Alignment-free RNA-seq workflow

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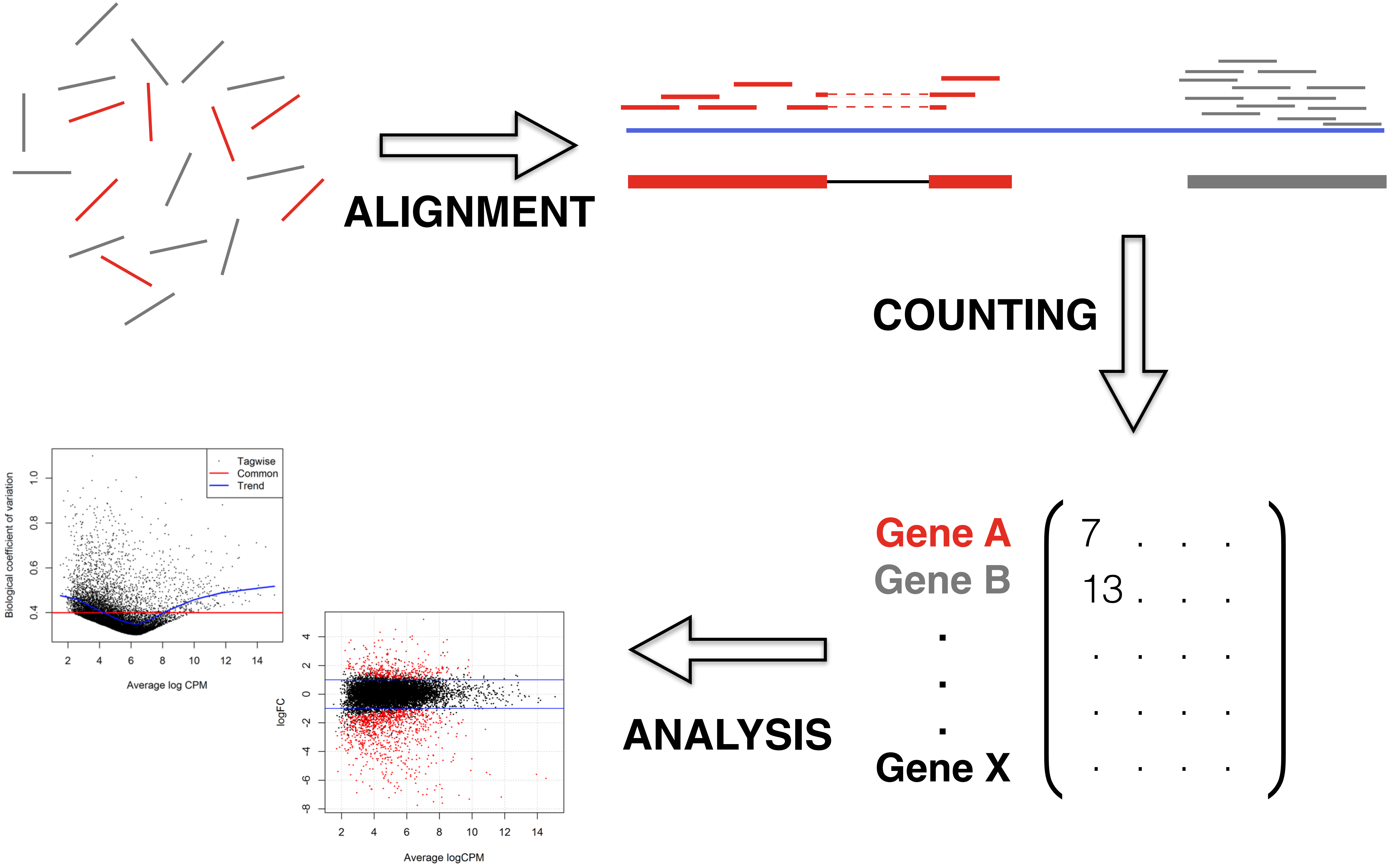
University of Zurich

Brixen 2017

The alignment-based workflow



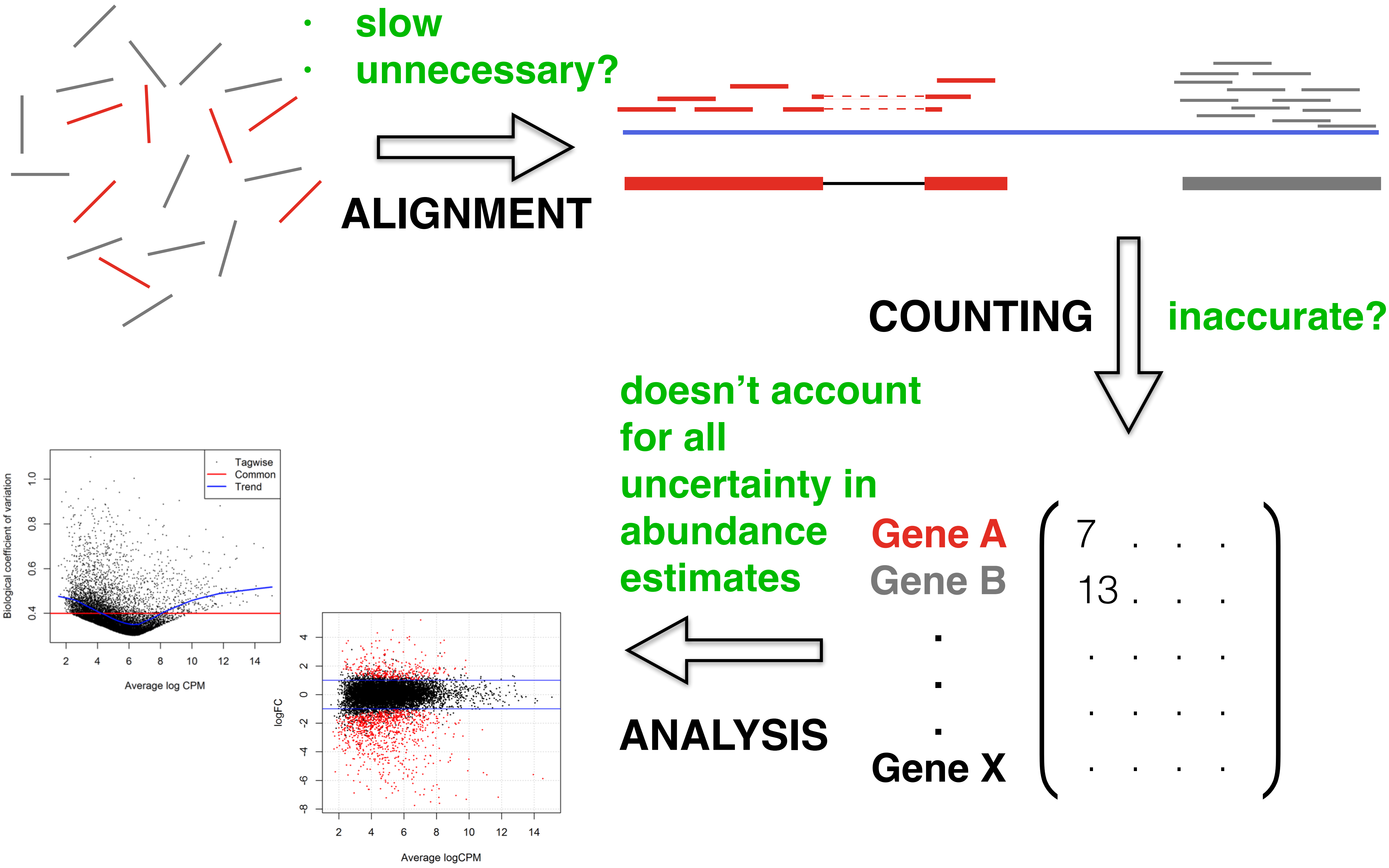
ALIGNMENT

COUNTING

$$\begin{array}{l} \text{Gene A} \\ \text{Gene B} \\ \vdots \\ \\ \text{Gene X} \end{array} \left(\begin{array}{cccc} 7 & . & . & . \\ 13 & . & . & . \\ . & . & . & . \\ . & . & . & . \\ . & . & . & . \end{array} \right)$$

ANALYSIS

The alignment-based workflow



- slow
- unnecessary?

ALIGNMENT

COUNTING

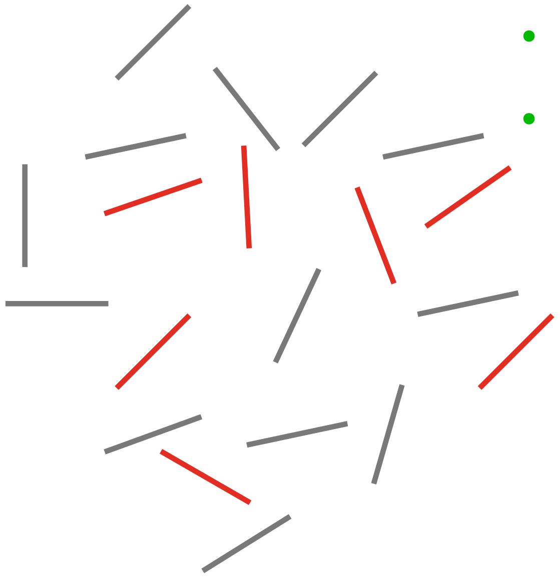
inaccurate?

doesn’t account for all uncertainty in abundance estimates

ANALYSIS

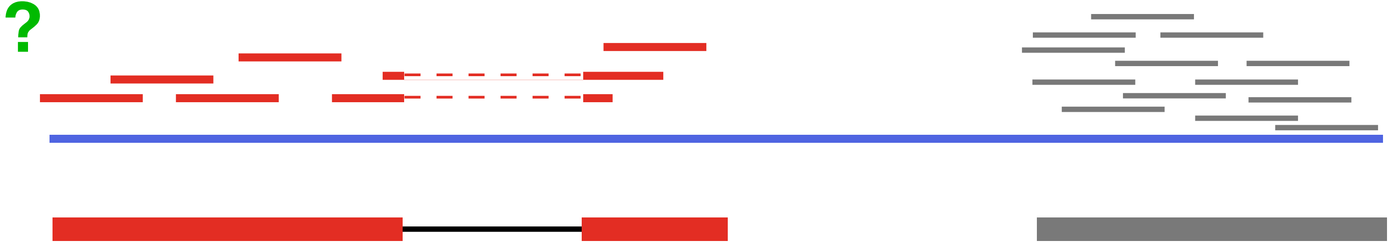
Gene A 7 . . .
Gene B 13 . . .
. . . . .
. . . . .
. . . . .
Gene X . . . .

The alignment-based workflow



- slow
- unnecessary?

ALIGNMENT



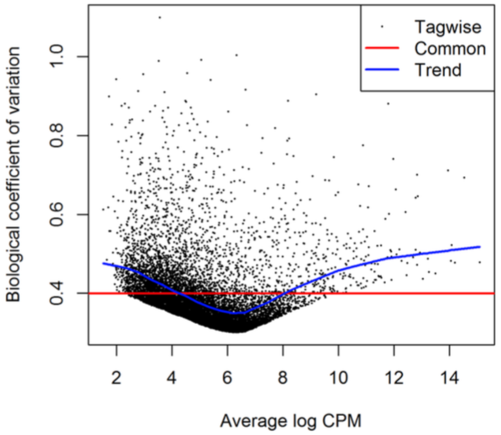
COUNTING

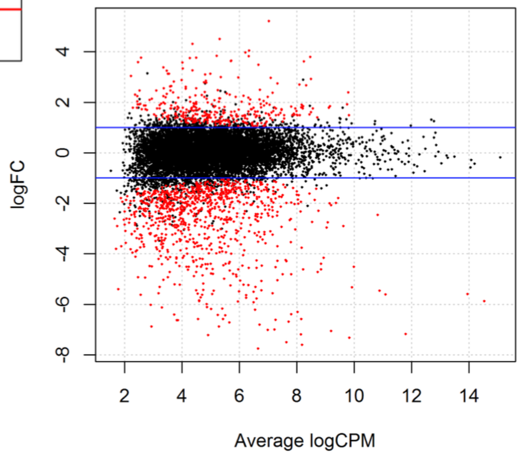
inaccurate?

doesn't account for all uncertainty in abundance estimates

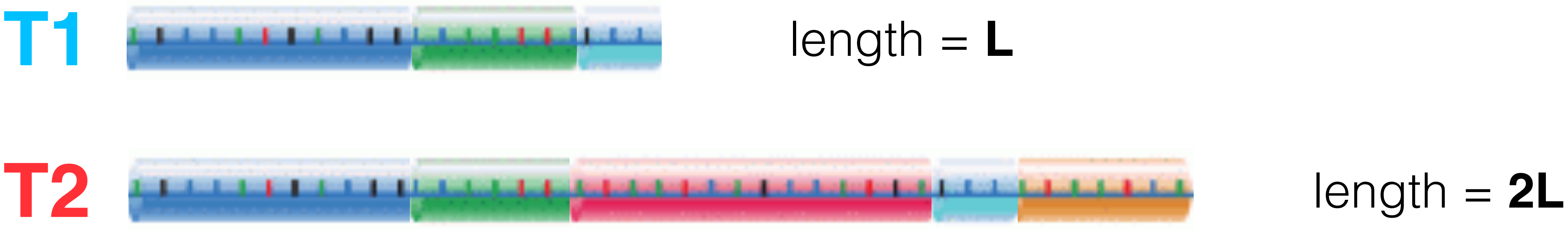
$$\begin{array}{lcccc} \text{Gene A} & 7 & . & . & . \\ \text{Gene B} & 13 & . & . & . \\ \vdots & . & . & . & . \\ & . & . & . & . \\ \text{Gene X} & . & . & . & . \end{array}$$

ANALYSIS





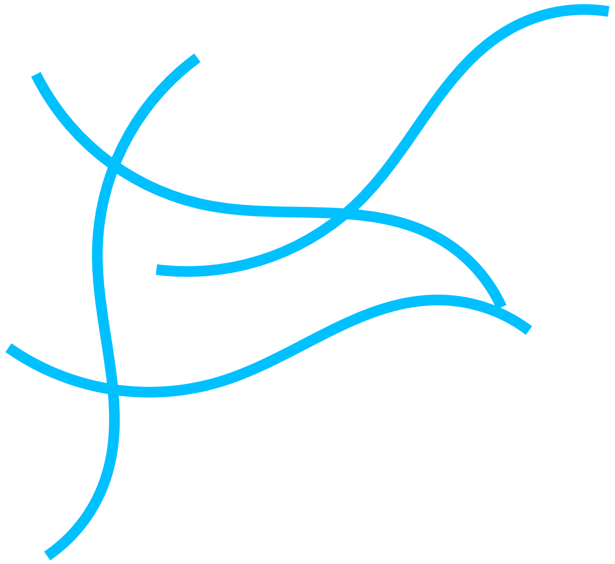
Impact of differential isoform usage on gene-level counts



