# Reference alignment of reads

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March 2025

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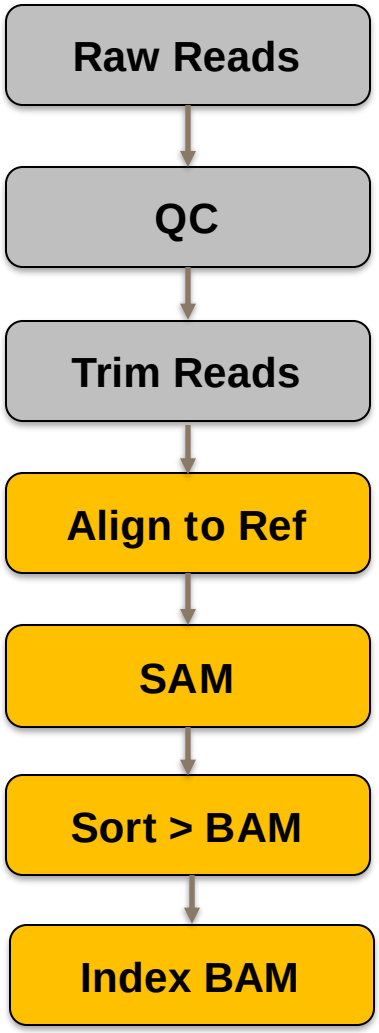
# Previously …




- Previous session we learnt about FASTQ reads and read cleaning/trimming
- Task now is to align these reads to a selected reference sequence
- 8:30 – 10:00
  - 8:30 – 9:15: Talk
  - 9:15 – 10:00: Practical

# Overview

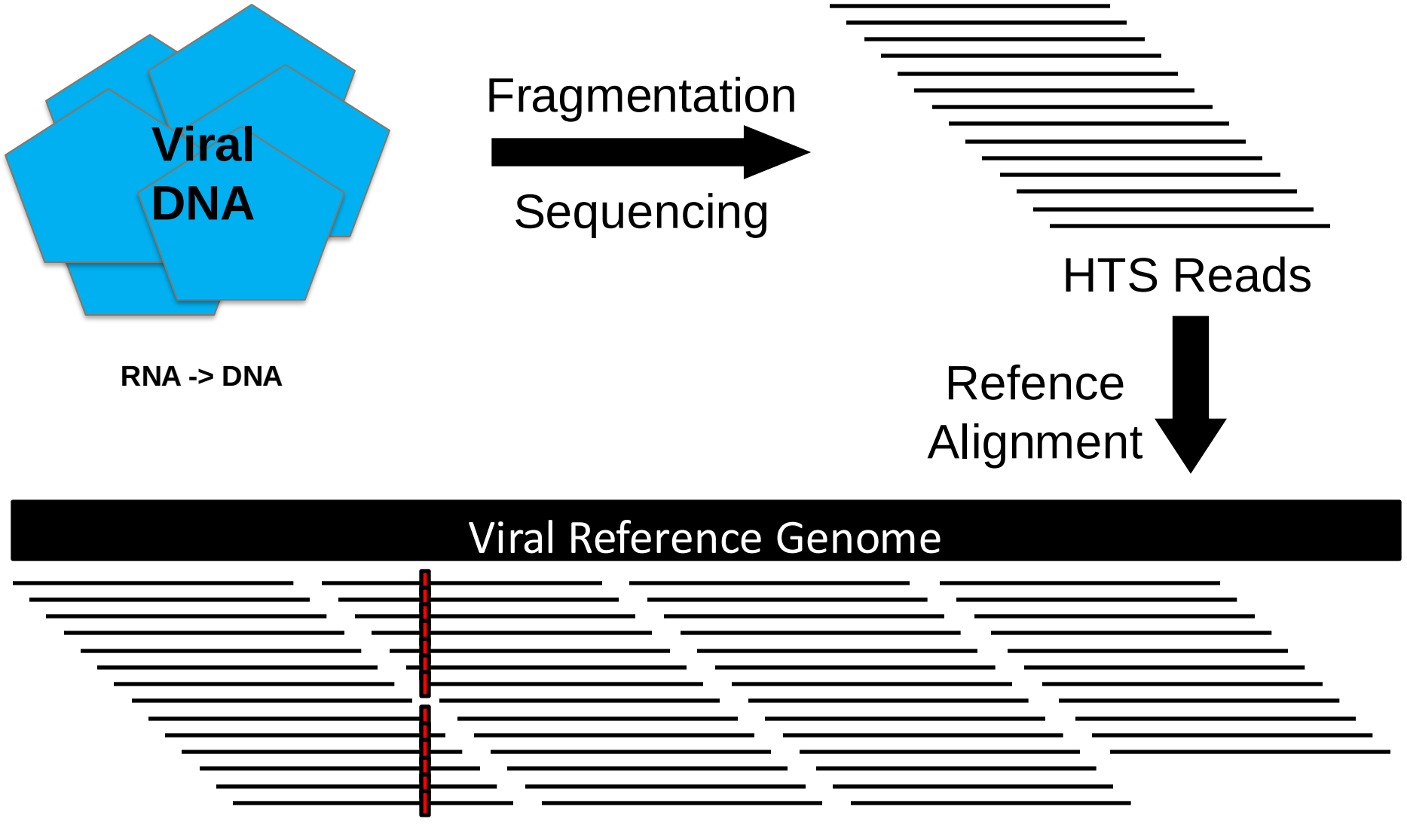
- **What is reference alignment?**
  - How does it work?
- **What tools can you use?**
- **What do the results look like?**
  - Basic statistics
  - Coverage plots
- **Reference alignment practical**
  - Learn the basic steps of refence alignment, SAM/BAM conversion, calculating basic mapping statistics and coverage plots.

# Reference alignment

- **Reference alignment:** want to know the **exact position** on the genome a read originates
  - And the **base-to-base** correspondence (to extract mutations, indels)

- **Reference assembly:** assemble reads back together to form a genome
  - Assemble from scratch – *de novo* assembly – using read overlaps, kmers

# Aligning reads to a reference genome



# Aligning reads to a reference - needs

- **Need Reads**
  - Single or paired, short or long
  - Typically pre-trimmed & filtered
  - But you can use your raw read files

- **Need a Reference**
  - A suitable reference
  - [More on this later]

- Trimmed reads were aligned to the HCV reference genome (GenBank accession NC_038882) with BWA {Li et, 2009}.

# Be careful – aligners tend not to complain

- **Sample**
  - Ebola virus sample from a human patient
- **Reads**
  - Reads were adapter trimmed and quality filtered using trim_galore (quality 25, length 50).
- **Reference**
  - Reads were aligned to the HCV reference genome (GenBank accession NC_038882
- **Result – SAM file of all the reads aligned to the reference**

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- **Sample**
  - Ebola virus sample from a human patient
- **Reads**
  - Reads were adapter trimmed and quality filtered using trim_galore (quality 25, length 50).
- **Reference**
  - Reads were aligned to the HCV reference genome (GenBank accession NC_038882
- **Result – SAM file of all the reads aligned to the reference**
  - **No errors**
  - **Number of mapped reads (0), coverage statistics (0 cov)**

# Unmapped reads

- Reads that could not be aligned to the reference sequence are marked as unmapped
- What are these reads?

# Unmapped reads

- Reads that could not be aligned to the reference sequence are marked as unmapped

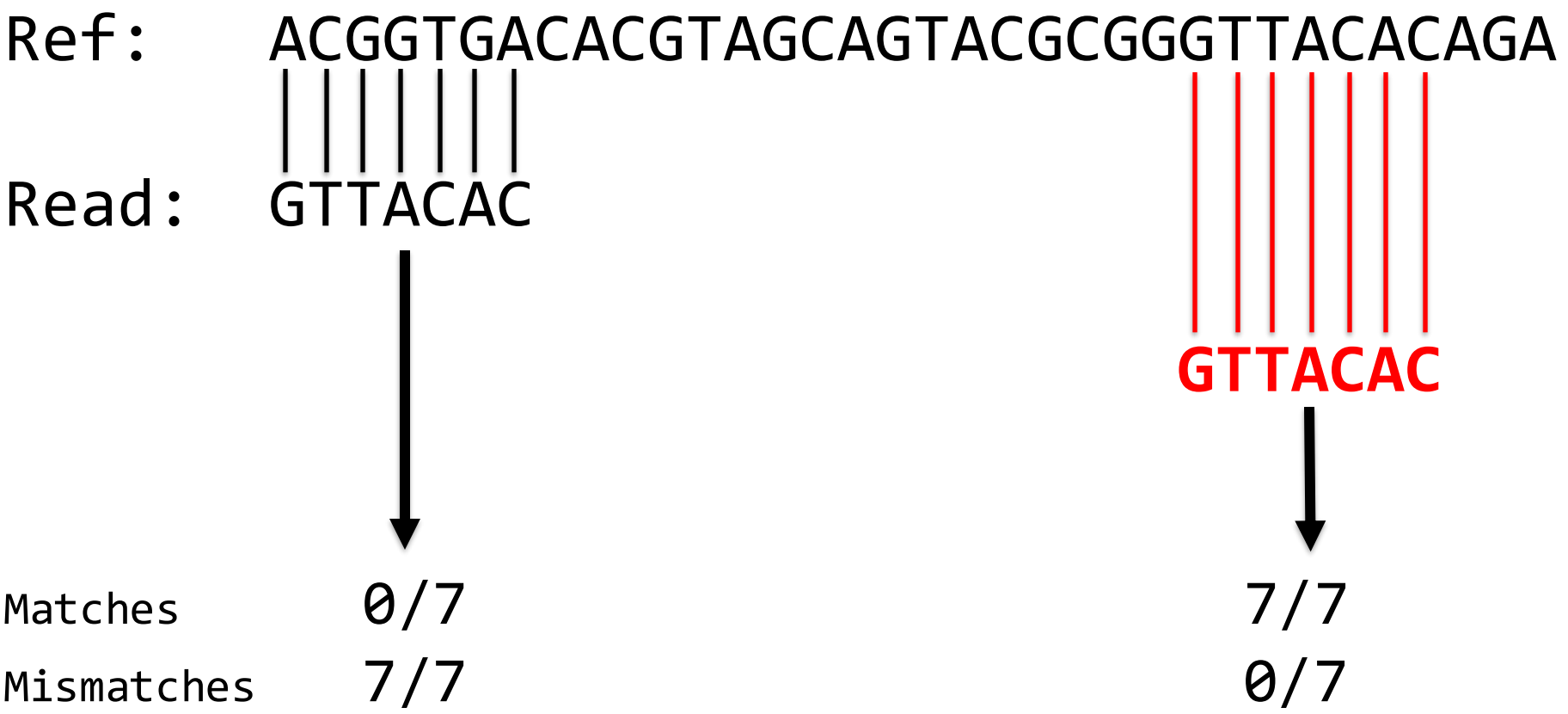
- What are these reads?
  - Host
  - Bacteria, Parasites,
  - Other viruses
  - Random ”low complexity” sequences

- This will be missed as we are “targeting” a specific reference sequence to align against
  - Possible solution: metagenomics

