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

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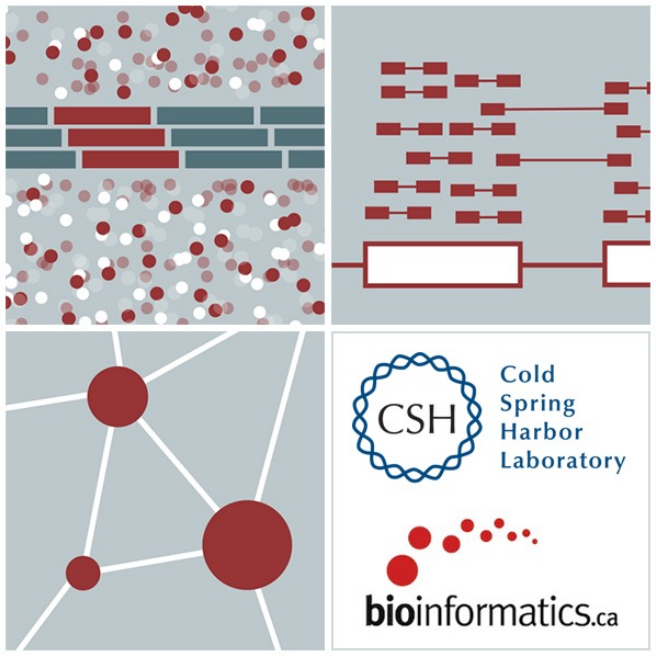
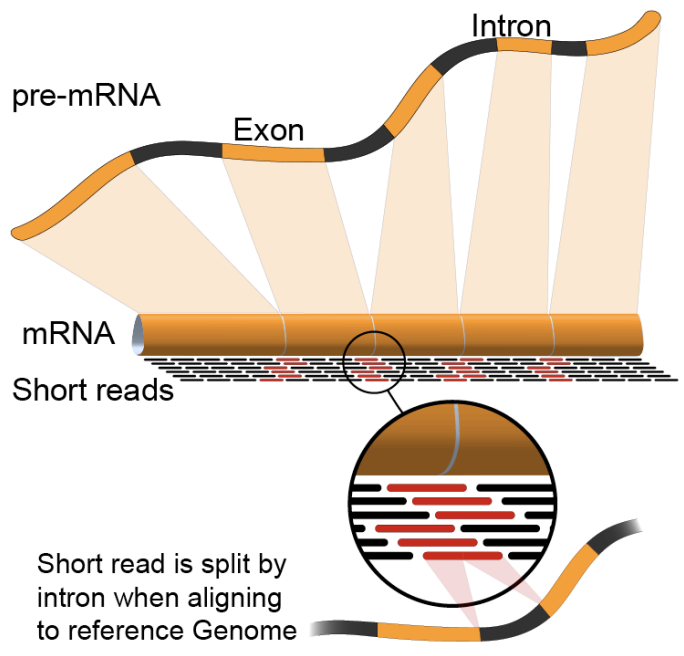


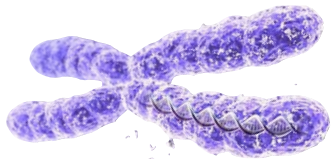


Washington University in St. Louis
School of Medicine

What is a sequence read? (a.k.a. “a read”)

Reads are the sequencer's best guess at what it saw for a given DNA molecule.

It's the "raw" data.



"base calling"
(mistakes happen)

cctaaccct
acccctgg
acccctgg
tcccctgg
cctaaccct
cctaaccct
ggccctgg
acccctgg
acccctgg
actaaccct
cctaaccct
cctaaccct
acccctgg
cctaaccct
acccctgg
cctaaccct

Slide courtesy of Andrew Farrell

Alignment is central to most genomics applications

FASTQ format

Align to genome

Quality control

Call SNPs

Find peaks

Quantify transcripts

Find SVs

. . .

etc.

Analysis, Interpretation, Discovery

Phase 0

Phase1

Phase 2-100

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Analysis, Interpretation, Discovery

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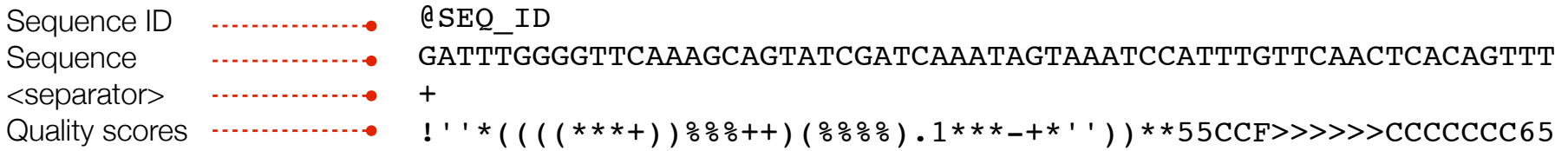
Phase1

Phase 2-100

The FASTQ format

A “standard” format for storing and defining sequences from next-generation sequencing technologies.



- FASTQ files are generally used to store short-read data from high-throughput sequencing experiments.
- The sequence and quality scores are usually put into a single line

Sequence IDs

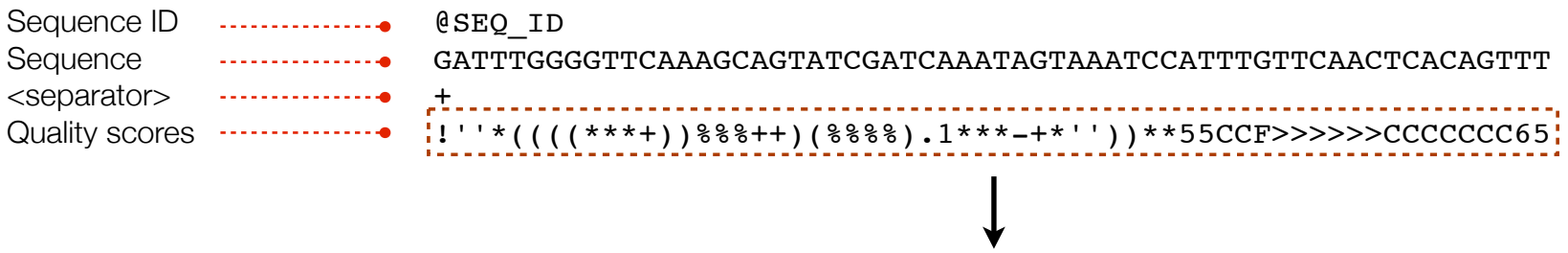
```
@HWUSI-EAS100R:6:73:941:1973#0/1
```

HWUSI-EAS100R	the unique instrument name
6	flowcell lane
73	tile number within the flowcell lane
941	'x'-coordinate of the cluster within the tile
1973	'y'-coordinate of the cluster within the tile
#0	index number for a multiplexed sample (0 for no indexing)
/1	the member of a pair, /1 or /2 *(paired-end or mate-pair reads only)*

```
@EAS139:136:FC706VJ:2:2104:15343:197393 1:Y:18:ATCACG
```

EAS139	the unique instrument name
136	the run id
FC706VJ	the flowcell id
2	flowcell lane
2104	tile number within the flowcell lane
15343	'x'-coordinate of the cluster within the tile
197393	'y'-coordinate of the cluster within the tile
1	the member of a pair, 1 or 2 *(paired-end or mate-pair reads only)*
Y	Y if the read is filtered, N otherwise
18	0 when none of the control bits are on, otherwise it is an even number
ATCACG	index sequence

