Short Read Mapping and File Formats

What are we doing today?

I. Examining Read Quality
II. Quality/Adapter Trimming
III. Read Mapping
IV. SAM, BAM and CRAM format

Quality Control

Goals

- Is my data of sufficient quality to use?
- The instrument assigns a confidence value to each base. Are the bases high quality overall?
- Does the complexity look normal?
  - PCR and library prep problems can lead to duplication of the same sequences over and over
- Are there adapters or other over-represented sequences?
- Are there lane batch effects?

FASTQC

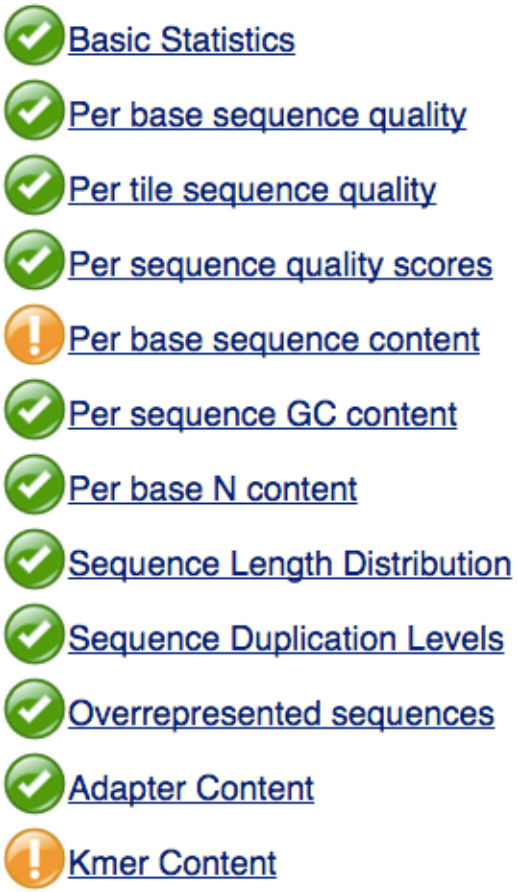
Babraham Bioinformatics

Accepts input formats:

- FastQ (all quality encoding variants)
- GZip compressed FastQ
- SAM
- BAM

Does a 12 point analysis of quality

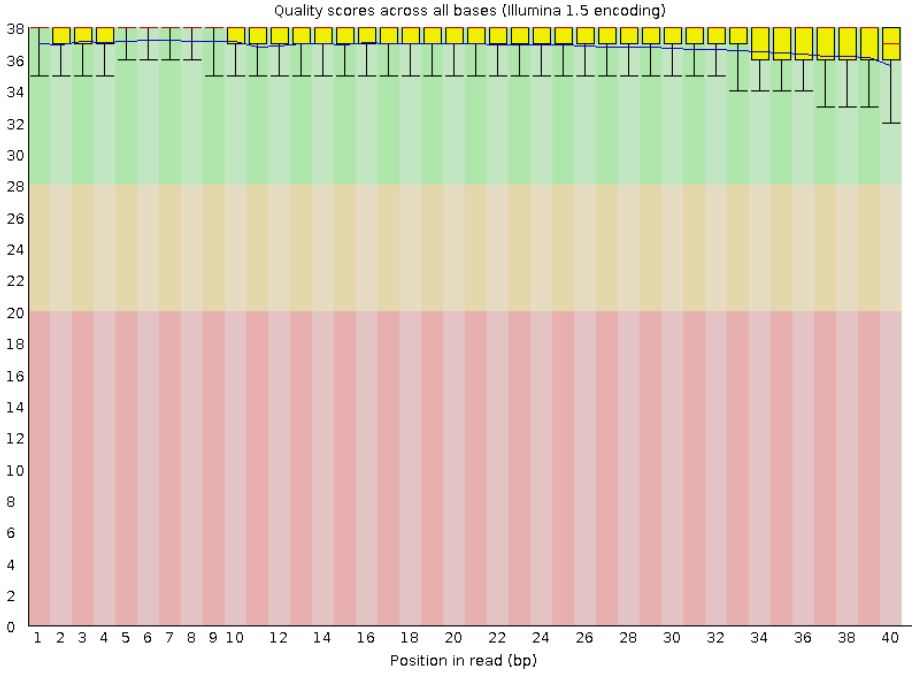
Generates an html output file

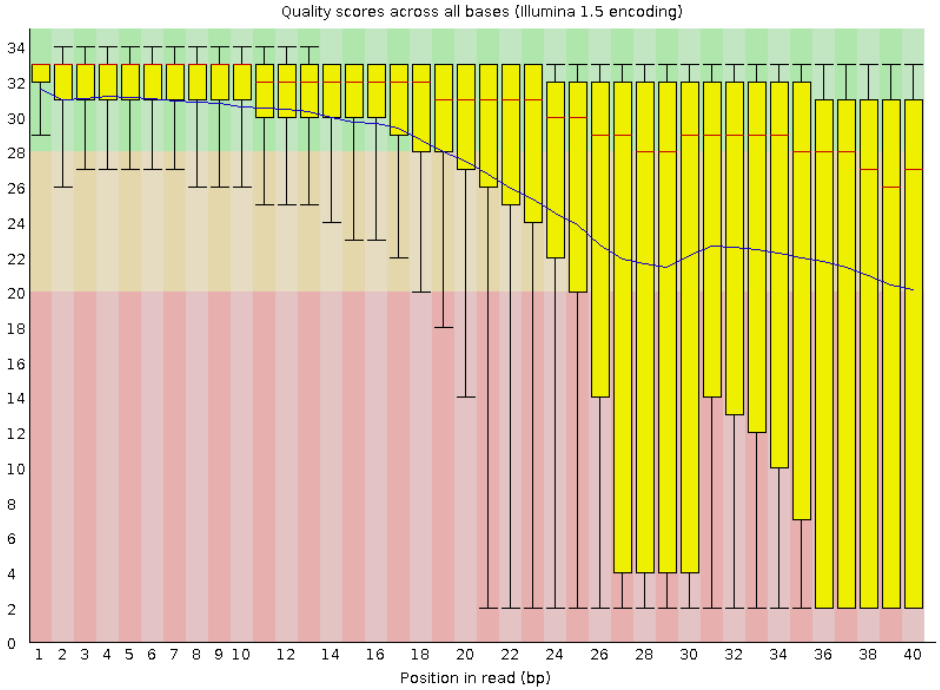


http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

FASTQC

Babraham Bioinformatics





http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

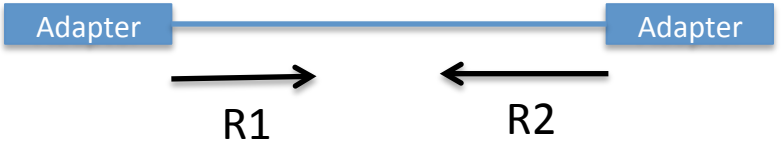
Trimming

- From the quality control step, we know where the problems are
- All illumina reads tend to have degrading quality at the end of the read
- Get rid of the bad data, keep the good data
  - Cut adapter and other Illumina-specific sequences from the read.
  - Trim off low quality bases
  - Drop a read entirely if is too low quality or too short

Trimmomatic

- Optimized for Illumina NGS
- **Very flexible**
- Handles paired end data well
- Threaded

- Detects adapter read through
- No read through:



- Read through:

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina Sequence Data. Bioinformatics, btu170.

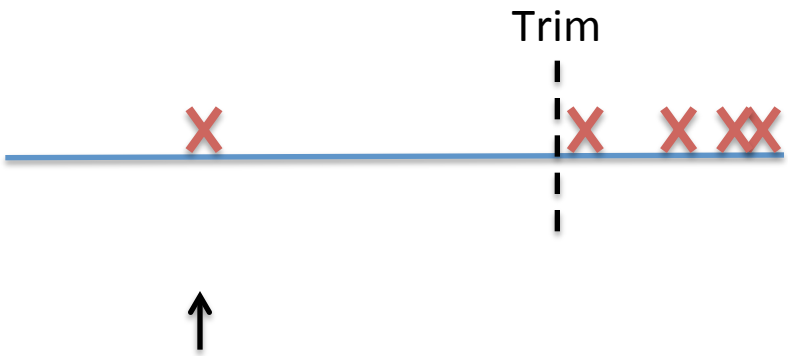
Trimmomatic

The current trimming steps are:

- ILLUMINACLIP: Cut adapter and other illumina-specific sequences from the read. Comes with basic Illumina adapters, make sure yours are in there or add yours!
- SLIDINGWINDOW: Perform a sliding window trimming, cutting once the average quality within the window falls below a threshold.
- LEADING: Cut bases off the start of a read, if below a threshold quality
- TRAILING: Cut bases off the end of a read, if below a threshold quality
- CROP: Cut the read to a specified length
- HEADCROP: Cut the specified number of bases from the start of the read
- MINLEN: Drop the read if it is below a specified length after trimming

Trimmomatic

- Maximum Information Quality Filtering:
- Retain low-quality bases early in a read in order to make sure the read is sufficiently long to be informative
- Trimming process becomes more strict later in the read



Skewer

- Faster than trimmomatic
- A bit less flexible
- If your data is in pretty good shape and needs only basic trimming, this is a great option

https://github.com/relipmoc/skewer

Jiang, H., Lei, R., Ding, S.W. and Zhu, S. (2014) Skewer: a fast and accurate adapter trimmer for next-generation sequencing paired-end reads. BMC Bioinformatics, 15, 182.

Trimming is critical for downstream success

- Trimming is important for all read sets, but truly critical for those with lower quality
- Resequencing applications
- Alignment of reads with and without trimming using BWA

Higher quality data
- 79% aligned if no trimming done
- 83% aligned after trimming

Lower quality data
- 7% aligned if no trimming done
- 80% aligned after trimming

Bolger et al., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics

Trimming is critical for downstream success

- Assembly applications
- Same trend

Higher quality data

- 58% increase in contig N50 size (from 60kbp to 95kbp)
- 28% increase in maximum contig size

Lower quality data

- 77% increase in contig N50 size (from 100kbp to 177kbp)
- 55% increase in maximum contig size

