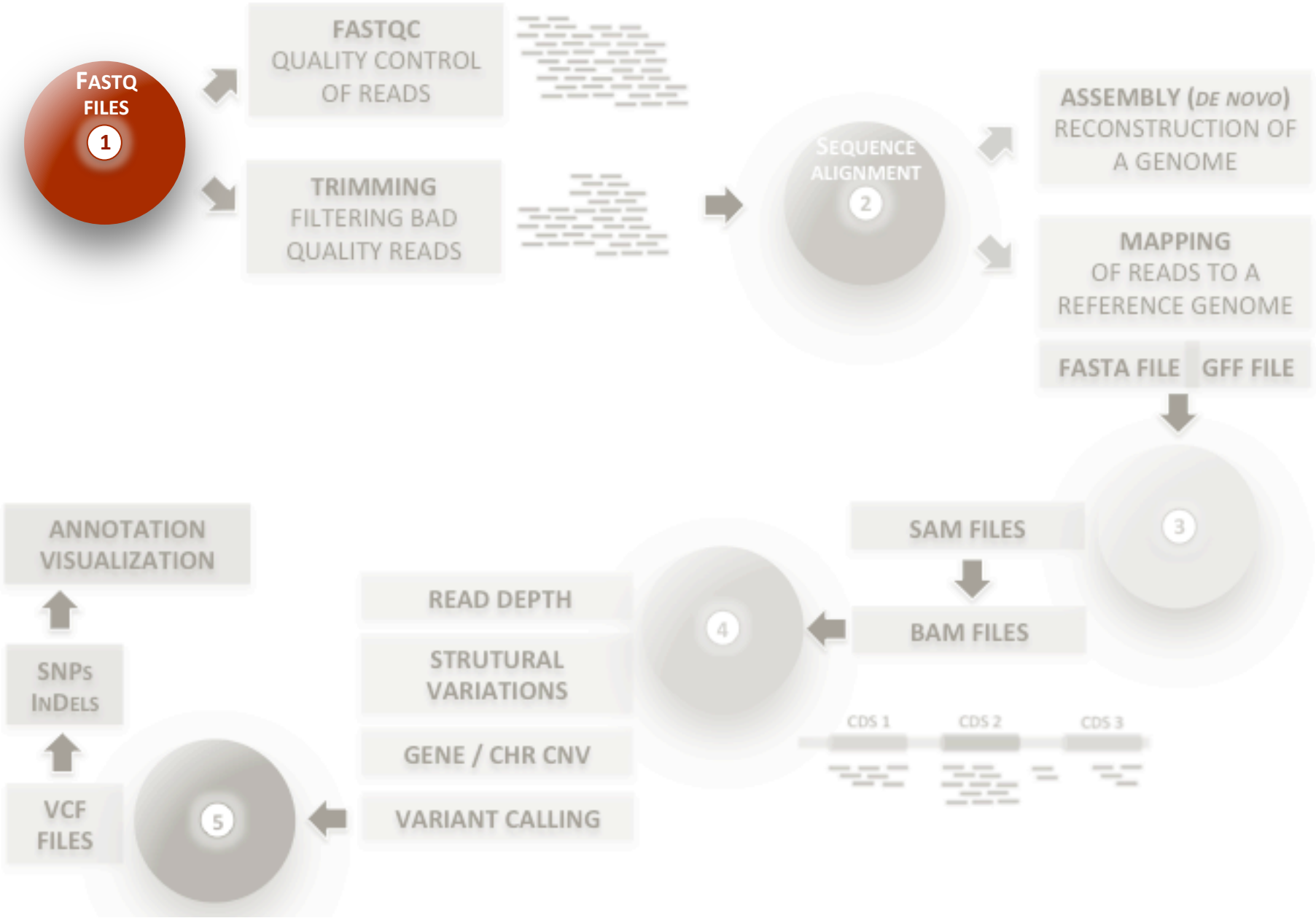
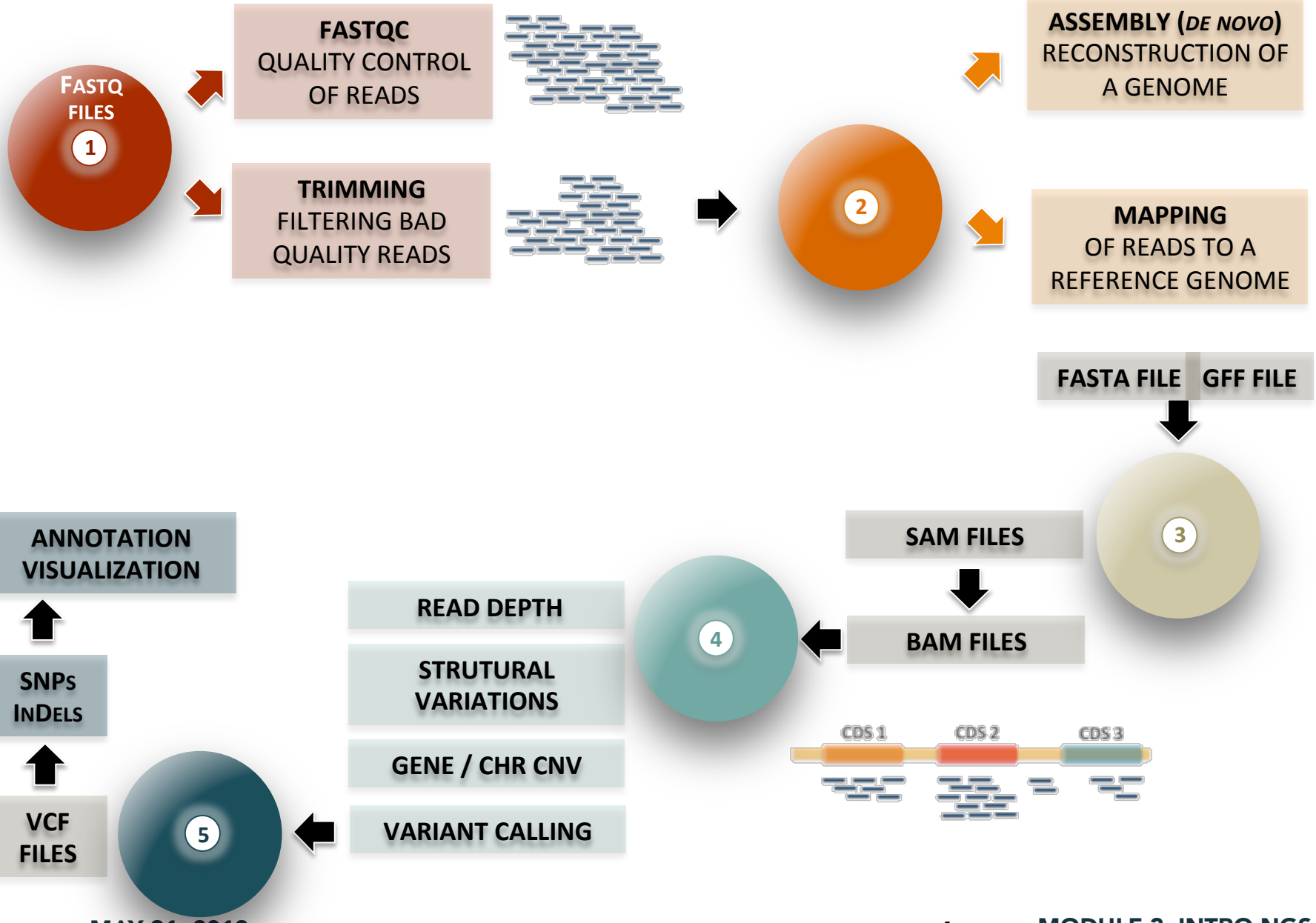
H3ABioNet
Pan African Bioinformatics Network for H3Africa

File formats
Understanding your mapping


FASTQ FILES
1
FASTQC
QUALITY CONTROL OF READS
TRIMMING
FILTERING BAD QUALITY READS
SEQUENCE ALIGNMENT
2
ASSEMBLY (DE NOVO)
RECONSTRUCTION OF A GENOME
MAPPING
OF READS TO A REFERENCE GENOME
FASTA FILE
GFF FILE
3
SAM FILES
BAM FILES
4
READ DEPTH
STRUTURAL VARIATIONS
GENE / CHR CNV
VARIANT CALLING
5
VCF FILES
SNPs InDels
ANNOTATION VISUALIZATION
CDS 1
CDS 2
CDS 3

Part I: File formats

Overview of analysis workflow



FASTQ FILE FORMAT

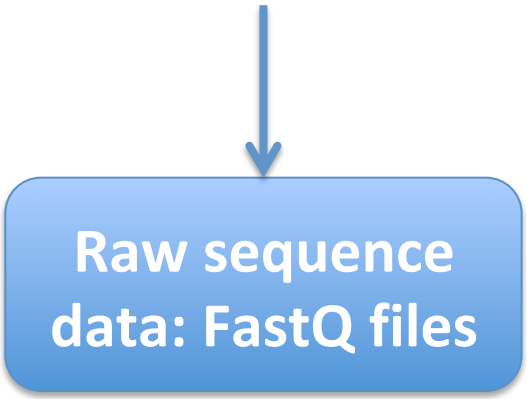
read file format

What is a FastQ file?