Quality scores



Qualities are based on the Phred scale and are *encoded*

$$Q = -10^{*}\log_{10}(P_{err})$$

- FASTQ files encodes phred scores as ASCII characters
- Phred quality scores characterize the quality of DNA sequences – these scores are assigned by the sequencer
- A quality score of 20 (Q20) represents an error rate of 1 in 100 (meaning every 100 bp sequencing read may contain an error); call accuracy of 99%.

$$Q=-10Log_{10}(P_{error})$$

Probability of Error		Q
1/1,000,000	0.000001	60
1/100,000	0.000010	50
1/10,000	0.000100	40
1/1,000	0.001000	30
1/100	0.010000	20
1/10	0.100000	10
1/1	1.000000	0

- **Higher Q scores** indicate a smaller probability of error.
- **Lower Q scores** indicate lower confidence in the called base.
  - Increased false-positive variant calls
- Q30 is a standard a benchmark for quality in next-generation sequencing

Quality score encoding

Dec	Hex	Char	Dec	Hex	Char	Dec	Hex	Char	Dec	Hex	Char
0	00	Null	32	20	Space	64	40	@	96	60	`
1	01	Start of heading	33	21	!	65	41	A	97	61	a
2	02	Start of text	34	22	"	66	42	B	98	62	b
3	03	End of text	35	23	#	67	43	C	99	63	c
4	04	End of transmit	36	24	$	68	44	D	100	64	d
5	05	Enquiry	37	25	%	69	45	E	101	65	e
6	06	Acknowledge	38	26	&	70	46	F	102	66	f
7	07	Audible bell	39	27	'	71	47	G	103	67	g
8	08	Backspace	40	28	(	72	48	H	104	68	h
9	09	Horizontal tab	41	29	)	73	49	I	105	69	i
10	0A	Line feed	42	2A	*	74	4A	J	106	6A	j
11	0B	Vertical tab	43	2B	+	75	4B	K	107	6B	k
12	0C	Form feed	44	2C	,	76	4C	L	108	6C	l
13	0D	Carriage return	45	2D	-	77	4D	M	109	6D	m
14	0E	Shift out	46	2E	.	78	4E	N	110	6E	n
15	0F	Shift in	47	2F	/	79	4F	O	111	6F	o
16	10	Data link escape	48	30	0	80	50	P	112	70	p
17	11	Device control 1	49	31	1	81	51	Q	113	71	q
18	12	Device control 2	50	32	2	82	52	R	114	72	r
19	13	Device control 3	51	33	3	83	53	S	115	73	s
20	14	Device control 4	52	34	4	84	54	T	116	74	t
21	15	Neg. acknowledge	53	35	5	85	55	U	117	75	u
22	16	Synchronous idle	54	36	6	86	56	V	118	76	v
23	17	End trans. block	55	37	7	87	57	W	119	77	w
24	18	Cancel	56	38	8	88	58	X	120	78	x
25	19	End of medium	57	39	9	89	59	Y	121	79	y
26	1A	Substitution	58	3A	:	90	5A	Z	122	7A	z
27	1B	Escape	59	3B	;	91	5B	[	123	7B	{
28	1C	File separator	60	3C	<	92	5C	\	124	7C	|
29	1D	Group separator	61	3D	=	93	5D	]	125	7D	}
30	1E	Record separator	62	3E	>	94	5E	^	126	7E	~
31	1F	Unit separator	63	3F	?	95	5F	_	127	7F	□

Formula for getting PHRED quality from encoded quality:

Q = ascii(char) - 33

Example:

!+EI

ASCII	33	43	69	73
-33	0	10	36	40

- ASCII = **A**merican **S**tandard **C**ode for **I**nformation **I**nterchange
- Every text symbol must have an integer value representing it inside the computer
- An ASCII code is the numerical representation of a character such as 'a' or '@'

FASTA format

We start with a reference genome to map to

The reference sequence (chromosome)

Sequence description

```
>20 dna:chromosome chromosome:GRCh37:20:1:63025520:1
NNNNNNNNNNTACTTCGATTGCGTATTTACGGACGTAGCGAGTCTTTAGAGTCTTTTAGTCTGTATC
GTCGTAGTGTCAGTTCGTAGTCTATGTCGTATTCGTAGGCGTACGTAGTCGTGTAGTCAGTCGTGTT
```

DNA sequence

http://en.wikipedia.org/wiki/FASTA_format

The goal. Easy, right?

FASTQ

cctaaccct
acccctgg
tcccctgg
acccctgg
ggccctgg
cctaaccct
cctaaccct
acccctgg
acccctgg
actaaccct
cctaaccct
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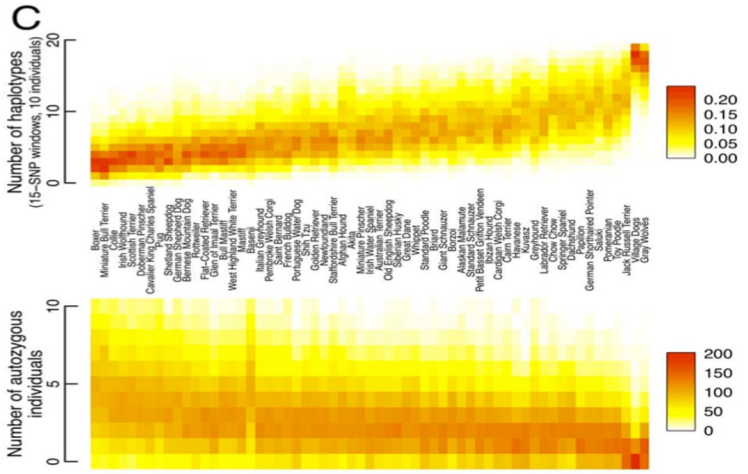
cctaaccct
acccctgg
cctaaccct



Sequence alignment is the crucial first step.

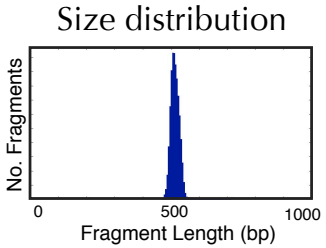
Aligning to a reference genome

This is like a jigsaw puzzle

Could fit here - but there are differences

Could fit here as well.

Single-end alignment



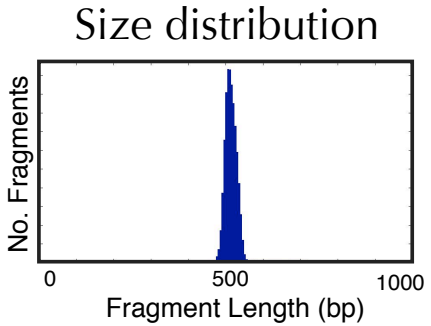
0. Size-selected DNA library (e.g., ~500bp)

1. Sequence the ***entire length*** of each molecule the library

472bp 499bp 519bp 503bp 561bp

2. Align each ***contiguous*** molecule to the reference genome.

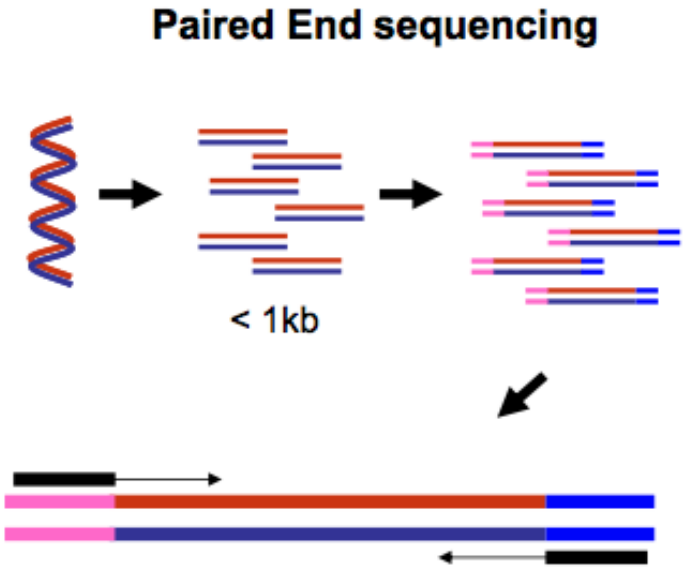
Paired-end alignment



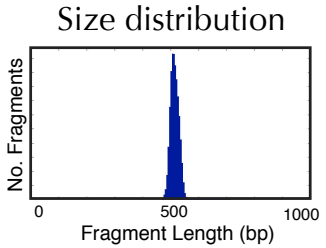
0. Size-selected DNA library (e.g., ~500bp)