Bolger et al., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics

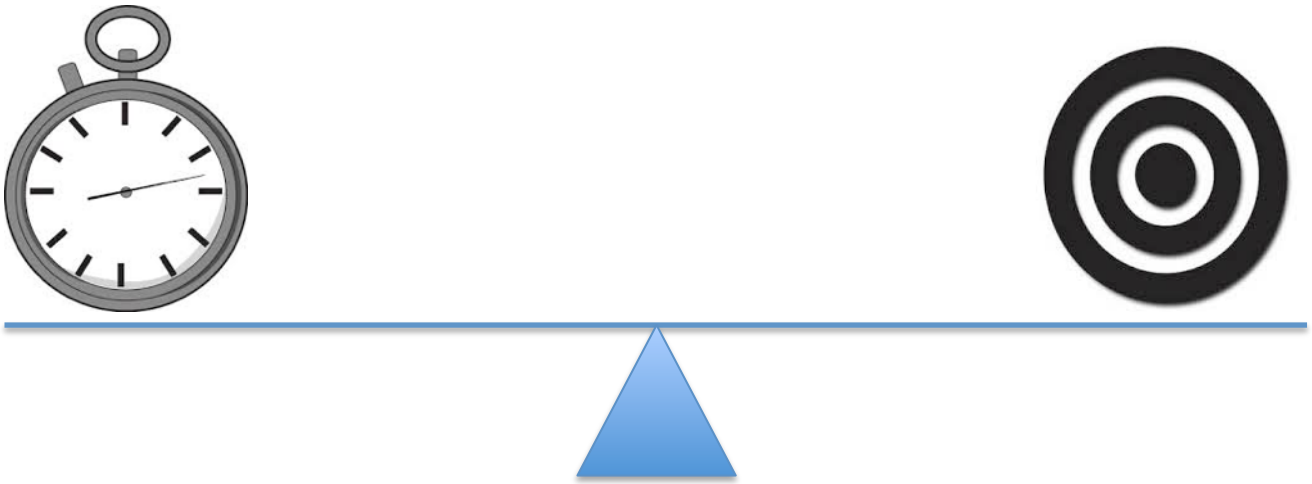
Trimming

- Current community wisdom:
  - Quality trimming reduces error
  - But also reduces content and contiguity
- Gentle trimming is preferred – many times the defaults are too stringent, you will lose lots of data!
- Application matters
  - For mapping and variant calling, possibly no trimming (phred 5)
  - For assembly, a bit more trimming is good (phred 10)

II. READ MAPPING

But we've already covered alignment.

- Alignment methods must have tradeoffs for speed vs accuracy
- Depending on the application, may want to make different tradeoffs
- Global vs local
- Different types of alignment objectives lead to different categories of aligners



Types of Alignment software

From wikipedia (open source and commercial):

- Database search only (23 including BLAST)
  - Aligning a query or set of queries to a set of database sequences
- Pairwise alignment (43 including dotplotters)
  - Aligning two sequences to each other
- Multiple sequence alignment (47)
  - Aligning 3 or more sequences together
- Genomic analysis (16)
  - Aligning genome length sequences to each other, or aligning cDNA to a genome
- Motif finding
  - Finding motifs in a database (17)

Many aligners fit into more than one category

https://en.wikipedia.org/wiki/List_of_sequence_alignment_software

Short Read Mappers

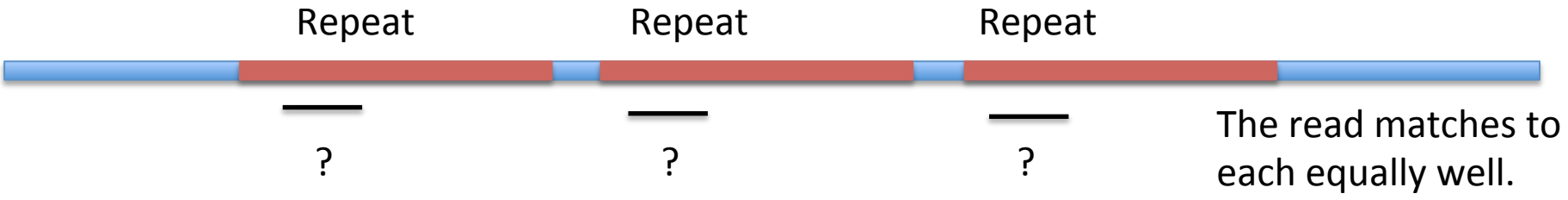
- BLAST is much faster than original algorithms (Smith Waterman for example)
- Still too slow for the amount of data produced by NGS technology
- Resequencing usually involves comparing very similar sequences (>90% identity in residues) to a reference genome
- Software that leverages this high percent identity can be faster
- Can generally utilize a global strategy instead of a local strategy

Short Read Mappers

- Orders of magnitude faster than BLAST
- several tens of millions of reads mapped per hour per CPU
- Only matches of 95% identity or greater are found
- Usually only output the best hit or the set of hits all equivalently good
  - The point is usually to find the origin in the reference genome
  - Other genomic regions of lower identity are not considered useful

Uniqueness

- Some reads can be mapped uniquely to the reference
- Some map to multiple locations
- Multiply mapped reads are difficult to apply to downstream applications
  - RNASeq – which gene do they represent?
  - SNP – which location carries the substitution?
- How to deal with multiply mapped reads?
  - Throw them away
  - This introduces bias and ignores real genomic regions that may be biologically important – will discuss more for RNASeq



Clever Tricks to find “Best” Alignments

- Use the quality values
  - Penalize mismatches at high quality bases more than mismatches at low quality bases
- Paired End information
  - If one read does not map uniquely, but the other does, use that information to place the non-unique one
  - Need to know your insert size

Decisions for the end user

- How many mismatches are allowed for a read to be considered mapped?
  - Heterozygosity between sample and reference
  - Incomplete/low quality reference
- How many matches to report?
  - Does your downstream analysis need/want to include multiple matches?

