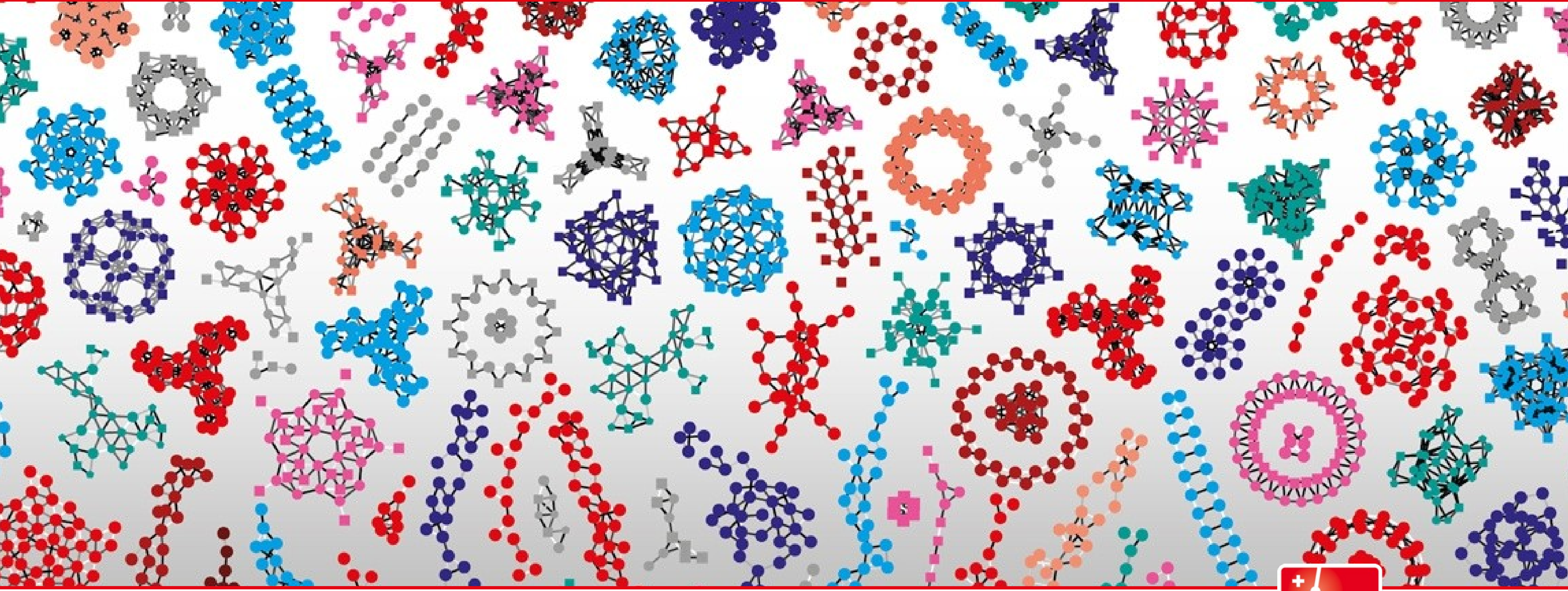


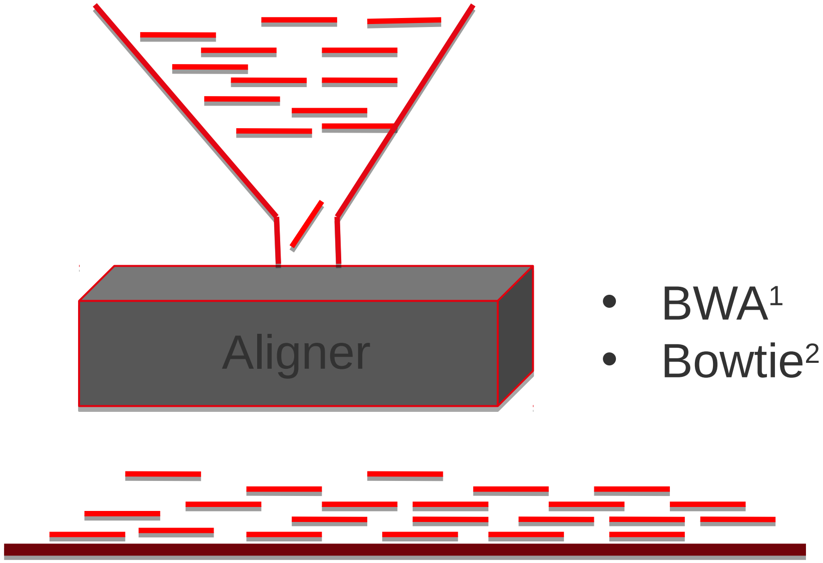
Swiss Institute of Bioinformatics

Introduction to RNA-Seq – Mapping & Aligning

Wandrille Duchemin

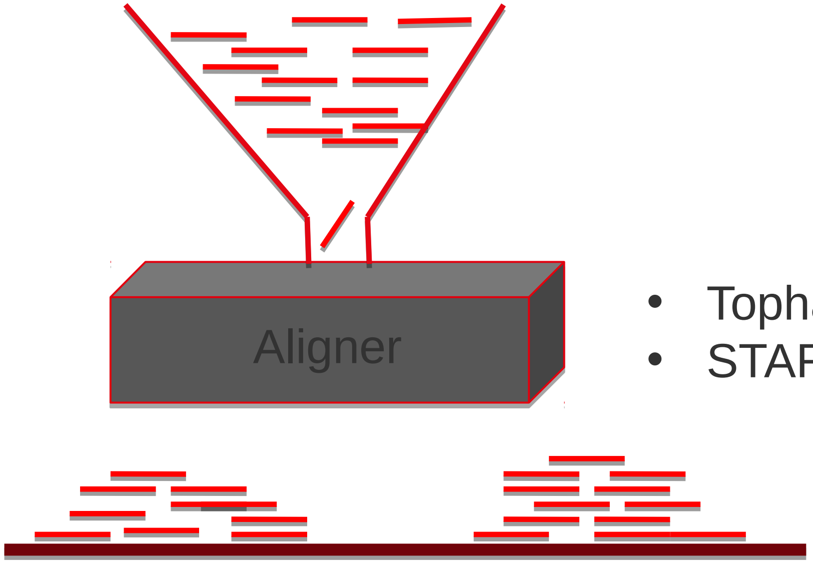
“Aligning” & “Mapping” Sequencing Reads

Whole genome re-sequencing



- BWA[1]
- Bowtie[2]

