@CSoneson

High-throughput sequencing data processing

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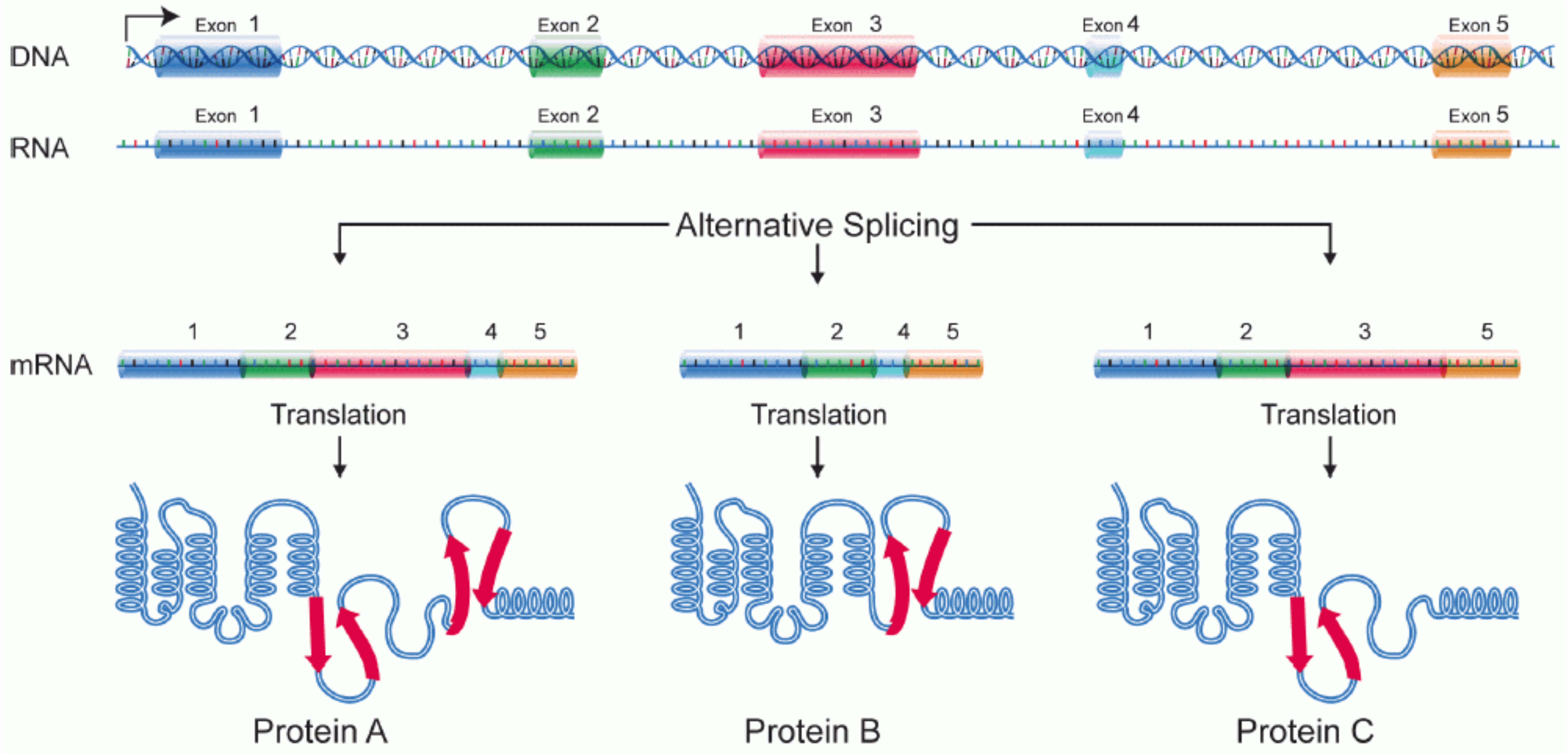
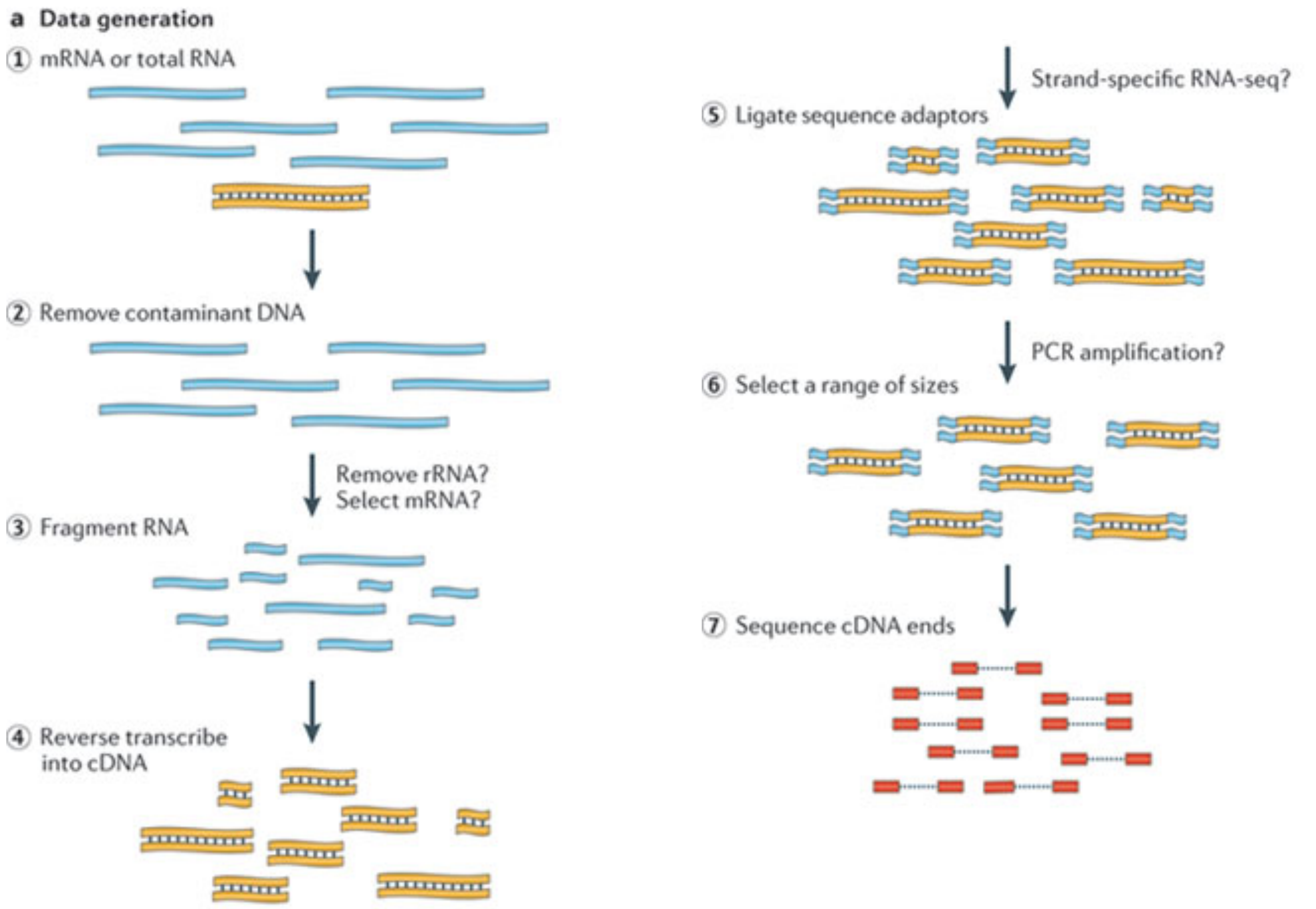


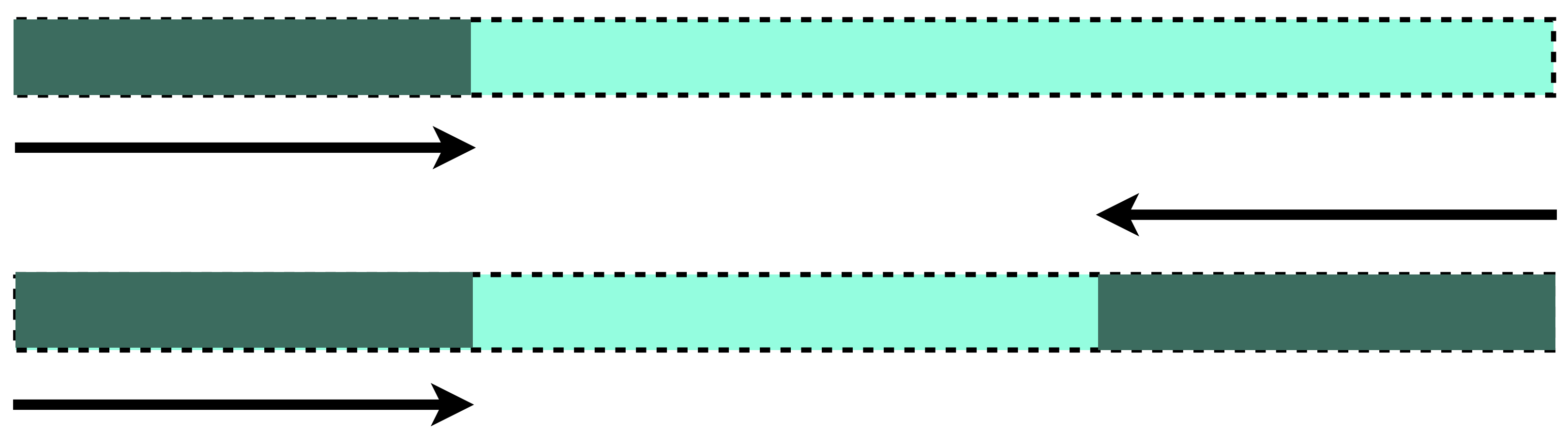
DNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
RNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Alternative Splicing
mRNA
1 2 3 4 5
1 2 4 5
1 2 3 5
Translation
Translation
Translation
Protein A
Protein B
Protein C

RNA-sequencing



Martin & Wang, Nat. Rev. Genetics (2011)

Single- vs paired-end sequencing



- Each fragment can be sequenced from one end only, or from both ends

- Single-end cheaper and faster

- Paired-end provide improved ability to localize the fragment in the genome and resolve mapping close to repeat regions - less multimapping reads

Strand-specificity

- In “standard” protocols, we don’t know from which strand a read stems
- Various “strand-specific” protocols allow us to keep this information
- Strand-specificity leads to lower number of ambiguous reads (overlapping multiple genes)

RESEARCH ARTICLE | OPEN ACCESS

Differentially expressed genes from RNA-Seq and functional enrichment results are affected by the choice of single-end versus paired-end reads and stranded versus non-stranded protocols

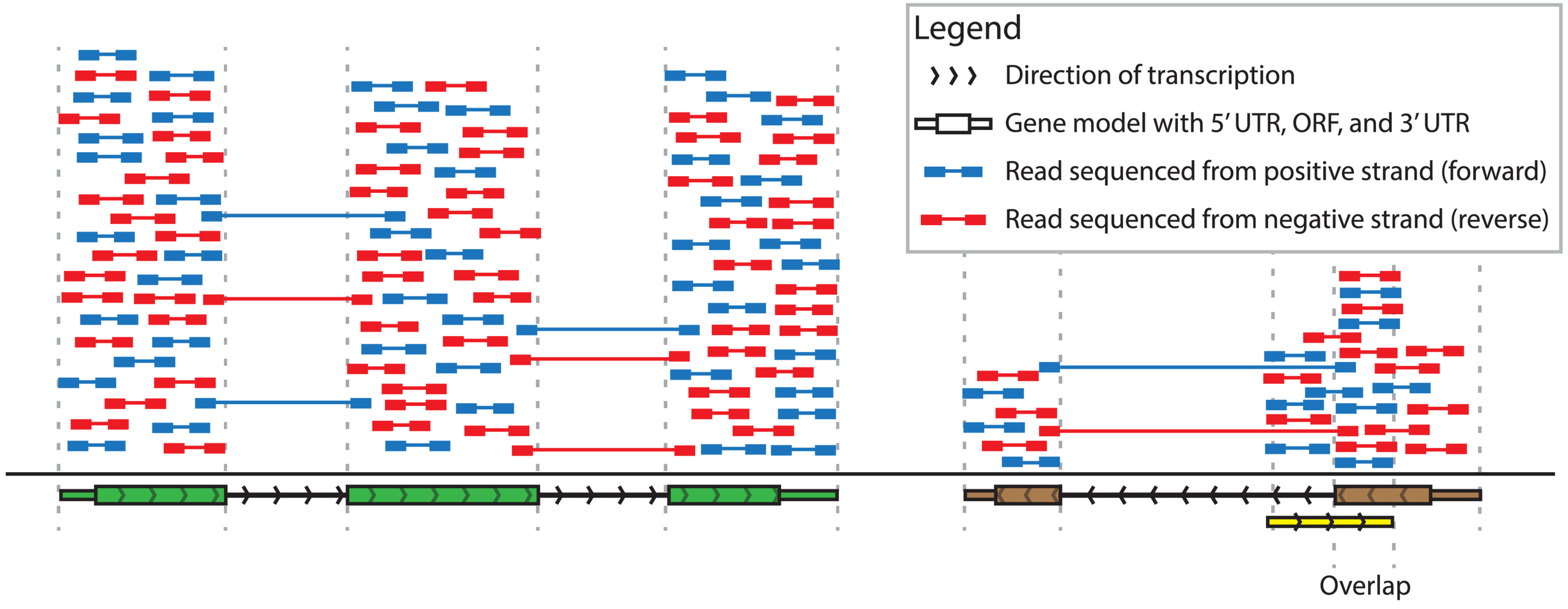
Susan M. Corley, Karen L. MacKenzie, Annemiek Beverdam, Louise F. Roddam and Marc R. Wilkins

