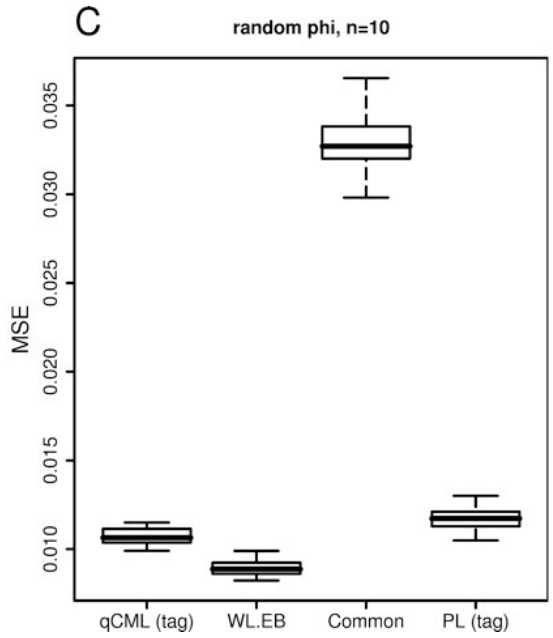


Robinson, M.D. & Smyth, G. (2008) *Biostatistics*

Shrinkage

- Genes do not share a common dispersion parameter

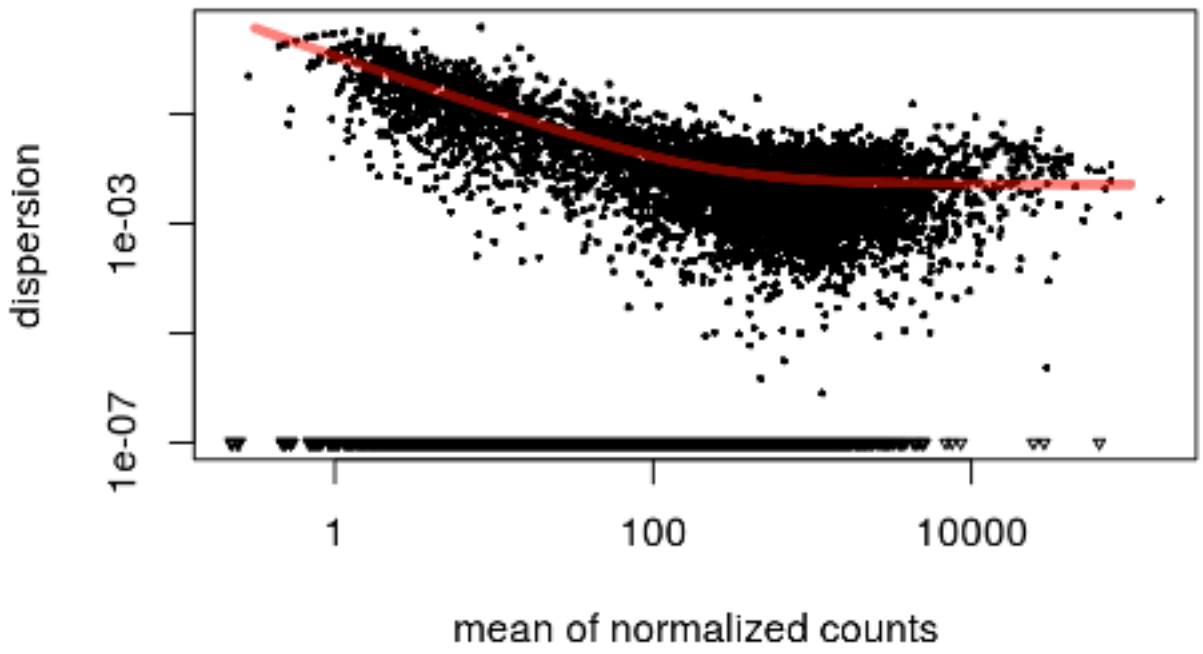
- 'Moderated' estimate – assign a per-gene weight to the combined estimate