1. Sequence ***solely the 5' ends*** of each molecule the library

472bp
499bp
519bp
503bp
1061bp



Paired-end alignment



0. Size-selected DNA library (e.g., ~500bp)

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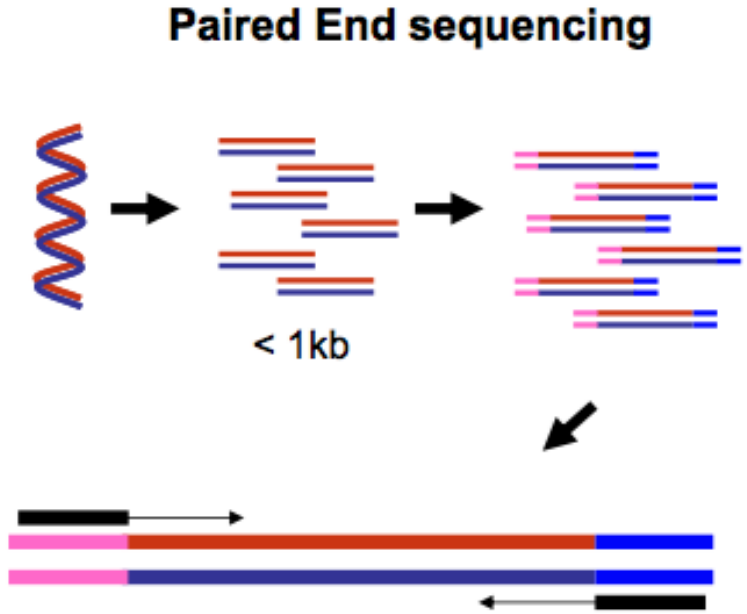
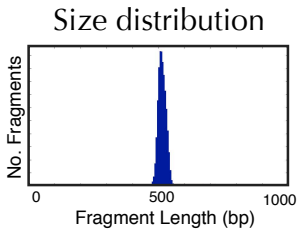
472bp 499bp 519bp 503bp 1061bp