BMC Genomics 2017 18:399 | DOI: 10.1186/s12864-017-3797-0 | ReadCube

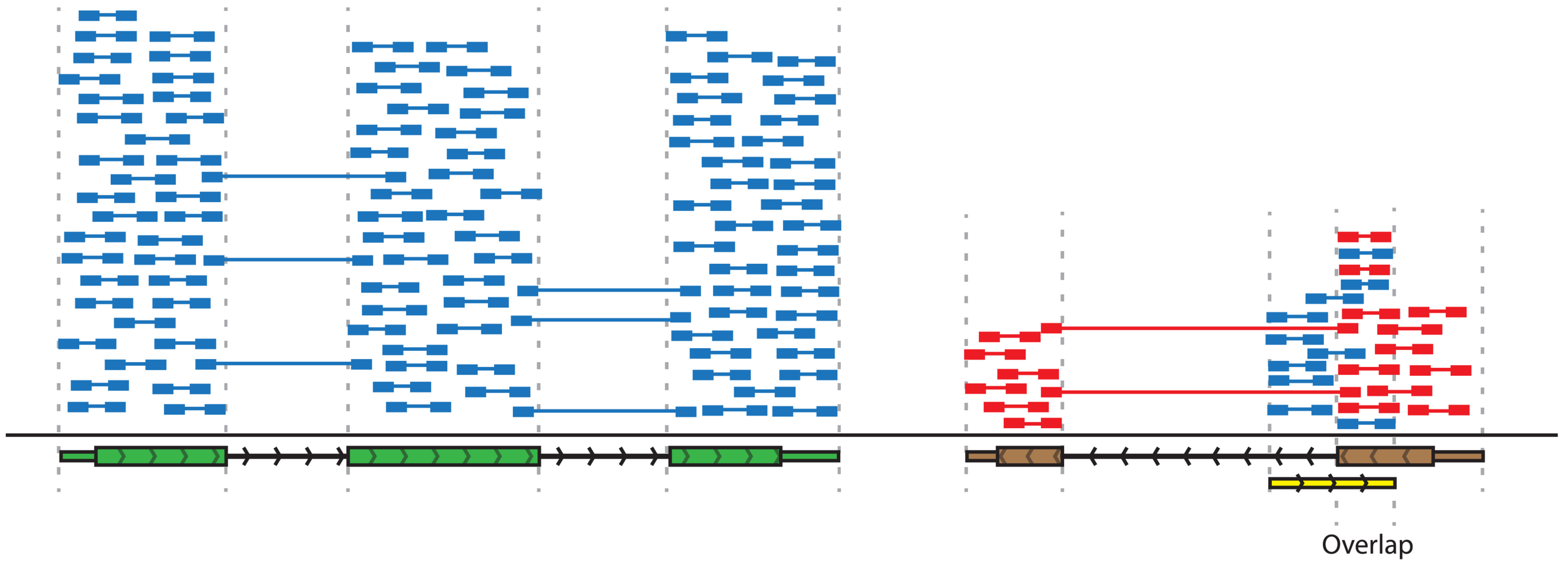
Received: 16 December 2016 | Accepted: 16 May 2017 | Published: 23 May 2017

Strand-specificity

A.

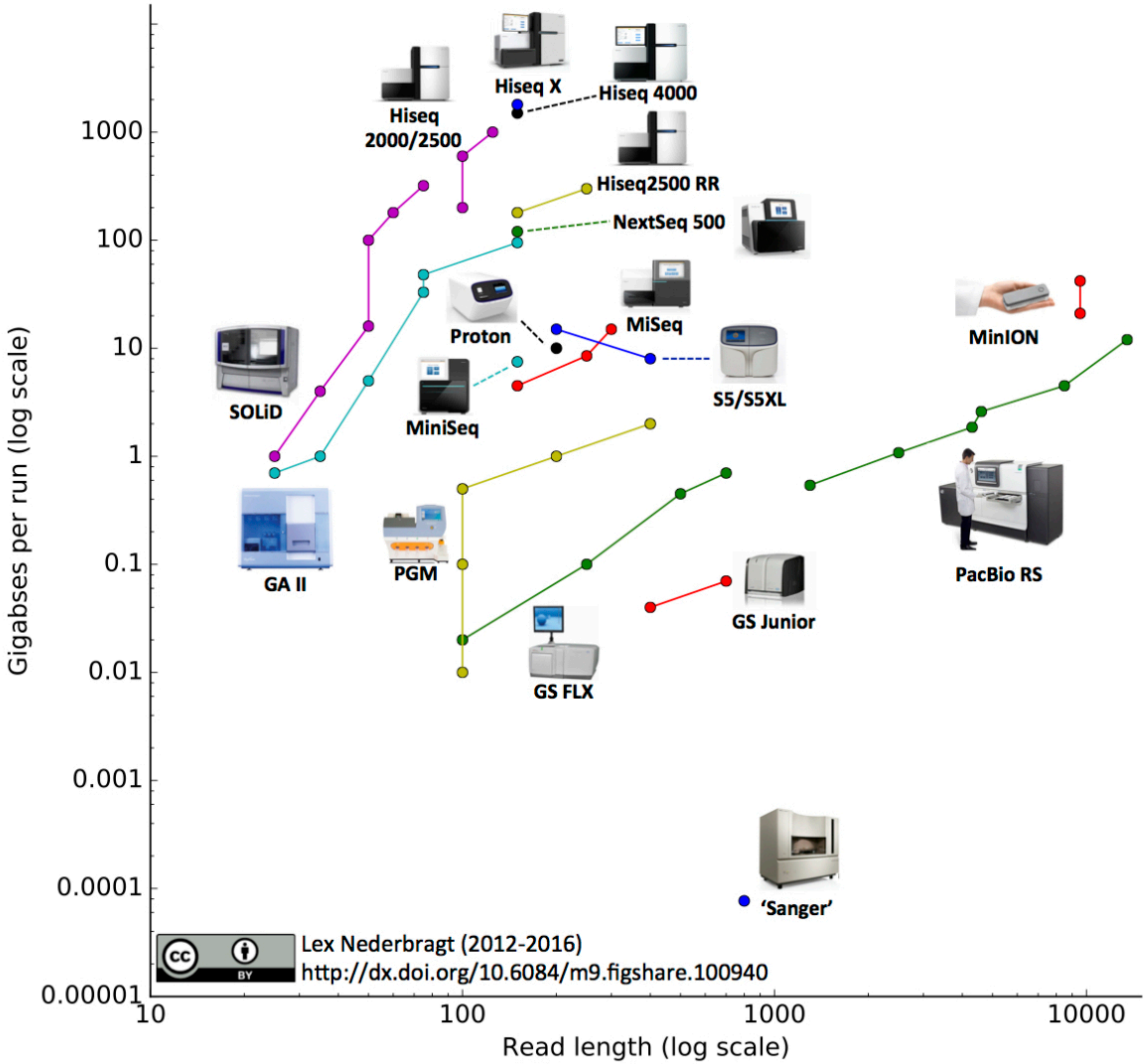


B.



https://doi.org/10.1371/journal.pcbi.1004393.g006

The world of sequencing technologies



Raw reads - FASTQ format

- Combines sequence and base quality information
- Four lines per sequence (read)
    - ID line (starting with @)
    - sequence
    - another ID line (starting with +)
    - base qualities
- For paired-end sequencing: one file for “first” reads and one for “second” reads

FASTQ format - sequence ID line

```
@D7MHBFN1:202:D1BUDACXX:4:1101:1340:1967 1:N:0:CATGCA
NATCTTCGGATCACTTTGGTCAAATTGAAACGATACAGAGAAGATTGTAAGTAACAATATTTACCAAGGTTCGAGTCATACTAACTCGTTGTCCTATAGT
+
#1=DDFFFHHHHHJJJJJJHIJIJJJIJIJGIIIJJJJJJIIJIJJJHIIFGIIIIJJJJJIIEHJIIHHGFFF@?ADFEDDEDCDDBDDBDCDDDDEC
```

- D7MHBFNI - unique instrument name
- 202 - run ID
- D1BUDACXX - flowcell ID
- 4 - flowcell lane
- 1101 - tile number within lane
- 1340 - x-coordinate of cluster within tile
- 1967 - y-coordinate of cluster within tile
- 1 - member of pair (1 or 2). Older versions: /1 and /2
- Y/N - whether the read failed quality control (Y = bad)
- 0 - none of the control bits are on
- CATGCA - index sequence (barcode)

FASTQ format - base qualities

- For each letter, estimate the probability of being erroneous (p)
- Phred score $Q = -10 \cdot \log_{10}(p)$

Phred score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10000	99.99%
50	1 in 100000	99.999%

Quality format encoding

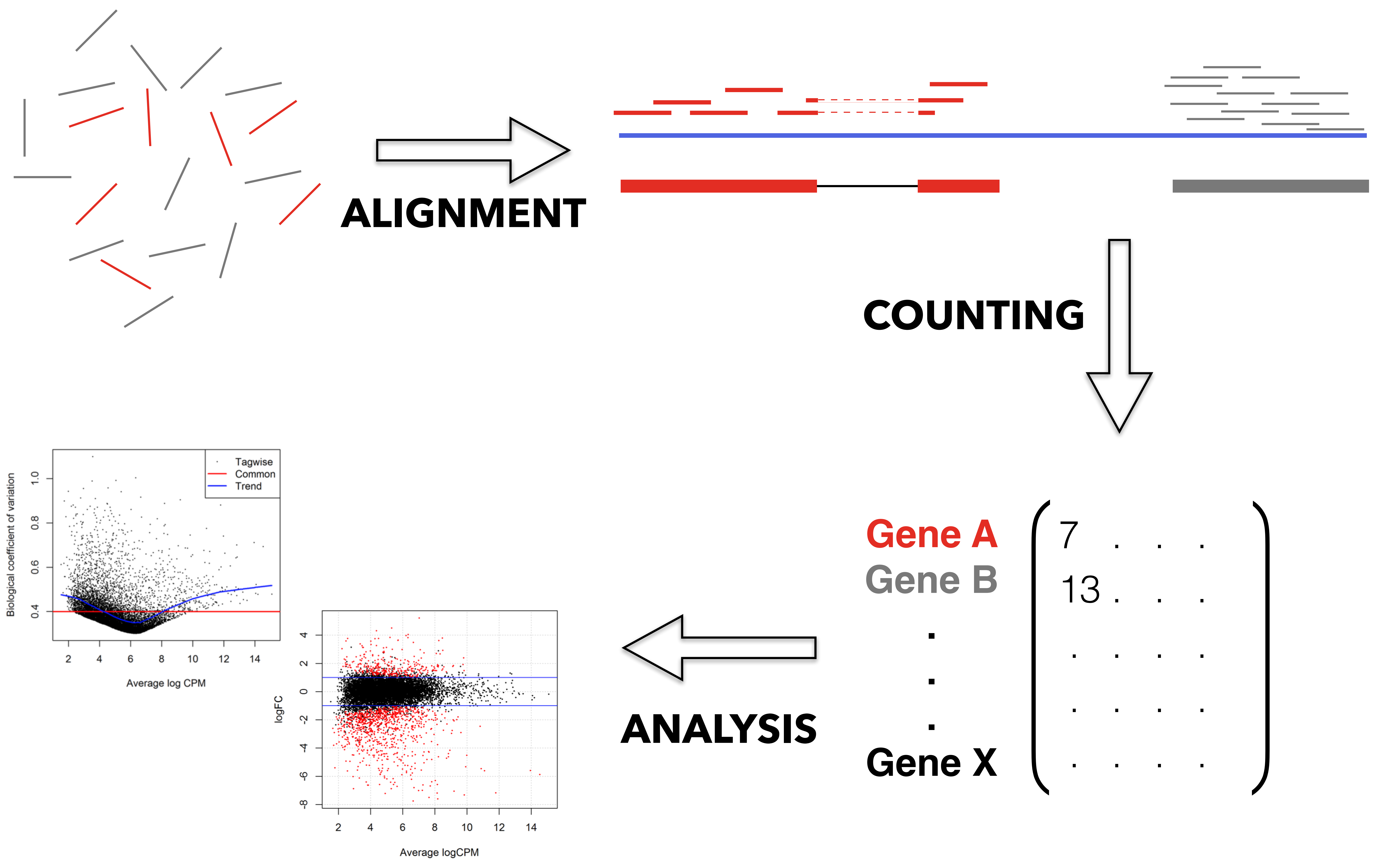
```
  SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS.....................................................
  ..........................XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX......................
  ...............................IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII......................
  ..................................JJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ......................
  LLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLLL....................................................
  !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
  |                         |    |        |                              |                     |
 33                        59   64       73                            104                   126
  0........................26...31.......40
                           -5....0........9.............................40
                                 0........9.............................40
                                    3.....9.............................40
  0........................26...31........41

 S - Sanger        Phred+33,  raw reads typically (0, 40)
 X - Solexa        Solexa+64, raw reads typically (-5, 40)
 I - Illumina 1.3+ Phred+64,  raw reads typically (0, 40)
 J - Illumina 1.5+ Phred+64,  raw reads typically (3, 40)
     with 0=unused, 1=unused, 2=Read Segment Quality Control Indicator (bold)
     (Note: See discussion above).
 L - Illumina 1.8+ Phred+33,  raw reads typically (0, 41)
```