FASTQ= FASTA + Quality

FastQ format is a text-based format for storing both a biological sequence and the corresponding per base quality scores

-> Most common output provided by sequencing platforms



FASTQ FILE FORMAT

read file format

What is a FastQ file?

FASTQ= FASTA + Quality

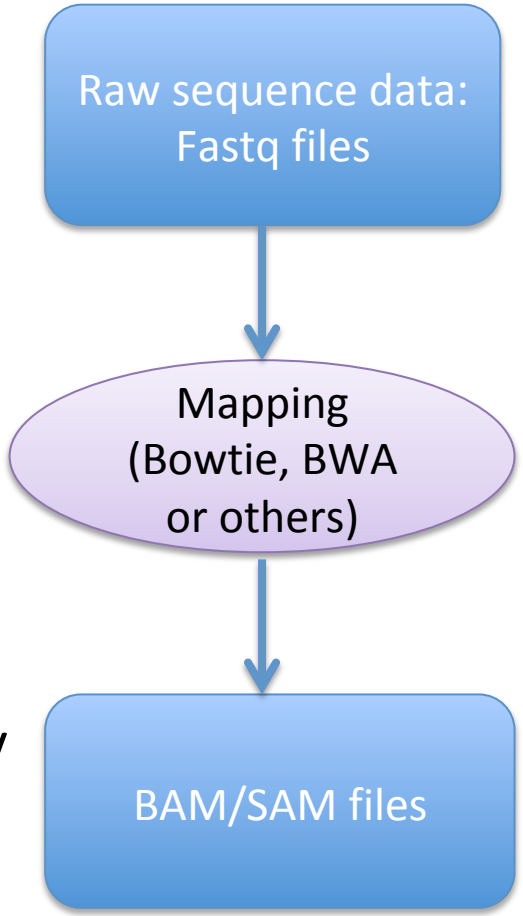
FastQ format is a text-based format for storing both a biological sequence and its corresponding quality scores

Raw sequence data: FastQ files

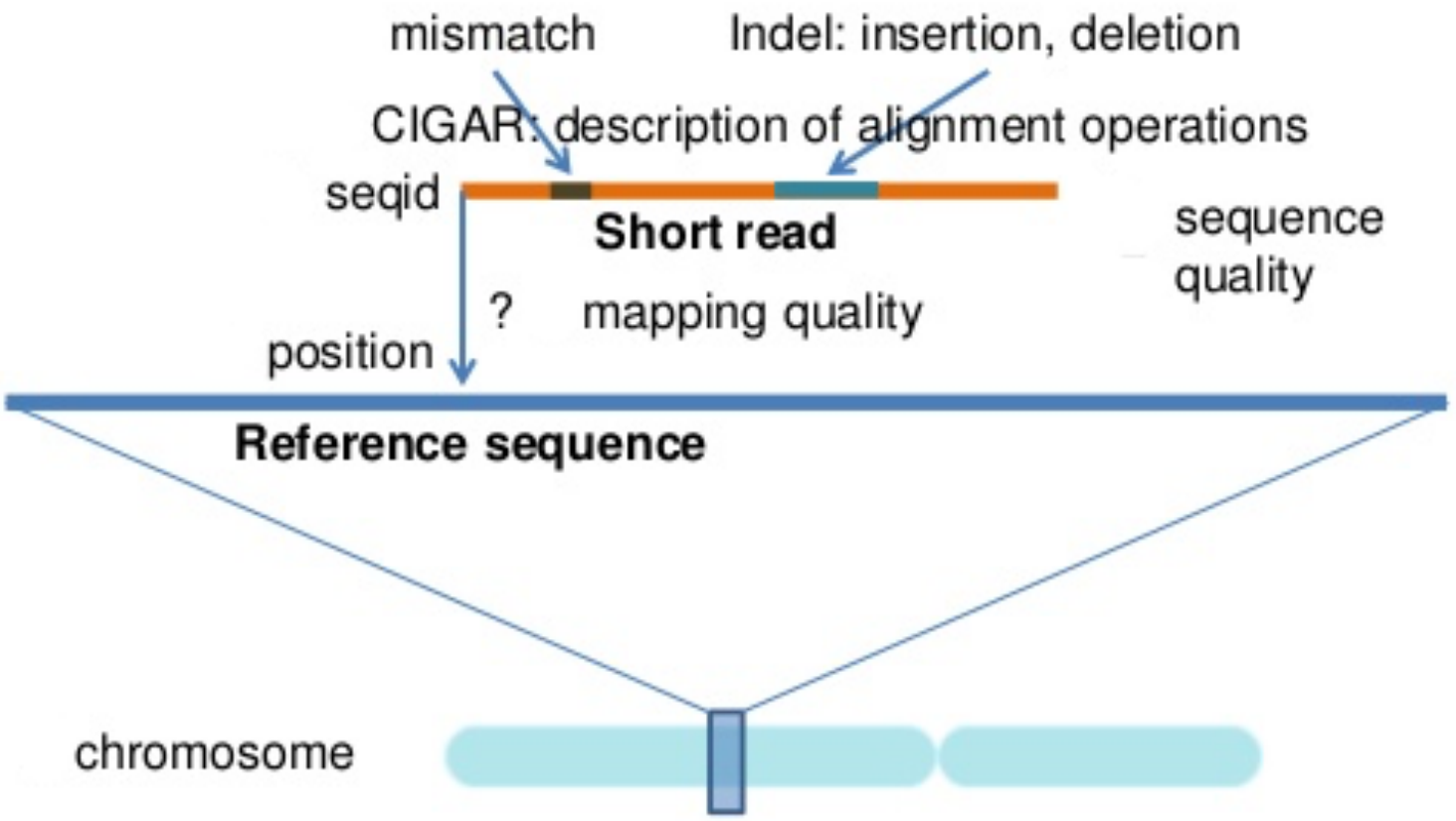
SAM and BAM formats

SAM/BAM files

- After mapping the FASTQ file to the reference genome you will end up with a SAM or BAM alignment file
- SAM stands for Sequence Alignment/Map format
- A single SAM file can store mapped, unmapped, and even QC-failed reads from a sequencing run, and indexed to allow rapid access. This means that the raw sequencing data can be fully recapitulated from the SAM/ BAM file.



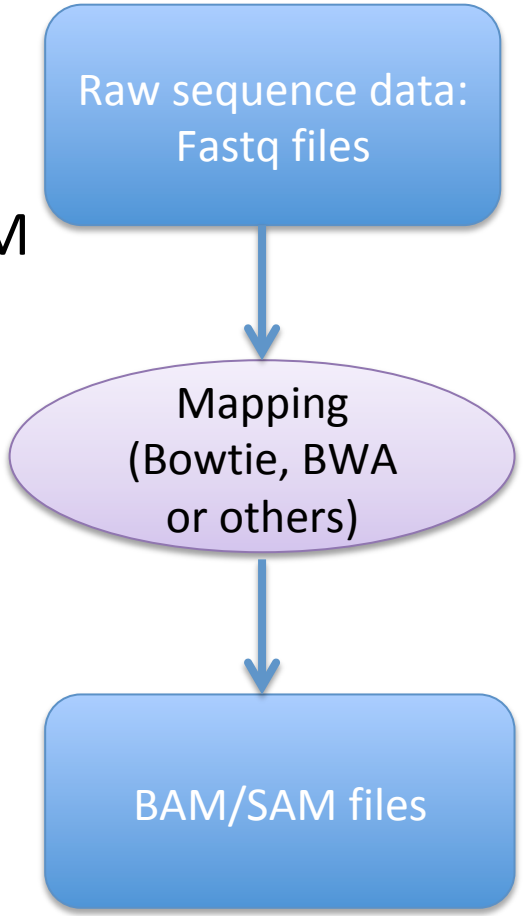
SAM/BAM files



Li Shen, 2014

SAM/BAM files

- SAM is rarely helpful and really takes up too much space which is why we use only the BAM in principle
- A BAM file (.bam) is the binary version of a SAM file (saving storage and faster manipulation)