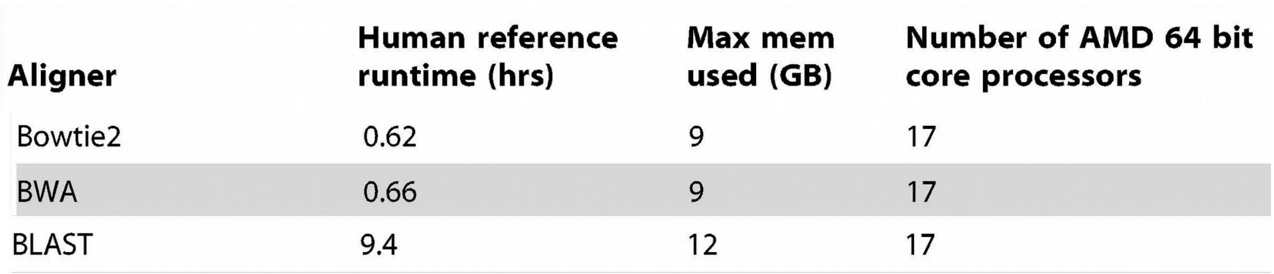
Transcriptome sequencing (RNA-seq)



- Tophat[3]
- STAR[4]

1. Li and Durbin 2009
2. Langemead et al. 2009
3. Trapenell et al. 2009; Kim et al. 2013
4. Dobin et al. 2013

Why not use BLAST?



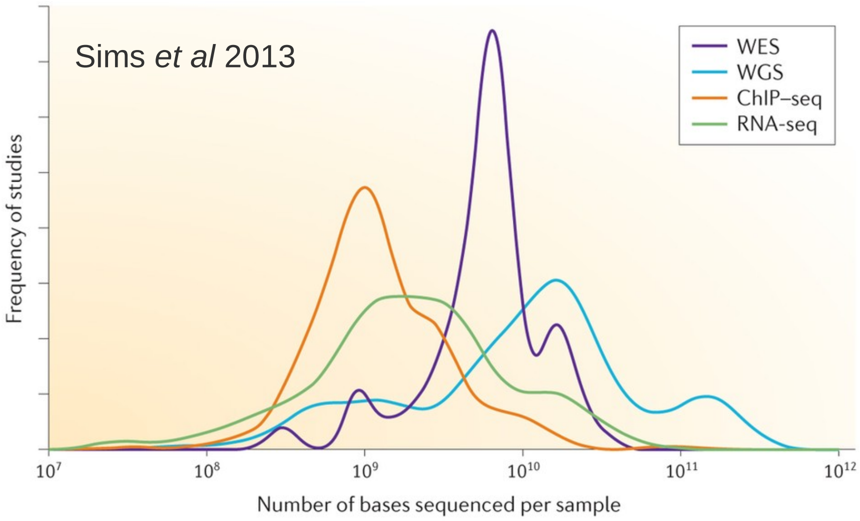
Aligner	Human reference runtime (hrs)	Max mem used (GB)	Number of AMD 64 bit core processors
Bowtie2	0.62	9	17
BWA	0.66	9	17
BLAST	9.4	12	17

The elapsed (wallclock) time needed to align 20 million Illlumina reads from a human transcriptome sample against a human genome (hg19). Adapted from Borozan *et al* 2013

~200 million reads on average per WGS study

$20e^{10}$ bases = $20e^{8}$ reads (100 bp)

~ 94 Hours using Blast = ~4 Days
~ 6.4 Hours using BWA or Bowtie2



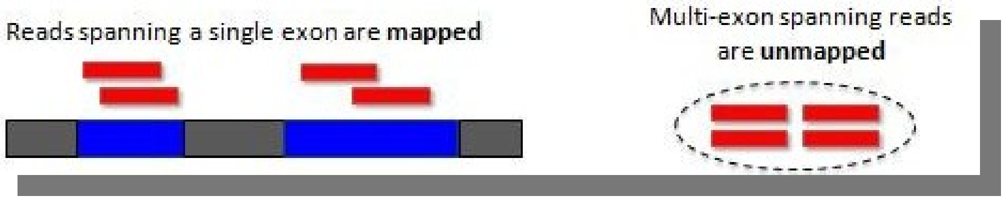
Sims *et al* 2013

Nature Reviews | Genetics

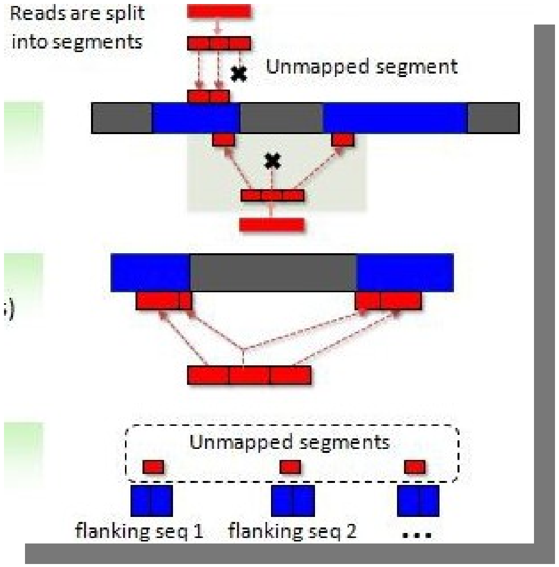
reads from 2012 to June 2013 for the Illumina platform in the European Nucleotide Archive

Alignment using TopHat2

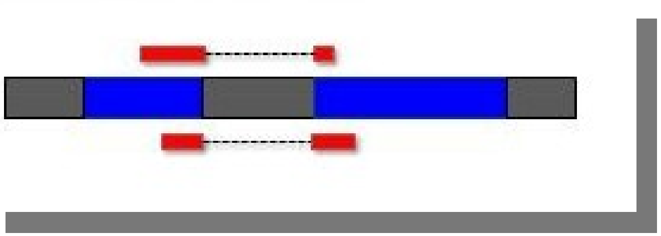
- **Phase 1 – mapping complete reads using Bowtie2 (Burrows-Wheeler algorithm)**


