Applied Computational Multi-Omics

Genetic Alterations and Functional Impact

Daniel Sobral, INSA

17/03/2025

Genetic Alterations and Functional Impact

Learning Objectives

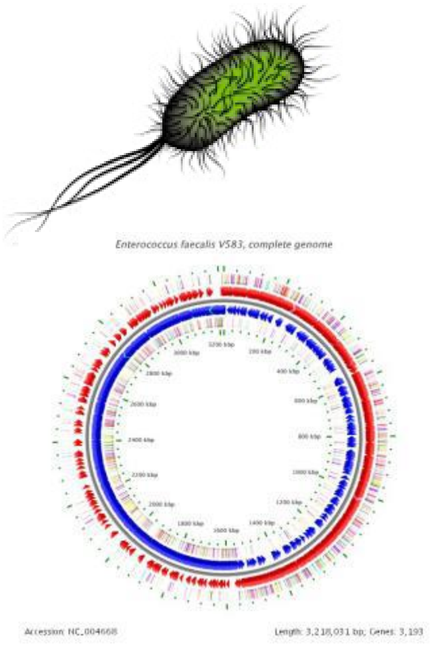
Introduction to variant calling

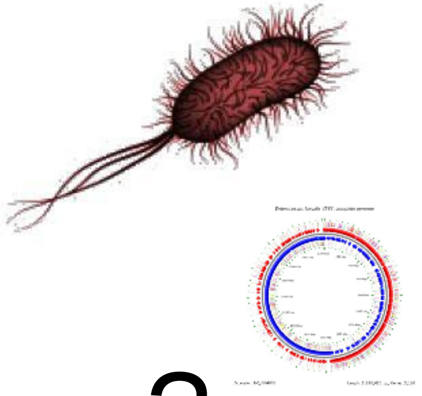
- 2 major classes: SNPs / indels and large structural variants
- Factors that influence the variant calling process
- Overview of the VCF file format
- Variant quality and Genotype quality

Introduction to Variant Annotation

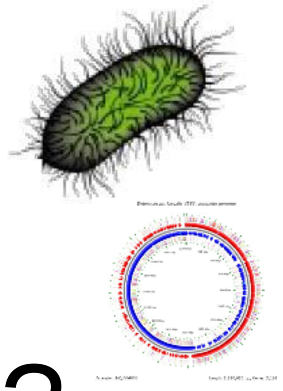
Variant Calling

Common question: find mutations underlying phenotypes





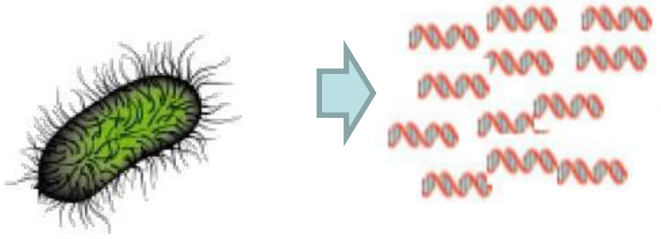
?



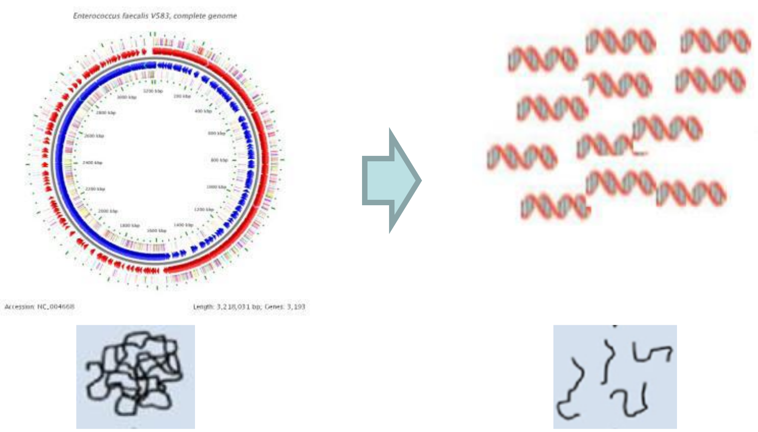
?

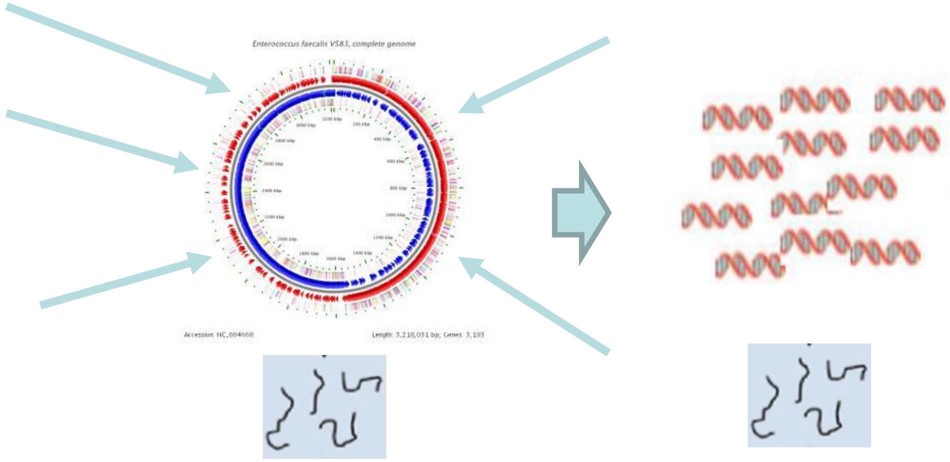
Data pre-processing for variant discovery

DNA Extraction

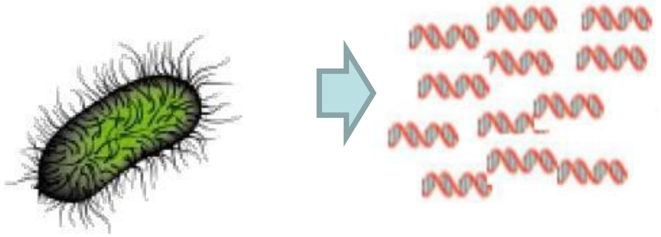


Whole Genome VS Targeted





DNA Extraction



Eg. TruSight One Enrichment Panel (Human)
Eg. SARS-CoV-2 Artic Amplicon panel

Note: Amplicon VS Enrichment

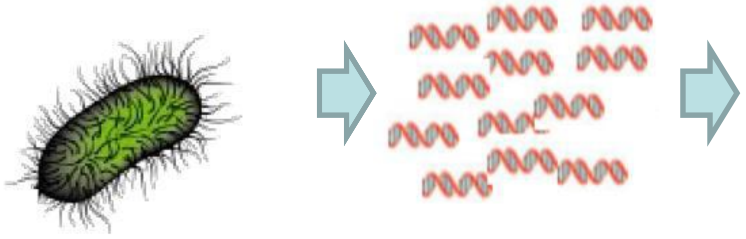
How to calculate coverage

Example for the Human Genome (~$3x10^9$ bp):

- WGS 30x coverage, 150bp read pairs
  - $30 \times 3x10^9 / (150x2) = 3x10^8$ = 300 million read pairs
- WES (~1-2% genome) 30x coverage, 150bp read pairs
  - 6 million read pairs (theoretical minimum, but usually more)

Data pre-processing for variant discovery

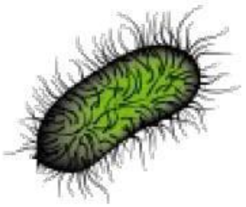
DNA Extraction **Sequencing**

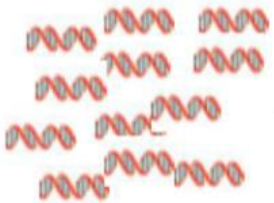


TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **("raw" fastQ)**

Data pre-processing for variant discovery

DNA Extraction **Sequencing**





TGCTCAGTT TGAC ATGGAGT GTTGT **(“clean” fastQ)**

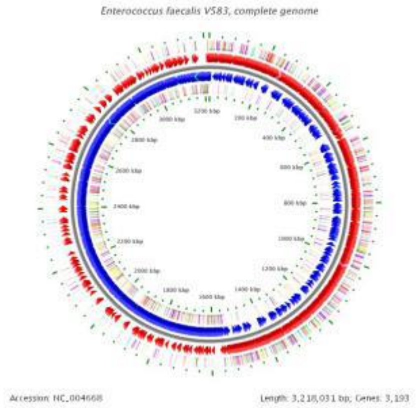
QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **(“raw” fastQ)**

Data pre-processing for variant discovery

(fasta file)

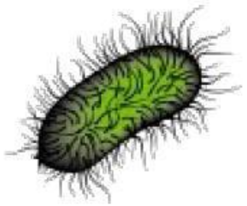
Reference Genome

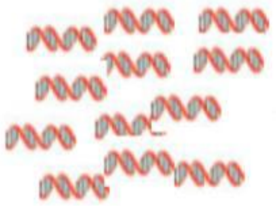


geneA geneB geneC

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

DNA Extraction **Sequencing**





TGCTCAGTT TGAC ATGGAGT GTTGT **(“clean” fastQ)**

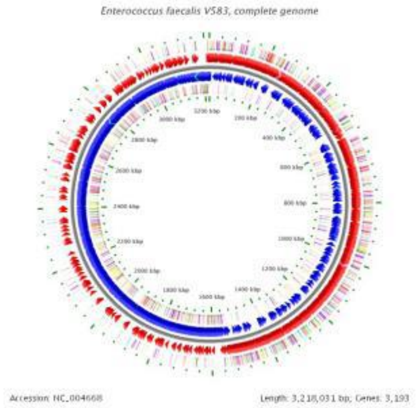
QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **(“raw” fastQ)**

Data pre-processing for variant discovery

(fasta file)