2. Separately align ***each end of each*** molecule to the reference genome.



Paired-end alignment



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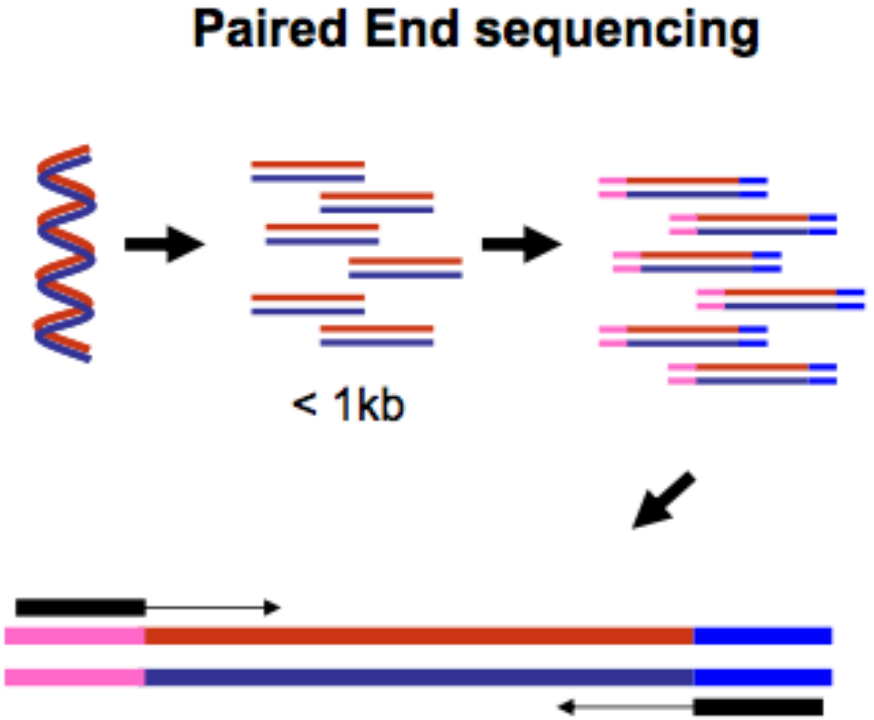
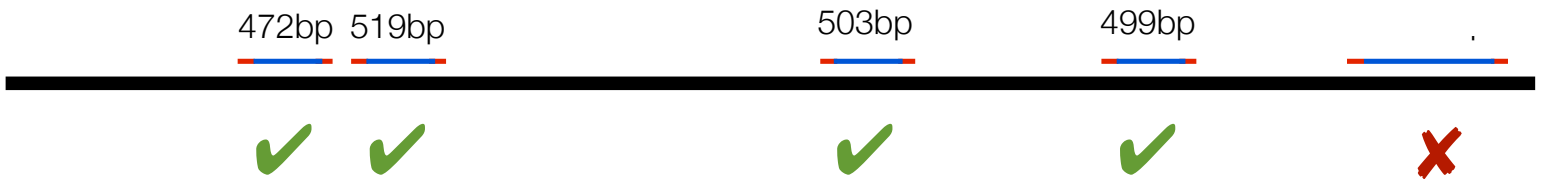
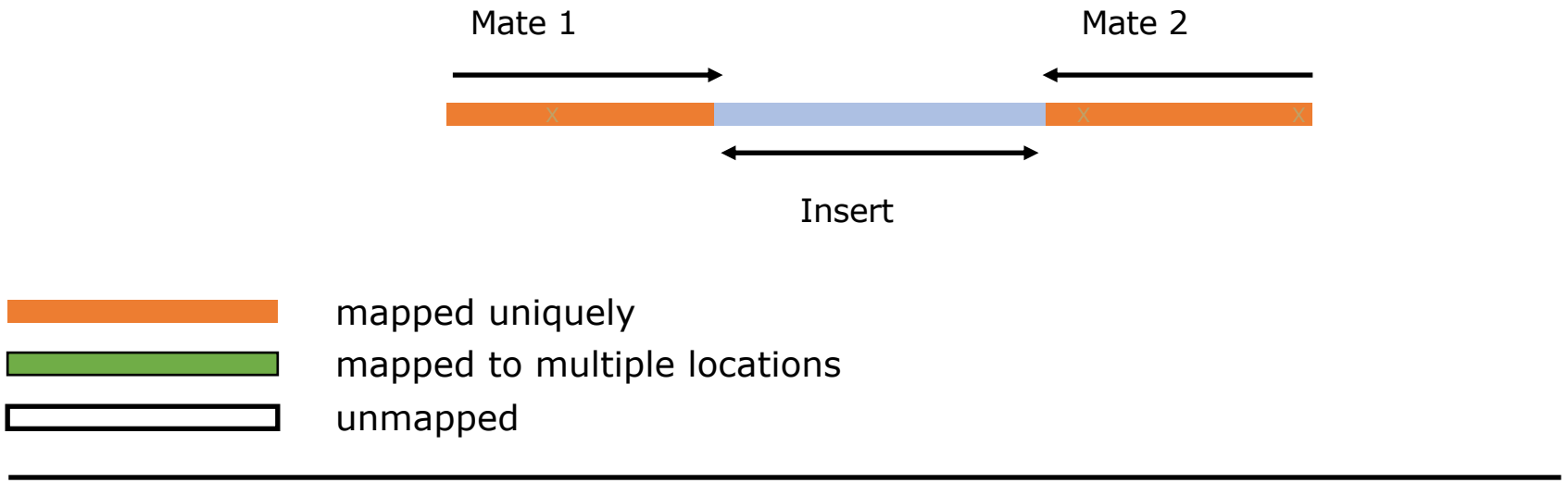
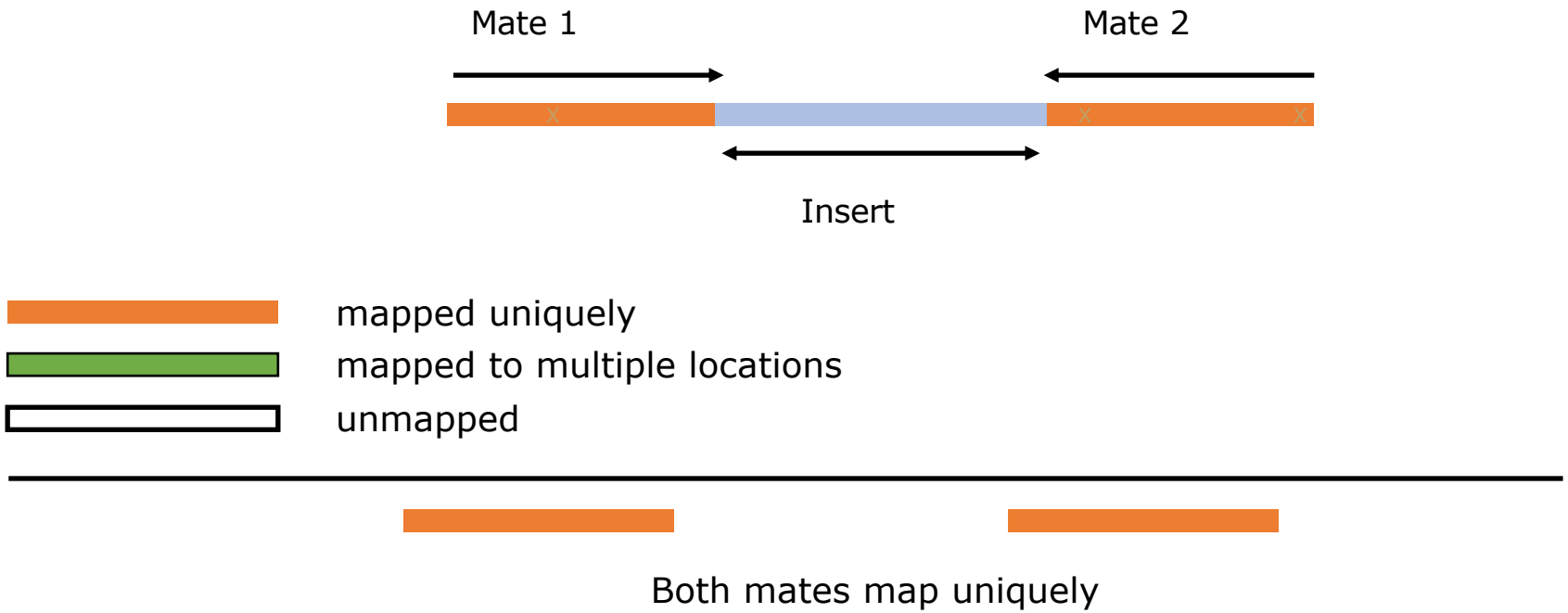