Reads spanning a single exon are **mapped**
Multi-exon spanning reads are **unmapped**

- **Phase 2 – “remapping”**



- **Phase 3 – “stitching”**



Kim *et al* 2013

Alignment using STAR

- **Phase 1 – Mapping using “Maximum Mappable Prefix”**
- **Phase 2 – “Stitching”**

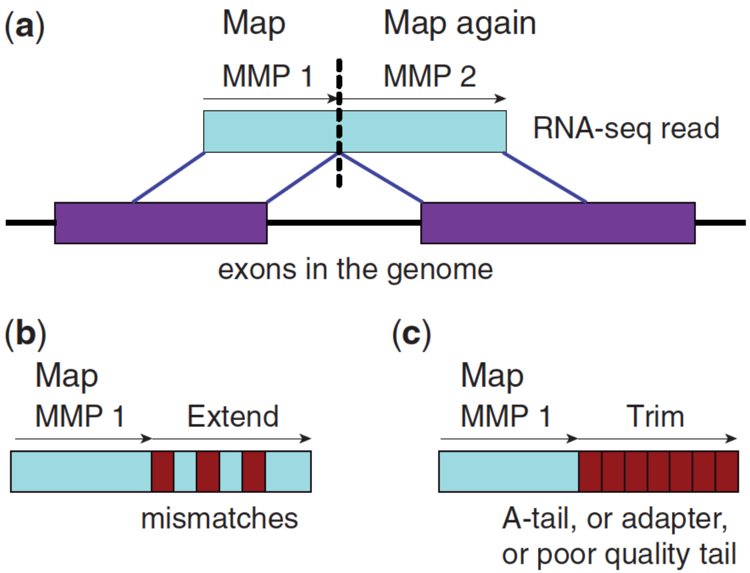
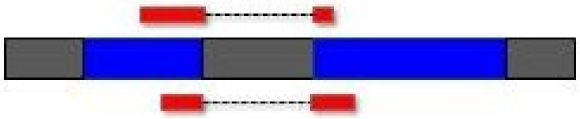


Fig. 1. Schematic representation of the Maximum Mappable Prefix search in the STAR algorithm for detecting (**a**) splice junctions, (**b**) mismatches and (**c**) tails



Dobin *et al* 2013

“Pseudo-aligners”

- **The basic idea is to determine the compatibility of reads with targets (genomic features) without the need for computationally expensive alignment**

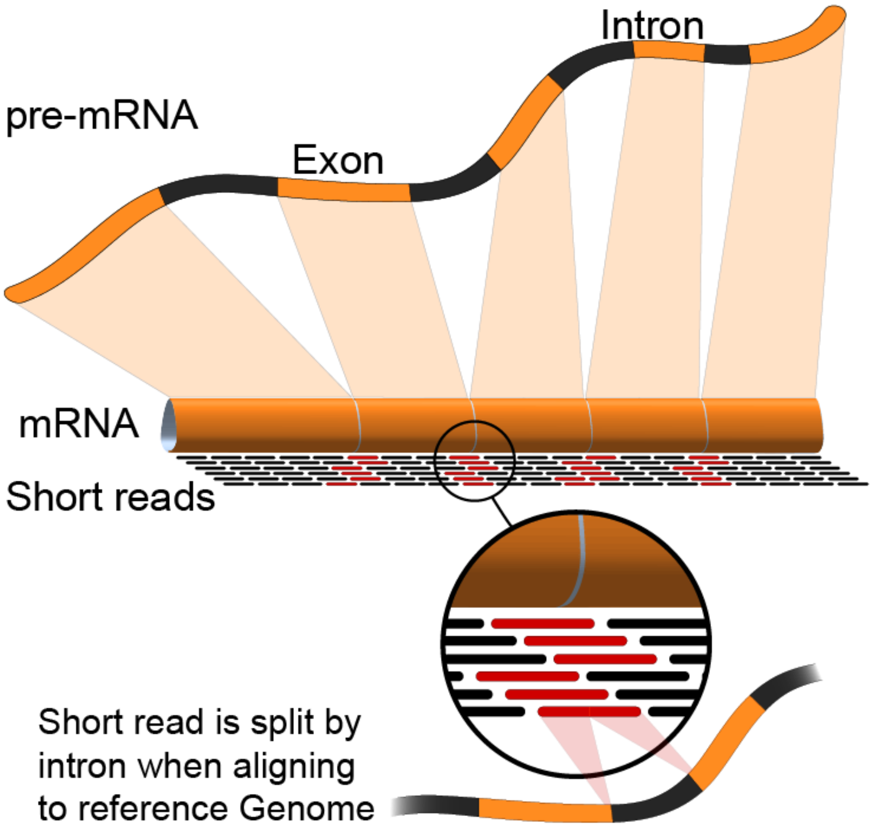
- **The reference is split into k-mers to construct a De Bruijn graph representation of the transcriptome**

