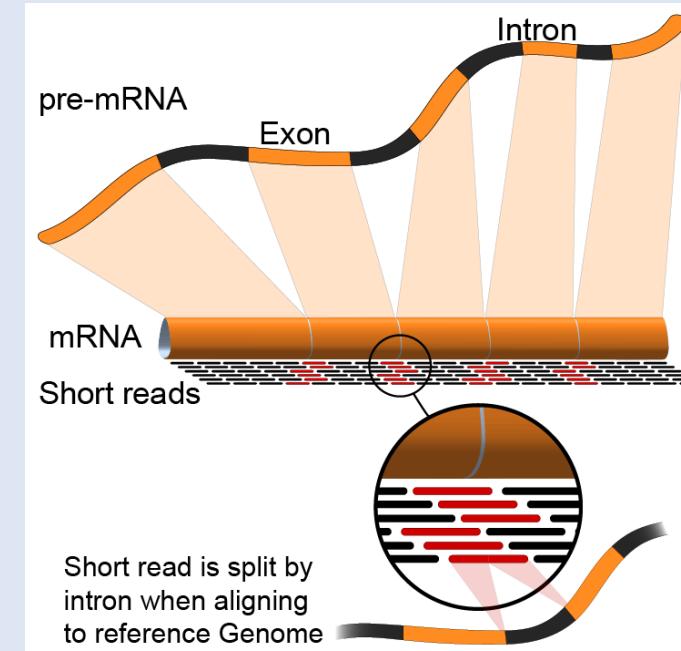
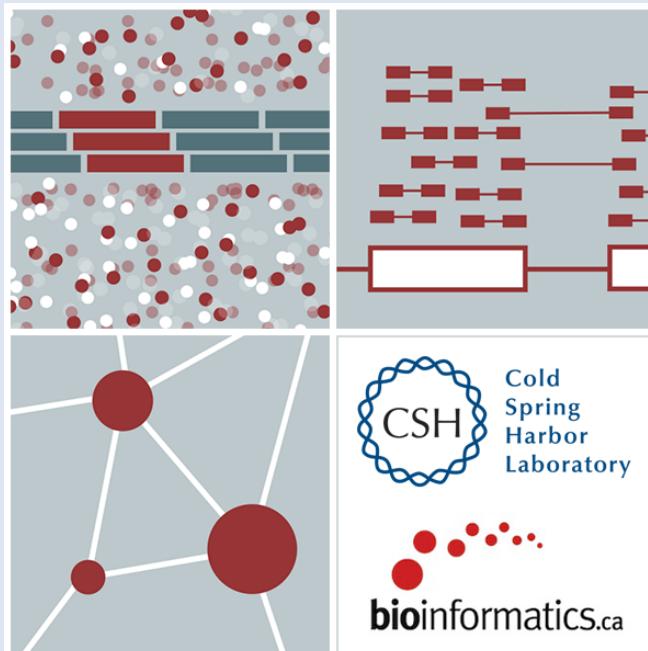




Cold
Spring
Harbor
Laboratory



RNA-Seq Module 2: SAM/BAM/BED file formats

Felicia Gomez, Charlz Jerold, Obi Griffith, Malachi Griffith,
My Hoang, Mariam Khanfar, Chris Miller, Kartik Singhal, Jennie Yao

Advanced Sequencing Technologies & Bioinformatics Analysis November 10-21, 2025

Washington University in St. Louis
SCHOOL OF MEDICINE

Introduction to the SAM/BAM format

- The specification
 - <http://samtools.sourceforge.net/SAM1.pdf>
- SAM is uncompressed text data
- BAM is a compressed version of SAM
 - lossless BGZF format
- BAM files are usually ‘indexed’
 - A ‘.bai’ file will be found beside the ‘.bam’ file
 - Indexing provides fast retrieval of alignments overlapping a specified region without going through all alignments.
 - BAM must be sorted by the reference ID and then the leftmost coordinate before indexing

