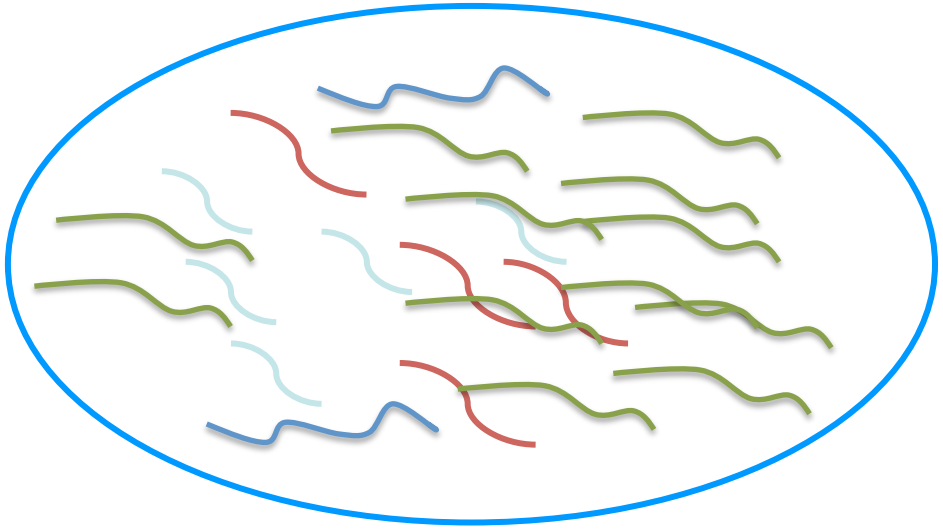
RNA-seq Quantification

Hubert Rehrauer

RNA-seq: A census of RNA molecules

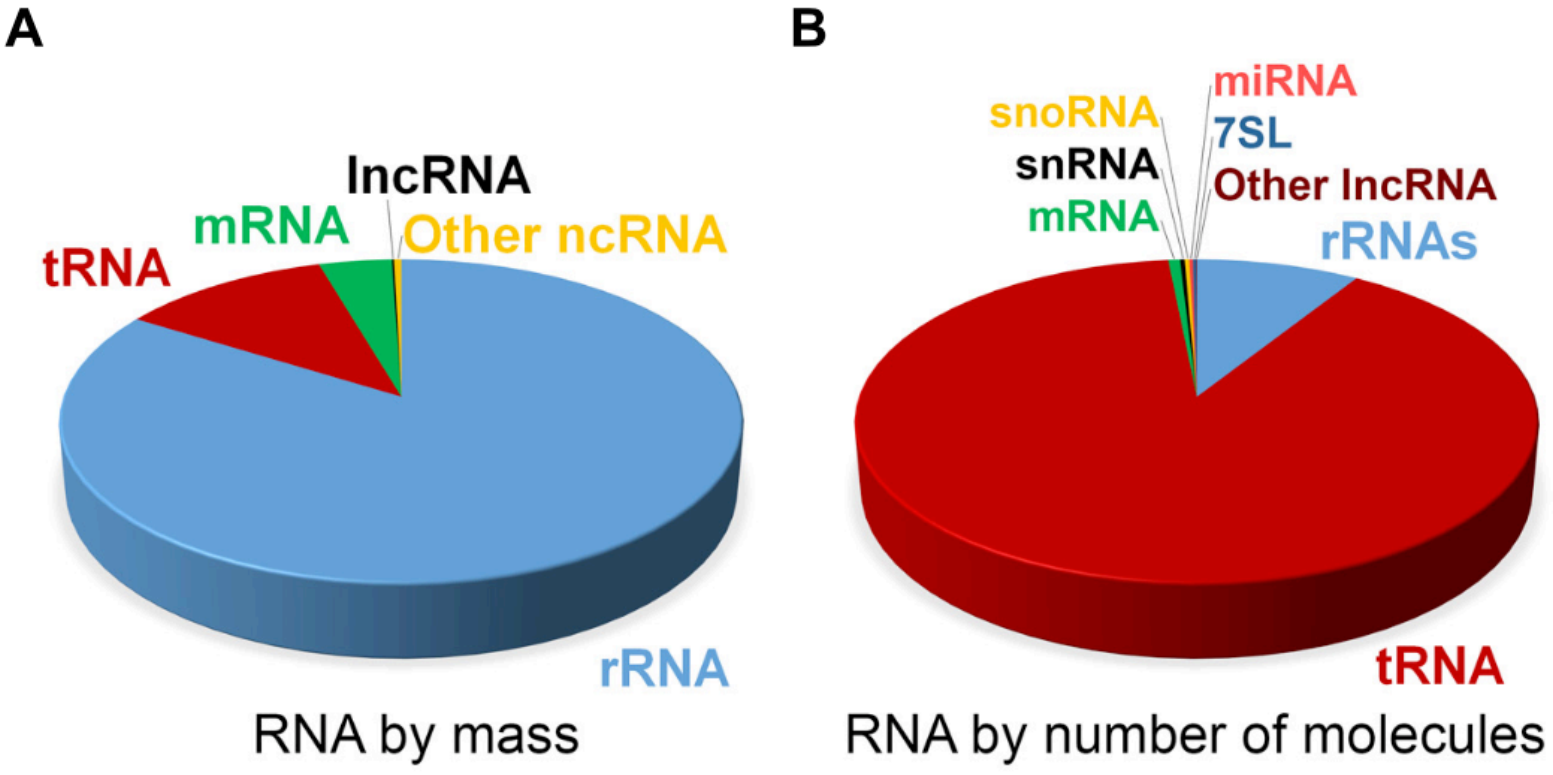


- RNA-seq aims at determining the transcriptional activity at each gene locus

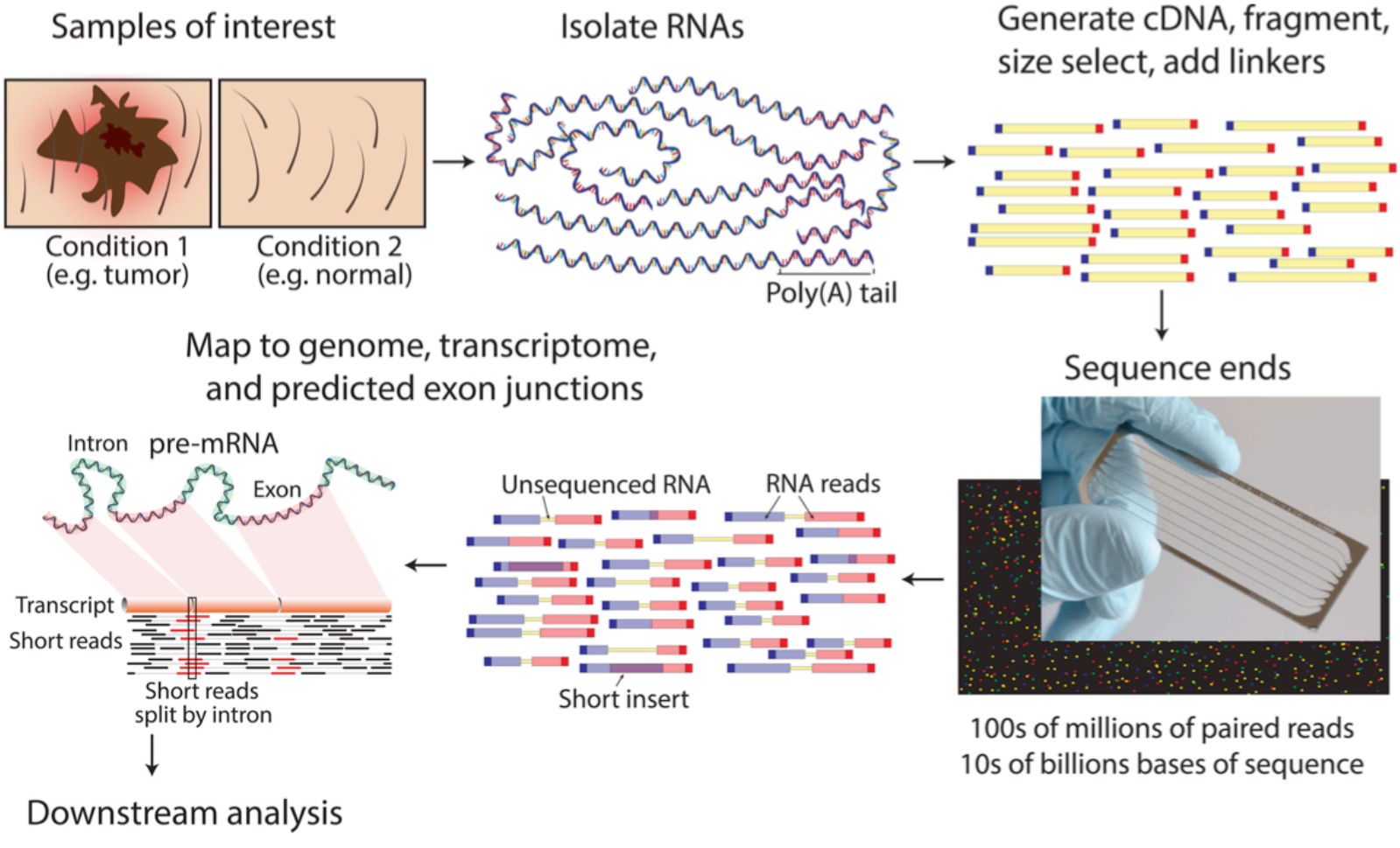
- A mammal cell has 20’000 – 30’000 gene loci
- They can give rise to >100’000 different RNA sequences
- In a typical cell at a given time there are between 100’000 to 500’000 mRNA molecules plus other RNA molecules
- This corresponds to 10-30 picograms
- For a bulk RNA-seq experiment we typically start with >= 100ng from 100’000 or more cells

