Session – Mapping against reference genome and Variant Calling

BU-ISCIII

Unidades Comunes Científico Técnicas – SGSAFI-ISCIII

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Mapping against reference genome and Variant Calling :

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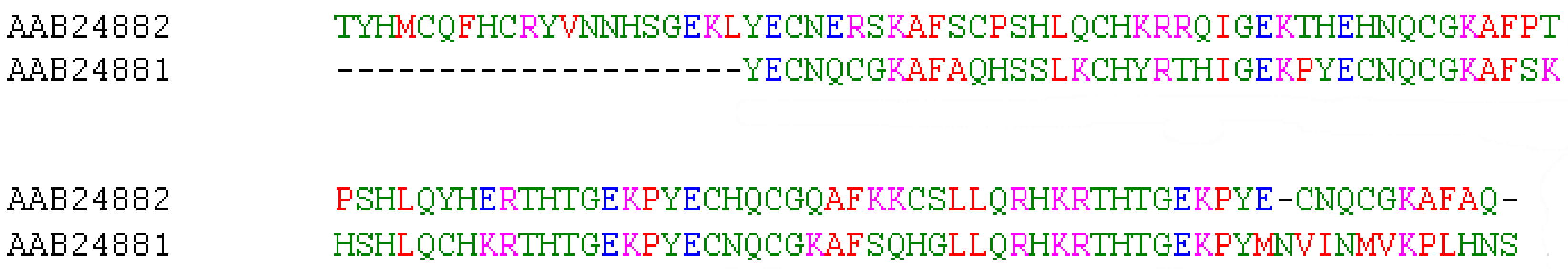
Alignment

Definition:

Arrange two or more nucleotide or aminoacid sequences to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships.

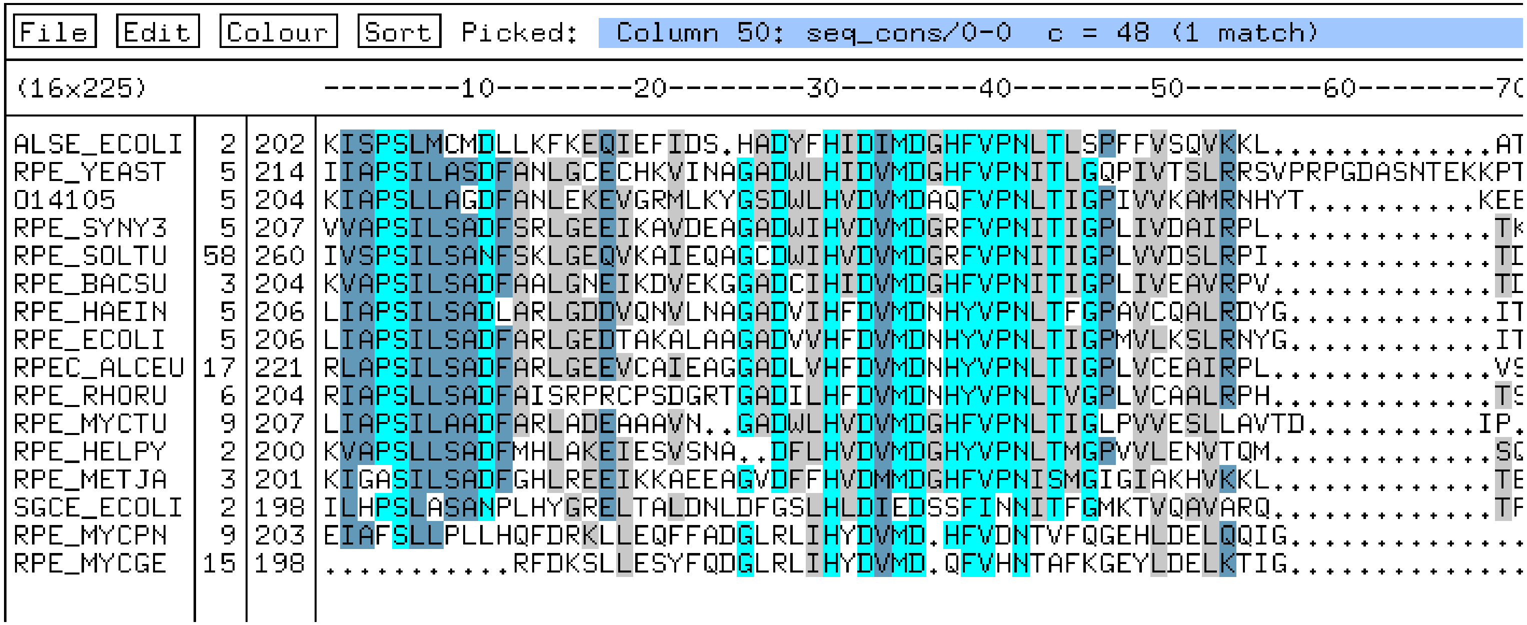


```
AAB24882        TYHMCQFHCRYVNNHSGEKLYECNERSKAFSCPSHLQCHKRRQIGEKTHEHNQCGKAFPT
AAB24881        --------------------YECNQCGKAFAQHSSLKCHYRTHIGEKPYECNQCGKAFSK

AAB24882        PSHLQYHERTHTGEKPYECHQCGQAFKKCSLLQRHKRTHTGEKPYE-CNQCGKAFAQ-
AAB24881        HSHLQCHKRTHTGEKPYECNQCGKAFSQHGLLQRHKRTHTGEKPYMNVINMVKPLHNS
```

Multiple alignment (MSA)

Definition:

A multiple alignment is a colection of three or more sequences partial or completely aligned.

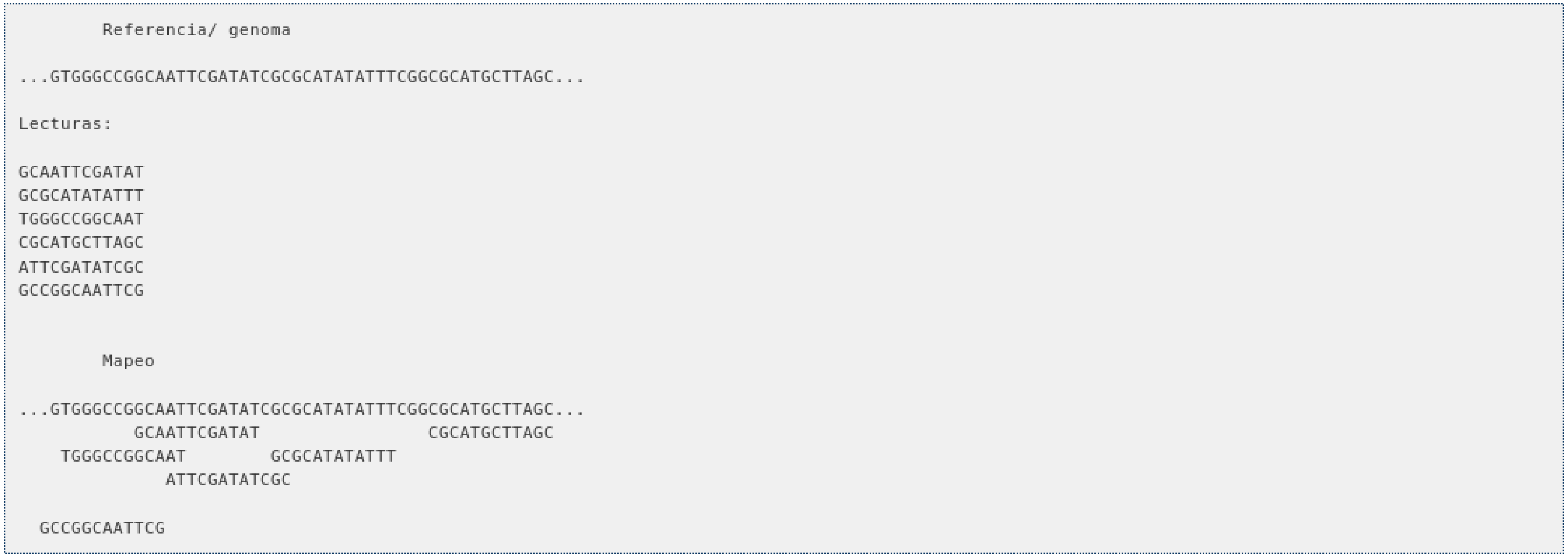


```
Picked: Column 50: seq_cons/0-0   c = 48 (1 match)

(16x225)                 --------10--------20--------30--------40--------50--------60--------7

ALSE_ECOLI   2 202 KISPSLMCMDLLKFKEQIEFIDS.HADYFHIDIMDGHFVPNLTLSPFFVSQVKKL.............AT
RPE_YEAST    5 214 IIAPSILASDFANLGCECHKVINAGADWLHIDVMDGHFVPNITLGQPIVTSLRRSVPRPGDASNTEKKPT
014105       5 204 KIAPSLLAGDFANLEKEVGRMLKYGSDWLHVDVMDAQFVPNLTIGPIVVKAMRNHYT..........KEE
RPE_SYNY3    5 207 VVAPSILSADFSRLGEEIKAVDEAGADWIHVDVMDGRFVPNITIGPLIVDAIRPL.............TK
RPE_SOLTU   58 260 IVSPSILSANFSKLGEQVKAIEQAGCDWIHVDVMDGRFVPNITIGPLVVDSLRPI.............TI
RPE_BACSU    3 204 KVAPSILSADFAALGNEIKDVEKGGADCIHIDVMDGHFVPNITIGPLIVEAVRPV.............TI
RPE_HAEIN    5 206 LIAPSILSADLARLGDDVQNVLNAGADVIHFDVMDNHYVPNLTFGPAVCQALRDYG............IT
RPE_ECOLI    5 206 LIAPSILSADFARLGEDTAKALAAGADVVHFDVMDNHYVPNLTIGPMVLKSLRNYG............IT
RPEC_ALCEU  17 221 RLAPSILSADFARLGEEVCAIEAGGADLVHFDVMDNHYVPNLTIGPLVCEAIRPL.............VS
RPE_RHORU    6 204 RIAPSLLSADFAISRPRCPSDGRTGADILHFDVMDNHYVPNLTVGPLVCAALRPH.............TS
RPE_MYCTU    9 207 LIAPSILAADFARLADEAAAVN..GADWLHVDVMDGHFVPNLTIGLPVVESLLAVTD..........IP.
RPE_HELPY    2 200 KVAPSLLSADFMHLAKEIESVSNA..DFLHVDVMDGHYVPNLTMGPVVLENVTQM.............SG
RPE_METJA    3 201 KIGASILSADFGHLREEIKKAEEAGVDFFHVDMMDGHFVPNISMGIGIAKHVKKL.............TE
SGCE_ECOLI   2 198 ILHPSLASANPLHYGRELTALDNLDFGSLHLDIEDSSFINNITFGMKTVQAVARQ.............TF
RPE_MYCPN    9 203 EIAFSLLPLLHQFDRKLLEQFFADGLRLIHYDVMD.HFVDNTVFQGEHLDELQQIG..............
RPE_MYCGE   15 198 ...........RFDKSLLESYFQDGLRLIHYDVMD.QFVHNTAFKGEYLDELKTIG..............
```

Mapping definition

Definición:

Place a sequence inside a larger sequence. For example, determine the position of a read inside a reference genome.



```
        Referencia/ genoma

...GTGGGCCGGCAATTCGATATCGCGCATATATTTCGGCGCATGCTTAGC...

Lecturas:

GCAATTCGATAT
GCGCATATATTT
TGGGCCGGCAAT
CGCATGCTTAGC
ATTCGATATCGC
GCCGGCAATTCG


        Mapeo

...GTGGGCCGGCAATTCGATATCGCGCATATATTTCGGCGCATGCTTAGC...
           GCAATTCGATAT                CGCATGCTTAGC
    TGGGCCGGCAAT        GCGCATATATTT
              ATTCGATATCGC

  GCCGGCAATTCG
```

Alignment vs mapping

Mapping:

- A mapping is regarded to be correct if it overlaps the true region.
- Each read maps independently
- From thousand to millions of sequences.

