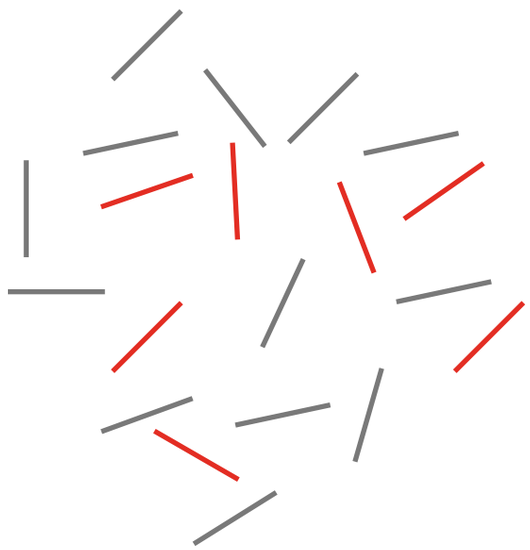
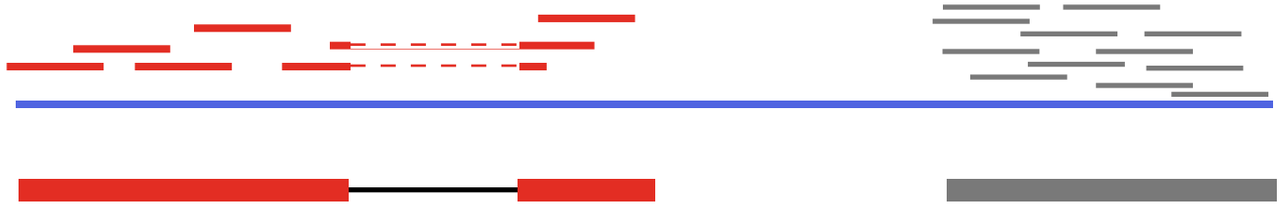
Modern RNA-seq analysis

Charlotte Soneson
University of Zurich
Brixen 2016

The traditional workflow



ALIGNMENT

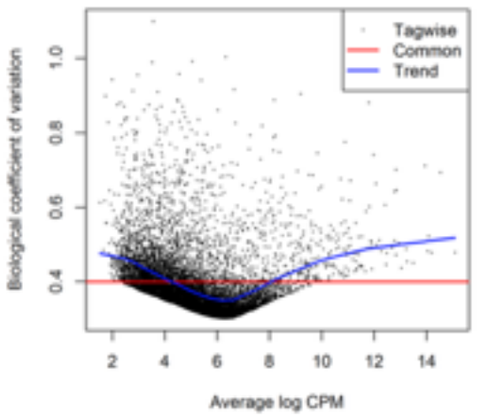


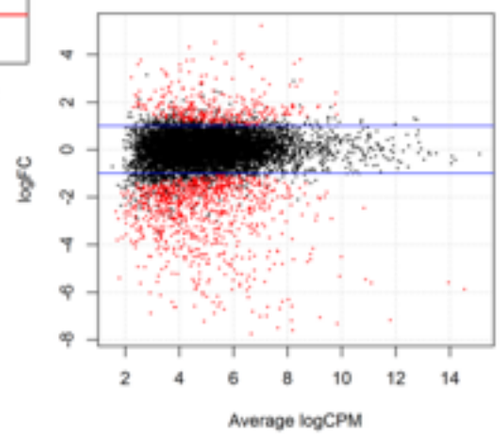
COUNTING

Gene A
Gene B
.
.
.
Gene X

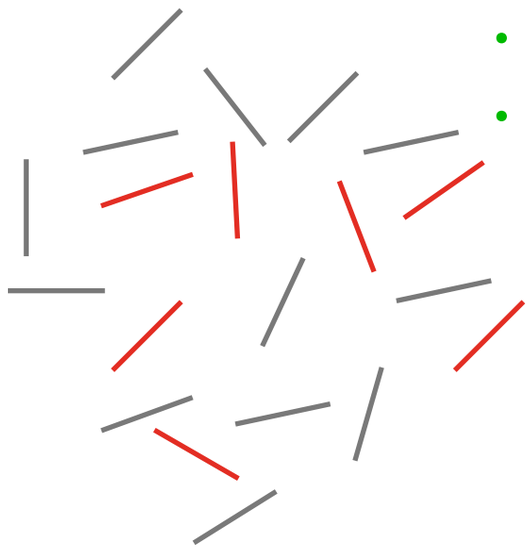
$$\begin{pmatrix} 7 & . & . & . \\ 13 & . & . & . \\ . & . & . & . \\ . & . & . & . \\ . & . & . & . \end{pmatrix}$$

ANALYSIS



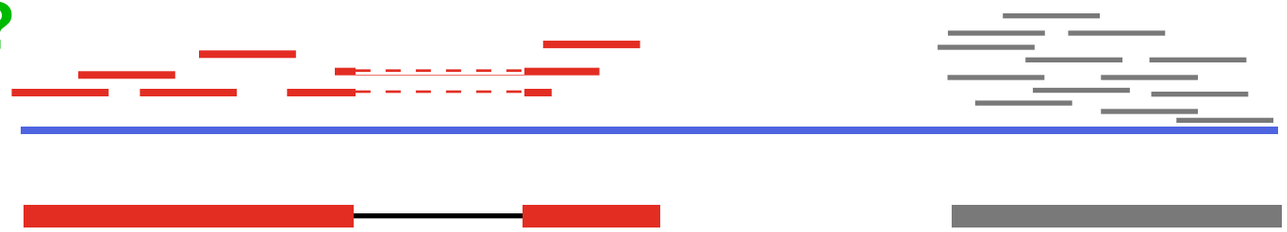


The traditional workflow



- slow
- unnecessary?

ALIGNMENT

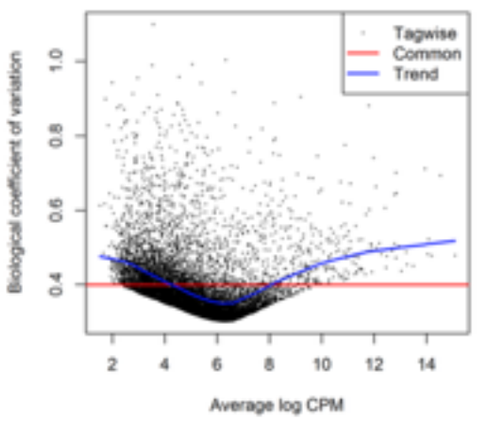


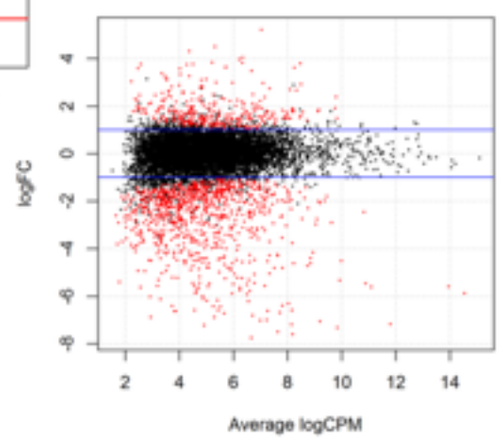
COUNTING

inaccurate?

doesn't account for all uncertainty in abundance estimates

ANALYSIS

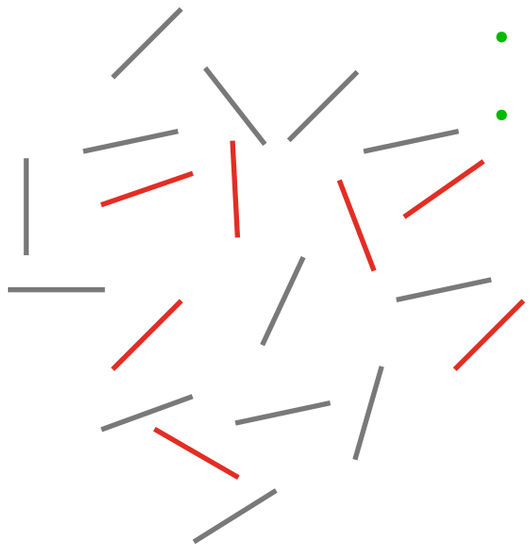




Gene A
Gene B
.
.
.
Gene X

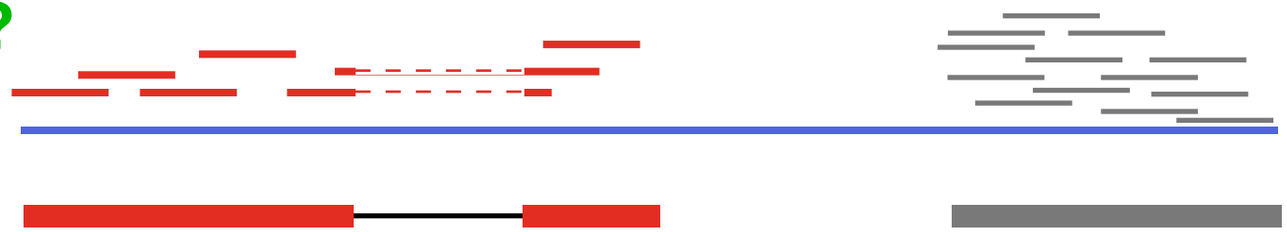
$$\begin{pmatrix} 7 & . & . & . \\ 13 & . & . & . \\ . & . & . & . \\ . & . & . & . \\ . & . & . & . \end{pmatrix}$$

The traditional workflow



- slow
- unnecessary?