RNA-seq: A census of RNA molecules

- library preparation includes fragmentation and amplification → billions of RNA fragments
- The sequencing is then a sampling process that “randomly” selects ~ 20 – 50 Mio fragments and determines their nucleotide sequence
- The typical mRNA length is 1000 – 3000 nt
- fragments to be sequenced are in the range 150 – 400 nt



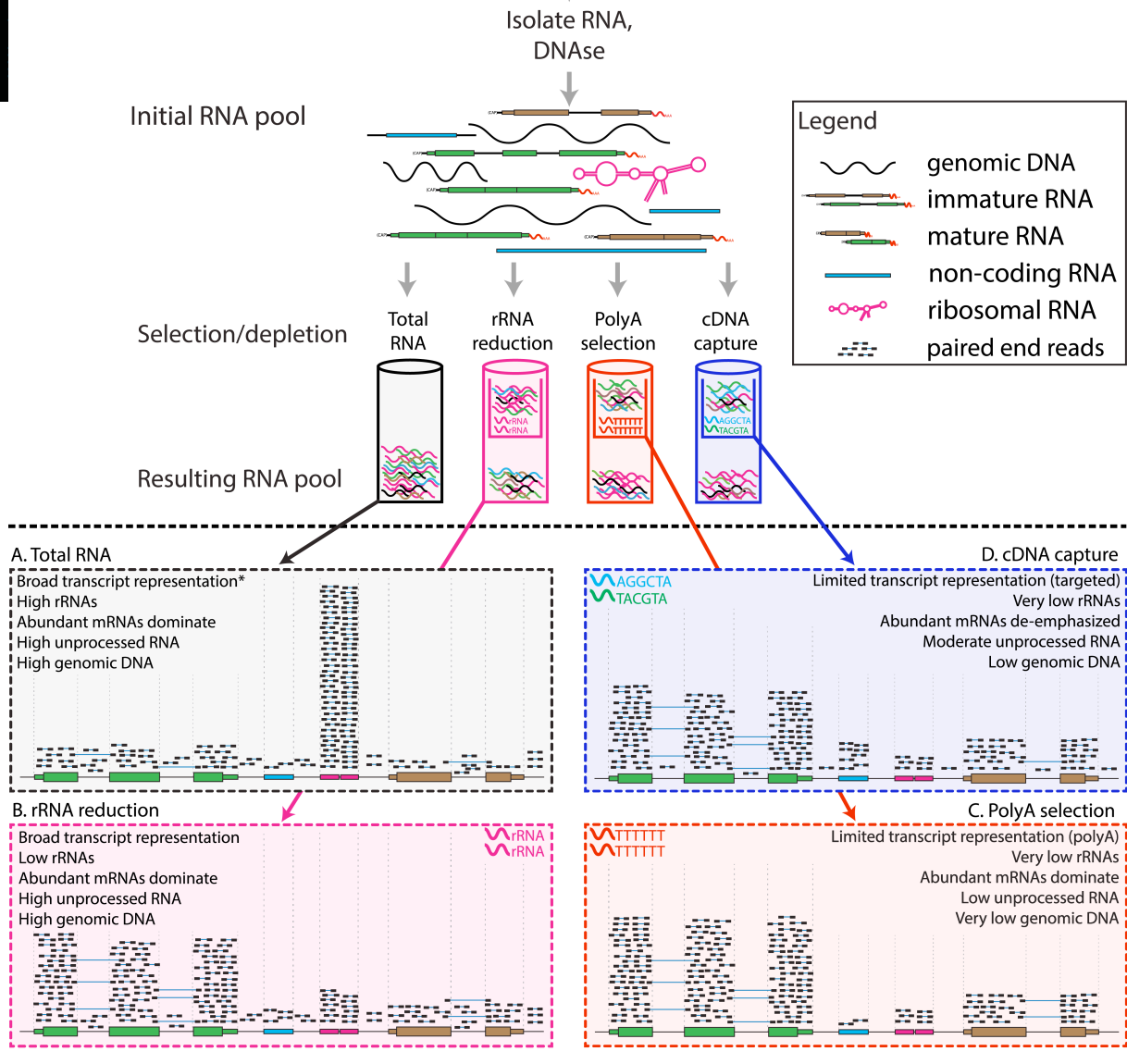
Palazzo et al. Frontiers in Genetics, 2015