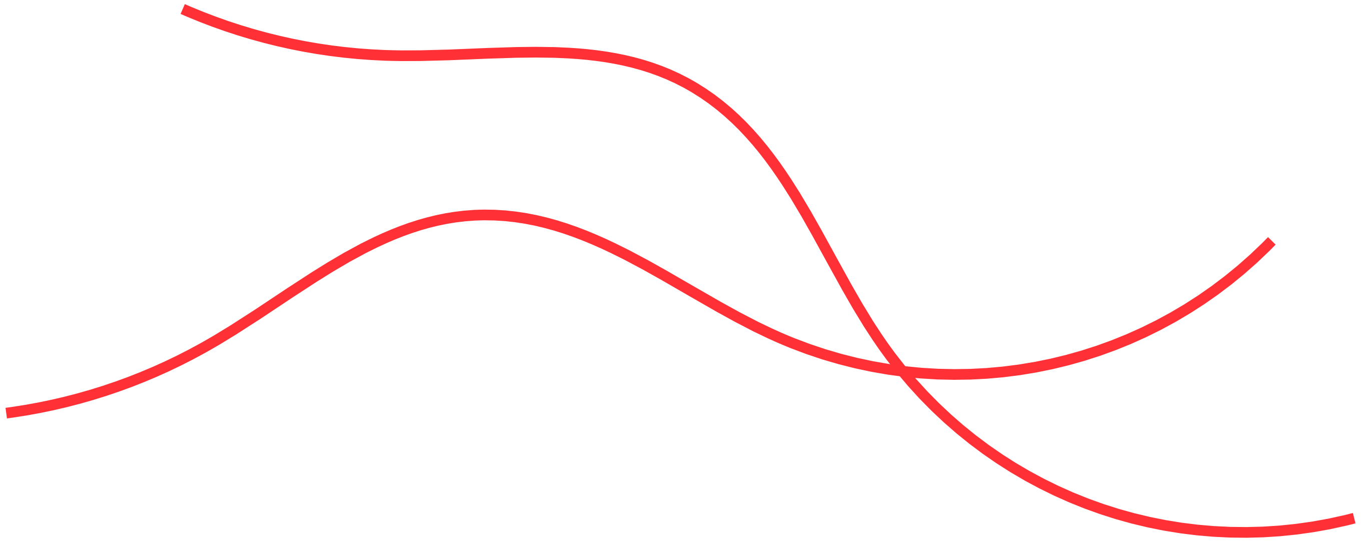
T1 length = **L**

T2 length = **2L**

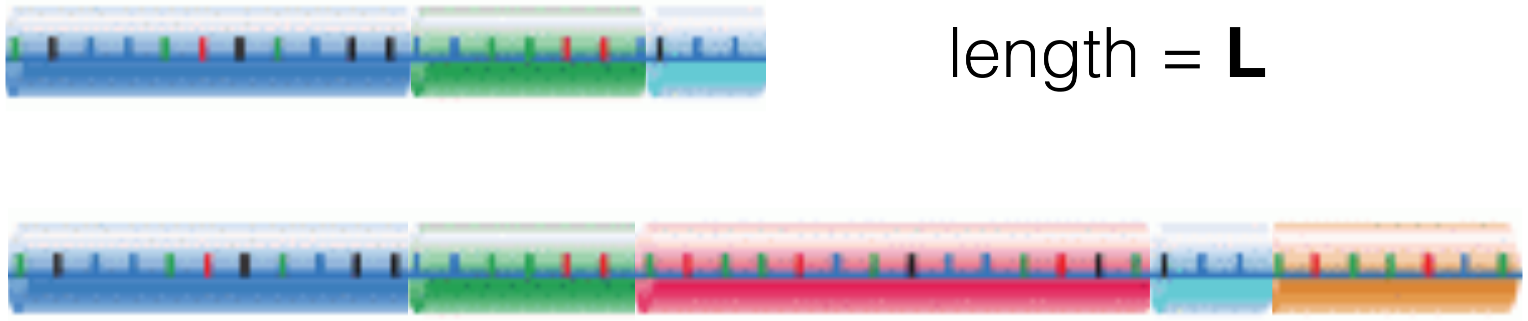
sample 1



sample 2



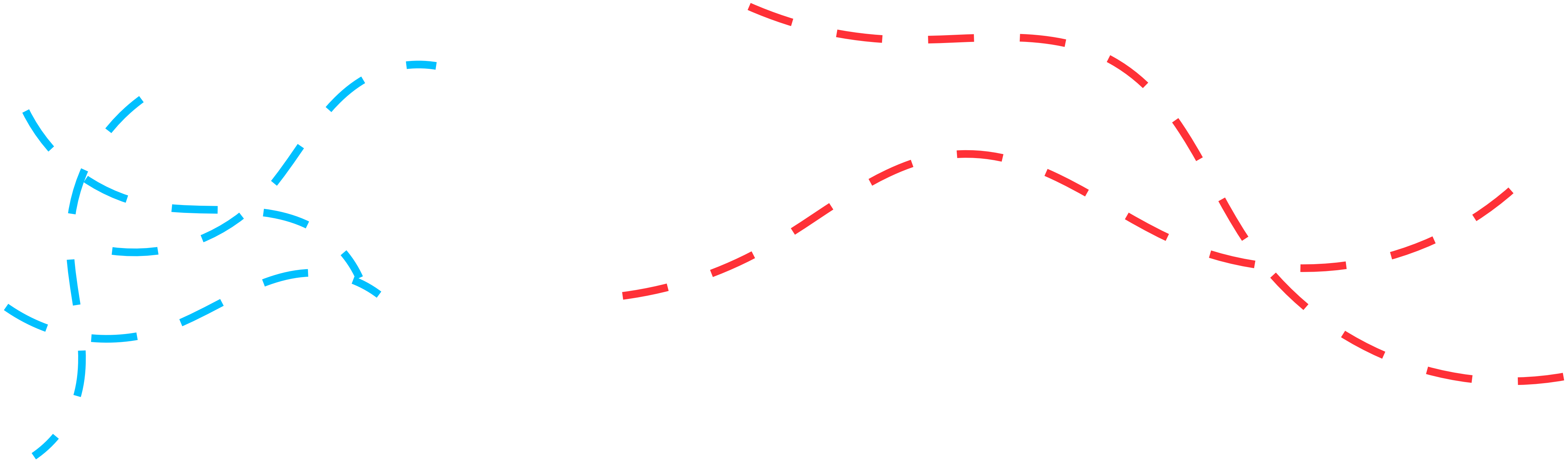
Impact of differential isoform usage on gene-level counts