ALIGNMENT

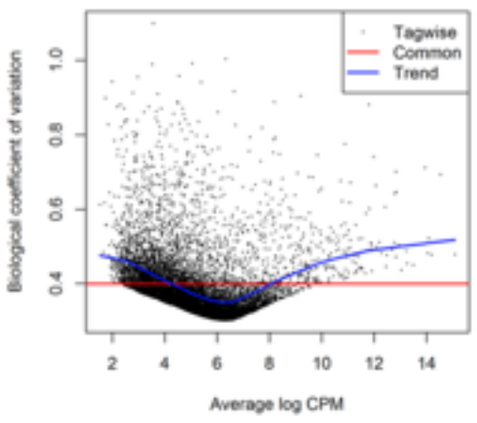


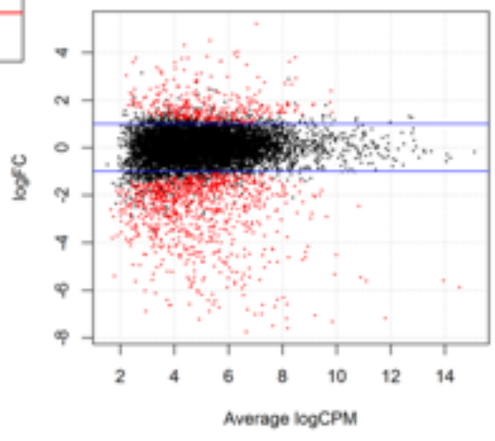
COUNTING

inaccurate?

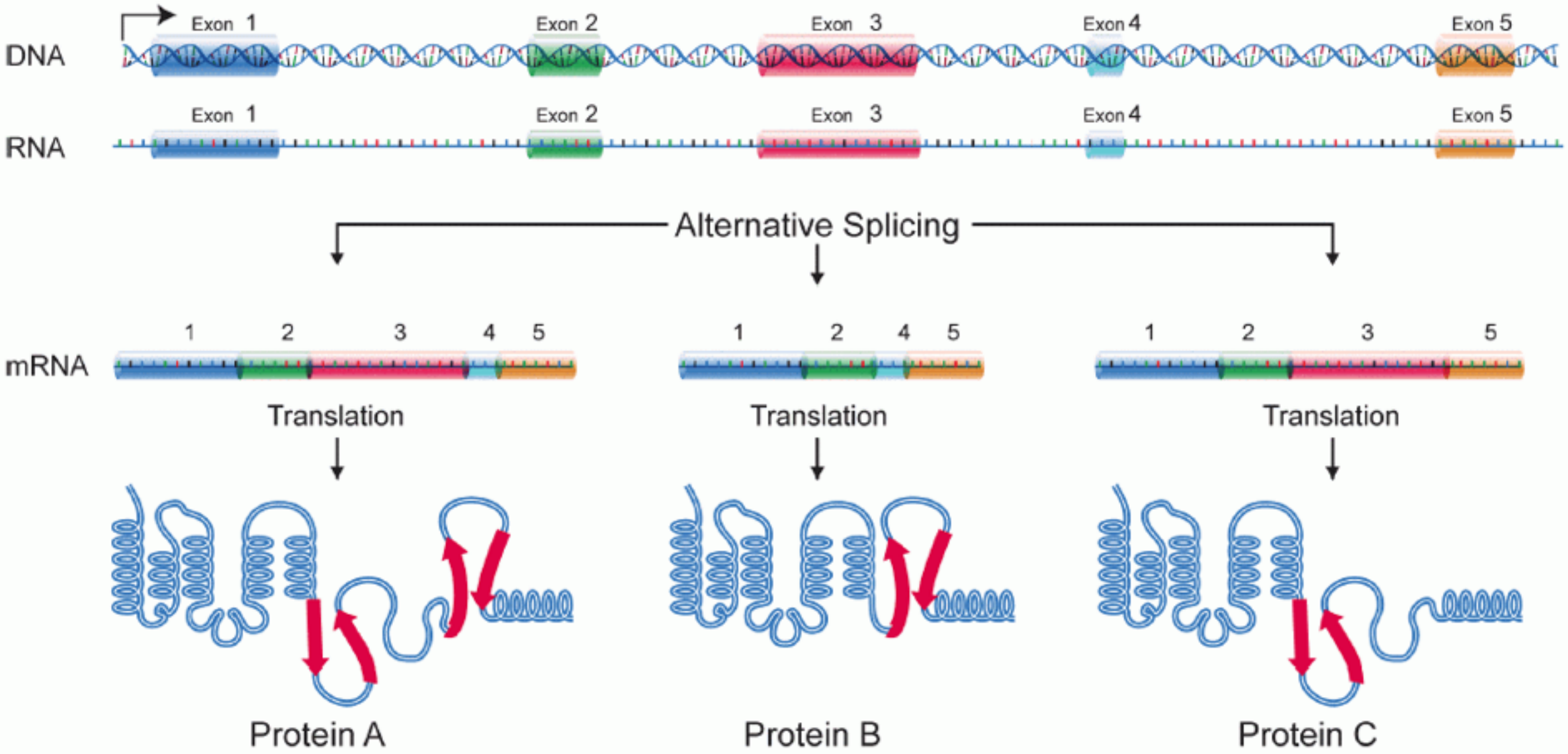
doesn't account for all uncertainty in abundance estimates

ANALYSIS



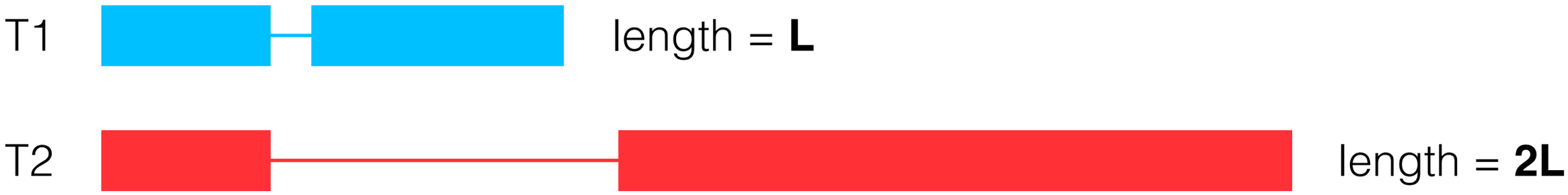


Gene A	7 . . .
Gene B	13 . . .
.	. . . .
.	. . . .
.	. . . .
Gene X	. . . .

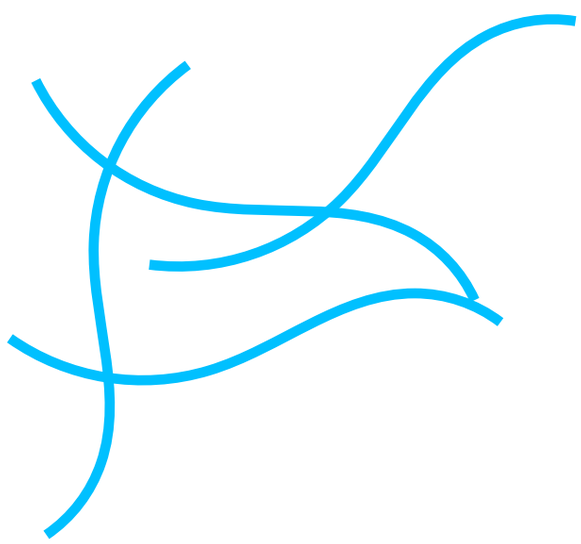

DNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
RNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Alternative Splicing
mRNA
1 2 3 4 5
1 2 4 5
1 2 3 5
Translation
Translation
Translation
Protein A
Protein B
Protein C