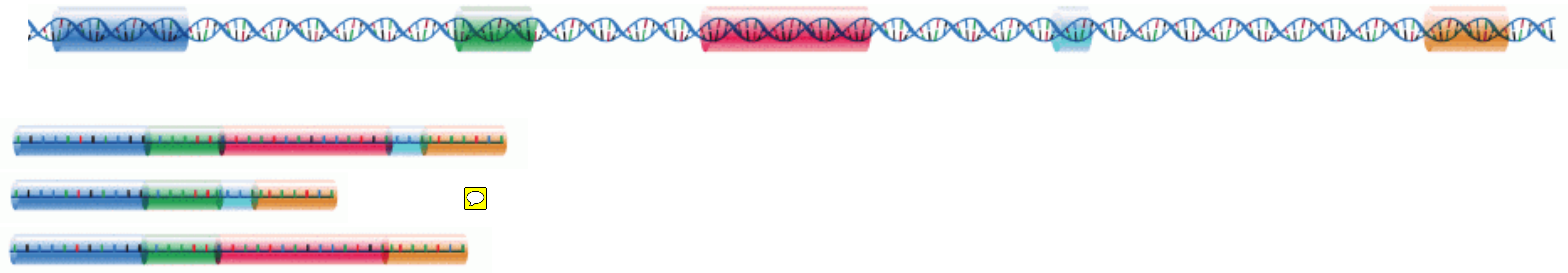
“Capital letters = good quality” (with Illumina 1.8+)

http://www.wikipedia.com/FASTQ_format

Alignment-based RNA-seq workflow

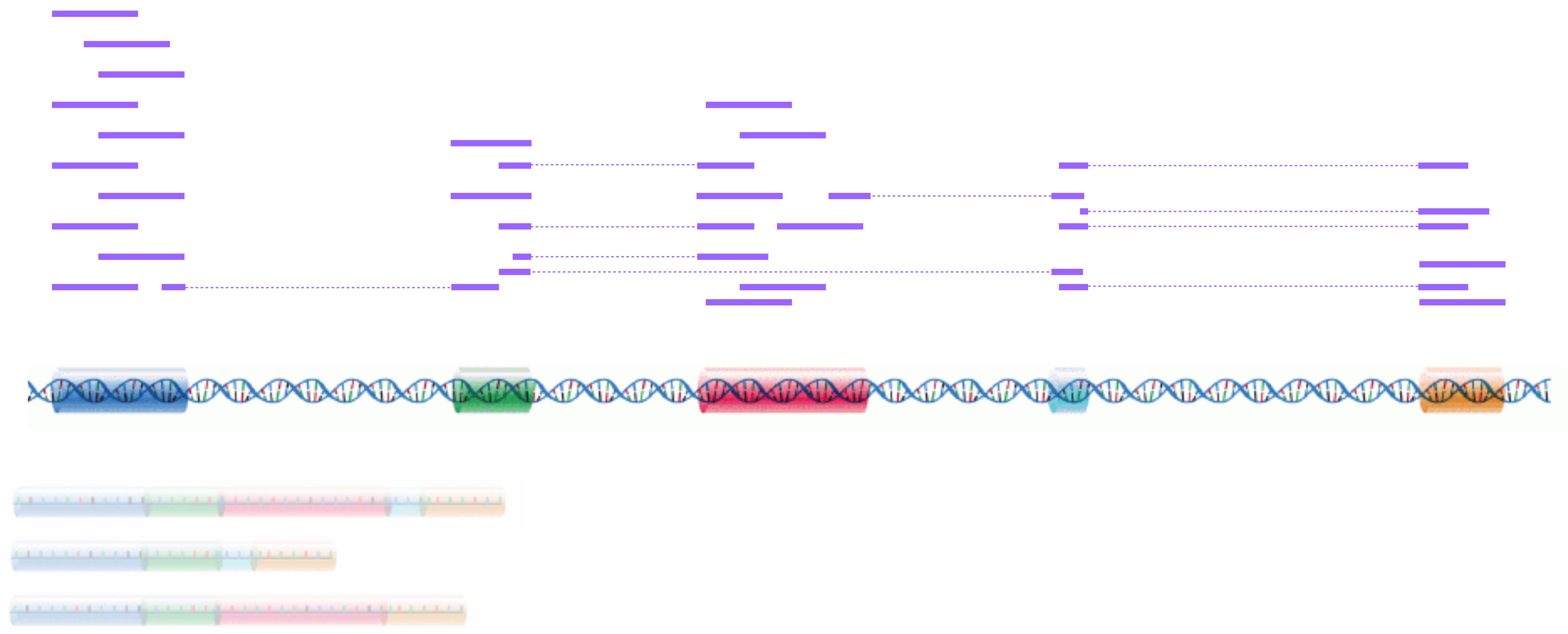


Abundance quantification



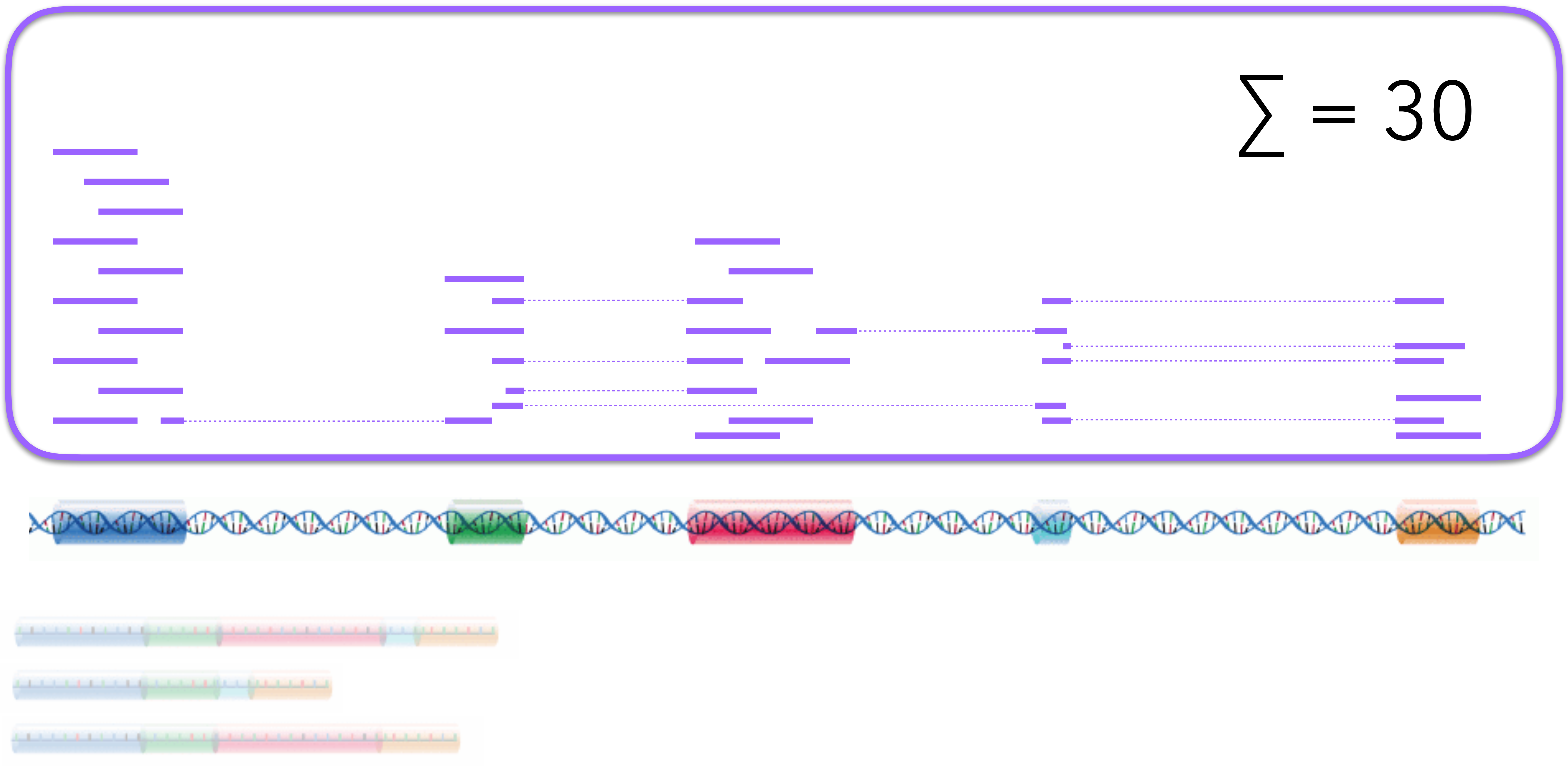
Abundance quantification

Gene-level counts, often obtained by genome alignment + overlap counting



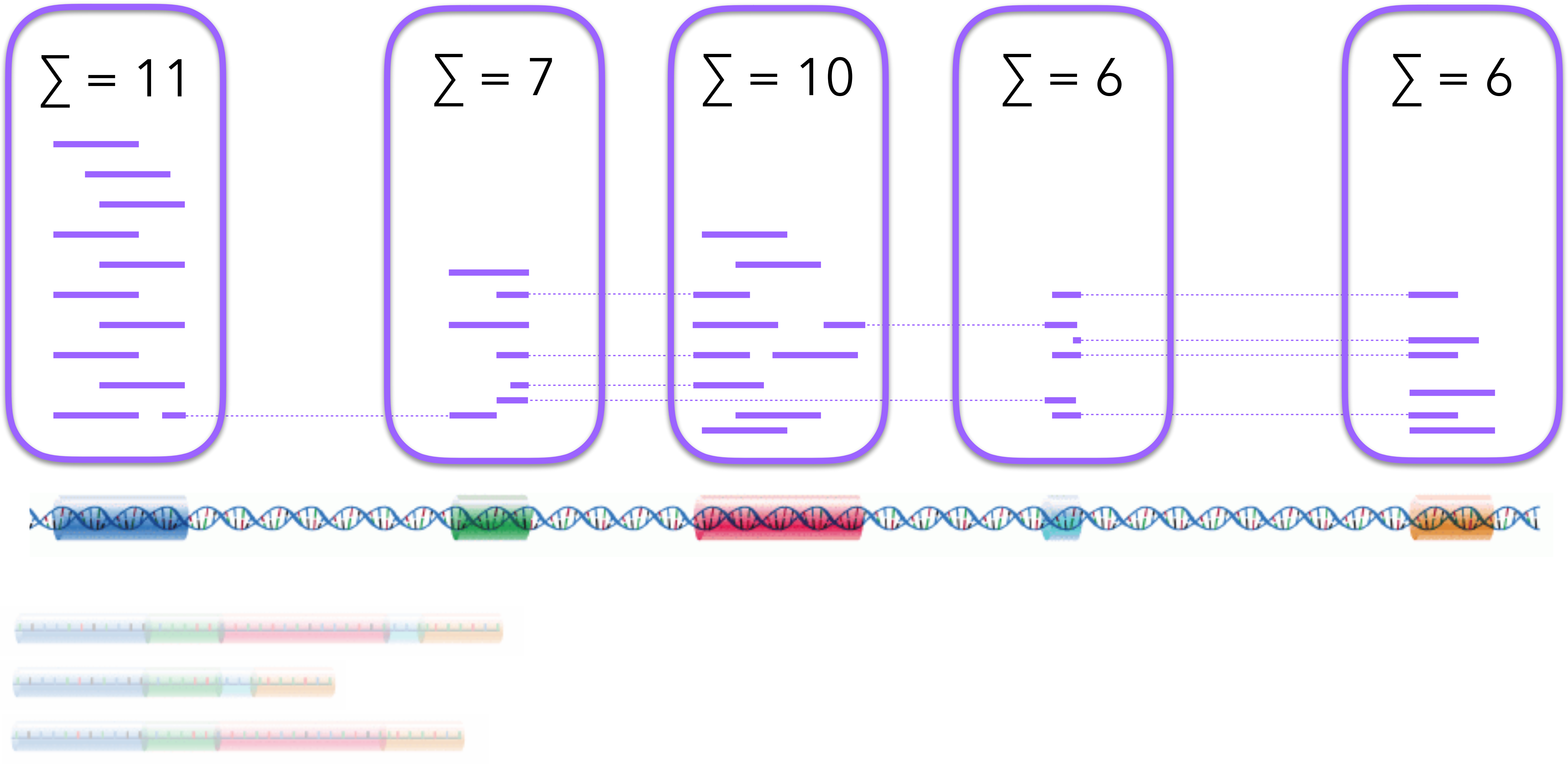
Abundance quantification

Gene-level counts, often obtained by genome alignment + overlap counting



Abundance quantification

Exon-level counts, often obtained by genome alignment + overlap counting



The human reference genome

- A “representative example” of the human genome sequence
- New versions are released periodically (the latest, GRCh38, in December 2013)
- Coordinates are not comparable across versions
- Typically provided as a *fasta* file (generic file format for biological sequences)

```
>chr1
......
GTTCTTGTTCTGTGTTCTTATAACCATACCAGAATTTTCTTCATCACAGA
CAGAGACTAAACTCTTTCTTCTCTTACCTTTCCTTTGATAATATTTTTGA
TCCAGGAATGGGGATAATTTTGCAGTTAAAATTTTCTTTTTATGATGGAA
GGTGAGGAGGAGAGAGAGGTTTACATTAGAAGTGACCCAACTCCATTTTC
TTCCAATGGTTTTTTTCAGTTTTATTTTTTTAAAGCGTGAACAGAGAATA
GTCACCTGATCAATTTAAATATGTCAAAAAGTGAAAGAAAAATCTCTCTT
TTAAAGGAAATGAGGGCAGTAACACAACCAAGGAATCAAAATTCAGGTTG
AGGCTGACCTTTGACCTGCAACTATGCTACTCCATGAACAGCAAGTAGGA
AATGGCTGATTTCATGAAGGTGGACTGGCATCAGAGGAGGCGAGGGATCC
AGGGTTCCTGATGAGTGGCAACATTCCTTGGTCTTTTGAGTTTGTTTGAT
TGGTGAATCAAATTTAGGTGACAGCCAGCTAAAGAGAGTGAGGGTGGCTG
TCTTGTGAATGGGAAGTGACCAAGCTTGAAAGCACAGACTgtggtggctc
......
```