Robinson, M.D. & Smyth, G. (2007) *Bioinformatics*

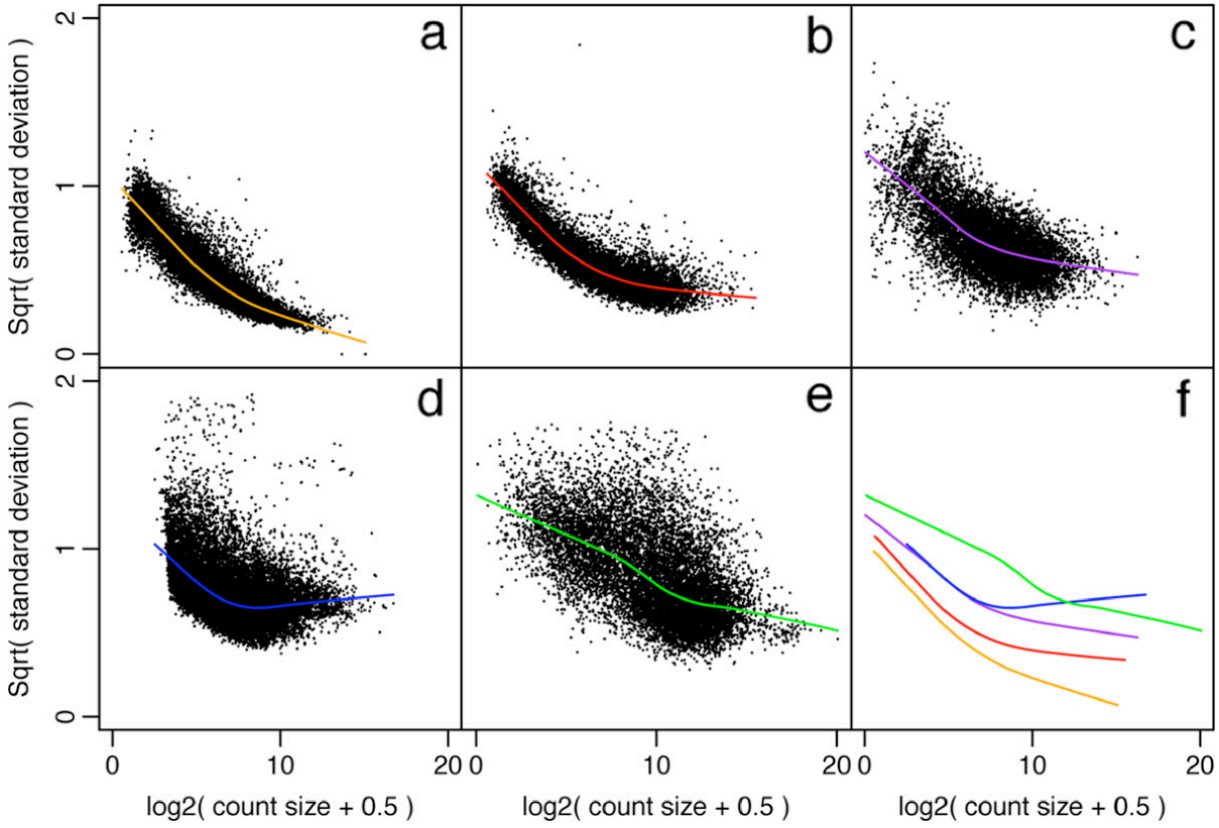
DESeq

- `DESeq` fits a mean/dispersion relationship model
- Shifts individual estimates to regression line



The mean-variance relationship

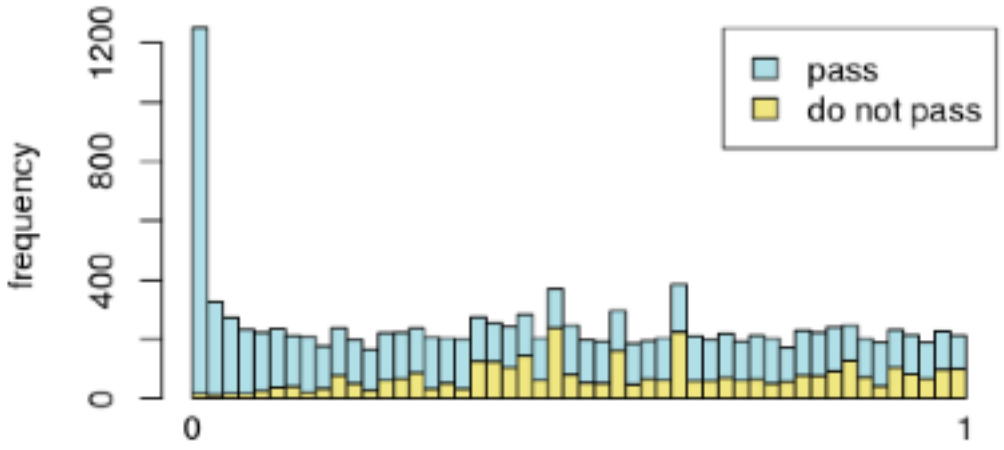
- Variance = Technical (variable) + Biological (constant)
- A=technical replicates ---> E =(very) biologically different replicates