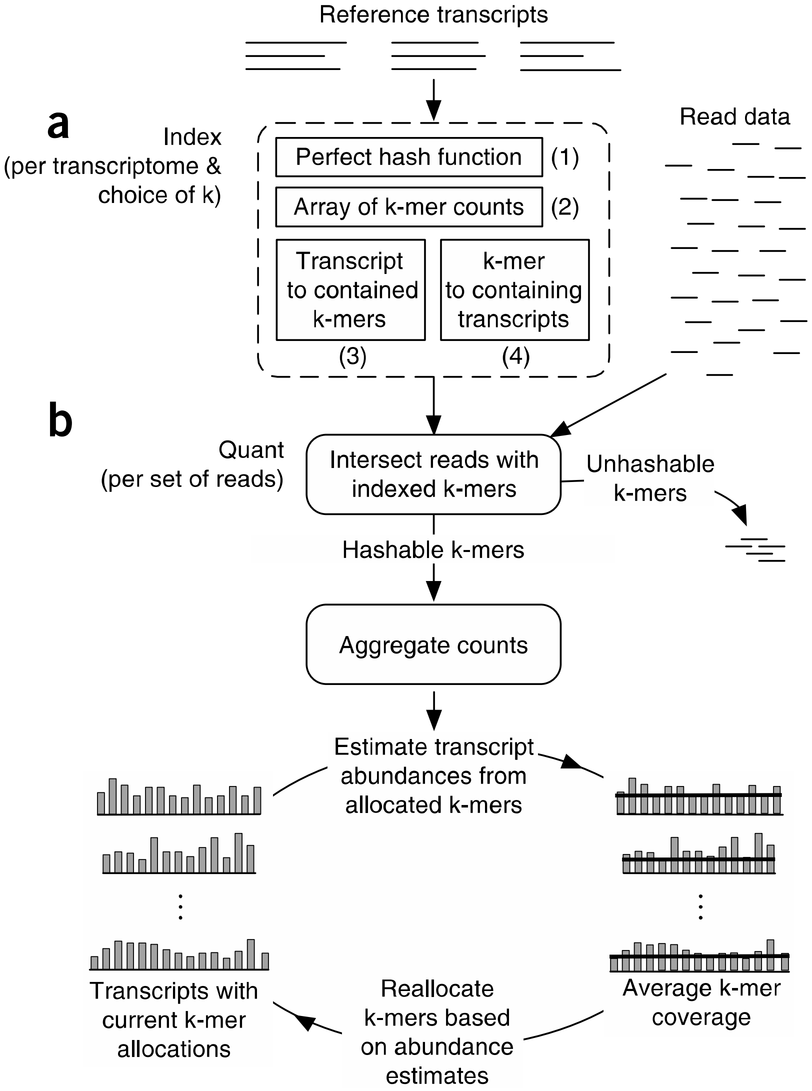
- **“Redundant” k-mers, *eg* the common region of multiple transcripts, are assigned to the same k-mer compatibility class. This substantially reduces the search space when reads are split into k-mers**

- **Primary advantage -> speed**

- **Primary disadvantage -> no alignment information**

Alignment vs. Pseudoalignment

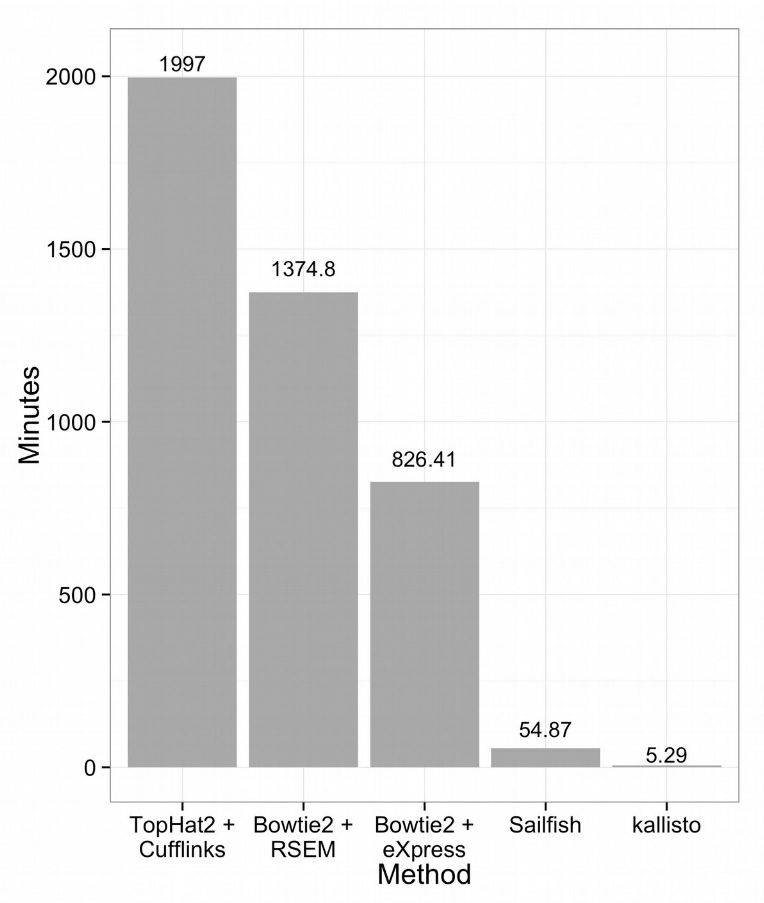


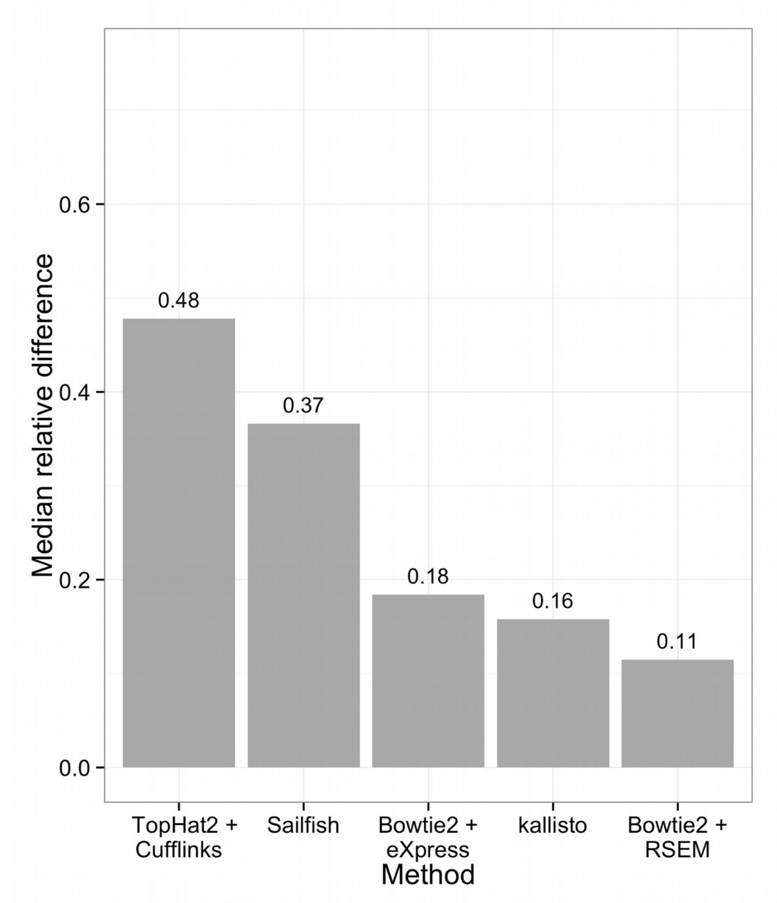


https://en.wikipedia.org/wiki/RNA-Seq

sailfish (Patro et al. 2014), see also “Kallisto” (Bray et al.2016)