Multiple alignment:

- An alignment is regarded to be correct only if each base is placed correctly.
- Minimizes differences among sequences
- From tens to hundred of sequences.

Consideratiosn:

- An algorithm can be good at mapping but may not be good aligning.
- This is because the true alignment minimizes differences between reads, but the read mapper only sees the reference.

Hen Li. Mapping, Alignment and SNP Calling. MPG Next Gen Workshop 2011

So in summary…

CTGACCTCATGTGATCCACCCGCCTTGGCC

Find best match for the read
in a reference sequence

TGATCCAC

Challenges

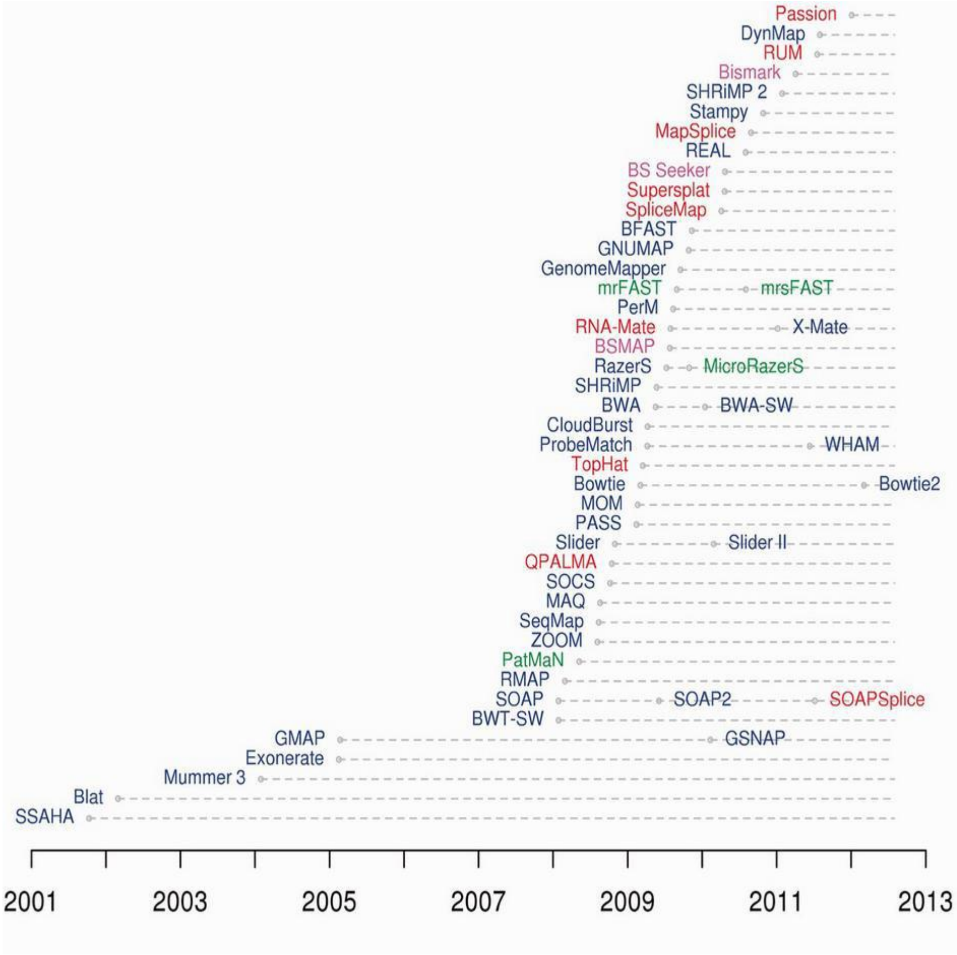
- Errors in reads
- Errors in libraries
- Repetitive regions (repeats, homologous regions)
- Homopolymers
- Individual polymorphisms

Pierre Lechat. Variants Calling lecture. Pasteur.fr

What mapper should I use?

Mappers:

- Más de 60 mappers available.
- Lots of papers reviewing its performamnce.



What mapper should I use?

Cosas a tener en cuenta:

- Computational resources vs sensibility
- Platform and type of experiment (Illumina/454/etc,paired-end,DNA/RNA/etc)
- Variation (indels allowance, mistmatch number,etc.)
- Repetitions (all regions, best match, random, user defined number…)

Importante:

- Default options don't have to be the best:

"… there is no tool that outperforms all of the others in all the tests. Therefore, the end user should clearly specify his needs in order to choose the tool that provides the best results." - Hatem et al *BMC Bioinformatics* 2013, **14**:184

What mapper should I use?

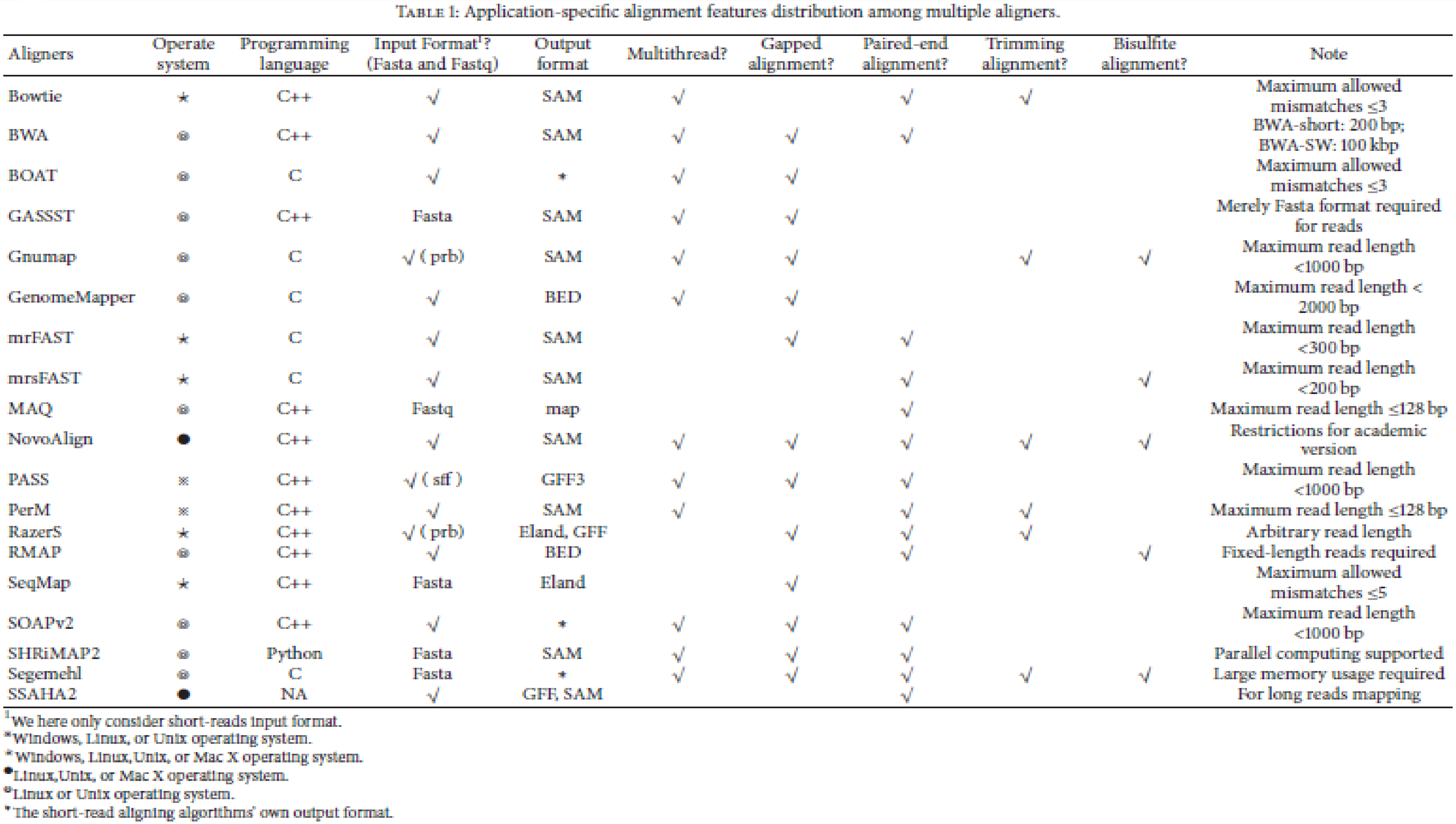


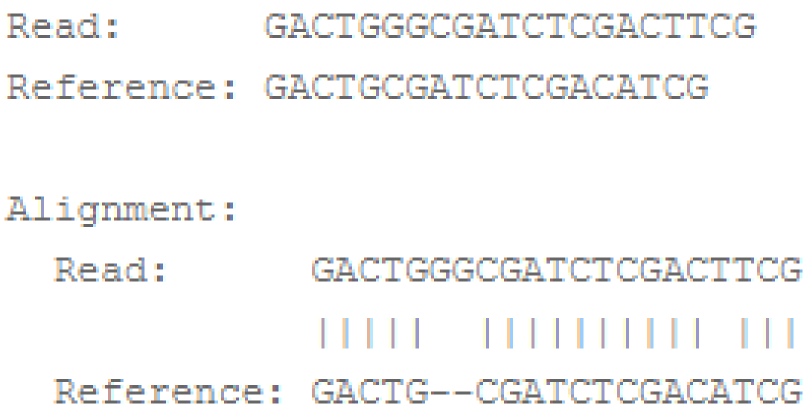
Table 1: Application-specific alignment features distribution among multiple aligners.