Wikipedia

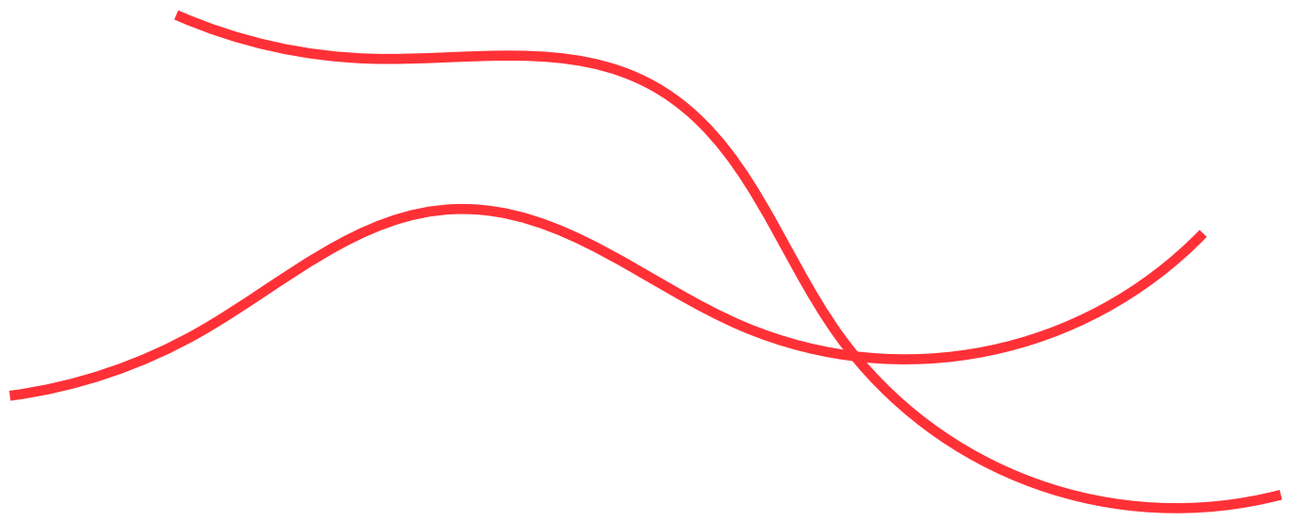
Abundance quantification



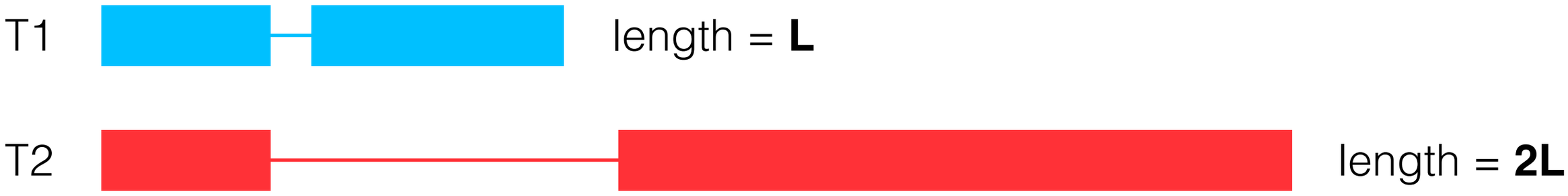
sample 1



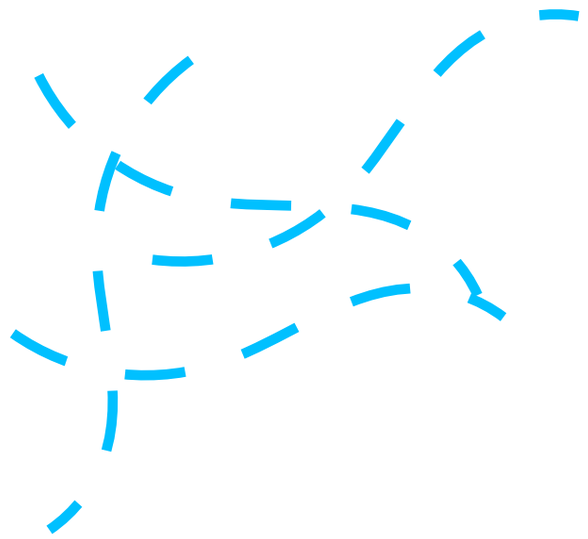
sample 2



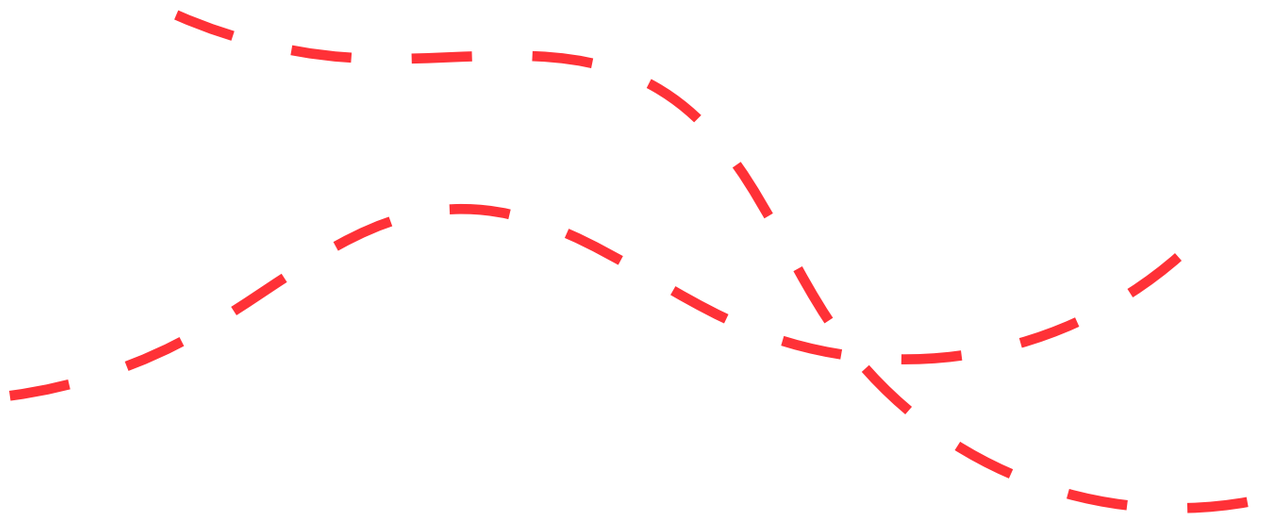
Abundance quantification



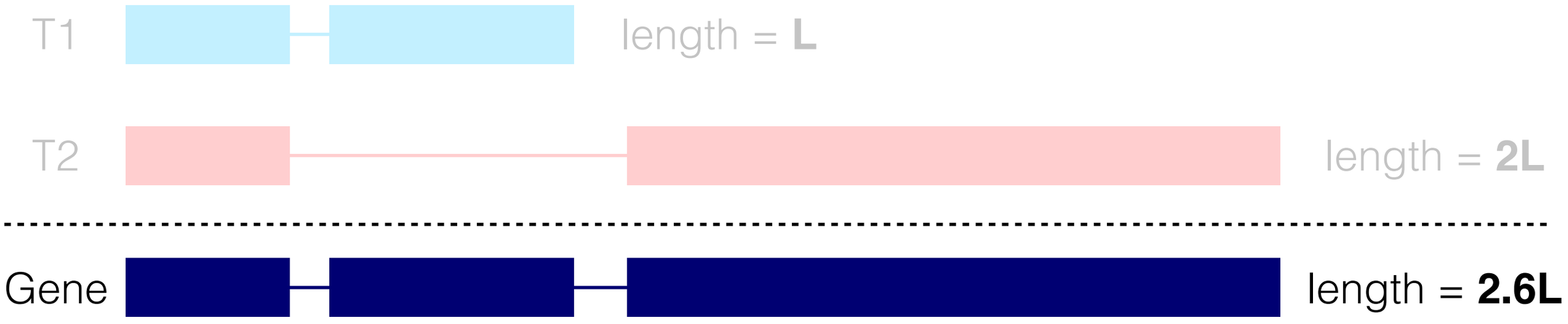
sample 1



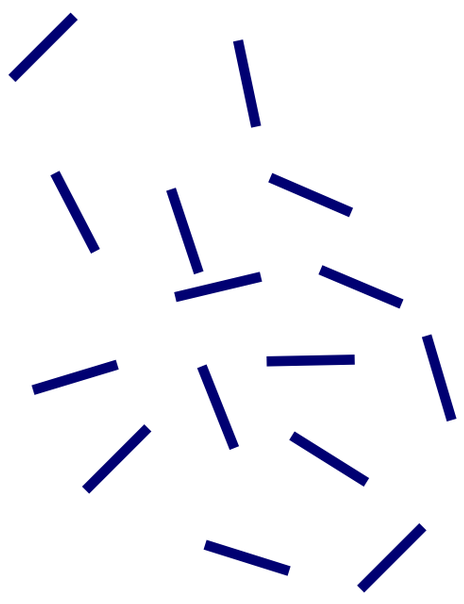
sample 2



Gene-level read counts

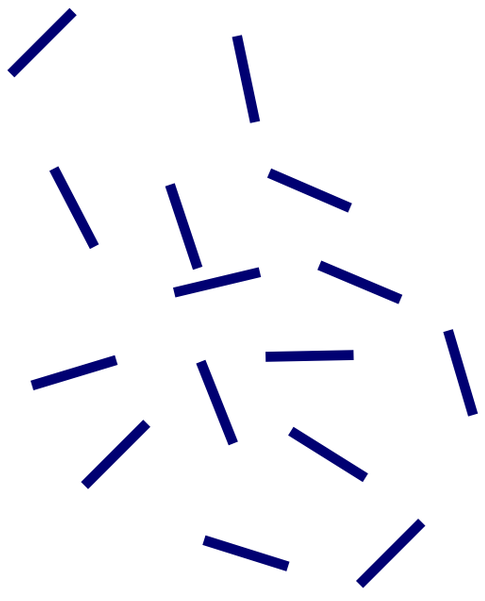


sample 1



150 reads

sample 2



150 reads

Gene	S1	S2
Count	150	150
RPK(M)	57.7	57.7

What can we do?

- Consider another abundance unit that better reflects the underlying abundances

- “Adjust” gene counts to reflect underlying isoform composition

What can we do?

How can we get such values?

Are they any good?

- Consider another abundance unit that better reflects the underlying abundances

- “Adjust” gene counts to reflect underlying isoform composition

How could such adjustment be done?

What can we do?

- Consider another
  reflects the
- "A
  con

How can we get such values?

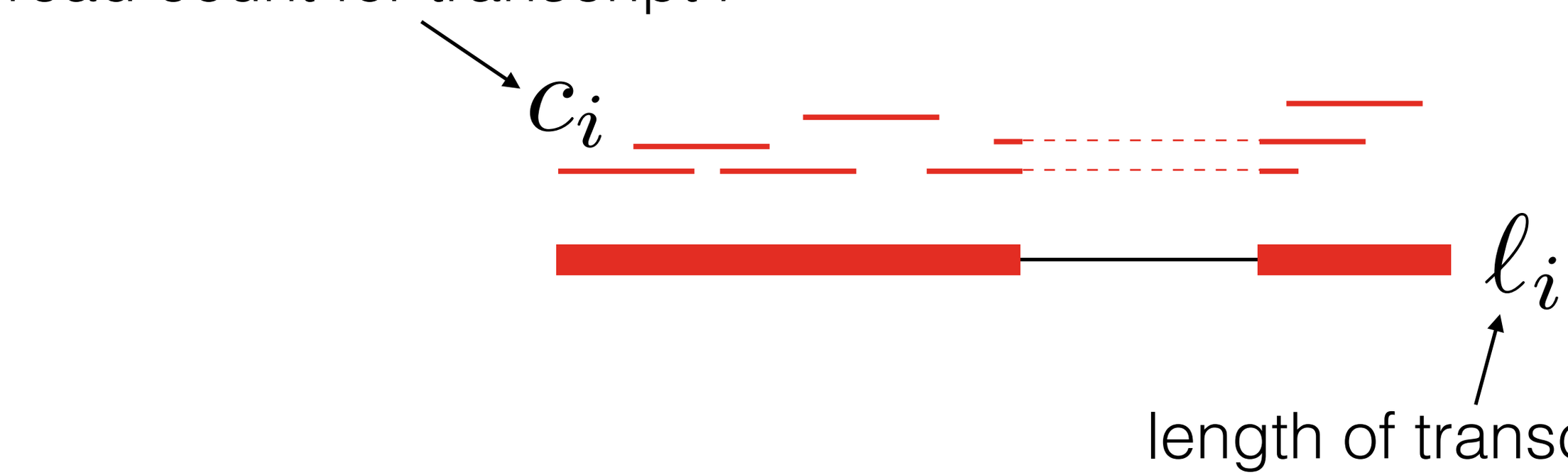
good?