Kallisto Pseudoalignment

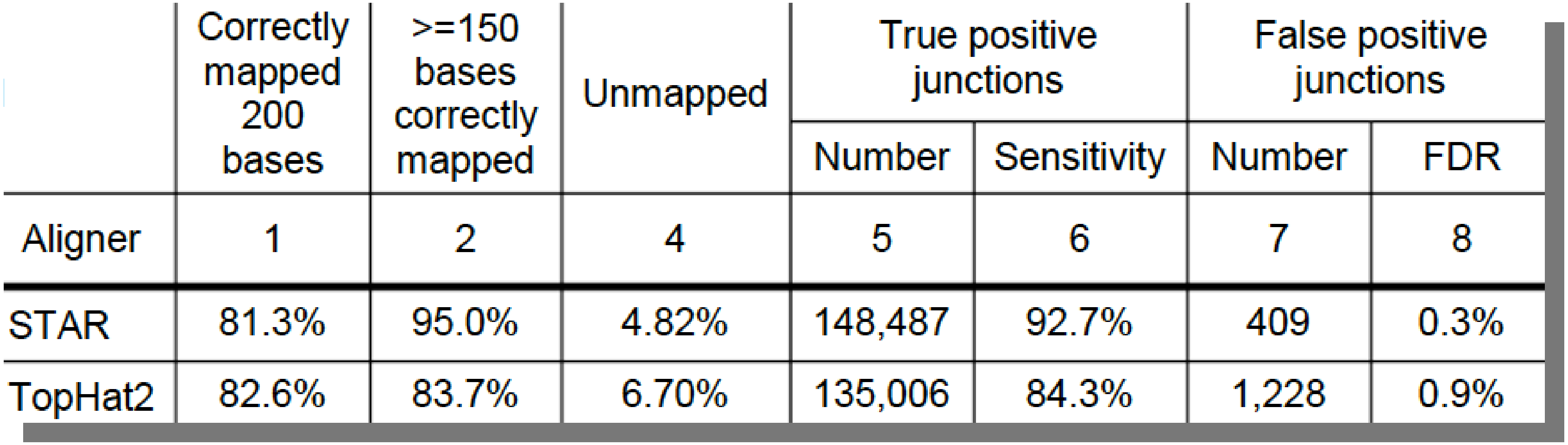




From the kallisto paper : https://arxiv.org/pdf/1505.02710.pdf

Also, kallisto or salmon ? https://github.com/mikelove/salmon_kallisto_diffs

Benchmarking the Aligners (simulated dataset)



	Correctly mapped 200 bases	>=150 bases correctly mapped	Unmapped	True positive junctions		False positive junctions	
				Number	Sensitivity	Number	FDR
Aligner	1	2	4	5	6	7	8
STAR	81.3%	95.0%	4.82%	148,487	92.7%	409	0.3%
TopHat2	82.6%	83.7%	6.70%	135,006	84.3%	1,228	0.9%

- Star is at least x20 faster than Tophat2 for similar parameters
- Tophat2 is at least x6 more memory efficient (can be run on recent laptops)

Dobin & Gingeras 2013

Essentially, if you have access to a cluster you should be using STAR

Reference Genome Preparation

- **Regardless of which aligner you choose to use, you must choose a suitable reference genome**
  - Consider the accompanying annotations
  - Consider which alternative scaffolds to use
- **The reference genome must be indexed in a manner specific to the algorithm of the chosen aligner**
  - STAR index != Bowtie2 index
  - Generally requires annotation file for RNA-Seq

Genome Annotation Files

- **Typically, these are column-based and tab-delimited text files describing genomic features**
  - Gene, CDS, exon, intron, miRNA, etc
  - Chromosome, start, end, strand, attributes, etc

- **The most common formats include:**
  - GFF (General Feature Format, latest is GFF3)
  - GTF (General Transfer Format, ~= GFF2)

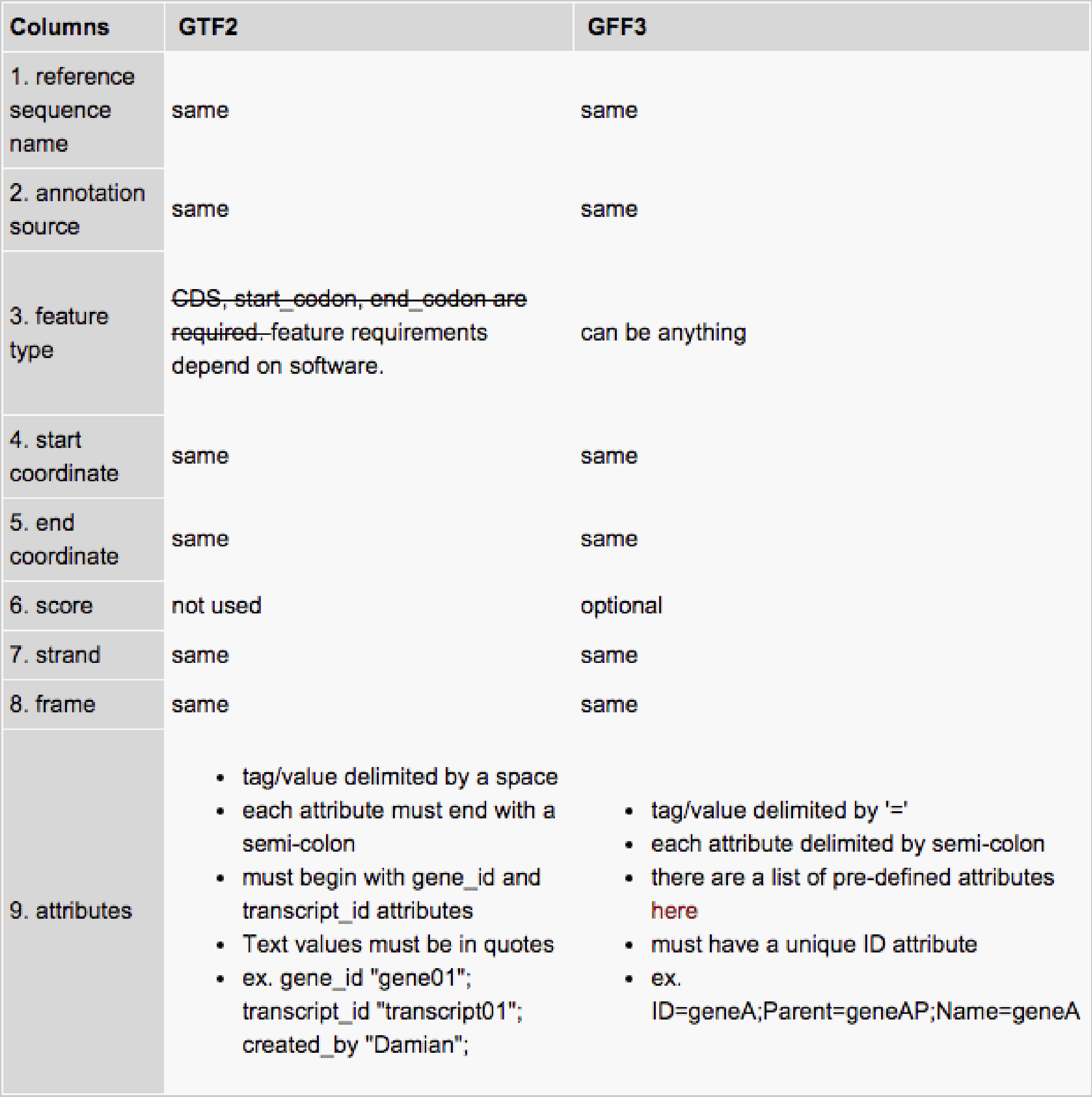
- **We will be working with GTF files**
  - Relatively simple, and bundled with iGenomes