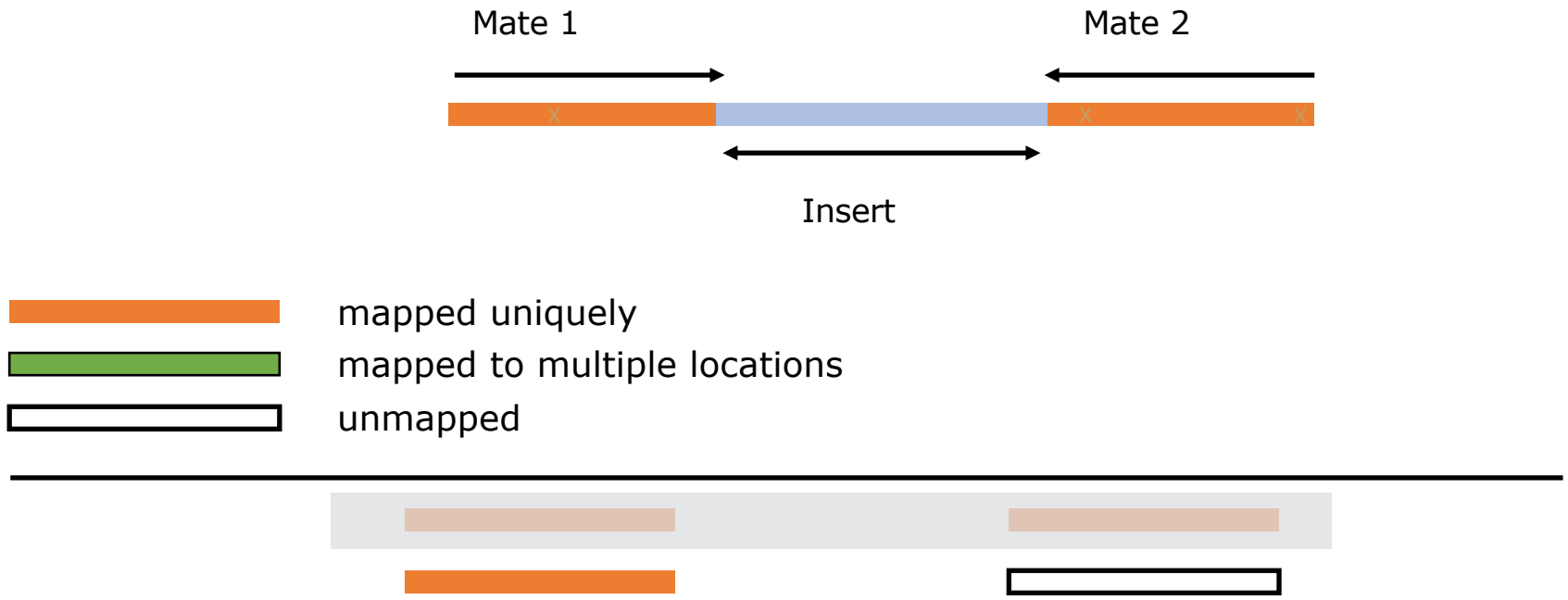
Paired end reads



Paired end reads

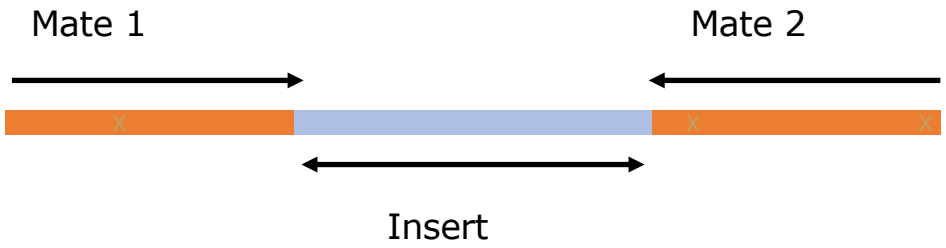


Paired end reads



One mate maps uniquely, the other is unmapped

Paired end reads



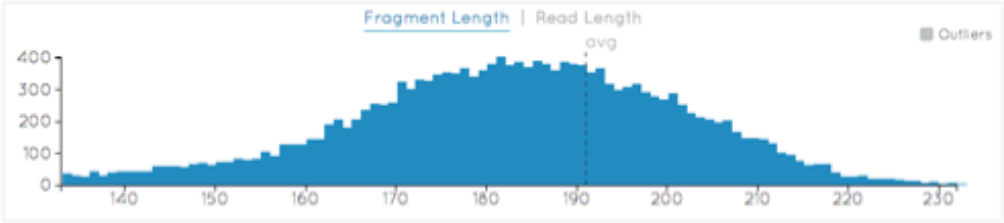
mapped uniquely

mapped to multiple locations

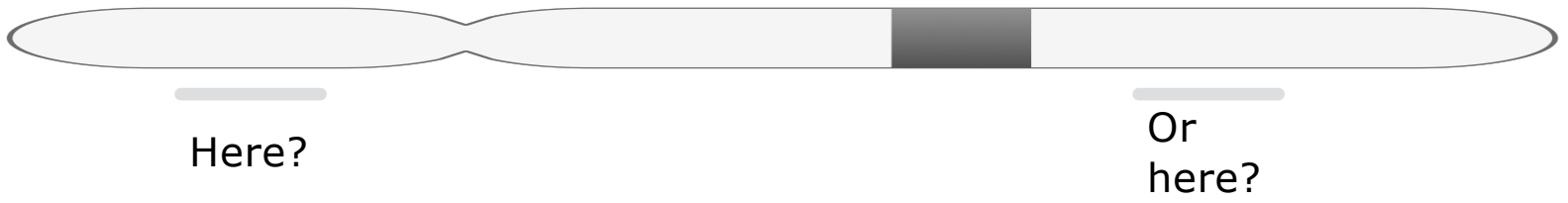
unmapped

One mate maps uniquely, the other maps to multiple locations

Use fragment length distribution to determine most likely location



What needs to be stored?



Where did the read map?
How confident are we that we are correct?
Which strand does the read come from?
Are there any differences with the reference?
What is the DNA sequence?
What are the quality scores for each base in the read?
What do we know about the mate?
Which read group does the read belong to?

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

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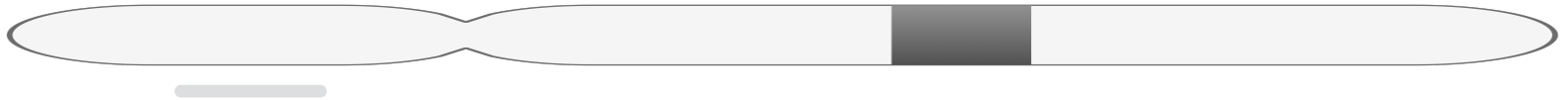

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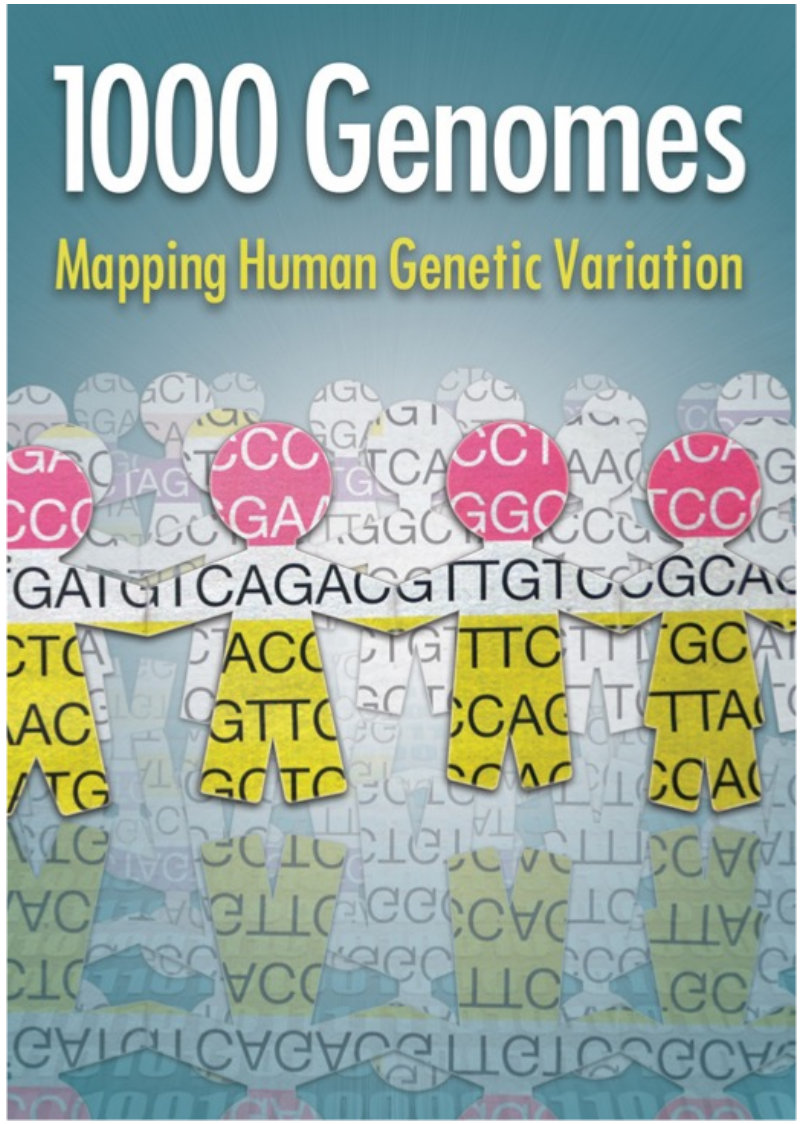
Store the alignment



Standardize alignment formats

SAM - Sequence Alignment/Map

- Compressed (BAM) saves space
- Can be indexed allowing fast access of regions
- Simple format
- Can represent single and paired end reads
- Many toolkits now available to process data

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

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/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-blad
e10-2-5.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-jw
alker-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-1543
4-136080019/staging-1iuJS/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_al
ignments/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=Mark
Duplicates 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]+):([0-9]+).* OPTICAL_DUPLICATE_PIXEL_DISTANCE=100 VERBOSITY=INFO
QUIET=false COMPRESSION_LEVEL=5 CREATE_INDEX=false CREATE_MD5_FILE=false
mgriffit@linus270 ~>
```

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