Example of SAM/BAM file format

Example SAM/BAM/CRAM header section (abbreviated)

```
mgriffit@linus270 ~> samtools view -H /gscmnt/gc13001/info/model_data/2891632684/build136494552	alignments/136080019.bam | grep -P "SN\|22\|HD\|RG\|PG"
@HD VN:1.4 SO:coordinate
@SQ SN:22 LN:51304566 UR:ftp://ftp.ncbi.nih.gov/genbank/Eukaryotes/vertebrates_mammals/Homo_sapiens/GRCh37/special_requests/GRCh37-lite.fa.gz AS:GRCh37-lite M5:a718acaa6135fdca8357d5bfe9
4211dd SP:Homo sapiens
@RG ID:2888721359 PL:illumina PU:D1BA4ACXX.3 LB:H_KA-452198-0817007-cDNA-3-lib1 PI:365 DS:paired end DT:2012-10-03T19:00:00-0500 SM:H_KA-452198-0817007 CN:WUGSC
@PG ID:2888721359 VN:2.0.8 CL:tophat --library-type fr--secondstrand --bowtie-version=2.1.0
@PG ID:MarkDuplicates PN:MarkDuplicates PP:2888721359 VN:1.85(exported) Cl:Net.sff.picard.sam.MarkDuplicates INPUT=[/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/scratch-Ilg6Y/H_KA-452198-0817007-cDNA-3-lib1-2888360300.bam] OUTPUT=/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/scratch-Ilg6Y/H_KA-452198-0817007-cDNA-3-lib1-2888360300-post_dup.bam METRICS_FILE=/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/staging-liuJS/H_KA-452198-0817007-cDNA-3-lib1-2888360300.metrics REMOVE_DUPLICATES=false ASSUME_SORTED=true MAX_FILE_HANDLES_FOR_READ_ENDS_MAP=9500 TMP_DIR=[/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/scratch-Ilg6Y] VALIDATION_STRICTNESS=SILENT MAX_RECORDS_IN_RAM=5000000 PROGRAM_RECORD_ID=MarkDuplicates PROGRAM_GROUP_NAME=MarkDuplicates MAX_SEQUENCES_FOR_DISK_READ_ENDS_MAP=50000 SORTING_COLLECTION_SIZE_RATIO=0.25 READ_NAME_REGEX=[a-zA-Z0-9]+:[0-9]+:[0-9]+:[0-9]+.* OPTICAL_DUPLICATE_PIXEL_DISTANCE=100 VERBOSITY=INFO QUIET=false COMPRESSION_LEVEL=5 CREATE_INDEX=false CREATE_MDS_FILE=false
mgriffit@linus270 ~>
```

Example SAM/BAM/CRAM alignment section (only 10 alignments shown)