Griffith et al. PLOS Computational Biology, 2015

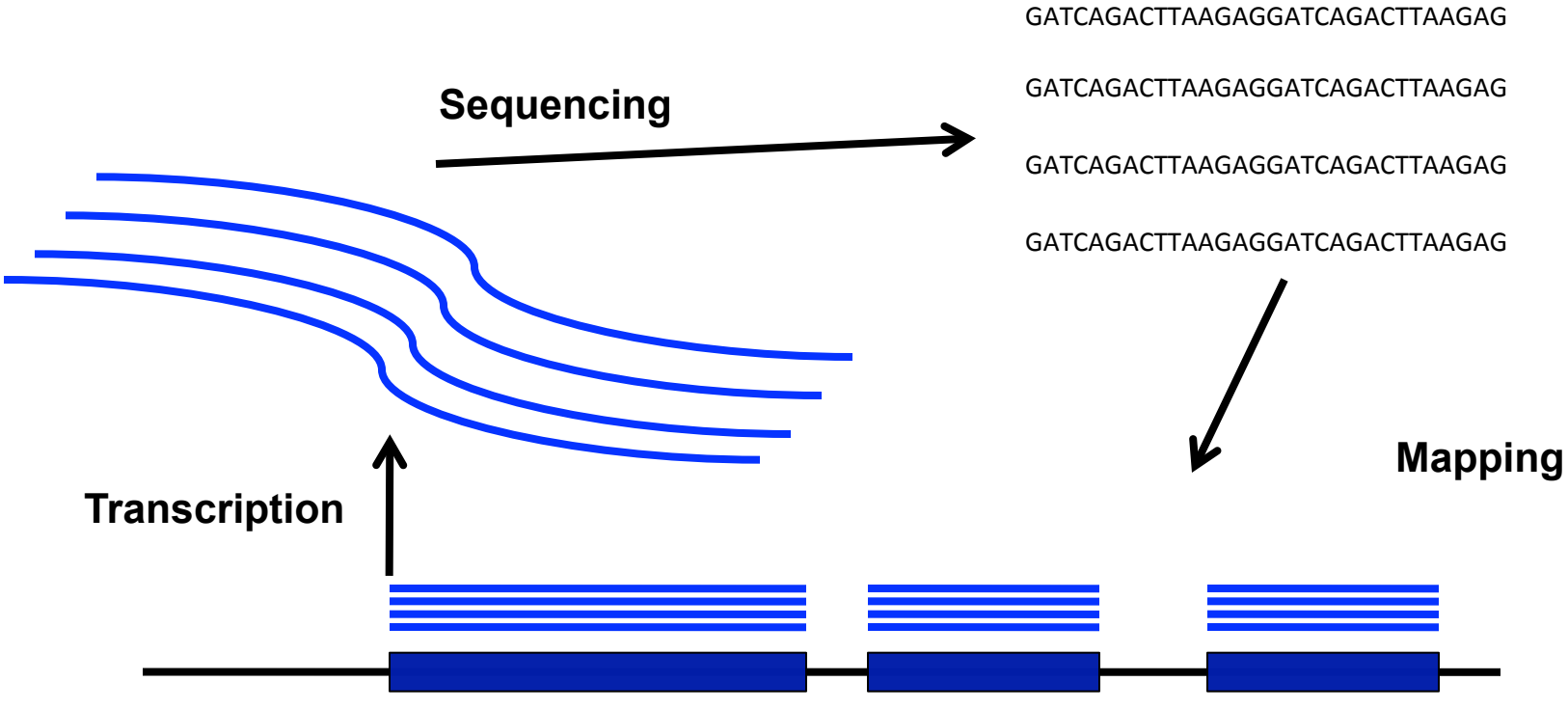
RNA-seq Experiment

- Note: DNAse eliminates the genomic DNA

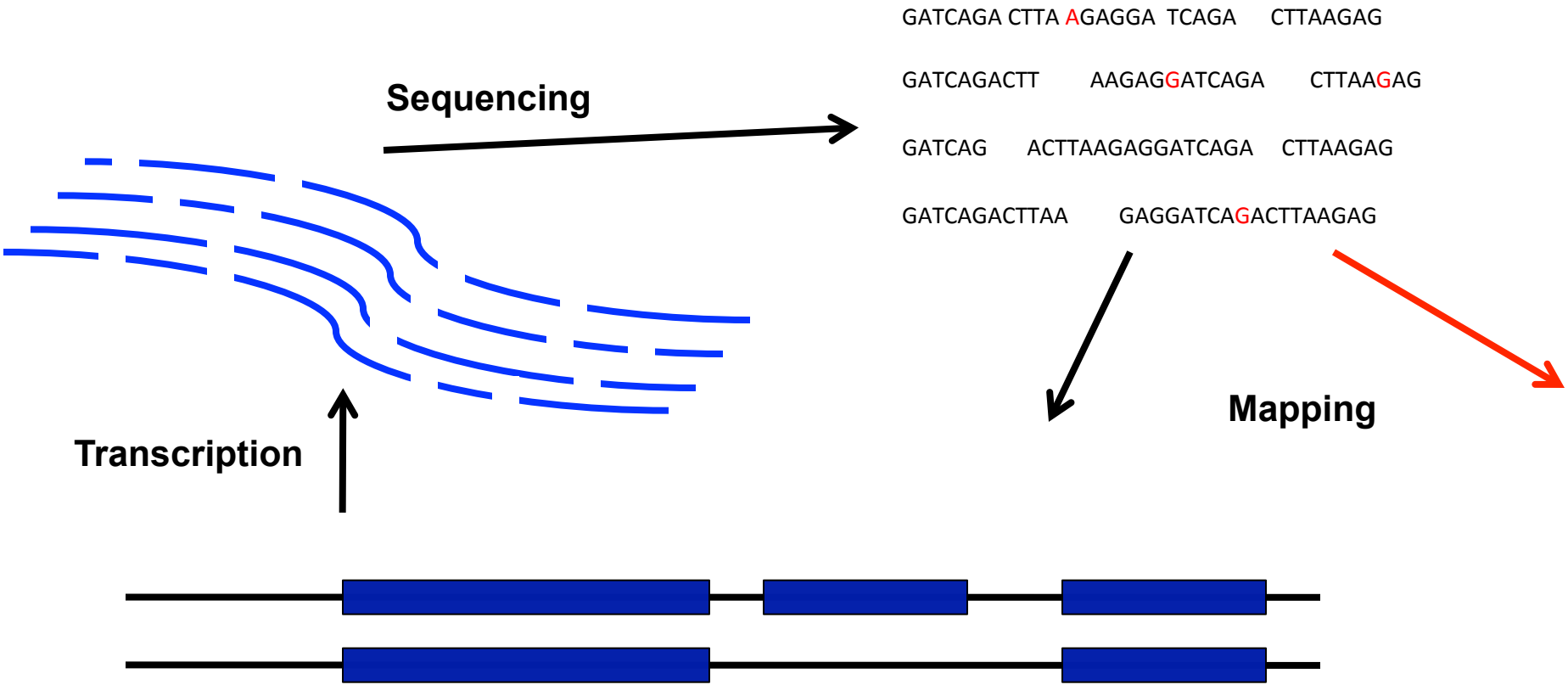


Griffith et al. PLOS Computational Biology, 2015

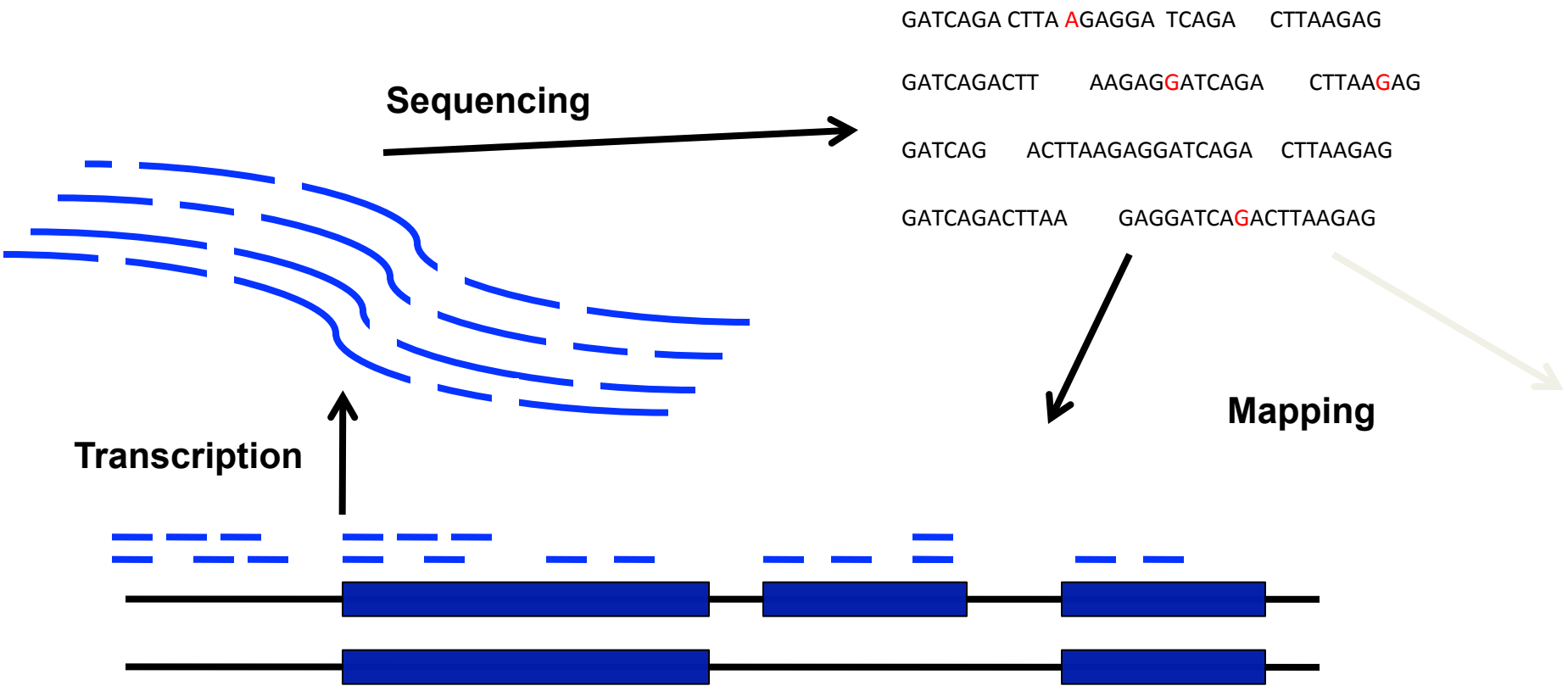
Read mapping and counting (ideal)



Read mapping and counting (today)



Read mapping and counting (today)



Abundance estimates

Abundance of what???

- Biologically relevant:
  - **gene level:**
    - # molecules transcribed from one gene locus
  - **isoform level:**
    - # molecules of a specific isoform transcribed from one gene
- Feasible with RNA-seq:
  - **relative fractions**

- Naïve approach for abundance estimate:
  - # reads that uniquely map to a gene locus
    → biased by length, discards information in multi-mapping reads
- More elaborate approaches consider multi-mappers and isoform lengths

RNA-seq comes with absolute counts but relative abundances

Gene	Sample 1 [Bn transcripts]	Sample 1 [Mio sequenced reads]	Sample 2 [Bn transcripts]	Sample 2 [Mio sequenced reads]
gene a	10	0.5	10	0.2
gene b	10	0.5	10	0.2
gene c	10	0.5	10	0.2
gene d	10	0.5	10	0.2
gene e	160	8.0	460	9.2
total	**200**	**10**	**500**	**10**

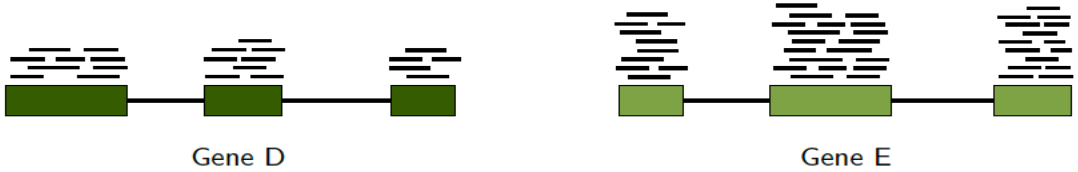
With RNA-seq different amounts of starting material will give the identical numbers of reads!
The read count for a gene is always relative to the counts for the other genes.

Number of reads and expression level

	Sample 1	Sample 2	Sample 3
Gene A	5	3	8
Gene B	17	23	42
Gene C	10	13	27
Gene D	752	615	1203
Gene E	1507	1225	2455

- Gene E has about twice as many reads aligned to it as Gene D
- What does it mean?

1) Gene E is expressed with twice as many transcripts as Gene D



2) Both genes are expressed with the same number of transcripts but Gene E is twice as long as Gene D

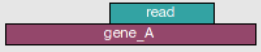
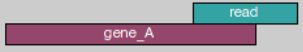
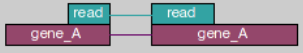
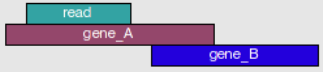
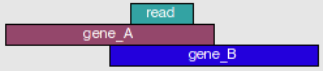
Number of Reads per Gene

- The number of reads **sequenced** per gene depends on
  - relative gene expression (relative to the other genes)
  - gene length
  - sequencing depth

- The number of reads that can be **aligned** to a gene depends additionally
  - expression of other genes that have identical sequences
  - sequencing errors
  - allele sequences
  - mapper settings, e.g. STAR

`--outFilterMultimapNmax` 20
max number of multiple alignments allowed for a read: if exceeded, the read is considered unmapped

Model-free Counting of Overlapping reads – Count Modes

	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

Straightforward Counting Modes

- unique reads
- unique reads + multi-reads randomly assigned
- unique reads + multi-reads proportionally assigned
- …

- **Do not count multi-reads towards all genes they align to.**
  In that case the sum of the counts could be larger than the number of reads

RNA-seq model

$$\alpha_t = \mathrm{P}[\text{read from transcript } t] = \frac{1}{Z}\rho_t l_t$$

with:

ρ_t expression level / abundance / fraction

l_t transcript length

$Z = \sum_t \rho_t l_t$ normalization factor

The normalization factor is the weighted mean length of the transcripts.