Aligners	Operate system	Programming language	Input Format[1]? (Fasta and Fastq)	Output format	Multithread?	Gapped alignment?	Paired-end alignment?	Trimming alignment?	Bisulfite alignment?	Note
Bowtie	★	C++	√	SAM	√		√	√		Maximum allowed mismatches ≤3
BWA	⊛	C++	√	SAM	√	√	√			BWA-short: 200 bp; BWA-SW: 100 kbp
BOAT	⊛	C	√	*	√	√				Maximum allowed mismatches ≤3
GASSST	⊛	C++	Fasta	SAM	√	√				Merely Fasta format required for reads
Gnumap	⊛	C	√ (prb)	SAM	√	√		√	√	Maximum read length <1000 bp
GenomeMapper	⊛	C	√	BED	√	√				Maximum read length < 2000 bp
mrFAST	★	C	√	SAM		√	√			Maximum read length <300 bp
mrsFAST	★	C	√	SAM			√		√	Maximum read length <200 bp
MAQ	⊛	C++	Fastq	map			√			Maximum read length ≤128 bp
NovoAlign	●	C++	√	SAM	√	√	√	√	√	Restrictions for academic version
PASS	※	C++	√ (sff)	GFF3	√	√	√			Maximum read length <1000 bp
PerM	※	C++	√	SAM	√		√	√		Maximum read length ≤128 bp
RazerS	★	C++	√ (prb)	Eland, GFF		√	√	√		Arbitrary read length
RMAP	⊛	C++	√	BED			√		√	Fixed-length reads required
SeqMap	★	C++	Fasta	Eland		√				Maximum allowed mismatches ≤5
SOAPv2	⊛	C++	√	*	√	√	√			Maximum read length <1000 bp
SHRiMAP2	⊛	Python	Fasta	SAM	√	√	√			Parallel computing supported
Segemehl	⊛	C	Fasta	*	√	√	√	√	√	Large memory usage required
SSAHA2	●	NA	√	GFF, SAM			√			For long reads mapping

[1] We here only consider short-reads input format.
※Windows, Linux, or Unix operating system.
★Windows, Linux,Unix, or Mac X operating system.
●Linux,Unix, or Mac X operating system.
⊛Linux or Unix operating system.
*The short-read aligning algorithms' own output format.

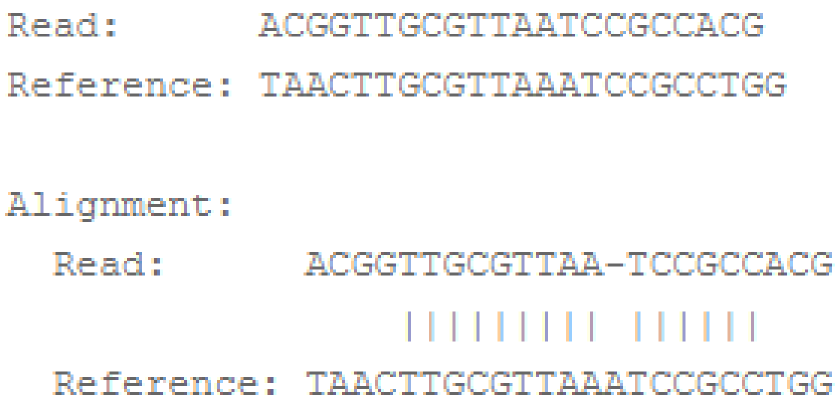
End-to-end vs local alignment

End-to-end



```
Read:      GACTGGGCGATCTCGACTTCG
Reference: GACTGCGATCTCGACATCG

Alignment:
  Read:      GACTGGGCGATCTCGACTTCG
             |||||  |||||||||| |||
  Reference: GACTG--CGATCTCGACATCG
```

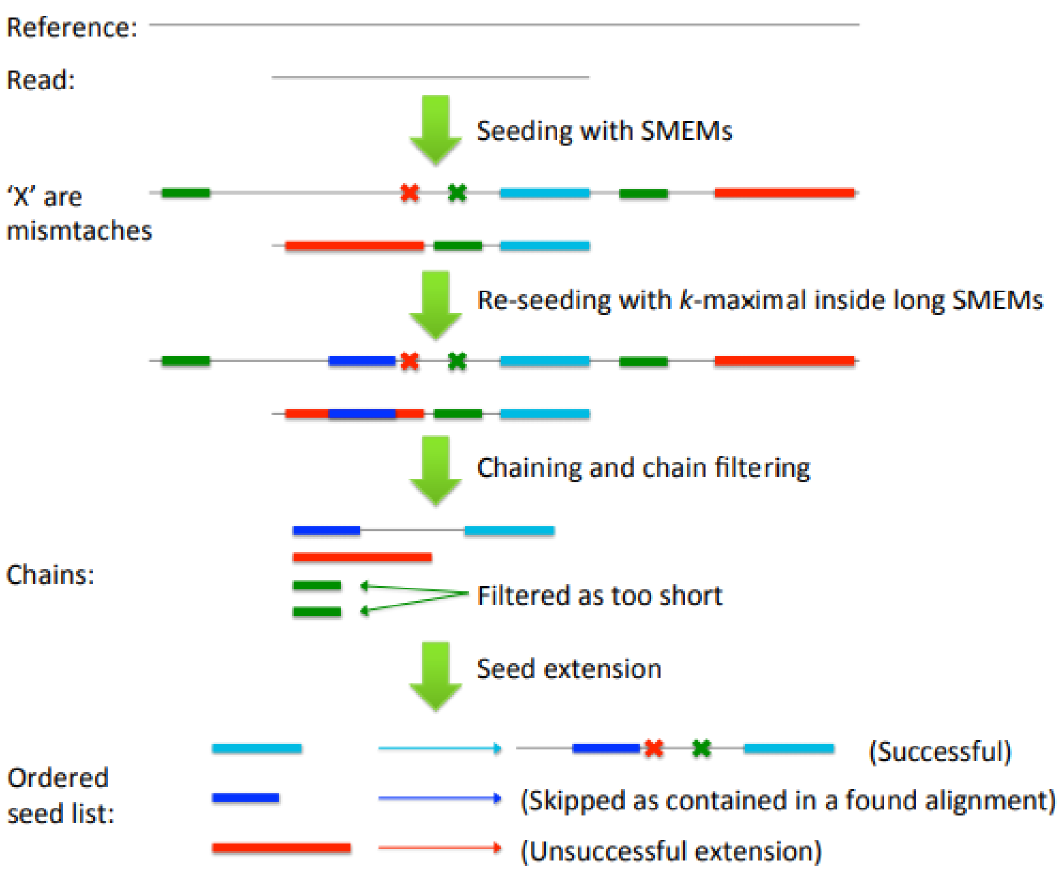
Local



```
Read:      ACGGTTGCGTTAATCCGCCACG
Reference: TAACTTGCGTTAAATCCGCCTGG

Alignment:
  Read:      ACGGTTGCGTTAA-TCCGCCACG
                 ||||||||| ||||||
  Reference: TAACTTGCGTTAAATCCGCCTGG
```

Bowtie2 manual.

BWA MEM



SMEM strategy

- Maximal exact match (MEM): an exact match that cannot be extended further in either direction
- Super-maximal exact match (SMEM): a MEM that is not contained in any other MEMs on the query coordinate (Li, 2012). At any query position, the longest exact match covering the position must be a SMEM.

Seed-and-extend algorithm

Local alignment

Hen LI. Aligning sequence reads, clone sequences and assembly con*gs with BWA-MEM. Poster. Broad Institute.

BOWTIE2

End-to-end alignment by default.

Three reporting modes:

- Best alignment
- K alignments
- All alignments

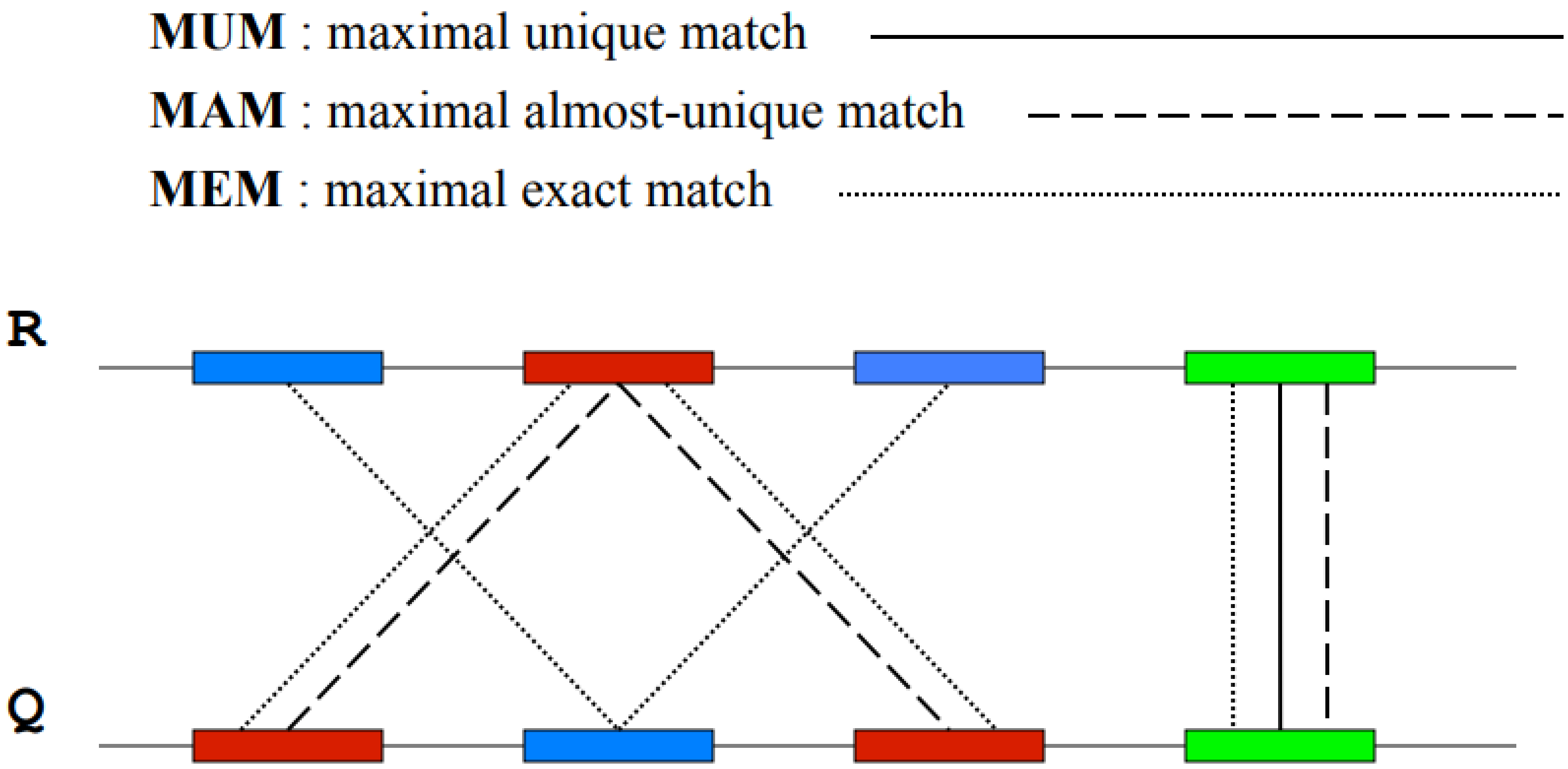
Lots of customizable parameters that change its performance.