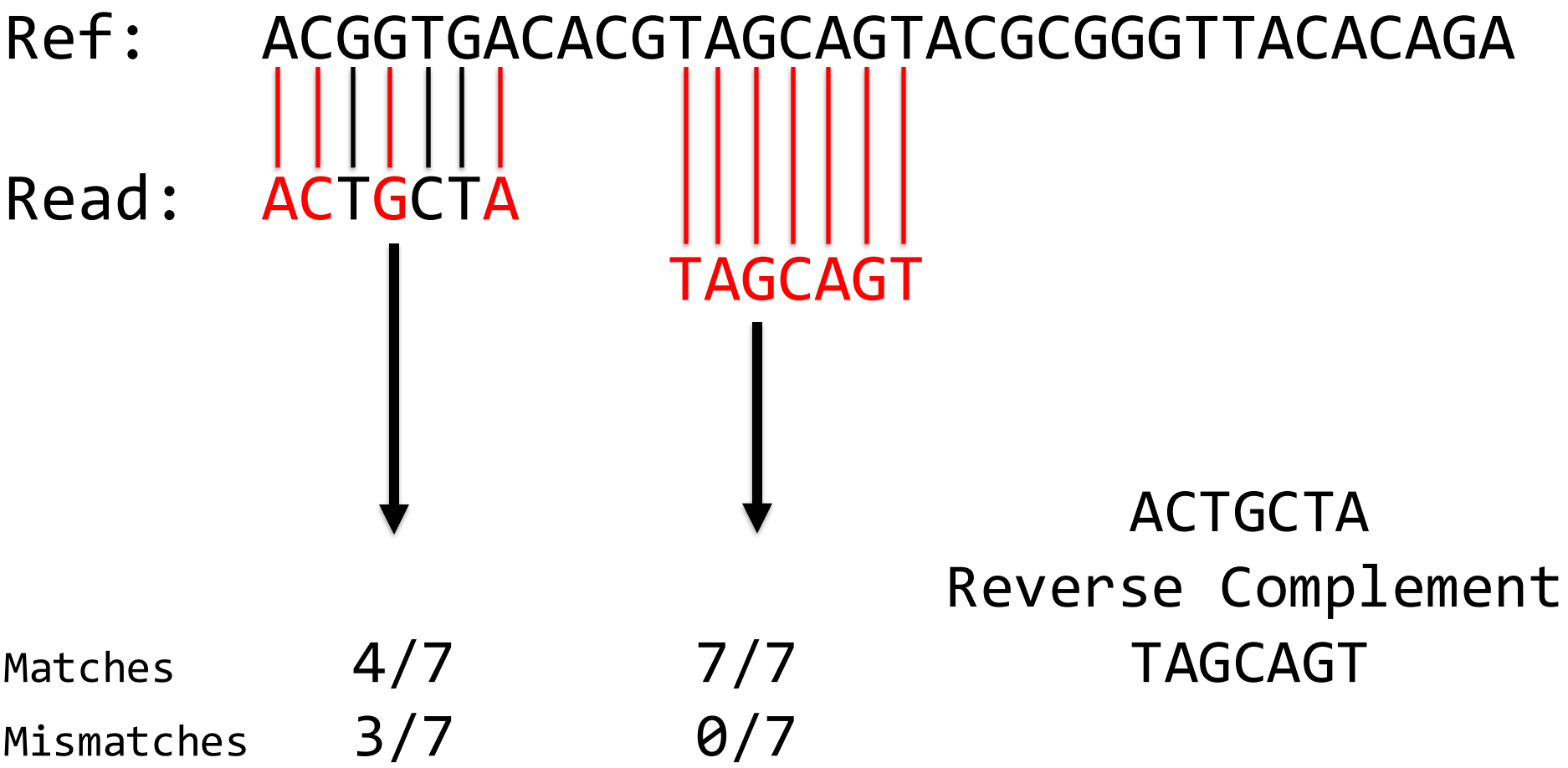
# Aligning reads to a references

```
Ref:    ACGGTGACACGTAGCAGTACGCGGGTTACACAGA

                                Read:  GTTACAC
```

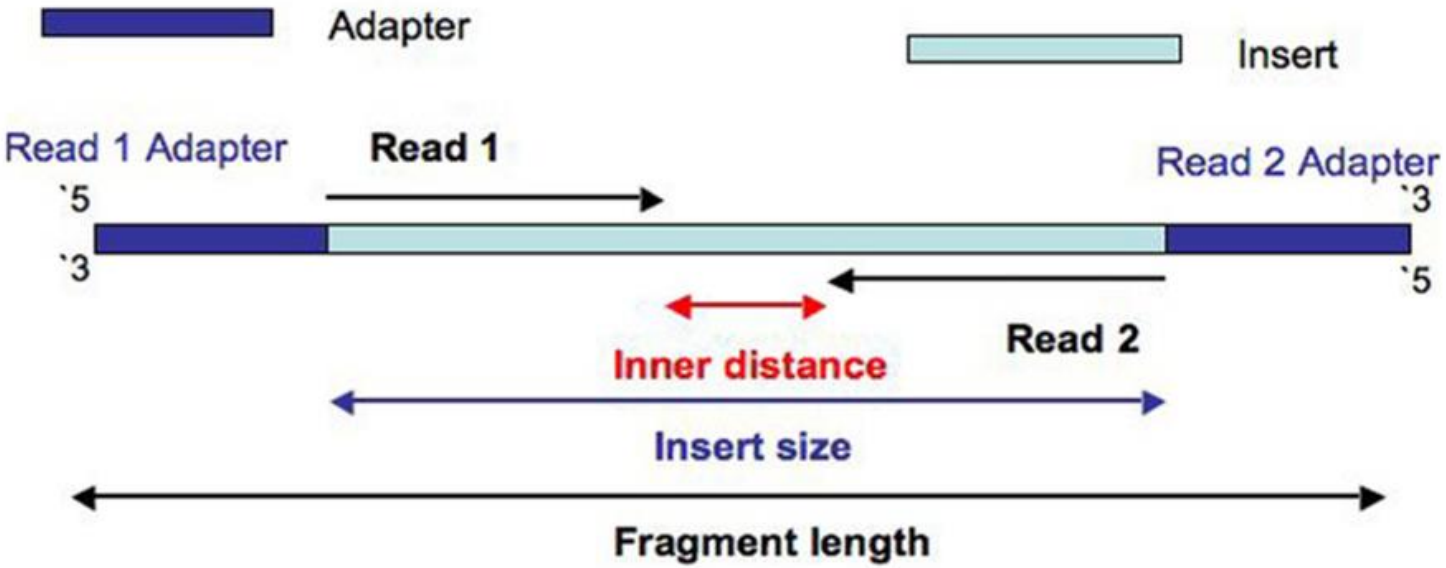
# Aligning reads to a references



# Aligners check the reverse complement

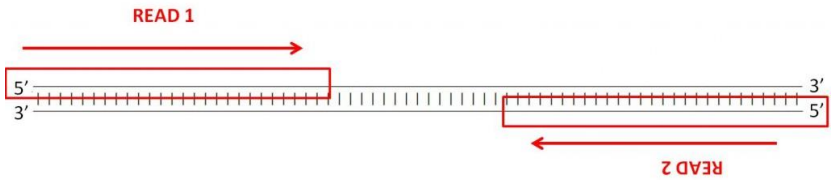


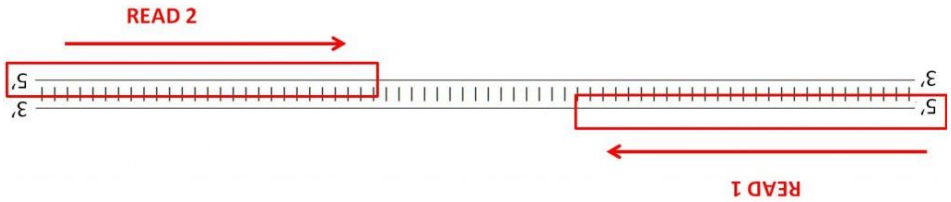
# Paired end … Insert Size



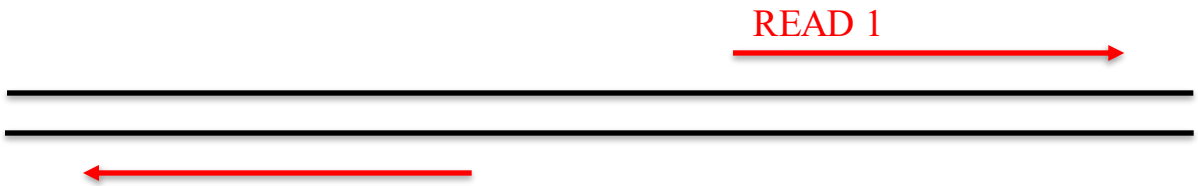

- Typically, the two reads do not overlap, but they can if the reads are long and fragments are short [redundant data, but can be used to correct errors]
- 500bp fragments + 2 x 300bp reads = 100bp overlap
- Turner 2014, Frontiers in Genetics

# Concordance & Discordance – paired reads



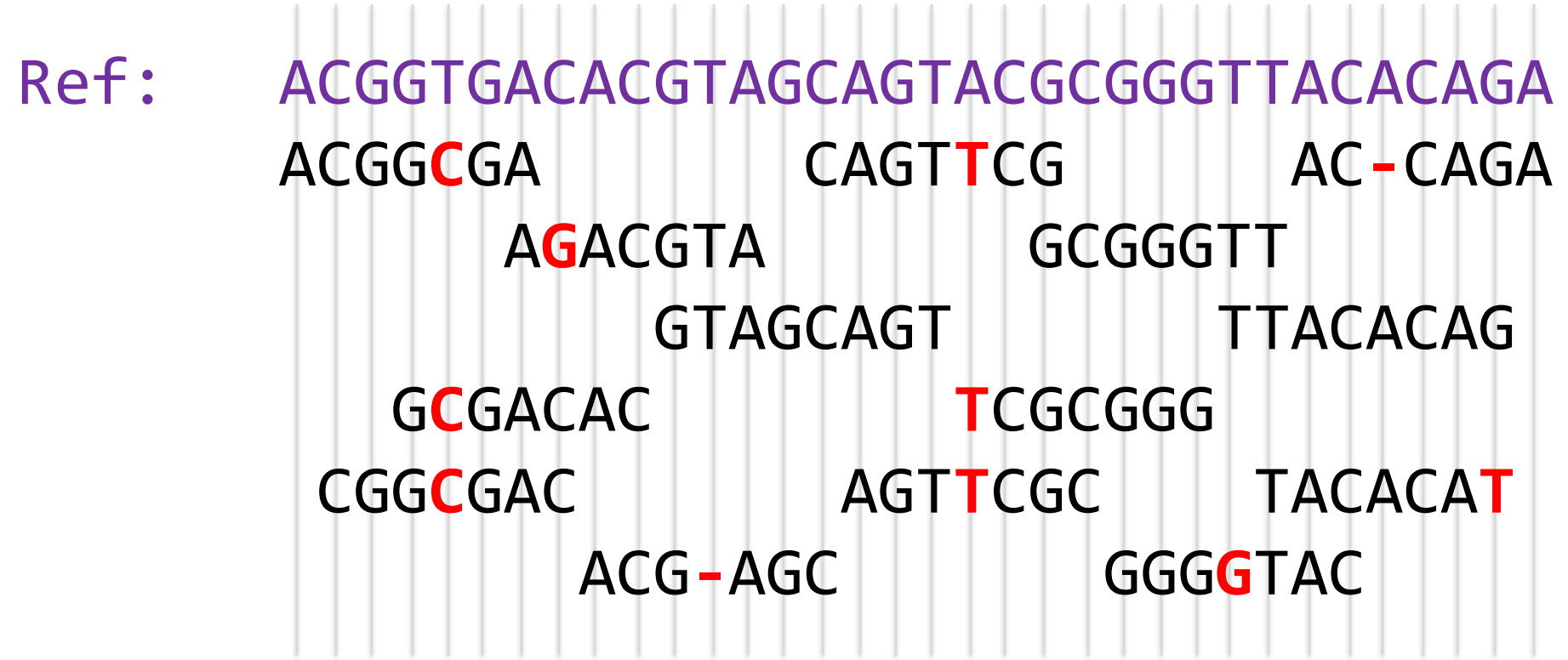




**Concordant:** consistent orientation of read pairs with respect to reference, have insert size within the expected range (depends on library)