We need transcript-level information!

How could such adjustment be done?

Abundance units

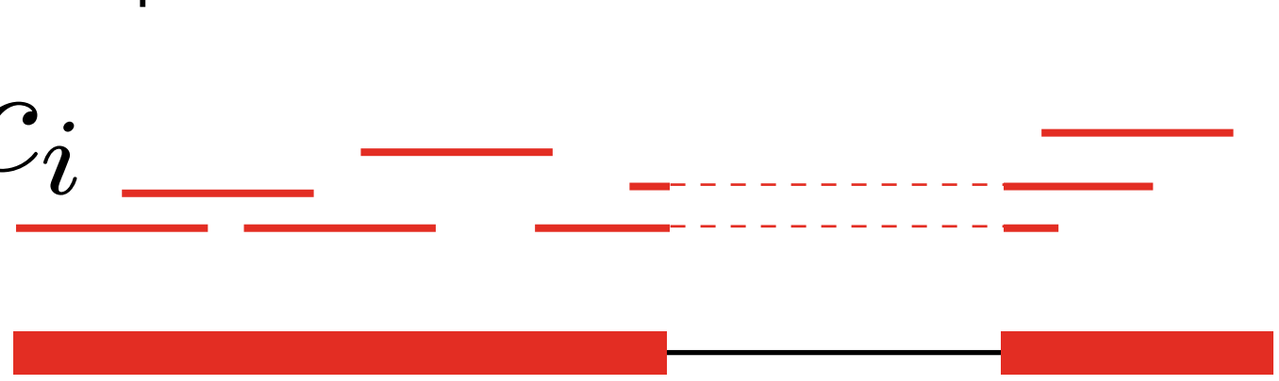
read count for transcript *i*



Abundance units

read count for transcript *i*

c_i



ℓ_i

fragment length

length of transcript *i*

$$t_i = \frac{c_i r}{\ell_i}$$

Abundance units

read count for transcript *i*

c_i

ℓ_i

fragment length

length of transcript *i*

$$t_i = \frac{c_i r}{\ell_i}$$

$$TPM_i = 10^6 \cdot \frac{t_i}{\sum_k t_k}$$

Abundance units

read count for transcript *i*

c_i

ℓ_i

length of transcript *i*

fragment length

$$t_i = \frac{c_i r}{\ell_i}$$

$$TPM_i = 10^6 \cdot \frac{t_i}{\sum_k t_k}$$

library size

$$RPKM_i = 10^9 \cdot \frac{c_i}{\ell_i \sum_k c_k} = 10^9 \cdot \frac{t_i}{\sum_k (t_k \ell_k)}$$

Abundance units

read count for transcript *i*

c_i

ℓ_i

length of transcript *i*

fragment length

$$t_i = \frac{c_i r}{\ell_i}$$

$$TPM_i \propto RPKM_i$$

$$\sum_i TPM_i = 10^6$$

$$TPM_i = 10^6 \cdot \frac{t_i}{\sum_k t_k}$$

library size

$$RPKM_i = 10^9 \cdot \frac{c_i}{\ell_i \sum_k c_k} = 10^9 \cdot \frac{t_i}{\sum_k (t_k \ell_k)}$$

Offsets (“average transcript lengths”)

- Similar to correction factors for library size, but sample-**and** gene-specific

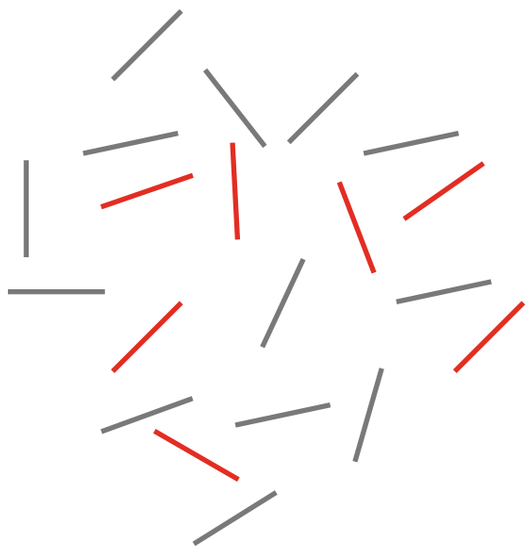
- Weighted average of transcript lengths, weighted by estimated abundances (TPMs)

- Average transcript length for gene *g* in sample *s*:

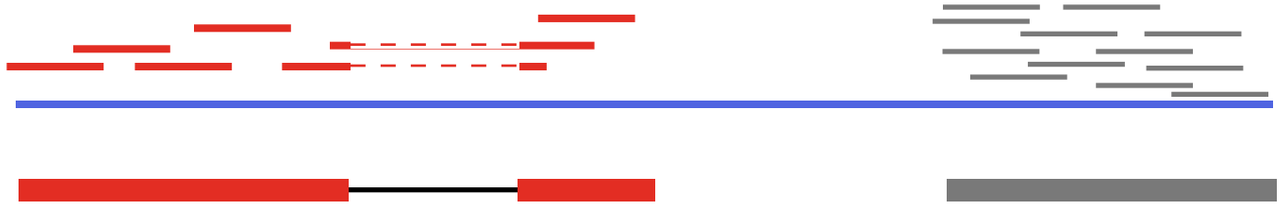
$$ATL_{gs} = \sum_{i \in g} \theta_{is} \bar{\ell}_{is}, \qquad \sum_{i \in g} \theta_{is} = 1$$

$\bar{\ell}_{is} =$ effective length of isoform i (in sample s)
$\theta_{is} =$ relative abundance of isoform i in sample s

The traditional workflow



ALIGNMENT

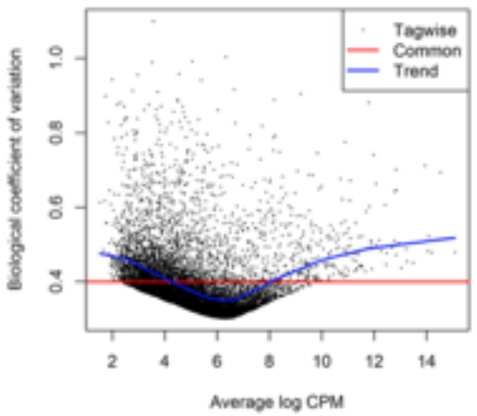


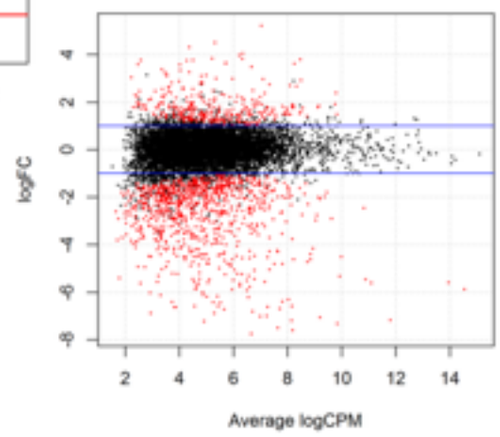
COUNTING

Gene A
Gene B
.
.
.
Gene X

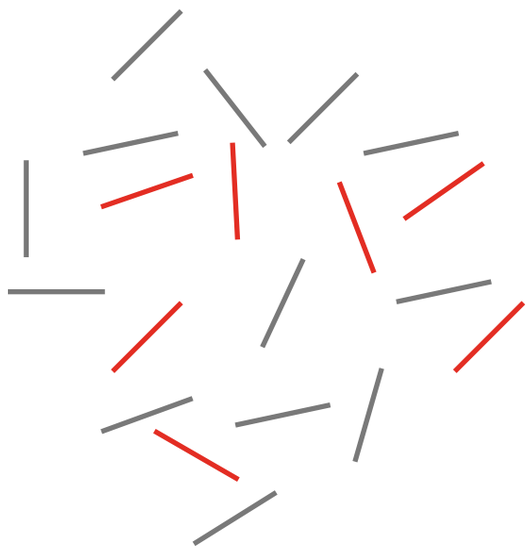
$$\begin{pmatrix} 7 & . & . & . \\ 13 & . & . & . \\ . & . & . & . \\ . & . & . & . \\ . & . & . & . \end{pmatrix}$$