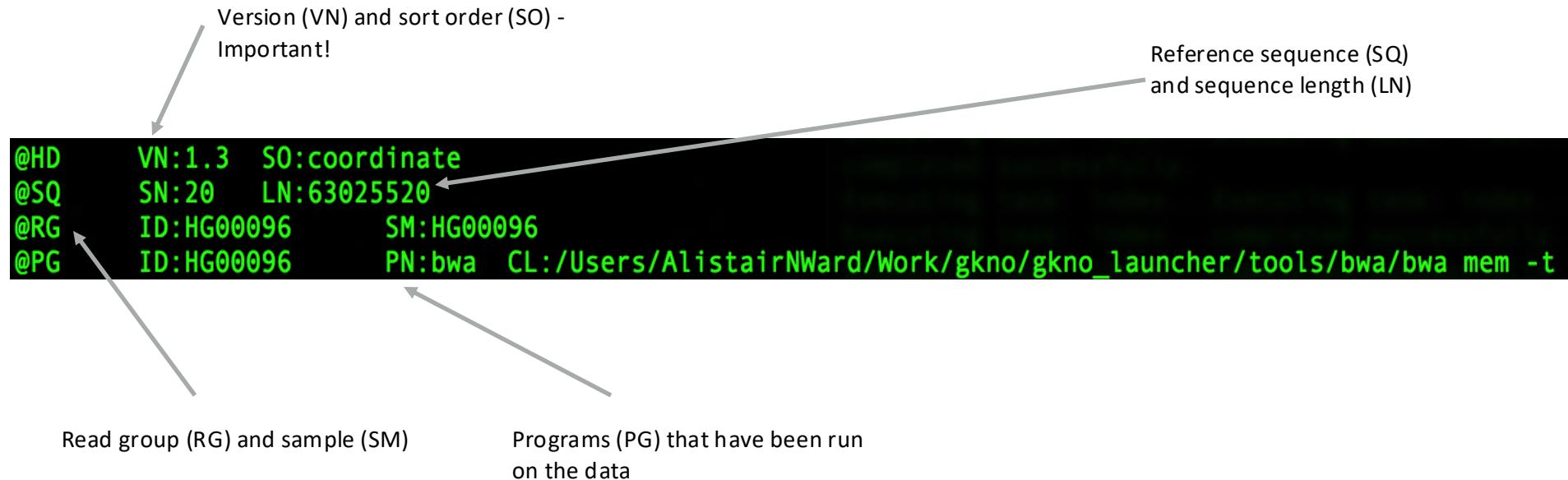
SAM/BAM header section

- Used to describe source of data, reference sequence, method of alignment, etc.
- Each section begins with character '@' followed by a two-letter record type code. These are followed by two-letter tags and values:
 - @HD The header line
 - VN: format version
 - SO: Sorting order of alignments
 - @RG Read group
 - ID: read group identifier
 - CN: name of sequencing center
 - SM: sample name
 - @SQ Reference sequence dictionary
 - SN: reference sequence name
 - LN: reference sequence length
 - SP: species
 - @PG Program
 - PN: program name
 - VN: program version

A BAM file is divided in header and alignment sections

Example SAM/BAM header section (abbreviated)

```
mgriffit@linus270 ~> samtools view -H /gscmnt/gc13001/info/model_data/2891632684/build136494552/alignments/136080019.bam | grep -P "SN\|:22|HD|RG|PG"
@HD VN:1.4 SO:coordinate
@SQ SN:22 LN:51304566 UR:ftp://ftp.ncbi.nih.gov/genbank/genomes/Eukaryotes/vertebrates_mammals/Homo_sapiens/GRCh37/special_requests/GRCh37-lite.fa.gz AS:GRCh37-lite M5:a718acaa6135fdca8357d5bfe9
4211dd SP:Homo sapiens
@RG ID:2888721359 PL:illumina PU:D1BA4ACXX.3 LB:H_KA-452198-0817007-cDNA-3-lib1 PI:365 DS:paired end DT:2012-10-03T19:00:00-0500 SM:H_KA-452198-0817007 CN:WUGSC
@PG ID:2888721359 VN:2.0.8 CL:tophat --library-type fr-secondstrand --bowtie-version=2.1.0
@PG ID:MarkDuplicates PN:MarkDuplicates PP:2888721359 VN:1.85(exported) CL:net.sf.picard.sam.MarkDuplicates INPUT=[/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/scratch-ILg6Y/H_KA-452198-0817007-cDNA-3-lib1-2888360300.bam] OUTPUT=/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/scratch-ILg6Y/H_KA-452198-0817007-cDNA-3-lib1-2888360300-post_dup.bam METRICS_FILE=/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/staging-liuJS/H_KA-452198-0817007-cDNA-3-lib1-2888360300.metrics REMOVE_DUPLICATES=false ASSUME_SORTED=true MAX_FILE_HANDLES_FOR_READ_ENDS_MAP=9500 TMP_DIR=[/gscmnt/gc13001/info/build_merged_alignments/merged-alignment-blade10-2-5.gsc.wustl.edu-jwalker-15434-136080019/scratch-ILg6Y] VALIDATION_STRINGENCY=SILENT MAX_RECORDS_IN_RAM=500000 PROGRAM_RECORD_ID=MarkDuplicates PROGRAM_GROUP_NAME=MarkDuplicates MAX_SEQUENCES_FOR_DISK_READ_ENDS_MAP=50000 SORTING_COLLECTION_SIZE_RATIO=0.25 READ_NAME_REGEX=[a-zA-Z0-9]+:[0-9]+([0-9]+):([0-9]+).* OPTICAL_DUPLICATE_PIXEL_DISTANCE=100 VERBOSITY=INFO QUIET=false COMPRESSION_LEVEL=5 CREATE_INDEX=false CREATE_MD5_FILE=false
mgriffit@linus270 ~>
```



A BAM file is divided in header and alignment sections

Example SAM/BAM alignment section (only 10 alignments shown)

SAM/BAM alignment section

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 ²⁹ -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 segment
8	PNEXT	Int	[0,2 ²⁹ -1]	Position of the mate/next segment
9	TLEN	Int	[-2 ²⁹ +1,2 ²⁹ -1]	observed Template LENGTH
10	SEQ	String	* [A-Za-z.=.]+	segment SEQuence
11	QUAL	String	[!-~]+	ASCII of Phred-scaled base QUALity+33