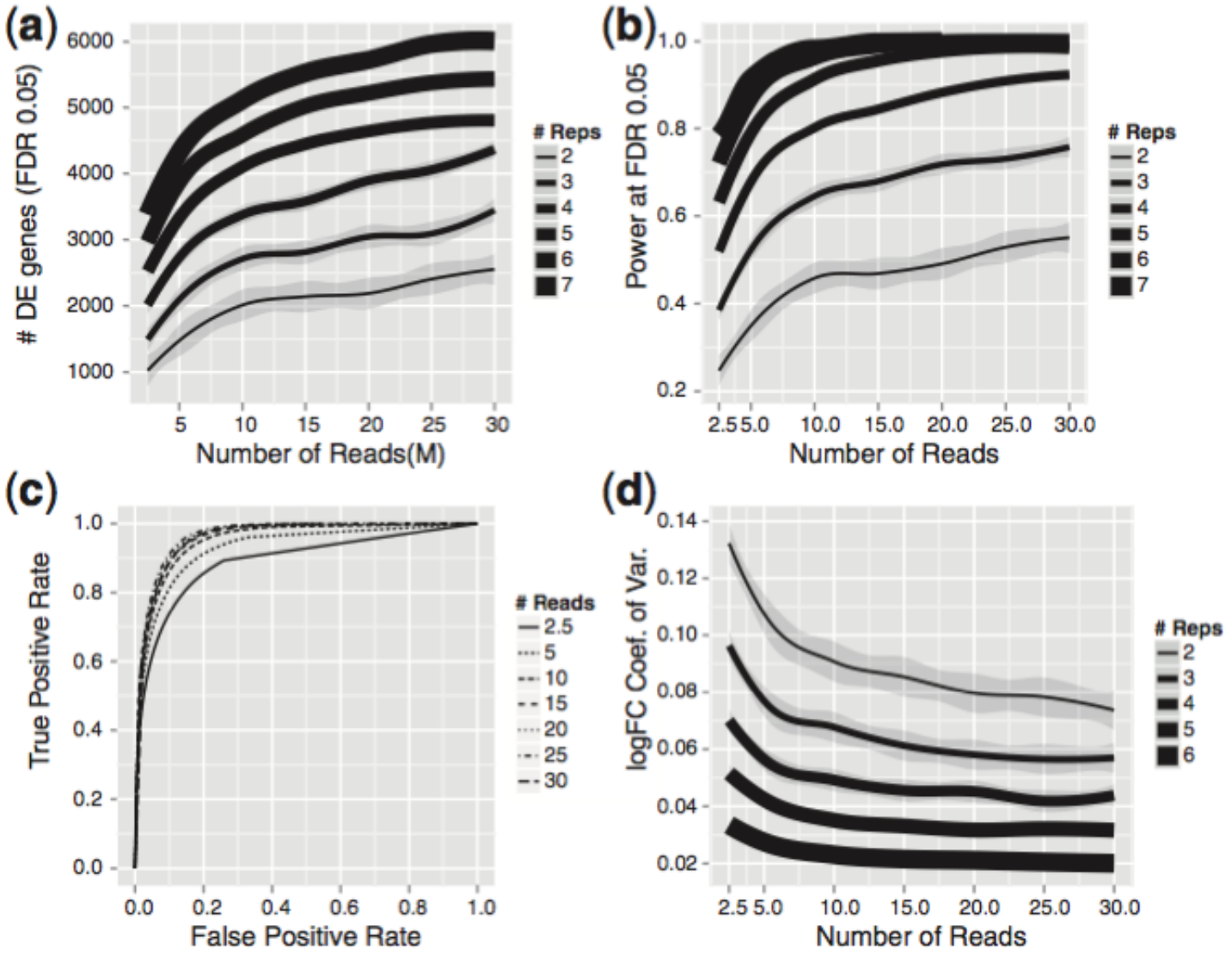
Law et al. (2010) *Genome Biology*

Filtering

- *Independent filtering* = remove genes that have little chance of showing DE
- Can use eg. total count