Reference Genome

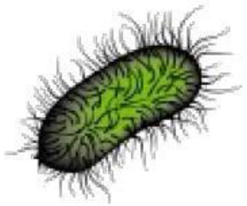


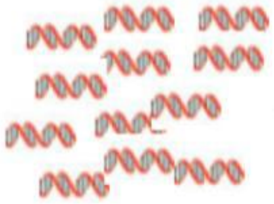
TGCTCAGTT

geneA geneB geneC

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

DNA Extraction **Sequencing**





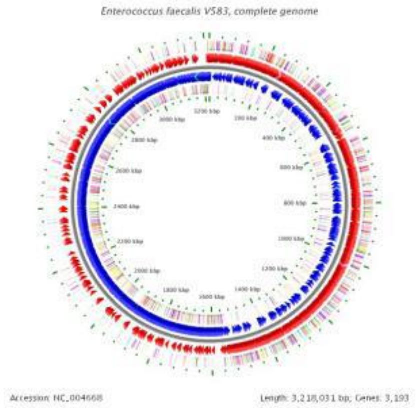
TGCTCAGTT TGAC ATGGAGT GTTGT **(“clean” fastQ)**

QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **(“raw” fastQ)**

Data pre-processing for variant discovery

(fasta file)
Reference Genome

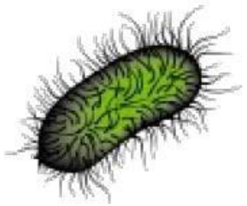


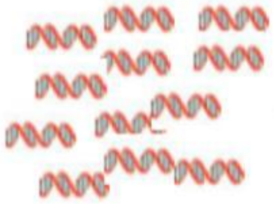
TGCTCAGTT

geneA geneB geneC

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

DNA Extraction **Sequencing** **Alignment**





TGCTCAGTT TGAC ATGGAGT GTTGT **(“clean” fastQ)**

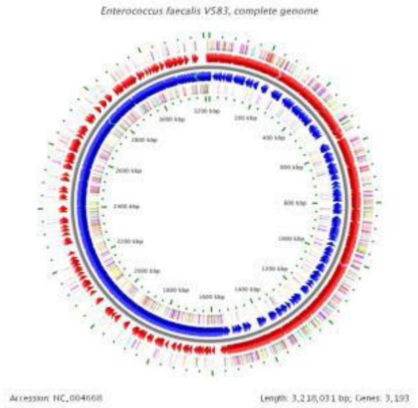
QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **(“raw” fastQ)**

Data pre-processing for variant discovery

(fasta file)

Reference Genome



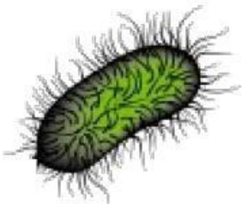
ACTCCAT GTTGT

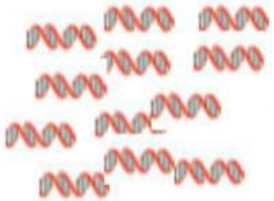
TGCTCAGTT

geneA **geneB** **geneC**

AAG**CGATGAC**TGCAT**GCACAG**TTGTGT**GTTTTCAC**GTGAC

DNA Extraction **Sequencing** **Alignment**





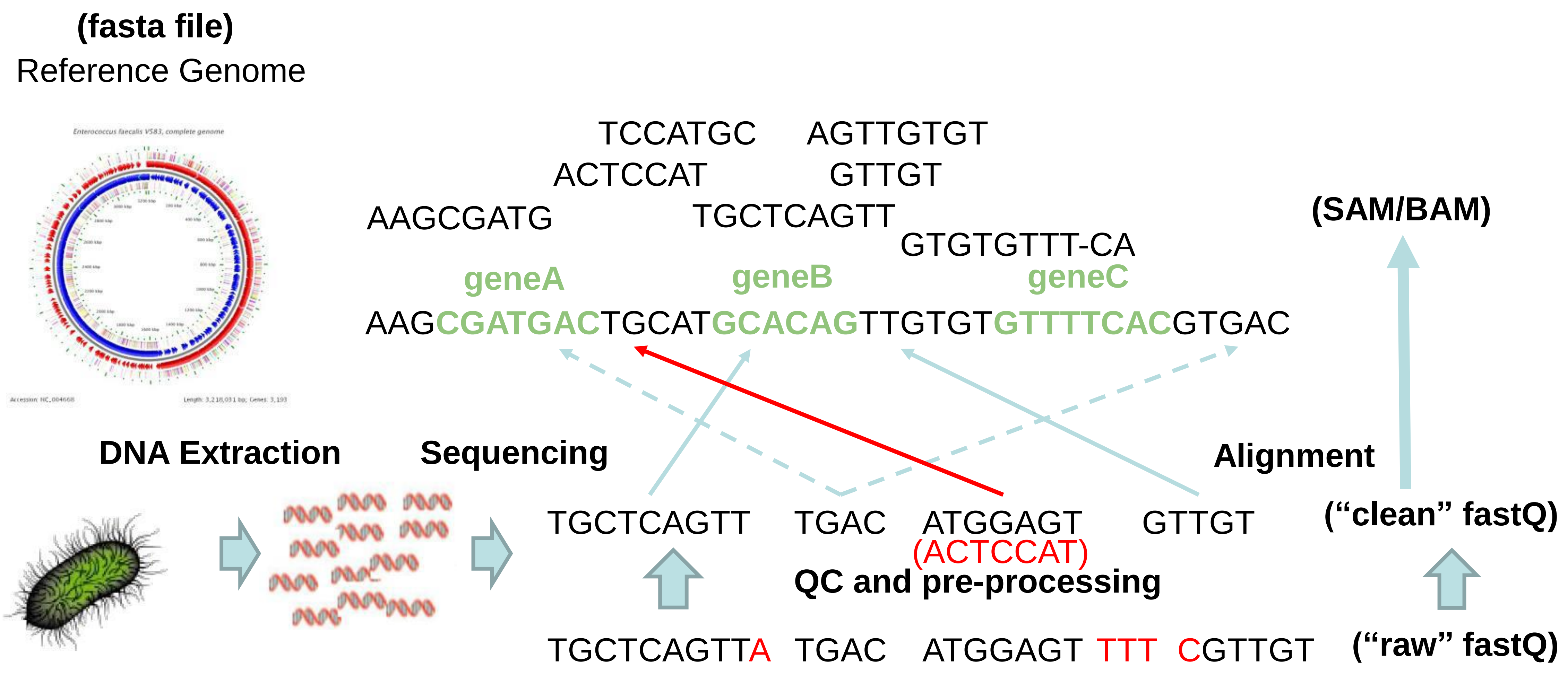
TGCTCAGTT TGAC ATGGAGT GTTGT **(“clean” fastQ)**

(ACTCCAT)

QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **(“raw” fastQ)**

Data pre-processing for variant discovery



SAM/BAM format

A file format to represent alignments

BAM -> binary form of SAM

```
Coor     12345678901234  5678901234567890123456789012345
ref      AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT

+r001/1        TTAGATAAAGGATA*CTG
+r002         aaaAGATAA*GGATA
+r003       gcctaAGCTAA
+r004                     ATAGCT..............TCAGC
-r003                            ttagctTAGGC
-r001/2                                        CAGCGGCAT
```

```
@HD VN:1.6 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
r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC
r003 2064 ref 29 17 6H5M       *  0   0 TAGGC
r001  147 ref 37 30 9M         =  7 -39 CAGCGGCAT
```

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

How alignment is made in practice

BWA (Burrows-Wheeler Aligner) is the most popular tool for WGS/WES

- Align millions of short reads to a human-sized genome in minutes

It is based on the FM-index of the Burrows-Wheeler Transform

"abracadabra$" $\xrightarrow{\text{BWT}}$ "ard$rcaaaabb" + C[c] + Occ(c, k)

(genome)

C[c] of "ard$rcaaaabb"

c	$	a	b	c	d	r
C[c]	0	1	6	8	9	10

Occ(c, k) of "ard$rcaaaabb"

	a	r	d	$	r	c	a	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

FM - index

https://en.wikipedia.org/wiki/FM-index

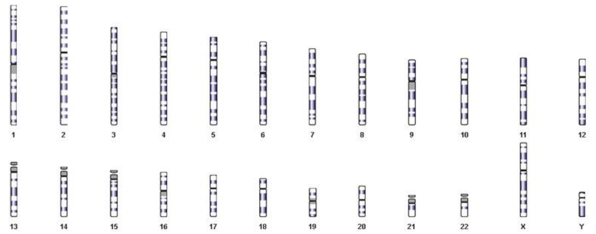
How alignment is made in practice

Genome BWT FM-índex needs to be created (only once)

$ bwa index genome.fasta

(genome.fasta file)

Reference Genome



$3x10^9$ bases

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

BWT

c	$	a	b	c	d	r
c[c]	0	1	6	8	9	10

	a	r	d	$	r	c	a	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

FM - index

How alignment is made in practice

Basic command to generate alignments with BWA:

$ bwa mem genome.fasta reads_R1.fastq(.gz) reads_R2.fastq(.gz)>output.sam

reads_R1.fastq
TGCTCAGTT

reads_R2.fastq
ACGTCCGA

BWT

c	$	a	b	c	d	r
C[c]	0	1	6	8	9	10

	a	r	d	$	r	c	a	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

Output in SAM format

TGCTCAGTT Chr2 position xxx

ACGTCCGA Chr2 position yyy

```
@HD VN:1.6 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
r004     0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC
r003  2064 ref 29 17 6H5M       *  0   0 TAGGC
r001   147 ref 37 30 9M         =  7 -39 CAGCGGCAT
```

How alignment is made in practice

Paired R1

TGCTCAGTT

TGC TCA GTT

BWT

c	$	a	b	c	d	r
c[c]	0	1	6	8	9	10

	a	r	d	$	r	c	a	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

Paired R2

ACGTCCGA

ACG TCC

TCC: chr1, chr2, chr3

TCA: chr2, chr3

GTT: chr2, chr3, chr6

→ chr2, chr3 (multimapping)