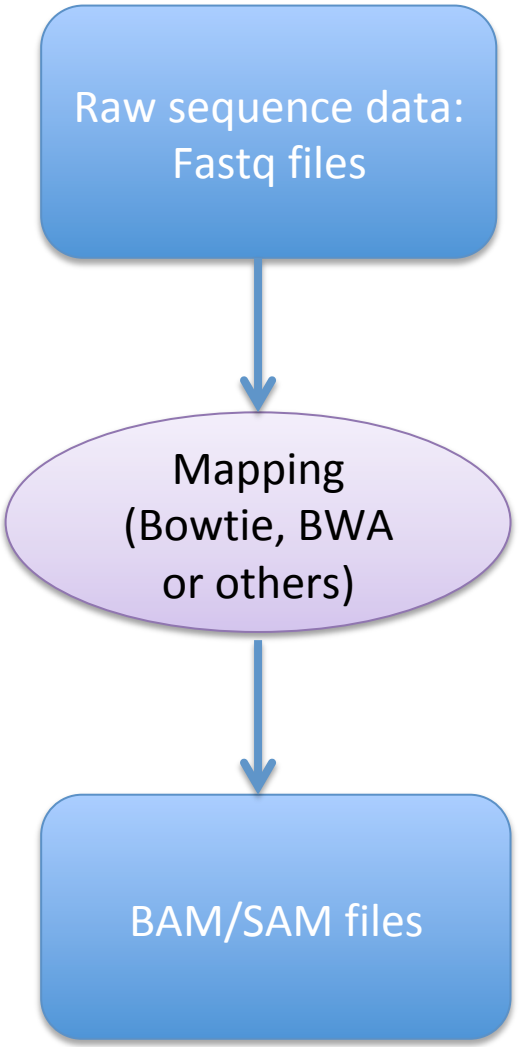
SAM/BAM files

- A SAM file (.sam) is a tab-delimited text file that contains sequence alignment data
- SAM files can be opened using a text editor or viewed using the UNIX "more" command

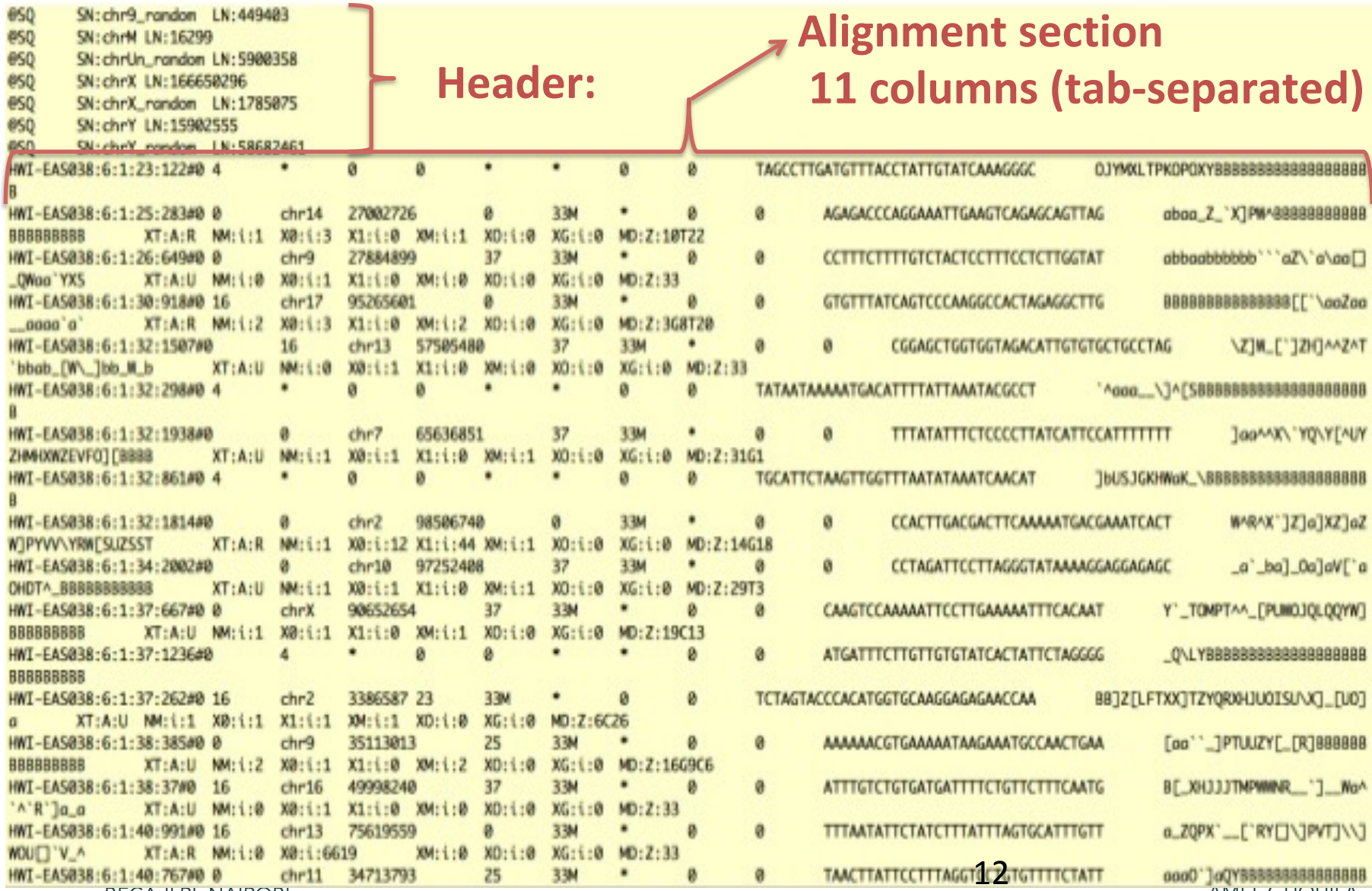
- Most alignment programs will supply:

- a header: describing the format version, sorting order of the reads, genomic sequences to which the reads were mapped

- an alignment section: contains the information for each sequence about where/ how it aligns to the reference genome