Explore the documentation and parameters for your software of choice

Is it doing what you think its doing?

Lots of choices

Many, many software packages

Blat
BWA
Eland
GSNAP
HISAT2
Maq
RMAP
Stampy
SHRiMP

SOAP Short Oligonucleotide Analysis Package

mosaik

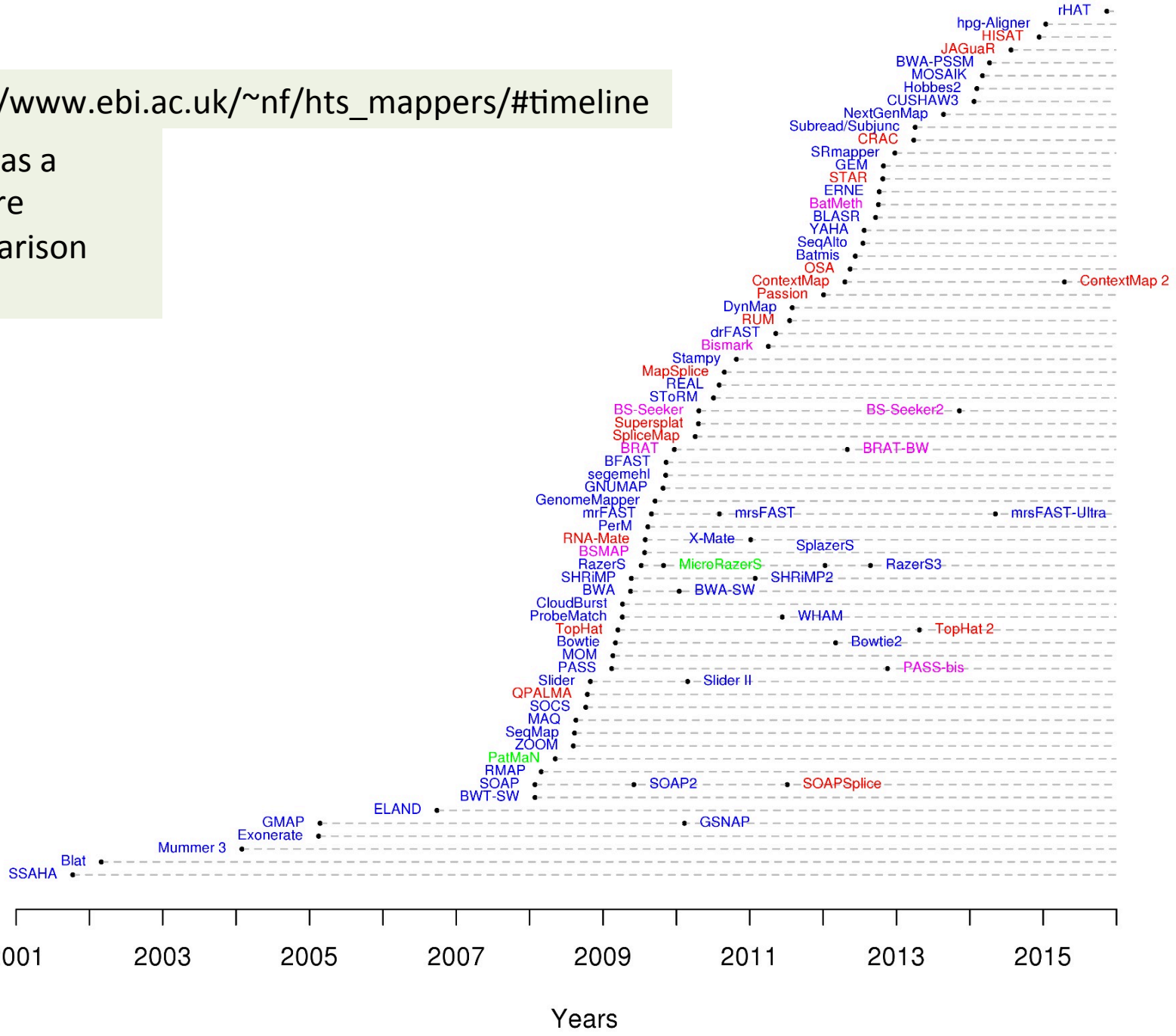
Bow TIE

Prize for best named:
VelociMapper

What's in the box matters.

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

Also has a Feature Comparison Table



How to choose?

- Good documentation
- Memory efficient
- Responsive mailing list or help forum
- Maintained and updated when bugs are found
- Too many emerge each year for the literature to keep up, but currently, most popular ones are very similar in mapping rate and time
  - BWA
  - HISAT2

What mappers have in common: Indexing Strategies

- Usually, the first step is to transform part of the data into a more suitable form for fast searching
- Indexing – creating a glossary or look up table
- Without indexing you would have to scan everything each time you did a search
- Consider web search engines

Burrows-Wheeler Aligner

- Has three algorithms
- Individual chromosomes cannot be longer than 2GB
- Output in SAM format

Burrows-Wheeler Aligner

http://bio-bwa.sourceforge.net/

- three algorithms, but one is the most common:
- BWA-MEM
  - Use for any sequences greater than 70bp up to 1Mb
  - Will work with reads with
    - 2% error for 100bp
    - 3% error for a 200bp
  - Has split read support – i.e. “It may produce multiple primary alignments for different part of a query sequence.”
    - structural variations, gene fusion or reference misassembly
  - By reporting multiple alignment locations, it does not always work well with other software
  - Can fix by using the –M flag