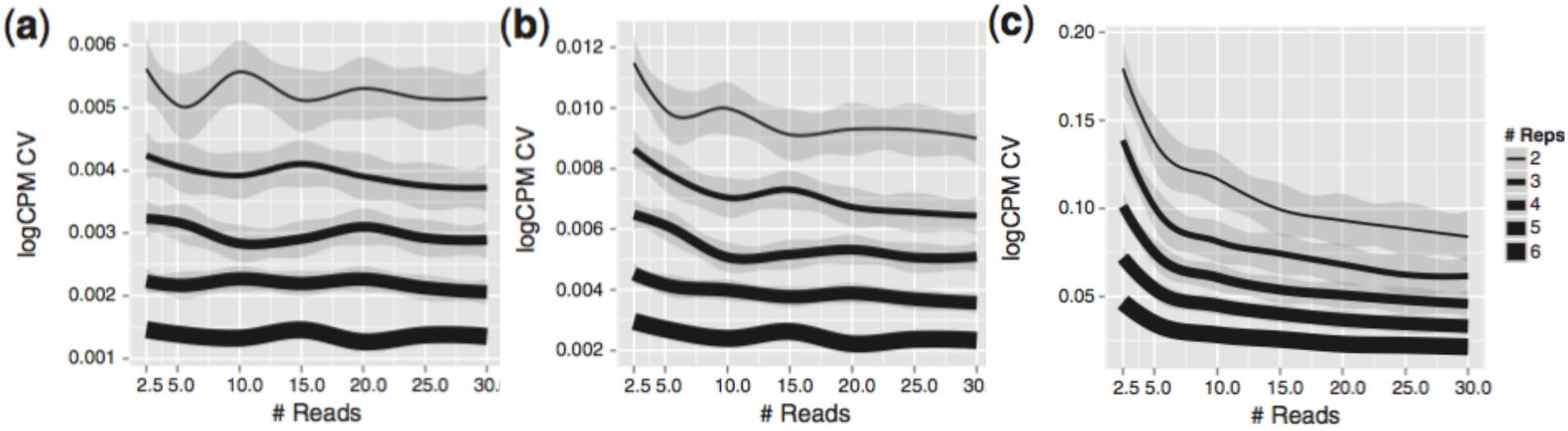


On replicates…



Liu et al. (2014) *Bioinformatics*

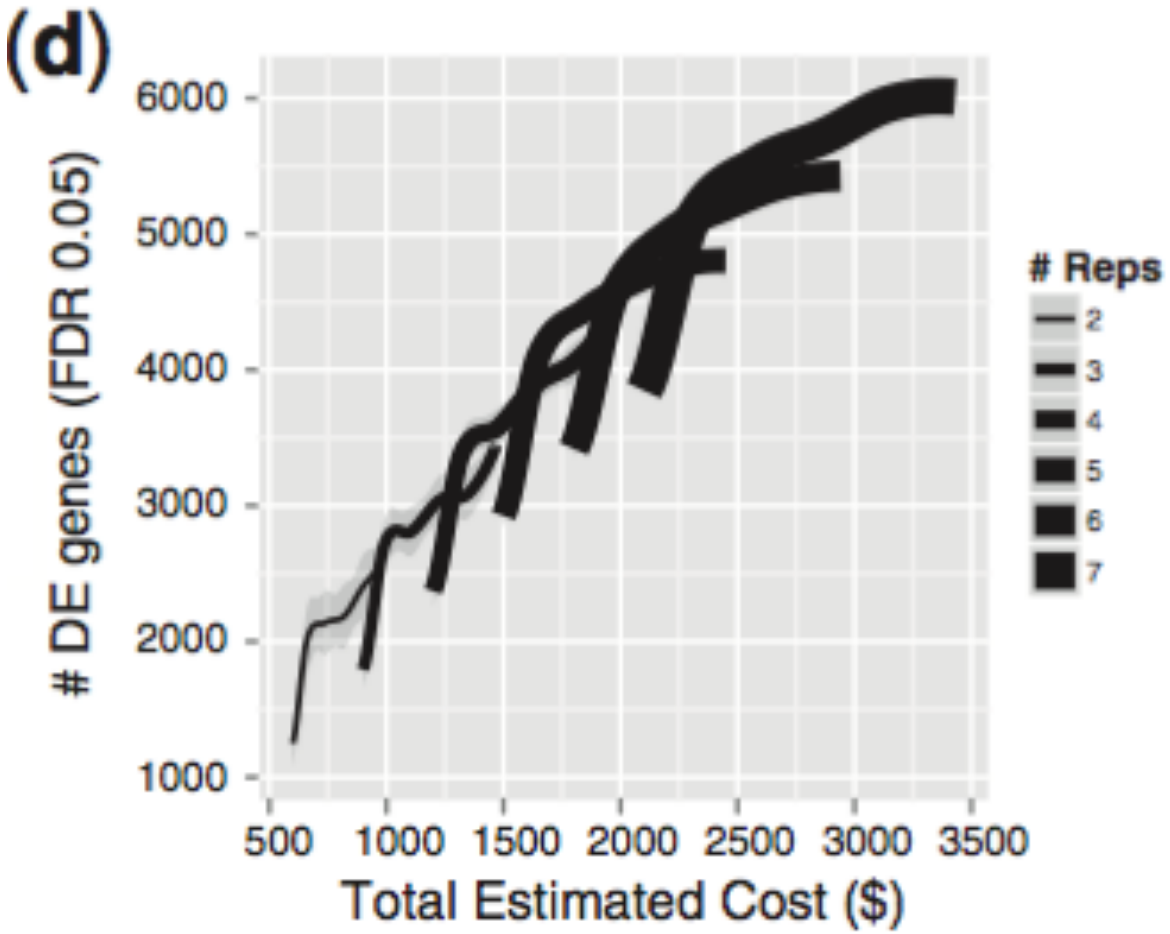
On replicates…



HIGH **MEDIUM** **LOW**