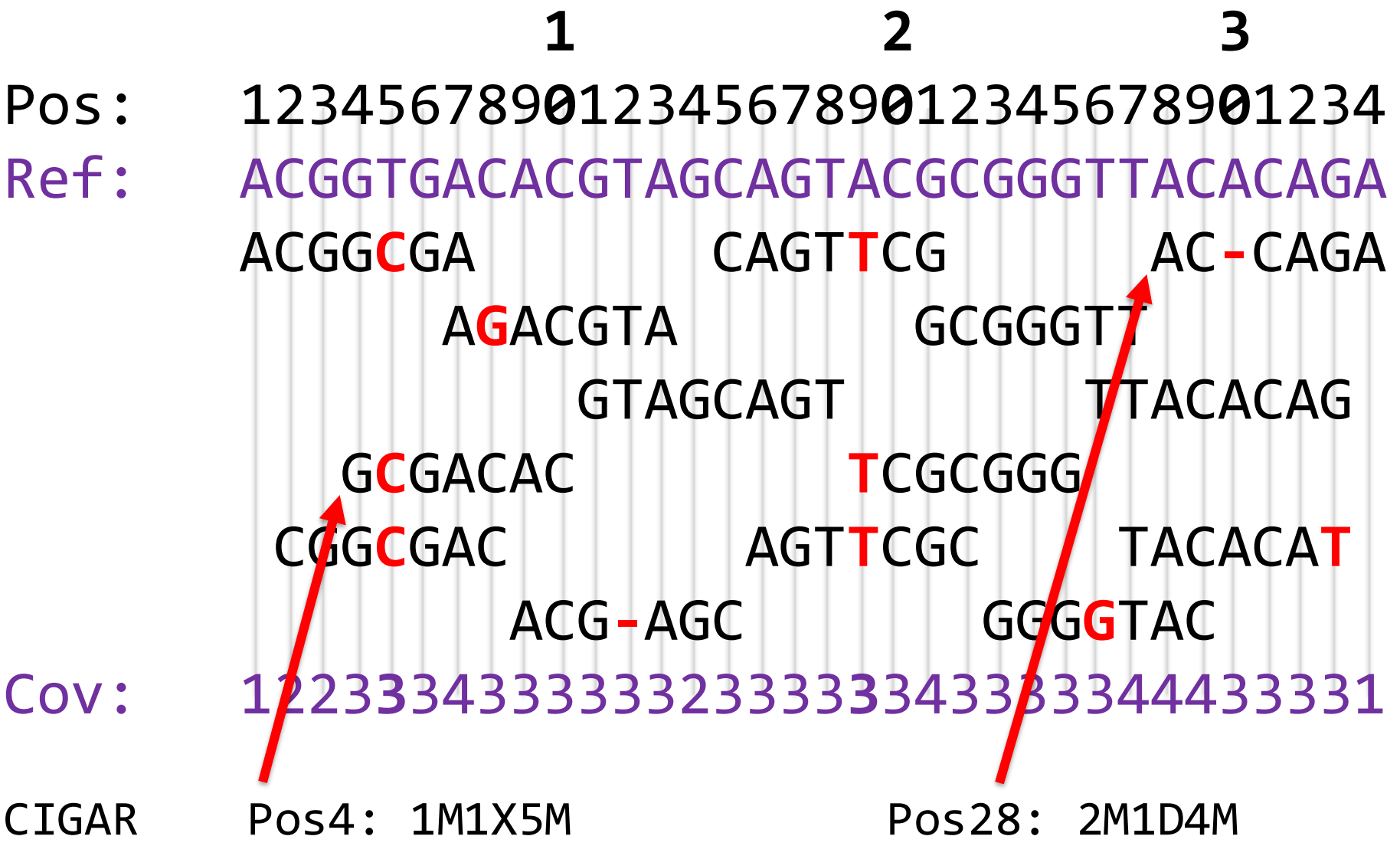



**Discordant:** inconsistent orientation (mixed up reads? reference issues? Abnormal insert size)

# Aligning reads to a reference: Mutations and Indels

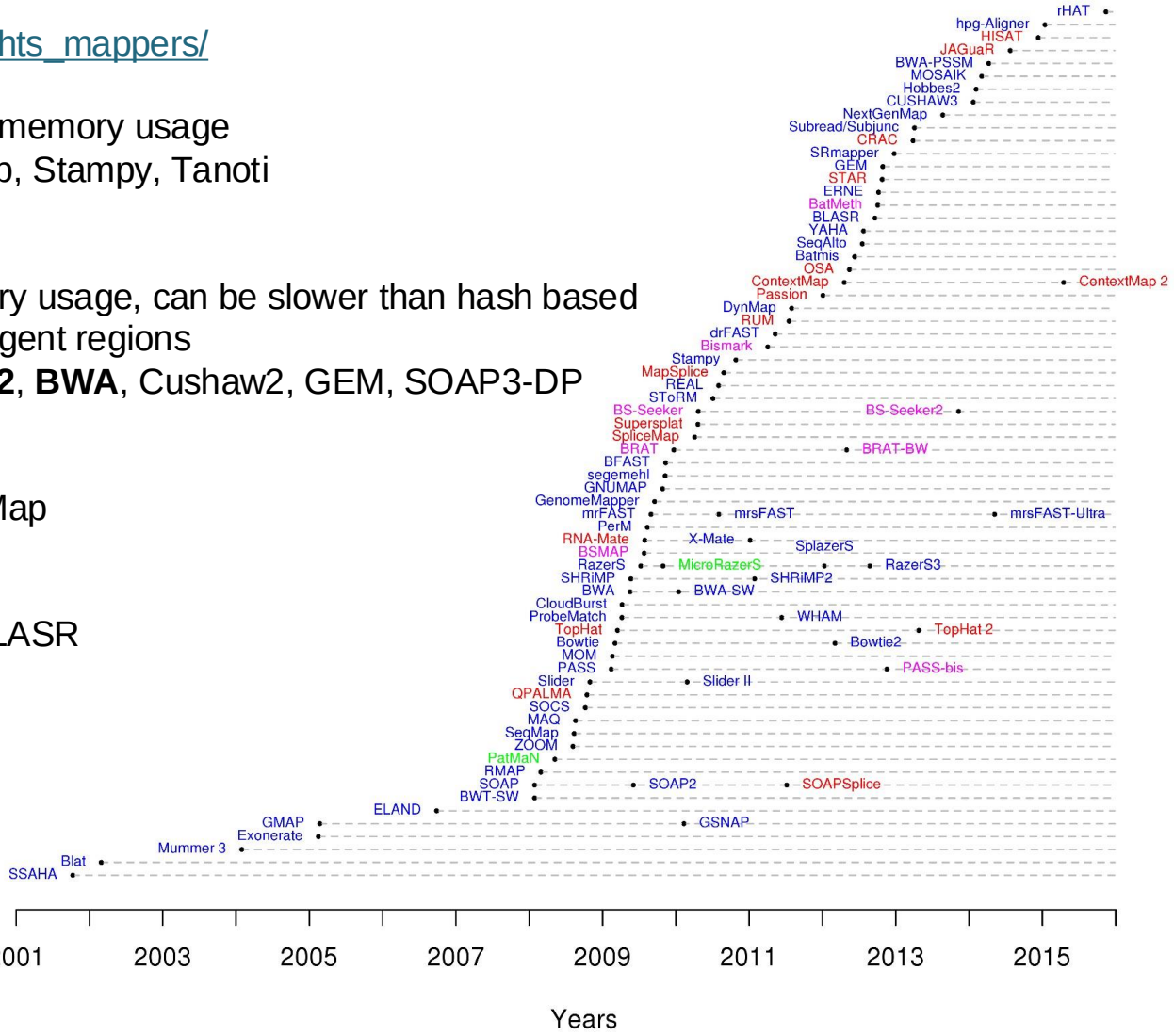


# CIGAR
## Concise Idiosyncratic Gapped Alignment Report



# Aligners – There are Lots

- https://www.ebi.ac.uk/~nf/hts_mappers/
- Hash based - faster, high memory usage
  - Mosaik, NextGenMap, Stampy, Tanoti
- Burrows-Wheeler based
  - Sensitive, low memory usage, can be slower than hash based
  - Can struggle in divergent regions
  - BarraCUDA, **Bowtie2**, **BWA**, Cushaw2, GEM, SOAP3-DP
- RNA-Seq Splice aware
  - HiSAT, TopHat, BBMap
- Long Reads
  - **Minimap2**, LAST, BLASR

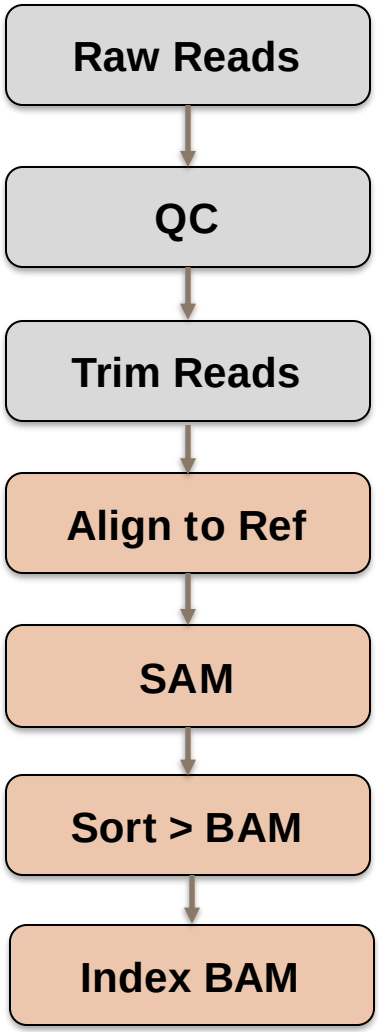


# Which aligner to use?

- Bowtie2
- BWA
- Tanoti
- BBMAP
- minimap2
- Mosaik
- …

- Sequencing technology – long vs short reads
- Library/Analysis – e.g. rna-seq
- **Short RNA viral genome - which aligner?**
  - In general aligners are quite consistent in terms of consensus sequence & coverage to a good (close) reference
  - Differences in aligner can be subtle – so may influence **low frequency** variants
- Starting out on a new virus - try a few aligners – not just about most reads aligned – consensus seq and variants

# Ref alignment commands - different tools




```
bwa index ref.fasta

bwa mem ref.fasta R1_qc.fq R2_qc.fq > my.sam
```

```
bowtie2-build ref.fasta ref.fasta

bowtie2 --local -x ref.fasta -1 R1_qc.fq -2 R2_qc.fq -S
my.sam
```

```
tanoti -r ref.fasta -i R1_qc.fq R2_qc.fq -o my.sam -p 1
```

```
samtools sort my.sam –o my.bam


samtools index my.bam
```

# Which reference sequence?

- **Selecting a suitable reference sequence is an important step.**
  - If your reference is too divergent from your sample if can affect read mapping and possibly the consensus sequence

- **First – obviously want to select the right virus!**
  - If you doing a reference assembly – you probably suspect a particular virus is present in your sample

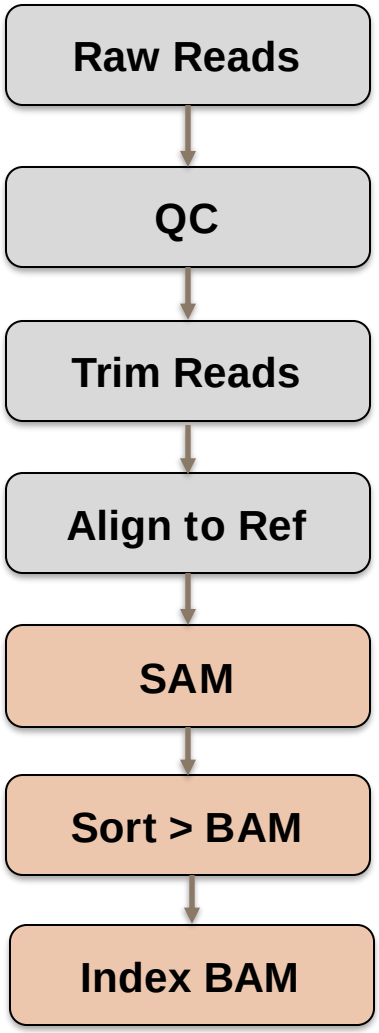
- Second – if a divergent virus e.g. HCV – select the right genotype:
  - Hepatitis C Virus (HCV) - want to select the right genotype – differ by 30–35% at the nucleotide level (subtypes can differ by 15-25% at nucleotide level)

- If unsure what virus is in the sample or suspect it is very divergent
  - **De novo assembly**
  - SHIVER (HIV)
  - Kraken
  - Panel alignment to all genotypes/subtypes – check stats

# Multiple Reference sequences

- The reference is in FASTA format: **Can be more than one sequence!**
  - Segmented virus
    - Influenza: PB1, PB2, PA, NP, HA, NA, M, NS
  - Host
    - Human chromosome 1, 2, 3, 4, 5 etc
  - Panel of viruses
    - HCV 1a, 1b, 1c, 2a, 2b
    - Respiratory viruses
  - Contigs from metagenomics

# SAM & BAM files




The result of the alignment step is typically a SAM file

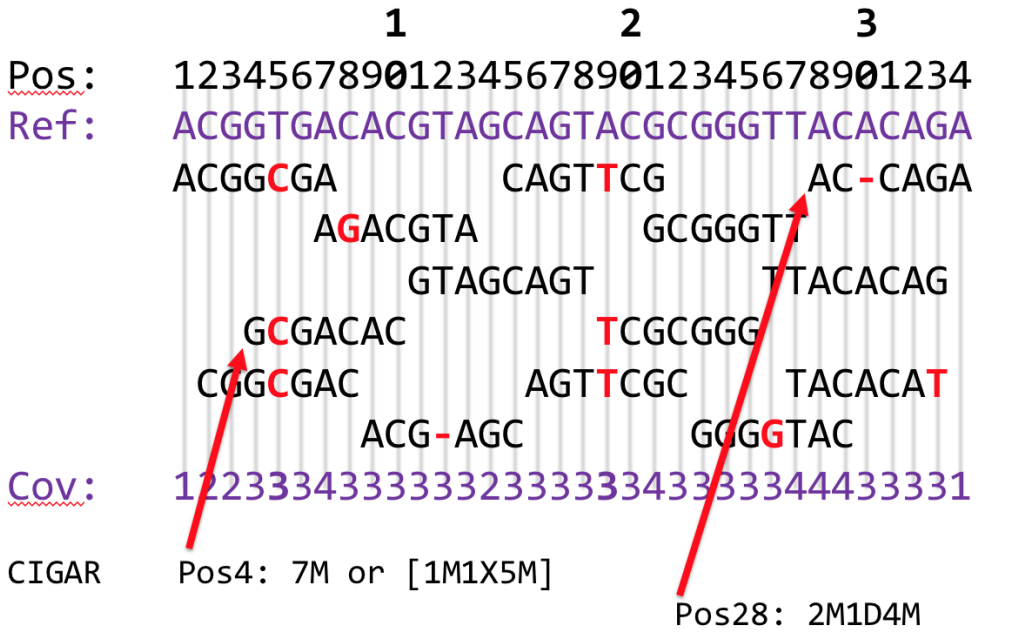
This is then sorted and converted to a BAM file, and indexed

```
samtools sort my.sam -o my.bam
```

```
samtools index my.bam
```