III. SAM, BAM AND CRAM FORMAT

SAM Format

- SAM = Sequence Alignment/Map format
- Tab delimited plain text
- Store large nucleotide sequence alignments
  - Alignment of every read
  - Including gaps, SNPs and structural variants
  - Pairing of reads
  - Can record more than one alignment location in the genome
  - Stores quality values
  - Stores information about duplication

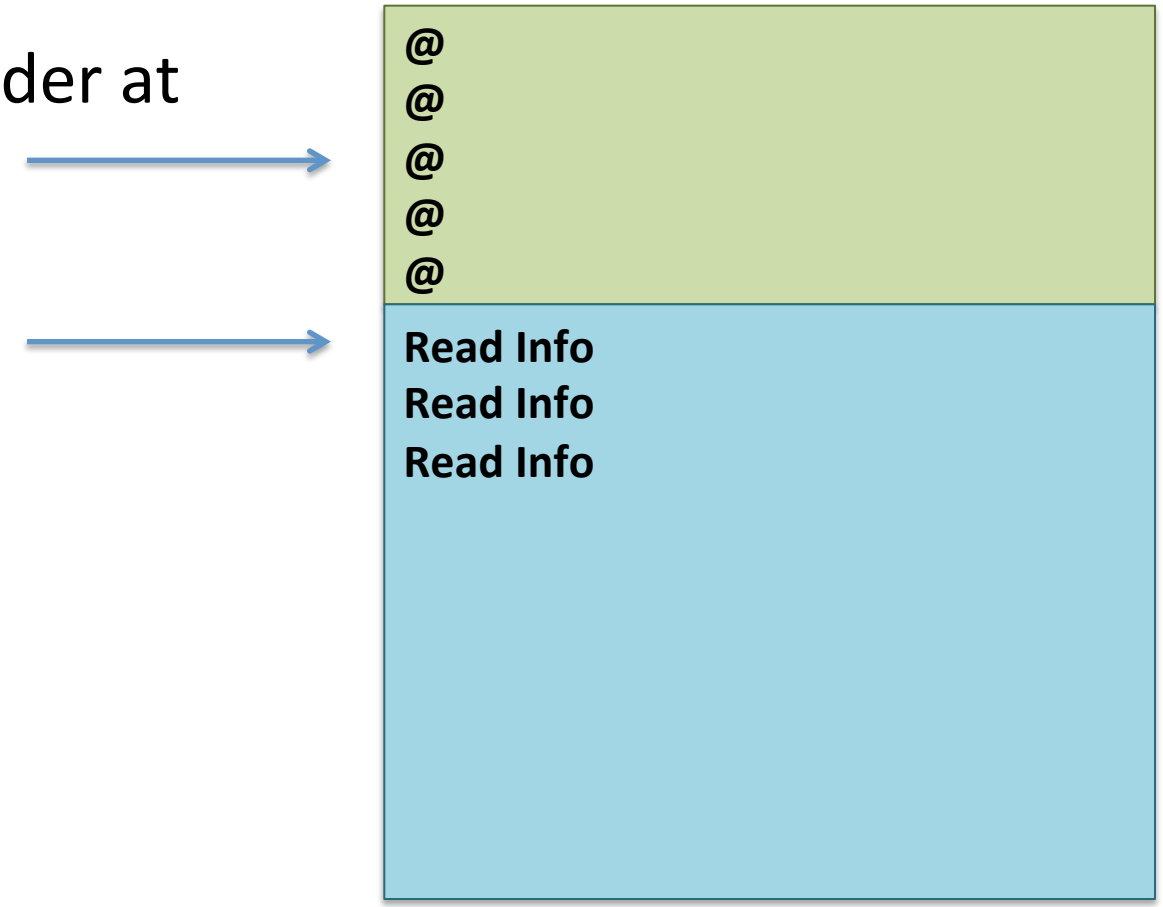
SAM Format

Strengths

- Flexible
- Useful for operations on very large sequences
- Extremely detailed documentation
  - https://samtools.github.io/hts-specs/SAMv1.pdf
- Manipulations can be done with the software samtools