RNA-seq model

Estimation of the probability that a read is from a specific transcript:

$$\hat{\alpha}_t = \frac{X_t}{N} = \frac{\#\text{reads mapping to transcript } t}{\#\text{mappable reads in total}}$$

Abundance estimates:

$$\hat{\rho}_t \propto \frac{\hat{\alpha}_t}{l_t}$$

Definition of expression levels

- Reads Per Kilobase per Million of mapped reads

$$\text{RPKM for transcript } t = 10^6 \times 10^3 \times \frac{X_t}{l_t N}$$

- Transcripts Per Million Transcripts

$$\text{TPM for transcript } t = 10^6 \times Z \times \frac{X_t}{l_t N}$$

- Preferable is TPM because it is independent of the transcriptome

Maximum Likelihood Estimation

- The estimated abundances represent unique MLE estimates

$$\text{with } \alpha = \{\alpha_t\}_{t \in T}$$

$$L[\alpha] = \prod_{t \in T} \prod_{f \in F_t} P[f \in t] \frac{1}{l_t}$$

$$= \prod_{t \in T} \prod_{f \in F_t} \alpha_t \frac{1}{l_t}$$

$$= \prod_{t \in T} \left(\frac{\alpha_t}{l_t} \right)^{X_t}$$

Effective Transcript Length

- Since fragments have a non-zero length the read probabilities depend actually on an effective length:

$$l_t := \text{transcript length - fragment length + 1}$$

- For simplicity we continue to use the symbol without tilde but will always assume it is the effective length
- The effective length represents the stretch of the transcript from which I can get a fragment that I can then map back to the transcript
- → The effective length must also consider mappability!
- → Mappability does depend on mapping algorithm, mutations, …

Multi-reads

- Reads that cannot be uniquely assigned to one transcript were ignored so far
- Multi-reads can occur
  - if a read aligns more than once in the genome
  - if at an alignment position there is more than one transcript defined
- Multi-reads do occur due to homology not due to pure chance

- Percentage of multi-reads is typically low < 5%
- Ignoring multi-reads leads to
  - loss of information
  - biased expression estimates (e.g. for genes in gene families)

Considering Multi-reads

- Define a compatibility matrix

$$\mathbf{Y} = \{y_{ft}\}_{f \in F, t \in T}$$

with

$$y_{ft} = \begin{cases} 1 \text{ if read } f \text{ aligns to transcript } t \\ 0 \text{ else} \end{cases}$$

- The likelihood is now:

$$\mathrm{L}[\alpha] = \prod_f \left(\sum_t y_{ft} \frac{\alpha_t}{l_t} \right)$$

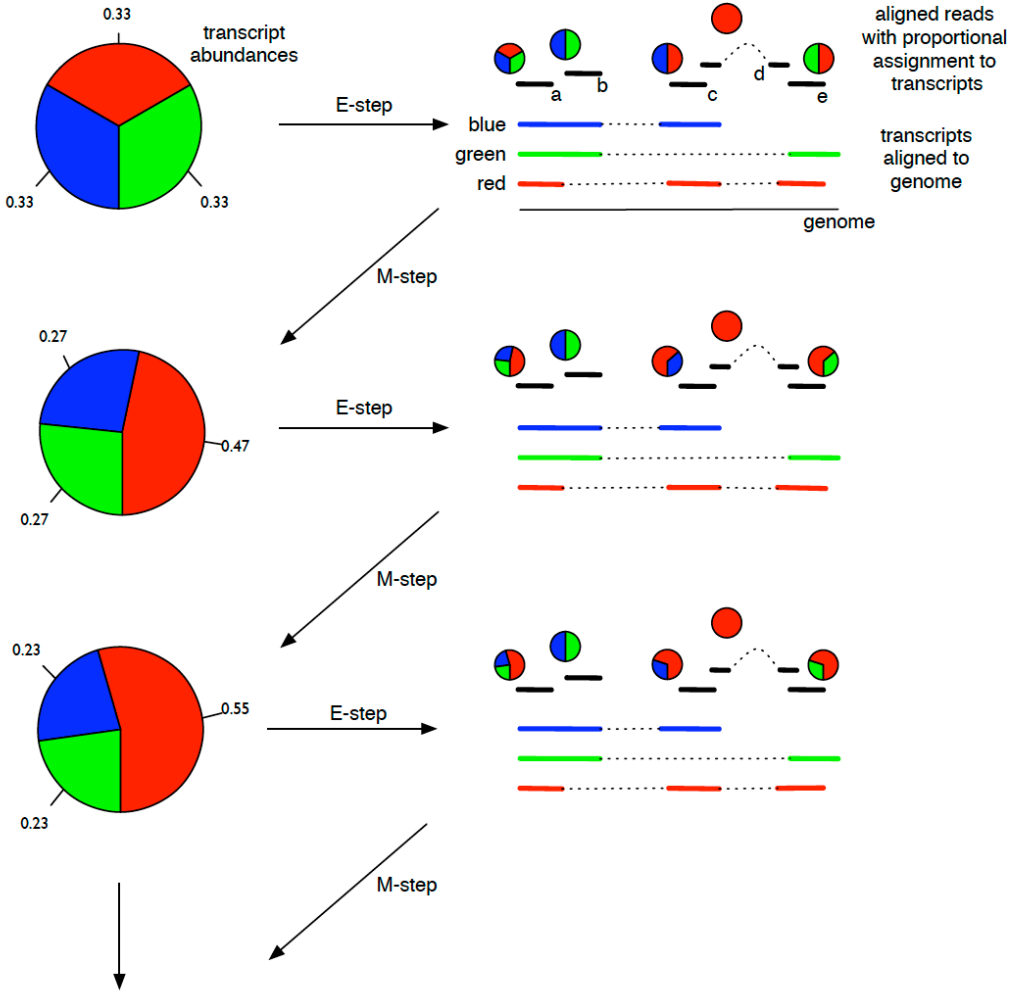
- but now abundances have to be estimated iteratively

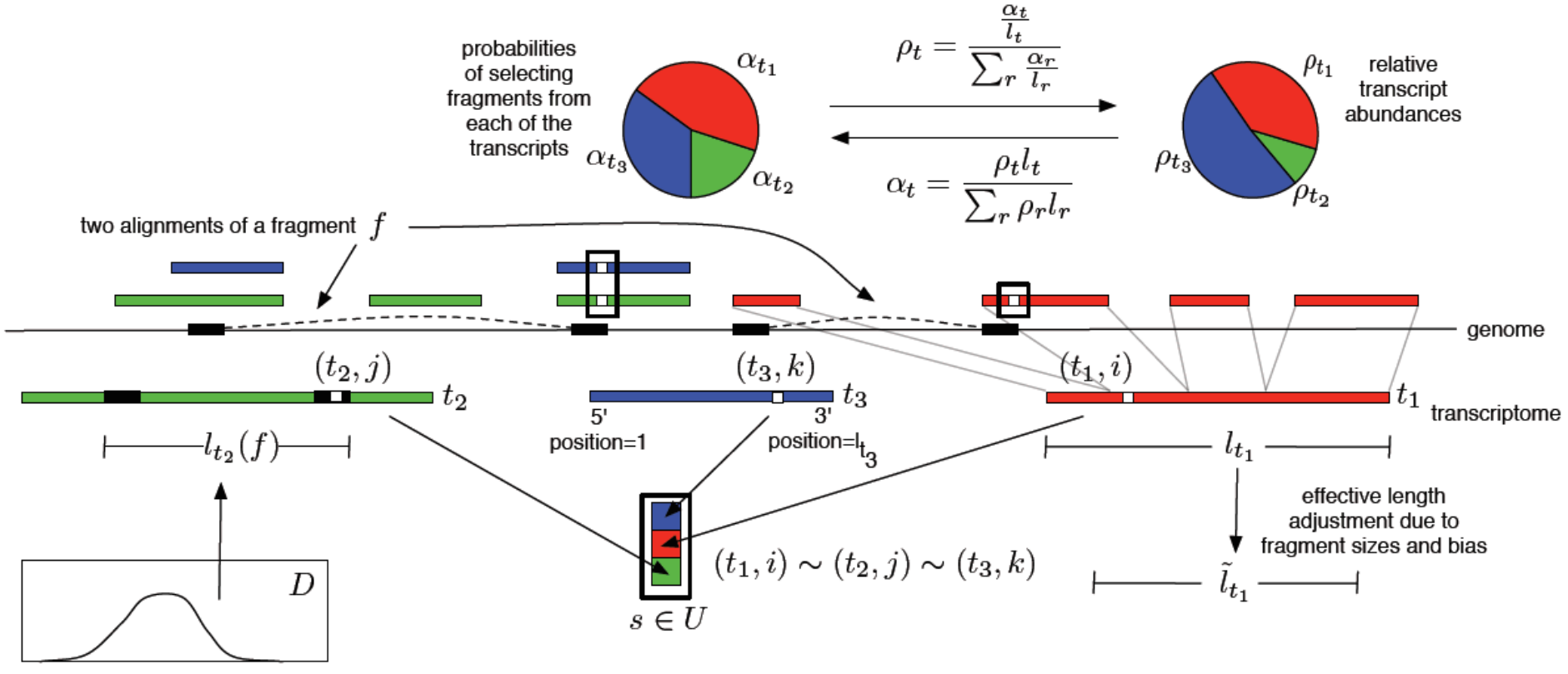
Iterative Estimation

Three step algorithm

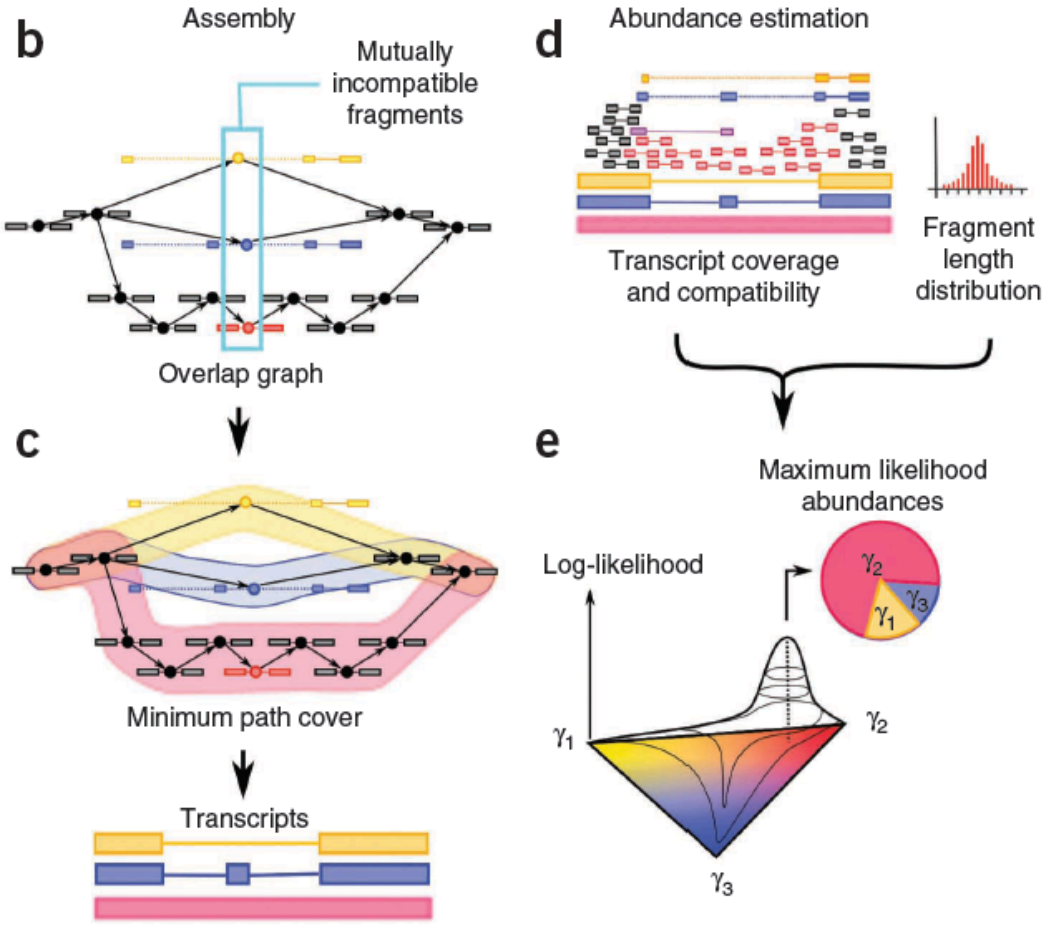
1. Estimate abundances based on uniquely mapping reads only
2. For each multi-read, divide it between the transcripts to which it maps, proportionally to their abundances estimated in the first step
3. Recompute abundances based on updated counts for each transcript
4. Continue with Step 2