# SAM files: Sequence Alignment MAP

- Virtually all aligners output results in **SAM** format
  - **S**equence **A**lignment/**M**ap
- Each line in the SAM file corresponds to a separate alignment
- Sequence and quality strings of the reads stored in the BAM
  - Can extract reads back out of SAM/BAM
  - But always keeps copies of your raw data




The name of the other read in pair

The position the other read in pair is aligned

Template Length/Insert Size

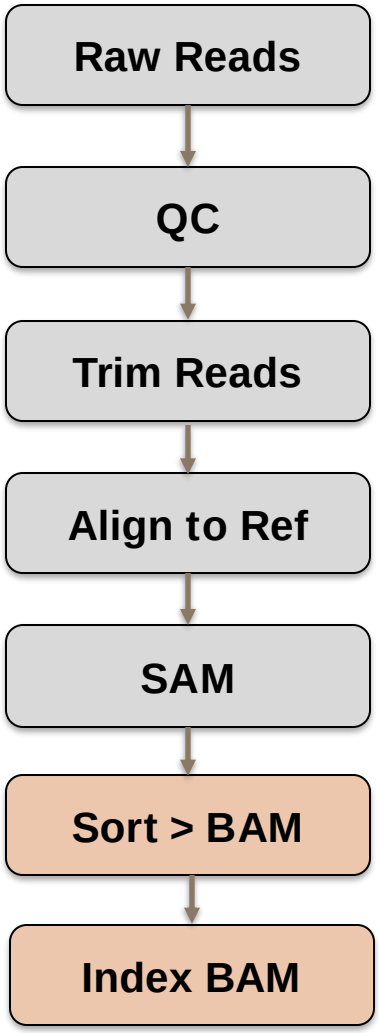
| QNAME | FLAG | RNAME | POS | MAPQ | CIGAR | RNEXT | PNEXT | TLEN | SEQ | QUALITY |
|---|---|---|---|---|---|---|---|---|---|---|
| Read3 | 10 | MyRefSeq | 28 | 52 | 2M1D4M | | | | ACCAGA | IHGFFF |
| Read8 | 10 | MyRefSeq | 4 | 57 | 1M1X5M | | | | GCGACAC | IIIHHGG |

# Samtools

- **One of the key HTS programs** - provides various utilities for manipulating alignments in the SAM/BAM [and CRAM] formats
  - sorting, merging, indexing and generating alignments in a per-position format.

- Links seamlessly to downstream tools such as VCFTools, BCFTools etc

# Converting SAMs to BAMs




- Convert your **SAM** file into **BAM** files
- **BAM B**inary **A**lignment/**M**ap
- BAM files are **compressed** binary versions of the same data (and **faster**)

- Initially the SAM/BAM is sorted by the order the reads were in their files

- **Sort** BAM file
  - All the reads where alignment starts at position 1 first
  - All the reads where alignment starts at position 2, then 3, then 4 etc

  - `samtools sort my.sam -o my.bam`

- **Index** the BAM file
  - Enables downstream tools (consensus/variant calling) to rapidly look up what is aligned to e.g. position 10,456

  - `samtools index my.bam`

# SAM Flags – Mapped/Unapped

- **4 =** Read unmapped
- Can be used to give you the most basic of statistics – how many reads are mapped to the reference and how many are unmapped
- Technically, it is counting how many mapped read alignments are in the SAM file

| # | Flag | Description |
|---|---|---|
| 1 | **1** | Read paired |
| 2 | **2** | Read mapped in proper pair |
| **3** | **4** | **Read unmapped** |
| 4 | **8** | Mate unmapped |
| 5 | **16** | Read reverse strand |
| 6 | **32** | Mate reverse strand |
| 7 | **64** | First in pair |
| 8 | **128** | Second in pair |
| 9 | **256** | Not primary alignment |
| 10 | **512** | Read fails platform/vendor quality checks |
| 11 | **1024** | Read is PCR or optical duplicate |
| 12 | **2048** | Supplementary alignment |

SAM Flag = 2nd filed of SAM file

| QNAME | FLAG | RNAME | POS | MAPQ | CIGAR | RNEXT | PNEXT | TLEN | SEQ | QUALITY |
|---|---|---|---|---|---|---|---|---|---|---|
| Read3 | 10 | MyRefSeq | 28 | 52 | 2M1D4M | | | | ACCAGA | IHGFFF |
| Read8 | 4 | * | 0 | 0 | | | | | GCGACAC | IIIHHGG |

# SAM Flags – Mapped/Unapped

- A read can sometimes have multiple alignments
- **256** = not primary = secondary = alternative alignments (equally good or not quite as good)
- **2048** = supplementary alignment = when read is split (spliced) and sections aligned separately

| # | Flag | Description |
|---|---|---|
| 1 | **1** | Read paired |
| 2 | **2** | Read mapped in proper pair |
| **3** | **4** | **Read unmapped** |
| 4 | **8** | Mate unmapped |
| 5 | **16** | Read reverse strand |
| 6 | **32** | Mate reverse strand |
| 7 | **64** | First in pair |
| 8 | **128** | Second in pair |
| 9 | **256** | Not primary alignment |
| 10 | **512** | Read fails platform/vendor quality checks |
| 11 | **1024** | Read is PCR or optical duplicate |
| 12 | **2048** | Supplementary alignment |

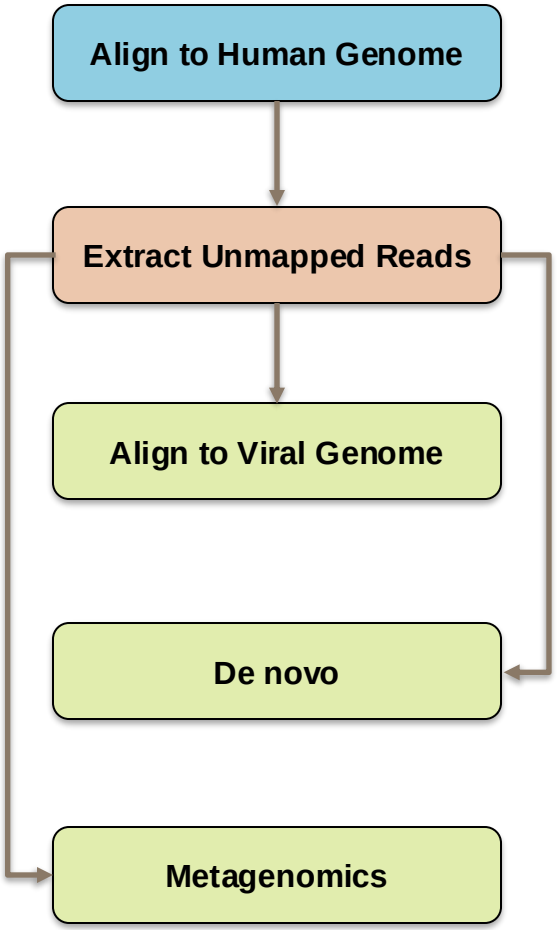
Typically, secondary/supplementary should be few for short RNA virus genome, but if louts it can indicate:

Repeat regions, Large deletions, Poor reference sequence

SAM Flag = 2nd filed of SAM file

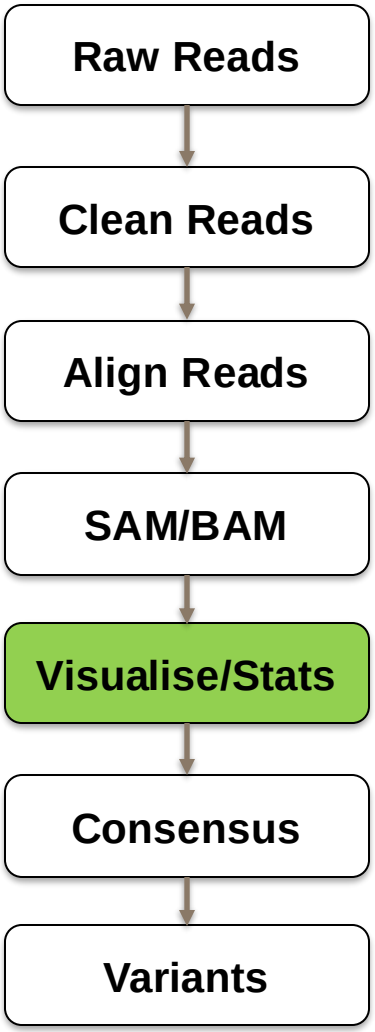
| QNAME | FLAG | RNAME | POS | MAPQ | CIGAR | RNEXT | PNEXT | TLEN | SEQ | QUALITY |
|---|---|---|---|---|---|---|---|---|---|---|
| Read3 | 10 | MyRefSeq | 28 | 52 | 2M1D4M | | | | ACCAGA | IHGFFF |
| Read8 | 4 | * | 0 | 0 | | | | | GCGACAC | IIIHHGG |

# Host filtering – exploiting flag4 (unmapped)



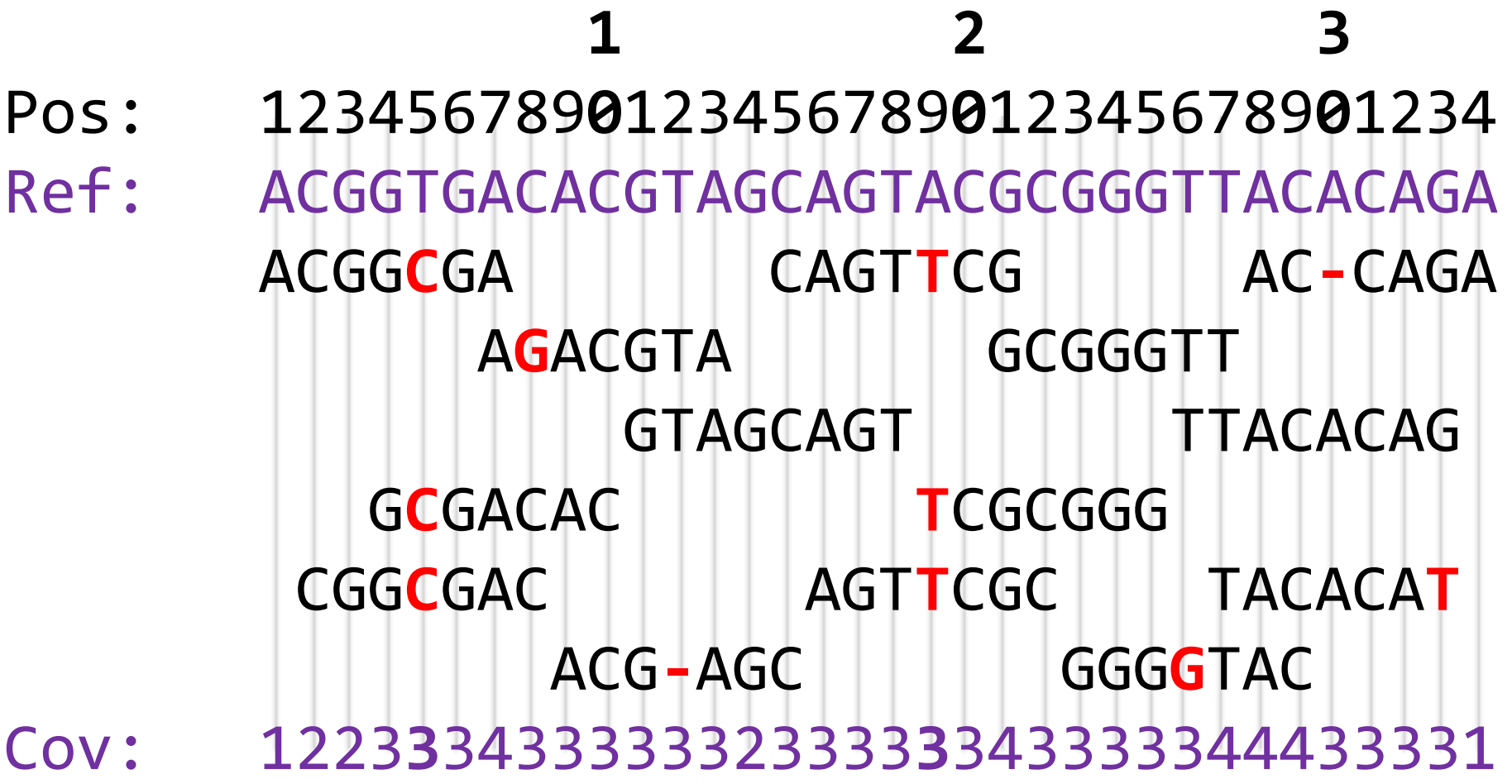

- Create read files without the human host
- samtools has a host of other function available:
  - samtools fastq
  - **samtools depth**
  - samtools stats
  - samtools ampliconclip
  - **samtools idxstats**
  - **samtools flagstat**
  - samtools consensus