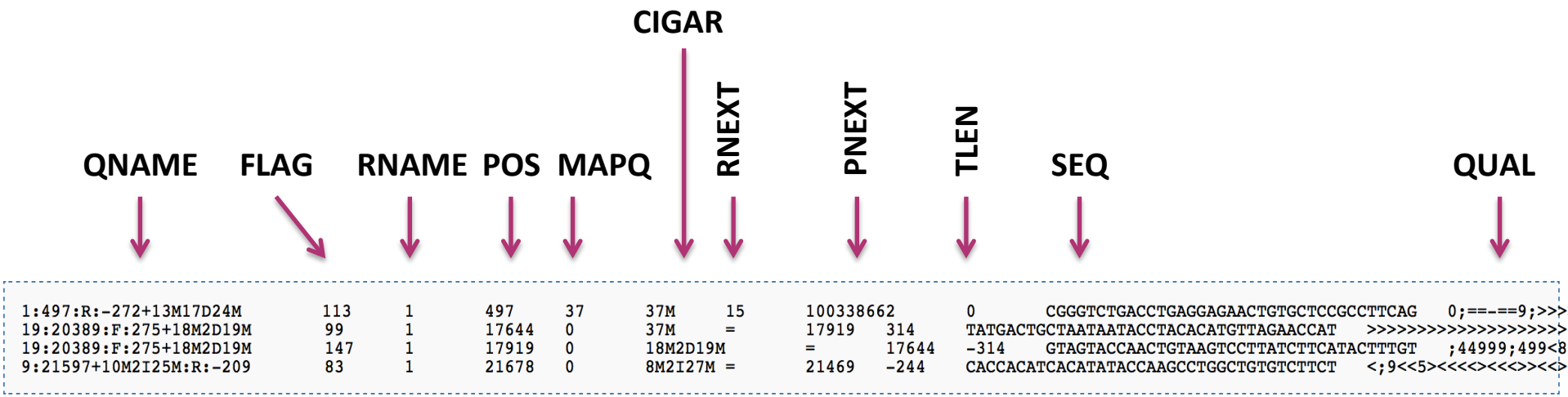


SAM files




```
@SQ     SN:chr9_random  LN:449403
@SQ     SN:chrM LN:16299
@SQ     SN:chrUn_random LN:5900358
@SQ     SN:chrX LN:166650296
@SQ     SN:chrX_random  LN:1785075
@SQ     SN:chrY LN:15902555
@SQ     SN:chrY_random  LN:58682461
HWI-EAS038:6:1:23:122#0 4       *       0       0       *       *       0       0       TAGCCTTGATGTTTACCTATTGTATCAAAGGGC        OJYMXLTPKDPOXYBBBBBBBBBBBBBBBBBBB
B
HWI-EAS038:6:1:25:283#0 0       chr14   27002726        0       33M     *       0       0       AGAGACCCAGGAAATTGAAGTCAGAGCAGTTAG        abaa_Z_`X]PW^BBBBBBBBBB
BBBBBBBBB       XT:A:R  NM:i:1  X0:i:3  X1:i:0  XM:i:1  XO:i:0  XG:i:0  MD:Z:18T22
HWI-EAS038:6:1:26:649#0 0       chr9    27884899        37      33M     *       0       0       CCTTTCTTTTGTCTACTCCTTTCCTCTTGGTAT        abbaabbbbbb```aZ\`a\aa[]
_QWaa`YX5       XT:A:U  NM:i:0  X0:i:1  X1:i:0  XM:i:0  XO:i:0  XG:i:0  MD:Z:33
HWI-EAS038:6:1:30:918#0 16      chr17   95265601        0       33M     *       0       0       GTGTTTATCAGTCCCAAGGCCACTAGAGGCTTG        BBBBBBBBBBBBBB[[`\aaZaa
__aaaa`a`       XT:A:R  NM:i:2  X0:i:3  X1:i:0  XM:i:2  XO:i:0  XG:i:0  MD:Z:3G8T20
HWI-EAS038:6:1:32:1507#0        16      chr13   57505480        37      33M     *       0       0       CGGAGCTGGTGGTAGACATTGTGTGCTGCCTAG        \Z]W_[`]ZH[]^^Z^T
`bbab_[W\_]bb_W_b       XT:A:U  NM:i:0  X0:i:1  X1:i:0  XM:i:0  XO:i:0  XG:i:0  MD:Z:33
HWI-EAS038:6:1:32:298#0 4       *       0       0       *       *       0       0       TATAATAAAAATGACATTTTATTAAATACGCCT        `^aaa__\]^[SBBBBBBBBBBBBBBBBBBB
B
HWI-EAS038:6:1:32:1938#0        0       chr7    65636851        37      33M     *       0       0       TTTATATTTCTCCCCTTATCATTCCATTTTTTT        ]aa^^X\`YQ\Y[^U\Y
ZHHXWZEVFQ][BBBB       XT:A:U  NM:i:1  X0:i:1  X1:i:0  XM:i:1  XO:i:0  XG:i:0  MD:Z:31G1
HWI-EAS038:6:1:32:861#0 4       *       0       0       *       *       0       0       TGCATTCTAAGTTGGTTTAATATAAATCAACAT        ]bUSJGKHWaK_\BBBBBBBBBBBBBBBBBB
B
HWI-EAS038:6:1:32:1814#0        0       chr2    98506740        0       33M     *       0       0       CCACTTGACGACTTCAAAAATGACGAAATCACT        W^R^X`]Z]a]XZ]aZ
W]PYVV\YRW[SUZSST       XT:A:R  NM:i:1  X0:i:12 X1:i:44 XM:i:1  XO:i:0  XG:i:0  MD:Z:14G18
HWI-EAS038:6:1:34:2002#0        0       chr10   97252488        37      33M     *       0       0       CCTAGATTCCTTAGGGTATAAAAGGAGGAGAGC        _a`_ba]_Oa]aV[`a
OHDT^_BBBBBBBBBB       XT:A:U  NM:i:1  X0:i:1  X1:i:0  XM:i:1  XO:i:0  XG:i:0  MD:Z:29T3
HWI-EAS038:6:1:37:667#0 0       chrX    90652654        37      33M     *       0       0       CAAGTCCAAAAATTCCTTGAAAAATTTCACAAT        Y`_TOMPT^^_[PUWOJQLQQYW]
BBBBBBBBB       XT:A:U  NM:i:1  X0:i:1  X1:i:0  XM:i:1  XO:i:0  XG:i:0  MD:Z:19C13
HWI-EAS038:6:1:37:1236#0        4       *       0       0       *       *       0       0       ATGATTTCTTGTTGTGTATCACTATTCTAGGGG        _Q\LYBBBBBBBBBBBBBBBBBB
BBBBBBBBB
HWI-EAS038:6:1:37:262#0 16      chr2    3386587 23      33M     *       0       0       TCTAGTACCCACATGGTGCAAGGAGAGAACCAA        BB]Z[LFTXX]TZYQRXHJUOISU\X]_[UO]
a       XT:A:U  NM:i:1  X0:i:1  X1:i:1  XM:i:1  XO:i:0  XG:i:0  MD:Z:6C26
HWI-EAS038:6:1:38:385#0 0       chr9    35113013        25      33M     *       0       0       AAAAAACGTGAAAAATAAGAAATGCCAACTGAA        [aa``_]PTUUZY[_[R]BBBBBB
BBBBBBBBB       XT:A:U  NM:i:2  X0:i:1  X1:i:0  XM:i:2  XO:i:0  XG:i:0  MD:Z:16G9C6
HWI-EAS038:6:1:38:37#0  16      chr16   49998240        37      33M     *       0       0       ATTTGTCTGTGATGATTTTCTGTTCTTTCAATG        B[_XHJJJTMPWWNR__`]__Wa^
`^`R`]a_a       XT:A:U  NM:i:0  X0:i:1  X1:i:0  XM:i:0  XO:i:0  XG:i:0  MD:Z:33
HWI-EAS038:6:1:40:991#0 16      chr13   75619559        0       33M     *       0       0       TTTAATATTCTATCTTTATTTAGTGCATTTGTT        a_ZQPX`__[`RY[]\]PVT]\\]
WOU[]`V_^       XT:A:R  NM:i:0  X0:i:6619       XM:i:0  XO:i:0  XG:i:0  MD:Z:33
HWI-EAS038:6:1:40:767#0 0       chr11   34713793        25      33M     *       0       0       TAACTTATTCCTTTAGGTTTGTTTTCTATT        aaaO`]aQYBBBBBBBBBBBBBBBB
```

SAM files



http://samtools.sourceforge.net/SAM1.pdf
http://genome.sph.umich.edu/wiki/SAM

SAM files

The following table gives an overview of the mandatory fields in the SAM format:

Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	[!-?A-~]{1,255}	Query template NAME
2	FLAG	Int	[0,2^{16}-1]	bitwise FLAG
3	RNAME	String	*|[!-()+-<>-~][!-~]*	Reference sequence NAME
4	POS	Int	[0,2^{31}-1]	1-based leftmost mapping POSition
5	MAPQ	Int	[0,2^{8}-1]	MAPping Quality
6	CIGAR	String	*|([0-9]+[MIDNSHPX=])+	CIGAR string
7	RNEXT	String	*|=|[!-()+-<>-~][!-~]*	Ref. name of the mate/next read
8	PNEXT	Int	[0,2^{31}-1]	Position of the mate/next read
9	TLEN	Int	[-2^{31}+1,2^{31}-1]	observed Template LENgth
10	SEQ	String	*|[A-Za-z=.]+	segment SEQuence
11	QUAL	String	[!-~]+	ASCII of Phred-scaled base QUALity+33

(http://samtools.github.io/hts-specs/SAMv1.pdf)

- QNAME: Query template NAME. Reads/segments having identical QNAME are regarded to come from the same template. A QNAME '*' indicates the information is unavailable.
- Used to group/identify alignments that are together, like paired alignments or a read that appears in multiple alignments.

SAM files

The following table gives an overview of the mandatory fields in the SAM format:

Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	[!-?A-~]{1,255}	Query template NAME
2	FLAG	Int	[0,2^{16}-1]	bitwise FLAG
3	RNAME	String	*|[!-()+-<>-~][!-~]*	Reference sequence NAME
4	POS	Int	[0,2^{31}-1]	1-based leftmost mapping POSition
5	MAPQ	Int	[0,2^{8}-1]	MAPping Quality
6	CIGAR	String	*|([0-9]+[MIDNSHPX=])+	CIGAR string
7	RNEXT	String	*|=|[!-()+-<>-~][!-~]*	Ref. name of the mate/next read
8	PNEXT	Int	[0,2^{31}-1]	Position of the mate/next read
9	TLEN	Int	[-2^{31}+1,2^{31}-1]	observed Template LENgth
10	SEQ	String	*|[A-Za-z=.]+	segment SEQuence
11	QUAL	String	[!-~]+	ASCII of Phred-scaled base QUALity+33

(http://samtools.github.io/hts-specs/SAMv1.pdf)

FLAG: FLAG: bitwise FLAG (ideal for compression).