ACG: chr2, chr6

TCC: chr1, chr2

→ chr2

Most likely:

chr2

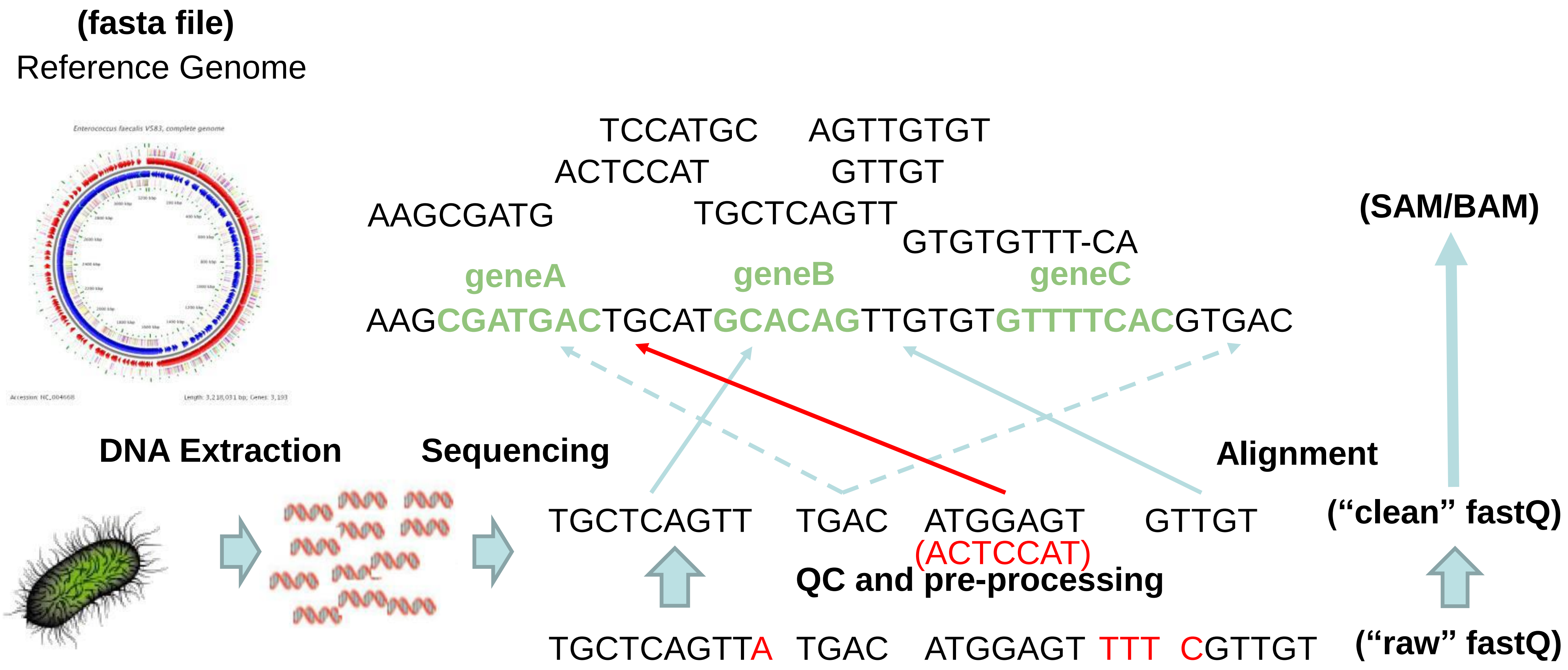
How alignment is made in practice: Summary

- Special algorithms are used to have fast alignments
  - They are not guaranteed to be perfect but most of the time they are very good

- A read can map equally well to multiple regions (multimappings)
  - BWA reports **one primary alignment (randomly chosen) with mapping quality of 0**
  - Depending on the software, it can generate **secondary alignments**
  - Information of paired reads are used to disambiguate multimappings if possible

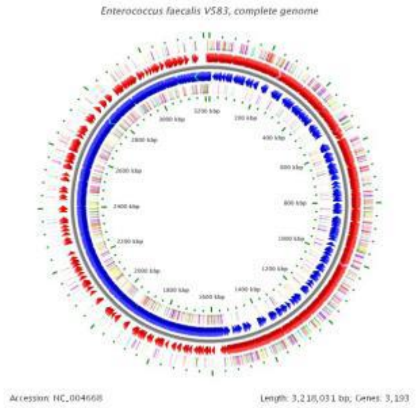
- Alignments are made piece-wise (a read is split in segments)
  - A read alignment can be split in a primary and **supplementary alignment(s)**
    - Eg. splicing in RNA-Seq; large deletions
  - Sometimes, only a part of the read is aligned (the rest is “masked”/hidden)
    - Particularly in repetitive areas this can lead to false alignments

Variant Calling



Variant Calling

(fasta file)
Reference Genome



SNV **SNV**

TCCATGC AGTTGTGT
ACTCCAT GTTGT
AAGCGATG TGCTCAGTT
GTGTGTTT-CA

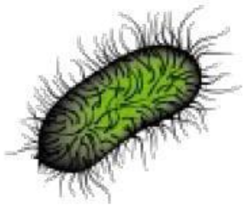
geneA **geneB** **geneC**

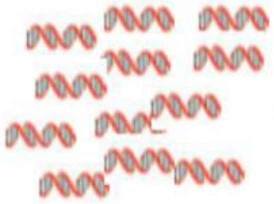
AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

(SAM/BAM)

Alignment

DNA Extraction **Sequencing**



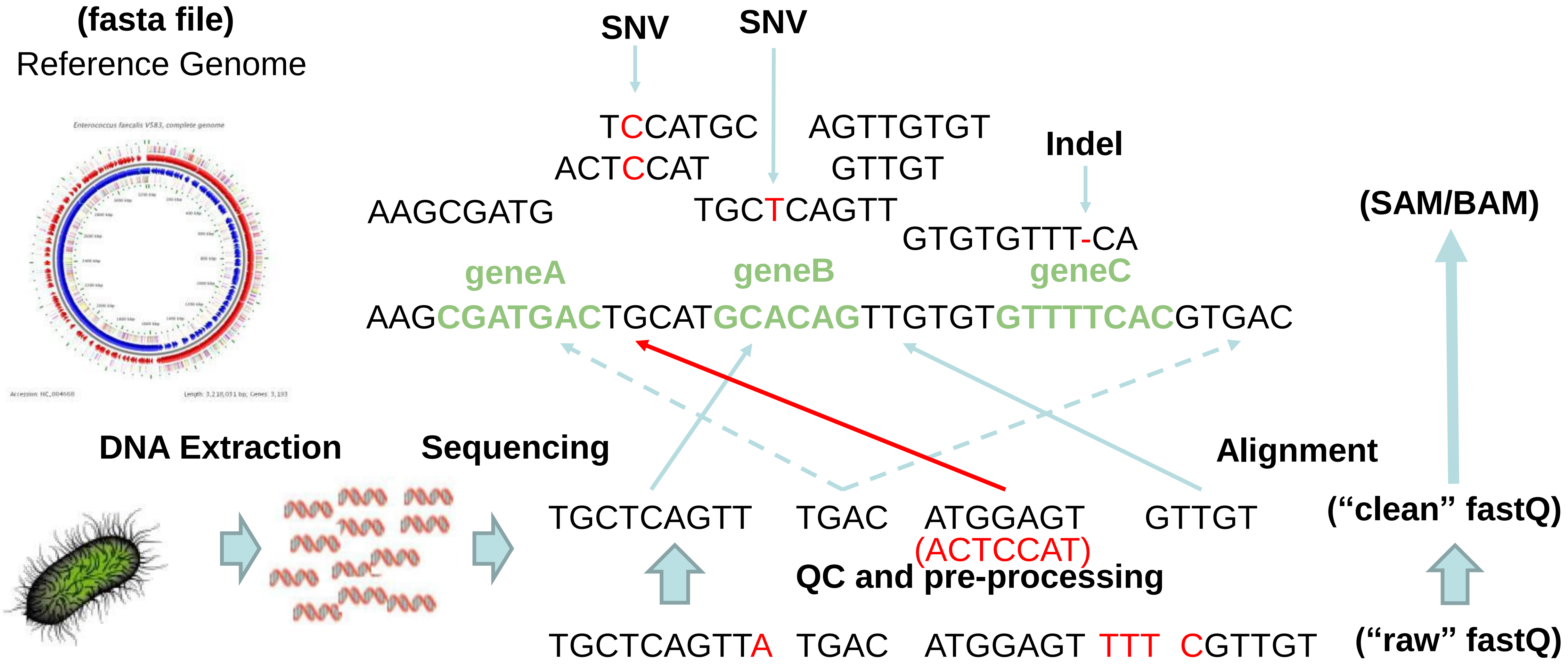


TGCTCAGTT TGAC ATGGAGT GTTGT **("clean" fastQ)**
(ACTCCAT)