# Post Assembly – after the BAM



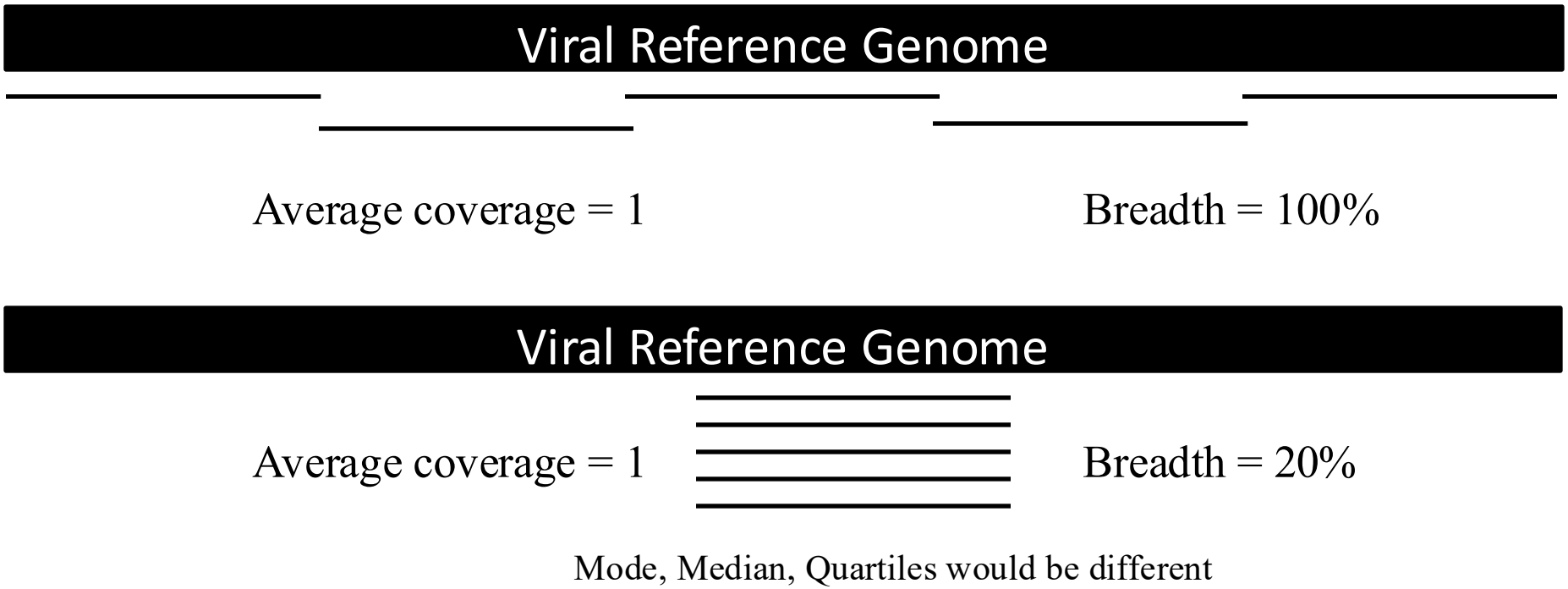

- Post assembly checks
  - Summary statistics:
    - Number of reads aligned
    - Number of reads unaligned
    - **Average depth of coverage**
    - **Breadth of coverage**
  - **Coverage plot**
  - **Visualisation of entire alignment**

# Coverage


1 2 3
Pos: 123456789012345678901234567890 1234
Ref: ACGGTGACACGTAGCAGTACGCGGGTTACACAGA
ACGGCGA
CAGTTCG
AC-CAGA
AGACGTA
GCGGGTT
GTAGCAGT
TTACACAG
GCGACAC
TCGCGGG
CGGCGAC
AGTTCGC
TACACAT
ACG-AGC
GGGGTAC
Cov: 122333433333323333334333333444333331

# Coverage Depth & Breadth

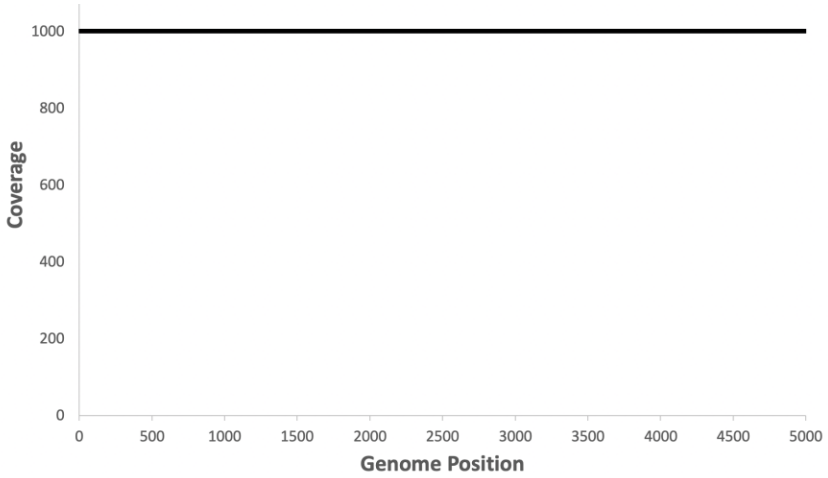
- **Coverage** is the number of reads that "cover" a particular genome coverage
  - **Depth**
- Average (mean) coverage: the average coverage across all genome positions
- Breadth of coverage: how much of the genome is actually covered



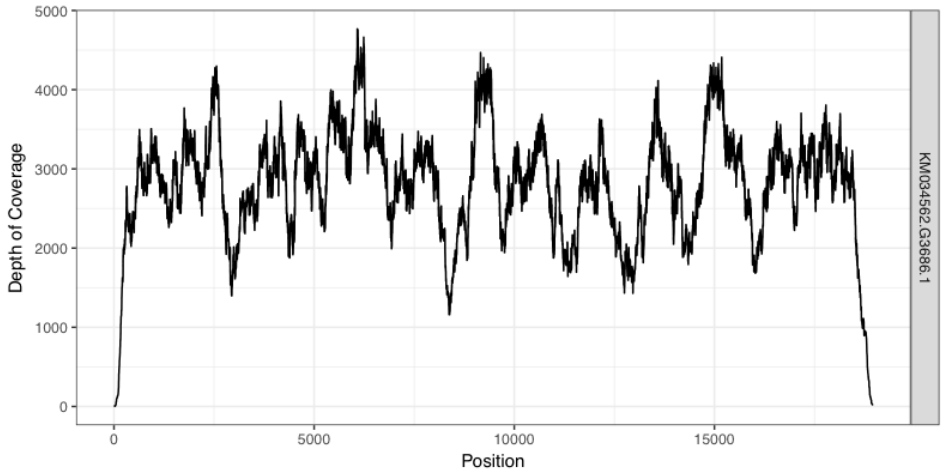
# Perfect Coverage Plots

- High uniform coverage across the entire genome
- Biases in library prep fragmentation and PCR (GC content)
  - the terminal ends are typically poorly covered
- Biases in bait capture, amplicon/primer efficiency, extraction methods

Don't think I've ever seen this

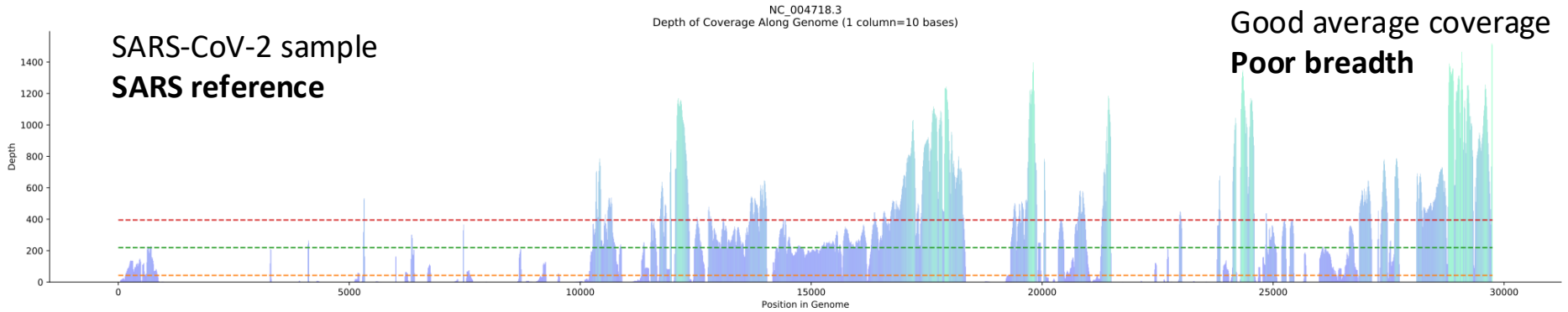



This is perfectly normal

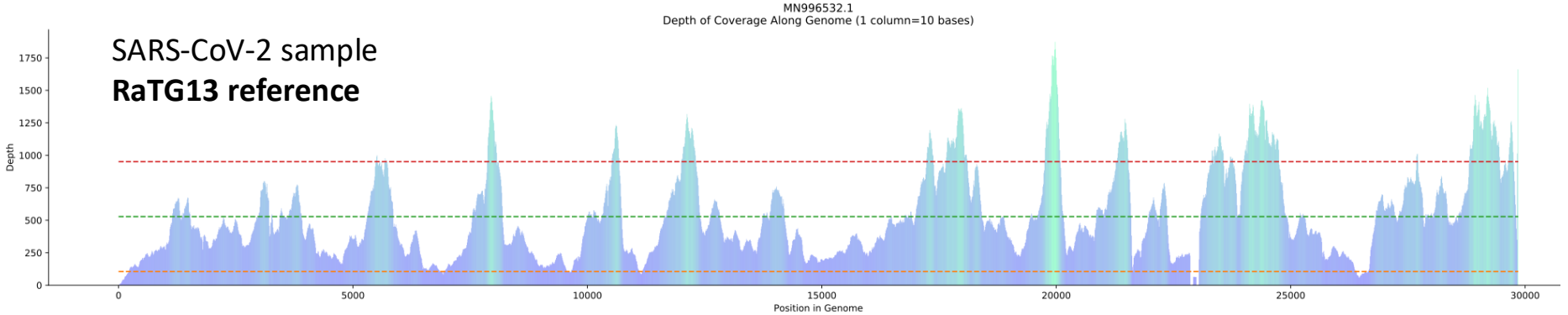


# Coverage plots – bad reference

- Sporadic coverage with frequent regions dropping down to zero can indicate a poor reference seq
- The reference is too divergent in many regions and reads can not be aligned at the nucleotide level



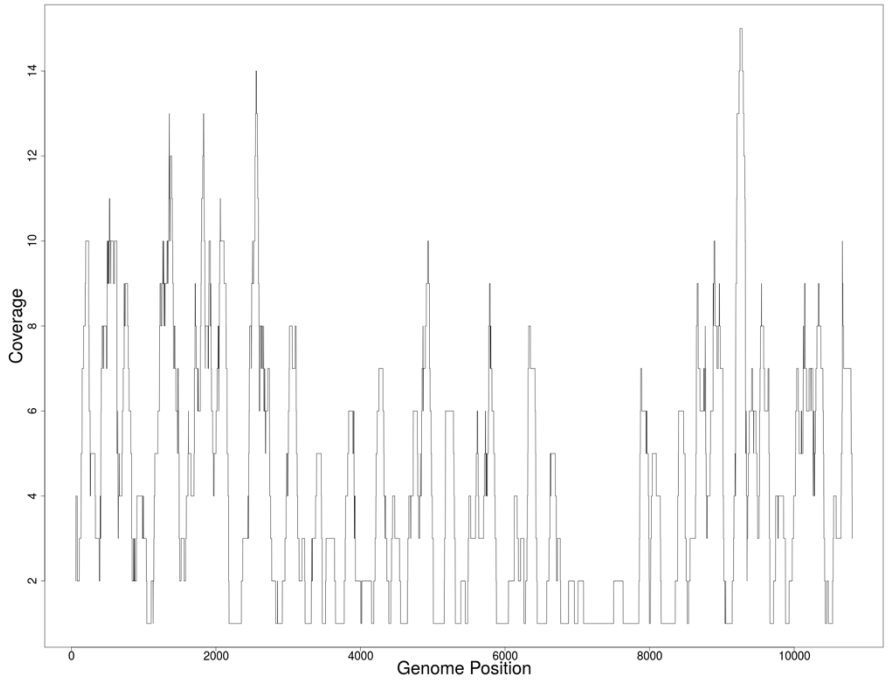

- Viruses can be very diverse – aligning to a different genotype/strain can give obscure results
- Align to different refs, genotype detection tools, **de novo assembly**