The human reference genome

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

https://www.gencodegenes.org/human/

Single species data

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

Show 10 entries | Show/hide column

★	Species	DNA (FASTA)	cDNA (FASTA)	CDS (FASTA)	ncRNA (FASTA)	Protein sequence (FASTA)
Y	**Human** *Homo sapiens*	FASTA	FASTA	FASTA	FASTA	FASTA
Y	**Mouse** *Mus musculus*	FASTA	FASTA	FASTA	FASTA	FASTA
Y	**Zebrafish** *Danio rerio*	FASTA	FASTA	FASTA	FASTA	FASTA

Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz 840 MB

Fasta files

Content	Regions	Description	Download
Transcript sequences	CHR	• Nucleotide sequences of all transcripts on the reference chromosomes	Fasta
Protein-coding transcript sequences	CHR	• Nucleotide sequences of coding transcripts on the reference chromosomes • Transcript biotypes: protein_coding, nonsense_mediated_decay, non_stop_decay, IG_*_gene, TR_*_gene, polymorphic_pseudogene	Fasta
Protein-coding transcript translation sequences	CHR	• Amino acid sequences of coding transcript translations on the reference chromosomes • Transcript biotypes: protein_coding, nonsense_mediated_decay, non_stop_decay, IG_*_gene, TR_*_gene, polymorphic_pseudogene	Fasta
Long non-coding RNA transcript sequences	CHR	• Nucleotide sequences of long non-coding RNA transcripts on the reference chromosomes	Fasta
Genome sequence (GRCh38.p12)	ALL	• Nucleotide sequence of the GRCh38.p12 genome assembly version on all regions, including reference chromosomes, scaffolds, assembly patches and haplotypes • The sequence region names are the same as in the GTF/GFF3 files	Fasta
Genome sequence, primary assembly (GRCh38)	PRI	• Nucleotide sequence of the GRCh38 primary genome assembly (chromosomes and scaffolds) • The sequence region names are the same as in the GTF/GFF3 files	Fasta

Genomic locations of genes and other features

- Typically provided in a **gtf** (gene transfer format) file
- Similar to **gff**, but more standardized

```
seqname  source  feature  start  end  score  strand  frame  attribute

2R   protein_coding   exon  5139815    5141712    .    -    .      gene_id "FBgn0020621"; transcript_id
"FBtr0112897"; exon_number "10"; gene_name "Pkn"; gene_biotype "protein_coding"; transcript_name "Pkn-RG";
exon_id "FBgn0020621:1";
2R   protein_coding   CDS   5141572    5141712    .    -    0      gene_id "FBgn0020621"; transcript_id
"FBtr0112897"; exon_number "10"; gene_name "Pkn"; gene_biotype "protein_coding"; transcript_name "Pkn-RG";
protein_id "FBpp0111810";
2R   protein_coding   stop_codon 5141569    5141571    .    -    0      gene_id "FBgn0020621"; transcript_id
"FBtr0112897"; exon_number "10"; gene_name "Pkn"; gene_biotype "protein_coding"; transcript_name "Pkn-RG";
```

http://www.ensembl.org/info/website/upload/gff.html

Genomic locations of genes and other features

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

https://www.gencodegenes.org/human/

e this list via our home page.

Show/hide columns

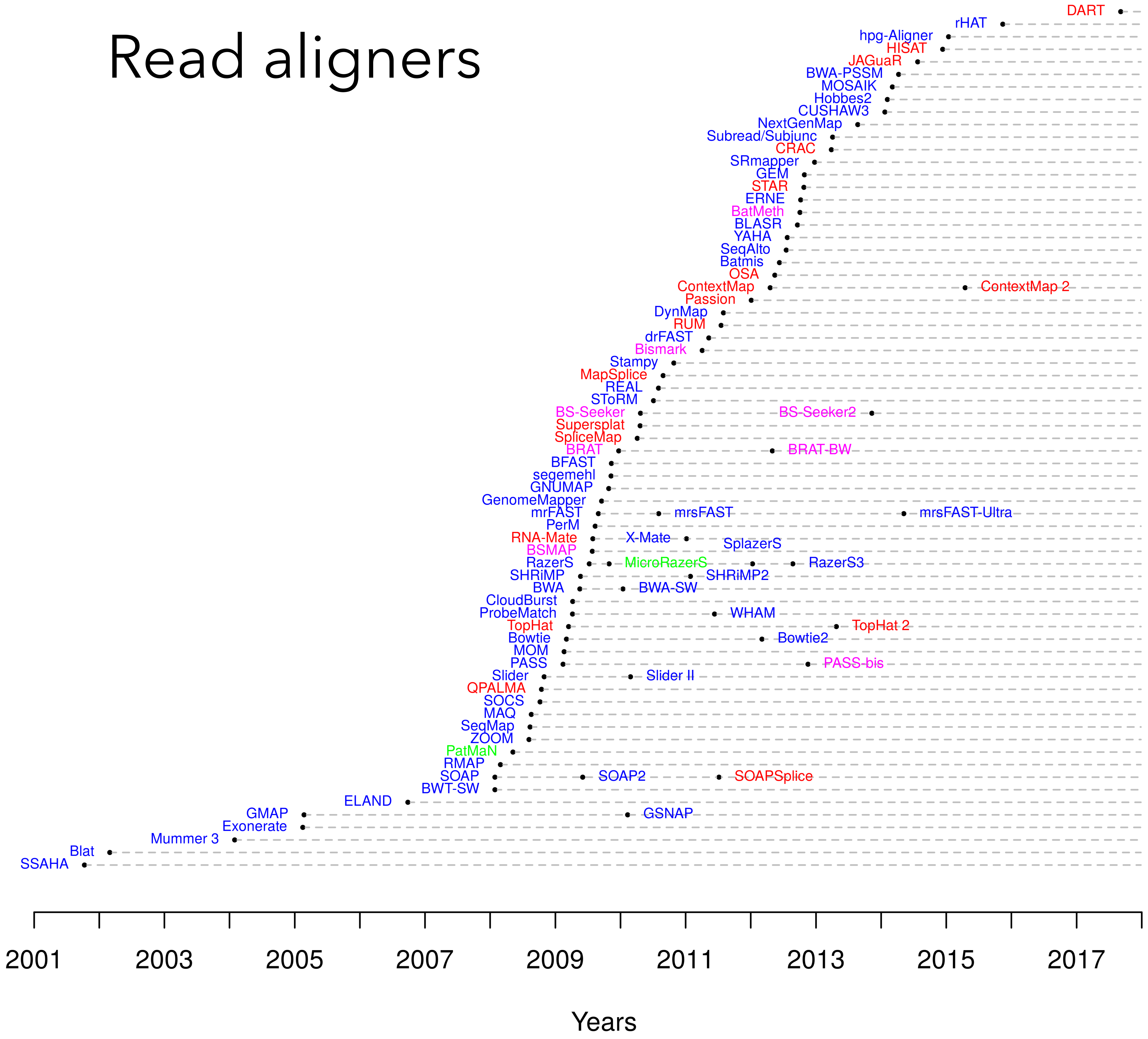
DS STA)	ncRNA (FASTA)	Protein sequence (FASTA)	Annotated sequence (EMBL)	Annotated sequence (GenBank)	Gene sets	Whole databases	Varia (G
TA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G
TA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G
TA	FASTA	FASTA	EMBL	GenBank	GTF GFF3	MySQL	G

GTF / GFF3 files

Content	Regions	Description	Download
Comprehensive gene annotation	CHR	• It contains the comprehensive gene annotation on the reference chromosomes only • This is the **main annotation file** for most users	GTF GFF3
Comprehensive gene annotation	ALL	• It contains the comprehensive gene annotation on the reference chromosomes, scaffolds, assembly patches and alternate loci (haplotypes) • This is a **superset** of the main annotation file	GTF GFF3
Comprehensive gene annotation	PRI	• It contains the comprehensive gene annotation on the primary assembly (chromosomes and scaffolds) sequence regions • This is a **superset** of the main annotation file	GTF GFF3
Basic gene annotation	CHR	• It contains the basic gene annotation on the reference chromosomes only • This is a **subset** of the corresponding comprehensive annotation, including only those transcripts tagged as 'basic' in every gene	GTF GFF3

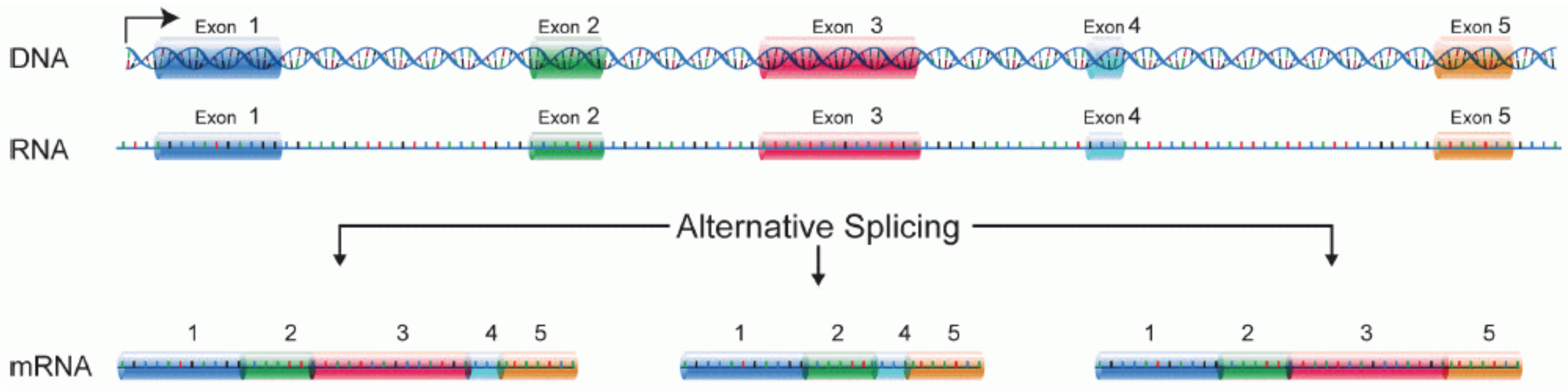
Read aligners

DNA
RNA
miRNA
bisulfite



http://www.ebi.ac.uk/~nf/hts_mappers/

Aligning RNA-seq reads



- Need a splice-aware aligner
- Common choices:
  - STAR
  - HISAT2

STAR: step 1 - indexing the genome

number of threads

output folder - name according to genome

```
$ STAR --runThreadN 24 \
       --runMode genomeGenerate \
       --genomeDir my_genome \
       --genomeFastaFiles my_genome.fa \
       --sjdbGTFfile my_genes.gtf \
       --sjdbOverhang 99
```

read length - 1

STAR: step 2 - aligning the reads

created index

read file(s)