Example whole genome aligner: MUMMER

- **Maximal Unique Matcher (MUM)**
  - match <- exact match of a minimum length
  - maximal <- cannot be extended in either direction without a mismatch
  - unique
    - occurs only once in both sequences (MUM)
    - occurs only once in a single sequence (MAM)
    - occurs one or more times in either sequence (MEM)

Adam M. Phillippy. Whole Genome Alignment with MUMmer. Presentation.

Example whole genome aligner: MUMMER



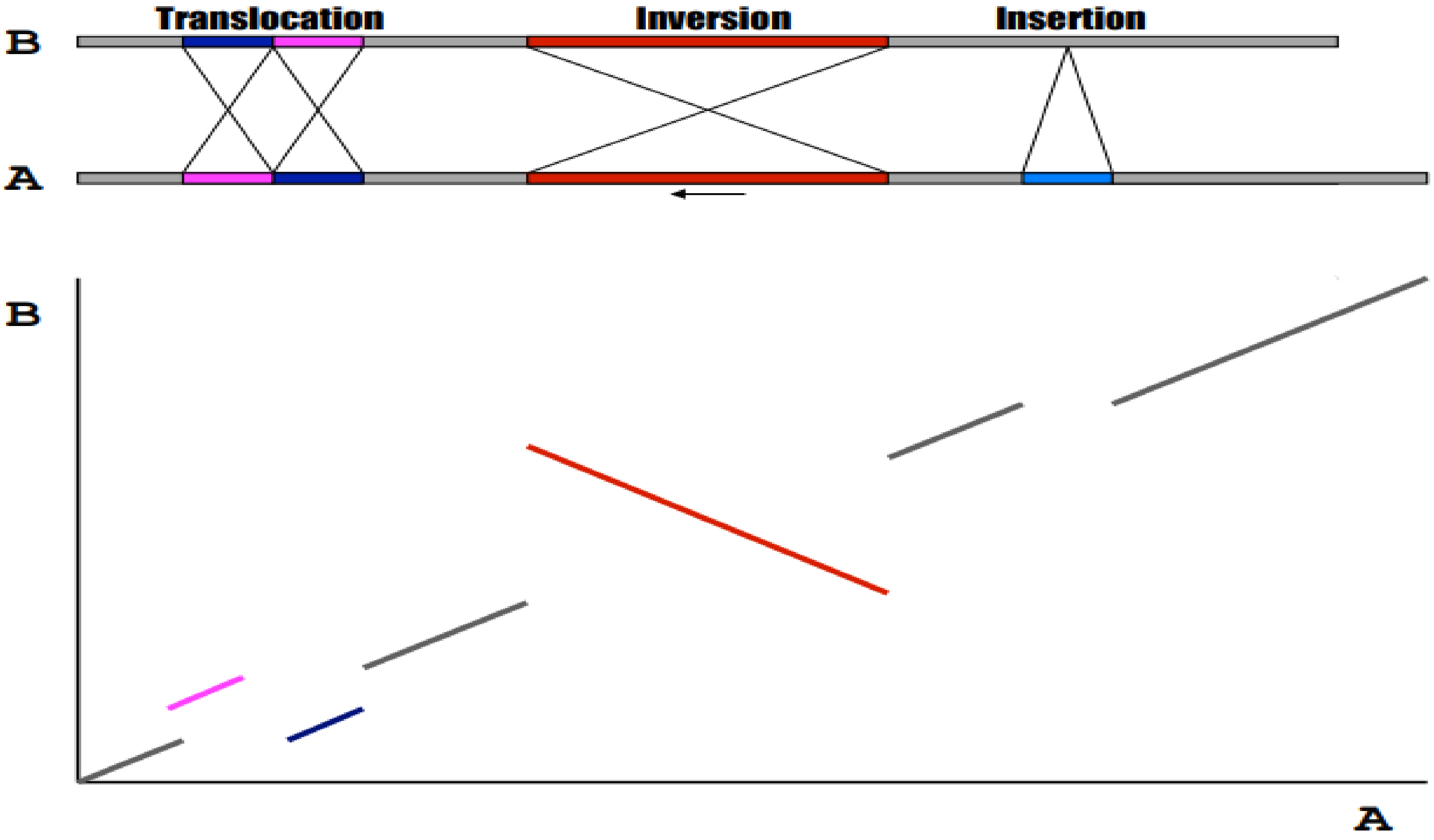
MUM : maximal unique match ———————

MAM : maximal almost-unique match – – – – – – – –

MEM : maximal exact match ··················

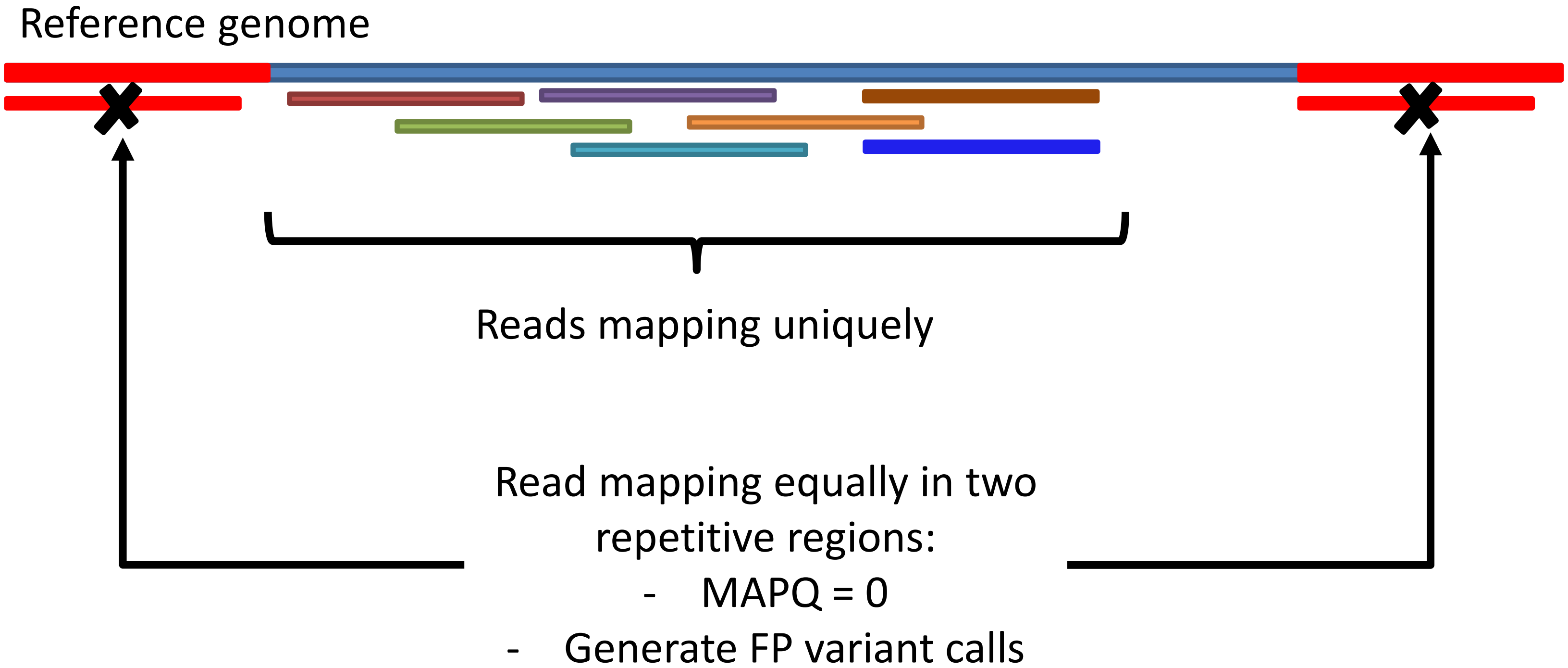
Adam M. Phillippy. Whole Genome Alignment with MUMmer. Presentation.

Example whole genome aligner: MUMMER



Adam M. Phillippy. Whole Genome Alignment with MUMmer. Lecture.

Which aligner should I use for aligning reads agains a complete genome for variant calling?