11 boolean flags all stotred in a singe column

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

SAM files

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

SAM file

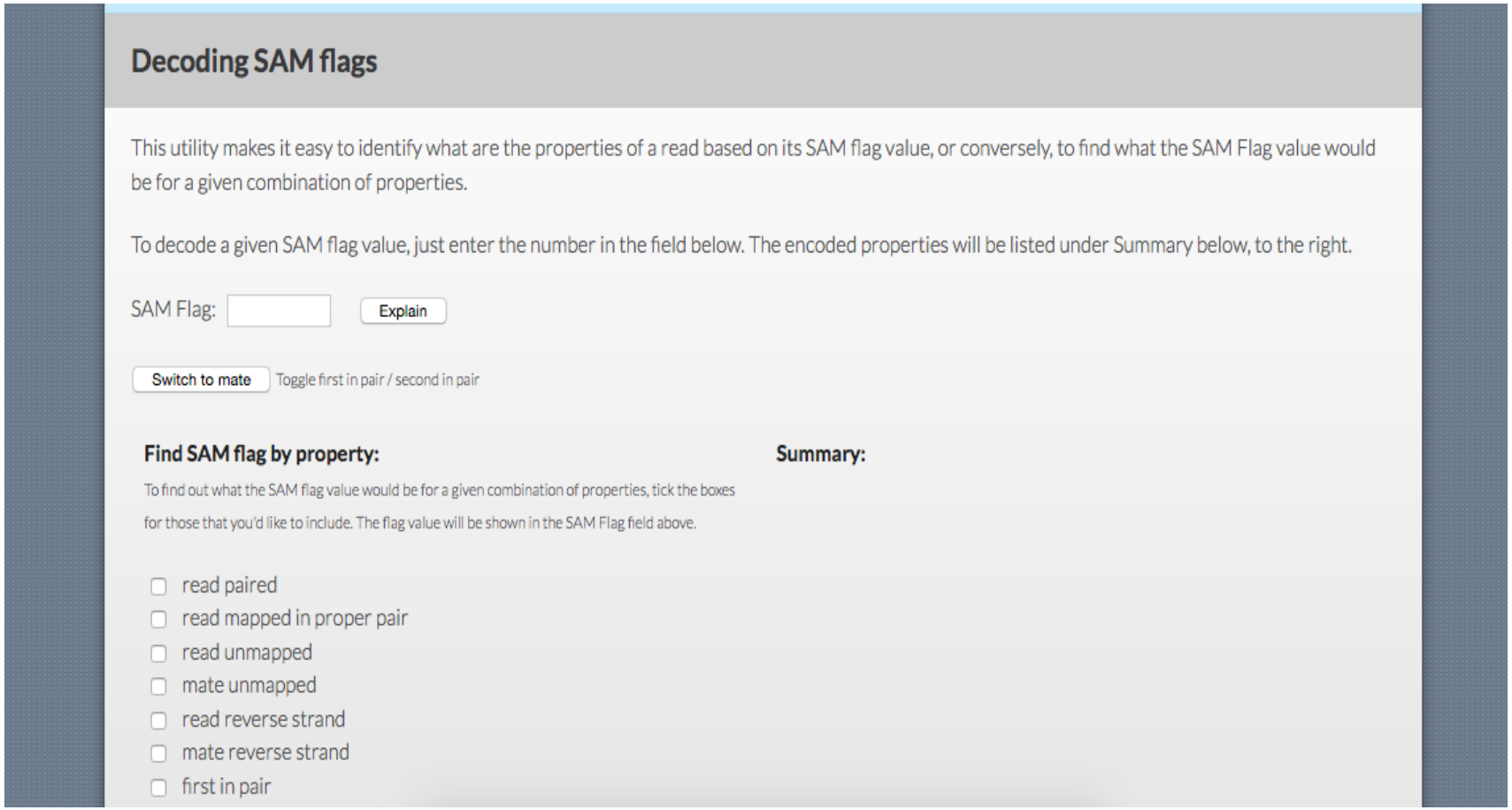
(b)
```
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTA *
```

read mapped to position 7:
FLAG 163 (=1 + 2 + 32 + 128):

- **Read is the second read in the pair (128)**
- **Read is properly paired (1 + 2)**
- **its mate is mapped to 37 on the reverse strand (32)**

SAM files

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



SAM files

The following table gives an overview of the mandatory fields in the SAM format:

Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	`[!-?A-~]{1,255}`	Query template NAME
2	FLAG	Int	$[0,2^{16}-1]$	bitwise FLAG
3	RNAME	String	`\*|[!-()+-<>-~][!-~]*`	Reference sequence NAME
4	POS	Int	$[0,2^{31}-1]$	1-based leftmost mapping POSition
5	MAPQ	Int	$[0,2^{8}-1]$	MAPping Quality
6	CIGAR	String	`\*|([0-9]+[MIDNSHPX=])+`	CIGAR string
7	RNEXT	String	`\*|=|[!-()+-<>-~][!-~]*`	Ref. name of the mate/next read
8	PNEXT	Int	$[0,2^{31}-1]$	Position of the mate/next read
9	TLEN	Int	$[-2^{31}+1,2^{31}-1]$	observed Template LENgth
10	SEQ	String	`\*|[A-Za-z=.]+`	segment SEQuence
11	QUAL	String	`[!-~]+`	ASCII of Phred-scaled base QUALity+33

(http://samtools.github.io/hts-specs/SAMv1.pdf)

It equals −10 log10 Pr{mapping position is wrong}, rounded to the nearest integer.

The MAPQ value can be used to figure out how unique an alignment is in the genome.

- ✓ Large number, >10 indicates it's likely the alignment is unique.
- ✓ 255 indicates that the mapping quality is not available

SAM files

- The CIGAR string is a **sequence of numbers and letters** representing the associated **information on bases alignment** used to indicate things like which bases align (either a match/ mismatch) with the reference, are deleted from the reference, and if there are insertions that are not in the reference

More information about these formats available here:
http://samtools.sourceforge.net
https://samtools.github.io/hts-specs/SAMv1.pdf

SAM files

Mapped and unmapped reads are imported into SAM/BAM format

The standard CIGAR description of pairwise alignment defines three operations:
'**M**' for **alignment match**, '**I**' for insertion compared with the reference and '**D**' for deletion.

(NB: The POS indicates that the read aligns starting at position 5 on the reference)

The CIGAR :
3M = 3 bases in the read sequence align with the reference.
1I = The next base in the read does not exist in the reference.
1D = The reference base does not exist in the read sequence

```
RefPos:      1  2  3  4  5  6  7  8  9 10 11 12 13
Reference:   C  C  A  T  A  C  T  G  A  A  C  T  G
Read: ACTAGAATG
```

```
RefPos:      1  2  3  4  5  6  7     8  9 10 11 12 13
Reference:   C  C  A  T  A  C  T     G  A  A  C  T  G
Read:                   A  C  T  A  G  A  A     T  G
```

POS: 5
CIGAR: 3M1I3M1D2M

http://genome.sph.umich.edu/wiki/SAM

SAM files

Examples of CIGAR strings for different types of alignments

Alignments

(a)

```
coor    12345678901234  5678901234567890123456789012345
ref     AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT

r001+         TTAGATAAAGGATA*CTG
r002+       aaaAGATAA*GGATA
r003+     gcctaAGCTAA
r004+                   ATAGCT..............TCAGC
r003-                         ttagctTAGGC
r001-                                      CAGCGCCAT
```