```
mgriffit@linus270 ~> samtools view -f 3 -F 1804 /gscmnt/gc13001/info/model_data/2891632684/build136494552/alignments/136080019.bam | head
HWI-ST495_129147882:3:2114:15769:38646  99      1       11306   3       100M    =       11508   302     ACTGCGGGGCCCTCTTGCTTACTGTATAGTGGTGGCACGCCGCCTGCTGGCAGCTAGGGACATTGCAGGGTCCTCTTGCTCAAGGTGTAGTGGCAGCACGC
CCFFFFFHHHGHJJJJJJIJJJHGIJJIJJHIIJJJJJJHFDDDDDDDDDDDDCDDDDDDDDDDDDD<CDDDDDDDDCCCC>@>CDDDDDD?1<B     CC:Z:15 MD:Z:5A94       PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102519765  AS:i:-5 XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:2114:15769:38646  147     1       11508   3       100M    =       11306   -302    ACTCCTAAATATGGGATTCCTGGGTTTAAAAGTATAAAATAAATATGTTTAATTTGTGAACTGATTACCATCAGAATTGTACTGTTCTGTATCCCACCAG5
;5:CDCDCDECEFCD@9E=?7EEIIIIHCEGGIJJJJIIJJIHF@?00IHHFFGG?*JJJIJGHGEIJJIJJJJJJJIHHCIEJJJHFHHGHFFEDFCCB     CC:Z:15 MD:Z:34A65      PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102519563  AS:i:-6 XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1210:1257:16203   163     1       11810   3       100M    =       12055   345     CCTGCATGTAGTTTAAACGAGATTGCCAGCACCGGGTATCATTCACCATTTTTCTTTTCGTTAACTTGCCGTCAGCCTTTTCTTTGACCTCTTCTTTCTGC
CCFFFFFHFHAFGGIIIJJJEEHGIGGGIJIJJGI?@EHIGIJDGHIHIGGIJJJJJJJJIJGHHHGHFFFCDDDDDDCDCCCCCA;>@>@AA@:AA>AA     CC:Z:15 MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:102519261  AS:i:0  XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:1210:1257:16203   83      1       12055   3       100M    =       11810   -345    GAGCACTGGAGTGGAGTTTTCCTGTGGAGAGGAGCCATGCCTAGAGTGGGATGGGCCATTGTTCATCTTCTGGCCCCTGTTGTCTGCATGTAACTTAATAC
CC>4C>DCCCACACDCC?BDCEE@ECFFFFFHHHHHIJJJIIJJIIIHHEHIIGJIJIJJIGHIIIJJJJJIIJJJJJIIJJIJIJJHGHHHDFEFFCCC     CC:Z:15 MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:102519016  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:2111:3117:78828   163     1       12634   3       100M    =       12746   212     GCCCTTCCCCAGCATCAGGTCTCCAGAGCTGCAGAAGACGACGGCCGACTTGGATCACACTCTTGTGAGTGTCCCCAGTGTTGCACAGGTGAGAGGAGAG<
@@FFFFFDHHHH9FHGIIFGAFDHEGII>GHIIIIIIIIIIIIIIIIFHDDFFEEECEECCACCCCCC:AADCCBCC>CAC<CCCCCC:@CB@@BAB##     CC:Z:15 MD:Z:85G14      PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102518437  AS:i:-5 XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:2111:3117:78828   83      1       12746   3       100M    =       12634   -212    GGGAGTGGCGTCGCCCCTAGGGCTCTACGGGGCCGGCATCTCCTGTCTCCTGGAGAGGCTTCGATGCCCCTCCACACCCTCTTGATCTTCCCTGTGATGTD
DCABDBDDDDDDDDDDCDDDDBDB@BDDDB@;CCCCCDEFD@;.?<HIGGEIGEHIGJJJIIGIGIIHEGFEHFJIIIIIGJJJJHHHHHFFFFFC@@     CC:Z:15 MD:Z:37G62      PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102518325  AS:i:-5 XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:1102:4242:26638   99      1       13503   3       100M    =       13779   376     CGCTGTGCCCTTCCTTTGCTCTGCCCGCTGGAGACGGTGTTTGTCATGGGCCTGGTCTGCAGGGATCCTGCTACAAAGGTGAAACCCAGGAGAGTGTGGAC
CCFFFFFHHHHHJJJIJJJJJJJJJJJJJGIIIIJJFHGGIJGIJJJEGIJIJJHHIHHGHFFEFDEEEECCCAACDDACDCDDDDDB?8?<B>A@CDC     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357414  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1309:15328:74082  99      1       13534   3       100M    =       13780   346     AGACGGTGTTTGTCATGGGCCTGGTCTGCAGGGATCCTGCTACAAAGGTGAAACCCAGGAGAGTGTGGAGTCCAGAGTGTTGCCAGGACCCAGGCACAGG@
CCFFFADHHHHFIJJJJJIJIJIHIJJJIHJJIJJJIIJJJIJJJJJJBFHIIJJJJJJIJHH=EEFFFFCEEECEDCDCDDDDDDDDDDDDDDDDBDCCD     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357383  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1308:10126:19636  99      1       13779   3       100M    =       14027   348     CCTCTGCAGGAGGCTGCCATTTGTCCTGCCCACCTTCTTAGAAGCGAGACGGAGCAGACCCATCTGCTACTGCCCTTTCTATAATAACTAAAGTTAGCTGC
CCFFFFFHHGHHJJJJJJJJJJIJJJJJJIJJJJJJJJJJIJJJJJJJJJJHJJJHGHHFFFFFFEEEEEEDDDDCDCDCDFADCACCACDDCCCCCCD     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357140  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1102:4242:26638   147     1       13779   3       100M    =       13503   -376    CCTCTGCAGGAGGCTGCCATTTGTCCTGCCCACCTTCTTAGAAGCGAGACGGAGCAGACCCATCTGCTACTGCCCTTTCTATAATAACTAAAGTTAGCTG#
##DCCDDDCCBBBABCCDDDCBDDBBDHC?=GIIJIIIIJIGIIIIJJHJJIJJIGCIIJJJJJIGHGJJIJIJJJJJIJIIIGGFGHHHHFFFFFCCC     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357140  AS:i:0  XS:A:+  YT:Z:UU
mgriffit@linus270 ~>
```

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
  - @SQ Reference sequence dictionary
    - SN: reference sequence name
    - LN: reference sequence length
    - SP: species
  - @RG Read group
    - ID: read group identifier
    - CN: name of sequencing center
    - SM: sample name
  - @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-blad
e10-2-5.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-jw
alker-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-1543
4-136080019/staging-1iuJS/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_al
ignments/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=Mark
Duplicates 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]+):([0-9]+).* OPTICAL_DUPLICATE_PIXEL_DISTANCE=100 VERBOSITY=INFO
QUIET=false COMPRESSION_LEVEL=5 CREATE_INDEX=false CREATE_MD5_FILE=false
mgriffit@linus270 ~>
```

Version (VN) and sort order (SO) - Important!

Reference sequence (SQ) and sequence length (LN)

```
@HD     VN:1.3  SO:coordinate
@SQ     SN:20   LN:63025520
@RG     ID:HG00096      SM:HG00096
@PG     ID:HG00096      PN:bwa  CL:/Users/AlistairNWard/Work/gkno/gkno_launcher/tools/bwa/bwa mem -t
```

Read group (RG) and sample (SM)