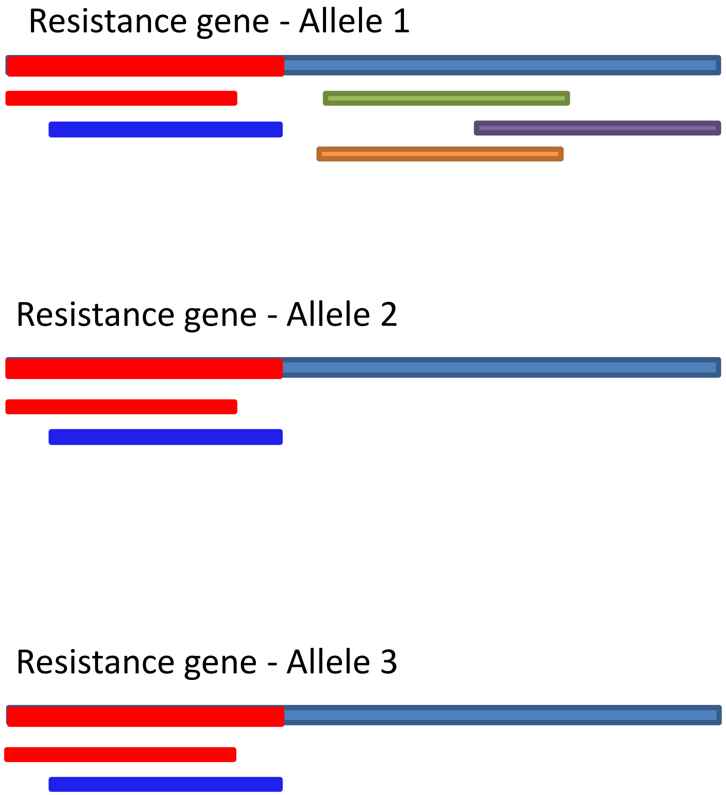
Read mapping equally in two repetitive regions:

- MAPQ = 0
- Generate FP variant calls

Which aligner should I use for aligning reads against a resistance gene database for determining with resistance genes I have in my sample?

🟥 Homologus/repetitive region

Reads mapping to the repetitive/homologus region map against all alleles.
We allow one read to map to **several locations**.

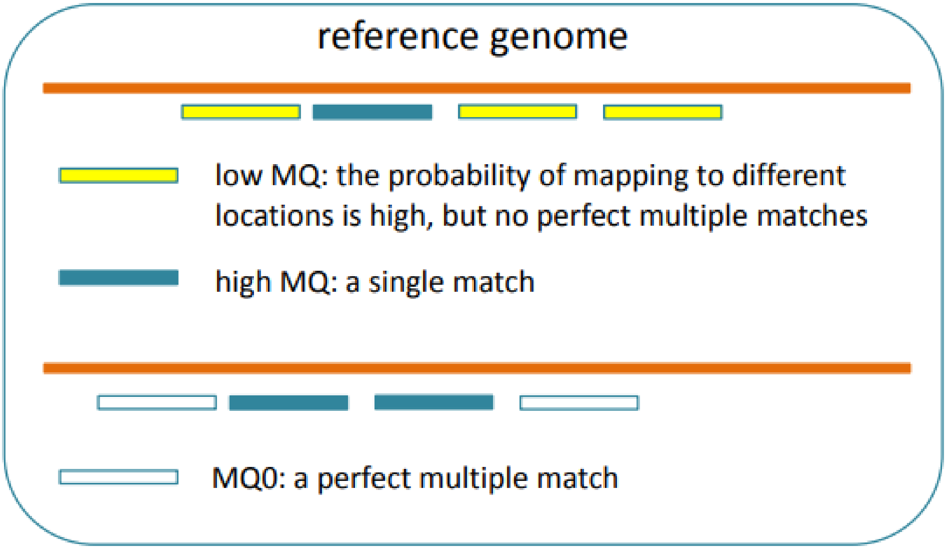

Resistance gene - Allele 1
Resistance gene - Allele 2
Resistance gene - Allele 3

Reads mapping uniquely only map in Allele 1. Which is the one more **covered**

MAPQ

- What if there are several possible places to align your sequencing read? This may be due to:
  - Repeated elements in the genome
  - Low complexity sequences
  - Reference errors and gaps

  MQ is a phredScore of the quality of the alignment


reference genome
low MQ: the probability of mapping to different locations is high, but no perfect multiple matches
high MQ: a single match
MQ0: a perfect multiple match

MAPQ

MAPQ is NOT comparable among mappers.

BWA:

- MAPQ represents the probability of the read to be mapped correctly.
- MAPQ = 0 identifies unmapped reads and…

Reads mapping to different locations!

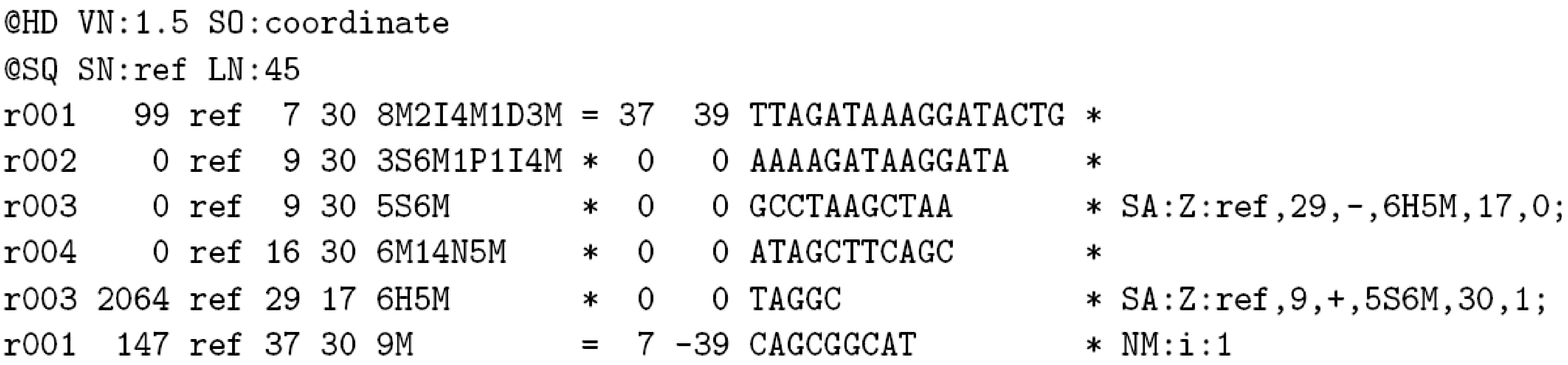
BOWTIE2:

- MAPQ represents the “uniqueness” of the read. A MAPQ < 10 indicates that there is at least a 1 in 10 chance that the read truly originated elsewhere
- MAPQ = 0 identifies unmapped reads

SAM format

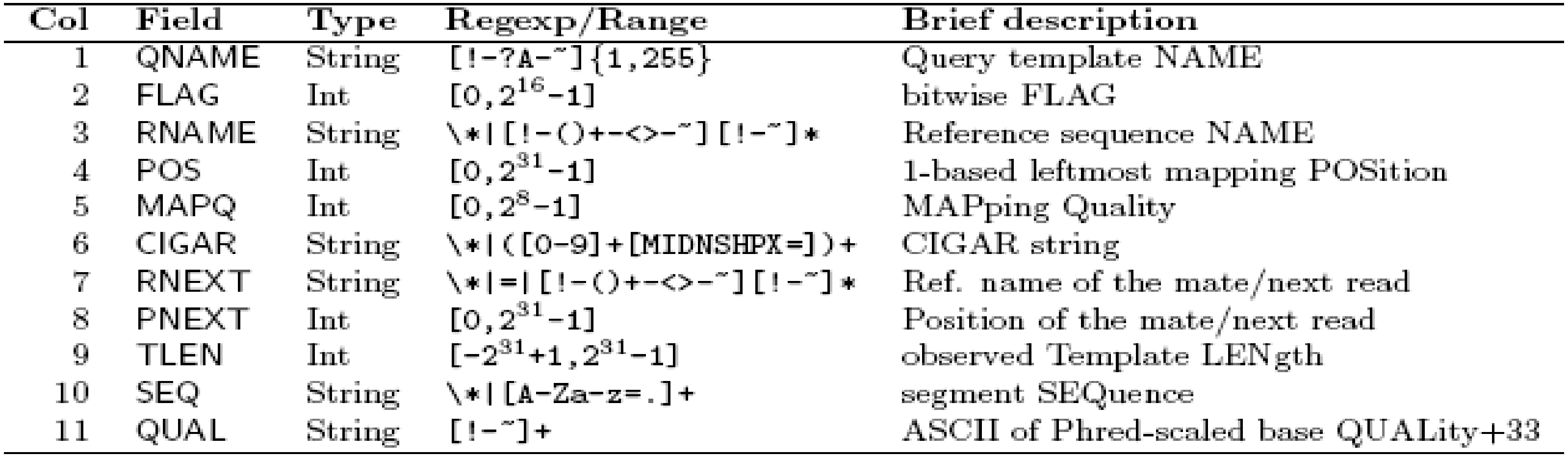
Definición:

It’s a specification that defines a generic format for storing nucleotide alignments. It describes a query alignment against a reference genome.

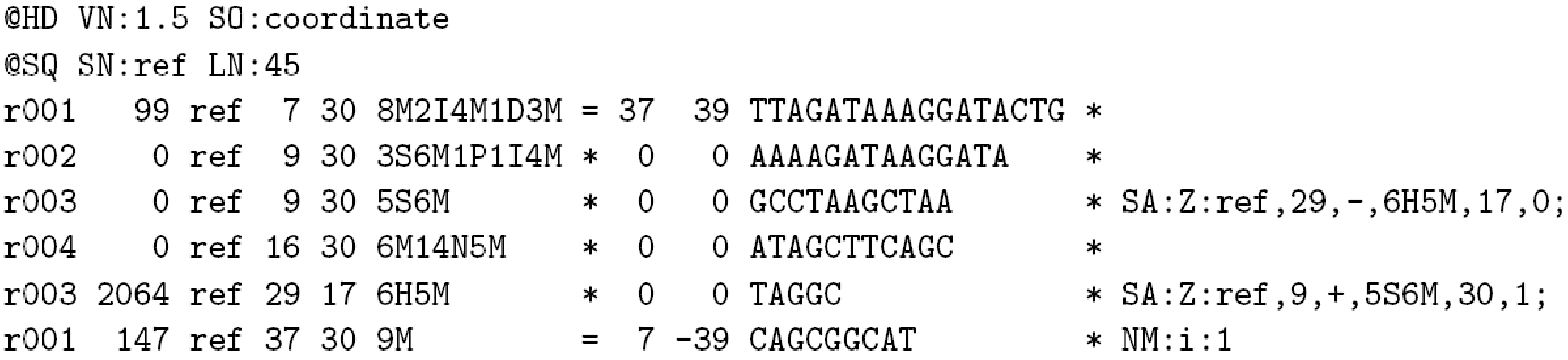


```
@HD VN:1.5 SO:coordinate
@SQ SN:ref LN:45
r001   99 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002    0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003    0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA       * SA:Z:ref,29,-,6H5M,17,0;
r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003 2064 ref 29 17 6H5M       *  0   0 TAGGC             * SA:Z:ref,9,+,5S6M,30,1;
r001  147 ref 37 30 9M         =  7 -39 CAGCGGCAT         * NM:i:1
```