SAM file

(b)

```
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTA *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

(Li et al., 2009)

SAM files

The following table gives an overview of the mandatory fields in the SAM format:

Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	[!-?A-~]{1,255}	Query template NAME
2	FLAG	Int	[0,2^{16}-1]	bitwise FLAG
3	RNAME	String	*|[!-()+-<>-~][!-~]*	Reference sequence NAME
4	POS	Int	[0,2^{31}-1]	1-based leftmost mapping POSition
5	MAPQ	Int	[0,2^{8}-1]	MAPping Quality
6	CIGAR	String	*|([0-9]+[MIDNSHPX=])+	CIGAR string
7	RNEXT	String	*|=|[!-()+-<>-~][!-~]*	Ref. name of the mate/next read
8	PNEXT	Int	[0,2^{31}-1]	Position of the mate/next read
9	TLEN	Int	[-2^{31}+1,2^{31}-1]	observed Template LENgth
10	SEQ	String	*|[A-Za-z=.]+	segment SEQuence
11	QUAL	String	[!-~]+	ASCII of Phred-scaled base QUALity+33

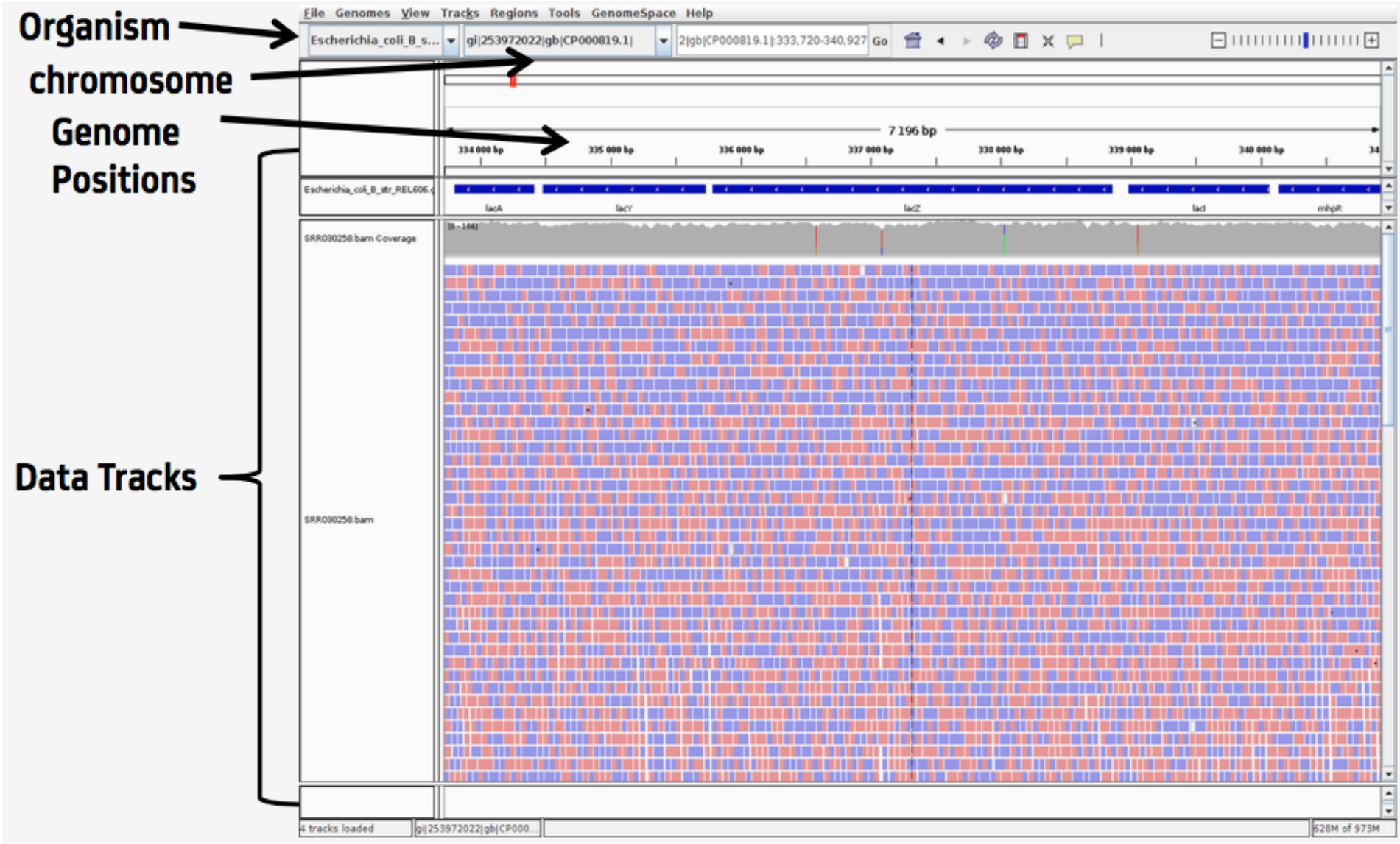
(http://samtools.github.io/hts-specs/SAMv1.pdf)

Name of mate (mate pair information for paired-end sequencing)
Position of mate (mate pair information)

Obviously, the chromosome and position are important. The CIGAR string is also important to know where insertions (i.e. introns) might exist in your read.

Part II: Visualizing mapping results

IGV: Integrated Genome Viewer


Organism
chromosome
Genome
Positions
Data Tracks
File Genomes View Tracks Regions Tools GenomeSpace Help
Escherichia_coli_B_s...
gi|253972022|gb|CP000819.1|
2|gb|CP000819.1|:333,720-340,927
Go
7 196 bp
334 000 bp
335 000 bp
336 000 bp
337 000 bp
338 000 bp
339 000 bp
340 000 bp
lacA
lacY
lacZ
lacI
mhpR
SRR030258.bam Coverage
SRR030258.bam
4 tracks loaded
628M of 973M

Part III: Exploring SAM/BAM files with Samtools

samtools

- A free software package for manipulating SAM/BAM files
- SAMtools provides the utilities for:
  - ✓ Viewing and formatting
  - ✓ Extracting statistics
  - ✓ Indexing
  - ✓ Manipulating SAM/BAM files
  - ✓ Editing

Usage: **samtools <command> [options]**

samtools

Different samtools options available:

http://www.htslib.org/doc/samtools.html

```
Usage:   samtools <command> [options]

Command: view         SAM<->BAM conversion
         sort         sort alignment file
         mpileup      multi-way pileup
         depth        compute the depth
         faidx        index/extract FASTA
         tview        text alignment viewer
         index        index alignment
         idxstats     BAM index stats (r595 or later)
         fixmate      fix mate information
         flagstat     simple stats
         calmd        recalculate MD/NM tags and '=' bases
         merge        merge sorted alignments
         rmdup        remove PCR duplicates
         reheader     replace BAM header
         cat          concatenate BAMs
         targetcut    cut fosmid regions (for fosmid pool only)
         phase        phase heterozygotes
```

samtools

One of the most used tools since BAM files are often the input files needed for many different analysis programs

samtools view

#from SAM to BAM
samtools view -b test.sam > test.bam

#from BAM to SAM
samtools view test.bam > test.sam

Use options –h and –H to deal with the header

samtools

Always sort your BAM files; many downstream programs only take sorted BAM files.

samtools sort

```
#sorting a bam file
samtools sort test.bam –o test.bam


#converting SAM directly to a sorted BAM file
samtools view test.sam |samtools sort –o test.bam
```

samtools

Some programs require that for faster access we need a companion file (often called index) for the different formats

- ✓ FASTA (.fa & .fai) (samtools faidx)
- ✓ BAM and BAI formats (suffixes .bam & .bai) (samtools index)
- ✓ VCF (.vcf & .vcf.idx)

samtools

- samtools flagstat file.bam
- Does a full pass through the input file to calculate and print statistics such as:
  - ✓ % reads mapped
  - ✓ % unmapped reads
  - ✓ % reads properly paired
  - ✓ Other information

1	6874858 + 0 in total (QC-passed reads + QC-failed reads)
2	90281 + 0 duplicates
3	6683299 + 0 mapped (97.21%)
4	6816083 + 0 paired in sequencing
5	3408650 + 0 read1
6	3407433 + 0 read2
7	6348470 + 0 properly paired (93.14NaV)
8	6432965 + 0 with itself and mate mapped
9	191559 + 0 singletons (2.81NaV)
10	57057 + 0 with mate mapped to a different chr
11	45762 + 0 with mate mapped to a different chr (mapQ>=5)

samtools

Some programs require that for faster access we need a companion file (often called index) for the different formats

FASTA (.fa & .fai)

BAM and BAI formats (suffixes .bam & .bai)

VCF (.vcf & .vcf.idx)

samtools

Many tools require a BAM Index file to more efficiently access reads in a BAM file.

To create a BAM index:

- You must first sort the BAM file to create a sorted.bam
- Run samtools index with the sorted.bam as input
- This will create a file named sorted.bam.bai which contains the index

Example:

```
samtools view  file.sam >file.bam
samtools sort file.bam -o file_sorted.bam
samtools index file_sorted.bam file_sorted.bai
```

samtools

Filtering out unmapped reads from BAM files

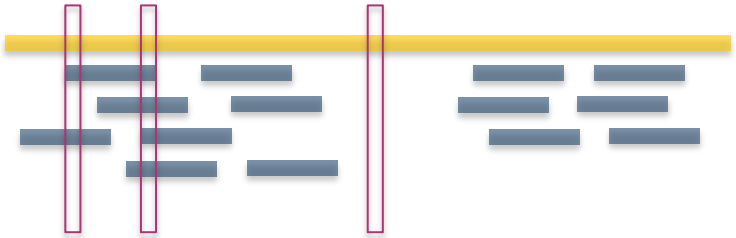
```
samtools view -h -F 4  file.bam > file_only_mapped.sam
# output back to BAM
samtools view -h -F 4 –b file.bam > file_only_mapped.bam
```

Extracting SAM entries mapping to a specific region