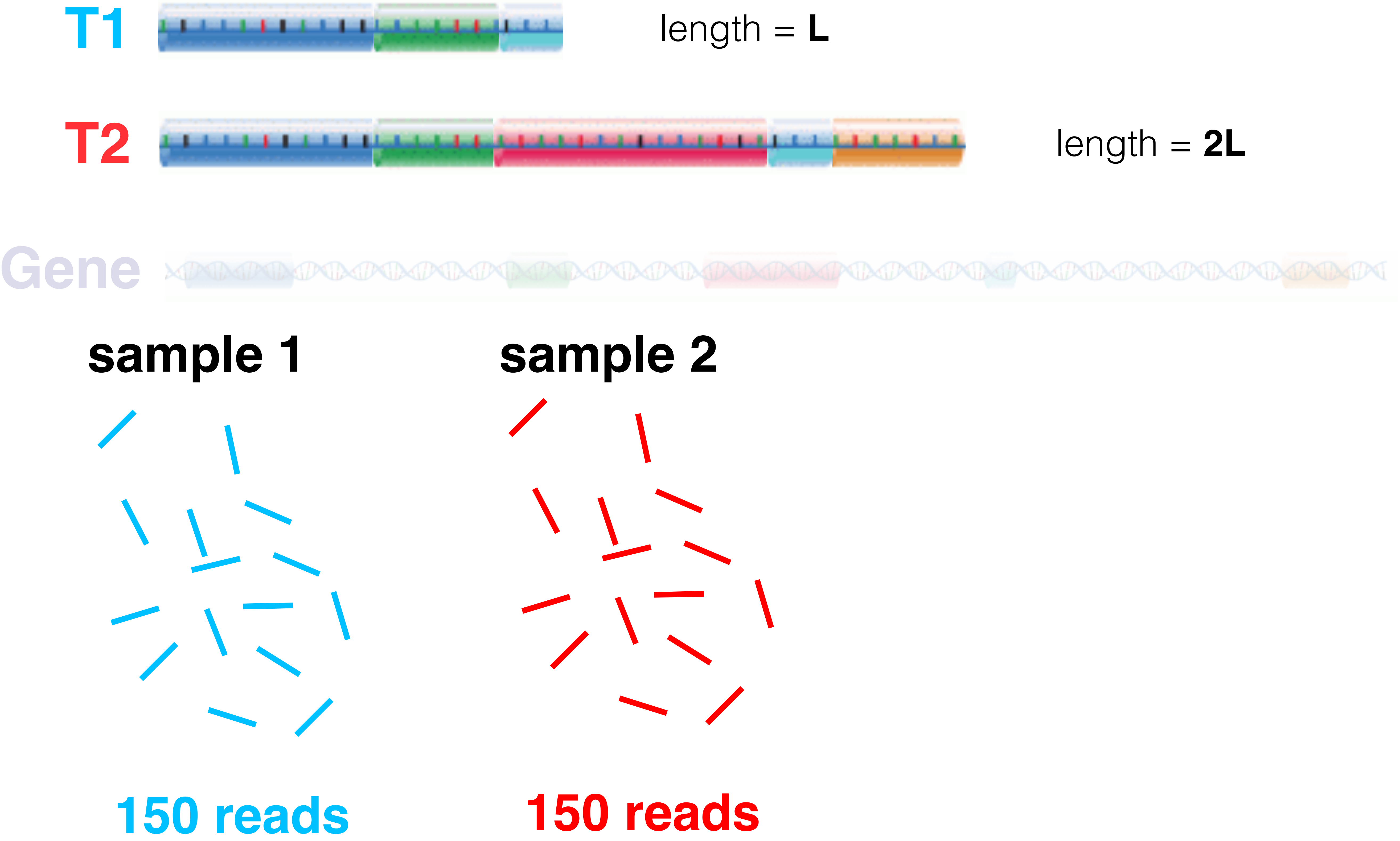
T1 length = **L**

T2 length = **2L**

sample 1 **sample 2**



Impact of differential isoform usage on gene-level counts



T1 length = **L**

T2 length = **2L**

Gene

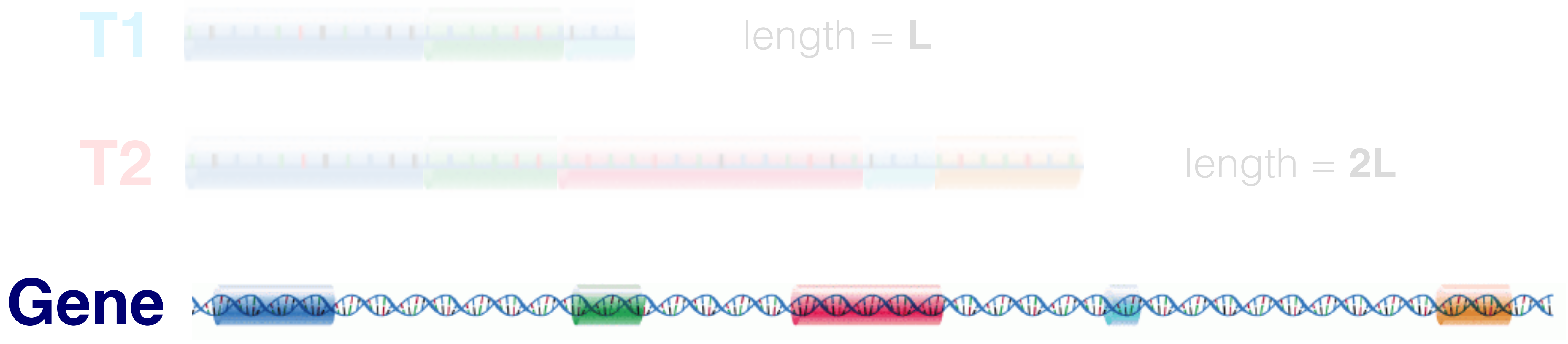
sample 1

sample 2

150 reads

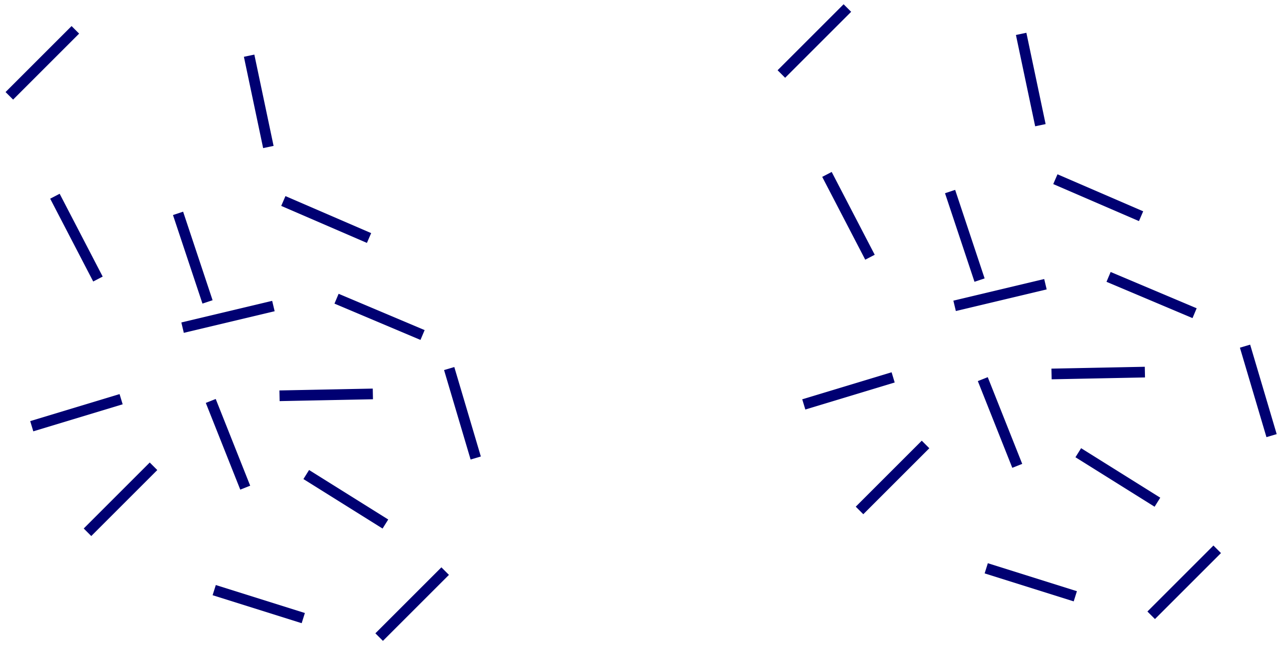
150 reads

Impact of differential isoform usage on gene-level counts



sample 1

sample 2



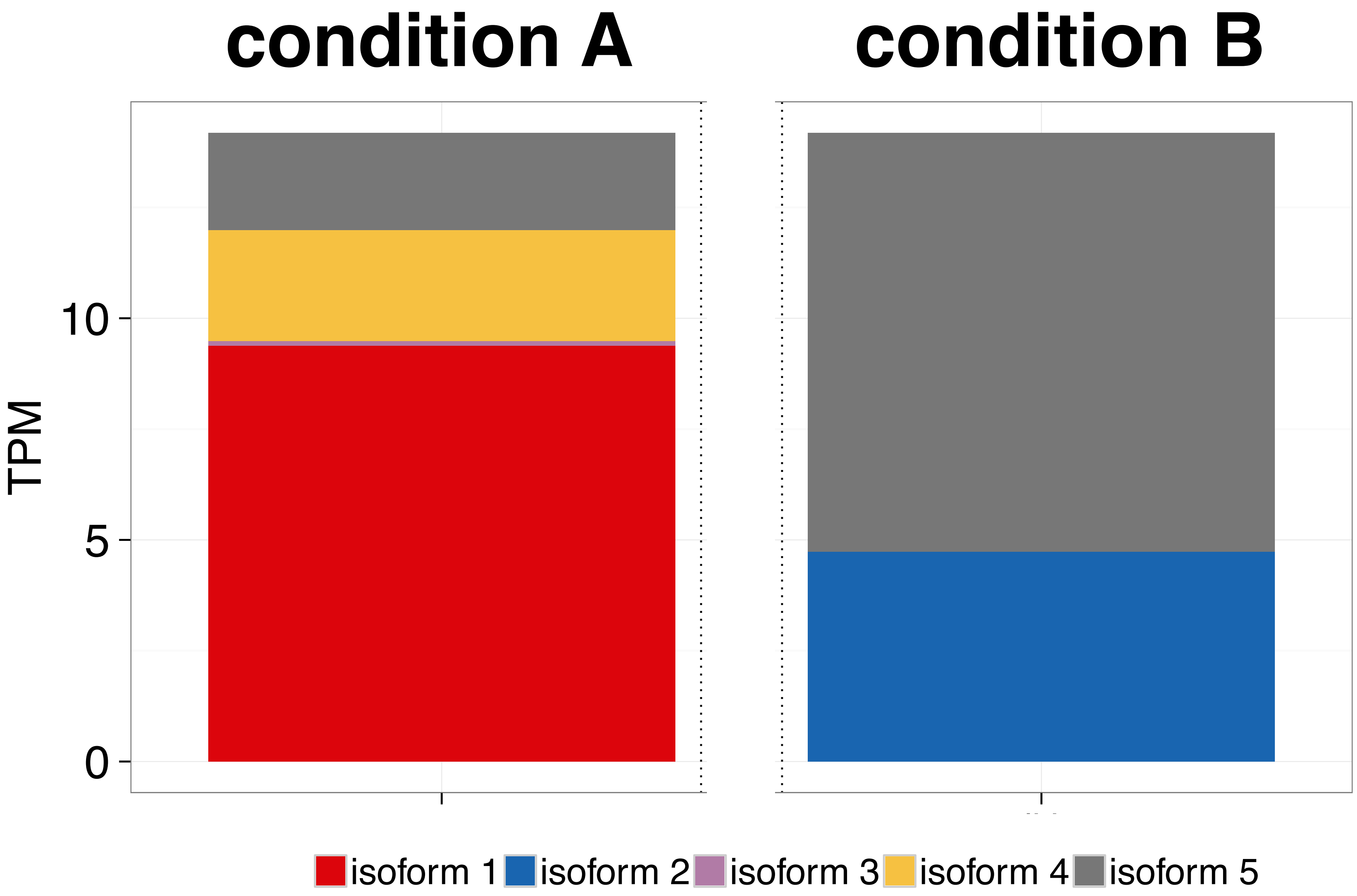
150 reads

150 reads

Gene	S1	S2
Count	150	150

Impact of differential isoform usage on gene-level counts

true abundance

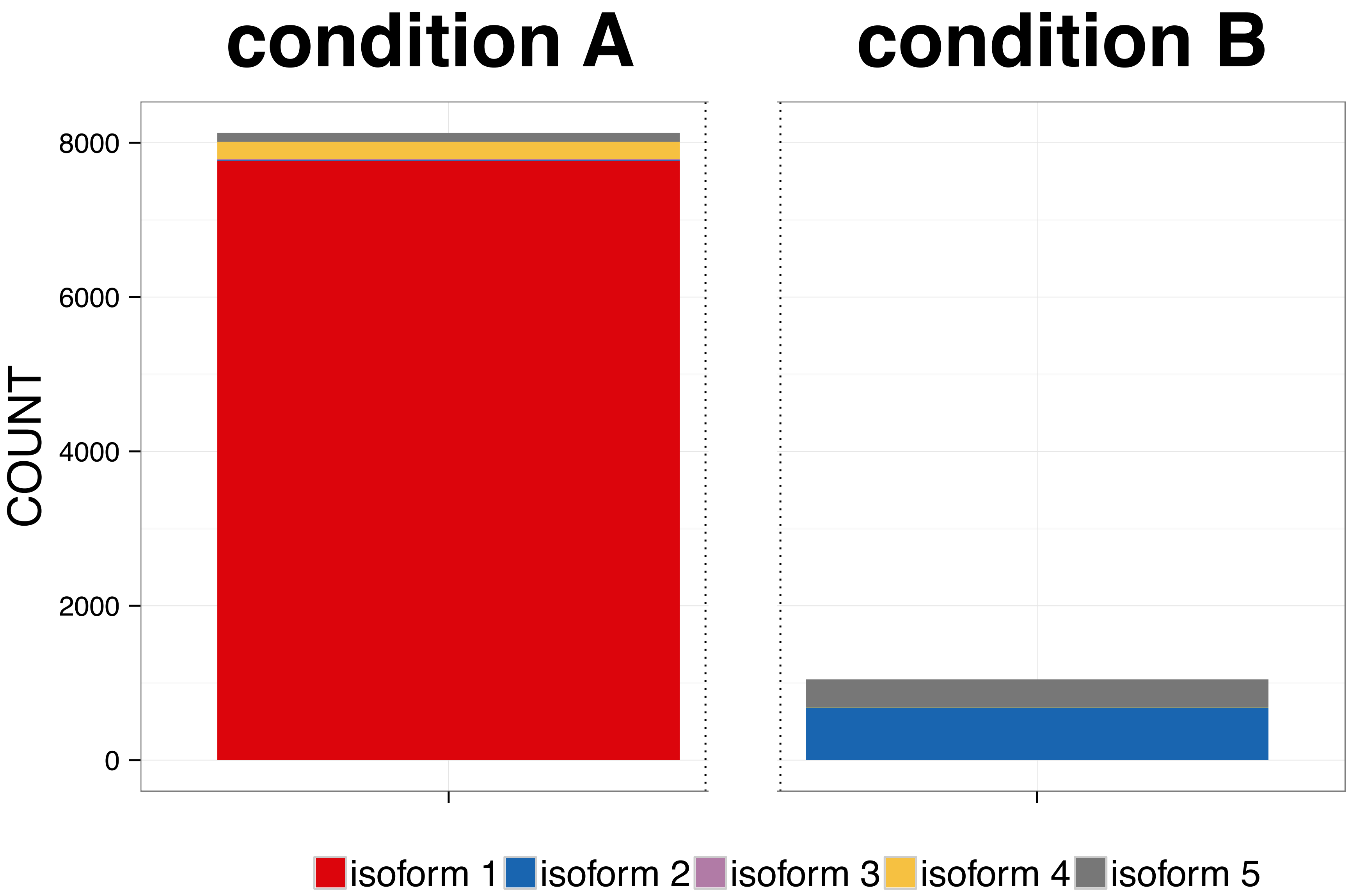


Lengths:

isoform 1:	12'232 bp
isoform 2:	1'733 bp
isoform 3:	891 bp
isoform 4:	1'404 bp
isoform 5:	543 bp

Impact of differential isoform usage on gene-level counts

read count



Lengths:

isoform 1:	12'232 bp
isoform 2:	1'733 bp
isoform 3:	891 bp
isoform 4:	1'404 bp
isoform 5:	543 bp

The isoform composition affects the observed read count for a gene

Differential isoform usage* can lead to false positives and false negatives in differential **gene** expression analyses

*differences in isoform composition between groups

What can we do?

- Consider another abundance unit that better reflects the underlying abundances (“number of transcript molecules”)
- Include “adjustment” of 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 (“number of transcript molecules”)

- Include “adjustment” of gene counts to reflect underlying isoform composition

How could such adjustment be done?

What can we do?

How can we get such values?

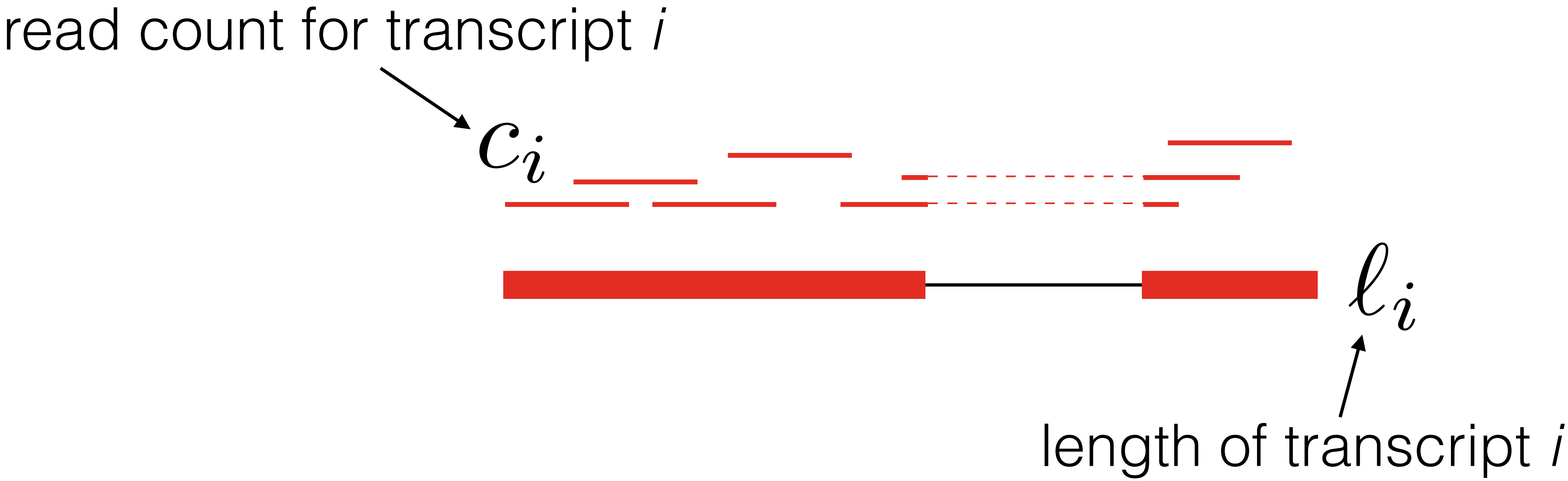
good?

- Consider another ab reflects the und umber of transcript
- Inc r gene counts to reflect und m composition

We need transcript-level information!

How could such adjustment be done?

Abundance units



read count for transcript *i*

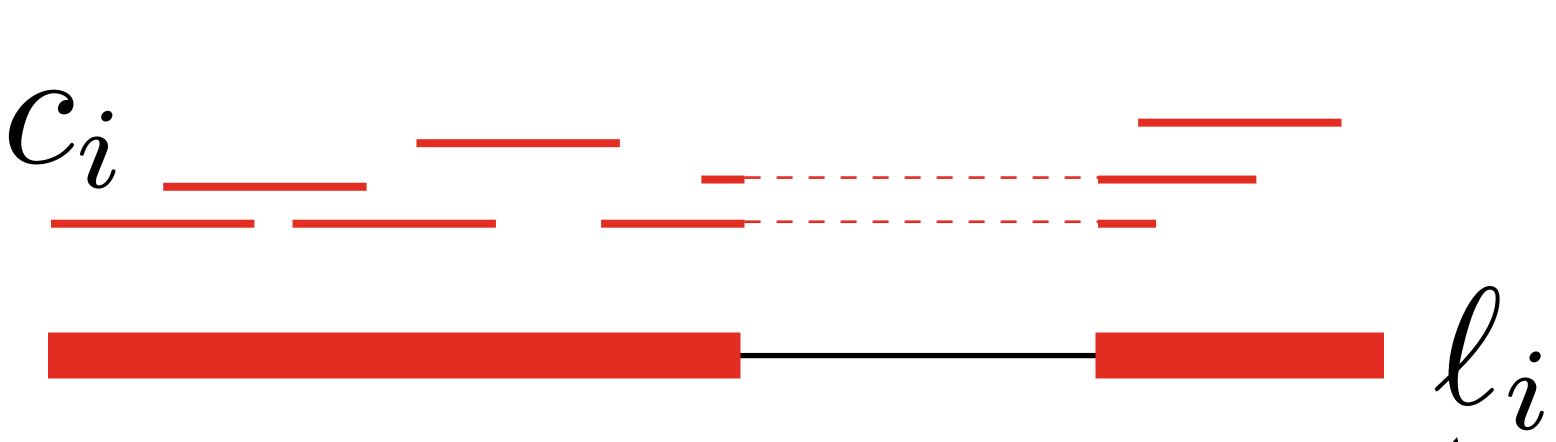
c_i

ℓ_i

length of transcript *i*

Abundance units

read count for transcript *i*



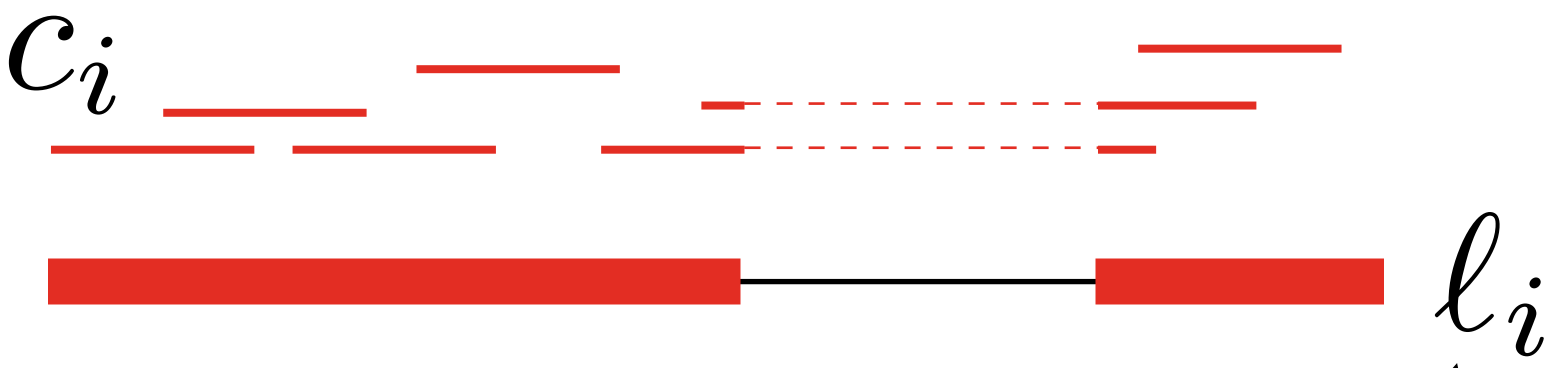
fragment length

length of transcript *i*

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

Abundance units

read count for transcript *i*



length of transcript *i*

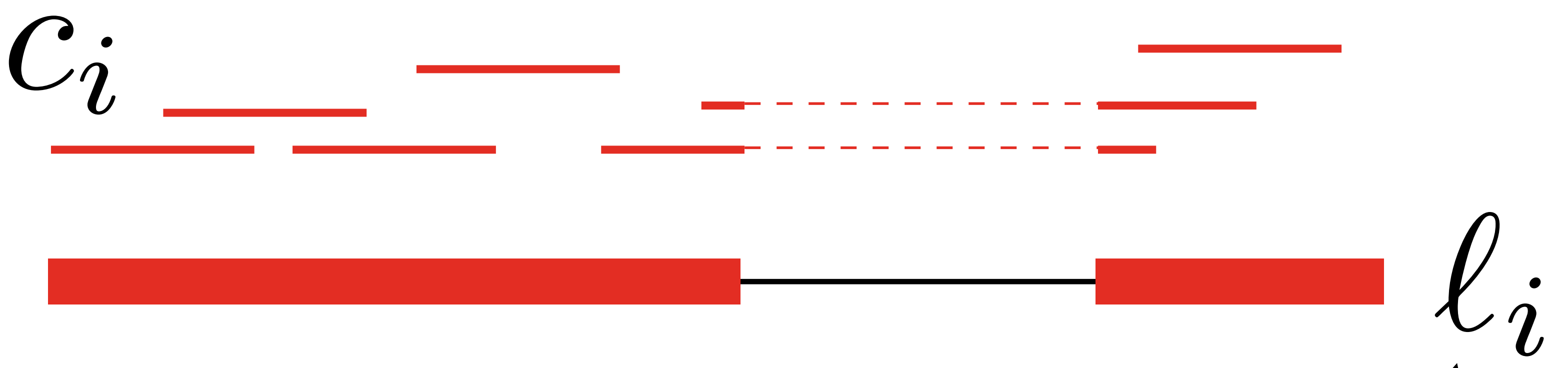
$$t_i = \frac{c_i r}{\ell_i}$$

fragment length

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

Abundance units

read count for transcript *i*



length of transcript *i*

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

fragment length

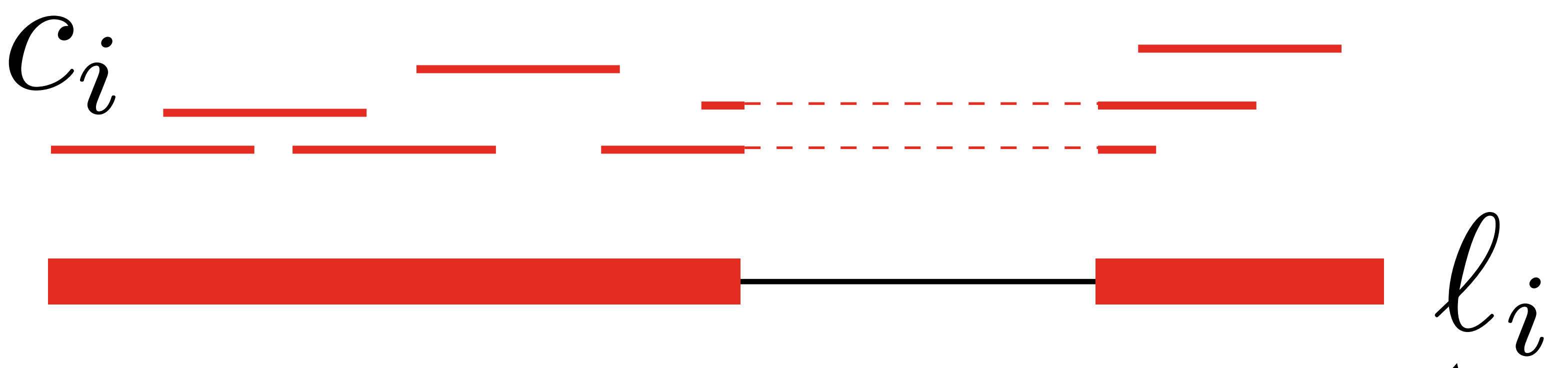
$$TPM_i = 10^6 \cdot \frac{t_i}{\sum_k t_k}$$

library size

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

Abundance units

read count for transcript *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

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

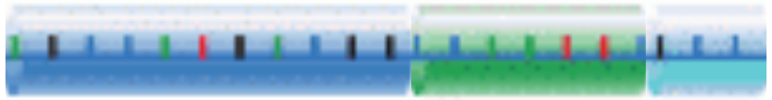
Average transcript lengths

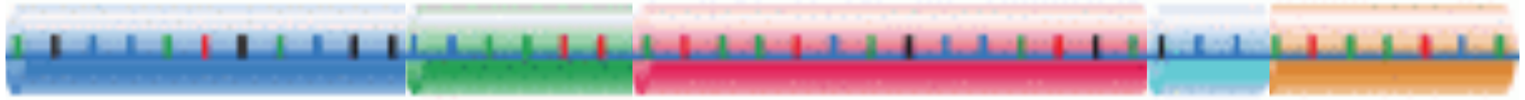
- Similar to correction factors for library size, but sample- **and** gene-specific
- Weighted average of transcript lengths, weighted by estimated abundances (TPMs)
- Average transcript length for gene *g* in sample *s*:

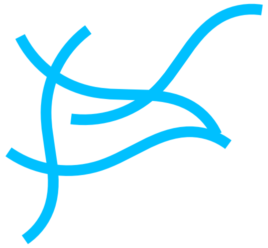
$$ATL_{gs} = \sum_{i \in g} \theta_{is} \bar{\ell}_{is}, \qquad \sum_{i \in g} \theta_{is} = 1$$