ANALYSIS

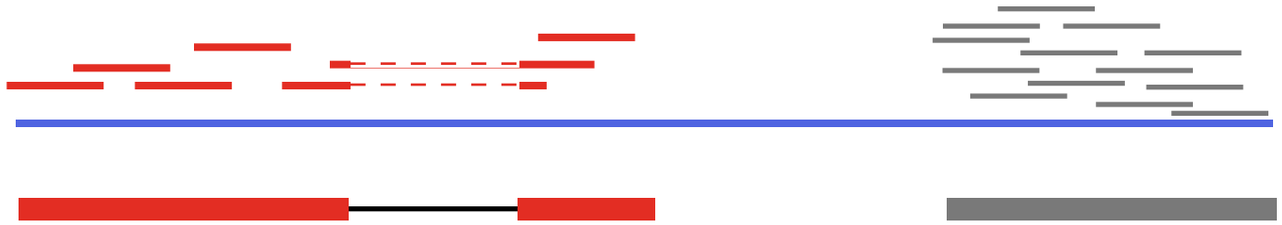




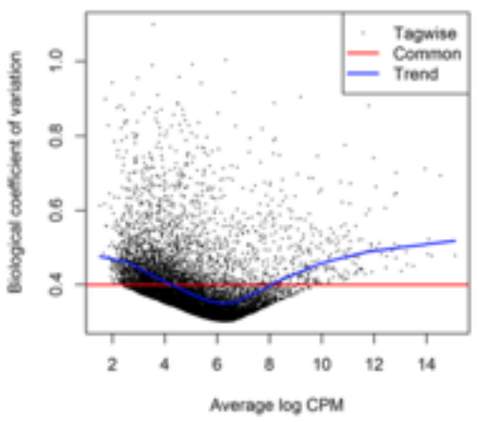
The “modern” workflow

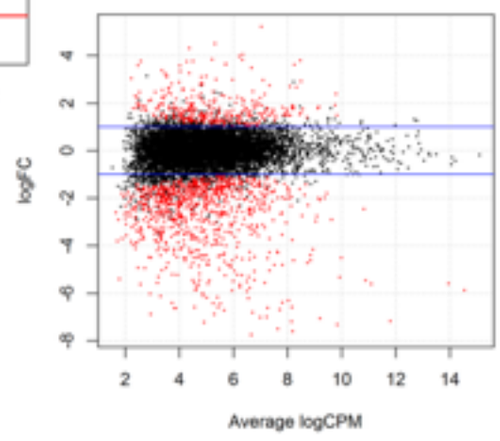


“MAPPING”



ESTIMATION





ANALYSIS

Gene A
Gene B
.
.
.
Gene X

$$\begin{pmatrix} 7 & . & . & . \\ 13 & . & . & . \\ . & . & . & . \\ . & . & . & . \\ . & . & . & . \end{pmatrix}$$

The “mapping” step

- Does not provide “full” alignment information (i.e., no exact base-by-base alignment).

- Rather, finds all transcripts (and positions) that a read is compatible with.

- Comes in various flavors:
  - pseudoalignment (kallisto)
  - lightweight alignment (Salmon)
  - quasimapping (Sailfish, RapMap)

Bray et al. 2016; Patro et al. 2014; Patro et al. 2015; Srivastava et al. 2016

The “estimation” step

- Input: for each read, the “equivalence class” of compatible transcripts
- Probabilistic modeling of read generation process, with transcript abundance as parameter
- EM algorithm
- Output: estimated abundance of each transcript

Step 1: build transcriptome index

kallisto

name of index

```
vpn-89-206-119-17:~ charlotte$ kallisto index -i transcriptome_index
.idx transcripts.fasta
```

transcriptome fasta file

Salmon

transcriptome fasta file

```
vpn-89-206-119-17:~ charlotte$ salmon index -t transcripts.fasta -i
transcriptome_index.idx -p 10
```

name of index

number of cores

Where to find transcript fasta?

www.ensembl.org/info/data/ftp/index.html

Single species data

Popular species are listed first. You can customise this list via our home page.

Show 10 entries Show/hide columns

★	Species	DNA (FASTA)	cDNA (FASTA)	CDS (FASTA)	ncRNA (FASTA)	Protein sequence (FASTA)	Annotated sequence (EMBL)	Annotated sequence (GenBank)	Gene sets	Whole databases	Varia (GV
Y	**Human** *Homo sapiens*	FASTA	FASTA	FASTA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G
Y	**Mouse** *Mus musculus*	FASTA	FASTA	FASTA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G
Y	**Zebrafish** *Danio rerio*	FASTA	FASTA	FASTA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G

Where to find transcript fasta?

www.ensembl.org/info/data/ftp/index.html

reference files for alignment-based workflow

Single species data

Popular species are listed first. You can customise this list via our home page.

Show 10 entries | Show/hide columns

★	Species	DNA (FASTA)	cDNA (FASTA)	CDS (FASTA)	ncRNA (FASTA)	Protein sequence (FASTA)	Annotated sequence (EMBL)	Annotated sequence (GenBank)	Gene sets	Whole databases	Varia (GV
Y	**Human** *Homo sapiens*	FASTA	FASTA	FASTA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G
Y	**Mouse** *Mus musculus*	FASTA	FASTA	FASTA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G
Y	**Zebrafish** *Danio rerio*	FASTA	FASTA	FASTA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G

Step 2: quantify

kallisto

name of index

```
vpn-89-206-119-17:~ charlotte$ kallisto quant -i transcriptome_index
.idx -o results/sample1 -b 30 -t 10 sample1_1.fastq sample1_2.fastq
```

output folder

bootstraps

number of cores

input fastq files

Salmon

name of index

libtype

```
vpn-89-206-119-17:~ charlotte$ salmon quant -i transcriptome_index.idx -l IU
-p 10 -1 sample1_1.fq -2 sample1_2.fq -o results/sample1 --numBootstraps 30
```

number of cores

input fastq files

output folder

bootstraps

Salmon LIBTYPE argument

http://salmon.readthedocs.io/en/latest/salmon.html#what-s-this-libtype

What's this `LIBTYPE`?

Salmon, like sailfish, has the user provide a description of the type of sequencing library from which the reads come, and this contains information about e.g. the relative orientation of paired end reads. However, we've replaced the somewhat esoteric description of the library type with a simple set of strings; each of which represents a different type of read library. This new method of specifying the type of read library is being back-ported into Sailfish and will be available in the next release.

The library type string consists of three parts: the relative orientation of the reads, the strandedness of the library, and the directionality of the reads.

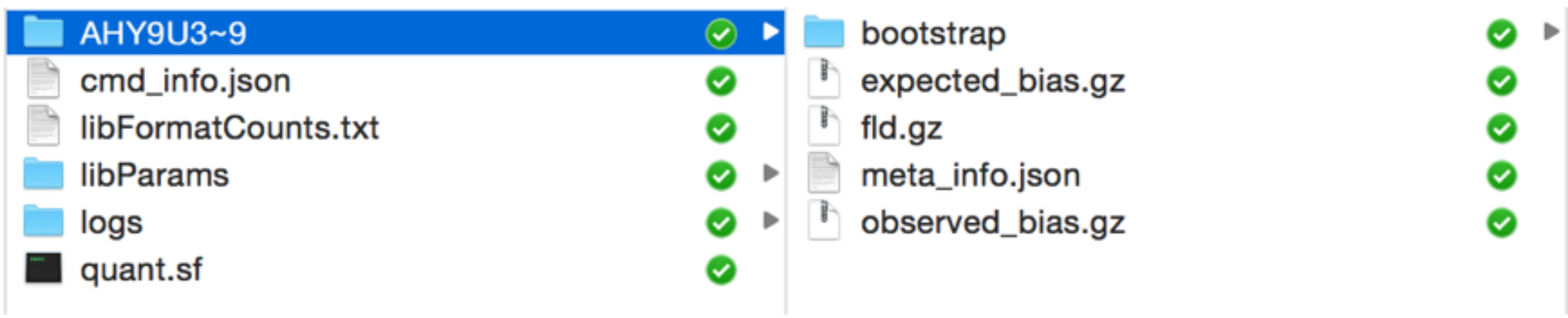
The first part of the library string (relative orientation) is only provided if the library is paired-end. The possible options are:

```
I = inward
O = outward
M = matching
```

output

kallisto

Salmon



output

kallisto

[abundance.tsv]

target_id	length	eff_length	est_counts	tpm
ENST00000406070	2025	1874.91	0	0
ENST00000446844	2227	2076.91	3.37465	0.129755
ENST00000599620	686	535.97	0	0
ENST00000471557	505	355.404	2.84168	0.638509
ENST00000338761	1456	1305.91	1.3122e-05	8.02414e-07
ENST00000417509	1444	1293.91	5.15988	0.318455
ENST00000484946	610	460.029	17.4159	3.02326
ENST00000490656	660	509.97	7.51996	1.17756
ENST00000439537	1161	1010.91	14.432	1.14006
ENST00000493251	641	491.006	2.63203	0.428073
ENST00000460127	408	259.526	0	0

Salmon

[quant.sf]

Name	Length	EffectiveLength	TPM	NumReads
ENST00000406070	2025	1869.81	0	0
ENST00000446844	2227	2071.81	0.137334	3.71695
ENST00000599620	686	530.936	0	0
ENST00000471557	505	350.256	0.731211	3.3457
ENST00000338761	1456	1300.81	0	0
ENST00000417509	1444	1288.81	7.58582e-08	1.27717e-06
ENST00000484946	610	455.039	2.87905	17.1142
ENST00000490656	660	504.969	1.46703	9.67744
ENST00000439537	1161	1005.81	1.47611	19.3952
ENST00000493251	641	485.994	0.597774	3.79512
ENST00000460127	408	253.708	0	0