# Coverage plots – low coverage

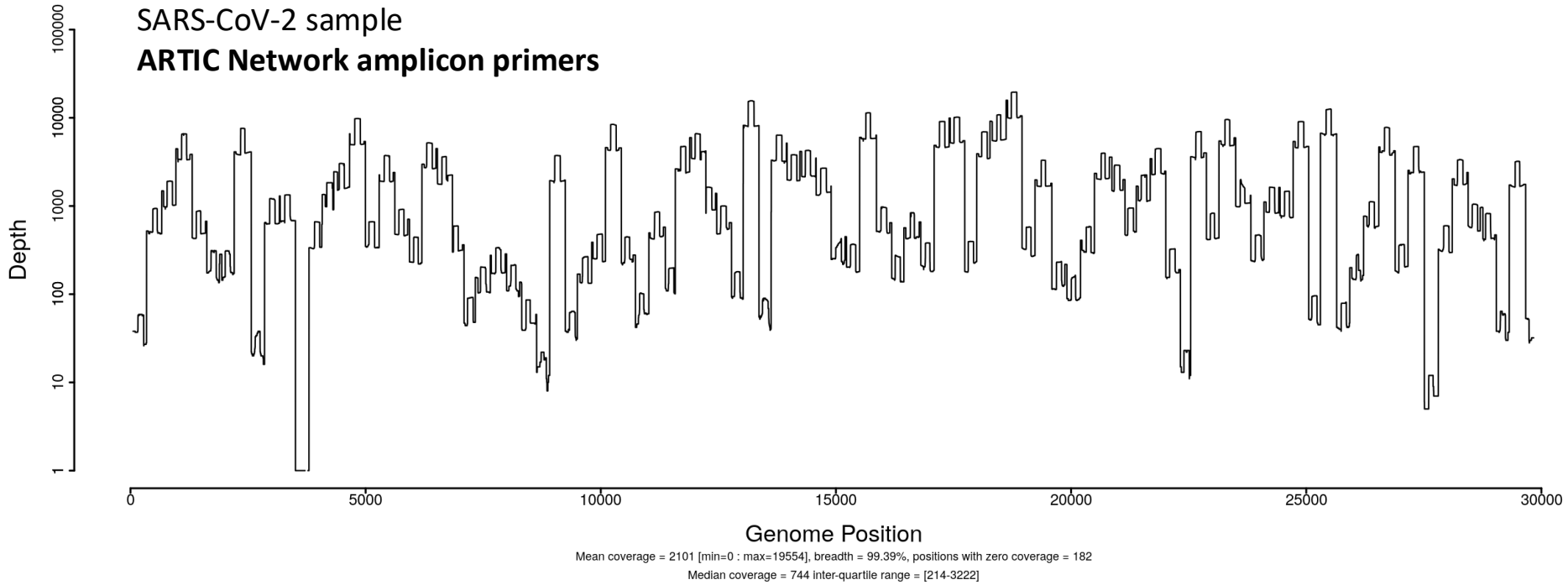
- Sometimes there is just not enough data present
  - Lower read trimming threshold
  - Just use the raw reads
    - Will be noisey
- Re-run the sample
  - Perhaps it was a bad run
  - Combine run data
- PCR amplification
- Bait capture

**Louping ill virus sample**



# Coverage plots – amplicons

- Amplicon data can give step like plots



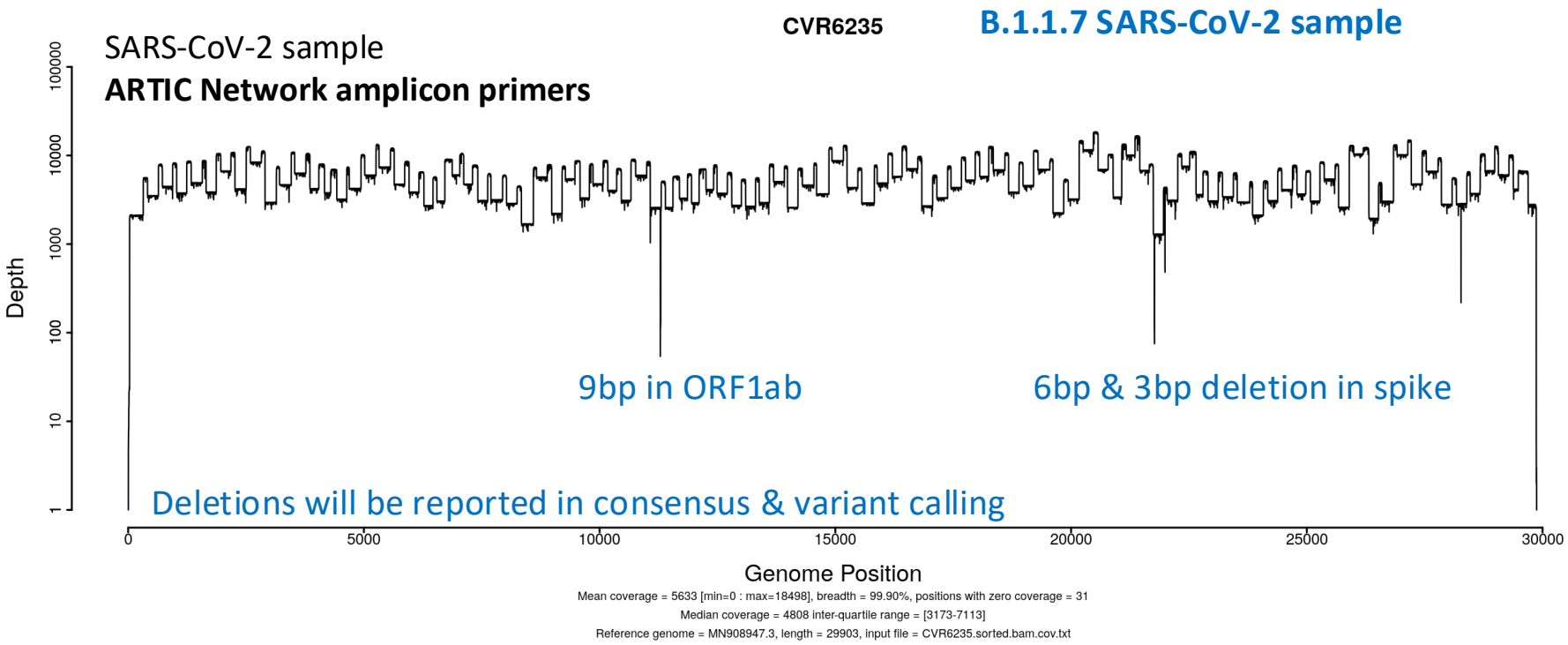

<- Illumina paired read2 250 bases

Illumina paired read1 250 bases->

400bp amplicon

# Coverage plots – deletions

- Sudden drops in coverage at a small number of sites can indicate deletions with respect to the reference

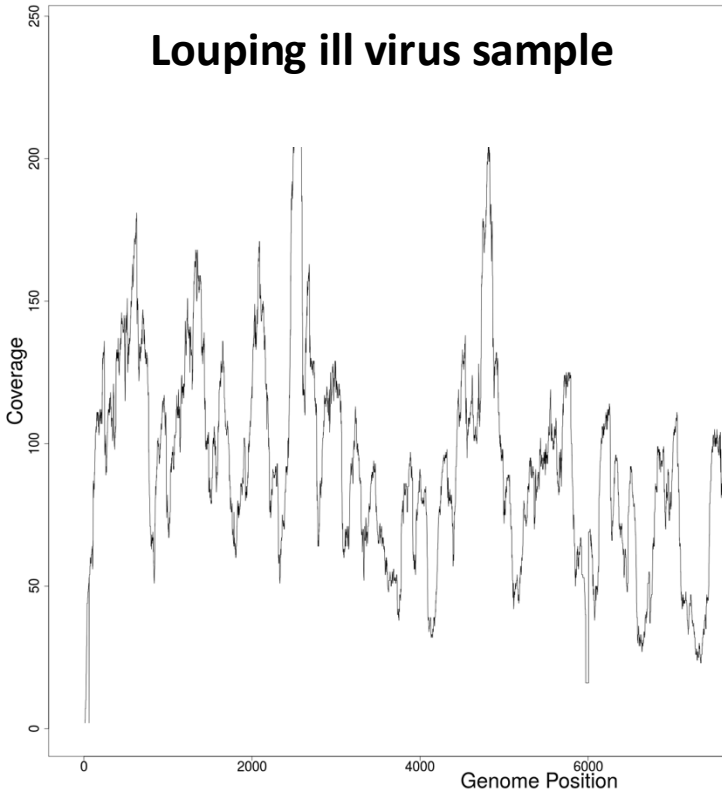


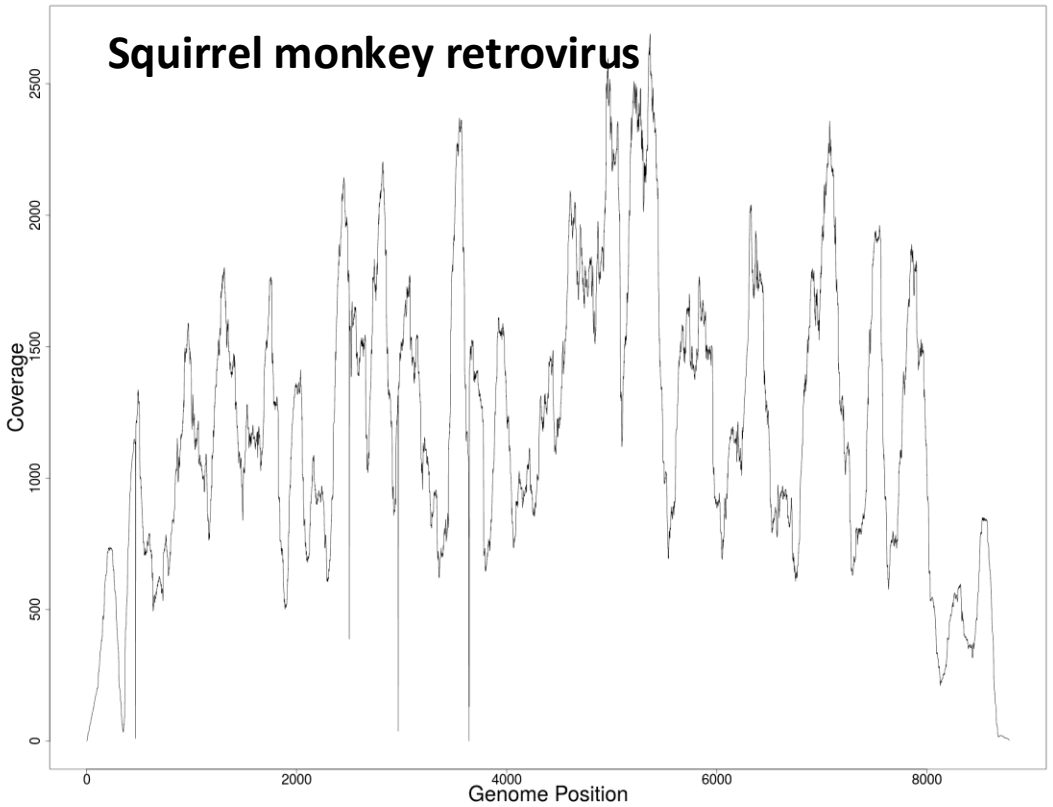

**This is a log plot and noisy minion data – the deleted sites still have some coverage but this is nothing compared to the other sites**