```
#index the bam file first
samtools index file.bam
samtools view file.bam chr1:200000-500000
#all reads mapping on chr1 as another bam
samtools view –b file.bam chr1 > file_chr1.bam
```

samtools

Samtools allows computing the depth at each position



Samtools depth

#syntax
samtools depth options file.bam

–a allows to output all positions (including those with zero depth)
samtools depth –a test.bam

#–q INT only count reads with base quality greater than *INT*
samtools depth –q int test.bam

samtools

Samtools allows computing the read depth per genomic region, as specified in the supplied BED file

Samtools bedcov

#syntax

samtools bedcov options region.bed file.bam

Exercises:

https://github.com/bixcop18/module_3_intro_NGS/blob/master/exploremappingresults.md

GFF and BED formats

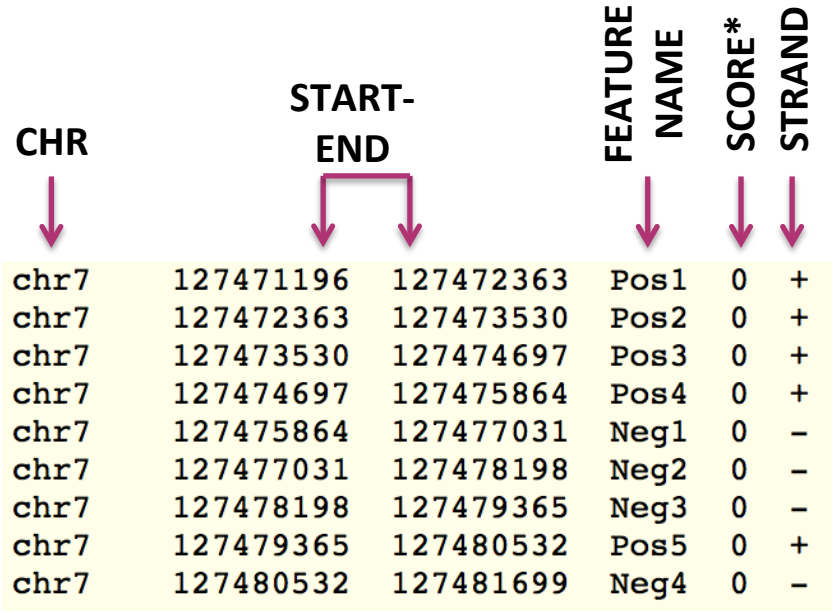
BED files

BED = (Browser Extensible Data)
(http://genome.ucsc.edu/FAQ/FAQformat)

BED starts are zero-based and BED ends are one-based

- Tab-delimited files
- 3 first fields required, others optional

File format (BEDPE): describes disjoint genome features, such as structural variations or paired-end sequence alignments.



chr7	127471196	127472363	Pos1	0	+
chr7	127472363	127473530	Pos2	0	+
chr7	127473530	127474697	Pos3	0	+
chr7	127474697	127475864	Pos4	0	+
chr7	127475864	127477031	Neg1	0	–
chr7	127477031	127478198	Neg2	0	–
chr7	127478198	127479365	Neg3	0	–
chr7	127479365	127480532	Pos5	0	+
chr7	127480532	127481699	Neg4	0	–

* Any string could be used (p-value, enrichment score…)

7. thickStart - The starting position at which the feature is drawn thickly.
8. thickEnd - The ending position at which the feature is drawn thickly.
9. itemRgb - An RGB value of the form R,G,B (e.g. 255,0,0).
10. blockCount - The number of blocks (exons) in the BED line.
11. blockSizes - A comma-separated list of the block sizes.
12. blockStarts - A comma-separated list of block starts.

BED files

(A) **BED3**: A BED file where each feature is described by **chrom**, **start**, and **end**.

For example: `chr1 11873 14409`

(B) **BED4**: A BED file where each feature is described by **chrom**, **start**, **end**, and **name**.

For example: `chr1 11873 14409 uc001aaa.3`

(C) **BED5**: A BED file where each feature is described by **chrom**, **start**, **end**, **name**, and **score**.

For example: `chr1 11873 14409 uc001aaa.3 0`

(D) **BED6**: A BED file where each feature is described by **chrom**, **start**, **end**, **name**, **score**, and **strand**.

For example: `chr1 11873 14409 uc001aaa.3 0 +`

(E) **BED12**: A BED file where each feature is described by all twelve columns listed above.

For example:
```
chr1 11873 14409 uc001aaa.3 0 + 11873
11873 0 3 354,109,1189, 0,739,1347,
```

BED files

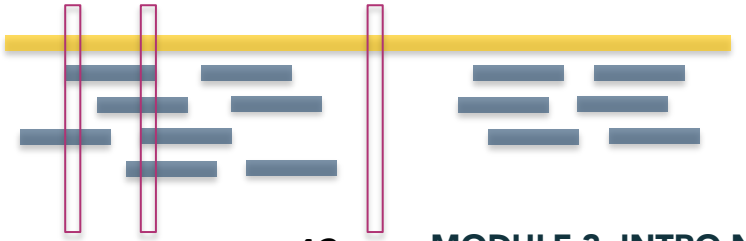
- **BEDTools** support a wide range of operations for interrogating and manipulating genomic features.

intersectBed
Returns overlapping features between two BED/GFF/VCF files.

genomeCoverageBed
Histogram or a “per base” report of genome coverage.
…

- Coverage

The number of times each nucleotide is « read »
→ Fold Coverage (numberX)



GFF files

GFF3

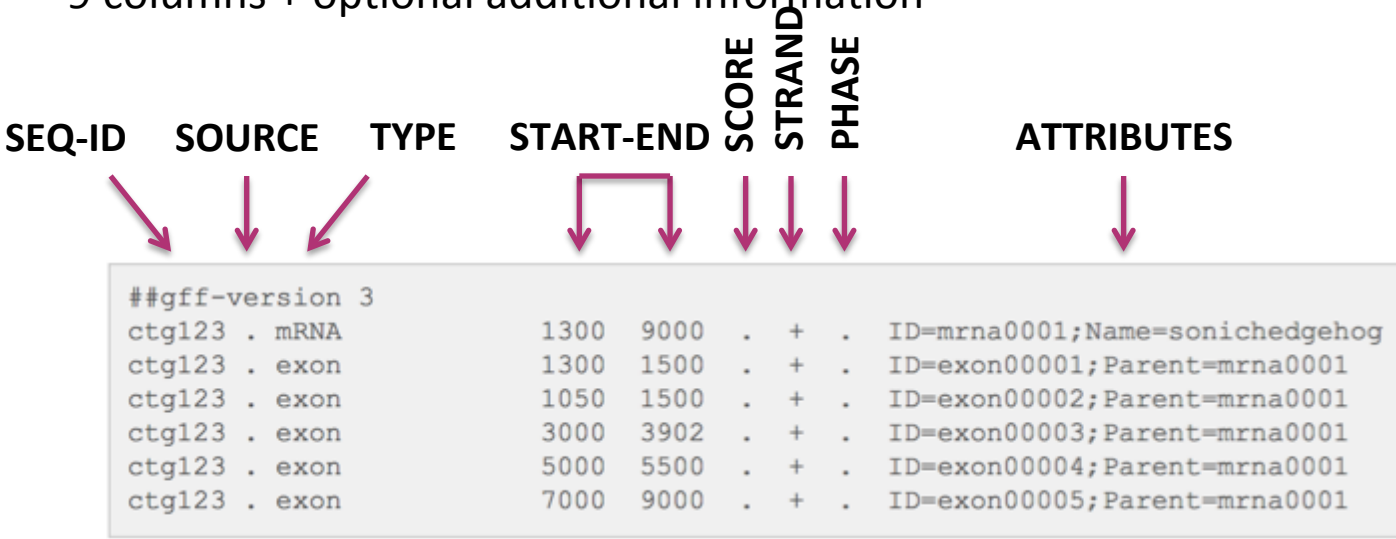
The two most widely used formats for representing genome features are the BED and GFF formats.

GFF (Generic Feature Format) is a standard file format for storing genomic features in a text file.

Careful : GFF has several versions, the most recent is *GFF3*.

GFF start at a 1-based position and ends at a 1-based position.

- 1 line for 1 feature
- tab-delimited file (tab-separated columns)
- 9 columns + optional additional information



http://gmod.org/wiki/GFF3
http://www.ensembl.org/

Bedtools genomecov

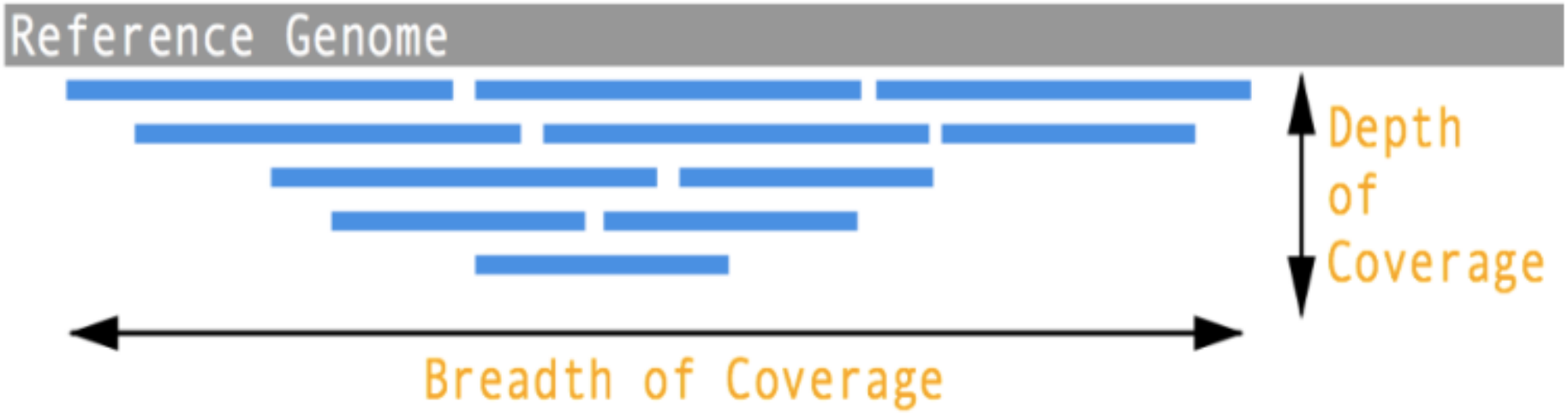
- **bedools genomecov** computes histograms per-base reports (-d) and BEDGRAPH (-bg) summaries of feature coverage

Command: bedtools genomecov −bg -ibam yourbam.bam -bg

Check out this link:
http://bedtools.readthedocs.io/en/latest/content/tools/genomecov.html

for more examples and different parameters

Depth and breadth of coverage



http://www.danielecook.com/calculate-depth-coverage-bam-file/

Sequencing depth and coverage

Theoretical sequencing coverage C= LN/G

G: length of the haploid genome

L: the read length

N: the number of reads

Coverage/read count calculator:
http://apps.bioconnector.virginia.edu/covcalc/

Compute depth coverage:

- http://www.danielecook.com/calculate-depth-coverage-bam-file/
- https://bioconductor.org/packages/devel/bioc/vignettes/CoverageView/inst/doc/CoverageView.pdf

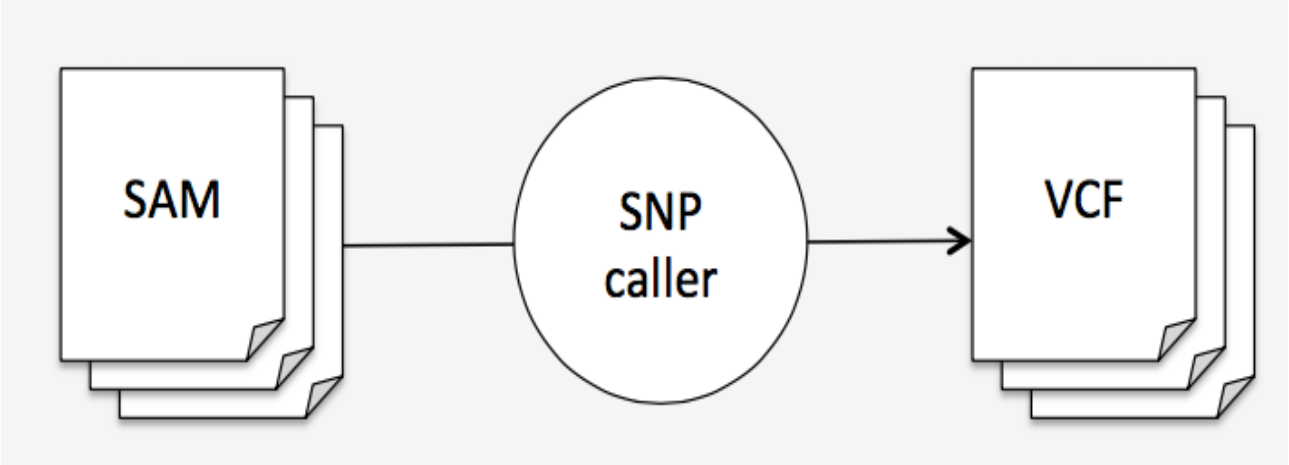
Sequencing depth and coverage

Check these papers:

- https://www.illumina.com/documents/products/technotes/technote_coverage_calculation.pdf
- https://www.nature.com/articles/nrg3642#df1
- https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5737511/

VCF format

VCF files



Many tools for variants detection: GATK, Samtools (mpileup), FreeBayes, PICARD, etc.

VCF files

VCF file (.vcf) = "Variant Calling format"

Tab-delimited text file format that can store information about variants: the differences between your sample and the reference genome.

All this information can be easily queried (**VCFtools**).

- Format validation.
- SNV annotation.
- VCF comparison.
- Statistics.
- …

VCF files

VCF Format coordinates are 1-based

Types of variants

SNPs

Alignment	*VCF representation*		
ACGT	POS	REF	ALT
ATGT	2	C	T

Insertions

Alignment	*VCF representation*		
AC-GT	POS	REF	ALT
ACTGT	2	C	CT

Deletions

Alignment	*VCF representation*		
ACGT	POS	REF	ALT
A--T	1	ACG	A

Complex events

Alignment	*VCF representation*		
ACGT	POS	REF	ALT
A-TT	1	ACG	AT

Large structural variants

VCF representation

POS	REF	ALT	INFO
100	T	<DEL>	SVTYPE=DEL;END=300

Example

VCF header