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

Average transcript lengths

T1  length = **L**

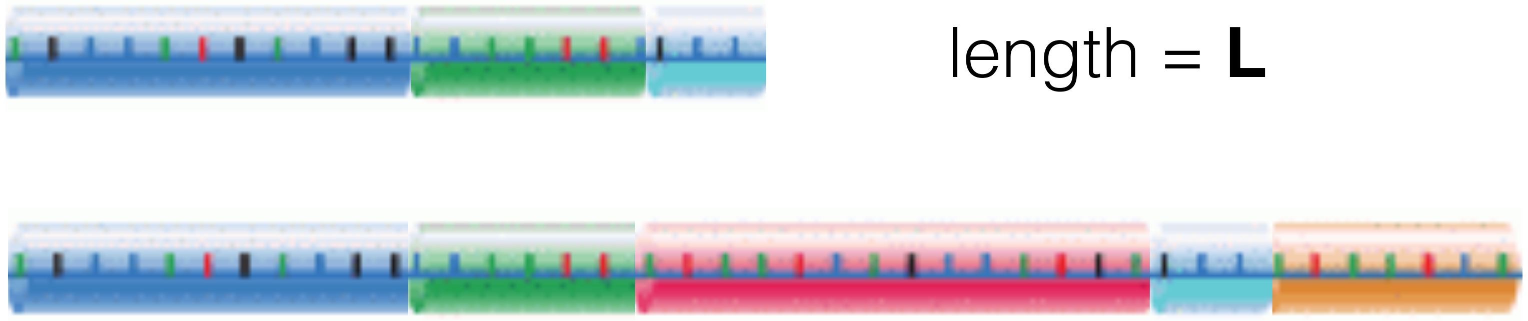
T2  length = **2L**



$$ATL_{g1} = 1 \cdot L + 0 \cdot 2L = L$$

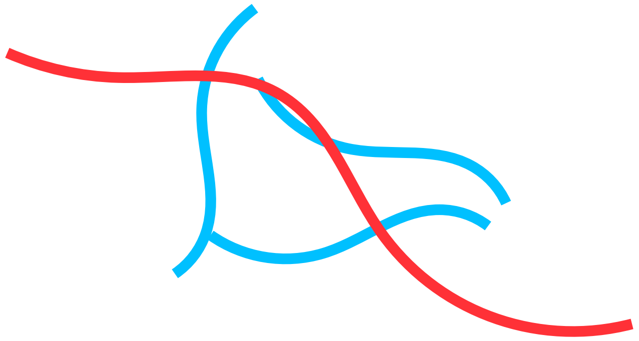
$$ATL_{g2} = 0 \cdot L + 1 \cdot 2L = 2L$$

Average transcript lengths

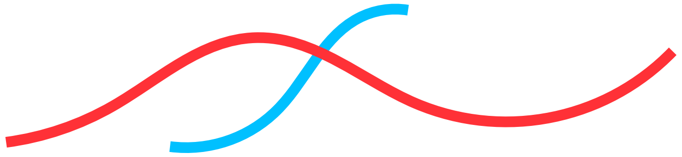


T1 length = **L**

T2 length = **2L**

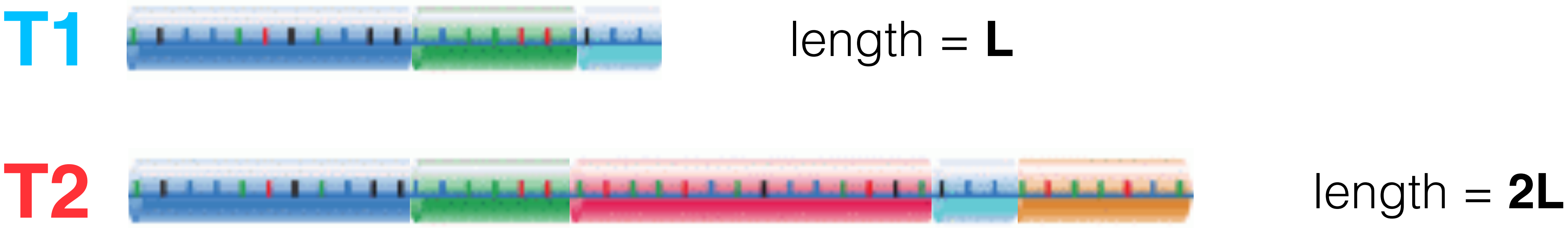


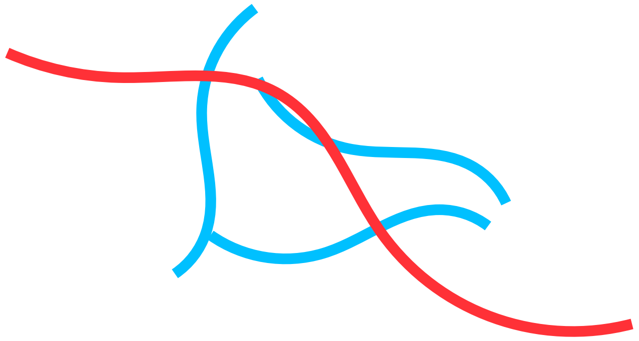
$$ATL_{g1} = 0.75 \cdot L + 0.25 \cdot 2L = 1.25L$$



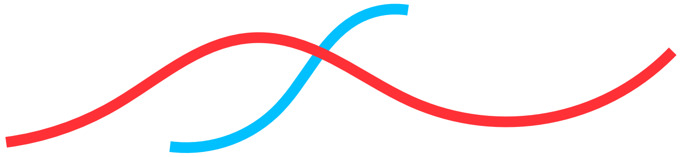
$$ATL_{g2} = 0.5 \cdot L + 0.5 \cdot 2L = 1.5L$$

Average transcript lengths





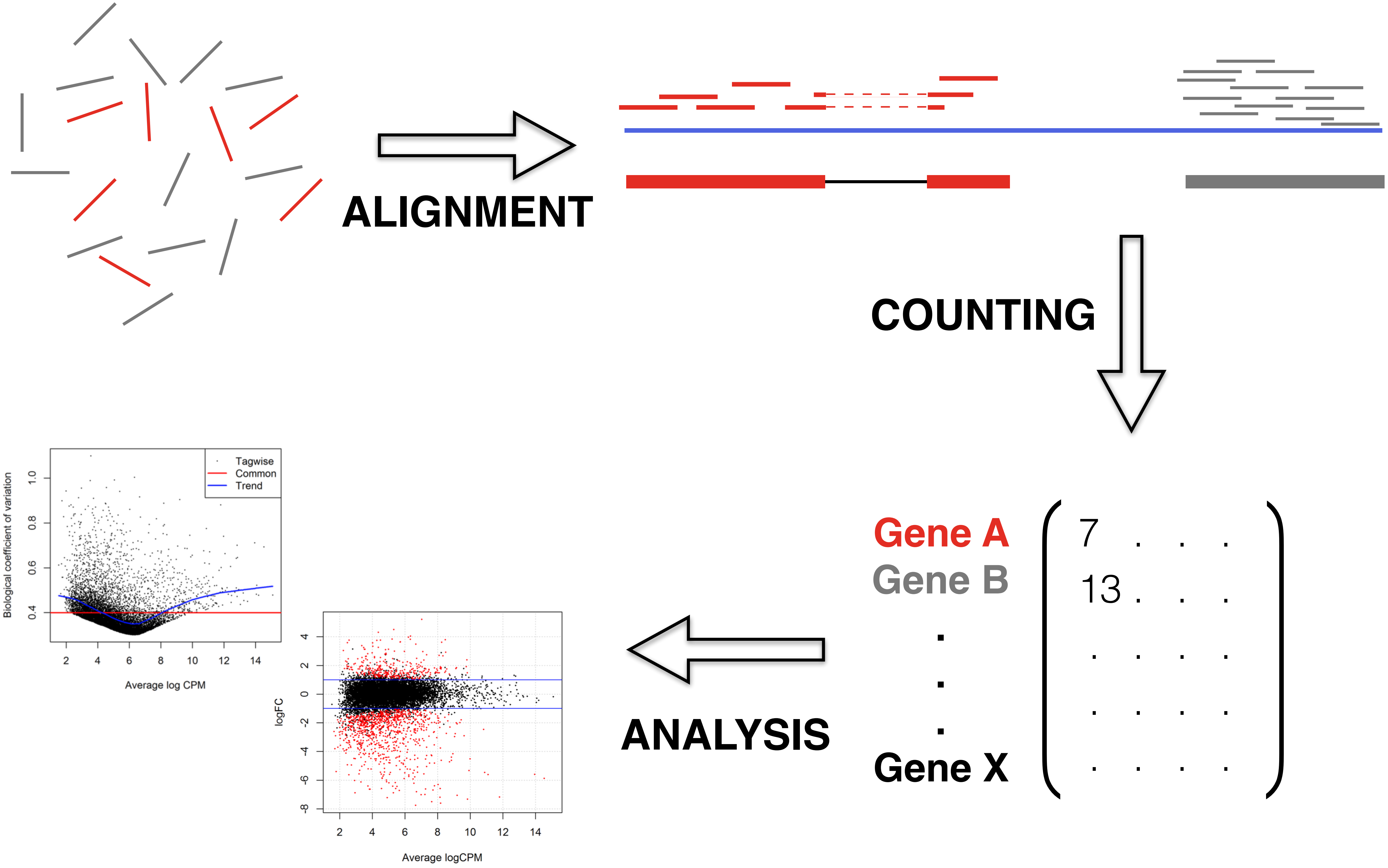
$$ATL_{g1} = \boxed{0.75} \cdot L + \boxed{0.25} \cdot 2L = 1.25L$$



$$ATL_{g2} = \boxed{0.5} \cdot L + \boxed{0.5} \cdot 2L = 1.5L$$

weights obtained from transcript TPM estimates

The alignment-based workflow



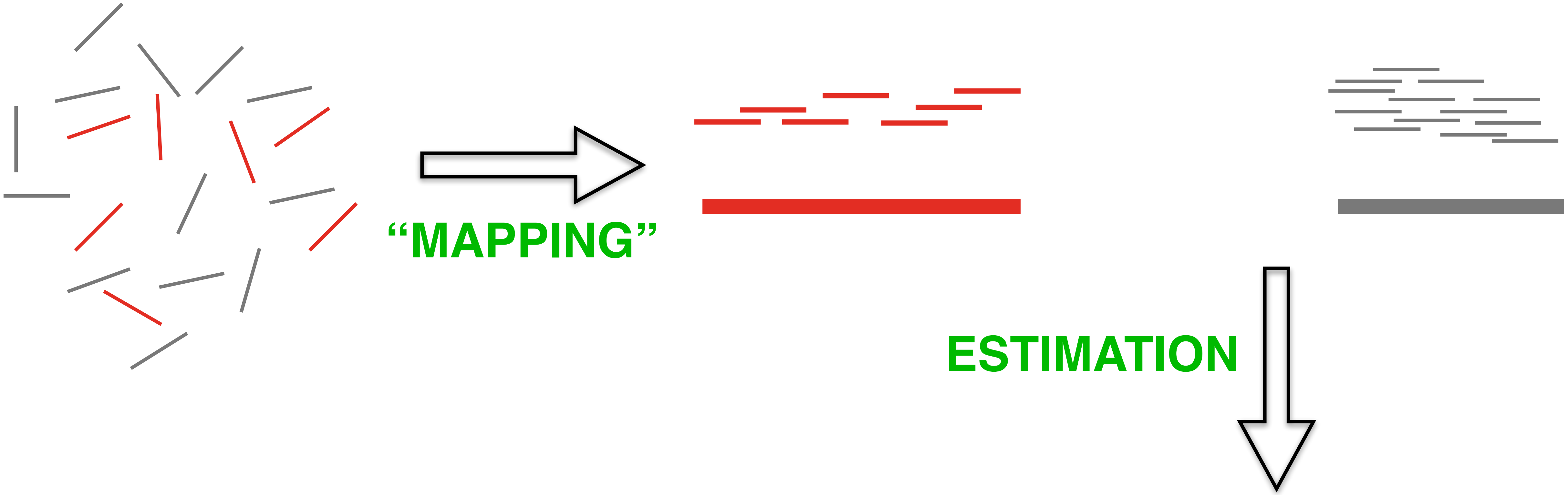
ALIGNMENT

COUNTING

$$\begin{array}{l} \text{Gene A} \\ \text{Gene B} \\ \cdot \\ \cdot \\ \cdot \\ \text{Gene X} \end{array} \begin{pmatrix} 7 & . & . & . \\ 13 & . & . & . \\ . & . & . & . \\ . & . & . & . \\ . & . & . & . \end{pmatrix}$$

ANALYSIS

The alignment-free workflow

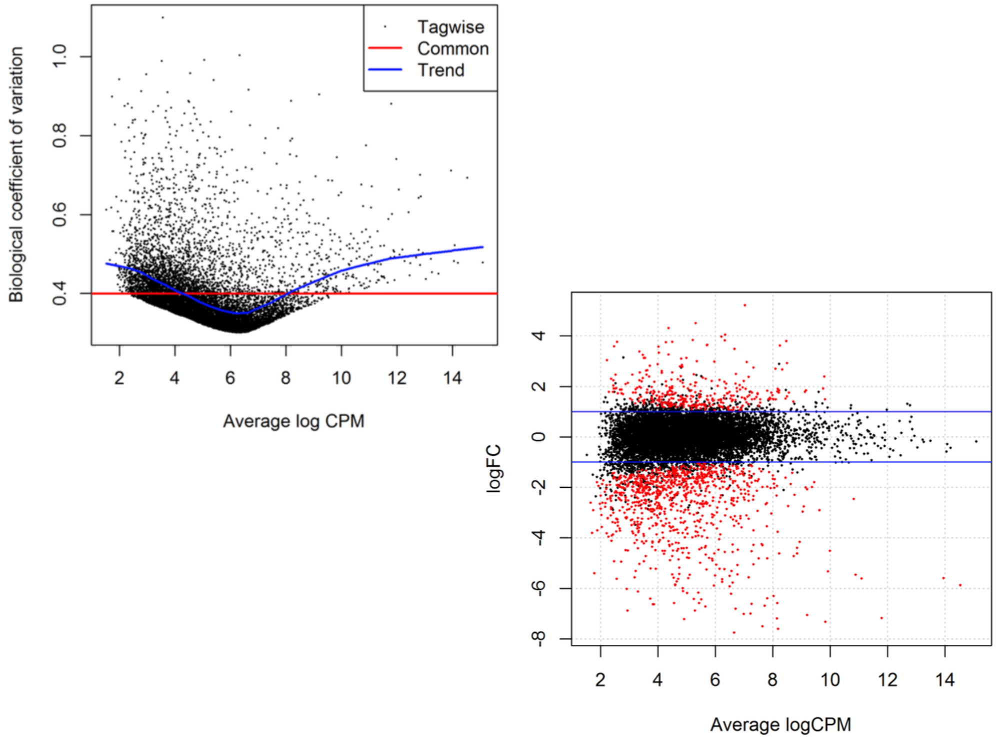


“MAPPING”

ESTIMATION

$$\begin{array}{l} \\ \text{Gene A} \\ \text{Gene B} \\ \cdot \\ \cdot \\ \cdot \\ \text{Gene X} \end{array} \left(\begin{array}{cccc} 7 & . & . & . \\ 13 & . & . & . \\ . & . & . & . \\ . & . & . & . \\ . & . & . & . \end{array}\right)$$