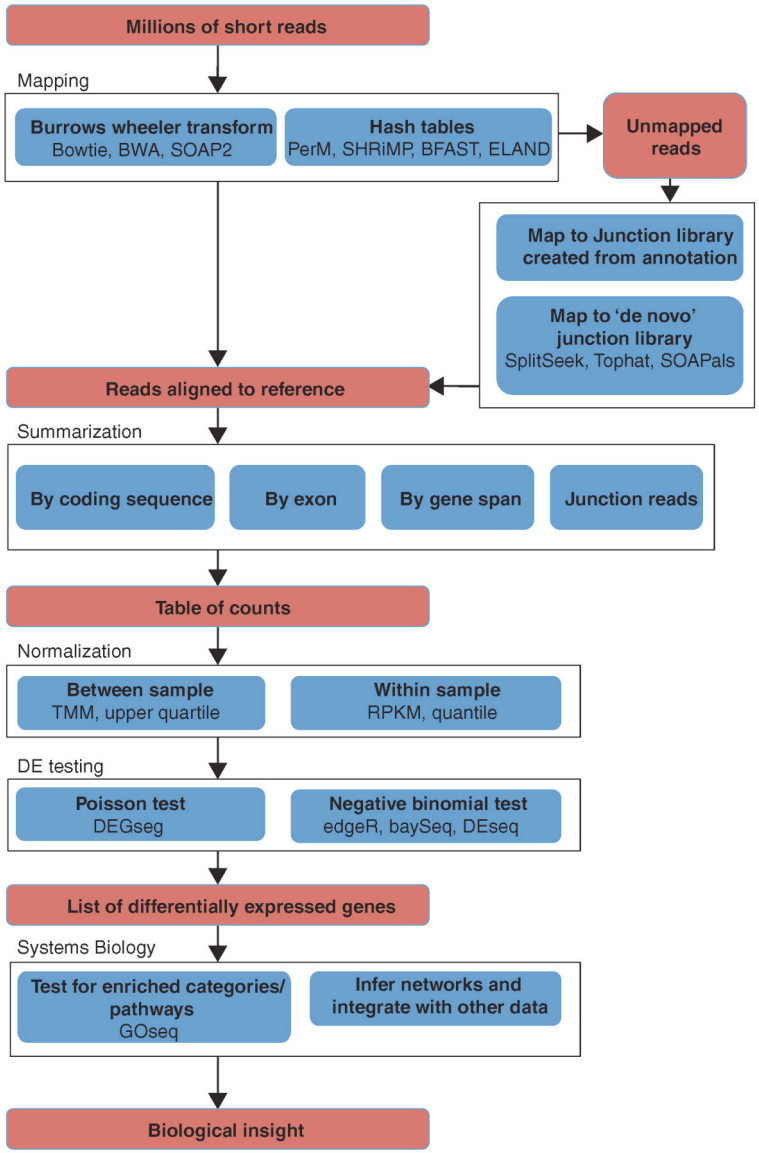
Liu et al. (2014) *Bioinformatics*

On replicates…



Liu et al. (2014) *Bioinformatics*

Summary



Oshlack, A. et al (2010) *Genome Biology*