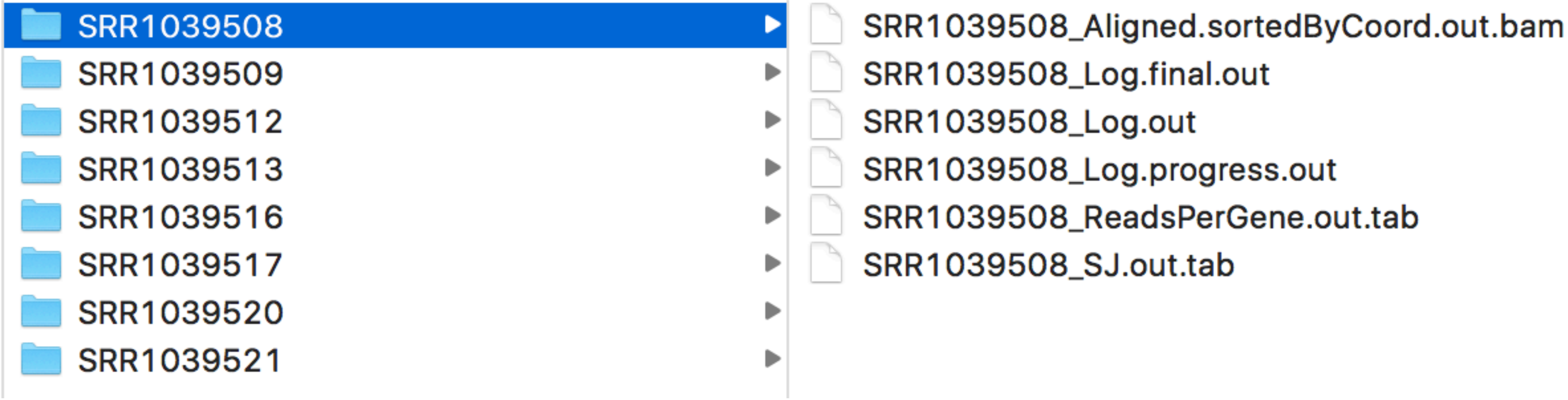
[include sample ID]

```
$ STAR --runThreadN 24 \
       --runMode alignReads \
       --genomeDir my_genome \
       --readFilesIn S1_read1.fq.gz \
         S1_read2.fq.gz \
       --readFilesCommand zcat \
       --outFileNamePrefix output/S1/ \
       --outSAMtype BAM SortedByCoordinate \
       --quantMode GeneCounts
```

count reads

for compressed read files

STAR: output



SRR1039508
SRR1039509
SRR1039512
SRR1039513
SRR1039516
SRR1039517
SRR1039520
SRR1039521
SRR1039508_Aligned.sortedByCoord.out.bam
SRR1039508_Log.final.out
SRR1039508_Log.out
SRR1039508_Log.progress.out
SRR1039508_ReadsPerGene.out.tab
SRR1039508_SJ.out.tab

Representing alignments - SAM format

- Header

```
@SQ  SN:chr1   LN:249250621
@SQ  SN:chr2   LN:243199373
@SQ  SN:chr3   LN:198022430
@SQ  SN:chr4   LN:191154276
```

- Body

```
seq.13906018   0    chr10     101948233 255  101M *    0    0
GTCCACAGTCCTTTCTCTGAAACCCTTGGGNNAAGTTGTTTCAGAATTANGNAA   CBCFFFFFHHHHHJJJJJJJJJJJJJJJ##11?
DHIIIIJJHIJJJJ#0#07     0L:A:F    IH:i:1    HI:i:1
```

- Typically, one line per alignment

- BAM = binary SAM

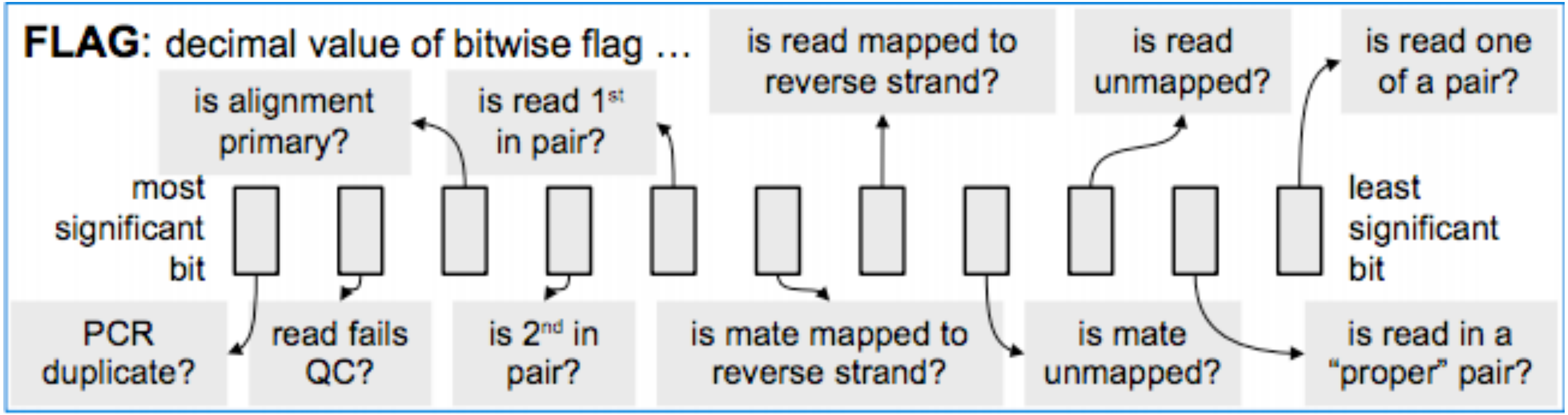
Representing alignments - SAM format

```
seq.13906018  0    chr10    101948233 255  101M *    0    0
GTCCACAGTCCTTTCTCTGAAACCCTTGGGNNAAGTTGTTTCAGAATTANGNAA   CBCFFFFFHHHHJJJJJJJJJJJJJJJ##11?
DHIIIIJJHIJJJJ#0#07     0L:A:F    IH:i:1    HI:i:1
```

- Column 1 - sequence ID
- Column 2 - flag. Ex:
  - 0 - non-paired read, mapping to forward strand
  - 16 - non-paired read, mapping to reverse strand
  - 4 - unmapped read
- Column 3 - reference sequence name for the alignment
- Column 4 - position of alignment
- Column 5 - mapping quality
  - 255 - not available
  - 0 - multiple best hits
- Column 6 - CIGAR string
- Column 7-8 - reference name/position of mate/next segment
- Column 9 - observed template length
- Column 10 - sequence (represented as mapped on the reference (forward) strand!)
- Column 11 - base quality
- Remaining columns are optional, and are of the type TAG:TYPE:VALUE

http://samtools.sourceforge.net/SAM1.pdf

The SAM flag



- ex: 83 = 00001010011 = first in pair, read on reverse strand, part of properly mapped pair

http://broadinstitute.github.io/picard/explain-flags.html

The CIGAR string

Op	BAM	Description
M	0	alignment match (can be a sequence match or mismatch)
I	1	insertion to the reference
D	2	deletion from the reference
N	3	skipped region from the reference
S	4	soft clipping (clipped sequences present in SEQ)
H	5	hard clipping (clipped sequences NOT present in SEQ)
P	6	padding (silent deletion from padded reference)
=	7	sequence match
X	8	sequence mismatch

- Describes the mapping in more detail
- See also the MD tag

The CIGAR string - example

```
RefPos:     1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19
Reference:  C  C  A  T  A  C  T  G  A  A  C  T  G  A  C  T  A  A  C
Read: ACTAGAATGGCT
```

Aligning these two:

```
RefPos:     1  2  3  4  5  6  7     8  9  10 11 12 13 14 15 16 17 18 19
Reference:  C  C  A  T  A  C  T     G  A  A  C  T  G  A  C  T  A  A  C
Read:                   A  C  T  A  G  A  A     T  G  G  C  T
```

With the alignment above, you get:

```
POS: 5
CIGAR: 3M1I3M1D5M
```

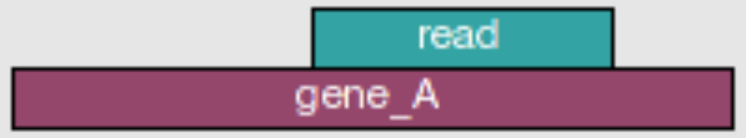
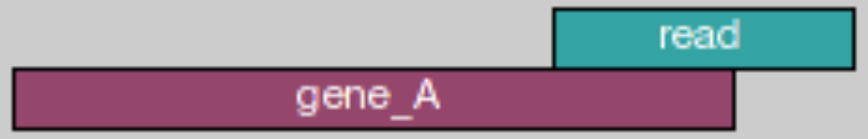
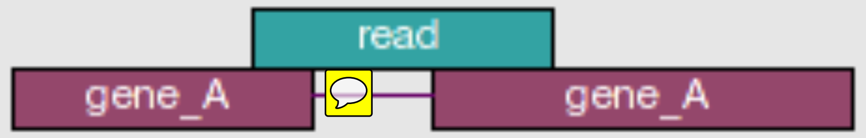
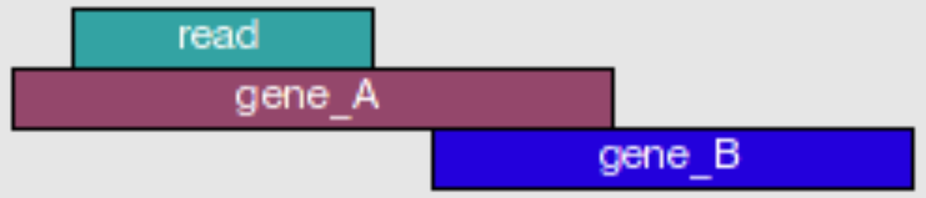
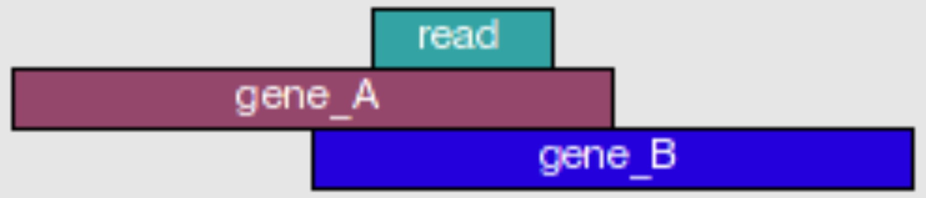
Working with SAM/BAM files

- SAMtools
  - convert between SAM/BAM
  - sort/index
  - view alignments
  - …
  - R interface in the Rsamtools package

Estimating abundances via overlap counting

- STAR
- HTseq-count (Python)
- Rsubread::featureCounts (R)
- GenomicAlignments::summarizeOverlaps (R)

Counting 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

http://www-huber.embl.de/HTSeq/doc/count.html

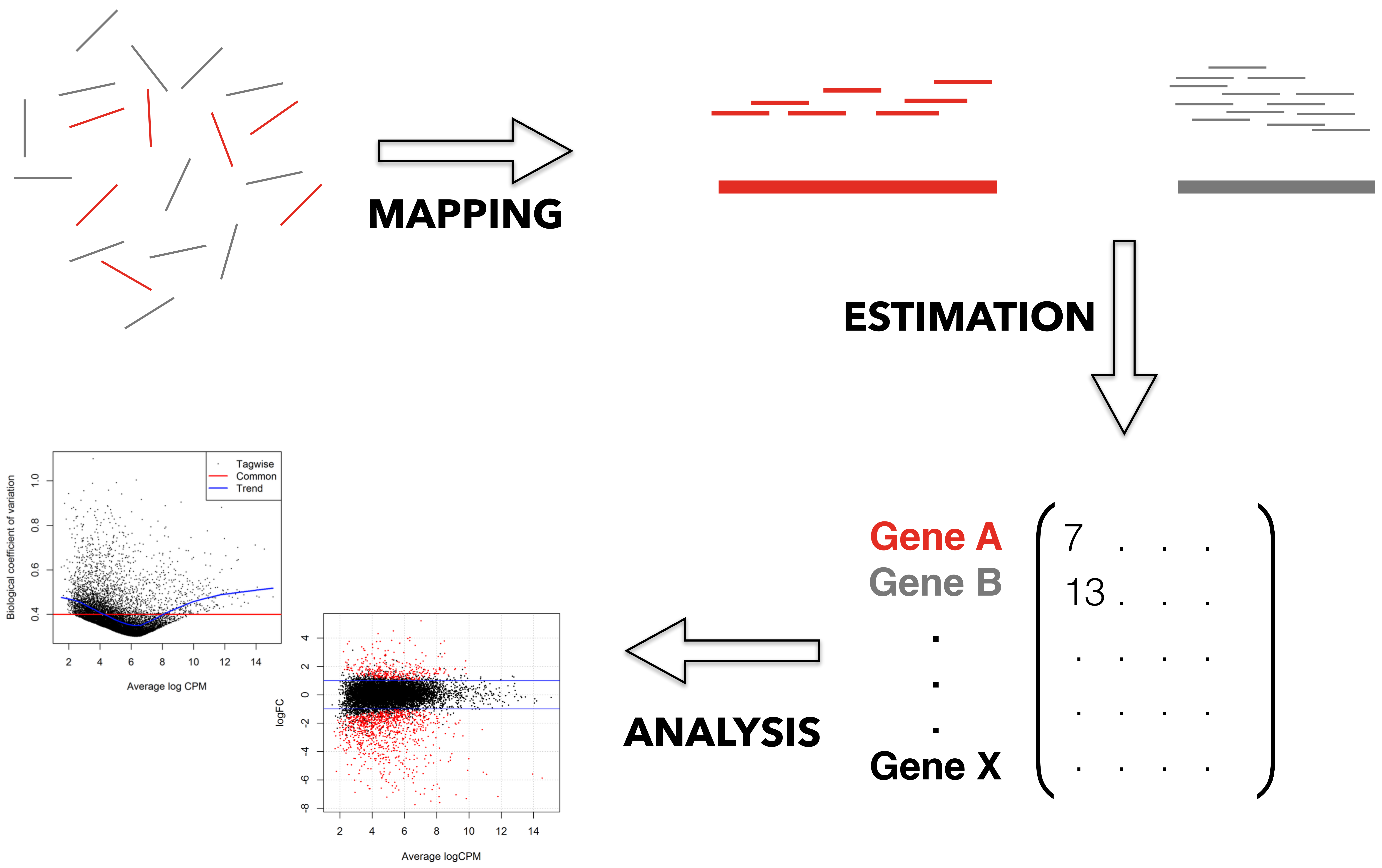
featureCounts

```
> featureCounts(files = bamfiles,
                annot.ext = "my_genes.gtf",
                isGTFAnnotationFile = TRUE,
                GTF.featureType = "exon",
                GTF.attrType = "gene_id",
                useMetaFeatures = TRUE,
                isPairedEnd = TRUE,
                strandSpecific = 0)
```

check your GTF file!