# Reference assembly – tunnel vision

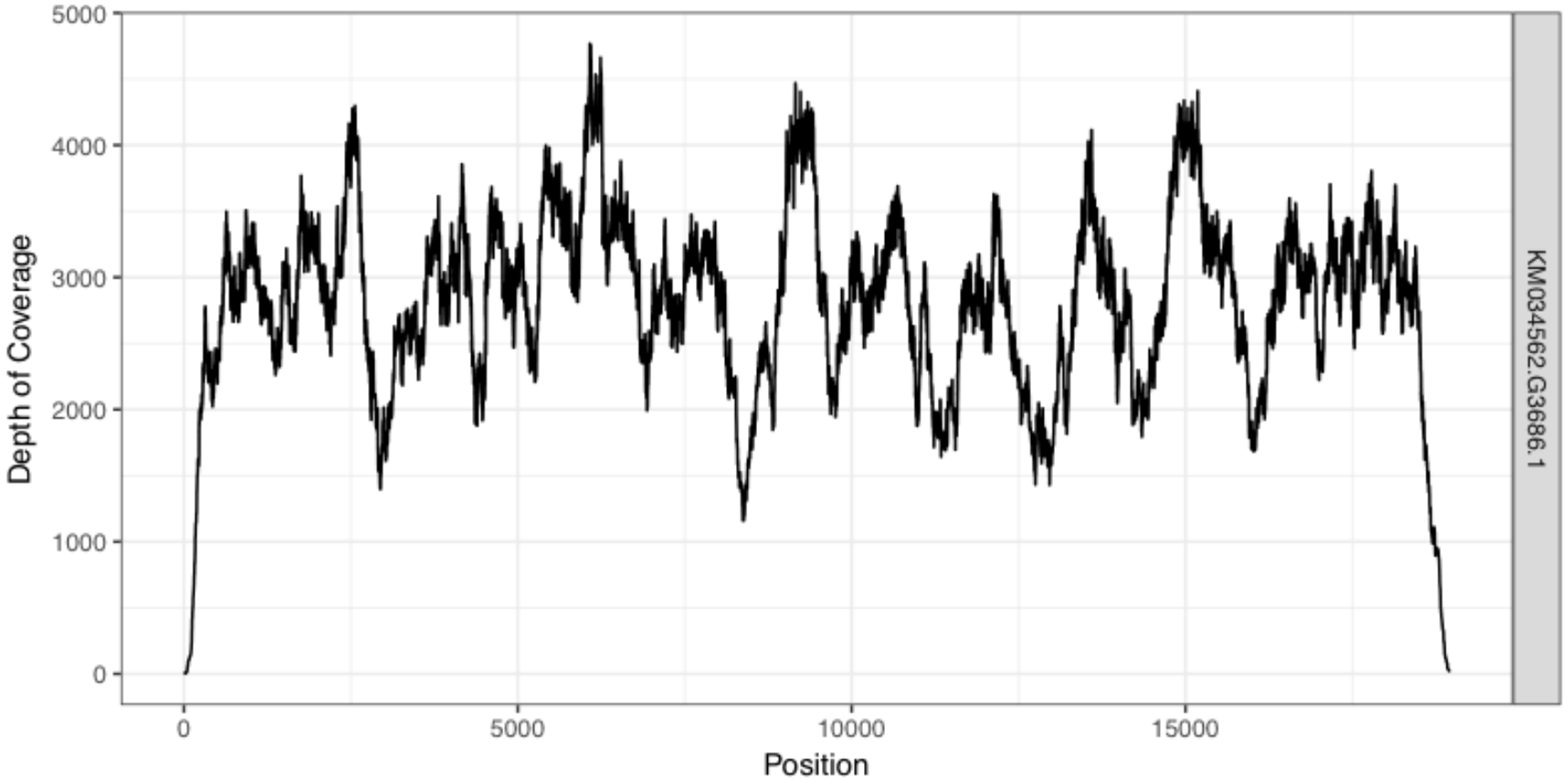
- With reference assembly you automatically focus on a single virus
  - You can align to multiple viruses in one go
  - But you will still need to decide what viruses to investigate

- **Good to run kraken/centriguge on your samples to (viral & mycoplasma contaminants)**



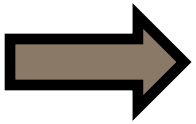
# How do you create coverage plots?

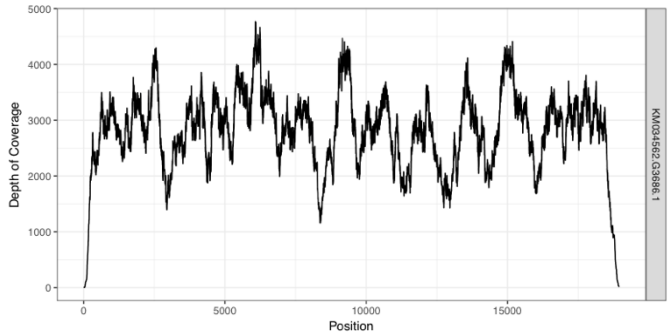


# samtools depth

- samtools has a built in function called ‘depth’
- `samtools depth -aa -d 0 my.bam > my_depth.txt`
- -aa: output data for absolutely all positions (even positions with zero coverage)
- -d 0: disable the maximum depth to report [default is 8000]
- 3 column text file:

| Chromosome | Position | Depth |
|---|---|---|
| MN908947.3 | 1 | 0 |
| MN908947.3 | 2 | 13 |
| MN908947.3 | 3 | 34 |
| … | … | … |



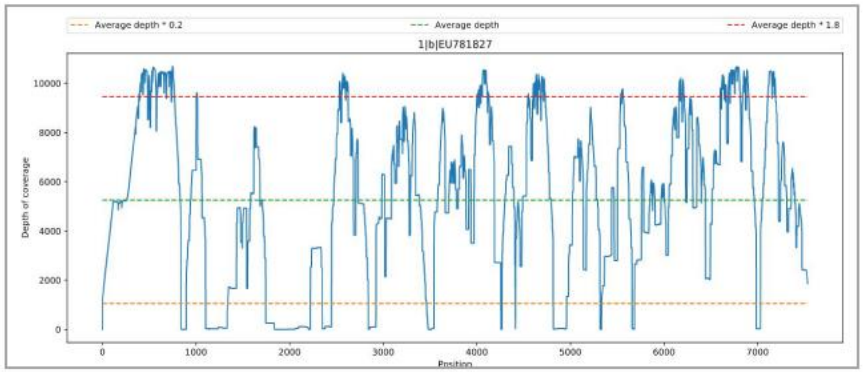



All chromosome will be reported in turn

# weeSam - https://github.com/centre-for-virus-research/weeSAM

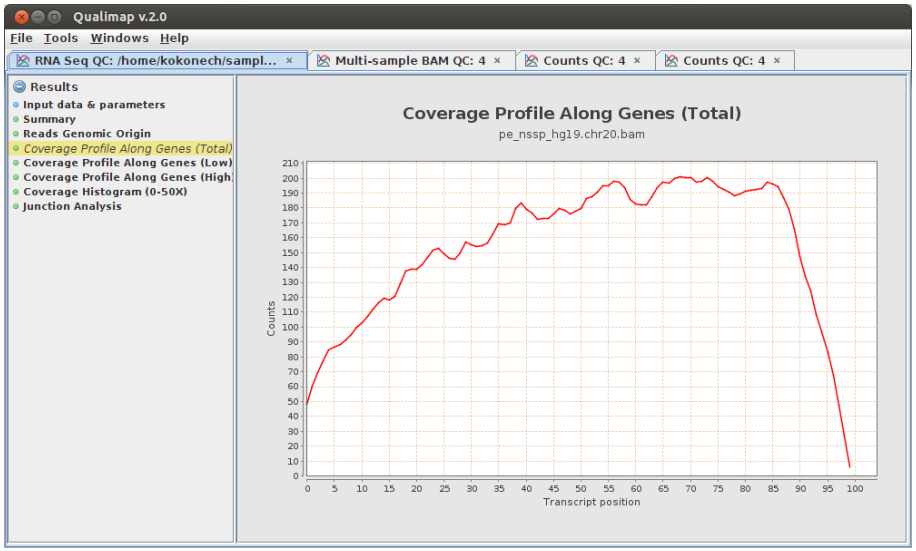
- weeSam is program that can give you information on breadth and depth of coverage as well as generate a coverage plot automatically
- `weeSAM --bam 1a.bam --html 1a`
- `1a_html_results/1a.html`

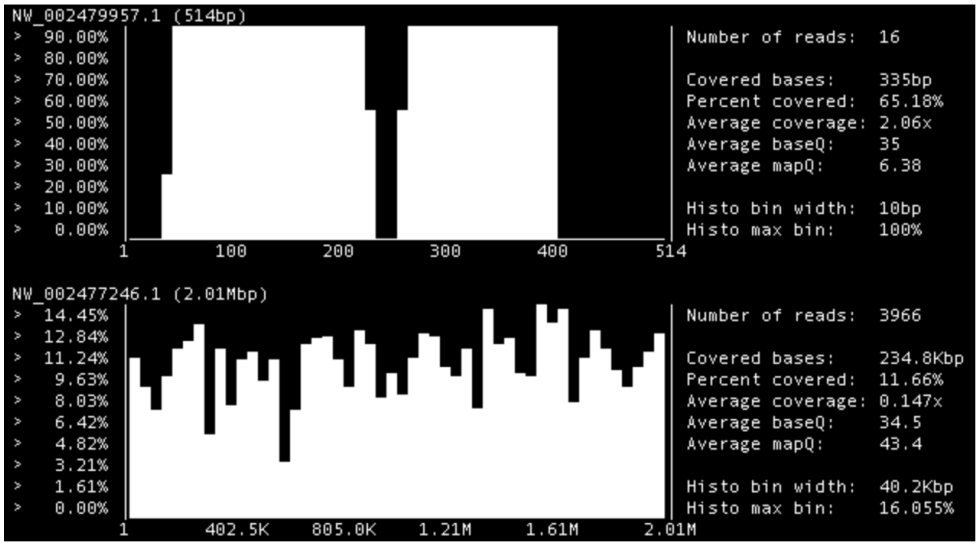
| Ref_Name | Ref_Len | Mapped_Reads | Breadth | %_Covered | Min_Depth | Max_Depth | Avg_Depth | Std_Dev | Above_0.2_Depth | Above_1_Depth | Above_1.8_Depth | Variation_Coefficient |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NC_004102.1 Hepatitis C virus genotype 1, complete genome | 9646 | 640000 | 9646 | 100.00 | 13 | 10729 | 9941.89 | 1699.34 | 98.82 | 90.91 | 0.00 | 0.17 |



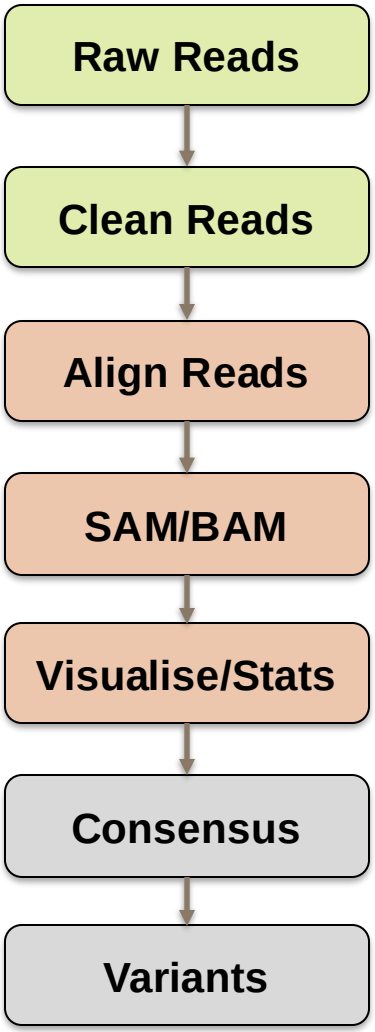
# Other tools

- Qualimap: http://qualimap.conesalab.org
- bamCov – https://github.com/fbreitwieser/bamcov



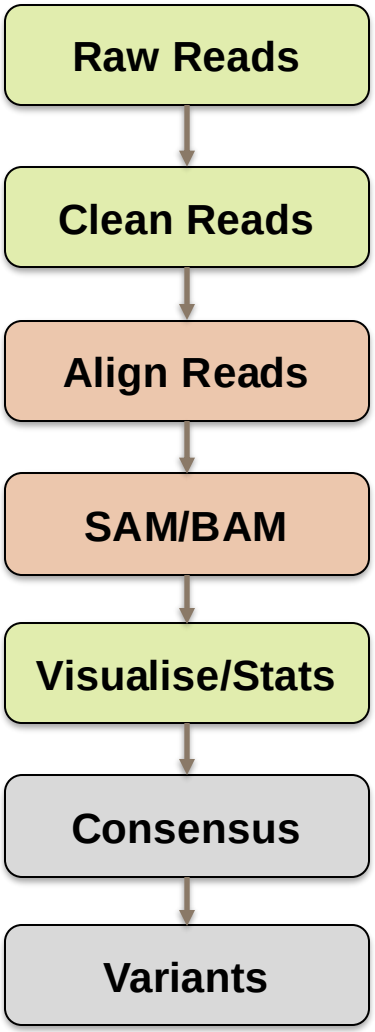



# Practical




- Hepatitis C virus (HCV) samples
- **Simulated Illumina paired end reads - R1 R2**
- **HCV Genotype 1a reference genome**
- First sample
  - High quality simulated data set
- 3 other samples
  - Real patient HCV samples (genotype 1a)
- BWA aligner, samtools, SAM/BAM, stats
- weeSAM covereage