ANALYSIS

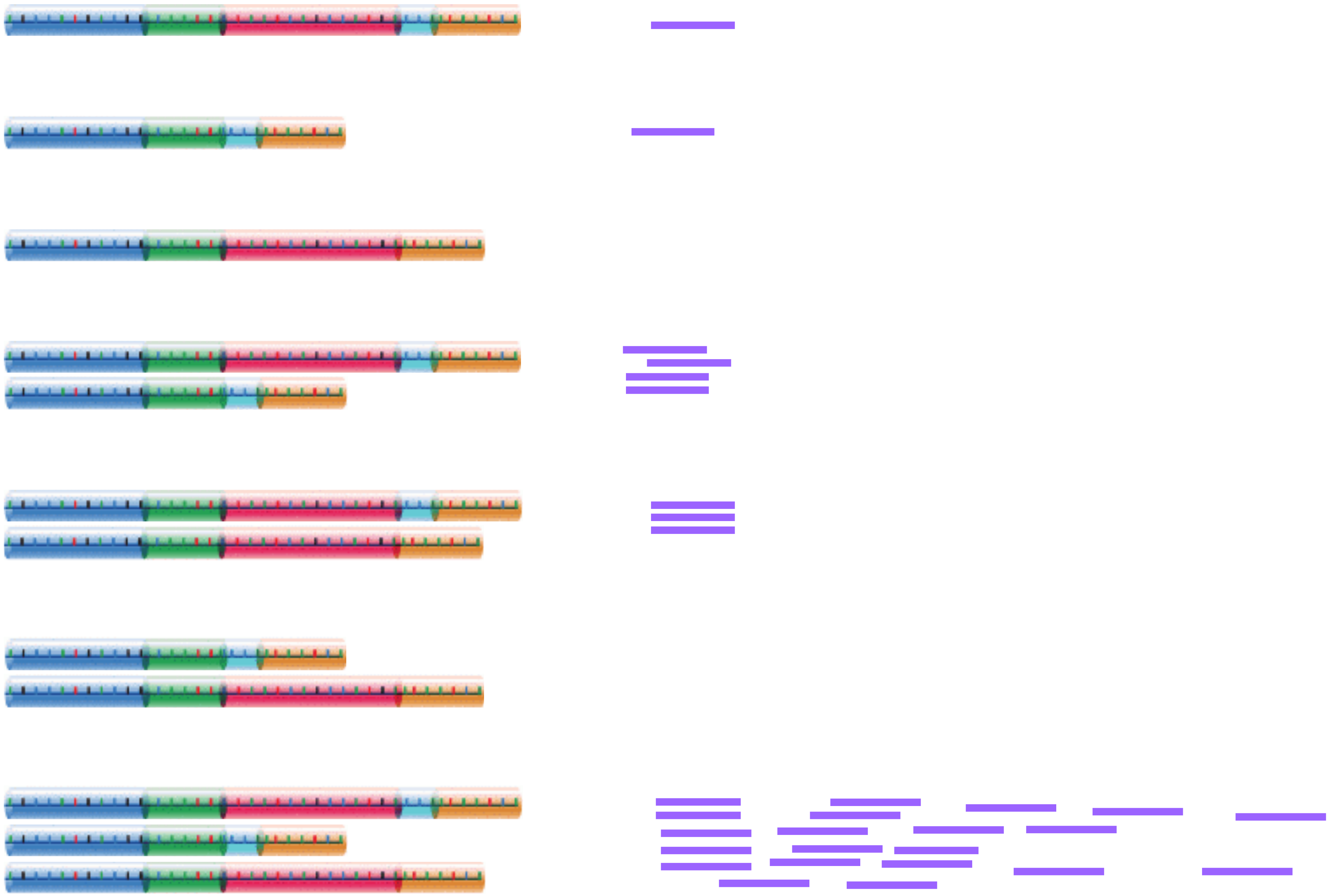


The “mapping” step

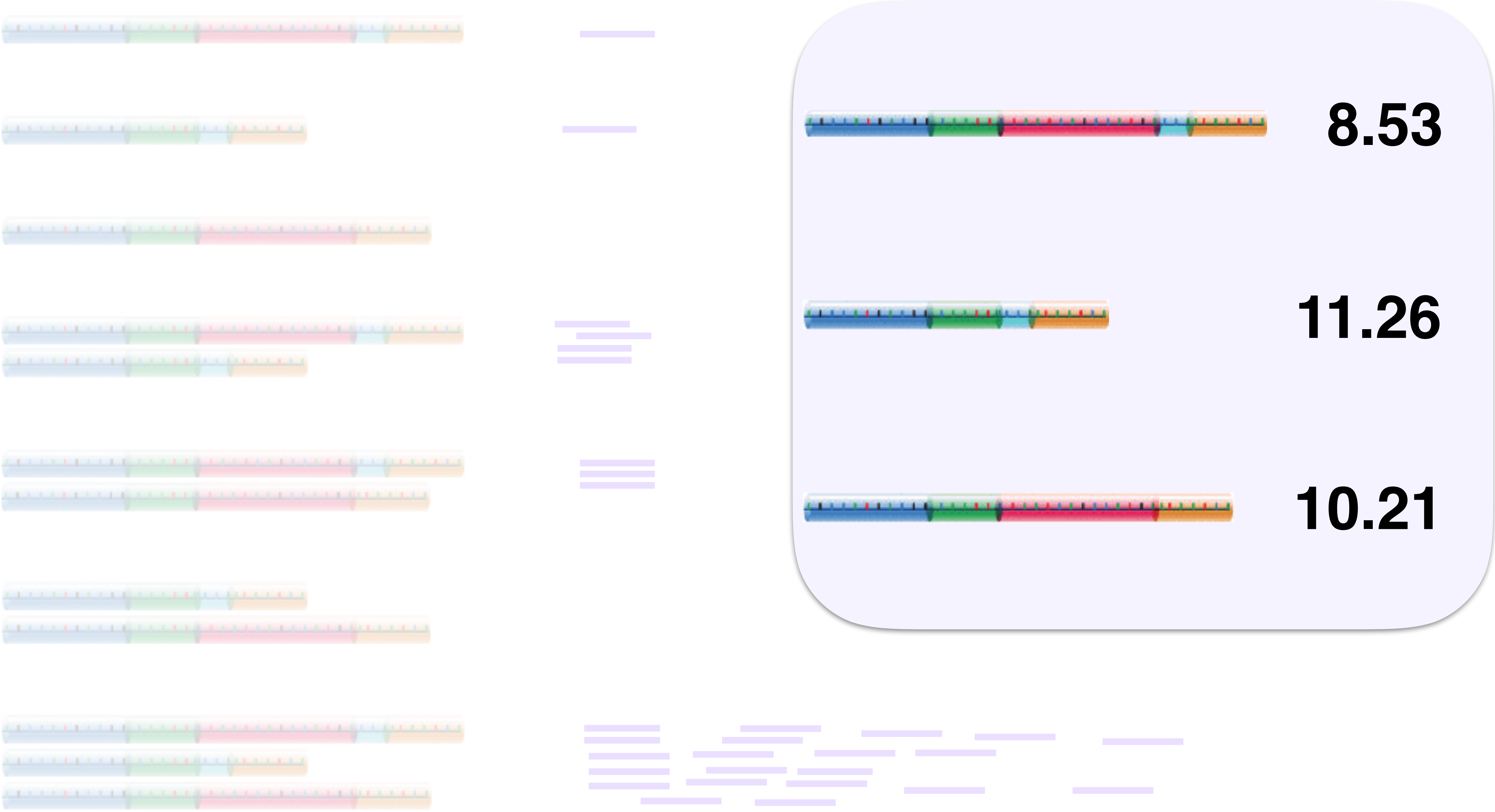
- Does not provide “full” alignment information (i.e., no exact base-by-base alignment).
- Rather, finds all transcripts (and positions) that a read is compatible with.
- Comes in various flavors:
    - pseudoalignment (kallisto)
    - lightweight alignment (Salmon)
    - quasimapping (Sailfish, RapMap)

Bray et al. 2016; Patro et al. 2014; Patro et al. 2017; Srivastava et al. 2016

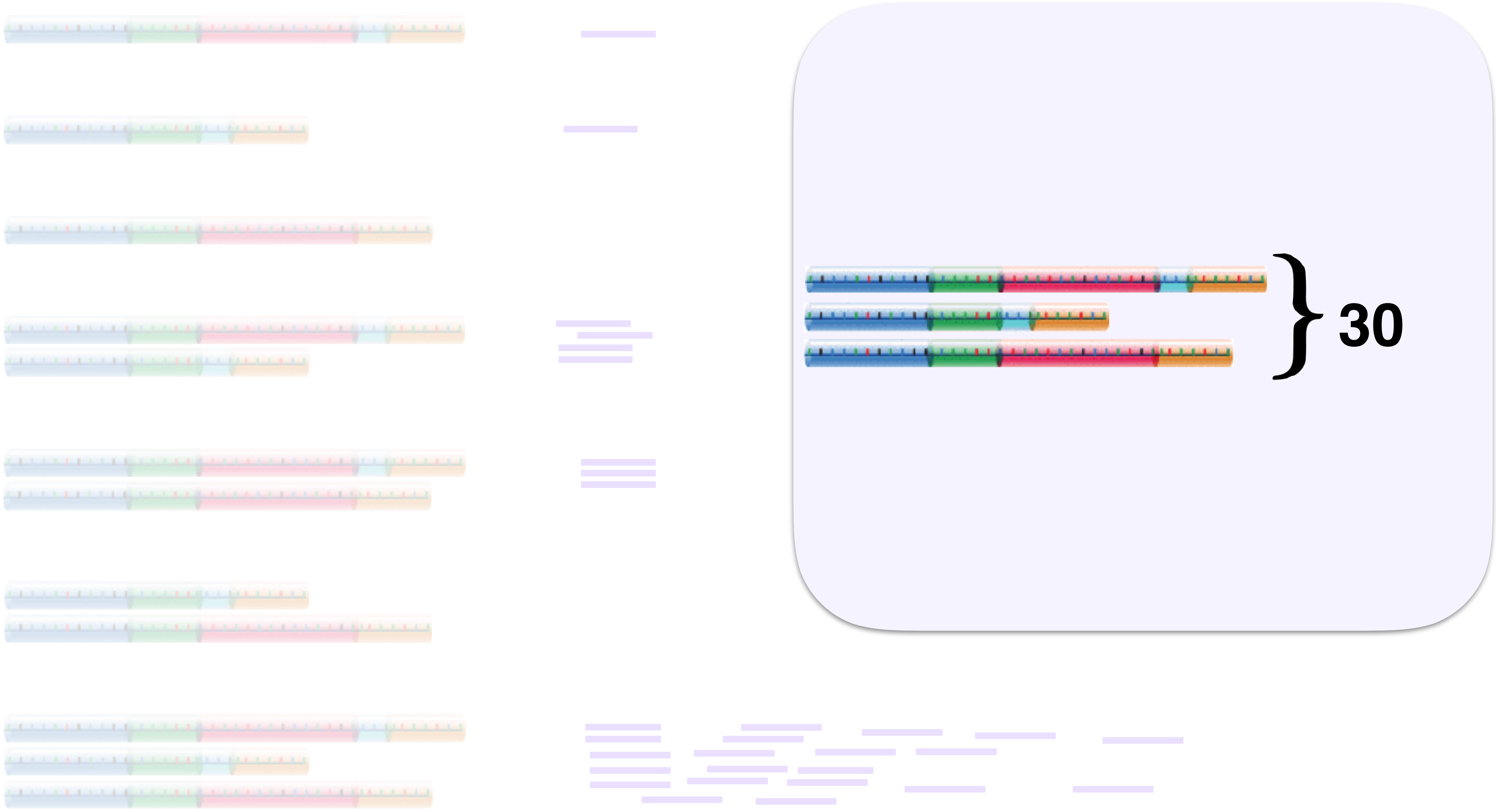
Transcript-level counts



Transcript-level counts



Gene-level counts



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

```
$ kallisto index -i my_transcripts.idx \
            my_transcripts.fasta
```

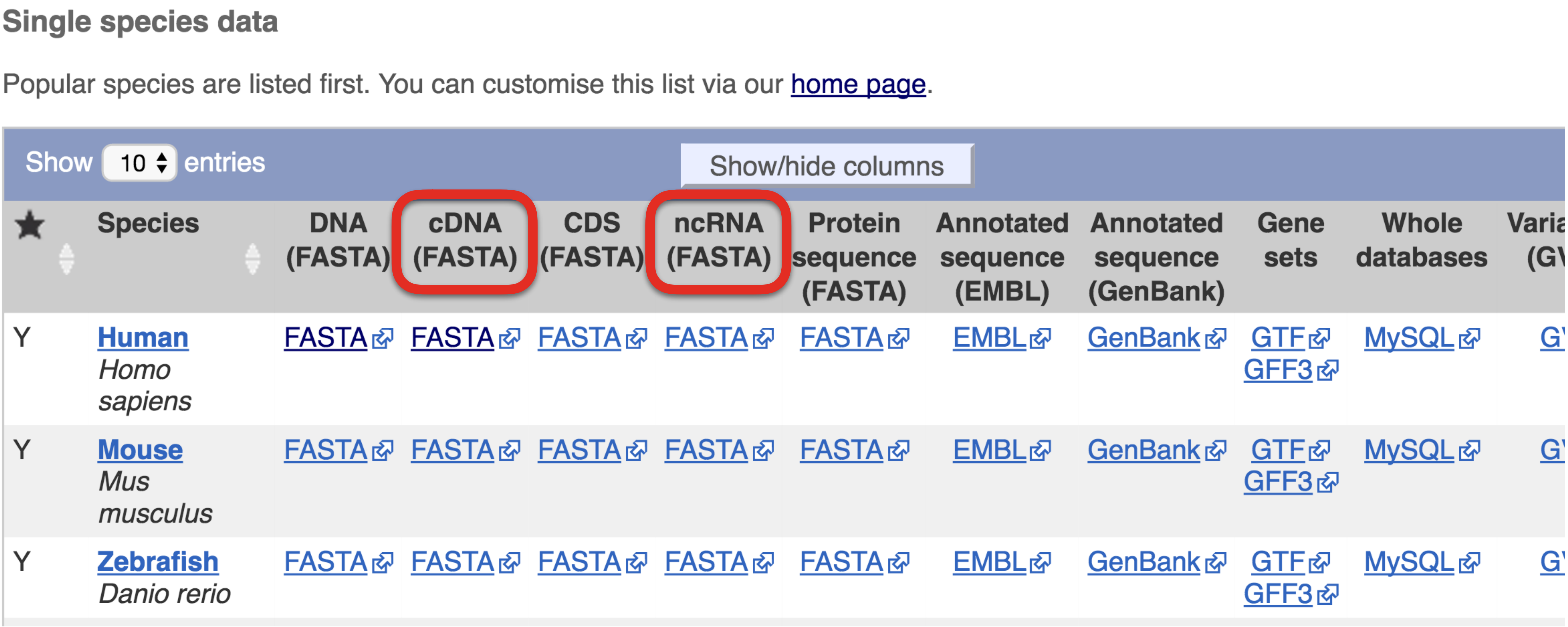
transcriptome fasta file

Salmon

```
$ salmon index -i my_transcripts.idx \
          -t my_transcripts.fasta
```

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.

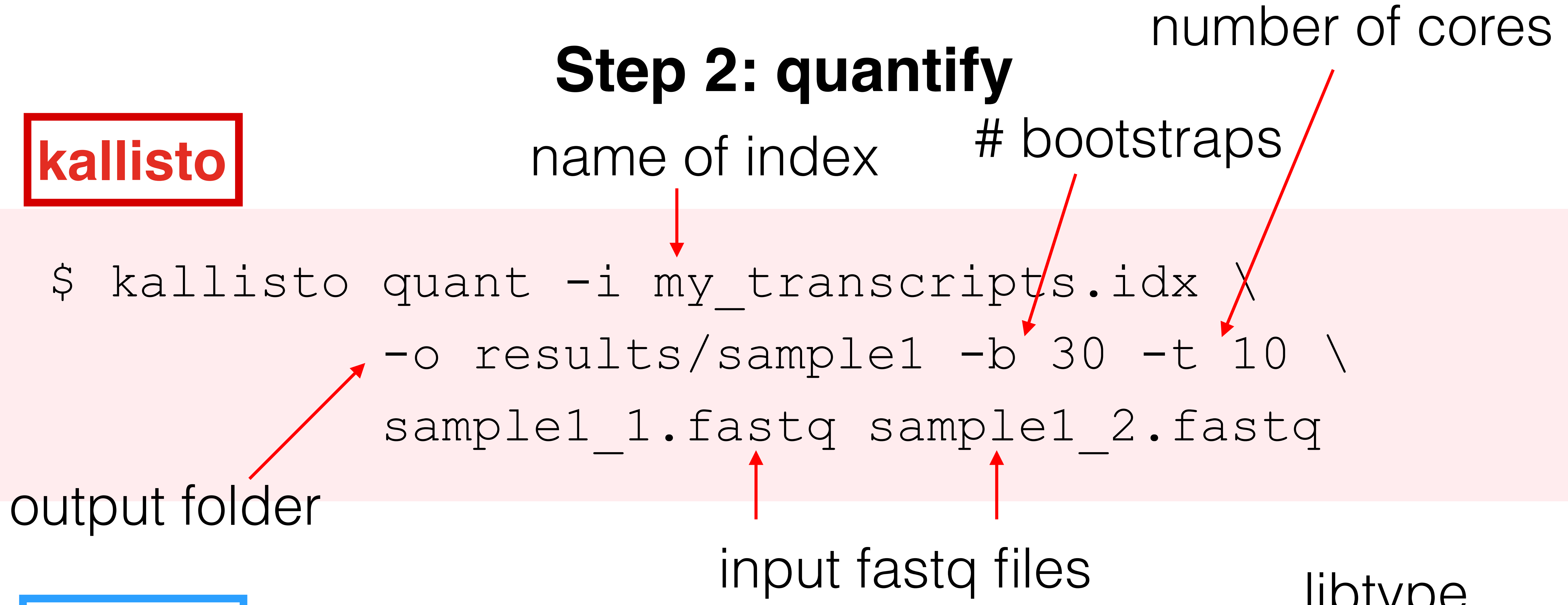
Show 10 entries

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 (G
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