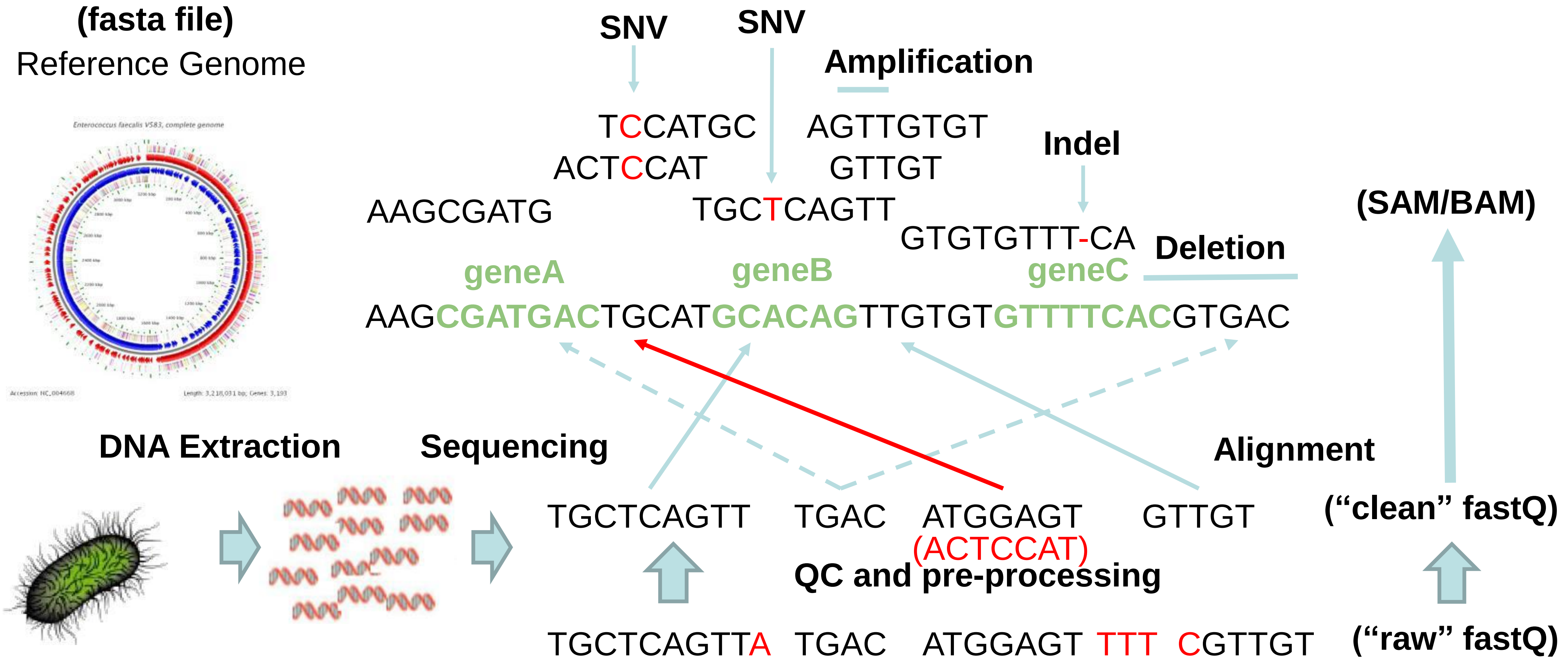
QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **("raw" fastQ)**

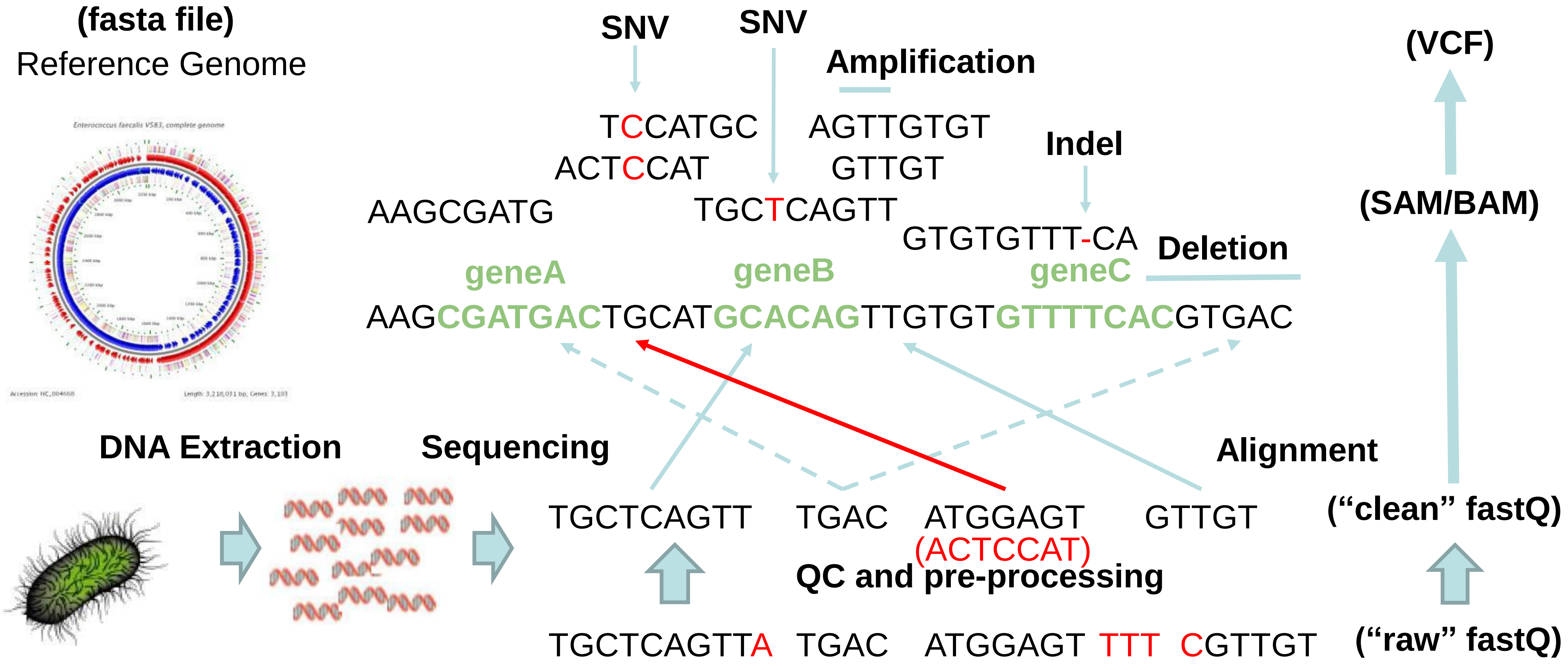
Variant Calling



Variant Calling



Variant Calling



The VCF format

```
##fileformat=VCFv4.4
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=file:///seq/references/1000GenomesPilot-NCBI36.fasta
##contig=<ID=20,length=62435964,assembly=B36,md5=f126cdf8a6e0c7f379d618ff66beb2da,species="Homo sapiens",taxonomy=x>
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS     ID        REF    ALT     QUAL FILTER INFO                              FORMAT      NA00001        NA00002        NA00003
20     14370   rs6054257 G      A       29   PASS   NS=3;DP=14;AF=0.5;DB;H2           GT:GQ:DP:HQ 0|0:48:1:51,51 1|0:48:8:51,51 1/1:43:5:.,.
20     17330   .         T      A       3    q10    NS=3;DP=11;AF=0.017               GT:GQ:DP:HQ 0|0:49:3:58,50 0|1:3:5:65,3   0/0:41:3
20     1110696 rs6040355 A      G,T     67   PASS   NS=2;DP=10;AF=0.333,0.667;AA=T;DB GT:GQ:DP:HQ 1|2:21:6:23,27 2|1:2:0:18,2   2/2:35:4
20     1230237 .         T      .       47   PASS   NS=3;DP=13;AA=T                   GT:GQ:DP:HQ 0|0:54:7:56,60 0|0:48:4:51,51 0/0:61:2
20     1234567 microsat1 GTC    G,GTCT  50   PASS   NS=3;DP=9;AA=G                    GT:GQ:DP    0/1:35:4       0/2:17:2       1/1:40:3
```

CHROM	chromosome
POS	position of the start of the variant
ID	unique identifier of the variant (e.g. rs number for SNPs)
REF	reference allele
ALT	comma separated list of alternate non-reference alleles
QUAL	phred-scaled quality score
FILTER	site filtering information
INFO	user extensible annotation (e.g. samtools and GATK may differ in this)

https://samtools.github.io/hts-specs/VCFv4.4.pdf

The VCF format

CHROM	chromosome
POS	position of the start of the variant
ID	unique identifier of the variant (e.g. rs number for SNPs)
REF	reference allele
ALT	comma separated list of alternate non-reference alleles
QUAL	phred-scaled quality score
FILTER	site filtering information
INFO	user extensible annotation (e.g. samtools and GATK may differ in this)

```
#CHROM POS     ID        REF    ALT     QUAL FILTER INFO                              FORMAT      NA00001        NA00002        NA00003
20     14370   rs6054257 G      A       29   PASS   NS=3;DP=14;AF=0.5;DB;H2           GT:GQ:DP:HQ 0|0:48:1:51,51 1|0:48:8:51,51 1/1:43:5:.,.
20     17330   .         T      A       3    q10    NS=3;DP=11;AF=0.017               GT:GQ:DP:HQ 0|0:49:3:58,50 0|1:3:5:65,3   0/0:41:3
20     1110696 rs6040355 A      G,T     67   PASS   NS=2;DP=10;AF=0.333,0.667;AA=T;DB GT:GQ:DP:HQ 1|2:21:6:23,27 2|1:2:0:18,2   2/2:35:4
20     1230237 .         T      .       47   PASS   NS=3;DP=13;AA=T                   GT:GQ:DP:HQ 0|0:54:7:56,60 0|0:48:4:51,51 0/0:61:2
20     1234567 microsat1 GTC    G,GTCT  50   PASS   NS=3;DP=9;AA=G                    GT:GQ:DP    0/1:35:4       0/2:17:2       1/1:40:3
```

https://samtools.github.io/hts-specs/VCFv4.4.pdf

Variant Quality vs Genotype Quality

- Variant Quality
  - Phred score estimating if variant is likely to be an artifact
- Genotype Quality
  - Phred score estimating accuracy of estimated sample genotype