https://support.illumina.com/sequencing/sequencing_software/igenome.html

GTF (GFF2) Annotation Format

- **http://www.ensembl.org/info/website/upload/gff.html**
- **Tab-delimited, empty columns denoted with “.”**
- **Column order:**
  - **seqname** – chromosome, scaffold, etc
  - **source** – origin of the annotation, db/project
  - **feature** – gene, transcript, exon, etc
  - **start** – feature start coordinate (1-based)
  - **end** – feature end coordinate (1-based)
  - **score** – floating point, *eg* quality score
  - **strand** – + (forward) or – (reverse)
  - **frame** – reading frame, 0, 1, or 2
  - **attribute** – semicolon-delimited feature descriptions

GTF vs GFF3



Columns	GTF2	GFF3
1. reference sequence name	same	same
2. annotation source	same	same
3. feature type	~~CDS, start_codon, end_codon are required.~~ feature requirements depend on software.	can be anything
4. start coordinate	same	same
5. end coordinate	same	same
6. score	not used	optional
7. strand	same	same
8. frame	same	same
9. attributes	• tag/value delimited by a space • each attribute must end with a semi-colon • must begin with gene_id and transcript_id attributes • Text values must be in quotes • ex. gene_id "gene01"; transcript_id "transcript01"; created_by "Damian";	• tag/value delimited by '=' • each attribute delimited by semi-colon • there are a list of pre-defined attributes here • must have a unique ID attribute • ex. ID=geneA;Parent=geneAP;Name=geneA

http://blog.nextgenetics.net/?e=27

Practical

Go to the website and do the reference genome index building practical

SAM Alignment Format - Header

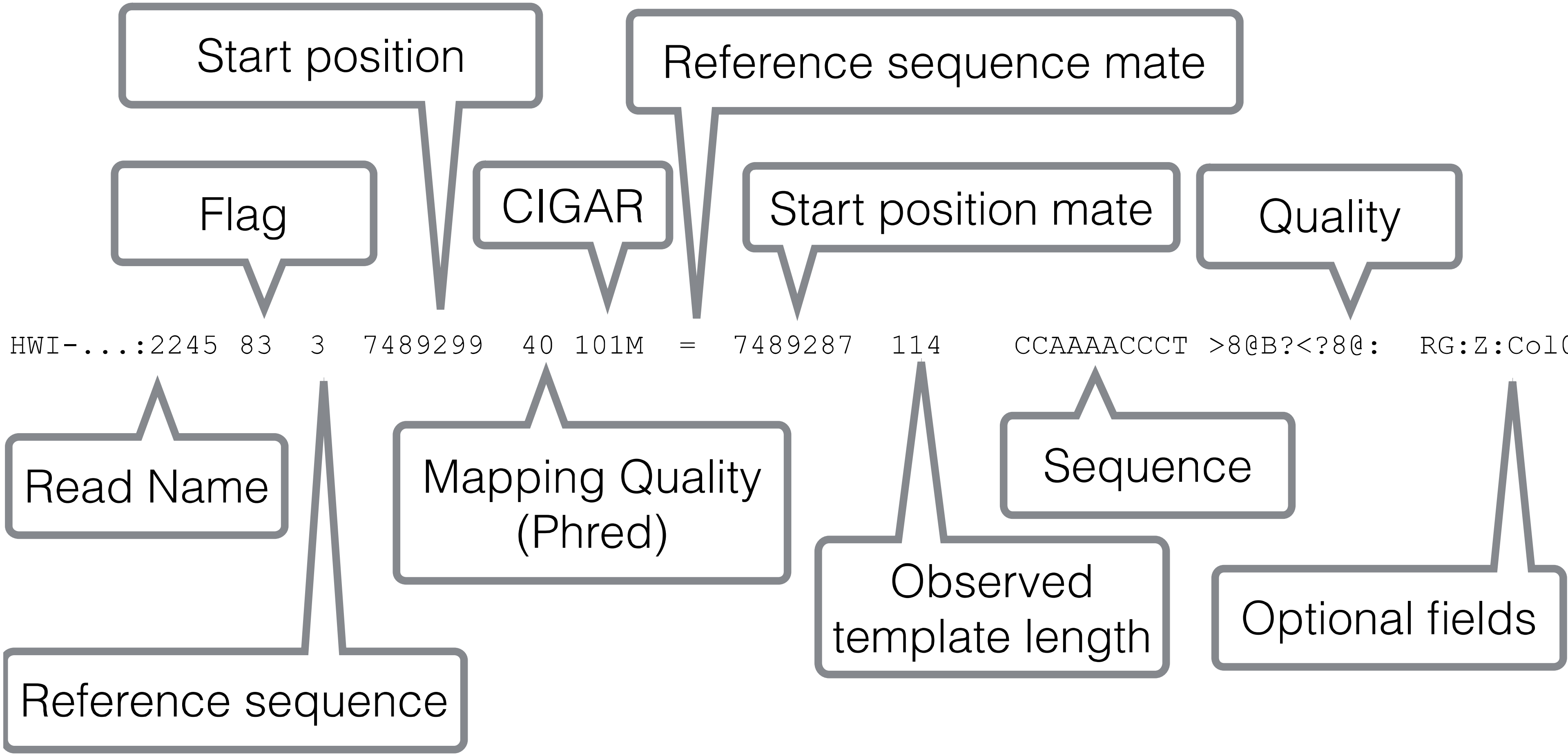
```
@SQ	SN:1 LN:30427671
@SQ	SN:2 LN:19698289
@SQ	SN:3 LN:23459830
@SQ	SN:4 LN:18585056
@SQ	SN:5 LN:26975502
@SQ	SN:M LN:366924
@SQ	SN:C LN:154478
@RG	ID:Col0_R1 PL:Illumina LB:1342 SM:Col0_R1
```