# bootstraps

number of cores

```
$ kallisto quant -i my_transcripts.idx \
           -o results/sample1 -b 30 -t 10 \
           sample1_1.fastq sample1_2.fastq
```

output folder

input fastq files

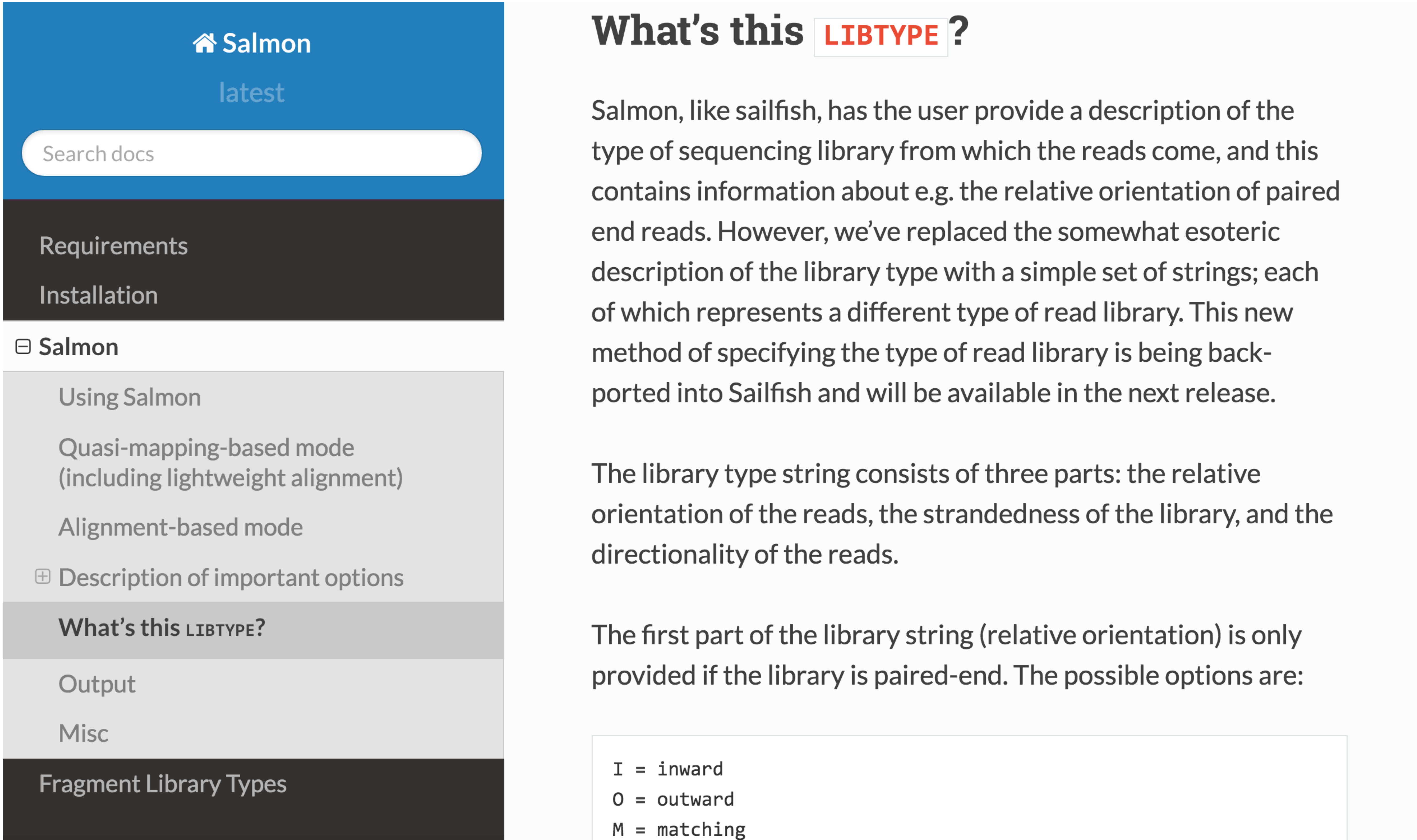
Salmon

libtype

```
$ salmon quant -i my_transcripts.idx -l A \
         -1 sample1_1.fastq -2 sample1_2.fastq \
         -p 10 -o results/sample1 \
         --numBootstraps 30 --seqBias --gcBias
```

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.

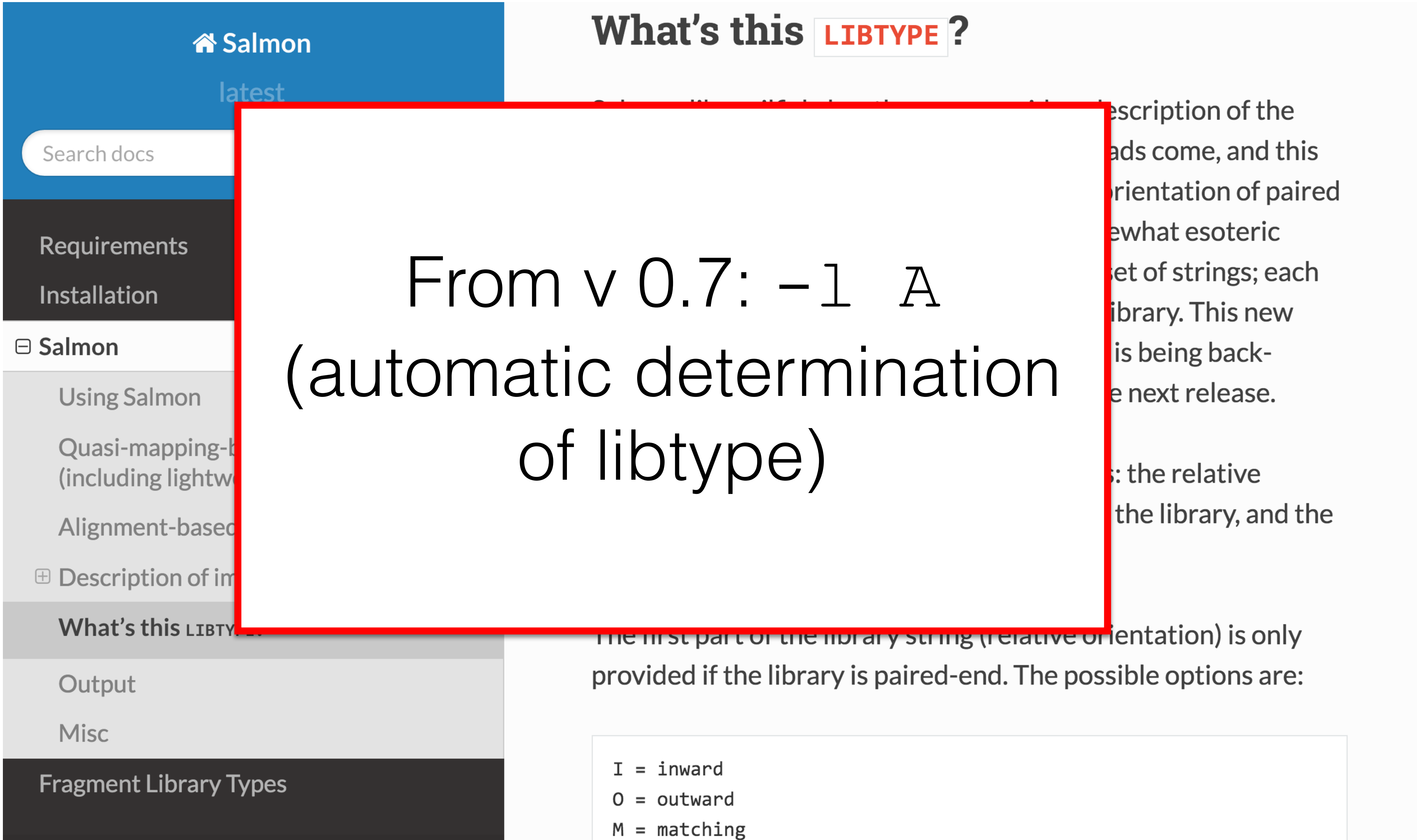
The library type string consists of three parts: the relative orientation of the reads, the strandedness of the library, and the directionality of the reads.

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
```

Salmon LIBTYPE argument

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



What's this `LIBTYPE`?

escription of the
ads come, and this
rientation of paired
ewhat esoteric
et of strings; each
ibrary. This new
is being back-
e next release.

: the relative
the library, and the

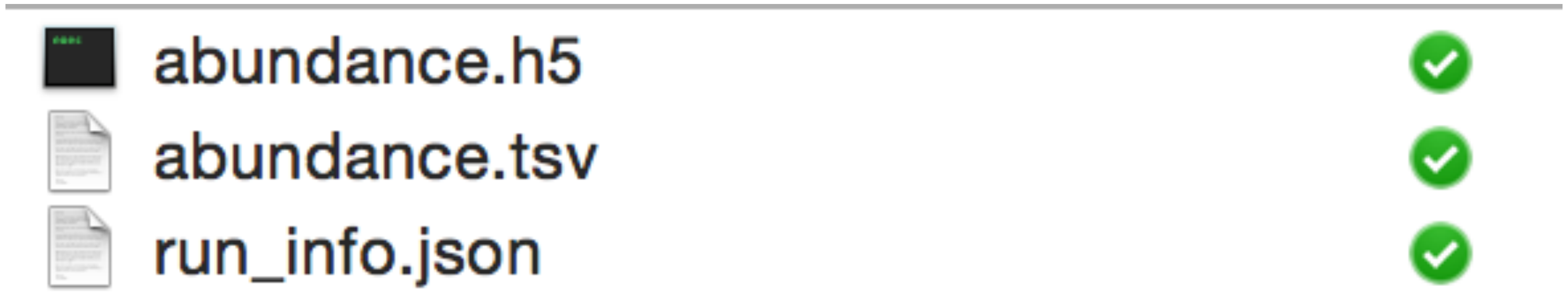
The first part of the library string (relative orientation) is only provided if the library is paired-end. The possible options are:

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

From v 0.7: `-l A`
(automatic determination of libtype)

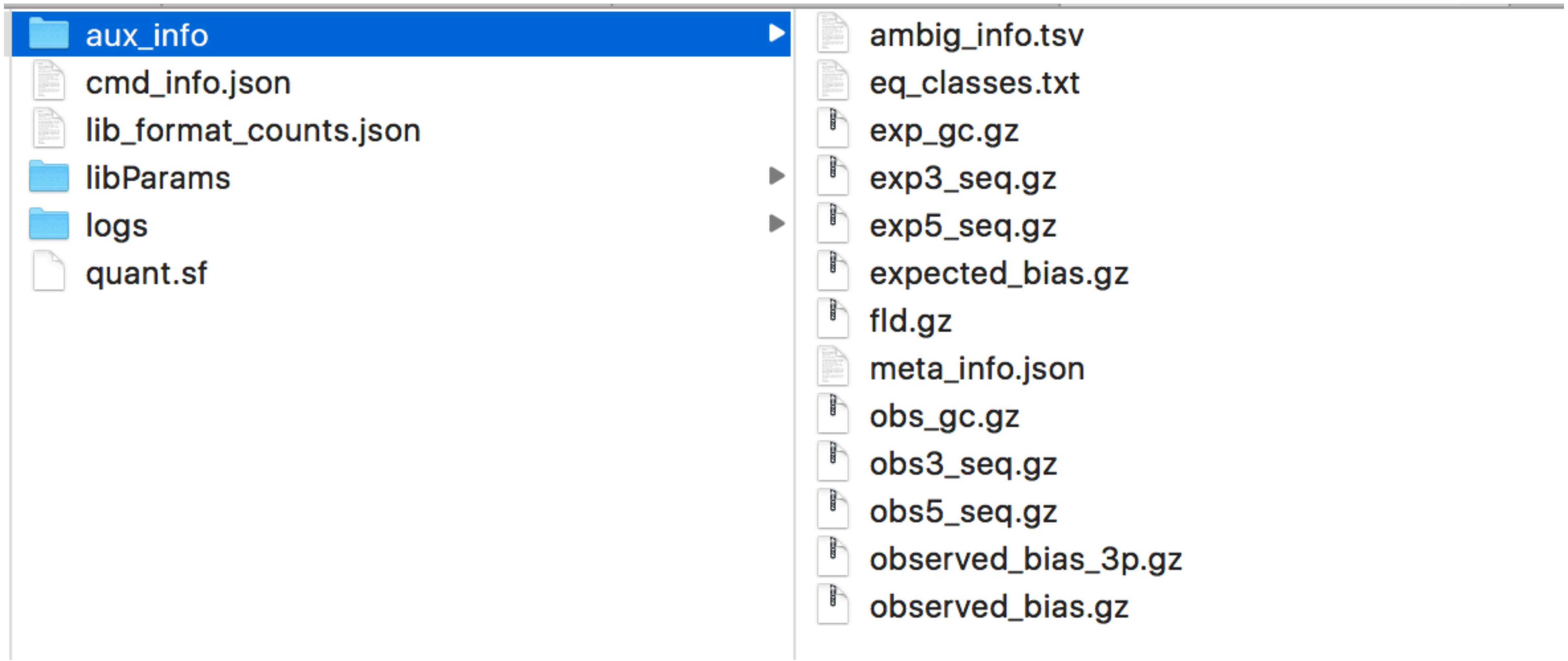
output

kallisto



abundance.h5 ✓
abundance.tsv ✓
run_info.json ✓

Salmon



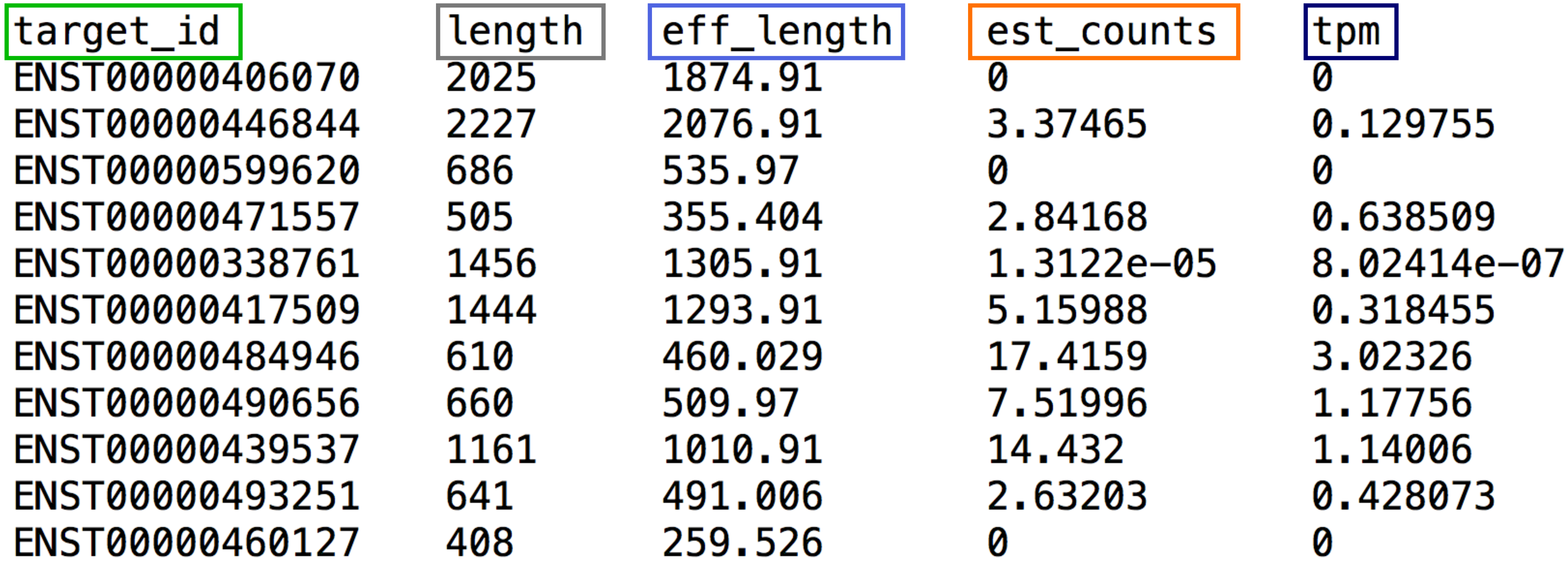
- aux_info ▶
- cmd_info.json
- lib_format_counts.json
- libParams ▶
- logs ▶
- quant.sf

- ambig_info.tsv
- eq_classes.txt
- exp_gc.gz
- exp3_seq.gz
- exp5_seq.gz
- expected_bias.gz
- fld.gz
- meta_info.json
- obs_gc.gz
- obs3_seq.gz
- obs5_seq.gz
- observed_bias_3p.gz
- observed_bias.gz

output

kallisto

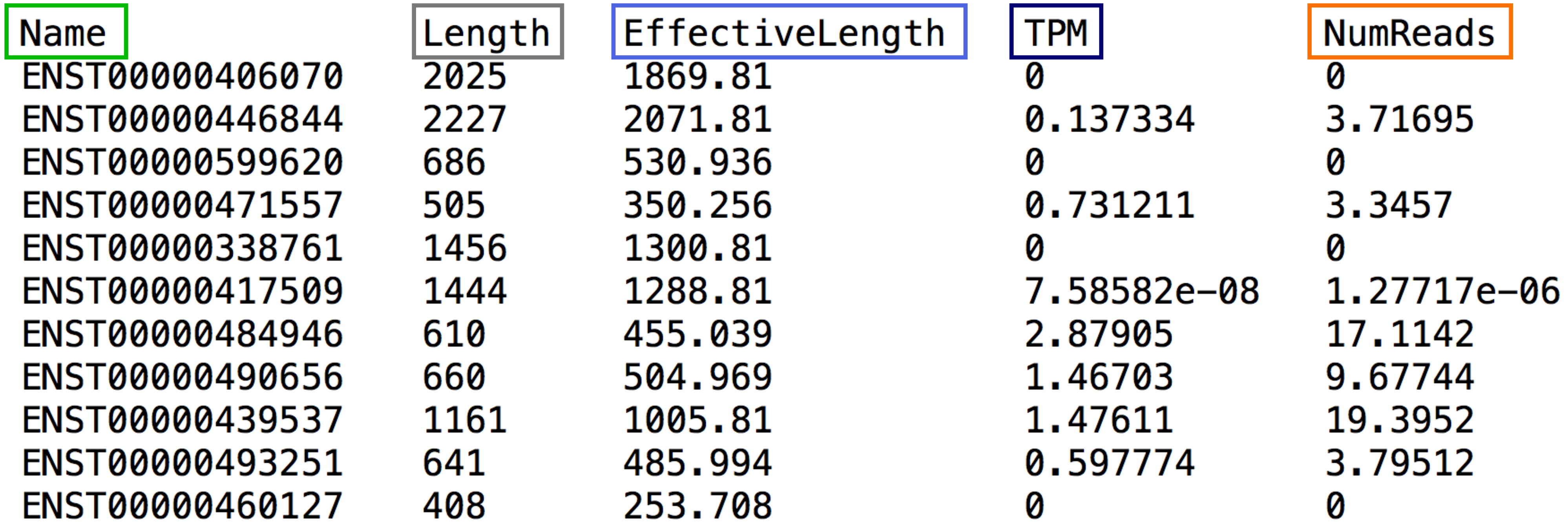
[abundance.tsv]