Expectation Maximization Estimation




probabilities of selecting fragments from each of the transcripts
α_{t_1}
α_{t_2}
α_{t_3}
$\rho_t = \frac{\frac{\alpha_t}{l_t}}{\sum_r \frac{\alpha_r}{l_r}}$
$\alpha_t = \frac{\rho_t l_t}{\sum_r \rho_r l_r}$
ρ_{t_1}
ρ_{t_2}
ρ_{t_3}
relative transcript abundances
two alignments of a fragment f
genome
(t_2, j)
t_2
(t_3, k)
t_3
(t_1, i)
t_1
transcriptome
5'
3'
position=1
position=l_{t_3}
$l_{t_2}(f)$
l_{t_1}
D
$(t_1, i) \sim (t_2, j) \sim (t_3, k)$
$s \in U$
effective length adjustment due to fragment sizes and bias
$\tilde{l}_{t_1}$

Transcript abundance estimation with Cufflinks



Trapnell 2010, Nature Biotechnology

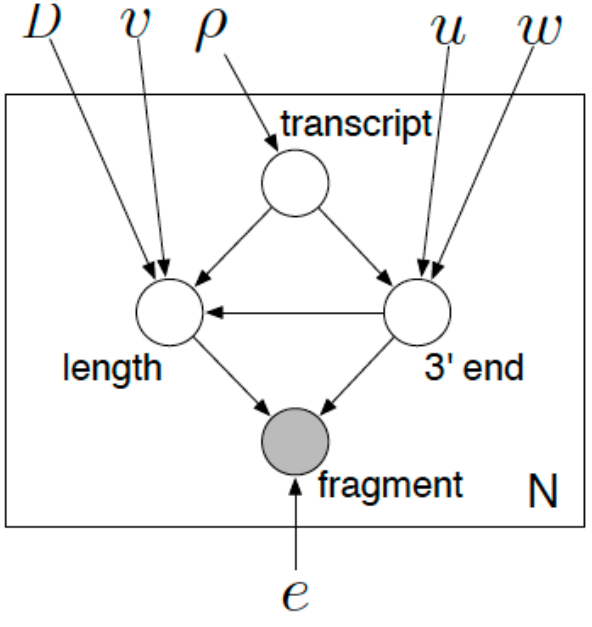
General Formulation of Abundance Estimation

A full model for the abundance estimation should consider:

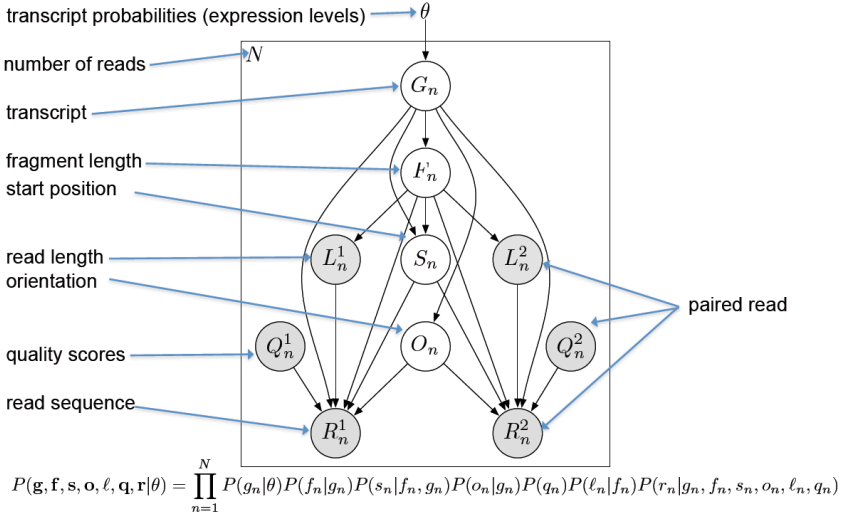
- position bias
- fragment-length distribution
- sequencing errors
- site-specific bias
- …