SAM format

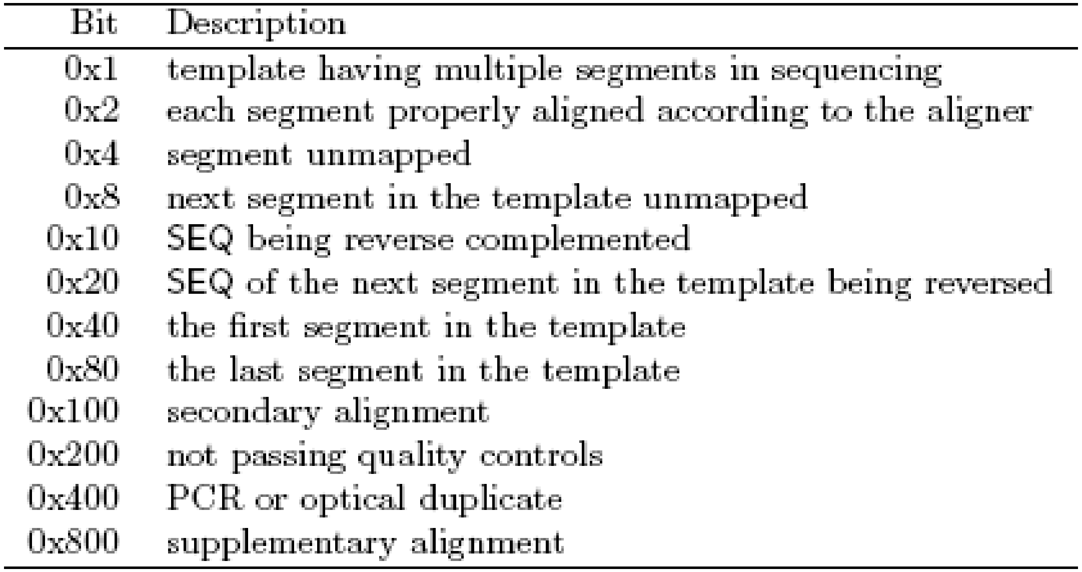


Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	[!-?A-~]{1,255}	Query template NAME
2	FLAG	Int	[0,2^{16}-1]	bitwise FLAG
3	RNAME	String	*|[!-()+-<>-~][!-~]*	Reference sequence NAME
4	POS	Int	[0,2^{31}-1]	1-based leftmost mapping POSition
5	MAPQ	Int	[0,2^{8}-1]	MAPping Quality
6	CIGAR	String	*|([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



```
@HD VN:1.5 SO:coordinate
@SQ SN:ref LN:45
r001   99 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002    0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003    0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA       * SA:Z:ref,29,-,6H5M,17,0;
r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003 2064 ref 29 17 6H5M       *  0   0 TAGGC             * SA:Z:ref,9,+,5S6M,30,1;
r001  147 ref 37 30 9M         =  7 -39 CAGCGGCAT         * NM:i:1
```

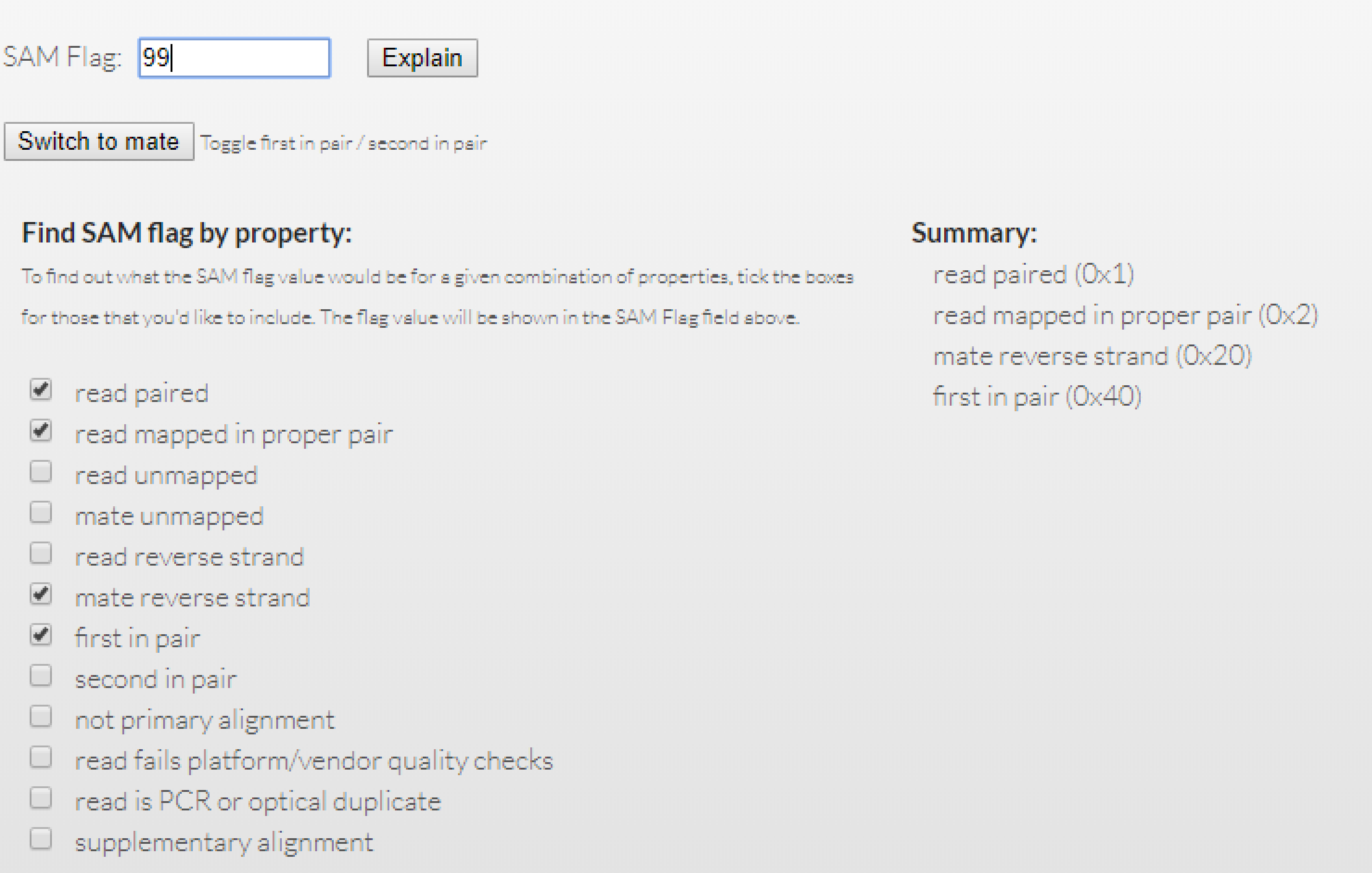
SAM format: flags



Bit	Description
0x1	template having multiple segments in sequencing
0x2	each segment properly aligned according to the aligner
0x4	segment unmapped
0x8	next segment in the template unmapped
0x10	SEQ being reverse complemented
0x20	SEQ of the next segment in the template being reversed
0x40	the first segment in the template
0x80	the last segment in the template
0x100	secondary alignment
0x200	not passing quality controls
0x400	PCR or optical duplicate
0x800	supplementary alignment

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

Flag explanation example 1



SAM Flag: 99

Toggle first in pair / second in pair

Find SAM flag by property:

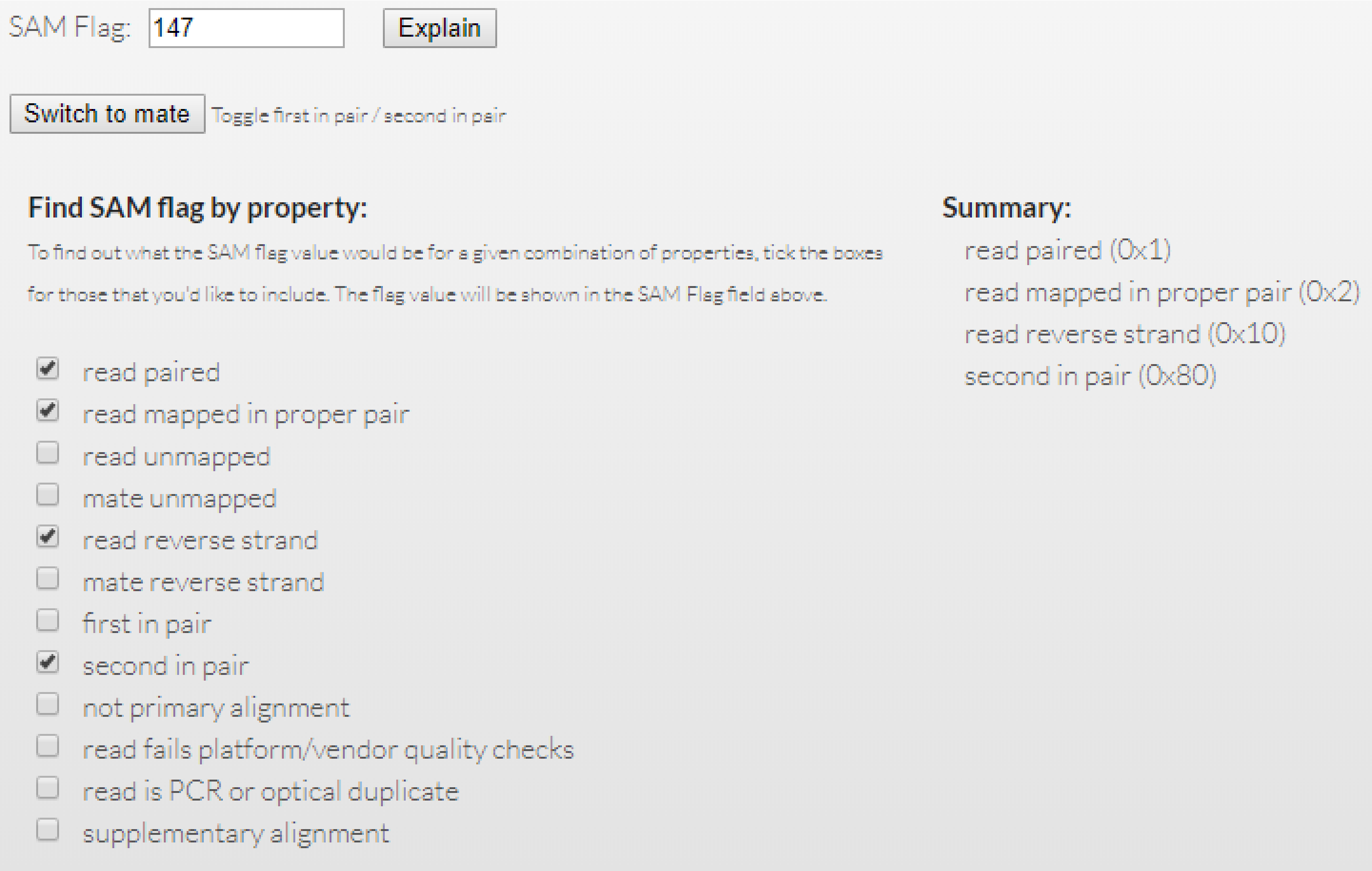
To find out what the SAM flag value would be for a given combination of properties, tick the boxes for those that you'd like to include. The flag value will be shown in the SAM Flag field above.