```
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 alignment-based workflow

Salmon/kallisto…

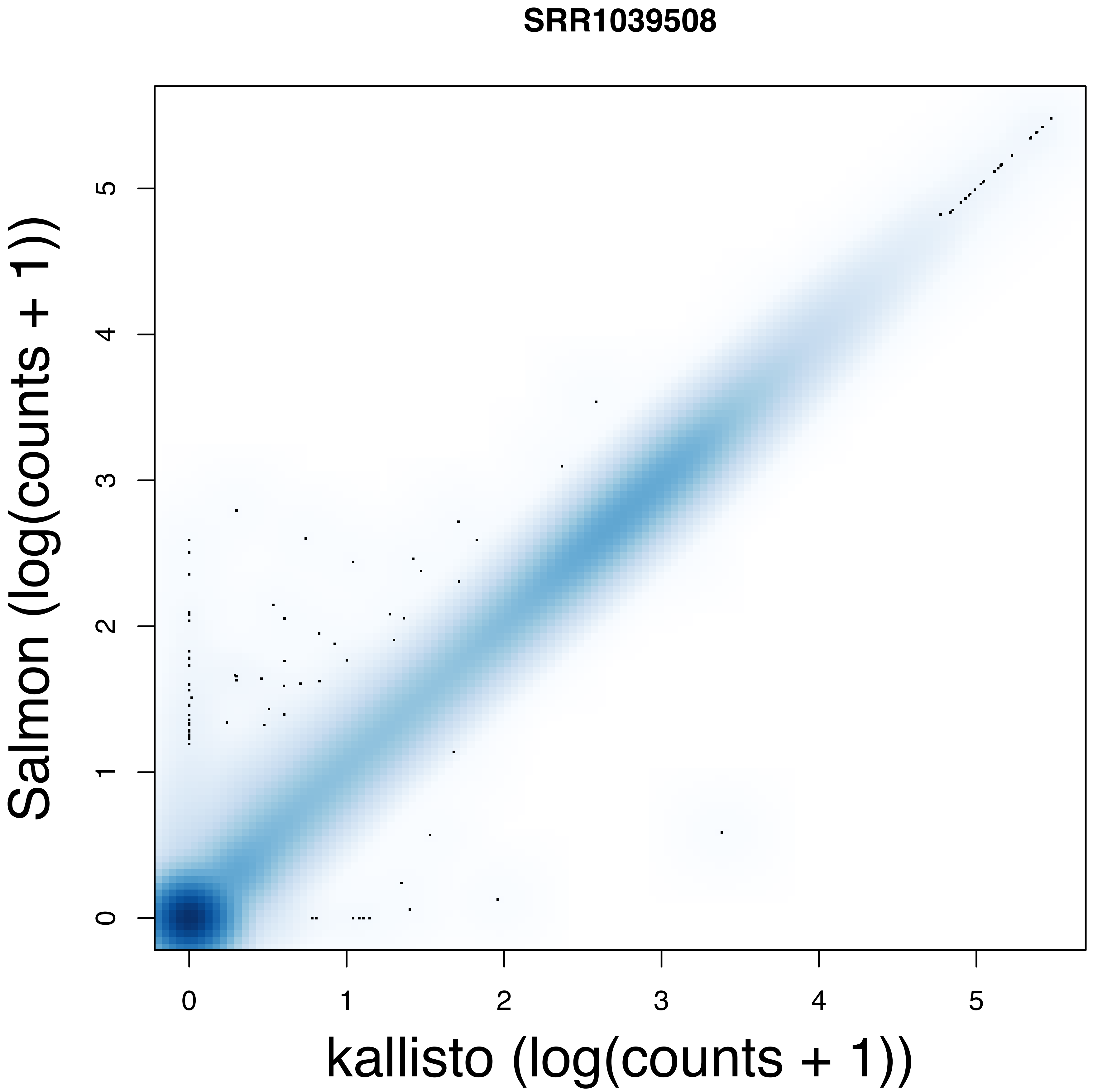
- … are considerably faster than traditional alignment+counting -> allow bootstrapping

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

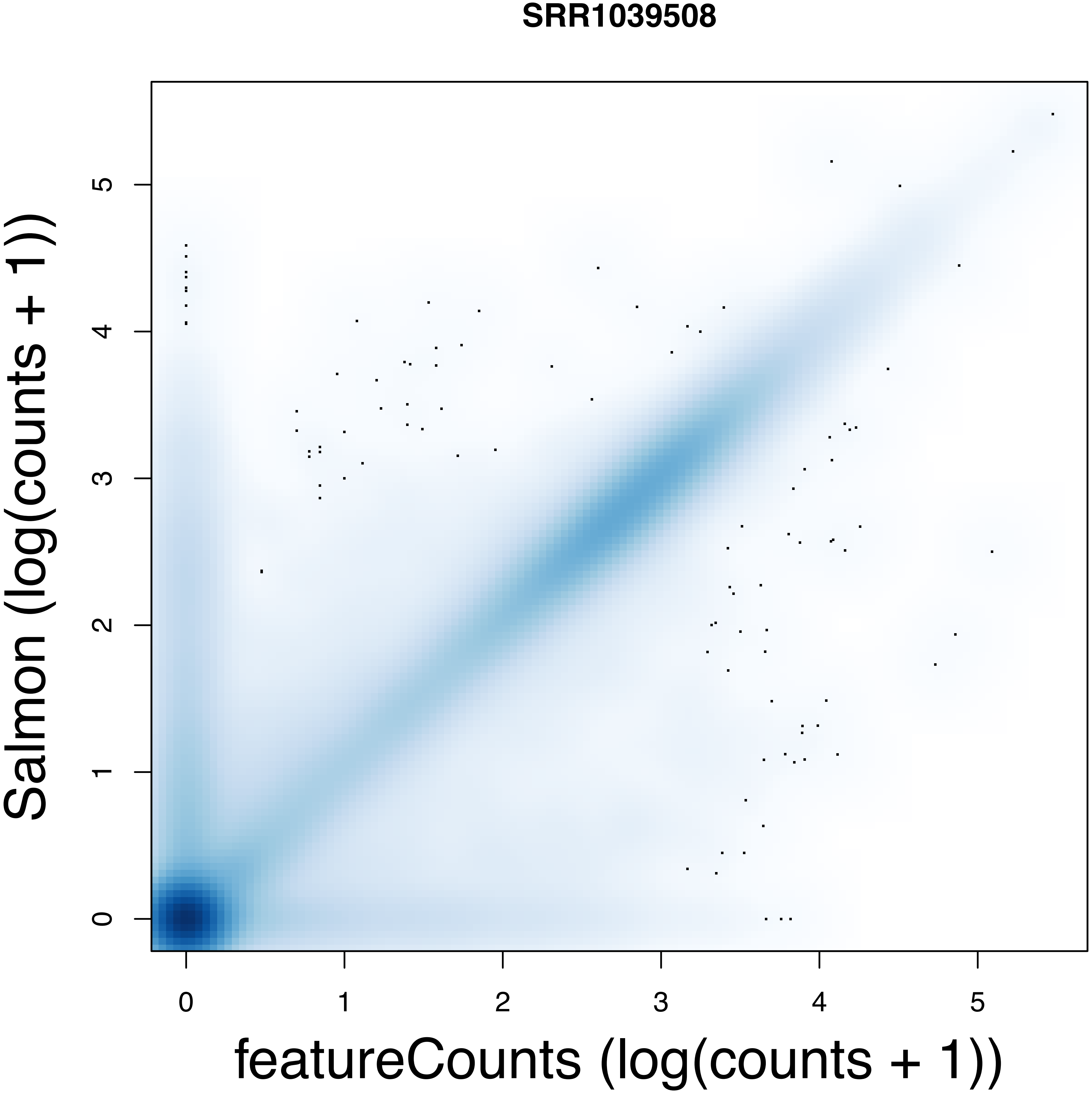
- … can use a larger fraction of the reads

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

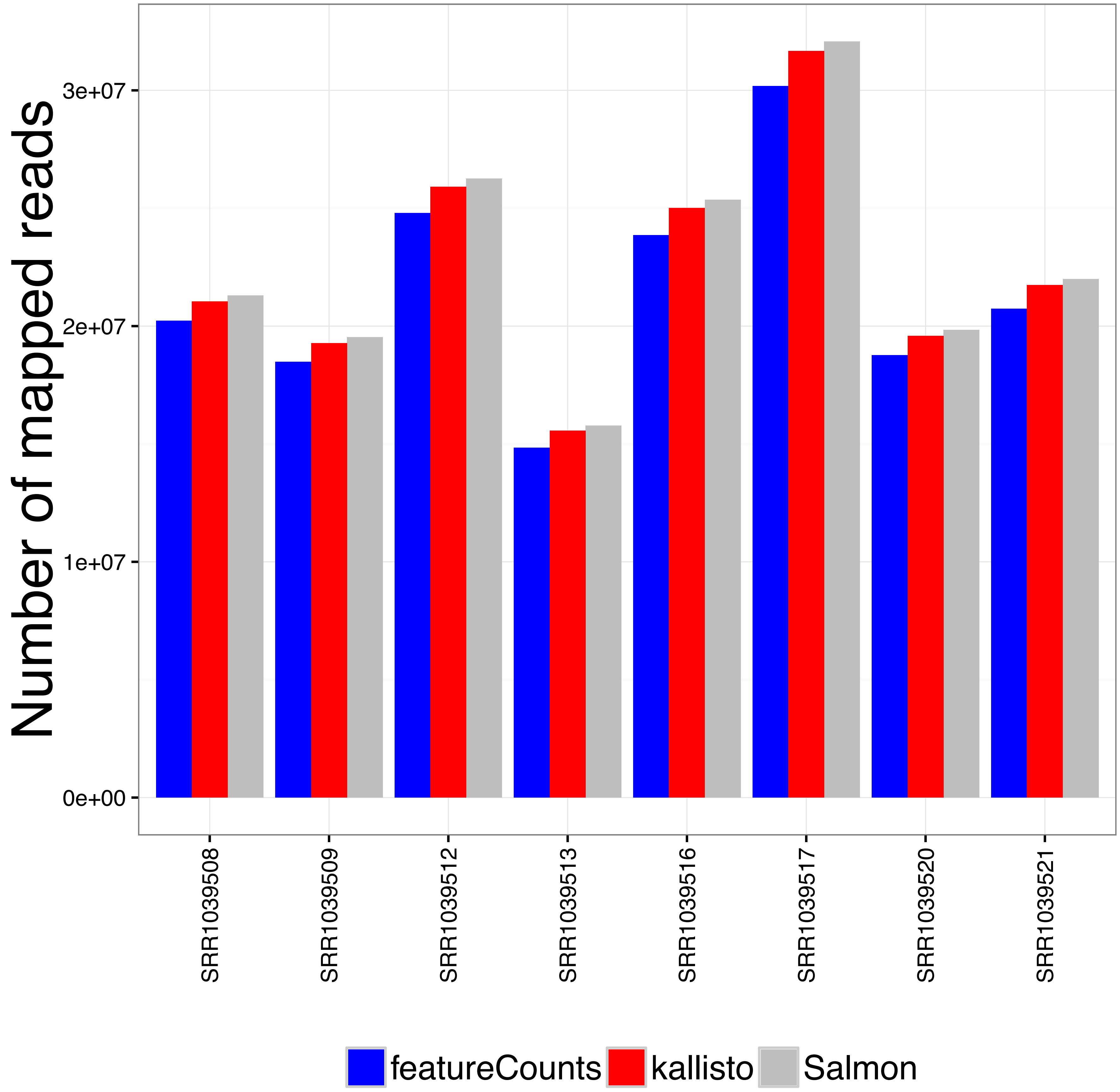
kallisto and Salmon gene counts overall similar



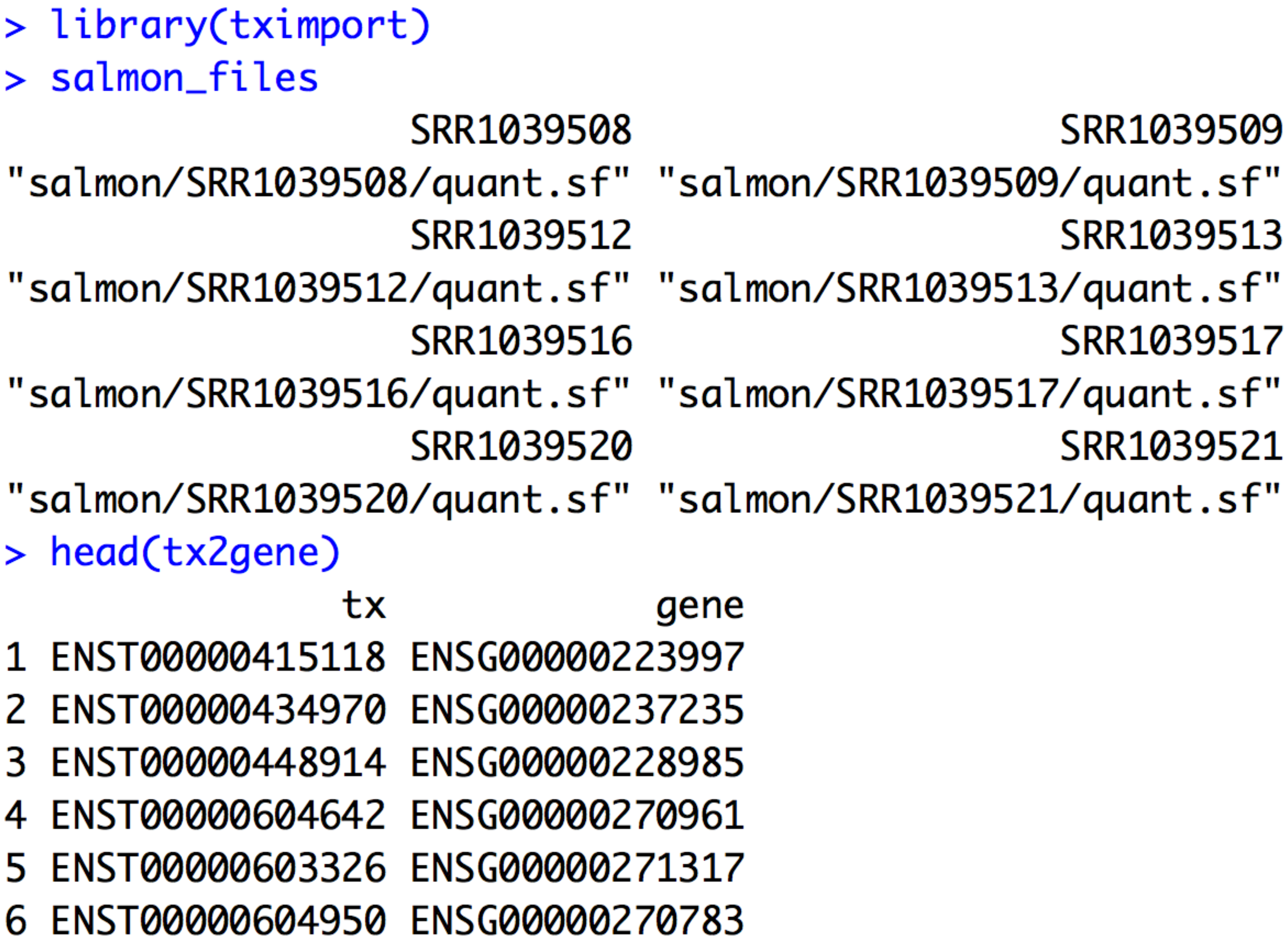
Gene-level counts mostly similar to alignment-based approach



kallisto and Salmon can use slightly more reads

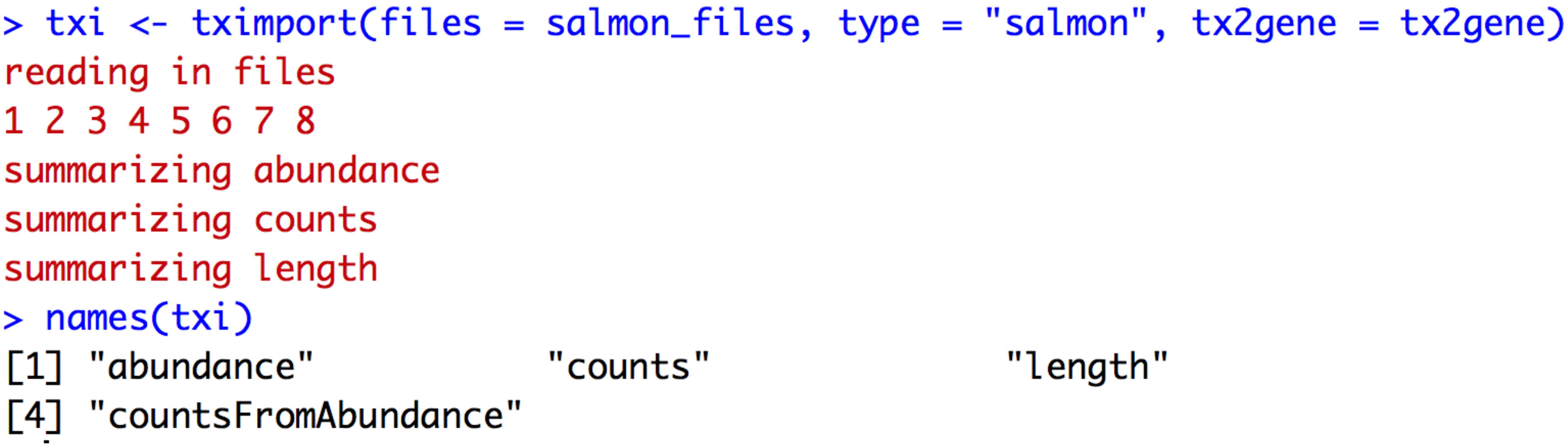


How to get the estimated values into R?