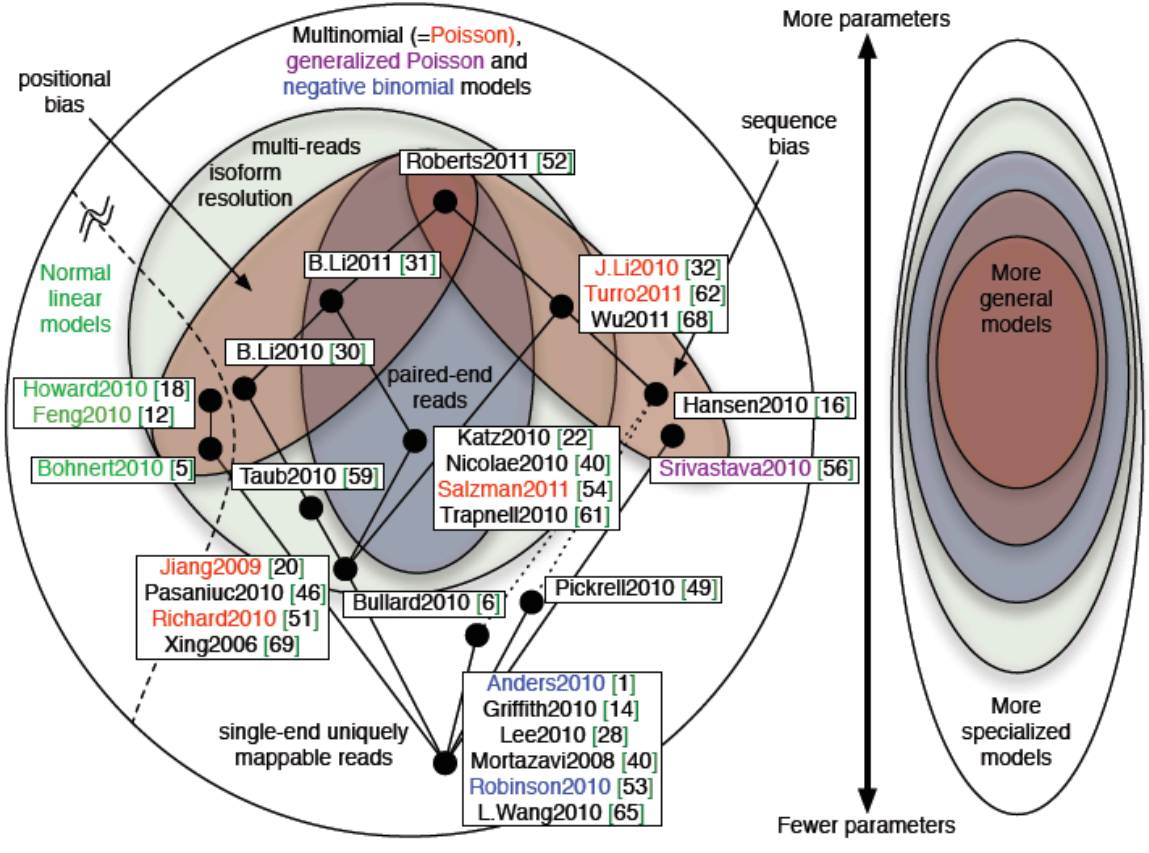
Example Implementations

Pachter: Cufflinks



Dewey: RSEM





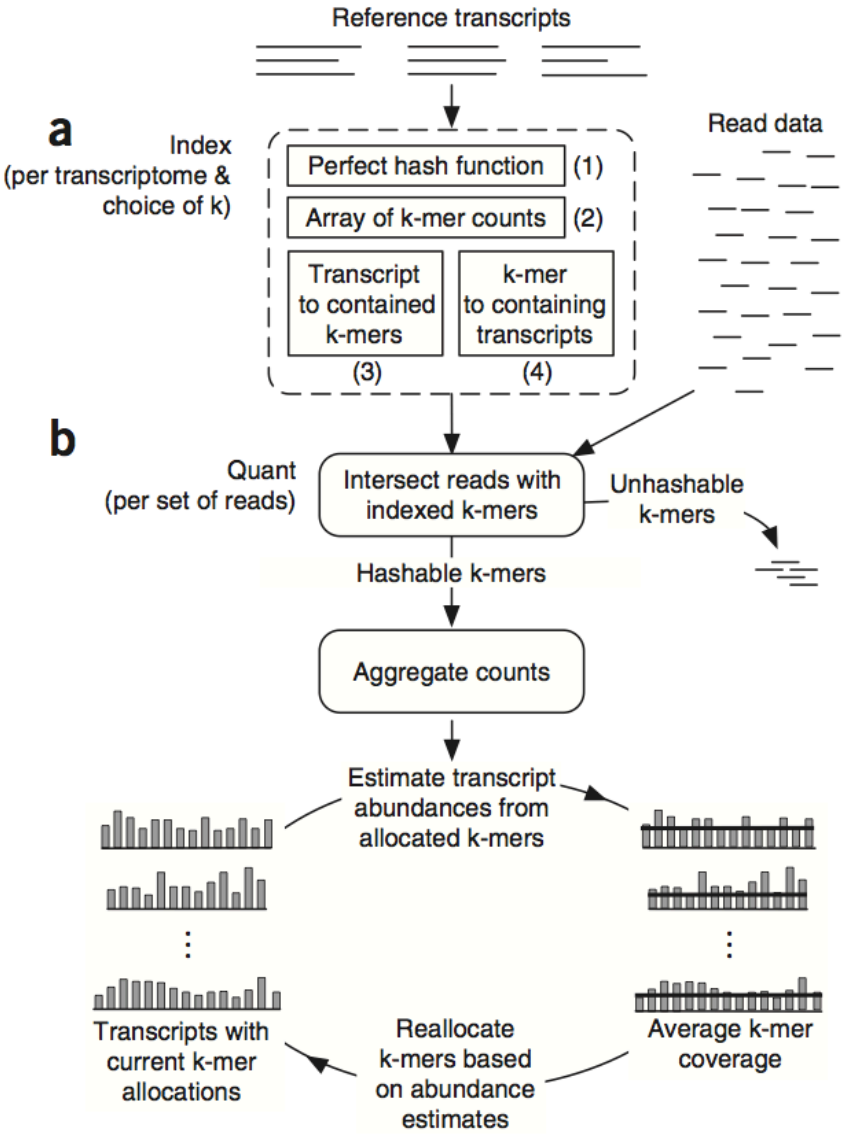
Pachter, 2011

Fast approaches to get the Read-Transcript Compatibility Matrix

- Sailfish: lightweight alignment
- Salmon: improvement of sailfish
- kallisto: pseudo-alignments

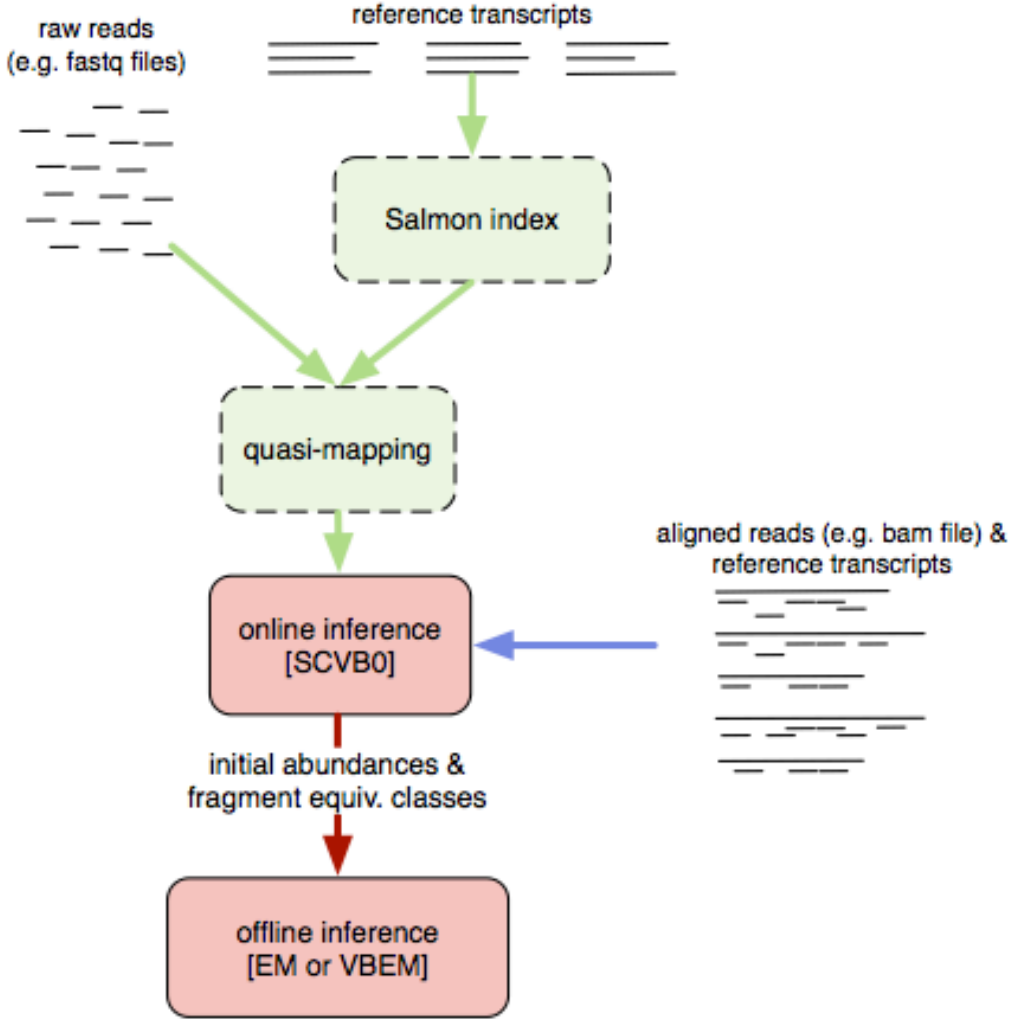
Sailfish

- No read alignment only k-mer lookup (very fast)
- Iterative resolution of ambiguous k-mers
- Original version treated k-mers of a read as independent



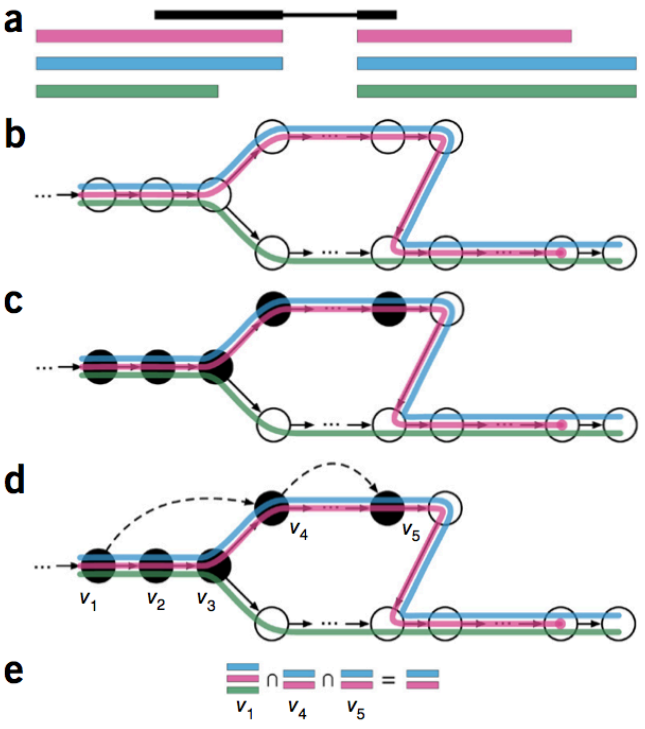
Patro et al. Sailfish …. Nature Biotechnology, 2014

Salmon


raw reads
(e.g. fastq files)
reference transcripts
Salmon index
quasi-mapping
aligned reads (e.g. bam file) &
reference transcripts
online inference
[SCVB0]
initial abundances &
fragment equiv. classes
offline inference
[EM or VBEM]

Quantification with pseudo-alignments

- Instead of hashing the transcriptome build a de Bruijn graph
- Find k-mer hits in the de Bruijn graph
- Identifies only transcripts that are consistent with all k-mer hits