SAM - Header

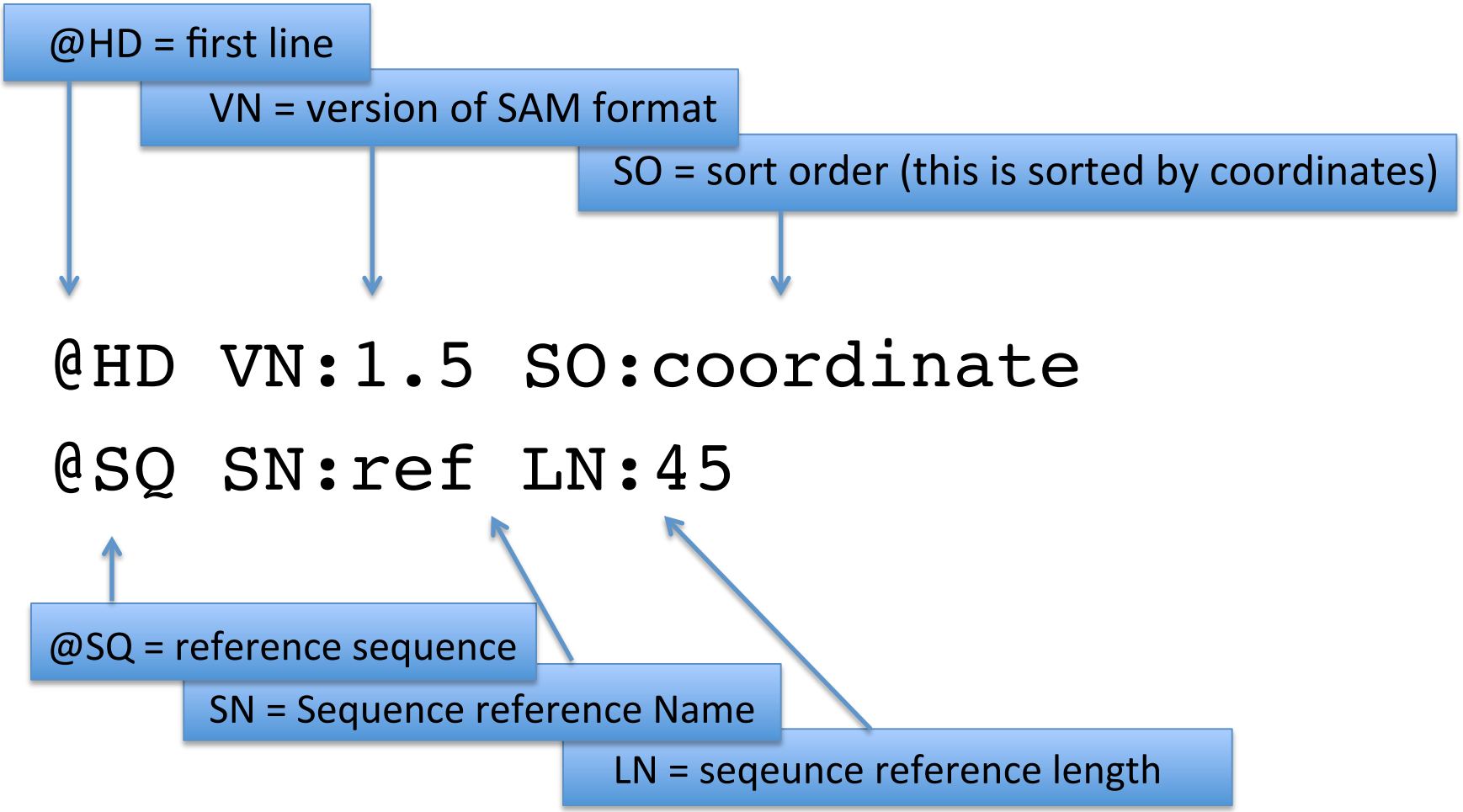
- Structure
  - Optional Header at top of file
  - Alignment information



SAM - Header

- Header lines start with @ symbol
- Always at top of file
- Contain lots of information about what was mapped, what it was mapped to, and how (metadata)
  - the version information for the SAM/BAM file
  - whether or not and how the file is sorted
  - information about the reference sequences
  - any processing that was used to generate the various reads in the file
  - software version

Simple Header



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

Alignment Line

- Below the headers are the alignment records
- Tab-delimited fields

1	QNAME	Query template/pair NAME
2	FLAG	bitwise FLAG
3	RNAME	Reference sequence NAME
4	POS	1-based leftmost POSition/coordinate of clipped sequence
5	MAPQ	MAPping Quality (Phred-scaled)
6	CIGAR	extended CIGAR string
7	MRNM	Mate Reference sequence NaMe (`=' if same as RNAME)
8	MPOS	1-based Mate POSistion
9	TLEN	inferred Template LENgth (insert size)
10	SEQ	query SEQuence on the same strand as the reference
11	QUAL	query QUALity (ASCII-33 gives the Phred base quality)
12+	OPT	variable OPTional fields in the format TAG:VTYPE:VALUE

Lets unpack this alignment line, taken from a SAM file:

SRR030257.2000020 83 gi|254160123|ref|
NC_012967.1| 329575260 36M = 3295706-82
TGCTGGCGGCGATATCGTCCGTGGTTCCGATCTGGT
?%<91<?>>??AAAAAAAAAAAAAAAAAAAAAAAA
XT:A: NM:i:0 SM:i:37 AM:i:37 X0:i:1 X1:i:0
XM:i:0 XO:i:0 XG:i:0 MD:Z:36

SAM Field 1

Query name

SRR030257.2000020

Field 2:
Flag

83

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 filters, such as platform/vendor quality controls
1024	0x400	PCR or optical duplicate
2048	0x800	supplementary alignment

```
64 + 16 + 2 + 1

1  = Read is paired
2  = Read mapped in proper pair
16 = Read mapped to reverse strand
64 = First in pair
```

Look up a SAM flag: https://broadinstitute.github.io/picard/explain-flags.html

SAM Field 3

Reference sequence name (useful especially if you have multiple chromosomes)

```
gi|254160123|ref|NC_012967.1|
```

SAM Field 4

Position- 1-based leftmost mapping POSition of the first matching base

```
3295752
```

SAM Field 5

Mapping Quality

- equals −10 log10 Pr{mapping position is wrong}, rounded to the nearest integer
- Same as phred!
- Probability of 99.9% = map quality of 30
- Probability of 0% = map quality of 0
- value 255 indicates that the mapping quality is not available.