@SQ Reference Sequence: SN name, LN length

@RG Read Group: e.g. grouping samples

https://samtools.github.io/hts-specs/SAMv1.pdf

SAM alignments



Start position

Reference sequence mate

Flag

CIGAR

Start position mate

Quality

```
HWI-...:2245 83  3  7489299  40 101M  =  7489287  114    CCAAAACCCT >8@B?<?8@:  RG:Z:Col0
```

Read Name

Mapping Quality (Phred)

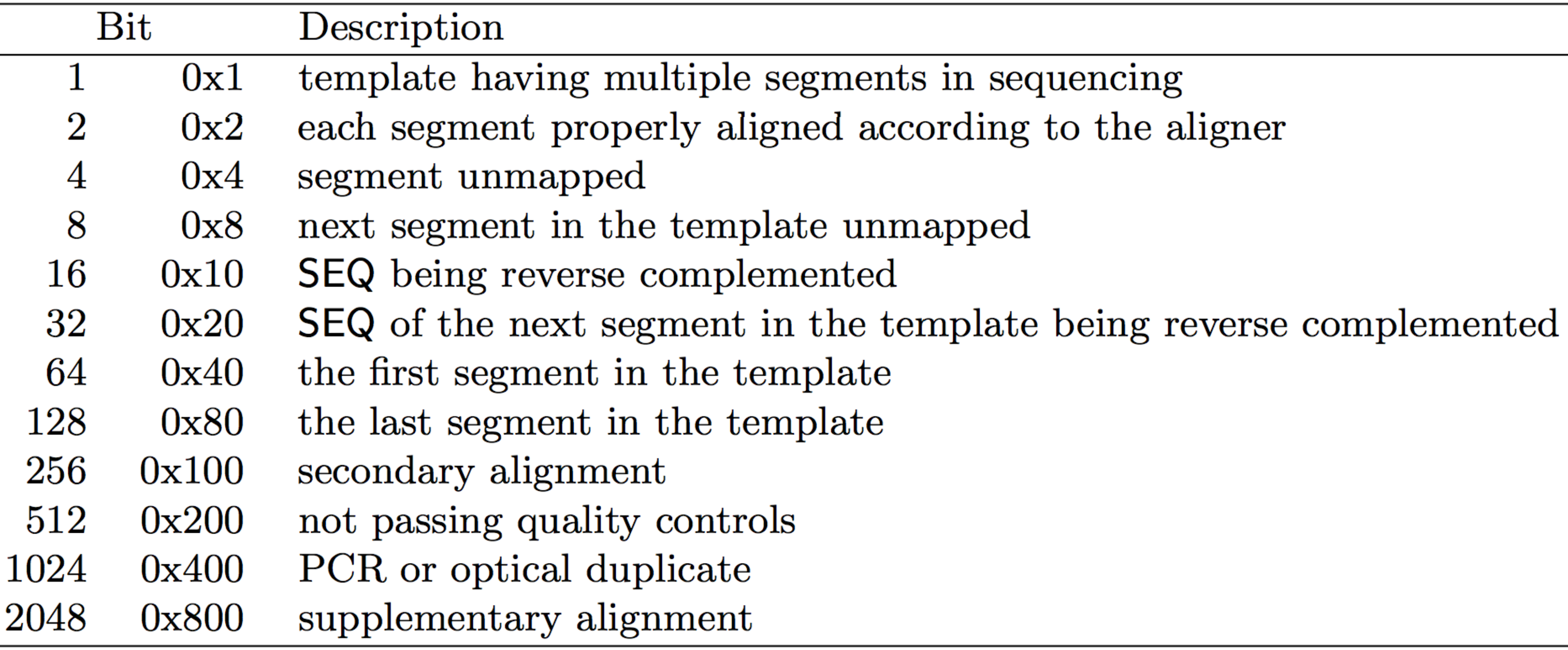
Sequence

Observed template length

Optional fields

Reference sequence

SAM Alignment Format: Flags



Bit		Description
1	0x1	template having multiple segments in sequencing
2	0x2	each segment properly aligned according to the aligner
4	0x4	segment unmapped
8	0x8	next segment in the template unmapped
16	0x10	SEQ being reverse complemented
32	0x20	SEQ of the next segment in the template being reverse complemented
64	0x40	the first segment in the template
128	0x80	the last segment in the template
256	0x100	secondary alignment
512	0x200	not passing quality controls
1024	0x400	PCR or optical duplicate
2048	0x800	supplementary alignment

Example, flag 83 = 64+16+2+1 means it's first read (0x40) of pair-end reads (0x1) and it's mapped on minus strand (0x10) and both reads mapped (0x2).