Bray et al. Nature Biotechnology, 2016

Performance comparison

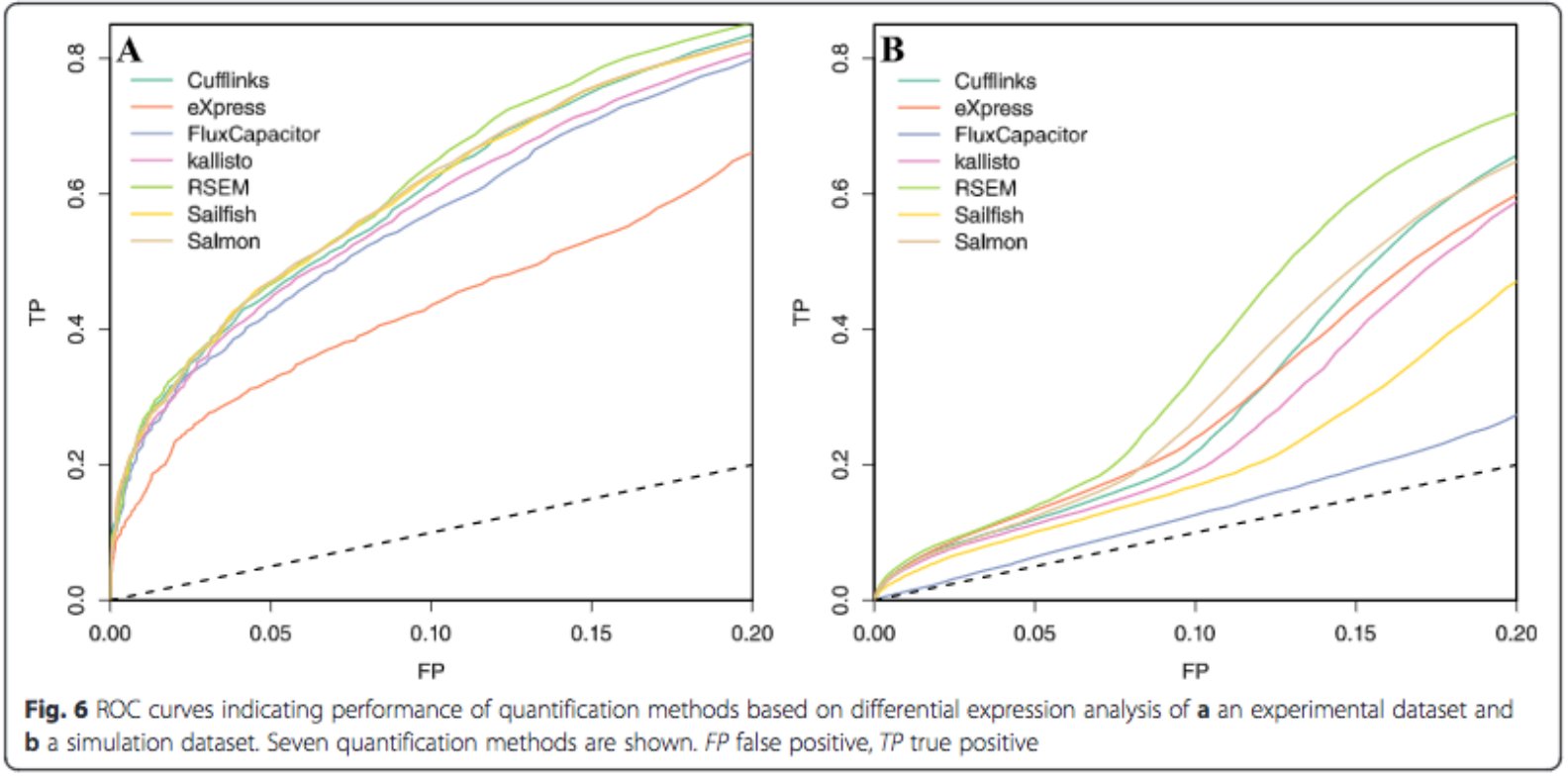
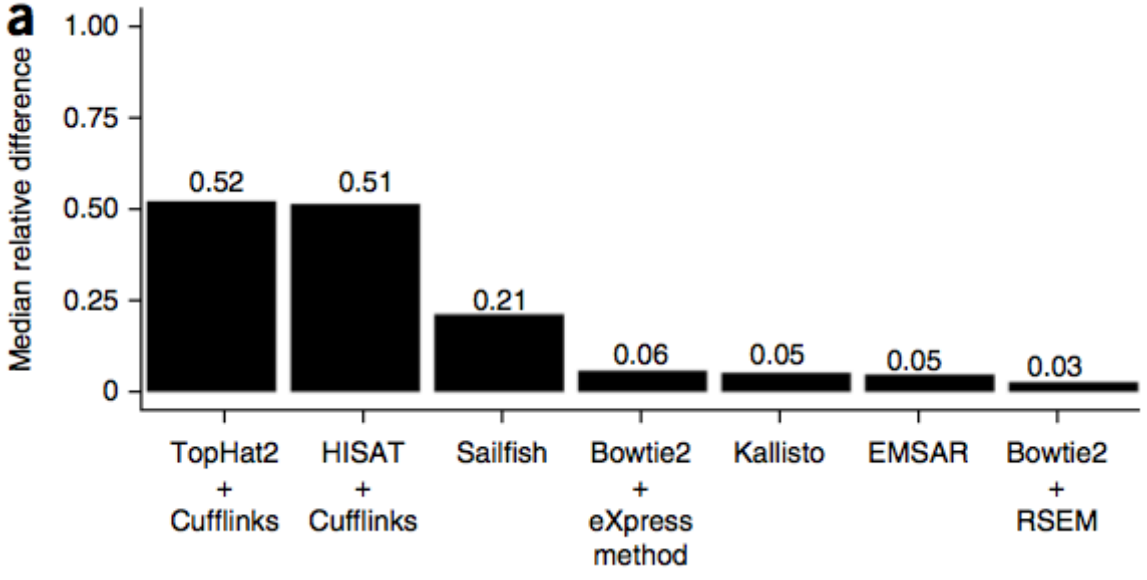
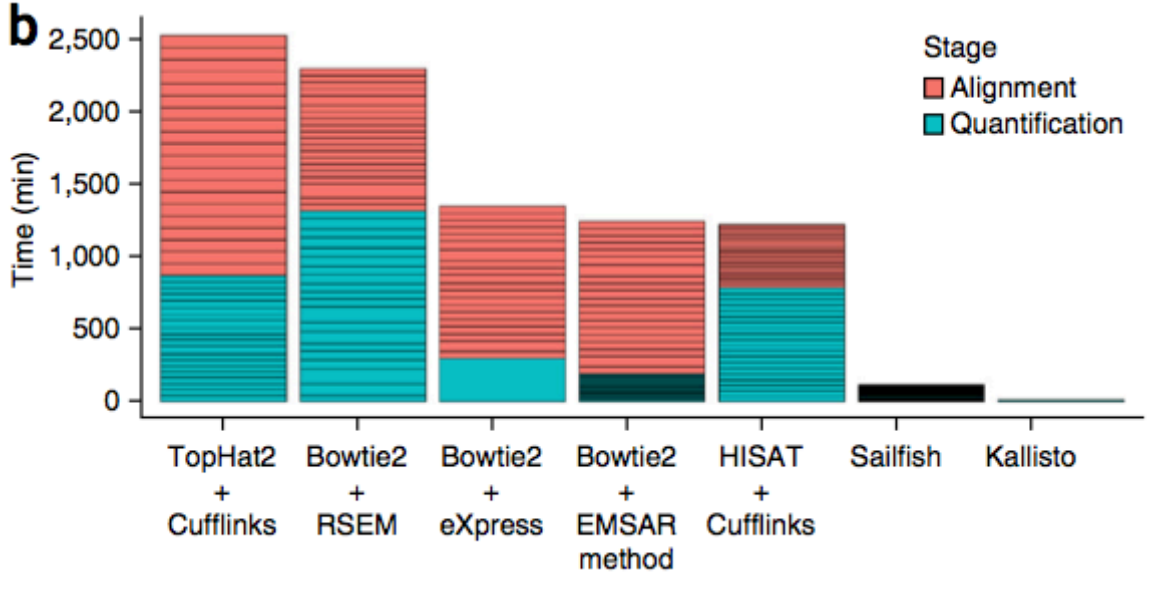


Fig. 6 ROC curves indicating performance of quantification methods based on differential expression analysis of **a** an experimental dataset and **b** a simulation dataset. Seven quantification methods are shown. *FP* false positive, *TP* true positive