VQ

GQ

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	NA00001	NA00002	NA00003
20	14370	rs6054257	G	A	29	PASS	NS=3;DP=14;AF=0.5;DB;H2	GT:GQ:DP:HQ	0|0:48:1:51,51	1|0:48:8:51,51	1/1:43:5:.,.
20	17330	.	T	A	3	q10	NS=3;DP=11;AF=0.017	GT:GQ:DP:HQ	0|0:49:3:58,50	0|1:3:5:65,3	0/0:41:3
20	1110696	rs6040355	A	G,T	67	PASS	NS=2;DP=10;AF=0.333,0.667;AA=T;DB	GT:GQ:DP:HQ	1|2:21:6:23,27	2|1:2:0:18,2	2/2:35:4
20	1230237	.	T	.	47	PASS	NS=3;DP=13;AA=T	GT:GQ:DP:HQ	0|0:54:7:56,60	0|0:48:4:51,51	0/0:61:2
20	1234567	microsat1	GTC	G,GTCT	50	PASS	NS=3;DP=9;AA=G	GT:GQ:DP	0/1:35:4	0/2:17:2	1/1:40:3

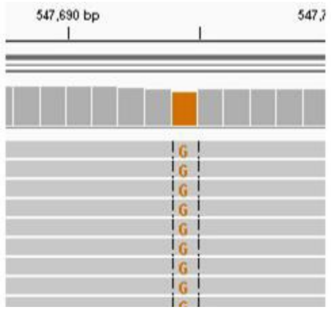
Variant Calling

- 2 Major types of Variants
  - Single Nucleotide Variants (SNV) and small Indels
    - Smaller than the size of one read
  - Large Structural Variants
    - Usually larger than the size of one read

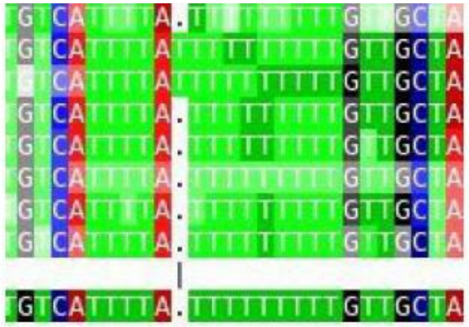
Single Nucleotide Variants (SNV) and small Indels

Variants detected within reads (smaller than size of read)

- SNVs:
  - – Change of a single nucleotide



- Indels:
  - – “Small” deletion or amplification



Single Nucleotide Variants (SNV) and small Indels

- Main factors affecting detection of SNVs and Indels
  - Number of reads (coverage supporting a variant)
  - Base quality (affects confidence in the SNVs)
  - PCR amplification bias (can generate duplicates and other biases)
  - Repetitive areas (mostly affects indels, but also affects SNVs)

Duplicated Reads

- Duplicate reads (same fragment) can appear
  - In library preparation during amplification (eg. WES)
  - In the amplification process while sequencing (optical duplicates)

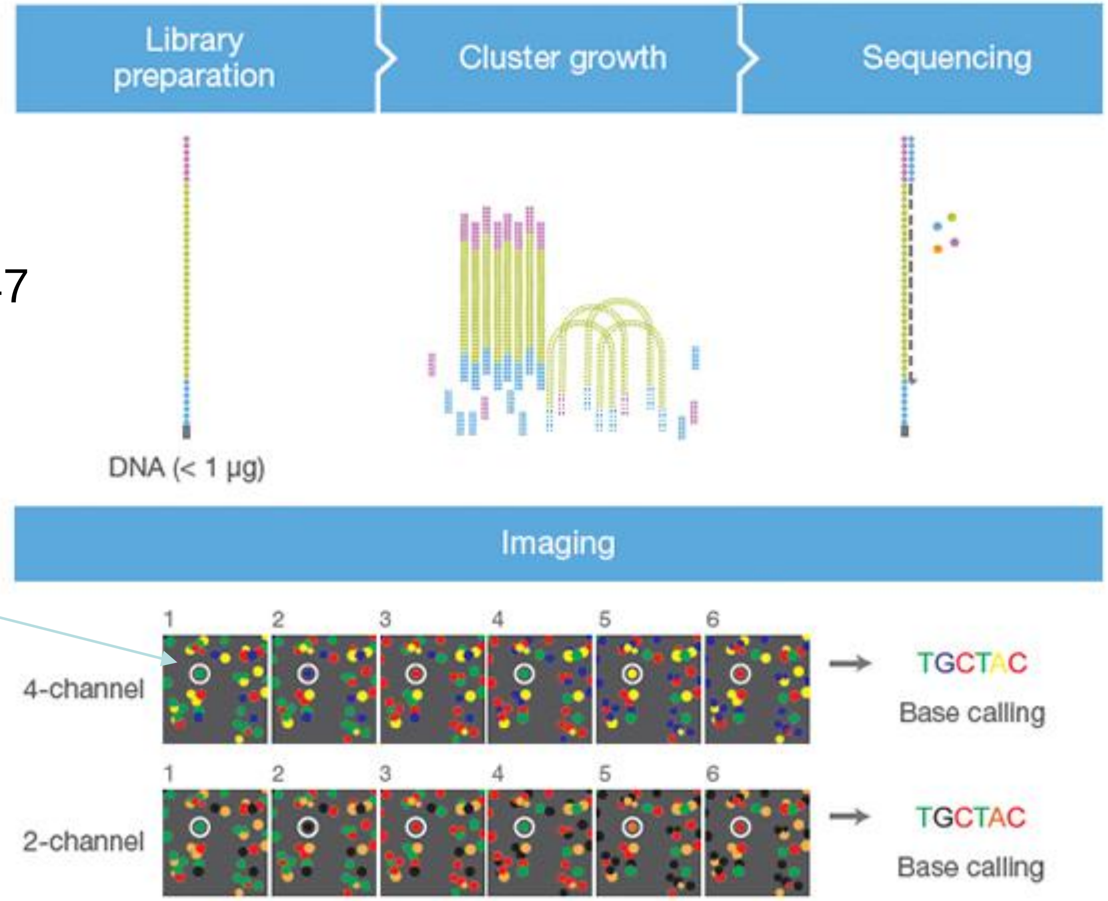
```
AAGCGATG                      AGTTGTGT
AAGCGATG   TCCATGC            AGTTGTGT
         ACTCCAT               GTTGT
                TGCTCAGTT
                            GTGTGTTT-CA

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC
     geneA         geneB         geneC
```

Duplicated Reads: optical duplicates

@MN00723:33:000H3MCVT:1:11102:7591:1087 1:N:0:47

Machine Flow Cell Lane Position

Optical duplicates are duplicate reads that are very close in the flow cell



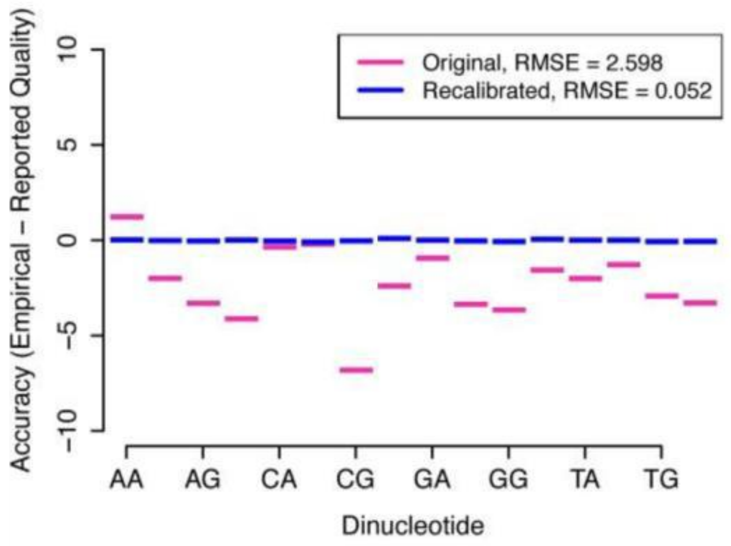
Duplicated Reads

- **The recommended practice is to ignore duplicates**
  - Only consider one of the duplicates for variant calling
    - Usually the one with the best quality
  - This may remove good information (eg. with high coverage, targeted)
  - Duplicates are marked and later ignored (or not)
  - Benefits of marking duplicates not always obvious
    - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4965708/
    - Eg. one can chose to only ignore reads marker as optical duplicates

Base Quality Recalibration

Base Quality Depends on several factors:

- Sample Quality (DNA)
- Nucleotide context
- Machine and cycle of sequencing
- Type of variant (SNP or Indel)

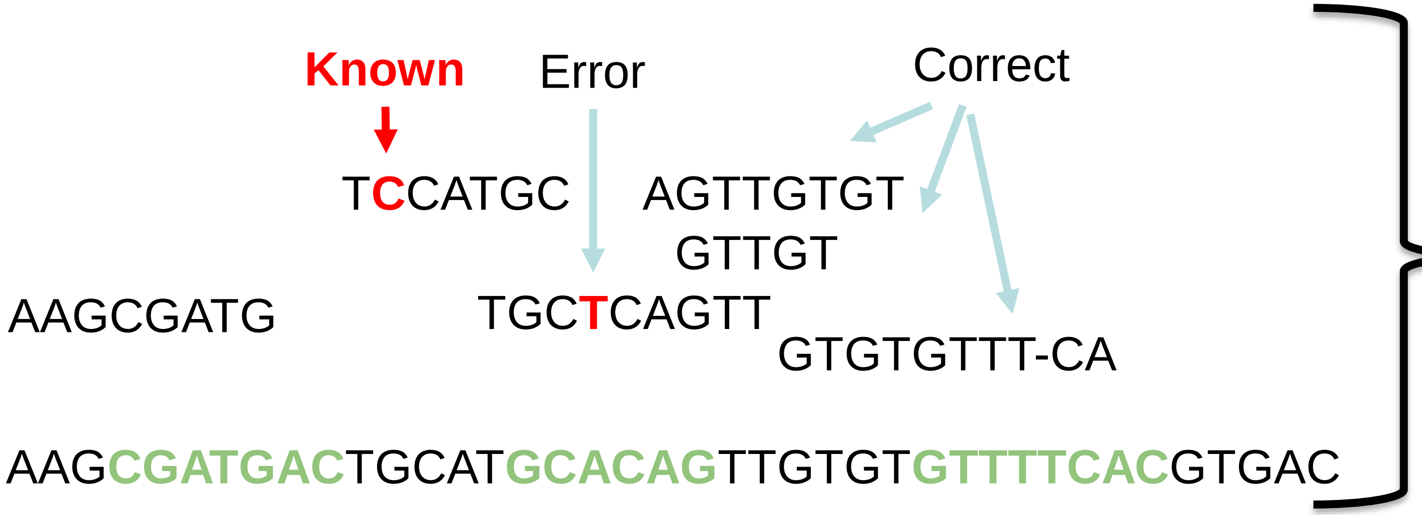


The machine may not estimate well the base quality score

Base Quality Recalibration

Use list of known variants to estimate correct quality values

- All bases different from reference **not in the provided list** of known variants are considered to be errors