Programs (PG) that have been run on the data

A BAM file is divided in header and alignment sections
Example SAM/BAM alignment section (only 10 alignments shown)

```
mgriffit@linus270 ~> samtools view -f 3 -F 1804 /gscmnt/gc13001/info/model_data/2891632684/build136494552/alignments/136080019.bam | head
HWI-ST495_129147882:3:2114:15769:38646  99      1       11306   3       100M    =       11508   302     ACTGCGGGGCCCTCTTGCTTACTGTATAGTGGTGGCACGCCGCCTGCTGGCAGCTAGGGACATTGCAGGGTCCTCTTGCTCAAGGTGTAGTGGCAGCACGC
CCFFFFFHHHGHJJJJJJJIJJJHGIJJIJJHIIJJJJJJHFDDDDDDDDDDDDCDDDDDDDDDDDDDD<CDDDDDDDDCCCC>@>CDDDDDD?1<B     CC:Z:15 MD:Z:5A94       PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102519765  AS:i:-5 XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:2114:15769:38646  147     1       11508   3       100M    =       11306   -302    ACTCCTAAATATGGGATTCCTGGGTTTAAAAGTATAAAATAAATATGTTTAATTTGTGAACTGATTACCATCAGAATTGTACTGTTCTGTATCCCACCAG5
;5:CDCDCDECEFCD@9E=?7EEIIIIHCEGGIJJJJIIJJIHF@?00IHHFFGG?*JJJIJGHGEIJJIJJJJJJJIHHCIEJJJHFHHGHFFEDFCCB     CC:Z:15 MD:Z:34A65      PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102519563  AS:i:-6 XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1210:1257:16203   163     1       11810   3       100M    =       12055   345     CCTGCATGTAGTTTAAACGAGATTGCCAGCACCGGGTATCATTCACCATTTTTCTTTTCGTTAACTTGCCGTCAGCCTTTTCTTTGACCTCTTCTTTCTGC
CCFFFFFHFHAFGGIIIIJJJEEHGIGGGIJIJJGI?@EHIGIJDGHIHIGGIJJJJJJJJIJGHHHGHFFFCDDDDDDCDCCCCCA;>@>@AA@:AA>AA     CC:Z:15 MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:102519261  AS:i:0  XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:1210:1257:16203   83      1       12055   3       100M    =       11810   -345    GAGCACTGGAGTGGAGTTTTCCTGTGGAGAGGAGCCATGCCTAGAGTGGGATGGGCCATTGTTCATCTTCTGGCCCCTGTTGTCTGCATGTAACTTAATAC
CC>4C>DCCCACACDCC?BDCEE@ECFFFFHHHHHIJJJIIJJIIIHHEHIIGJIJIJJIGHIIIJJJJJIIJJJJJIIJJIJIJJHGHHHDFEFFCCC     CC:Z:15 MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:102519016  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:2111:3117:78828   163     1       12634   3       100M    =       12746   212     GCCCTTCCCCAGCATCAGGTCTCCAGAGCTGCAGAAGACGACGGCCGACTTGGATCACACTCTTGTGAGTGTCCCCAGTGTTGCACAGGTGAGAGGAGAG<
@@FFFFFDHHHH9FHGIIFGAFDHEGII>GHIIIIIIIIIIIIIIIFHDDFFEEECEECCCACCCCCC:AADCCBCC>CAC<CCCCCC:@CB@@BAB##     CC:Z:15 MD:Z:85G14      PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102518437  AS:i:-5 XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:2111:3117:78828   83      1       12746   3       100M    =       12634   -212    GGGAGTGGCGTCGCCCCTAGGGCTCTACGGGGCCGGCATCTCCTGTCTCCTGGAGAGGCTTCGATGCCCCTCCACACCCTCTTGATCTTCCCTGTGATGTD
DCABDBDDDDDDDDDDDCDDDDBDB@BDDDB@;CCCCCDEFD@;.?<HIGGEIGEHIGJJJIIGIGIIHEGFEHFJIIIIIGJJJJHHHHHFFFFFC@@     CC:Z:15 MD:Z:37G62      PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:1  XM:i:
1       XN:i:0  XO:i:0  CP:i:102518325  AS:i:-5 XS:A:-  YT:Z:UU
HWI-ST495_129147882:3:1102:4242:26638   99      1       13503   3       100M    =       13779   376     CGCTGTGCCCTTCCTTTGCTCTGCCCGCTGGAGACGGTGTTTGTCATGGGCCTGGTCTGCAGGGATCCTGCTACAAAGGTGAAACCCAGGAGAGTGTGGAC
CCFFFFFHHHHHJJJIJJJJJJJJJJJJJJGIIIIJJFHGGIJGIJJJEGIJIJJHHIHHGHFFEFDEEEECCCAACDDACDCDDDDDB?8?<B>A@CDC     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357414  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1309:15328:74082  99      1       13534   3       100M    =       13780   346     AGACGGTGTTTGTCATGGGCCTGGTCTGCAGGGATCCTGCTACAAAGGTGAAACCCAGGAGAGTGTGGAGTCCAGAGTGTTGCCAGGACCCAGGCACAGG@
CCFFFADHHHHFIJJJJJIJIJIHIJJJIHJJIJJIIJJIJIJJJJJBFHIIJJJJJJIJHH=EEFFFFCEEECEDCDCDDDDDDDDDDDDDDDBDCCD     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357383  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1308:10126:19636  99      1       13779   3       100M    =       14027   348     CCTCTGCAGGAGGCTGCCATTTGTCCTGCCCACCTTCTTAGAAGCGAGACGGAGCAGACCCATCTGCTACTGCCCTTTCTATAATAACTAAAGTTAGCTGC
CCFFFFFHHGHHJJJJJJJJJJIJJJJJJIJJJJJJJJJIJIJJJJJJJJJHJJJHGHHFFFFFFEEEEEDDDDCDCDCDFADCACCACDDCCCCCCD     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357140  AS:i:0  XS:A:+  YT:Z:UU
HWI-ST495_129147882:3:1102:4242:26638   147     1       13779   3       100M    =       13503   -376    CCTCTGCAGGAGGCTGCCATTTGTCCTGCCCACCTTCTTAGAAGCGAGACGGAGCAGACCCATCTGCTACTGCCCTTTCTATAATAACTAAAGTTAGCTG#
##DCCDDDCCBBBABCCDDDCBDDBBDHC?=GIIJIIIIJIGIIIIJJHJJIJJIGCIIJJJJJIGHGJJIJIJJJJJIJIIIGGFGHHHHFFFFFCCC     CC:Z:2  MD:Z:100        PG:Z:MarkDuplicates     RG:Z:2888721359 XG:i:0  NH:i:2  HI:i:0  NM:i:0  XM:i:
0       XN:i:0  XO:i:0  CP:i:114357140  AS:i:0  XS:A:+  YT:Z:UU
mgriffit@linus270 ~>
```

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.  CCTGTTTCTCCACAAAGTGTTTACTTTTGGATTTTTGCCAGTCTAACAGGTGAAGCCCTGGAGATTCTTATTAGTGATTTGGGCTGGGGCCTGGCCATGT
11    QUAL   e.g.  CCCFFFFFHHHHHJJIJFIJJJJJJJJJJHIJJJJJJJIJJJJJGGHIJHIJJJJJJJJJGHGGIJJJJJJIJEEHHHHFFFFCDCDDDDDDDB@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  TTCCCCAGTAGCTGGGATTACAGGCATACGCCACCATG
```

? ?

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  TTCCCCAGTAGCTGGGATTACAGGCATACGCCACCATG
```

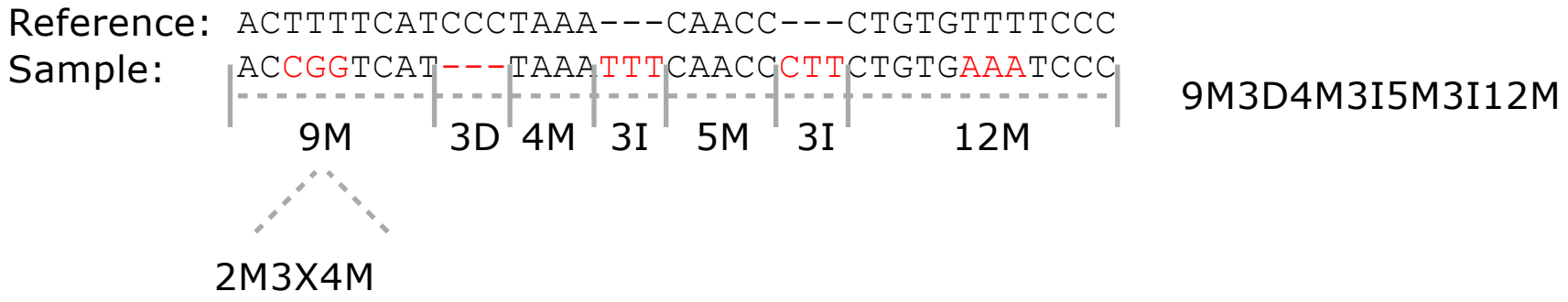
? ?