Teng et al. Genome Biology, 2016

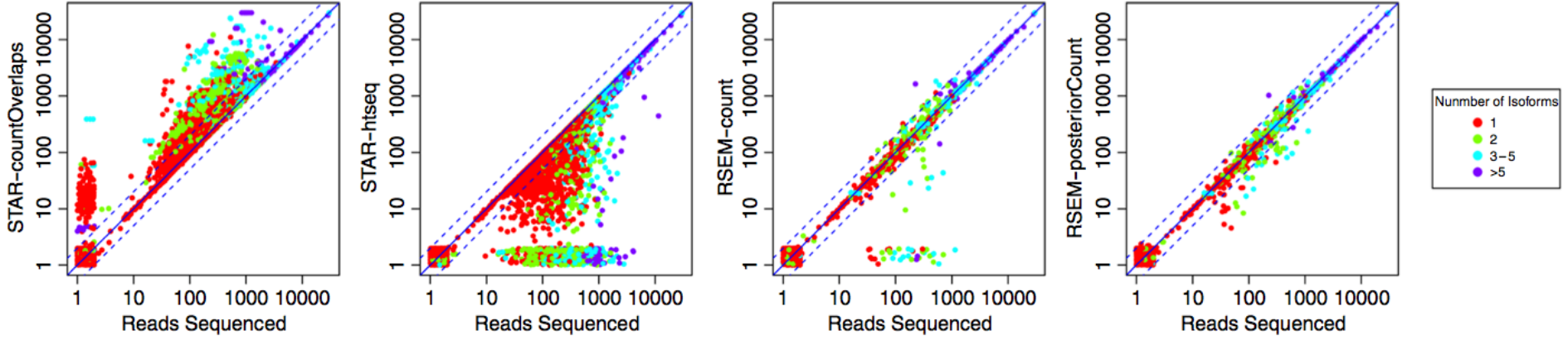
Performance Comparison





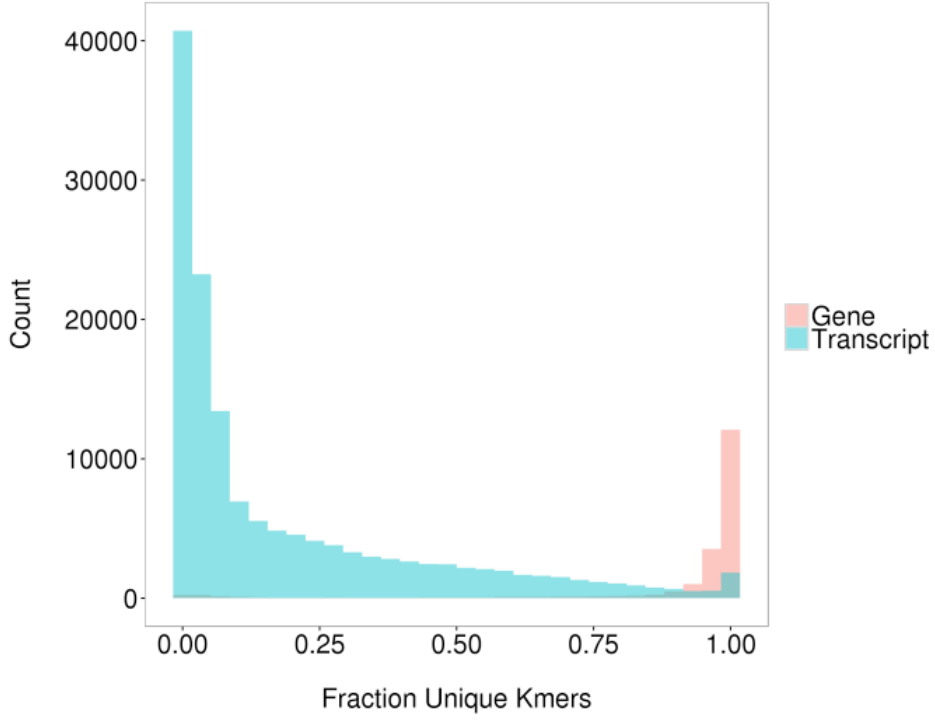
Bray et al. Nature Biotechnology, 2016

Read Counting Accuracy

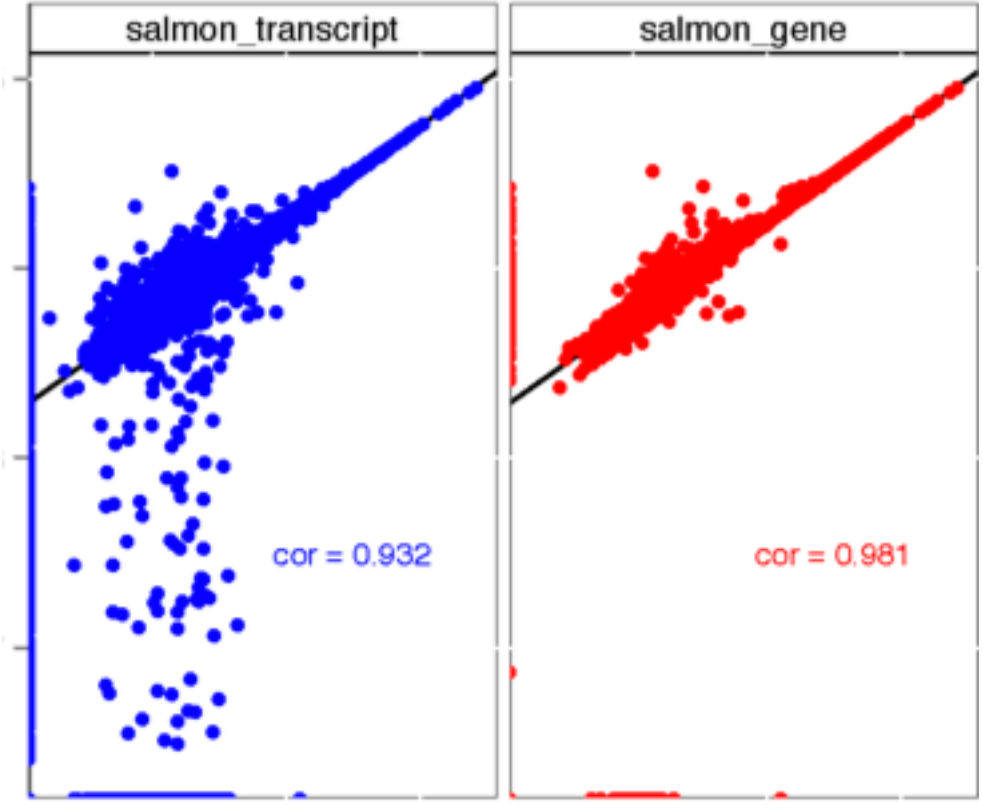


Rehrauer et al.BMC Bioinformatics, 2014

Uniqueness: Isoform-level vs gene-level



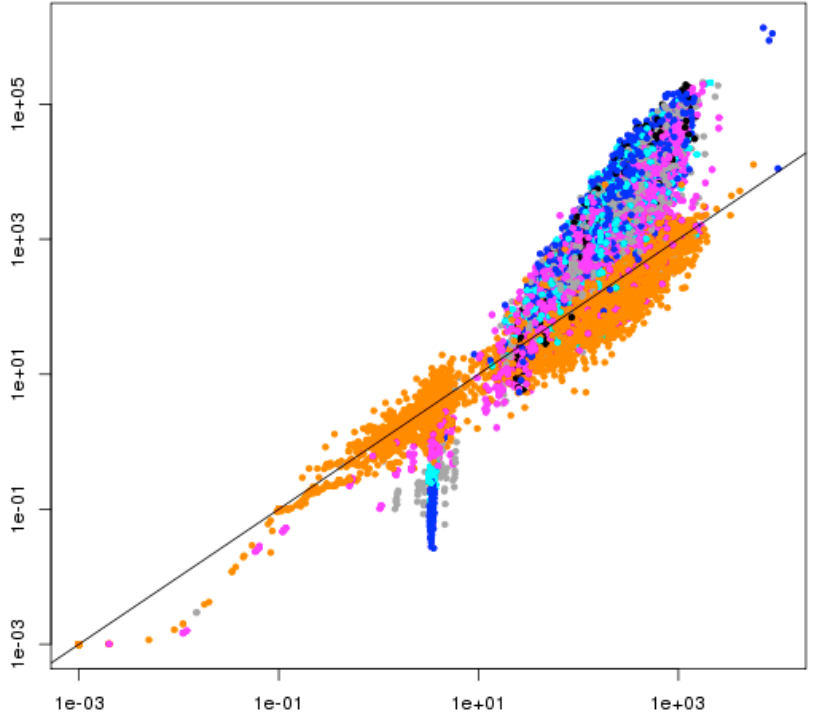
Accuracy: Isoform-level vs gene-level



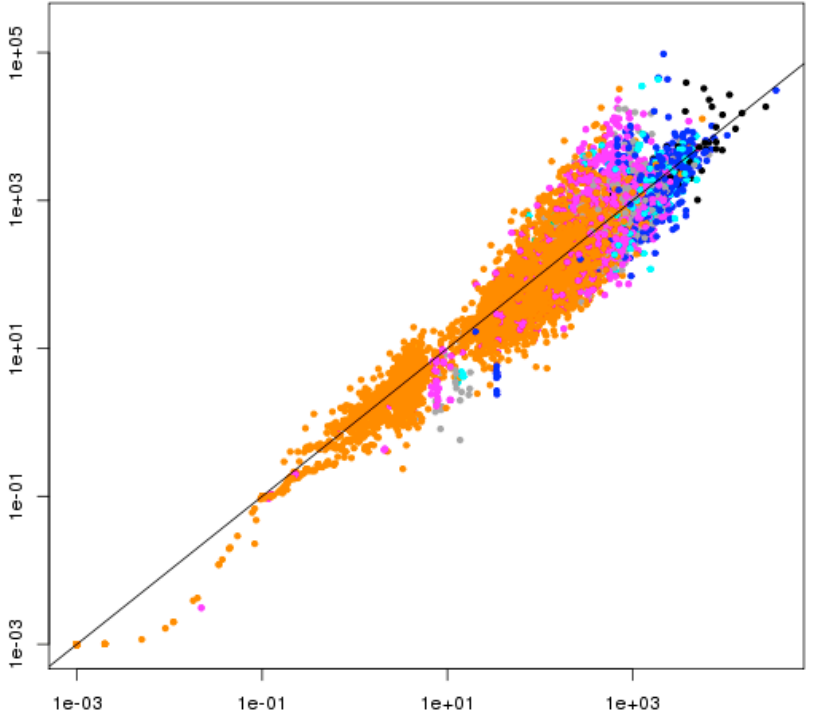
Soneson et al. F1000, 2015

Isoform level has higher variability

isoform level

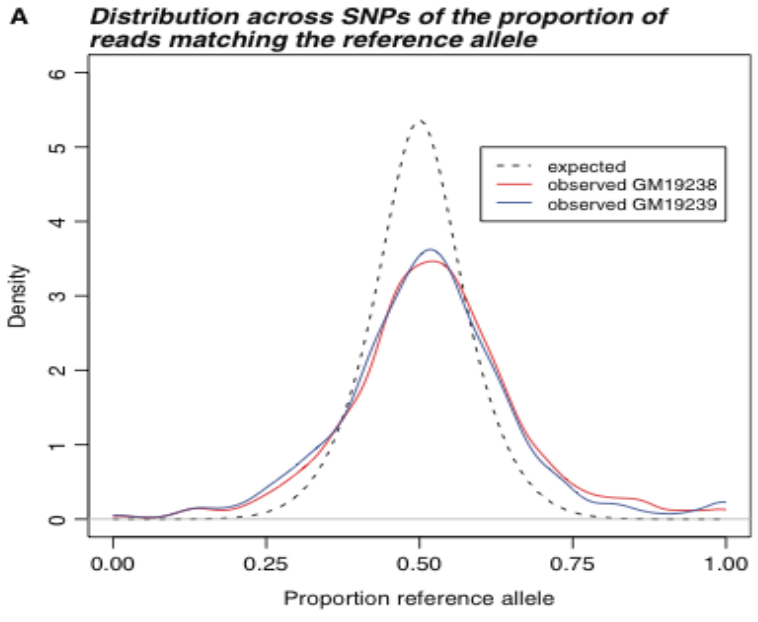


gene level



Biological Issue: SNPs

- SNPs may lead to false positives in differential expression because fewer reads map to non-reference alleles
- Note: Sequencing enables allele-specific expression values



Cufflinks and Related

- Pachter, L. Models for transcript quantification from RNA-Seq. *arXiv preprint arXiv:1104.3889* (2011).
- Trapnell C, Williams BA, Pertea G, Mortazavi AM, Kwan G, van Baren MJ, Salzberg SL, Wold B, Pachter L. **Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation** *Nature Biotechnology* doi:10.1038/nbt.1621
- Roberts A, Trapnell C, Donaghey J, Rinn JL, Pachter L. **Improving RNA-Seq expression estimates by correcting for fragment bias** *Genome Biology* doi:10.1186/gb-2011-12-3-r22
- Roberts A, Pimentel H, Trapnell C, Pachter L. **Identification of novel transcripts in annotated genomes using RNA-Seq** *Bioinformatics* doi:10.1093/bioinformatics/btr355

- **RSEM:**
  Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).
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