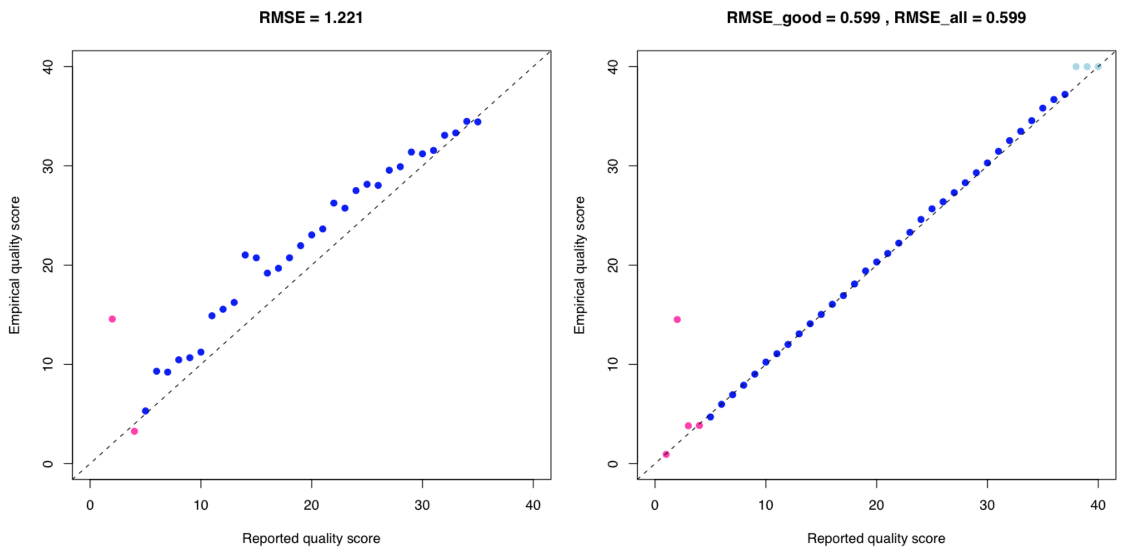
Count occurrences and compare Q value marked by the machine with the observed % errors

Base Quality Recalibration

Base Quality Recalibration:

The covariates being used here:

- ReadGroupCovariate
- QualityScoreCovariate
- ContextCovariate
- CycleCovariate



Original Data

After GATK Recalibration

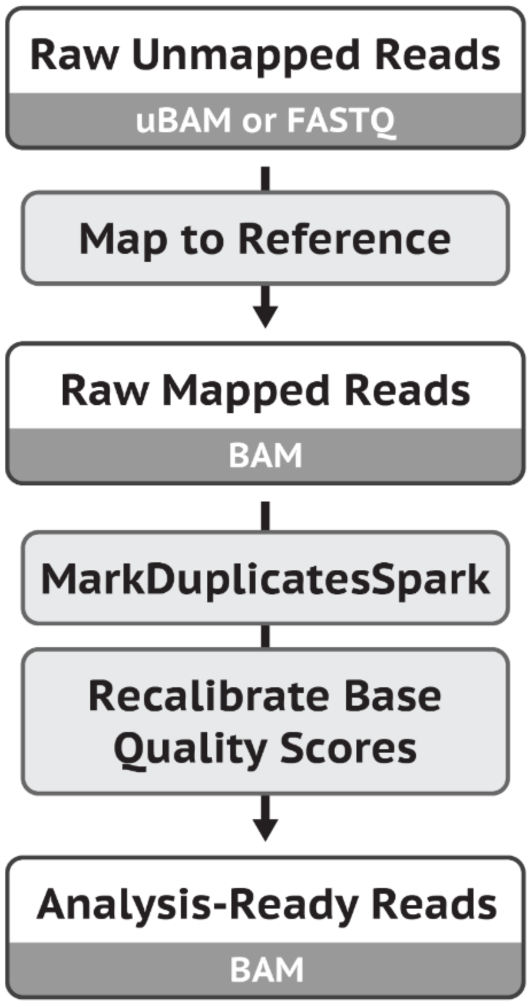
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3083463/

https://www.youtube.com/watch?v=L4D1dwES9s8

https://gatk.broadinstitute.org/hc/en-us/articles/360035890531-Base-Quality-Score-Recalibration-BQSR-

Data pre-processing for variant discovery

GATK
Best
Practices



https://gatk.broadinstitute.org/hc/en-us/articles/360035535912-Data-pre-processing-for-variant-discovery

Example estimating Genotype Quality

- These calculations are software-dependent
  - Example Genotype Likelihood (GATK)

$$\mathcal{L}(g) = \frac{1}{m^k} \prod_{j=1}^{l} \left[(m-g)\epsilon_j + g(1-\epsilon_j) \right] \prod_{j=l+1}^{k} \left[(m-g)(1-\epsilon_j) + g\epsilon_j \right]$$

g: genotype (i.e. 0, 1 or 2)
m: ploidy (2 for human)
ϵ: base error
k: number of bases at the site
l: number of bases that equal reference

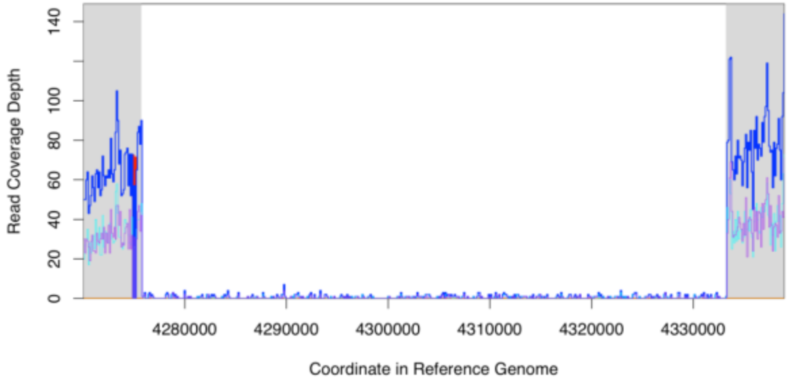
Large Structural Variants

Variants larger than the size of reads

- Large Deletions and Amplifications
  - Gene Deletions and Duplications
- Other Structural Variants
  - Fusions; Inversions; Transposons...
- Horizontal Transfer
  - Novel genomic regions

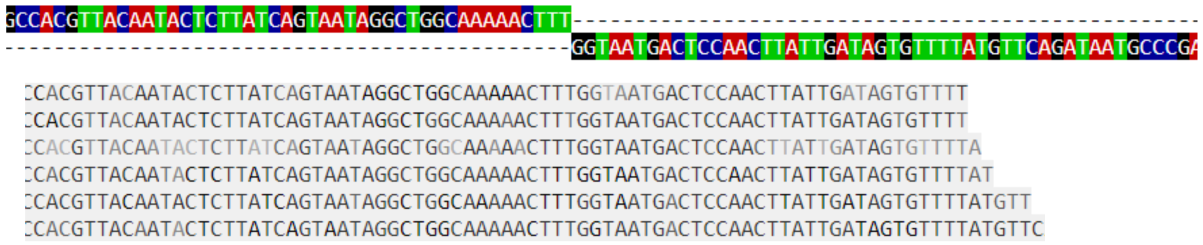
Large Structural Variants

- Evidence used to detect Structural Variants
  - Differences in Coverage
    - Most commonly used
    - Particularly with targeted sequencing
    - Although there’s still amplification bias



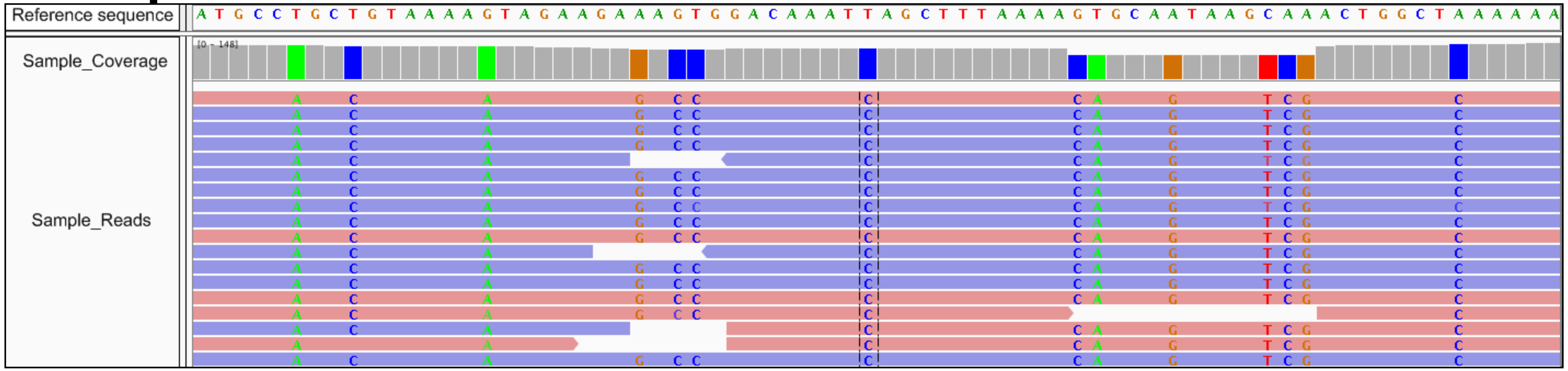
Large Structural Variants

- Evidence used to detect Structural Variants
  - Junction evidence (difficult in targeted sequencing)
    - Can use paired read information (namely, expected fragment length –noisier)
    - Can use information within reads (more precise - requires bigger reads)