- ☑ read paired
- ☑ read mapped in proper pair
- □ read unmapped
- □ mate unmapped
- □ read reverse strand
- ☑ mate reverse strand
- ☑ first in pair
- □ second in pair
- □ not primary alignment
- □ read fails platform/vendor quality checks
- □ read is PCR or optical duplicate
- □ supplementary alignment

Summary:

read paired (0x1)
read mapped in proper pair (0x2)
mate reverse strand (0x20)
first in pair (0x40)

Flag explanation example 2



SAM Flag: 147

Toggle first in pair / second in pair

Find SAM flag by property:

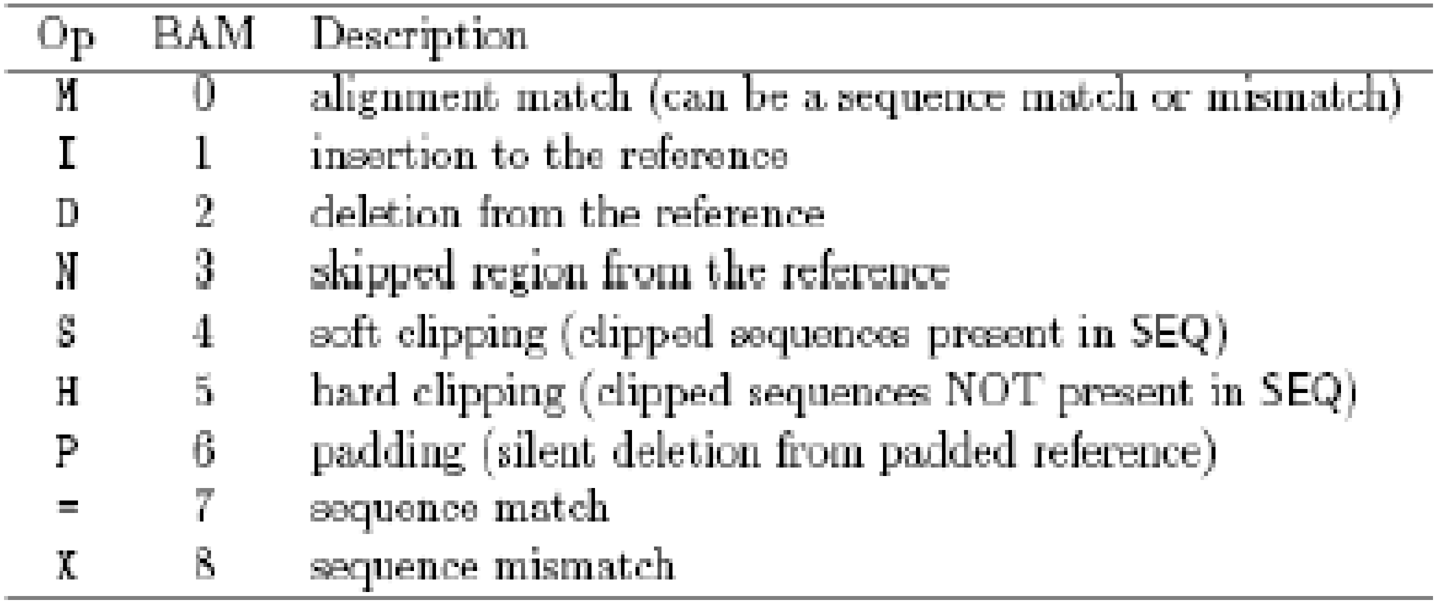
To find out what the SAM flag value would be for a given combination of properties, tick the boxes for those that you'd like to include. The flag value will be shown in the SAM Flag field above.

- ☑ read paired
- ☑ read mapped in proper pair
- □ read unmapped
- □ mate unmapped
- ☑ read reverse strand
- □ mate reverse strand
- □ first in pair
- ☑ second in pair
- □ not primary alignment
- □ read fails platform/vendor quality checks
- □ read is PCR or optical duplicate
- □ supplementary alignment

Summary:

read paired (0x1)
read mapped in proper pair (0x2)
read reverse strand (0x10)
second in pair (0x80)

SAM format: 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

SAM vs BAM format

- SAM and BAM format are exactly the same.
  - SAM is a tabular plain text file.
  - BAM is its binary format. Binary meaning is in a compress format not human readable.
  - We **MUST** always use BAM format because it is optimized for computer-reading

AND

BECAUSE IT SAVES A LOT OF DISK SPACE!!

<u>Typical bam and sam format files weights from a S. grumpensis</u>

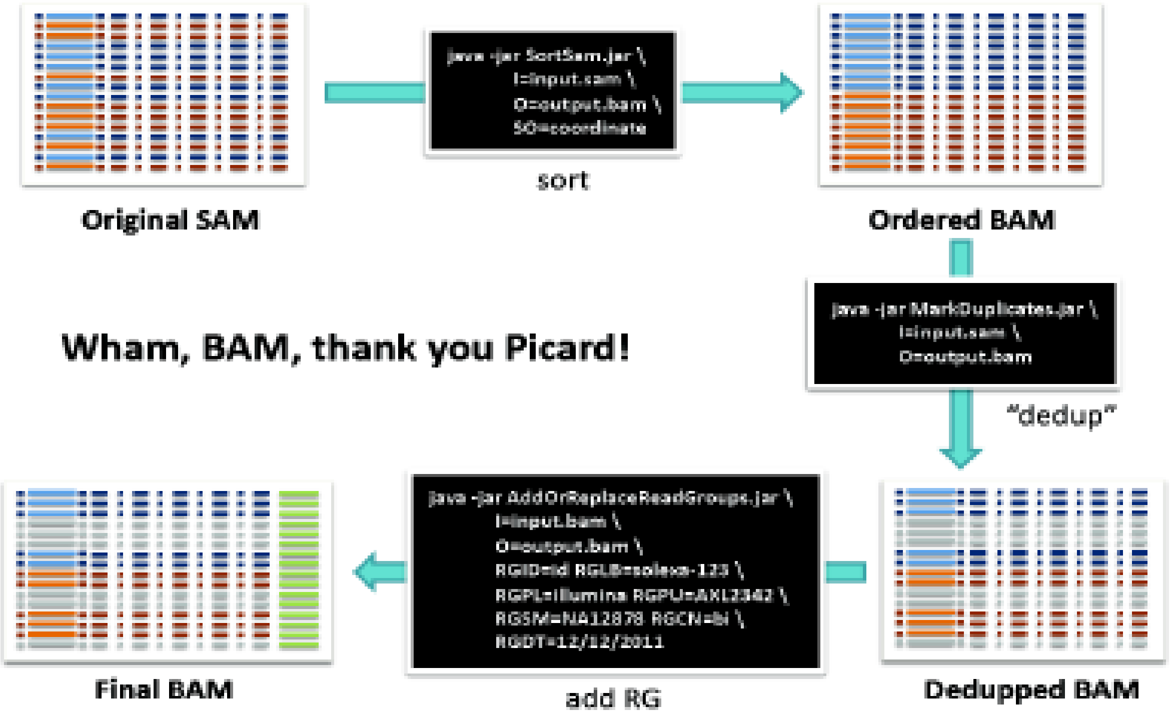
SAM format file: 3.6 GB

BAM format file: 689 M

Duplicate filter

- Duplicates are non-independent measurements of a sequence
  - Sampled from the exact same template of DNA
  - Violates assumptions of variant calling
- Errors in sample/library prep will get propagated to all the duplicates
- Just pick the “best” copy – mitigates the effects of errors
- **Definition**: sequences starting and finishing in the exact same coordinates. Both pairs if paired-end.

Duplicate filter



```
java -jar SortSam.jar \
        I=input.sam \
        O=output.bam \
        SO=coordinate
```

```
java -jar MarkDuplicates.jar \
        I=input.sam \
        O=output.bam
```

```
java -jar AddOrReplaceReadGroups.jar \
        I=input.bam \
        O=output.bam \
        RGID=id RGLB=solexa-123 \
        RGPL=illumina RGPU=AXL2342 \
        RGSM=NA12878 RGCN=bi \
        RGDT=12/12/2011
```

Wham, BAM, thank you Picard!

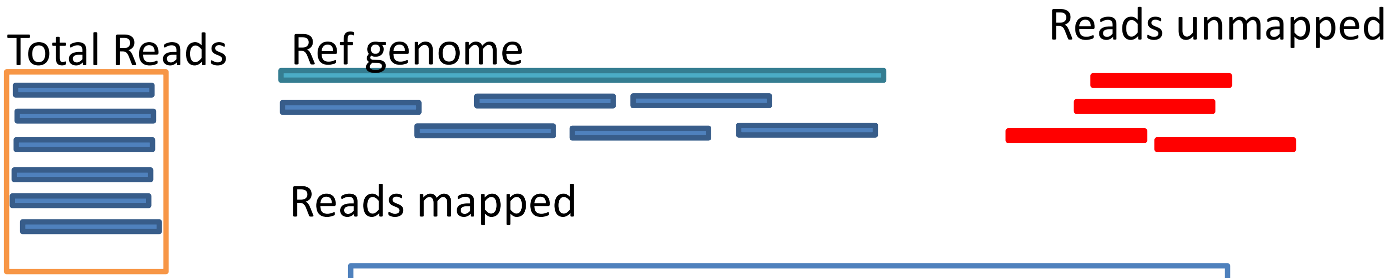
Mapping statistics

- % mapped: reads mapped/total reads
- % unmapped: reads unmapped/total reads
- % duplicates: reads belonging to same template/total reads
- Mean depth of coverage
- Coverage: % genome with at least one read mapped.

Mapping quality control

- **% mapping:** number of reads mapping againts reference genome.