# Practical – HCV_SIM commands – adapt for another sample



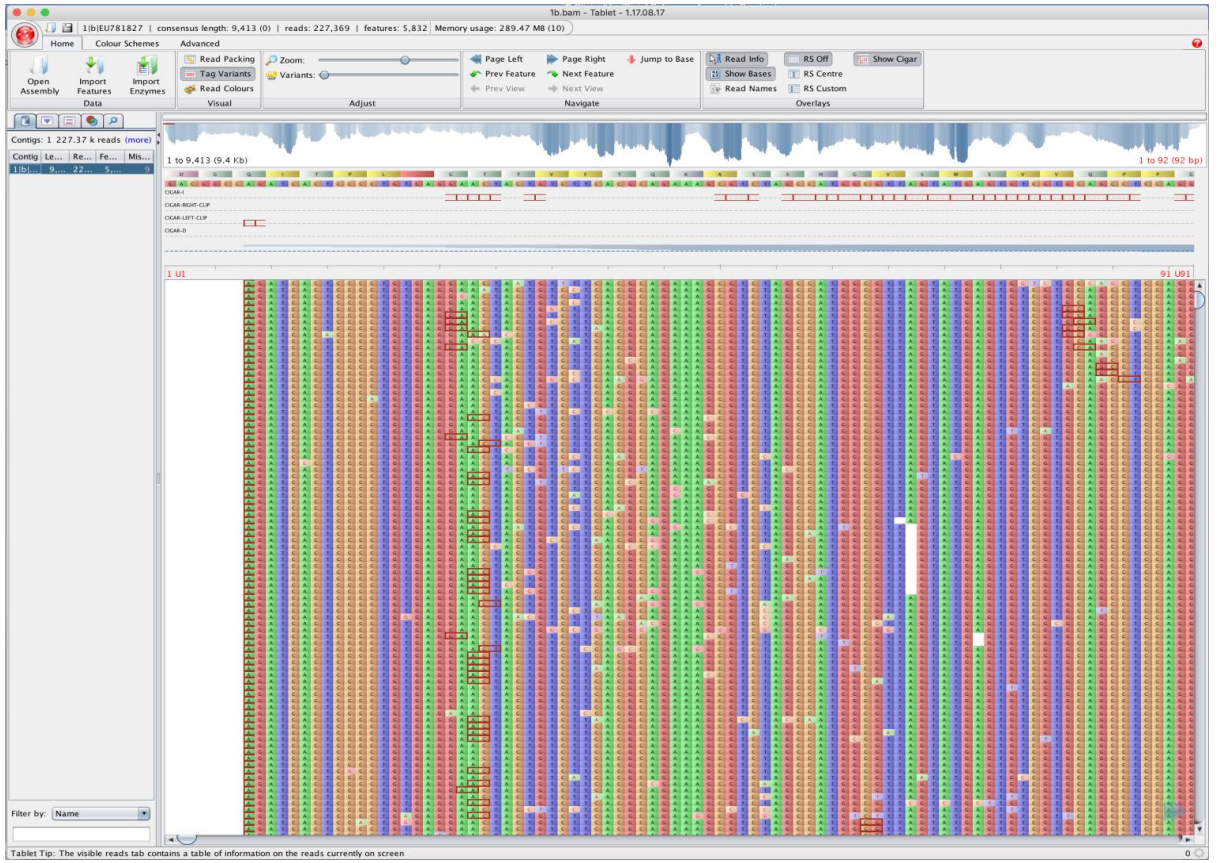

```
bwa index ../1a_hcv_ref.fasta

bwa mem ../1a_hcv_ref.fasta hcv_sim_R1.fq
hcv_sim_R2.fq > 1b.sam

samtools view -b 1b.sam

samtools sort -o 1b.bam

samtools index 1b.bam

rm 1b.sam

samtools view -c -F4 1b.bam

samtools view -c -f4 1b.bam
```

# The End … Tablet

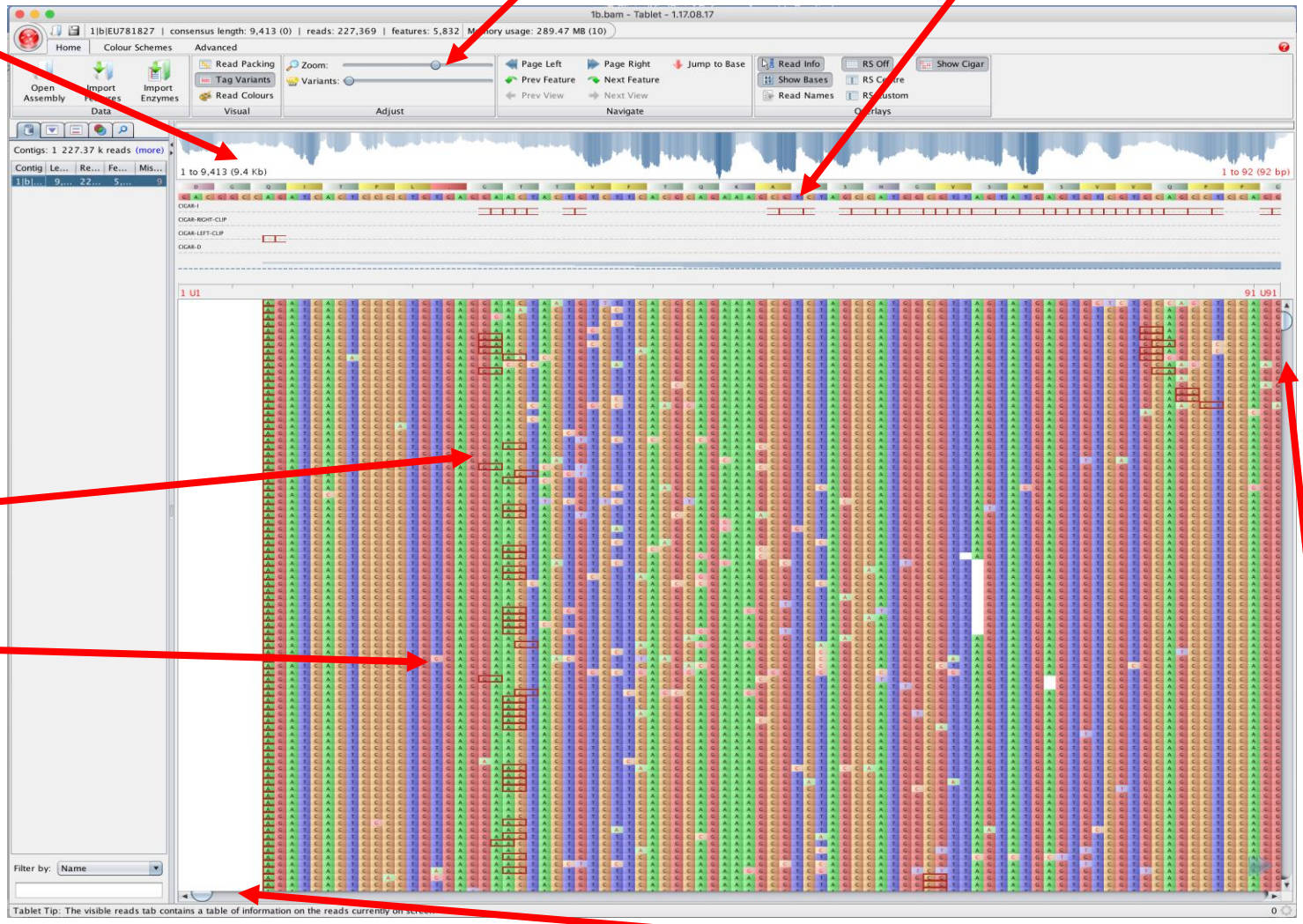
- Tablet demo if time later on

# Tablet: https://ics.hutton.ac.uk/tablet/

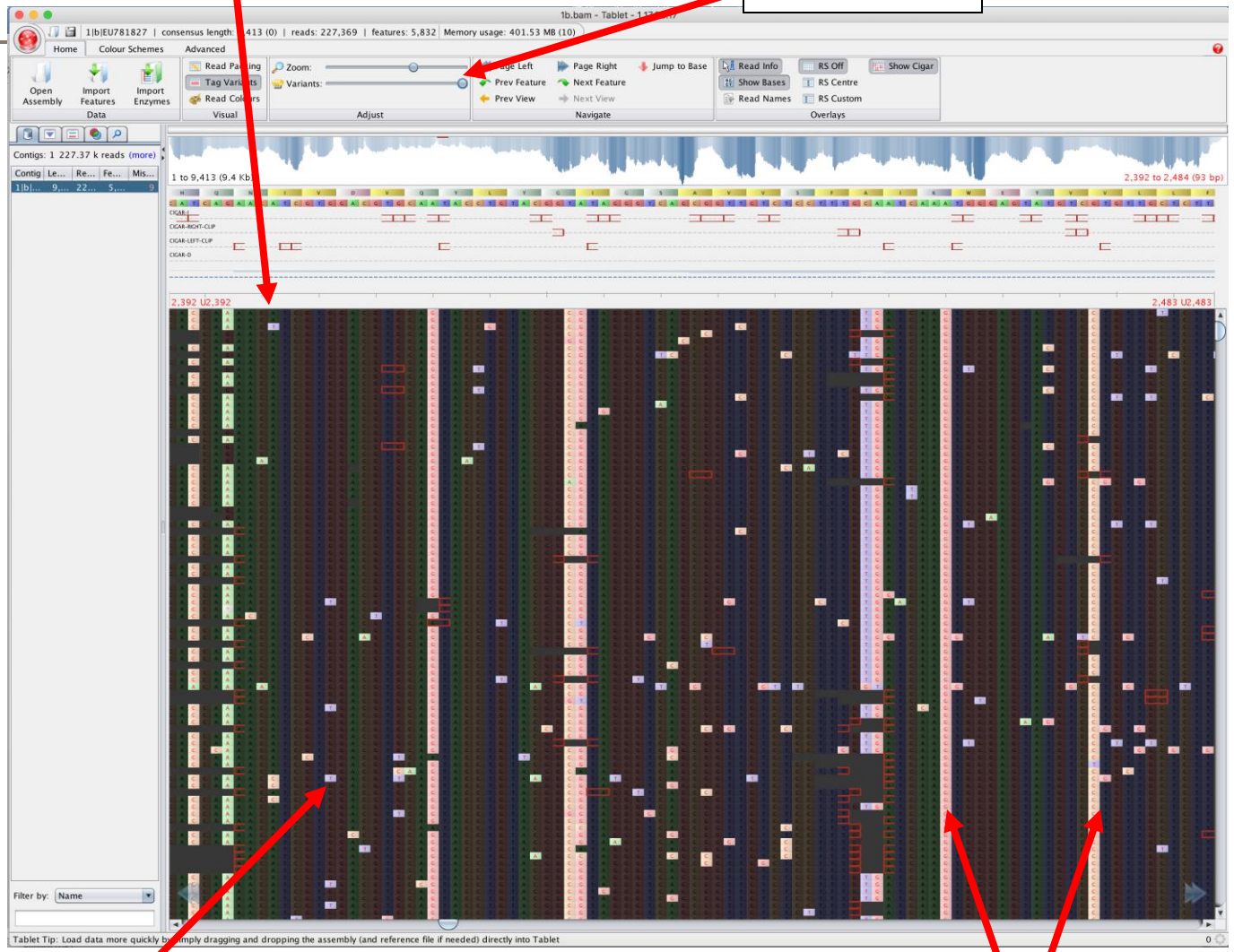
- **`tablet`**
- Zoom, scroll, colour schemes: nucleotides, direction, mutations
- Tablet is a lightweight, high-performance graphical viewer for next generation sequence assemblies and alignments.
  - BAM file
  - Reference file



# Tablet


Zoom slider
Reference sequence
Coverage overview
Read display
Mutations
Scroll bars
1b.bam - Tablet - 1.17.08.17
Home
Colour Schemes
Advanced
Open Assembly
Import Features
Import Enzymes
Read Packing
Tag Variants
Read Colours
Zoom:
Variants:
Page Left
Page Right
Jump to Base
Prev Feature
Next Feature
Prev View
Next View
Read Info
Show Bases
Read Names
RS Off
Show Cigar
Contigs: 1 227.37 k reads (more)
1 to 9,413 (9.4 Kb)
1 to 92 (92 bp)
Filter by: Name

# Tablet


Mouse Genome Position
Variants slider
Minority variants
Consensus level variants