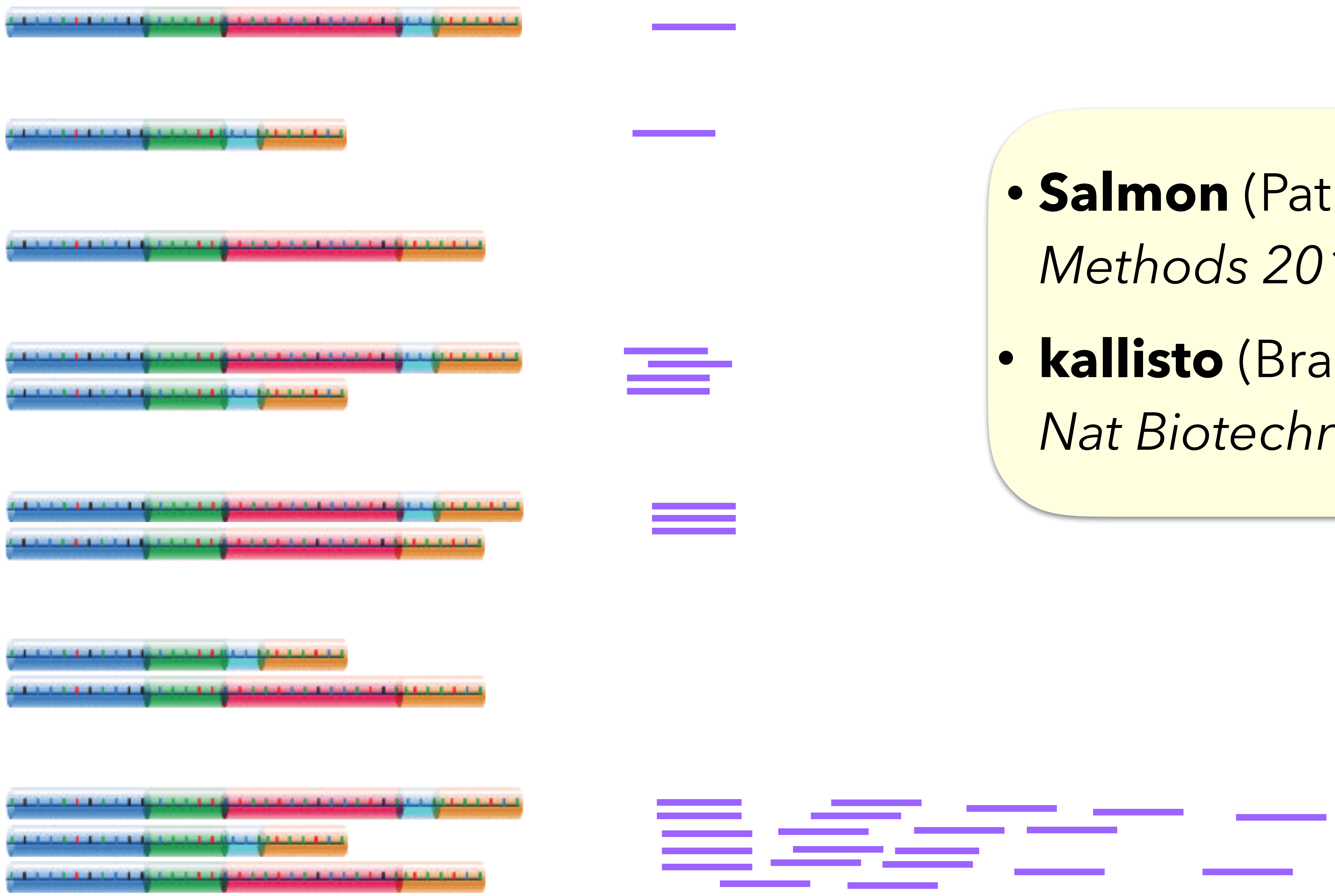
```
2R	protein_coding	exon	5139815	5141712	.	-	.	gene_id "FBgn0020621"; transcript_id
"FBtr0112897"; exon_number "10"; gene_name "Pkn"; gene_biotype "protein_coding"; transcript_name "Pkn-RG";
exon_id "FBgn0020621:1";
```

Alignment-free RNA-seq workflow



Abundance quantification

Equivalence class counts, often obtained by "alignment-free" estimation methods



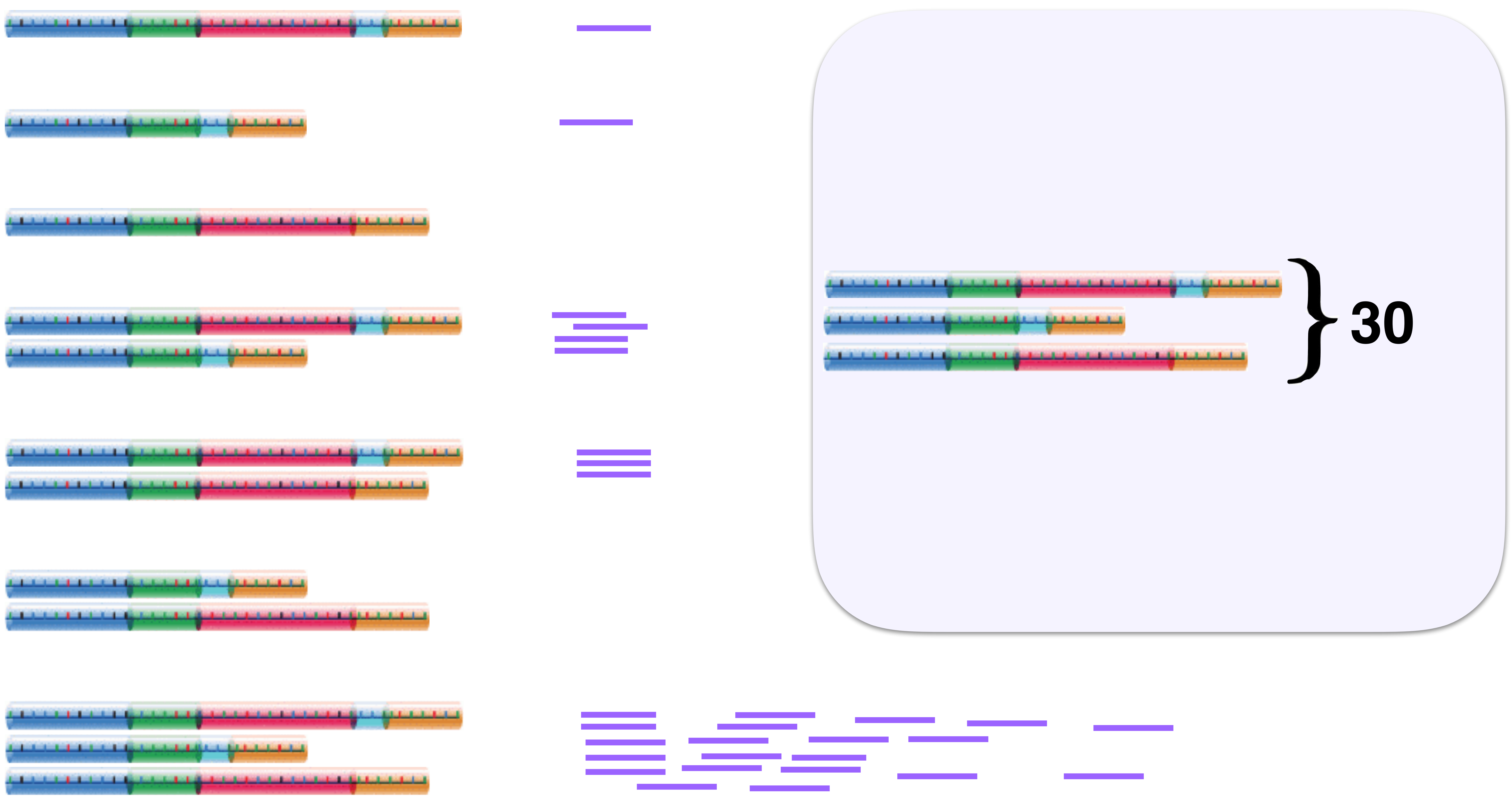
- **Salmon** (Patro et al, *Nat Methods 2017*)
- **kallisto** (Bray et al, *Nat Biotechnol* 2016)

Abundance quantification

Transcript-level counts, often obtained by "alignment-free" estimation methods

Abundance quantification

Gene-level counts, obtained by summation of transcript-level counts



Reference transcript sequences

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 column

★	Species	DNA (FASTA)	cDNA (FASTA)	CDS (FASTA)	ncRNA (FASTA)	Protein sequence (FASTA)
Y	**Human** *Homo sapiens*	FASTA	FASTA	FASTA	FASTA	FASTA
Y	**Mouse** *Mus musculus*	FASTA	FASTA	FASTA	FASTA	FASTA
Y	**Zebrafish** *Danio rerio*	FASTA	FASTA	FASTA	FASTA	FASTA

https://www.gencodegenes.org/human/

Fasta files

Content	Regions	Description	Download
Transcript sequences	CHR	• Nucleotide sequences of all transcripts on the reference chromosomes	Fasta
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Long non-coding RNA transcript sequences	CHR	• Nucleotide sequences of long non-coding RNA transcripts on the reference chromosomes	Fasta
Genome sequence (GRCh38.p12)	ALL	• Nucleotide sequence of the GRCh38.p12 genome assembly version on all regions, including reference chromosomes, scaffolds, assembly patches and haplotypes • The sequence region names are the same as in the GTF/GFF3 files	Fasta
Genome sequence, primary assembly (GRCh38)	PRI	• Nucleotide sequence of the GRCh38 primary genome assembly (chromosomes and scaffolds) • The sequence region names are the same as in the GTF/GFF3 files	Fasta

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
```

Step 2: estimate transcript abundances

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

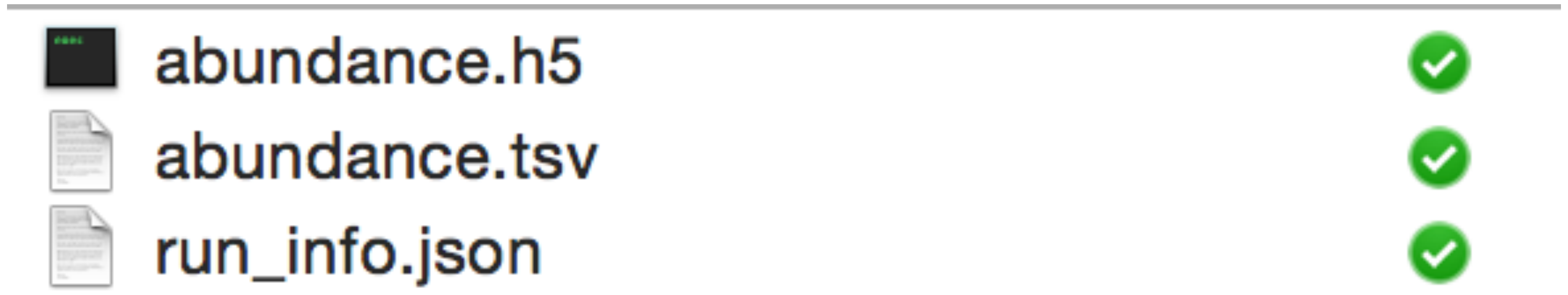
Salmon

libtype

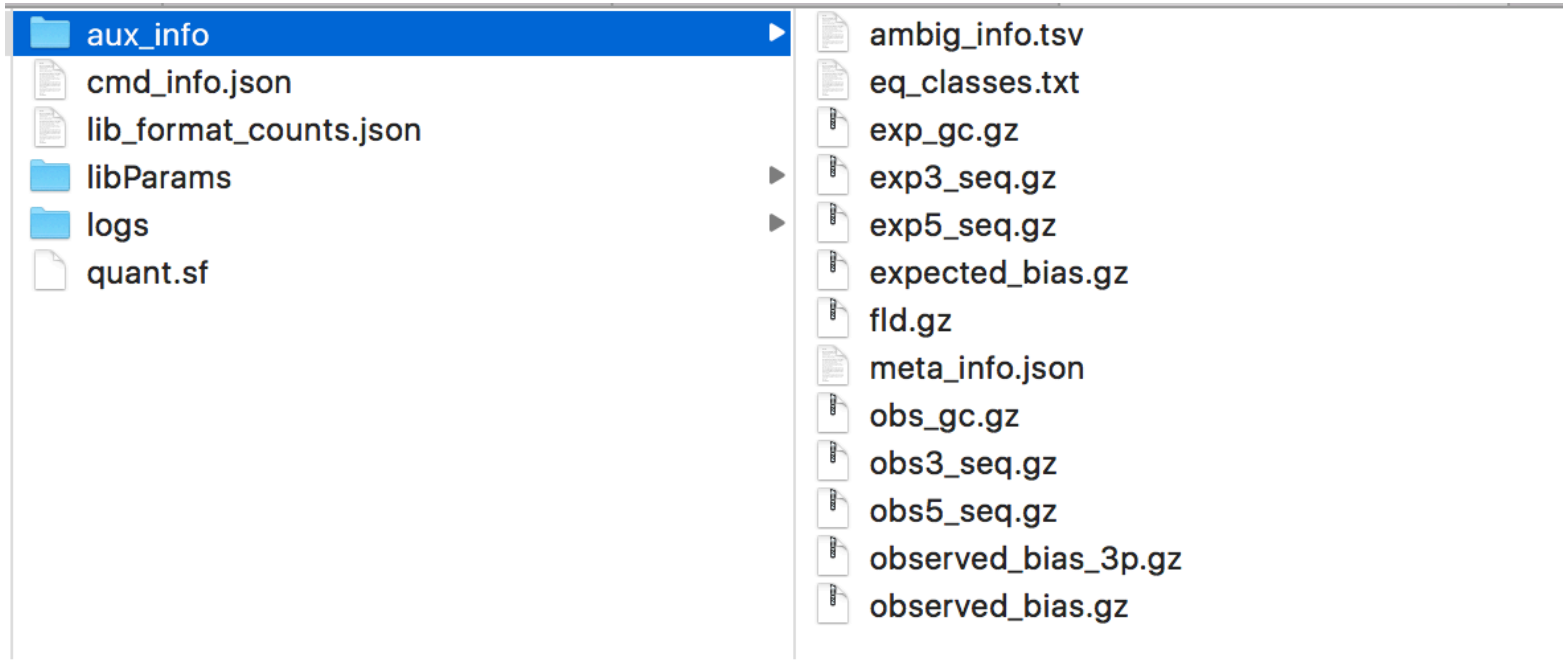
```
$ salmon quant -i my_transcripts.idx -l A \
        -1 sample1_1.fastq -2 sample1_2.fastq \
        -p 10 -o results/sample1 --validateMappings \
        --numBootstraps 30 --seqBias --gcBias
```

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

Reading the estimated values into R