Comparison to traditional workflow

Salmon/kallisto…

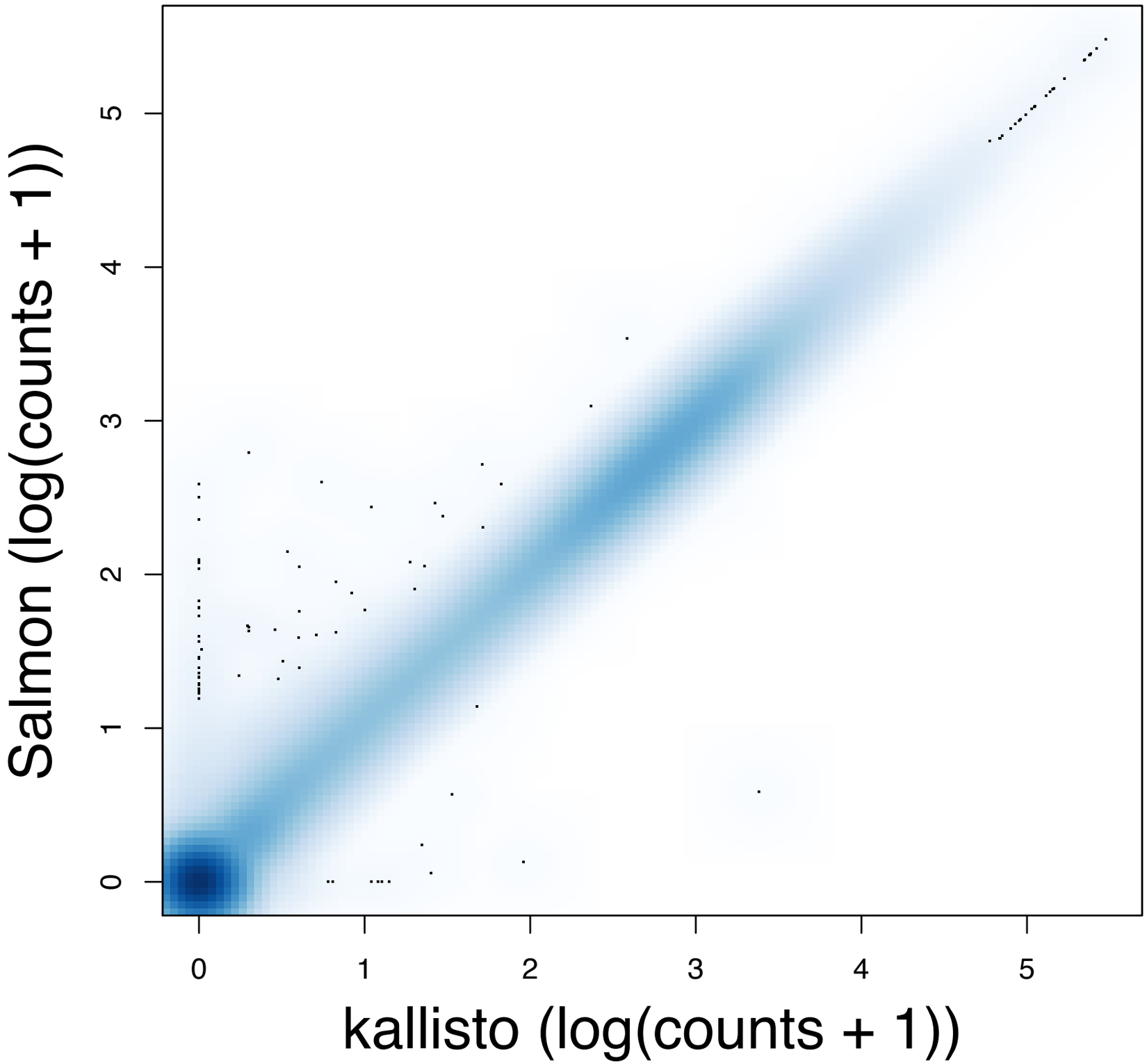
- … are considerably faster than traditional alignment +counting -> allow bootstrapping

- … provide more highly resolved estimates (transcripts rather than gene) - can be aggregated to gene level

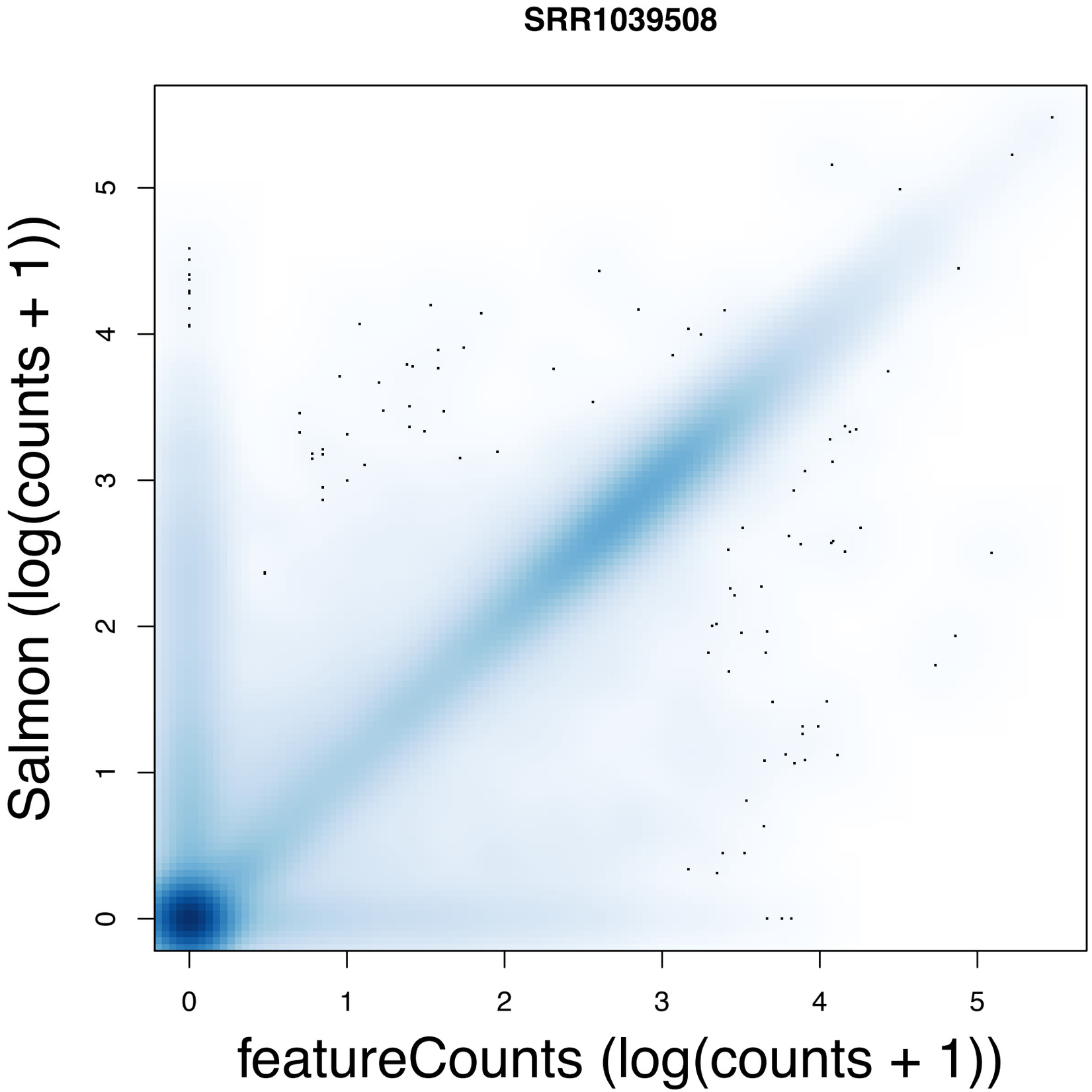
- … can use a larger fraction of the reads

- … don’t give precise alignments (for e.g. visualization in genome browser) - but avoid large alignment files

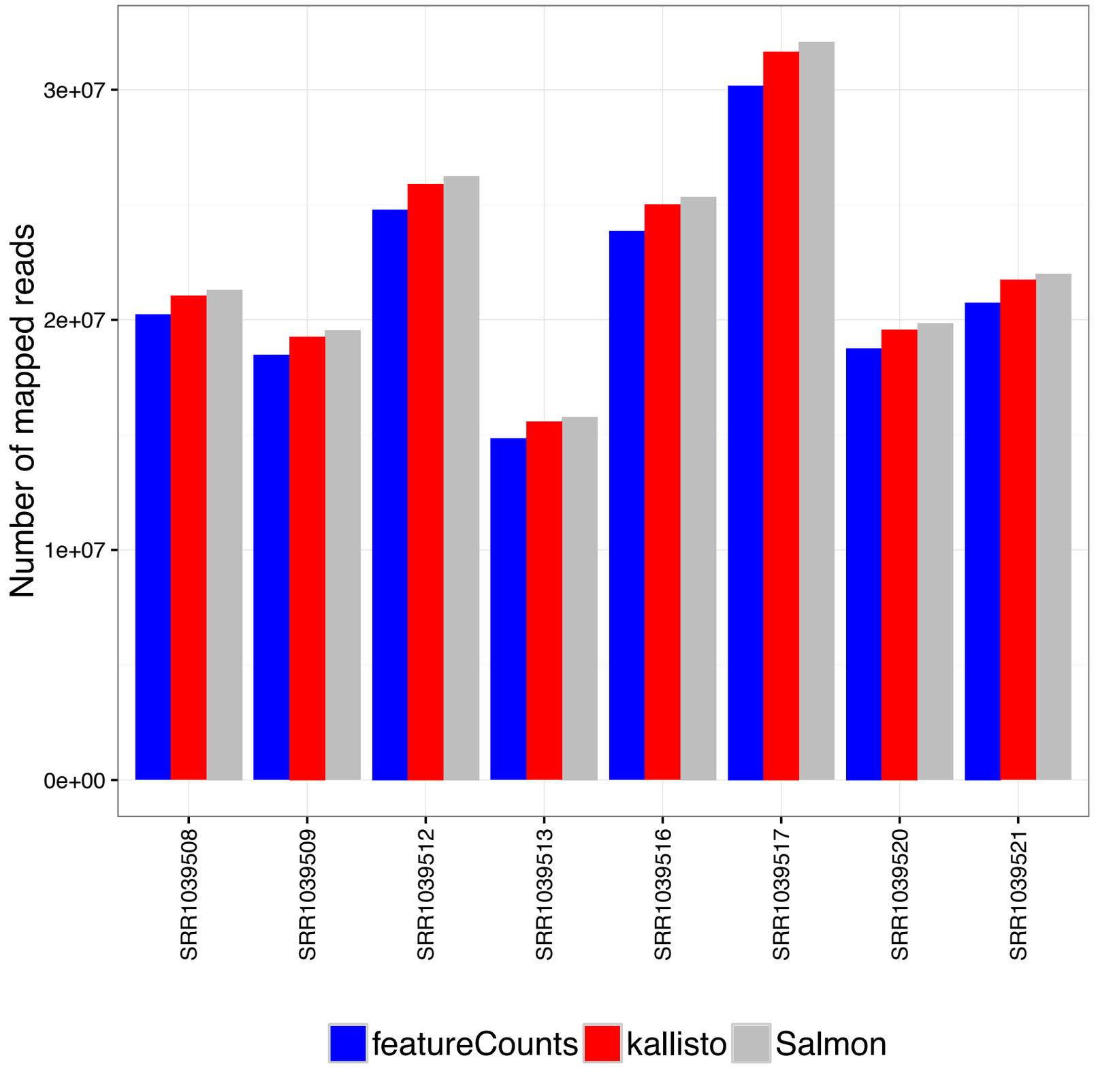
kallisto and Salmon gene counts overall similar