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

https://samtools.github.io/hts-specs/SAMv1.pdf

SAM format: CIGAR string

- **Summary of alignment to the reference**
- ***eg*, 101M, 1S92M, 15M87N70M90N16M**

Code	Description	
M	Alignment match	Base-level match + mismatch
I	Insertion	
D	Deletion	
N	Skipped	*eg* intron
S	Soft clipping	Kept in SAM
H	Hard clipping	Not in SAM

SAM format: optional fields

- **Used by some aligners to encode additional information for downstream analyses**
- **Can cause incompatibilities among workflows**

Code	Description
RG	Read Group e.g. sample or lane
MD	String for mismatching positions
NH	Number of reported alignments that contains the query in the current record
HI	Query hit index, indicating the alignment record is the i-th one stored in SAM

BAM format

- **Binary SAM format**
- **Lossless compression of SAM format**
- **~4-fold smaller file size**
- **Genome viewers and many downstream applications require the BAM file to be sorted and have an index (typically .bai extension)**

Practical

- Go to the website and do the mapping and QC practicals

REFERENCES

Li H & Durbin R (2009) “Fast and accurate short read alignment with Burrows-Wheeler transform” Bioinformatics 25(14):1754-60

Langmead *et al* (2009) “Ultrafast and memory-efficient alignment of short DNA sequences to the human genome” Genome Biology 10(3):R25.

Trapnell *et al* (2009) “TopHat: discovering splice junctions with RNA-Seq” Bioinformatics 25(9):1105-11.

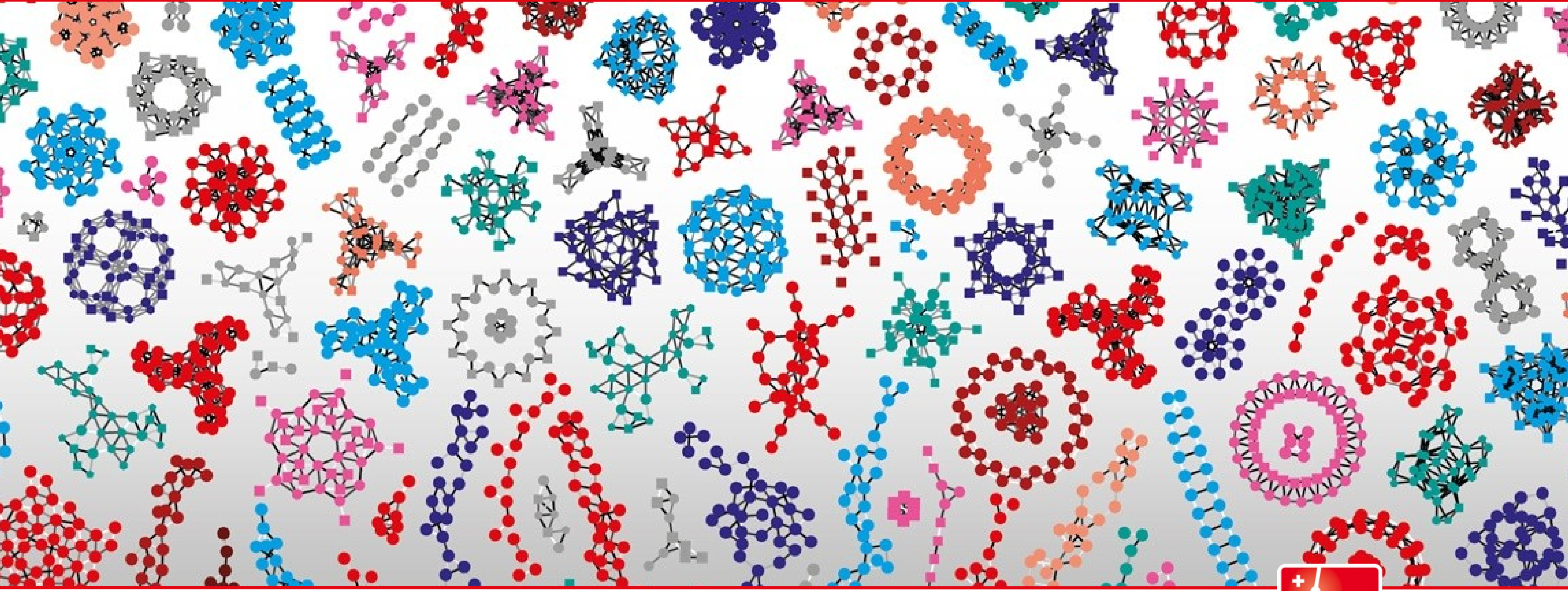
Kim *et al* (2013) “TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions” Genome Biology 14(4):R36.

Dobin *et al* (2013) “STAR: ultrafast universal RNA-seq aligner” Bioinformatics 29(1):15-21.

Patro *et al* (2014) “Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms” Nature Biotechnology 32(5):462-4.

Patro *et al* (2017) “Salmon provides fast and bias-aware quantification of transcript expression” Nature Methods 14(4):417-419.

Bray *et al* (2016) “Near-optimal probabilistic RNA-seq quantification (Kallisto)” Nature Biotechnology 34(5):525-7.



Contributors:

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Annex : Assessing read coverage for biases

- **The RSeQC package includes a function for evaluating “gene body coverage”**

- **This can be used to assess 5’ or 3’ bias, which might happen if your RNA is degraded or otherwise biased**

- **Requirements:**

  - Genome annotations in the 12-column BED format
  - Index (.bai) for sorted BAM file, which can be generated using the SAMtools package

```
samtools index sample1_sorted.bam

geneBody_coverage.py -r /data/GRCm38/Mus_musculus.GRCm38.89.bed12 \

                     -i sample1_sorted.bam \

                     -f pdf \

                     -o output_prefix
```

Annex - CRAM format

- **Binary SAM format, significantly improved over BAM lossless compression**
- **Compatible with BAM files**
- **Both lossless and lossy compression possible**
- **https://samtools.github.io/hts-specs/CRAMv3.pdf**

Annex - Other relevant formats: BED

- **Tab-delimited text file used to describe intervals**
- **Minimally:**
  - Sequence ID
  - Start
  - End
- **Optional:**
  - Name
  - Score
  - Strand
- **For large files, use binary index format bigBED**
- **BEDtools (http://code.google.com/p/bedtools)**

Annex - Other relevant formats: VCF (Variant Call Format)

- **Tab-delimited text to describe SNPs, structural variants, indels etc**
- **Contains:**
  - Chromosome
  - Position
  - Reference allele, alternative allele(s)
  - Various statistical metrics
- **BCF: indexed binary format**
- **https://samtools.github.io/hts-specs/VCFv4.2.pdf**