```
> library(tximport)
> salmon_files
                  SRR1039508                   SRR1039509                   SRR1039512 
"salmon/SRR1039508/quant.sf" "salmon/SRR1039509/quant.sf" "salmon/SRR1039512/quant.sf" 
                  SRR1039513                   SRR1039516                   SRR1039517 
"salmon/SRR1039513/quant.sf" "salmon/SRR1039516/quant.sf" "salmon/SRR1039517/quant.sf" 
                  SRR1039520                   SRR1039521 
"salmon/SRR1039520/quant.sf" "salmon/SRR1039521/quant.sf" 
> head(tx2gene)
                tx               gene
1 ENST00000456328.2 ENSG00000223972.5
2 ENST00000450305.2 ENSG00000223972.5
3 ENST00000488147.1 ENSG00000227232.5
4 ENST00000619216.1 ENSG00000278267.1
5 ENST00000473358.1 ENSG00000243485.5
6 ENST00000469289.1 ENSG00000243485.5
```

Reading the estimated values into R

```
> txi <- tximport::tximport(files = salmon_files, type = "salmon",
+                           tx2gene = tx2gene)
reading in files with read_tsv
1 2 3 4 5 6 7 8
summarizing abundance
summarizing counts
summarizing length
> names(txi)
[1] "abundance"           "counts"              "length"              "countsFromAbundance"
```

Reading the estimated values into R

```
> head(txi$counts, 3)
                   SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517
ENSG00000000003.14     707.21    463.445    896.252    421.186    1185.87   1086.289
ENSG00000000005.5        0.00      0.000      0.000      0.000       0.00      0.000
ENSG00000000419.12     454.00    508.999    606.000    352.999     583.00    773.000
                   SRR1039520 SRR1039521
ENSG00000000003.14        802    596.220
ENSG00000000005.5           0      0.000
ENSG00000000419.12        410    500.001
> head(txi$abundance, 3)
                   SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517
ENSG00000000003.14   29.87883   21.13732   30.40148   23.50615   38.90846   31.20858
ENSG00000000005.5     0.00000    0.00000    0.00000    0.00000    0.00000    0.00000
ENSG00000000419.12   44.97429   54.55385   47.13428   44.61619   44.52030   51.50911
                   SRR1039520 SRR1039521
ENSG00000000003.14   36.73055   25.60277
ENSG00000000005.5     0.00000    0.00000
ENSG00000000419.12   43.01582   49.56923
> head(txi$length, 3)
                   SRR1039508 SRR1039509 SRR1039512 SRR1039513 SRR1039516 SRR1039517
ENSG00000000003.14  1869.3397  1871.3781  1924.5532  2029.1670  2001.5240  1919.7958
ENSG00000000005.5    783.9375   783.9375   783.9375   783.9375   783.9375   783.9375
ENSG00000000419.12   797.2506   796.3534   839.3260   895.9974   859.9590   827.7119
                   SRR1039520 SRR1039521
ENSG00000000003.14  1969.7615  1973.4448
ENSG00000000005.5    783.9375   783.9375
ENSG00000000419.12   859.8489   854.7984
```

counts

TPMs

“ATL” offsets

Even cooler: tximeta

```
> library(tximeta)
> meta
           SampleName    cell   dex albut      names avgLength Experiment    Sample
SRR1039508 GSM1275862  N61311 untrt untrt SRR1039508       126  SRX384345 SRS508568
SRR1039509 GSM1275863  N61311   trt untrt SRR1039509       126  SRX384346 SRS508567
SRR1039512 GSM1275866 N052611 untrt untrt SRR1039512       126  SRX384349 SRS508571
SRR1039513 GSM1275867 N052611   trt untrt SRR1039513        87  SRX384350 SRS508572
SRR1039516 GSM1275870 N080611 untrt untrt SRR1039516       120  SRX384353 SRS508575
SRR1039517 GSM1275871 N080611   trt untrt SRR1039517       126  SRX384354 SRS508576
SRR1039520 GSM1275874 N061011 untrt untrt SRR1039520       101  SRX384357 SRS508579
SRR1039521 GSM1275875 N061011   trt untrt SRR1039521        98  SRX384358 SRS508580
              BioSample                        files
SRR1039508 SAMN02422669 salmon/SRR1039508/quant.sf
SRR1039509 SAMN02422675 salmon/SRR1039509/quant.sf
SRR1039512 SAMN02422678 salmon/SRR1039512/quant.sf
SRR1039513 SAMN02422670 salmon/SRR1039513/quant.sf
SRR1039516 SAMN02422682 salmon/SRR1039516/quant.sf
SRR1039517 SAMN02422673 salmon/SRR1039517/quant.sf
SRR1039520 SAMN02422683 salmon/SRR1039520/quant.sf
SRR1039521 SAMN02422677 salmon/SRR1039521/quant.sf
```

Even cooler: tximeta