Gene-level counts mostly similar to traditional approach



kallisto and Salmon can use slightly more reads



How to get the estimated values into R?

```
> library(tximport)
> salmon_files
                    SRR1039508                     SRR1039509
"salmon/SRR1039508/quant.sf" "salmon/SRR1039509/quant.sf"
                    SRR1039512                     SRR1039513
"salmon/SRR1039512/quant.sf" "salmon/SRR1039513/quant.sf"
                    SRR1039516                     SRR1039517
"salmon/SRR1039516/quant.sf" "salmon/SRR1039517/quant.sf"
                    SRR1039520                     SRR1039521
"salmon/SRR1039520/quant.sf" "salmon/SRR1039521/quant.sf"
> head(tx2gene)
                tx            gene
1 ENST00000415118 ENSG00000223997
2 ENST00000434970 ENSG00000237235
3 ENST00000448914 ENSG00000228985
4 ENST00000604642 ENSG00000270961
5 ENST00000603326 ENSG00000271317
6 ENST00000604950 ENSG00000270783
> txi <- tximport(files = salmon_files, type = "salmon", tx2gene = tx2gene)
reading in files
1 2 3 4 5 6 7 8
summarizing abundance
summarizing counts
summarizing length
> names(txi)
[1] "abundance"           "counts"              "length"
[4] "countsFromAbundance"
```

How to get the estimated values into R?

```
> head(txi$abundance, n = 3)
                 SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003   26.95182   19.62924   28.33082   23.24692   36.71688
ENSG00000000005    0.00000    0.00000    0.00000    0.00000    0.00000
ENSG00000000419   38.51888   46.10853   42.34674   43.38094   40.21257
                 SRR1039517 SRR1039520 SRR1039521
ENSG00000000003   29.09426   34.83193   24.20944
ENSG00000000005    0.00000    0.00000    0.00000
ENSG00000000419   45.72329   39.29645   44.80912
> head(txi$counts, n = 3)
                 SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003   698.4915   463.0251   895.6865   420.4502  1154.6804
ENSG00000000005     0.0000     0.0000     0.0000     0.0000     0.0000
ENSG00000000419   465.9998   515.5963   625.0002   365.6836   590.0994
                 SRR1039517 SRR1039520 SRR1039521
ENSG00000000003  1078.464   780.3976   589.2203
ENSG00000000005     0.000     0.0000     0.0000
ENSG00000000419   797.987   419.6755   510.9196
> head(txi$length, n = 3)
                 SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516
ENSG00000000003  1983.8737  1947.5904  1978.7880  1993.6675  1963.7941
ENSG00000000005   783.3978   783.3978   783.3978   783.3978   783.3978
ENSG00000000419   926.0907   923.2618   923.7694   929.2005   916.3488
                 SRR1039517 SRR1039520 SRR1039521
ENSG00000000003  1967.1231  1951.0682  1986.9260
ENSG00000000005   783.3978   783.3978   783.3978
ENSG00000000419   926.1689   930.0241   930.8409
```

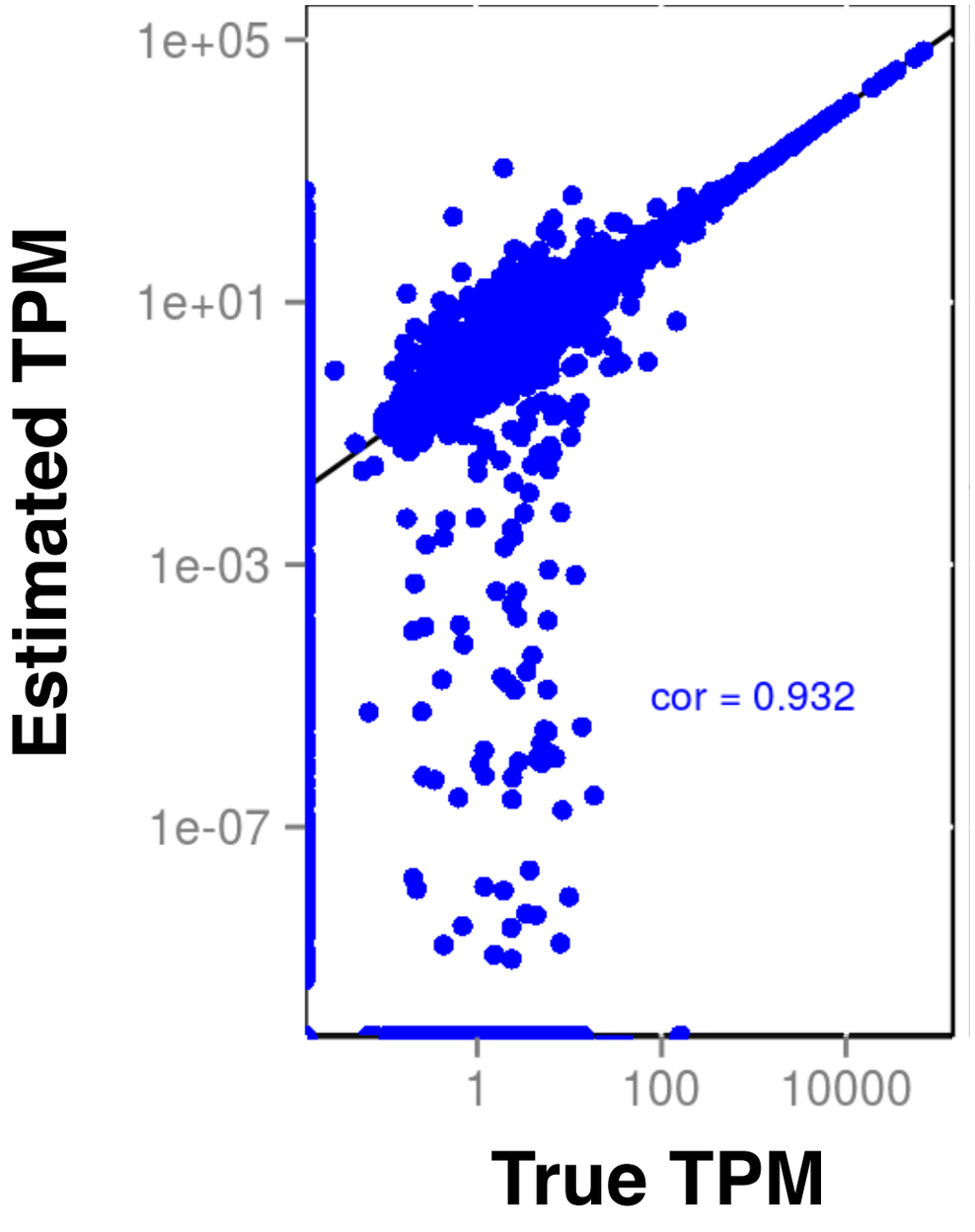
TPMs

counts

“ATL” offsets

A word of warning

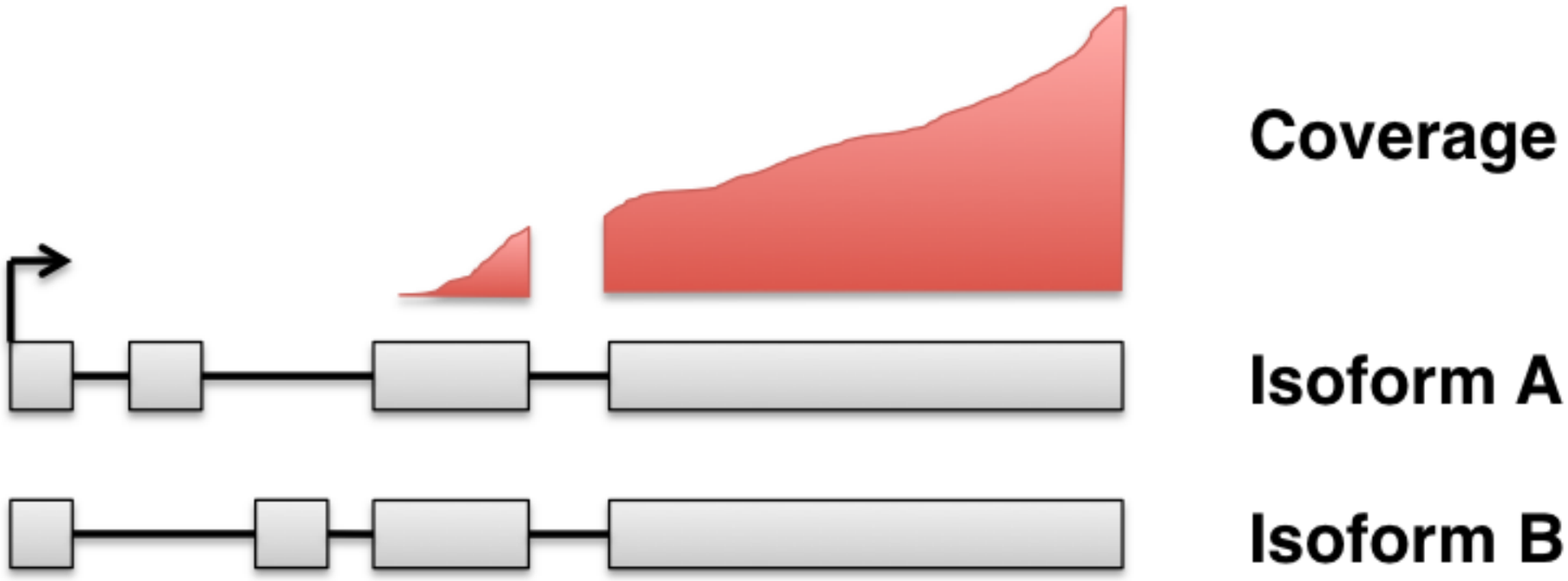
- Abundance estimates for lowly expressed transcripts are highly variable and should be interpreted with caution