Example values

1 QNAME e.g. HWI-ST495_129147882:1:2302:10269:12362
2 FLAG e.g. 99
3 RNAME e.g. 1
4 POS e.g. 11623
5 MAPQ e.g. 3
6 CIGAR e.g. 100M
7 RNEXT e.g. =
8 PNEXT e.g. 11740
9 TLEN e.g. 217
10 SEQ e.g. CCTGTTCTCCACAAAGTGTACTTTGGATTTGCCAGTCTAACAGGTGAAGCCCTGGAGATTCTTATTAGTGATTGGCTGGGCCTGGCCATGT
11 QUAL e.g. CCCFFFFFHHHHJJIJFIJJJJJJJJHJJJJJJJJJJGGHJJHJJJJJJJJHGGIJJJJJJJJJGHHGJJJEEHHHFFFFCDCDDDDDB@ACDD

SAM Format – Information Fields

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

1

2

3

4

5

6

7

8

9

10

SRR062634.14576120

163

20

899919 60

100M

= 900037 218

TTCCCCAGTAGCTGGGATTACAGGCATAAGCCACCATC

?

?

SAM/BAM flags explained

- 12 bitwise flags describing the alignment
- Stored as a binary string of length 12 instead of 12 columns of data
- Value of '1' indicates the flag is set. e.g.
001000000000
- All combinations can be represented as a number from 0 to 4095 (i.e. $2^{12}-1$). This number is used in the BAM/SAM file.
- You can specify 'required' or 'filter' flags in samtools view using the '-f' and '-F' options respectively

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

Note that to maximize confusion, each bit is described in the SAM specification using its hexadecimal representation (i.e., '0x10' = 16 and '0x40' = 64).

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

SAM Format – Information Fields

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

1

2

3

4

5

6

7

8

9

10

SRR062634.14576120

163

20

899919 60

100M

=

900037 218

TTCCCCAGTAGCTGGGATTACAGGCATAAGCCACCATC

?

?

CIGAR strings explained

- The ‘CIGAR’ (Compact Idiosyncratic Gapped Alignment Report)
- The CIGAR string is a sequence of base lengths and associated ‘operations’ indicating which bases align to the reference (either a match or mismatch), are deleted, are inserted, represent introns, etc.

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

- e.g. 81M859N19M

- A 100 bp read consists of: 81 bases of alignment to reference, 859 bases skipped (an intron), 19 bases of alignment

CRAM files

- CRAM is an ultra-compressed version of a BAM file
 - Usually between 30-60% smaller than the corresponding BAM
- Stores “diffs” from the reference genome
 - requires the matching reference genome to restore original data!
- Base quality binning may be used as well
- Some tools still require conversion back to bam

Quality Score Bins	Example of Empirically Mapped Quality Scores*
N (no call)	N (no call)
2–9	6
10–19	15
20–24	22
25–29	27
30–34	33
35–39	37
≥ 40	40

By replacing the quality scores between 19 and 25 with a new score of 22, data storage space is conserved.

*The mapped quality score of each bin (except “N”) is subject to change depending on individual Q-tables.

Introduction to the BED format

- When working with BAM files, it is very common to want to examine a focused subset of the reference genome
 - e.g. the exons of a gene
- These subsets are commonly specified in ‘BED’ files
 - <https://genome.ucsc.edu/FAQ/FAQformat.html#format1>
- Many BAM manipulation tools accept regions of interest in BED format
- Basic BED format (tab separated):
 - Chromosome name, start position, end position (BED3)
 - Coordinates in BED format are 0 based

Introduction to the BED format

- There are several flavors of BED format: BED3, BED4, BED6, BED8, etc
- First 3 fields always required: chr, start, stop
- Followed by up to 9 additional optional fields: name, score, strand, thickStart, thickEnd, itemRGB, blockCount, blockSizes, blockStarts

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	-

Manipulation of SAM/BAM and BED files

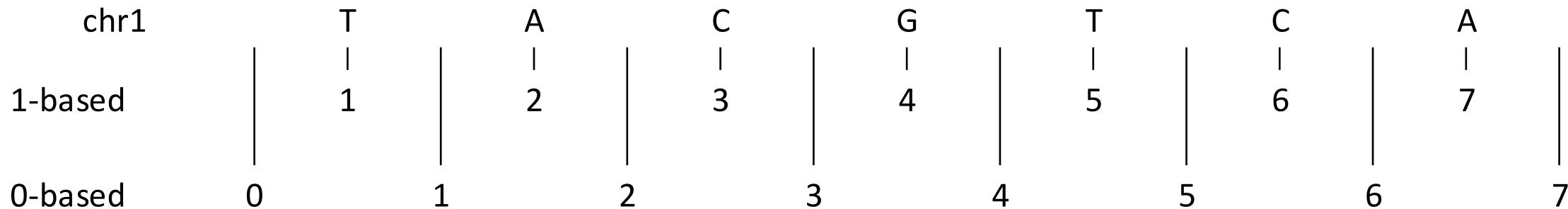
- Several tools are used ubiquitously in sequence analysis to manipulate these files
- SAM/BAM files
 - samtools
 - bamtools
 - Picard
- BED files
 - bedtools
 - bedops