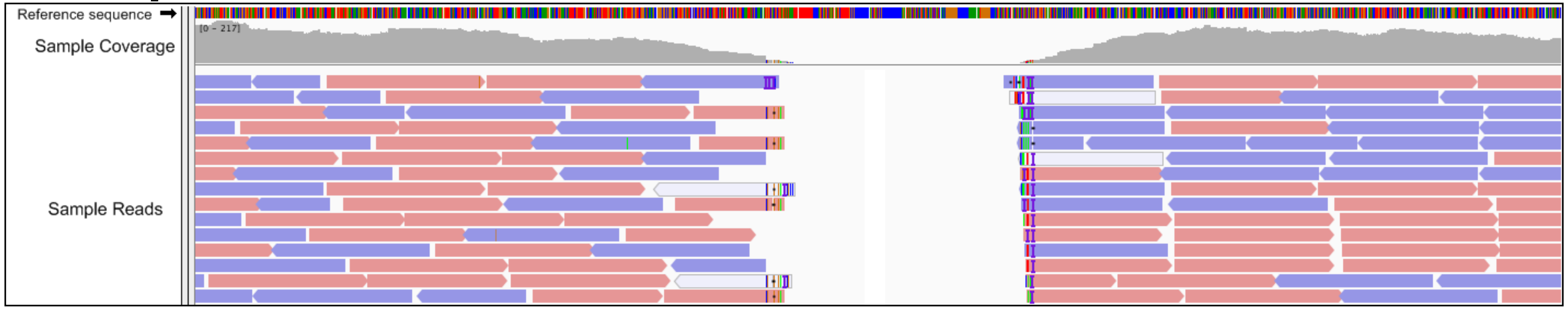


Examples of variants

Example mutations

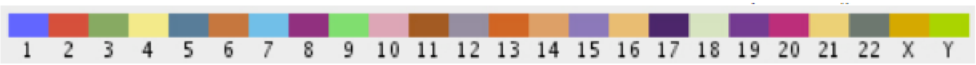


Example deletion



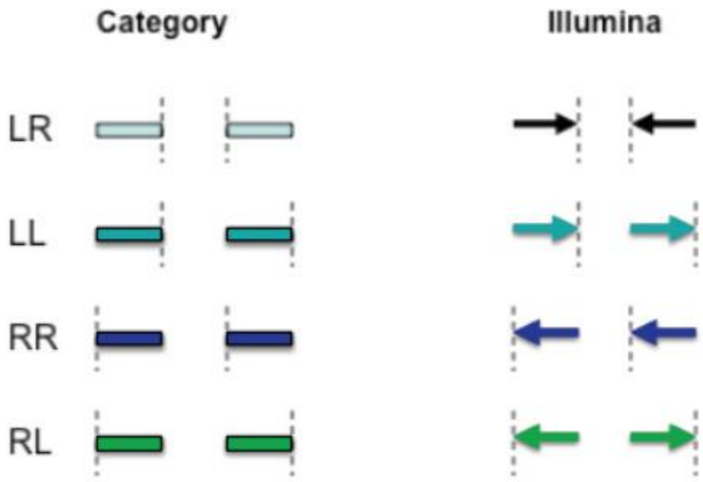
- IGV provides colors to signal unusual situations
  - Besides mutations, information from paired-end is also there

Bigger than expected
Smaller than expected



Pairs on different chromosomes

Insert Size Lengths



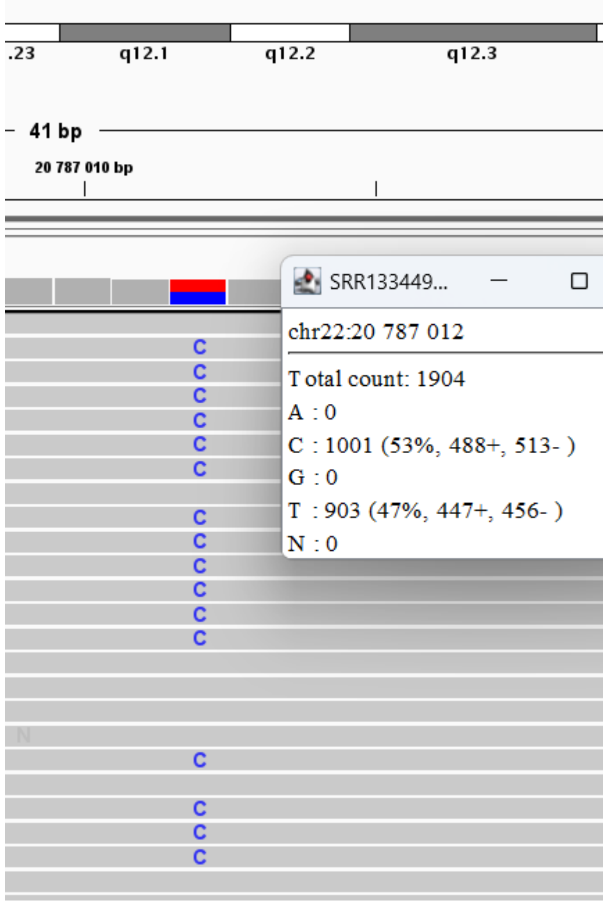
Normal
Unusual
Unusual
Unusual

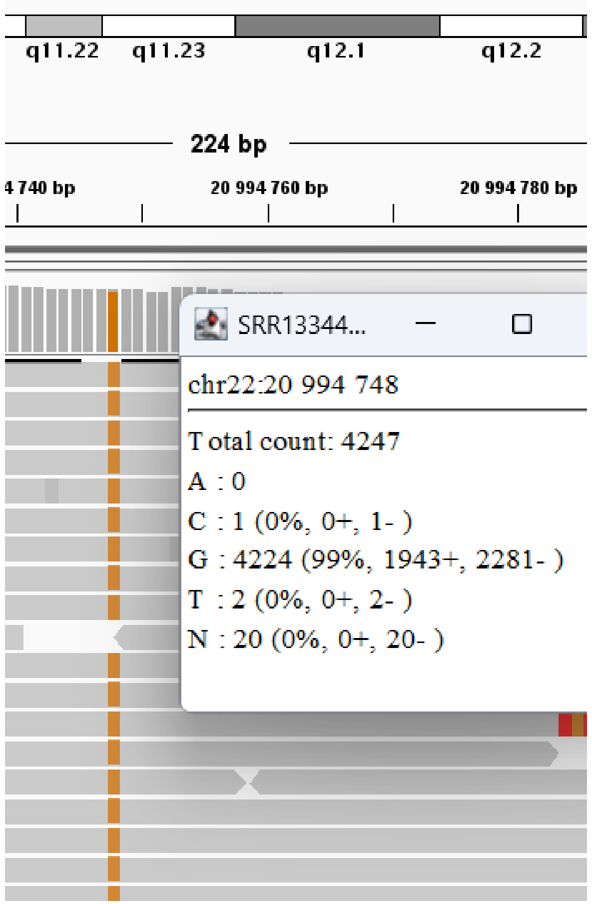
Pair orientation

https://software.broadinstitute.org/software/igv/

Visualization of Read Mappings

- Example of mutations





Variant Selection

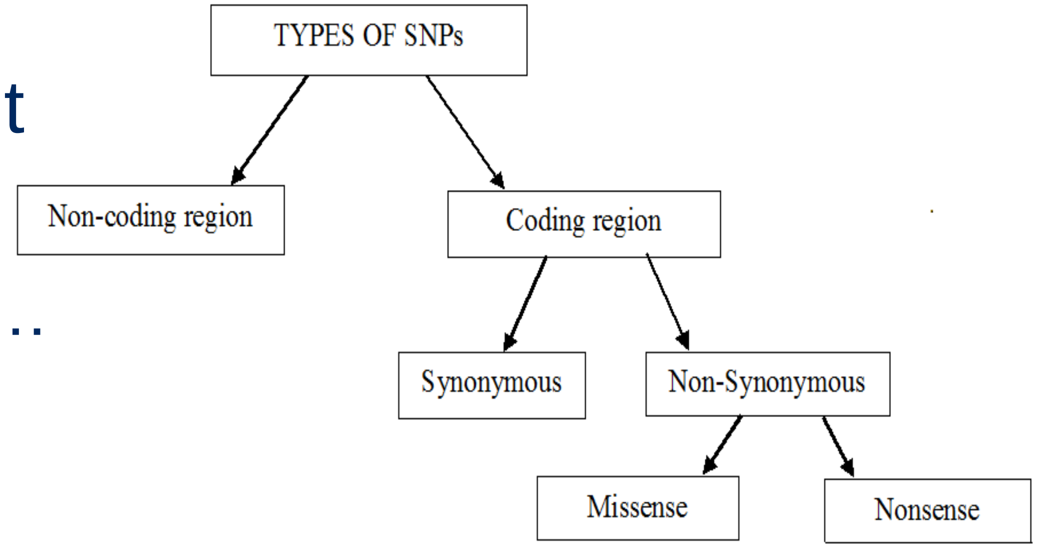
Criteria to select “valid” variants:

- Higher Number of reads (coverage supporting a variant)
- Low Bias in the Base quality supporting the variant
- Low bias in the strand of the reads supporting the variant
- Avoid variants only at the end of reads (repetitive areas)
- Avoid duplicate reads
- Etc…