Soneson, Love & Robinson, F1000 Research 2016

A word of warning

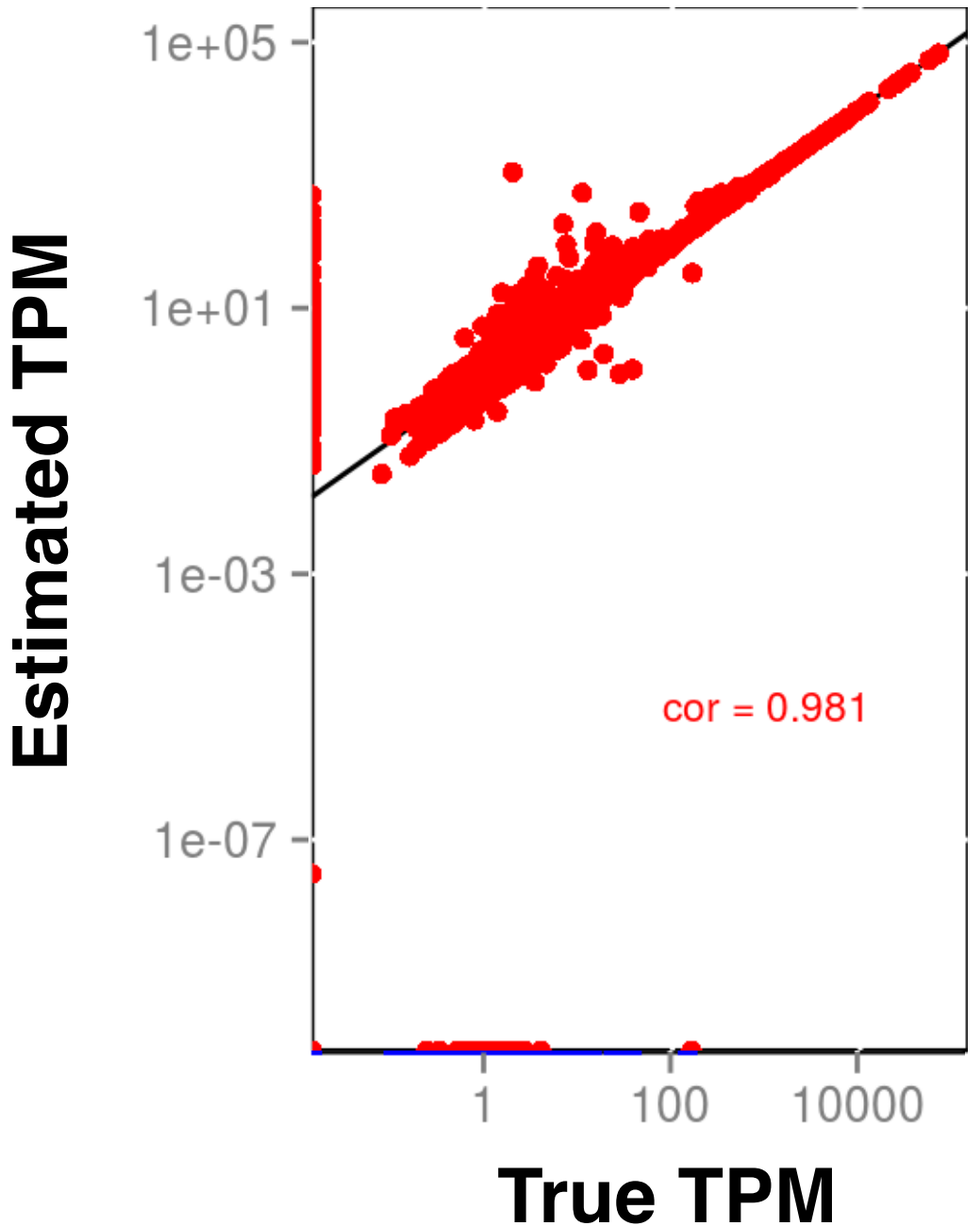
- Problematic when coverage of region defining an isoform is low



Soneson, Love & Robinson, F1000 Research 2016

A word of warning

- When aggregated to the gene level, abundance estimates are less variable



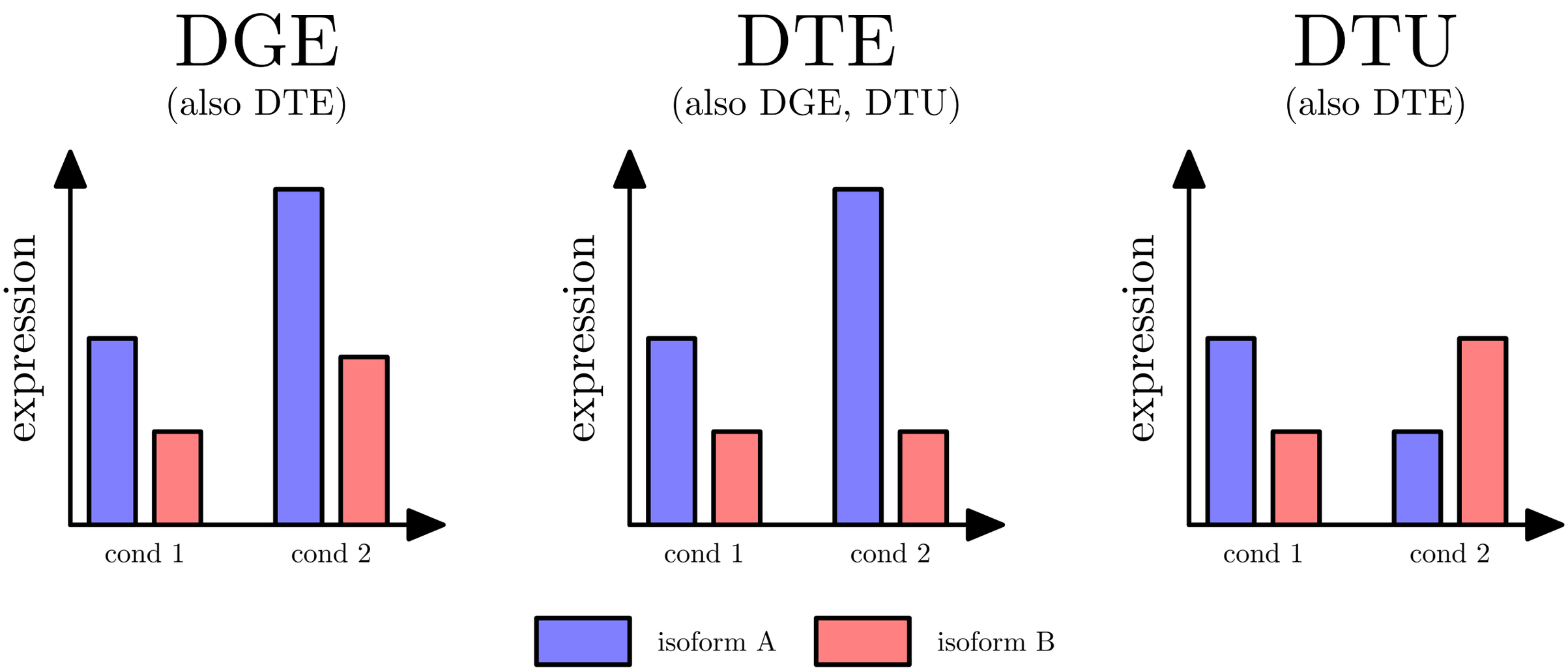
Soneson, Love & Robinson, F1000 Research 2016

Differential analysis types for RNA-seq

- Has the total output of a gene changed? **DGE**
- Has the expression of individual transcripts changed? **DTE**
- Has *any* isoform of a given gene changed? **DTE+G**
- Has the isoform composition for a given gene changed? **DTU/DEU**

- need **different** abundance quantification of transcriptomic features (genes, transcripts, exons)

Differential analysis types for RNA-seq



References

- Srivastava et al.: RapMap: a rapid, sensitive and accurate tool for mapping RNA-seq read to transcriptomes. Bioinformatics 32:i192-i200 (2016) - **RapMap**
- Patro et al.: Accurate, fast, and model-aware transcript expression quantification with Salmon. bioRxiv http://dx.doi.org/10.1101/021592 (2015) - **Salmon**
- Bray et al.: Near-optimal probabilistic RNA-seq quantification. Nature Biotechnology 34(5):525-527 (2016) - **kallisto**
- Patro et al.: Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms. Nature Biotechnology 32:462-464 (2014) - **Sailfish**
- Pimentel et al.: Differential analysis of RNA-Seq incorporating quantification uncertainty. bioRxiv http://dx.doi.org/10.1101/058164 (2016) - **sleuth**
- Wagner et al.: Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. Theory in Biosciences 131:281-285 (2012) - **TPM vs FPKM**
- Soneson et al.: Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Research 4:1521 (2016) - **ATL offsets (tximport package)**
- Li et al.: RNA-seq gene expression estimation with read mapping uncertainty. Bioinformatics 26(4):493-500 (2010) - **TPM, RSEM**