Picard
Samtools



% mapping = Reads mapped / Total Reads

Mandatory parameter for microbial genomics!! It indicates us how many reads we have from our organism of interest. In human genomics this is almost always 99.99% unless something terrible happens. Not here!!!

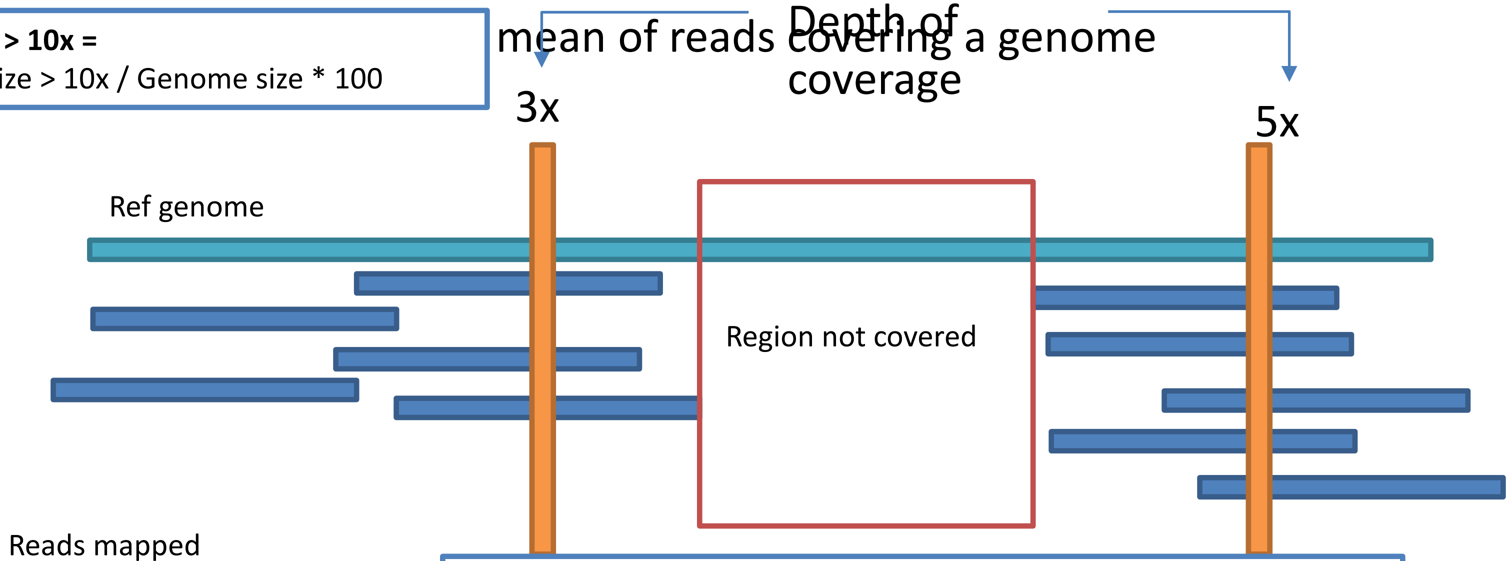
Mapping quality control

- **% genome > 10x:** percentage of genome covered with more than 10 reads.

Picard
Samtools

% genome > 10x =
Genome size > 10x / Genome size * 100

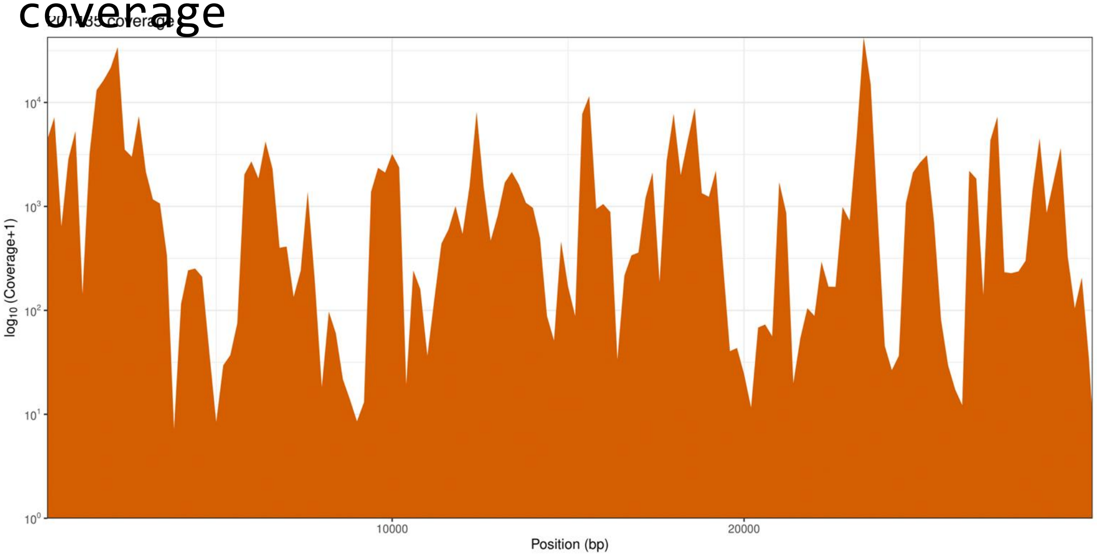
mean of reads covering a genome



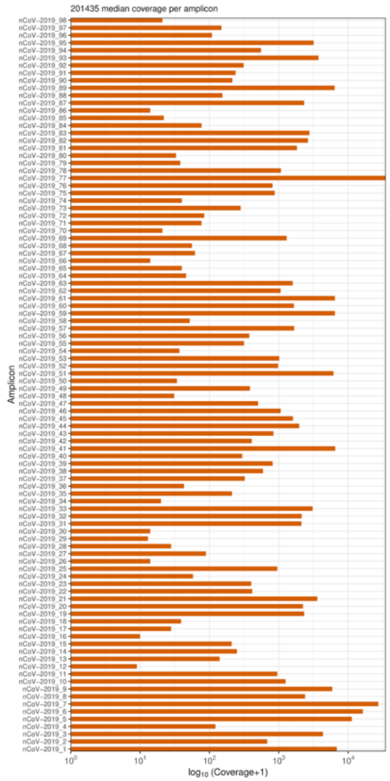
Mean Depth of coverage =
Depth of coverage in position 1, 2, 3 / Genome size

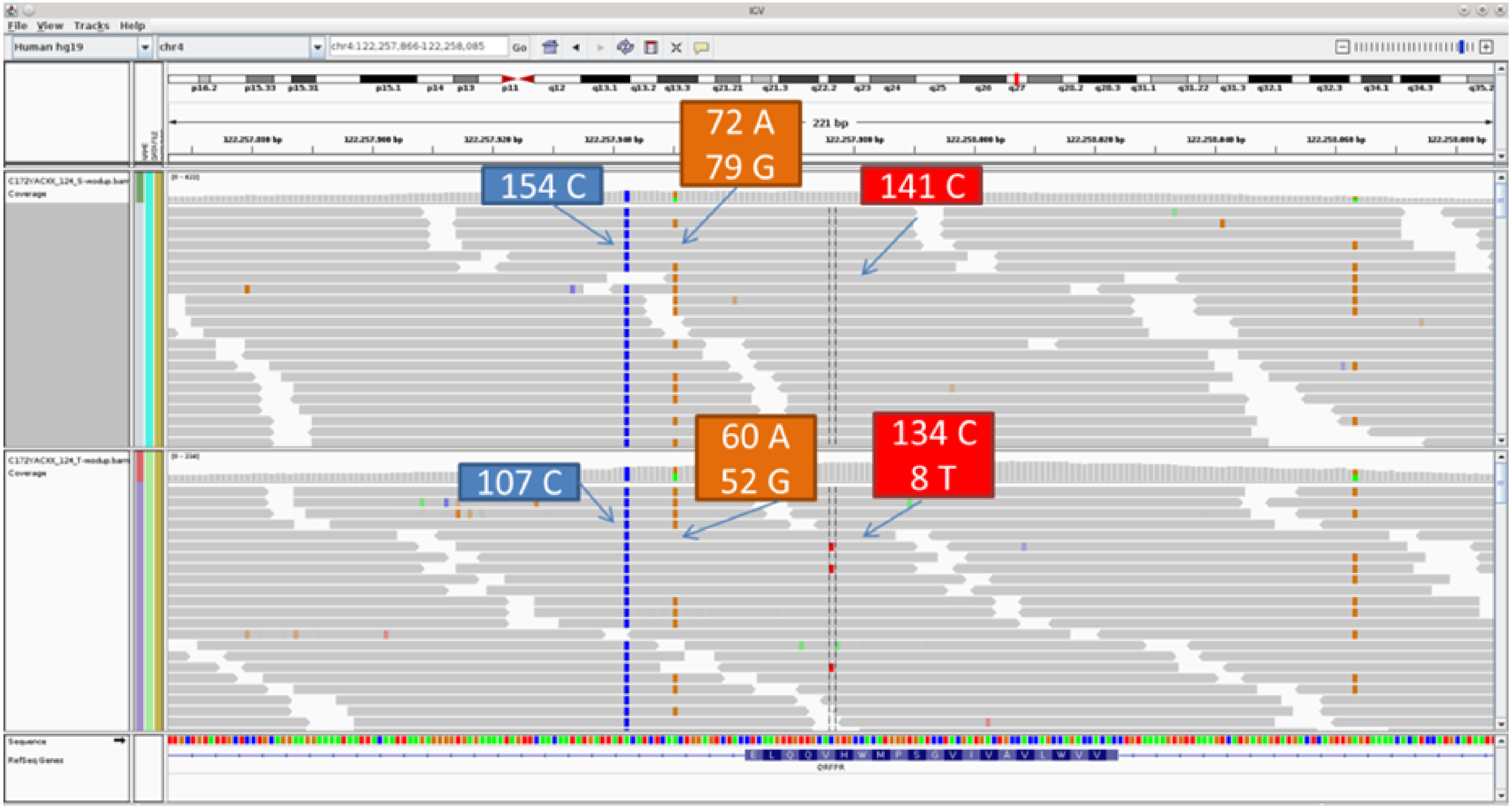
Amplicon QC results

Genome coverage



Amplicon coverage




IGV
File View Tracks Help
Human hg19
chr4
chr4:122,257,866-122,258,085
Go
221 bp
C172YACXX_124_S-nodup.bam Coverage
C172YACXX_124_T-nodup.bam Coverage
Sequence
RefSeq Genes
QRFPR

72 A
79 G

154 C

141 C

60 A
52 G

107 C

134 C
8 T

Thanks for your attention!