60

.000001% probability wrong

SAM Field 6

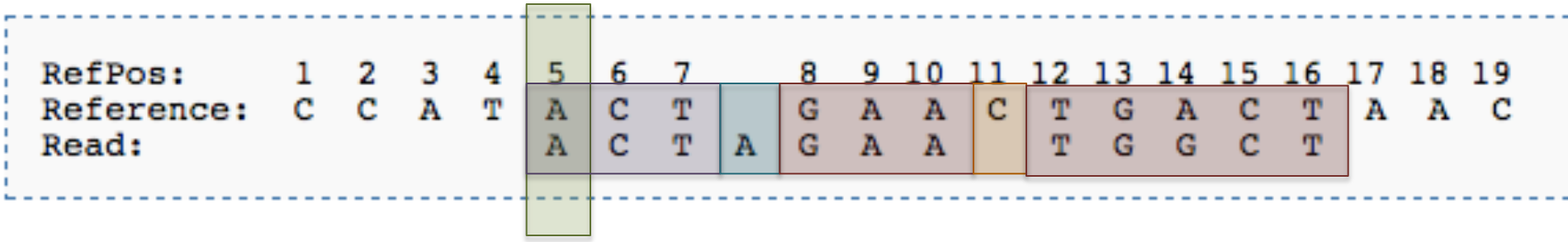
CIGAR String

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

```
36M   36 nucleotides match (perfect match)
8S28M 8 nucleotides clipped, 28 match
```

More CIGAR

Aligning these two:



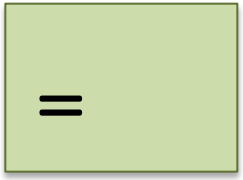
Position: 5

CIGAR: 3M1I3M1D5M

SAM Field 7

Reference sequence for the next read in the template

- For a forward read, this is the reference where the reverse read maps
- For a reverse read, this is the reference where the forward read maps



`=  reverse read maps on the same reference`

SAM Field 8

Position where the next read maps

```
3295706
```

```
(Forward read mapped at 3295752.
Remember the forward read mapped
to the reverse strand)
```

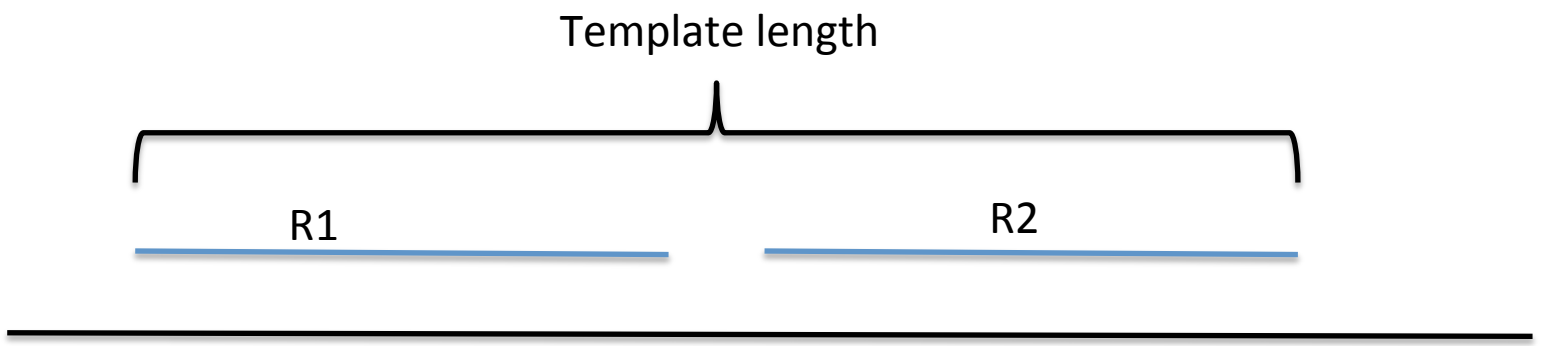
SAM Field 9

Observed template length

From leftmost base to rightmost base

Negative if this read is the rightmost read

-82



SAM Field 10

Sequence of the read

```
TGCTGGCGGCGATATCGTCCGTGGTTCCGATCTGGT
```

SAM Field 11

Quality of the read

```
?%<91<?>>??AAAAAAAAAAAAAAAAAAAAAAAAA
```

SAM Field 12

Optional MORE information

TAG:TYPE:VALUE format

```
XT:A:U  NM:i:0  SM:i:37 AM:i:37 X0:i:1
X1:i:0  XM:i:0  XO:i:0  XG:i:0  MD:Z:36
```

Anything with an X is specified by the user or by the mapping software, and is not part of the SAM spec.

Decipher the last fields

```
XT:A:U   One of Unique/Repeat/N/Mate-sw
NM:i:0   Edit distance to the reference
SM:i:37  Template-independent mapping quality
AM:i:37  Smallest template-independent mapping
            quality of other segments
X0:i:1   Number of best hits
X1:i:0   Number of suboptimal hits found by BWA
XM:i:0   Number of mismatches in the alignment
XO:i:0   Number of gap opens
XG:i:0   Number of gap extentions
MD:Z:36  String for mismatching positions
```

SAM

Example sam with one read:

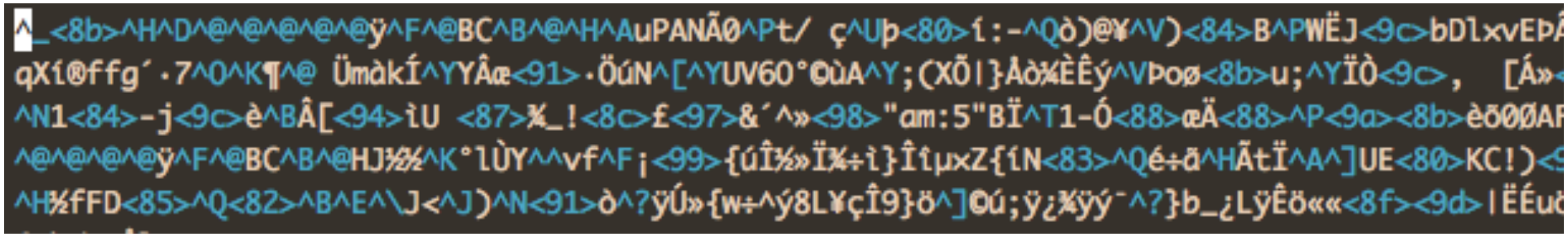
@SQ SN:gi|254160123|ref|NC_012967.1| LN:4629812

@PG ID:bwaPN:bwa VN:0.7.12-r1039 CL:/lustre/projects/rnaseq_ws/apps/bwa-0.7.12/bwa sampe ../raw_data/NC_012967.1.fasta aln_SRR030257_1.sai aln_SRR030257_2.sai ../raw_data/SRR030257_1.fastq ../raw_data/SRR030257_2.fastq

SRR030257.1 99 gi|254160123|ref|NC_012967.1| 950180 60 36M = 950295 151 TTACACTCCTGTTAATCCATACAGCAACAGTATTGG AAA;A;AA?A?AAAAA?;?A?1A;;????566)=*1 XT:A:U NM:i:1 SM:i:37 AM:i:25 X0:i:1 X1:i:0 XM:i:1 XO:i:0 XG:i:0 MD:Z:32C3

BAM Format

- Sister format to SAM
- BAM – Binary version of SAM
- compressed BGZF (Blocked GNU Zip Format) - a variant of GZIP (GNU ZIP),
- files are bigger than GZIP files, but they are much faster for random access
- Can index and then look up information embedded in the file with decompressing the whole file
- up to 75% smaller in size
- Not readable by people

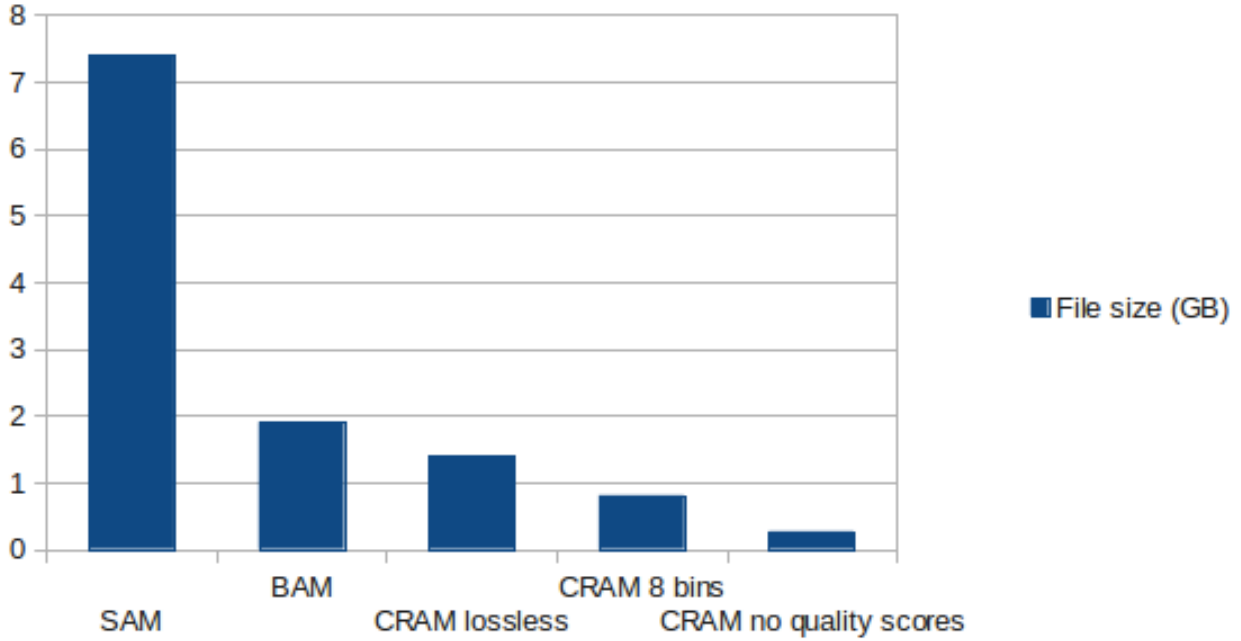


http://blastedbio.blogspot.com/2011/11/bgzf-blocked-bigger-better-gzip.html

CRAM

- Introduced in 2011 by EMBL/EBI
- Even smaller and more efficient than BAM files
- Rare

EBI has a cram toolkit
https://www.ebi.ac.uk/ena/software/cram-toolkit



Fritz, Markus Hsi-Yang, et al. "Efficient storage of high throughput DNA sequencing data using reference-based compression." Genome research 21.5 (2011): 734-740.

http://www.uppmax.uu.se/using-cram-to-compress-bam-files-on-uppnex