CIGAR strings explained

•The 'CIGAR' (**C**ompact **I**diosyncratic **G**apped **A**lignment **R**eport)

•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



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

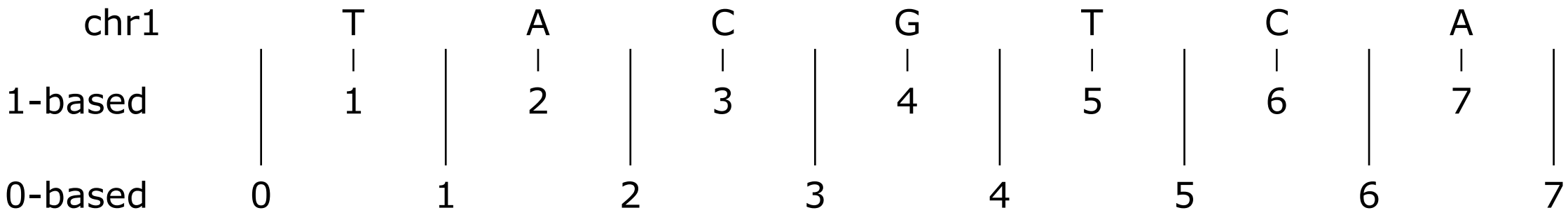
- SAM/BAM files
  - samtools
  - bamtools
  - Picard

- 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

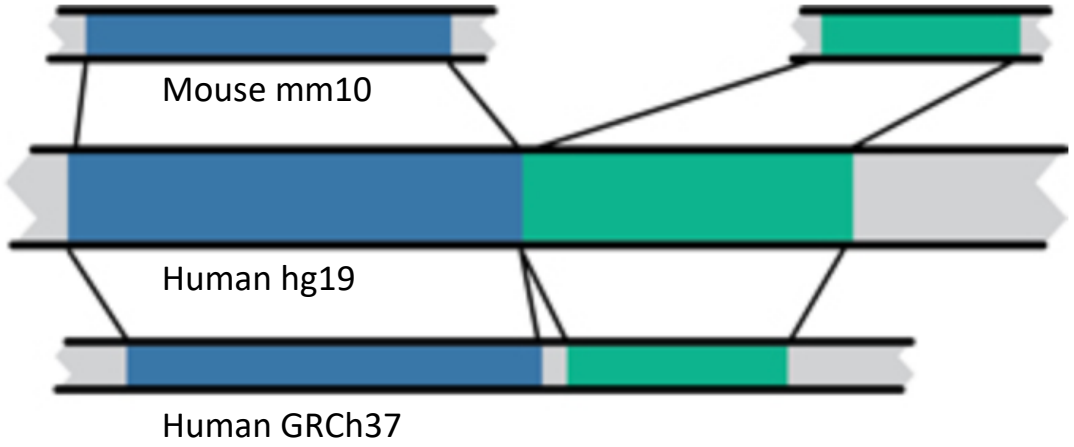
Reference Genome builds

Current human: GRCh38, hg38, b38
alternates: GRCh38v2_ccdg,
GRCh38_full_analysis_set_plus_decoy_hla

Previous human: GRCh37, hg19, b37

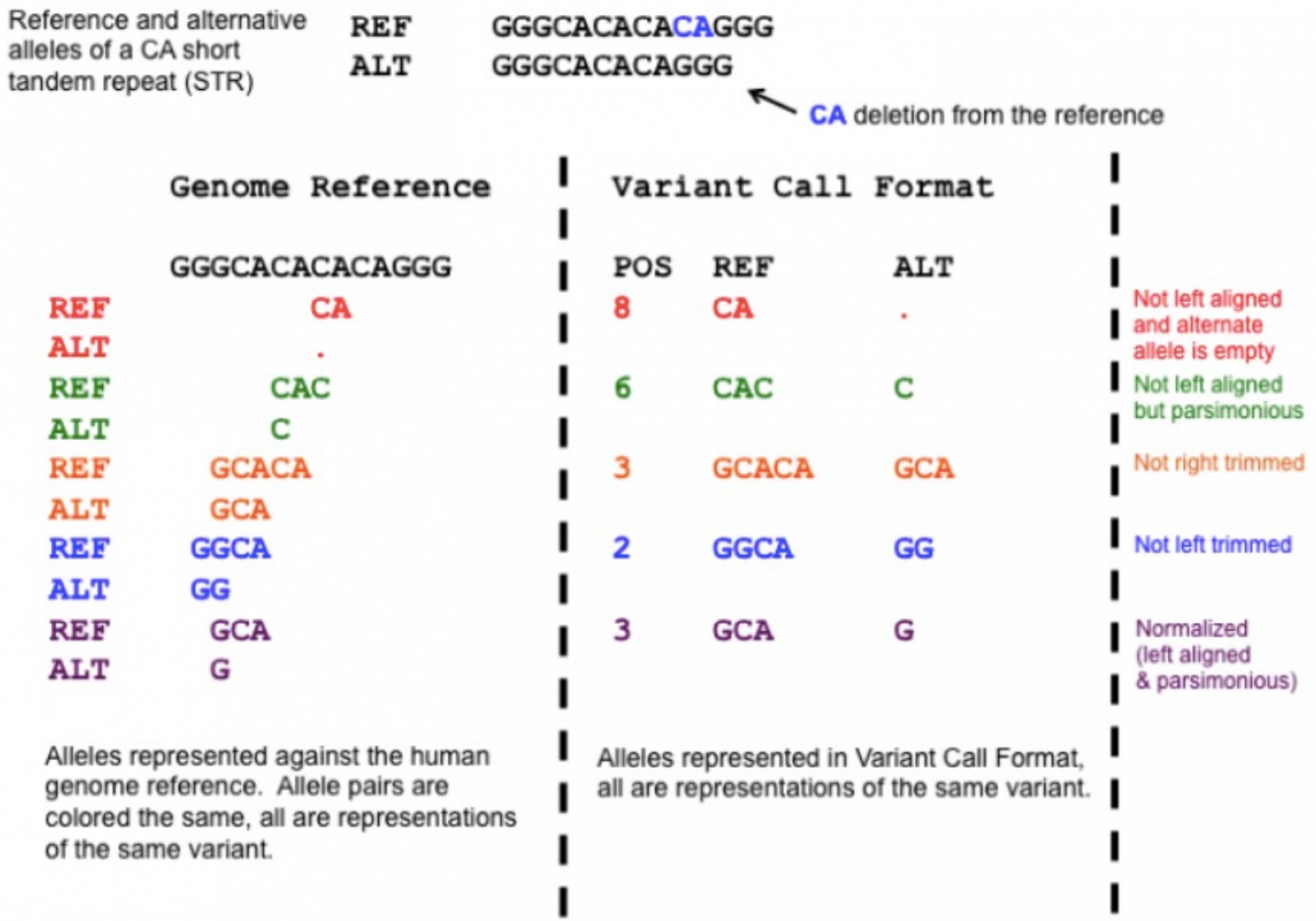
Current mouse: GRCm38, mm10

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



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