Common sources of confusion

- Genomic coordinate systems
- Genome builds
- Variant representation

Genomic coordinates – 1 vs 0 based



	1-based	0-based
Indicate a single nucleotide	chr1:4-4 G	chr1:3-4 G
Indicate a range of nucleotides	chr1:2-4 ACG	chr1:1-4 ACG
Indicate a single nucleotide variant	chr1:5-5 T/A	chr1:4-5 T/A

- 1-based : Single nucleotides, variant positions, or ranges are specified directly by their corresponding nucleotide numbers
 - GFF, SAM, VCF, Ensembl browser, ...
- 0-based: Single nucleotides, variant positions, or ranges are specified by the coordinates that flank them
 - BED, BAM, UCSC browser, ...

Genome builds

Reference Genome builds

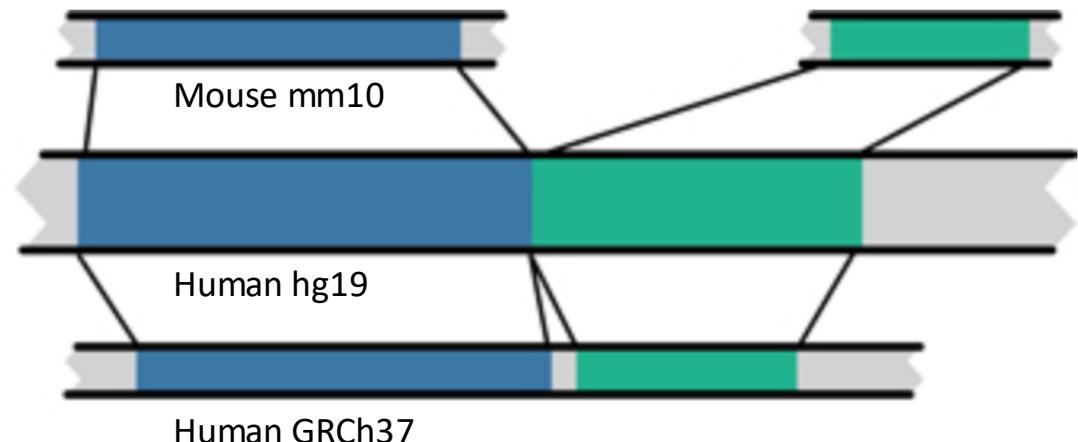
Current human: GRCh38, hg38, b38

alternates: GRCh38v2_ccdg,
GRCh38_full_analysis_set_plus_decoy_hla

Previous human: GRCh37, hg19, b37

Current mouse: GRCm38, mm10

Lift-over



For a detailed discussion of various human reference genome flavors refer here:
https://pmbio.org/module-02-inputs/0002/02/01/Reference_Genome/

Variant shifting (alignment) and parsimony/trimming

Reference and alternative alleles of a CA short tandem repeat (STR)	REF	GGGCACACAC A GGG	CA deletion from the reference
	ALT	GGGCACACAGGG	
REF	CA	8 CA	Not left aligned and alternate allele is empty
ALT	.	6 CAC	Not left aligned but parsimonious
REF	CAC	3 GCACA	Not right trimmed
ALT	C	2 GGCA	Not left trimmed
REF	GCACA	3 GCA	Normalized (left aligned & parsimonious)
ALT	GCA		
REF	GGCA		
ALT	GG		
REF	GCA		
ALT	G		

Alleles represented against the human genome reference. Allele pairs are colored the same, all are representations of the same variant.

Alleles represented in Variant Call Format, all are representations of the same variant.

Parsimony: representing variant in as few nucleotides as possible without reducing the length of any allele to 0

Left (right) aligning =
shifting the start position of a variant as far to the left (right) as possible

How should I sort my SAM/BAM file?

- Generally BAM files are sorted by position
 - This is for performance reasons
 - When sorted and indexed, arbitrary positions in a massive BAM file can be accessed rapidly
- Certain tools require a BAM sorted by read name
 - Usually this is when we need to easily identify both reads of a pair
 - The insert size between two reads may be large
 - In fusion detection we are interested in read pairs that map to different chromosomes

We are on a Coffee Break & Networking Session