```
##fileformat=VCFv4.0
##fileDate=20100707
##source=VCFtools
##reference=NCBI36
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality (phred score)">
##FORMAT=<ID=GL,Number=3,Type=Float,Description="Likelihoods for RR,RA,AA genotypes (R=ref,A=alt)">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##ALT=<ID=DEL,Description="Deletion">
##INFO=<ID=SVTYPE,Number=1,Type=String,Description="Type of structural variant">
##INFO=<ID=END,Number=1,Type=Integer,Description="End position of the variant">
```

Body

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	SAMPLE1	SAMPLE2
1	1	.	ACG	A,AT	.	PASS	.	GT:DP	1/2:13	0/0:29
1	2	rs1	C	T,CT	.	PASS	H2;AA=T	GT:GQ	0|1:100	2/2:70
1	5	.	A	G	.	PASS	.	GT:GQ	1|0:77	1/1:95
1	100	.	T	<DEL>	.	PASS	SVTYPE=DEL;END=300	GT:GQ:DP	1/1:12:3	0/0:20

Mandatory header lines

Optional header lines (meta-data about the annotations in the VCF body)

Reference alleles (GT=0)

Alternate alleles (GT>0 is an index to the ALT column)

Deletion

SNP

Large SV

Insertion

Other event

Phased data (G and C above are on the same chromosome)

http://wiki.bits.vib.be/index.php/NGS-formats

VCF files

No need to write your own parser, you can use GATK Variants to table

https://software.broadinstitute.org/gatk/documentation/tooldocs/current/org_broadinstitute_gatk_tools_walkers_variantutils_VariantsToTable.php

http://wiki.bits.vib.be/index.php/NGS-formats

Variants annotation

Variant annotation programs: SnpEff

- A variant annotation and effect prediction tool.
- Annotates and predicts the effects of variants on genes: Are they in a gene? In an exon? Do they change protein coding? Do they cause premature stop codons?

```
$ java -jar snpEff.jar
SnpEff version SnpEff 4.1 (build 2015-01-07), by Pablo Cingolani
Usage: snpEff [command] [options] [files]

Run 'java -jar snpEff.jar command' for help on each specific command

Available commands:
        [eff|ann]                       : Annotate variants / calculate effects (you can use either 'ann' or 'eff
        build                           : Build a SnpEff database.
        buildNextProt                   : Build a SnpEff for NextProt (using NextProt's XML files).
        cds                             : Compare CDS sequences calculated form a SnpEff database to the one in a
        closest                         : Annotate the closest genomic region.
        count                           : Count how many intervals (from a BAM, BED or VCF file) overlap with eac
        databases                       : Show currently available databases (from local config file).
        download                        : Download a SnpEff database.
        dump                            : Dump to STDOUT a SnpEff database (mostly used for debugging).
        genes2bed                       : Create a bed file from a genes list.
        len                             : Calculate total genomic length for each marker type.
        protein                         : Compare protein sequences calculated form a SnpEff database to the one
        spliceAnalysis                  : Perform an analysis of splice sites. Experimental feature.
```

Variants annotation

Here is an example of a file before and after being annotated using SnpEff:

VCF file before annotations

```
#CHROM POS     ID        REF    ALT     QUAL FILTER INFO
1      889455  .       G       A       100.0   PASS    AF=0.0005
1      897062  .       C       T       100.0   PASS    AF=0.0005
```

VCF file after being annotated using SnpEff

```
#CHROM POS     ID        REF    ALT     QUAL FILTER INFO
1      889455  .       G       A       100.0   PASS    AF=0.0005;EFF=STOP_GAINED(HIGH|NONSENSE|Cag/Tag|Q236*|
1      897062  .       C       T       100.0   PASS    AF=0.0005;EFF=STOP_GAINED(HIGH|NONSENSE|Cag/Tag|Q141*|
```

A you can see, SnpEff added an 'EFF' tag to the INFO field (eigth column).

```
|749|NOC2L||CODING|NM_015658|)
|642|KLHL17||CODING|NM_198317|)
```

http://wiki.bits.vib.be/index.php/NGS-formats