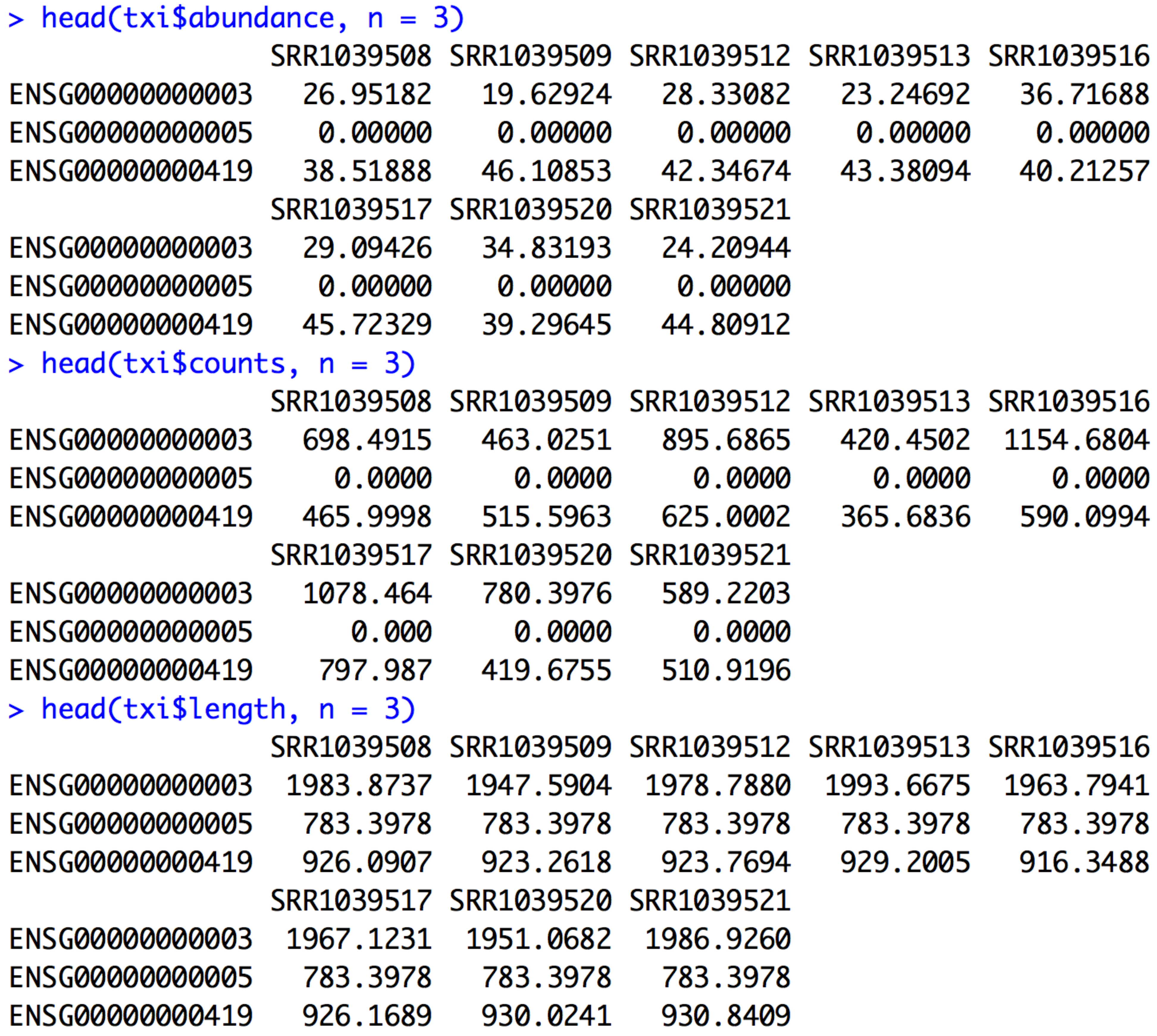
```
> library(tximport)
> salmon_files
                  SRR1039508                   SRR1039509 
"salmon/SRR1039508/quant.sf" "salmon/SRR1039509/quant.sf" 
                  SRR1039512                   SRR1039513 
"salmon/SRR1039512/quant.sf" "salmon/SRR1039513/quant.sf" 
                  SRR1039516                   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
```

How to get the estimated values into R?



```
> 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
```

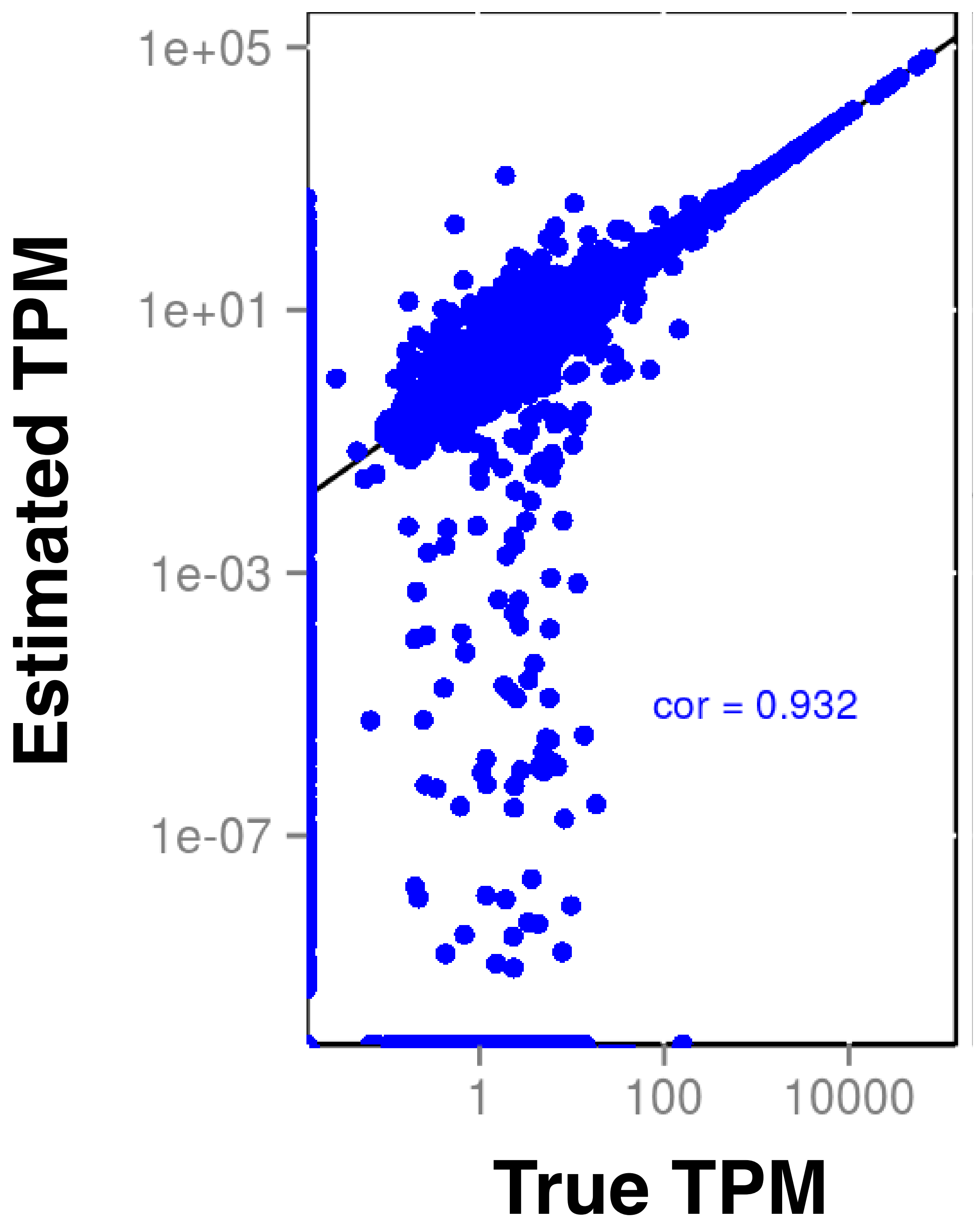
TPMs

counts

“ATL” offsets

A word of warning

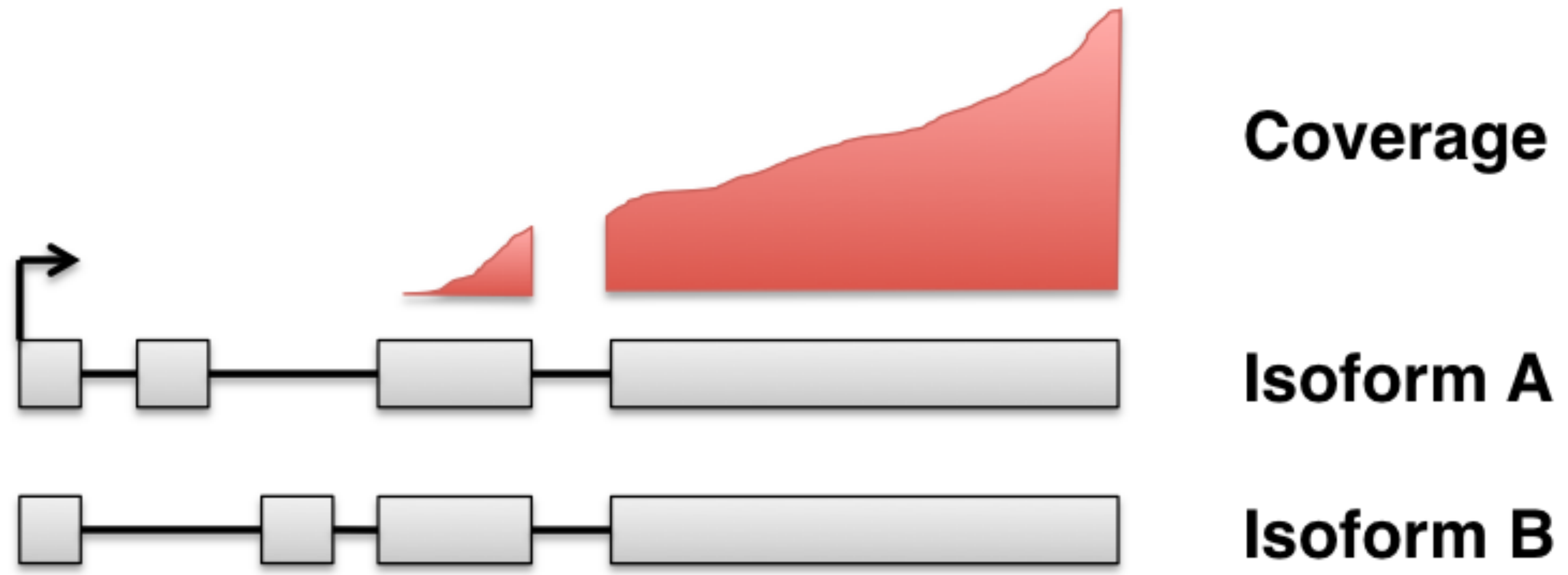
- Abundance estimates for lowly expressed transcripts are highly variable and should be interpreted with caution



Soneson, Love & Robinson, F1000 Research 2016

A word of warning

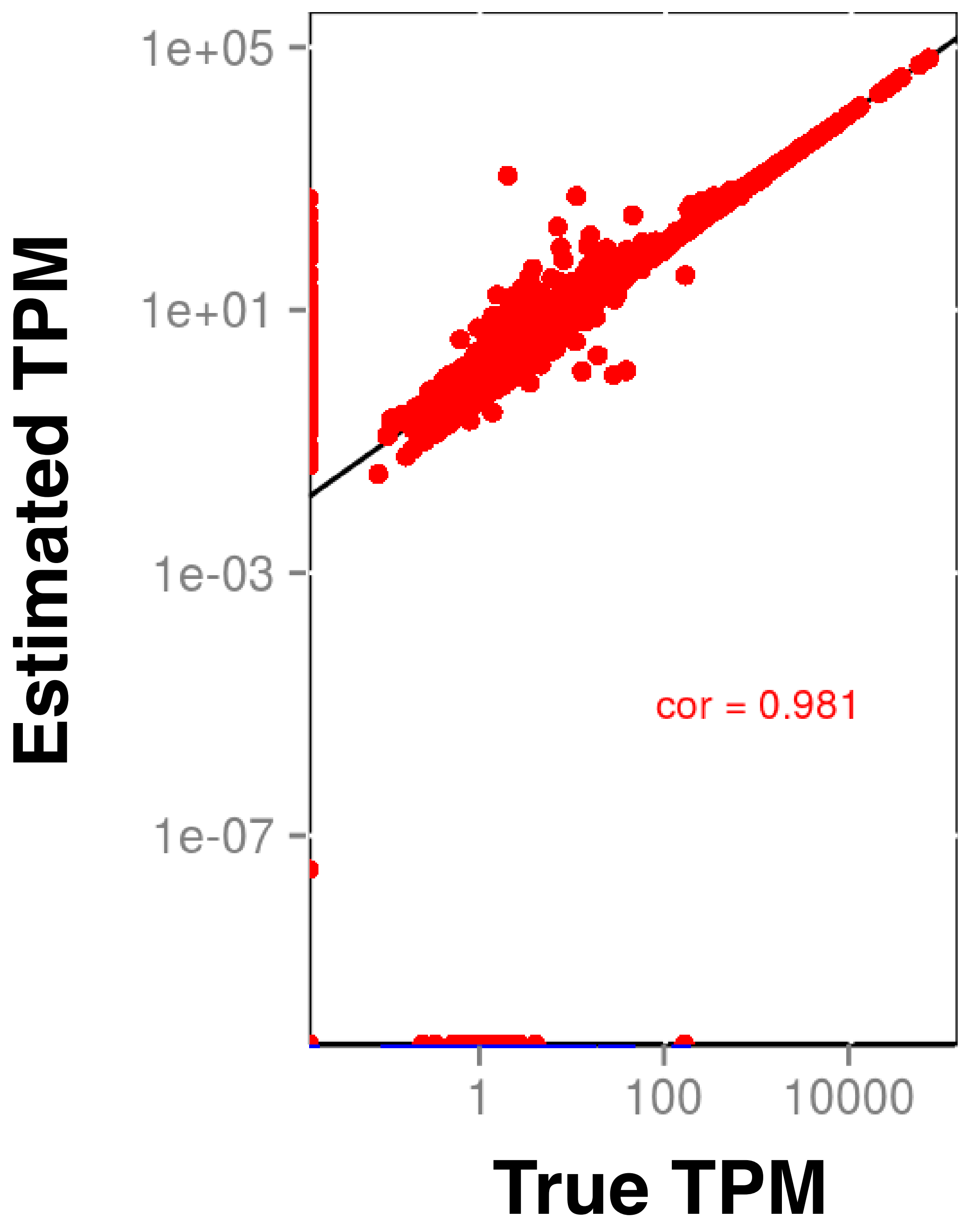
- Problematic when coverage of region defining an isoform is low



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

References

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- Patro et al.: Accurate, fast, and model-aware transcript expression quantification with Salmon. bioRxiv http://dx.doi.org/10.1101/021592 (2015) - **Salmon**
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- Pimentel et al.: Differential analysis of RNA-Seq incorporating quantification uncertainty. bioRxiv http://dx.doi.org/10.1101/058164 (2016) - **sleuth**
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- 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**