```
> st <- tximeta::tximeta(meta)
importing quantifications
reading in files with read_tsv
1 2 3 4 5 6 7 8
found matching transcriptome:
[ Gencode - Homo sapiens - release 29 ]
loading existing TxDb created: 2019-03-24 16:32:30
generating transcript ranges
fetching genome info
> sg <- tximeta::summarizeToGene(st)
loading existing TxDb created: 2019-03-24 16:32:30
obtaining transcript-to-gene mapping from TxDb
summarizing abundance
summarizing counts
summarizing length
```

Even cooler: tximeta

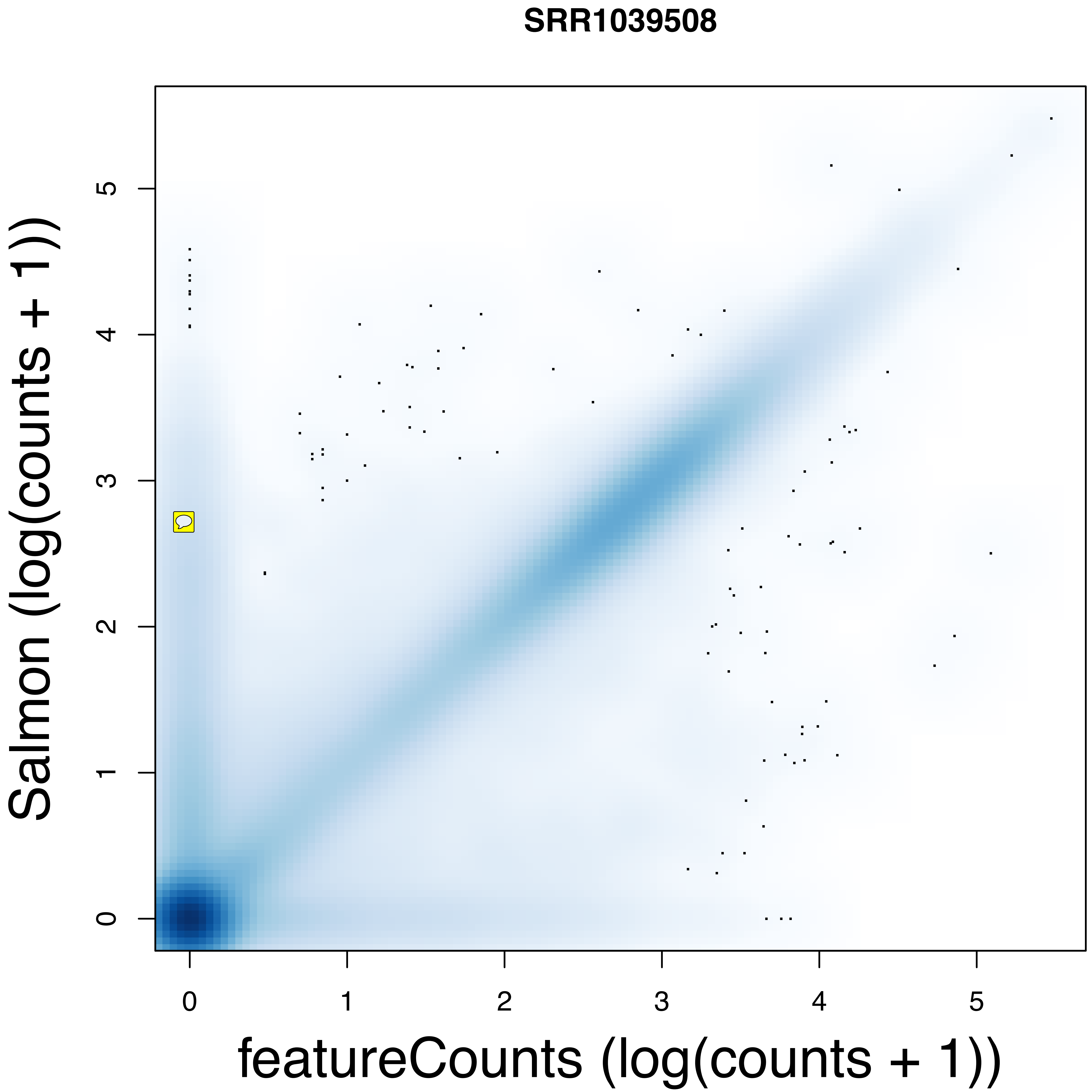
```
> st
class: RangedSummarizedExperiment
dim: 205870 8
metadata(5): tximetaInfo quantInfo countsFromAbundance txomeInfo txdbInfo
assays(3): counts abundance length
rownames(205870): ENST00000456328.2 ENST00000450305.2 ... ENST00000387460.2
  ENST00000387461.2
rowData names(3): tx_id gene_id tx_name
colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
colData names(9): SampleName cell ... Sample BioSample
> sg
class: RangedSummarizedExperiment
dim: 58294 8
metadata(5): tximetaInfo quantInfo countsFromAbundance txomeInfo txdbInfo
assays(3): counts abundance length
rownames(58294): ENSG00000000003.14 ENSG00000000005.5 ... ENSG00000285993.1
  ENSG00000285994.1
rowData names(1): gene_id
colnames(8): SRR1039508 SRR1039509 ... SRR1039520 SRR1039521
colData names(9): SampleName cell ... Sample BioSample
```

Comparison to alignment-based workflow

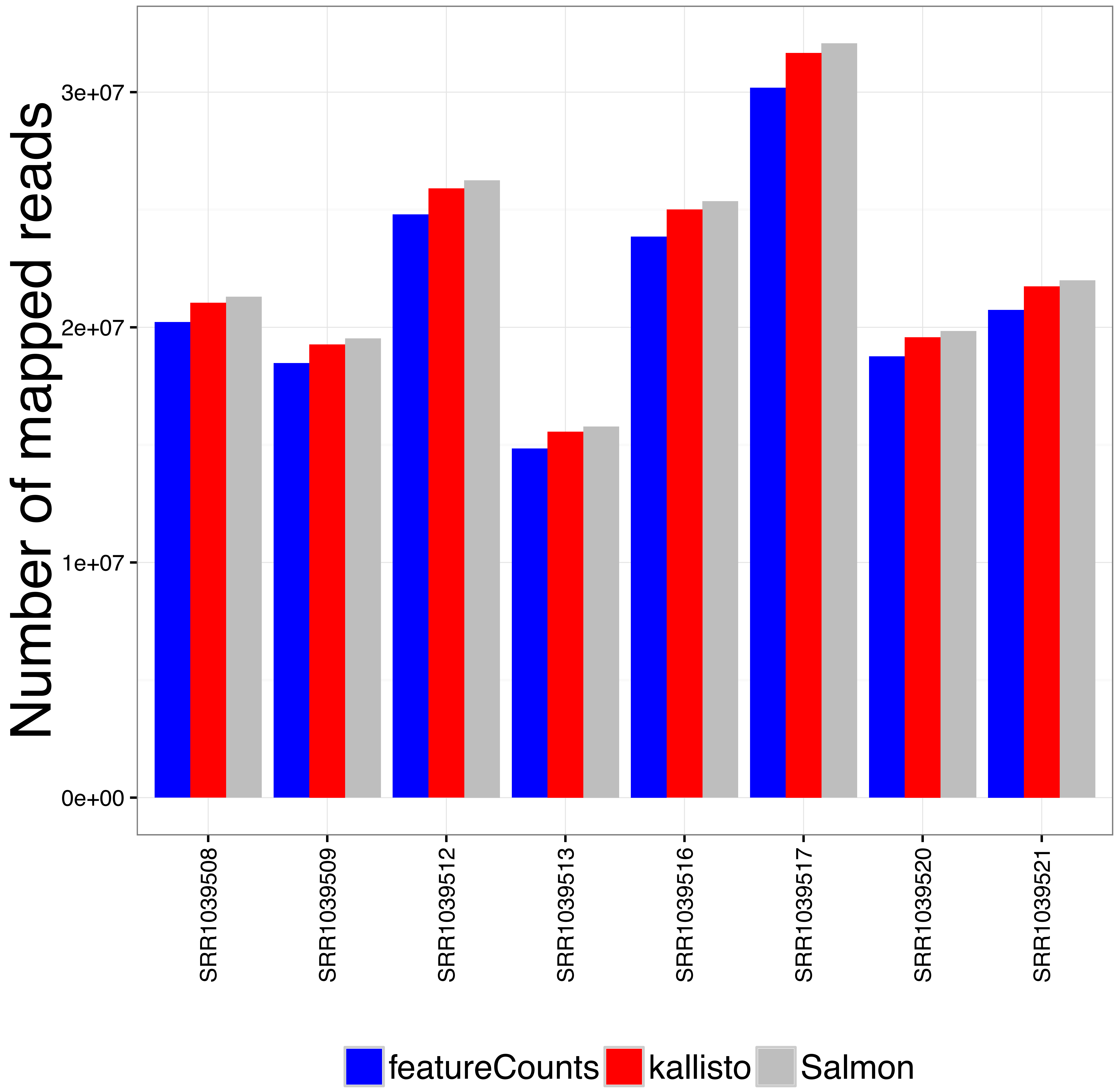
Alignment-free methods…

- … 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

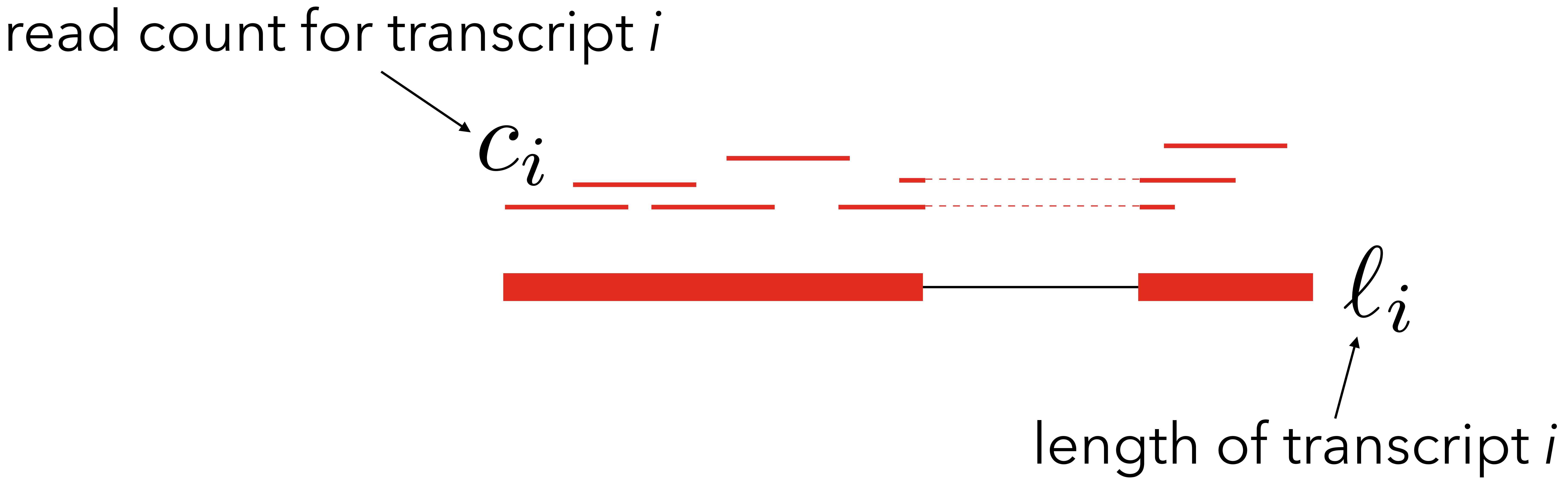
Counts are overall similar between workflows



Alignment-free methods can use slightly more reads



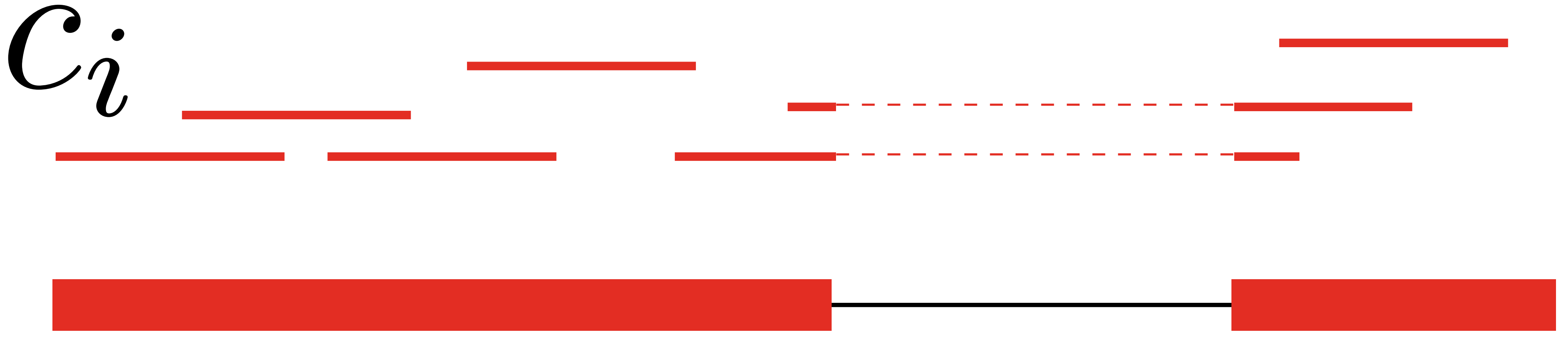
Abundance units



Abundance units

read count for transcript *i*

c_i



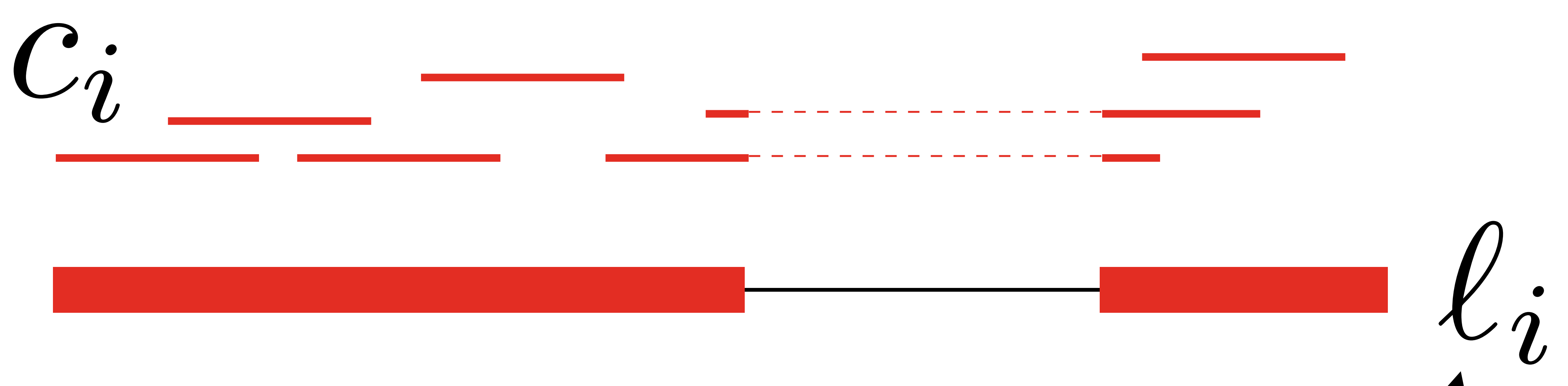
ℓ_i

length of transcript *i*

fragment length

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

Abundance units



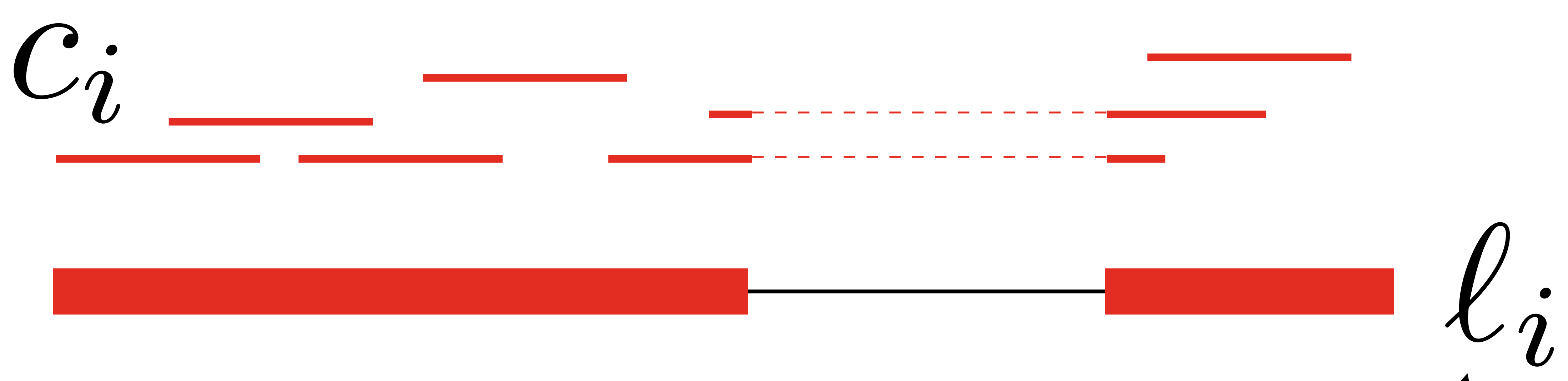
$$t_i = \frac{c_i r}{\ell_i}$$

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*

fragment length

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

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

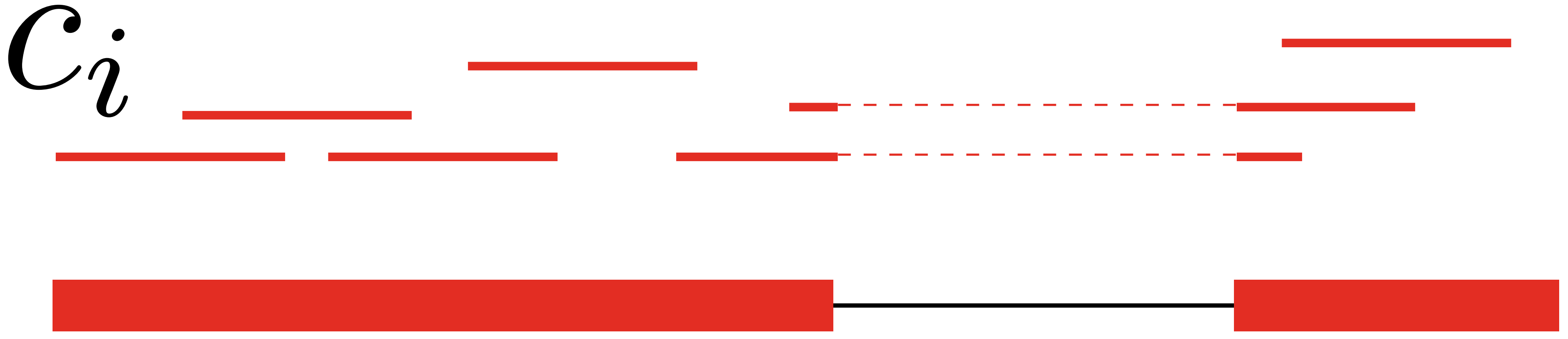
library size

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

Abundance units

read count for transcript *i*

c_i



ℓ_i

length of transcript *i*

fragment length

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

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

library size

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

$$TPM_i \propto RPKM_i$$

$$\sum_i TPM_i = 10^6$$