Variant Annotation

Criteria to select "relevant" variants:

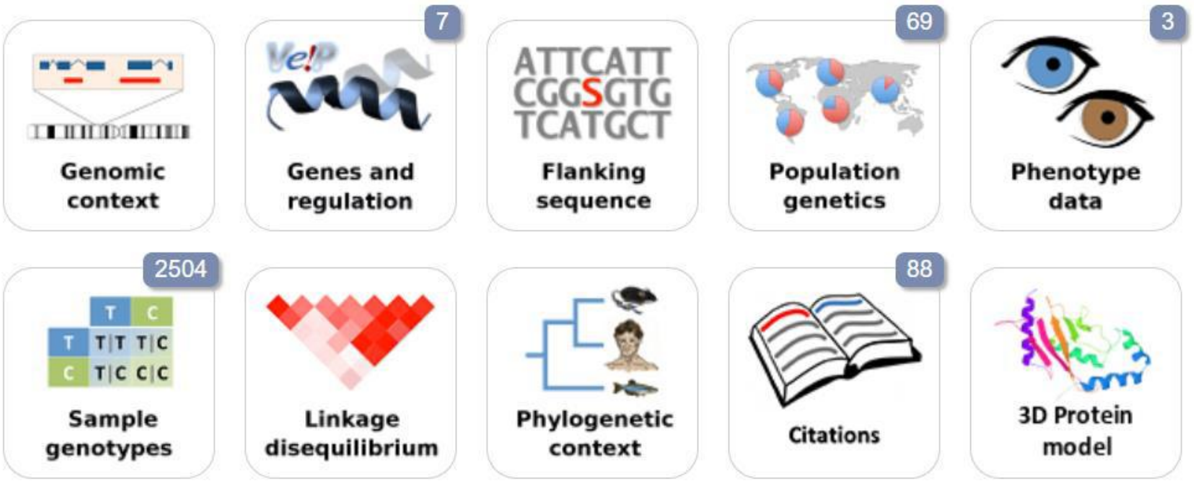
- Coding versus non-coding
  - Coding: Silent versus non-silent
  - Non-coding: can be complex
    - splice-sites, regulatory regions,…



Variant Annotation

Criteria to select "relevant" variants:

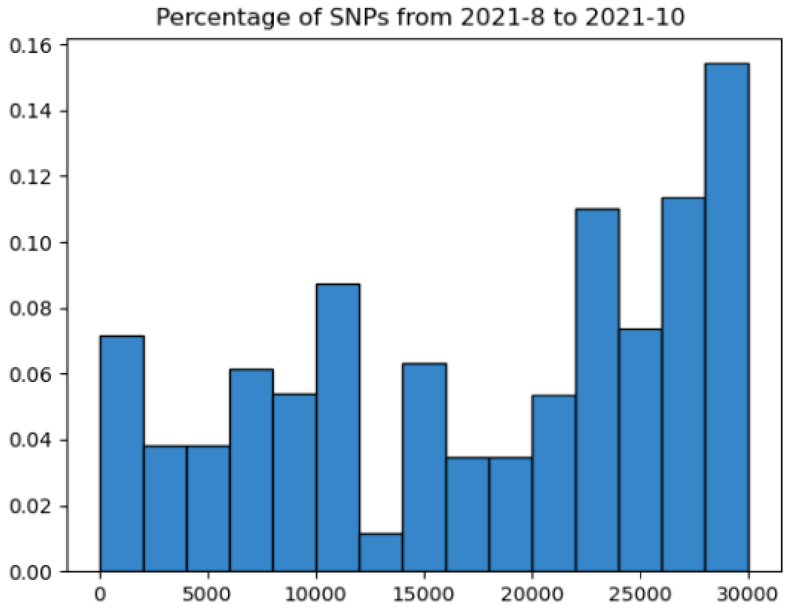
- Population frequency
- Disease-association

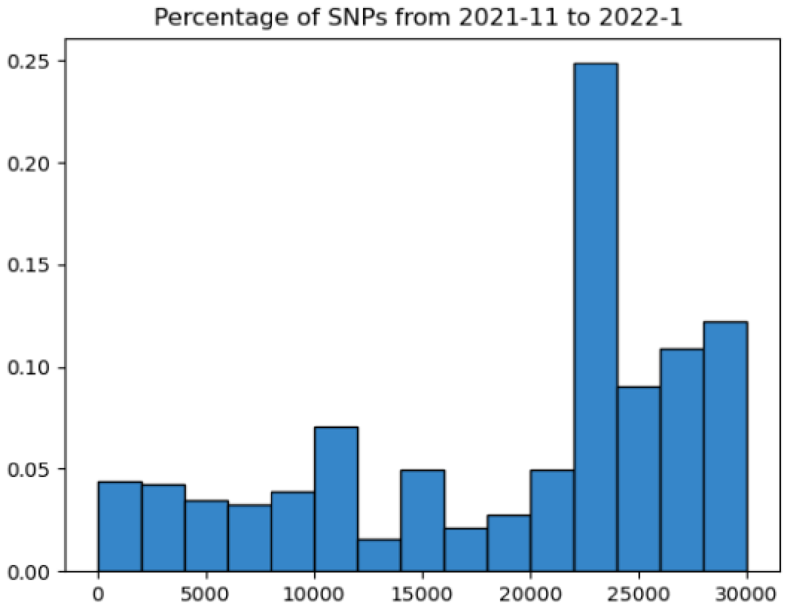


Only works for species with a lot of data, such as human

Variant Annotation

- Criteria to select "relevant" variants:
  - Simple exemple with SARS-CoV-2 (Omicron)





A few optional references:

- Single Nucleotide Variants and small Indels
  - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3083463/
- DeepVariant: Using AI to find variants
  - https://github.com/google/deepvariant; https://www.nature.com/articles/nbt.4235
- Copy Number Alterations and other Structural Variants
  - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4300727/
- Finding clonal vs subclonal variants
  - https://www.sciencedirect.com/science/article/pii/S2001037017300946
- Variant annotation
  - https://www.nature.com/articles/nprot.2015.105
  - https://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-0974-4

Practical Exercise

Sequencing of Monkeypox Samples from Portugal

https://github.com/dsobral/MBCB/blob/main/README.md

Mpox multi-country outbreak

National Institute of Health (INSA), Portugal

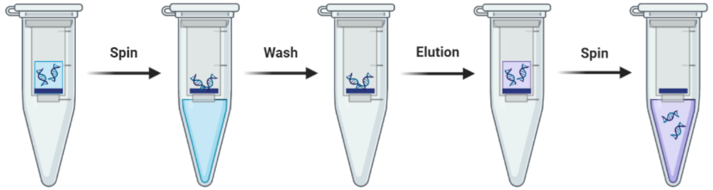
Virus genome sequencing is crucial to characterize the virus and better understand the epidemiology, sources of infection, and transmission patterns of the outbreak

All suspected samples are sent to the Portuguese NIH for diagnosis

First confirmed cases on 17th May 2022 (+20 suspected/confirmed cases on next day)

1st samples selected for sequencing

- 2 samples (skin exudates) with high viral load (Ct=17)
- DNA extraction using QIamp DNA Mini Kit (Qiagen)
- No human DNA depletion prior to DNA extraction



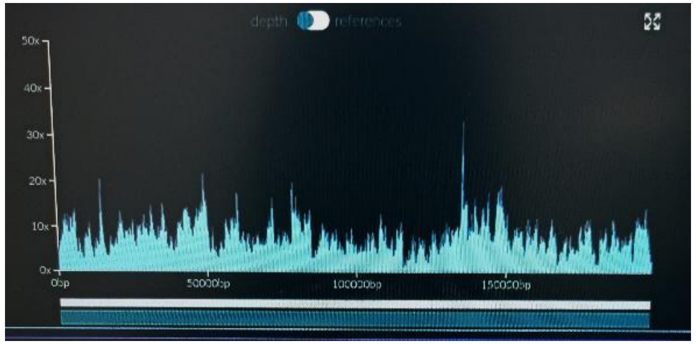
18 May 2022 – Sequencing

- Shotgun metagenomics on Oxford Nanopore MinION (Mk1B)

ONT MinION Mk1B

Run length 18h

~800k reads generated



Real-time reads mapping against ref genome
https://github.com/artic-network/rampart

19 May 2022 - Bioinformatics

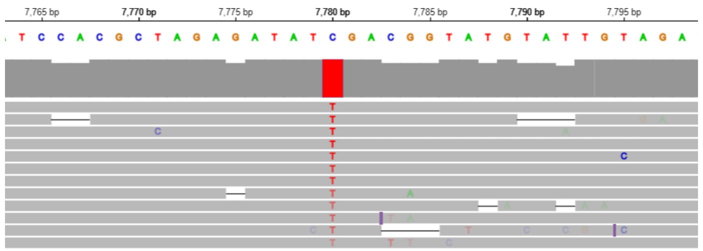
Analysis was performed using the **INSaFLU online platform** (https://insaflu.insa.pt/)

~0.5% Monkeypox reads

Success for one sample (Monkeypox/PT0001/2022)

~92% of the reference sequence 7-fold depth of coverage

197kb genome sequence was manually inspected to validate variant positions



Shotgun metagenomics allowed the **rapid reconstruction and phylogenomic characterization of the first MPXV outbreak genome sequences**

https://virological.org/c/monkeypox/46

V vborges

First draft genome sequence of Monkeypox virus associated with the suspected multi-country outbreak, May 2022 (confirmed case in Portugal)

Monkeypox Genome Reports

May, 19th, 2022

https://virological.org/t/first-draft-genome-sequence-of-monkeypox-virus-associated-with-the-suspected-multi-country-outbreak-may-2022-confirmed-case-in-portugal/799

Multi-country outbreak of Monkeypox virus: genetic divergence and first signs of microevolution

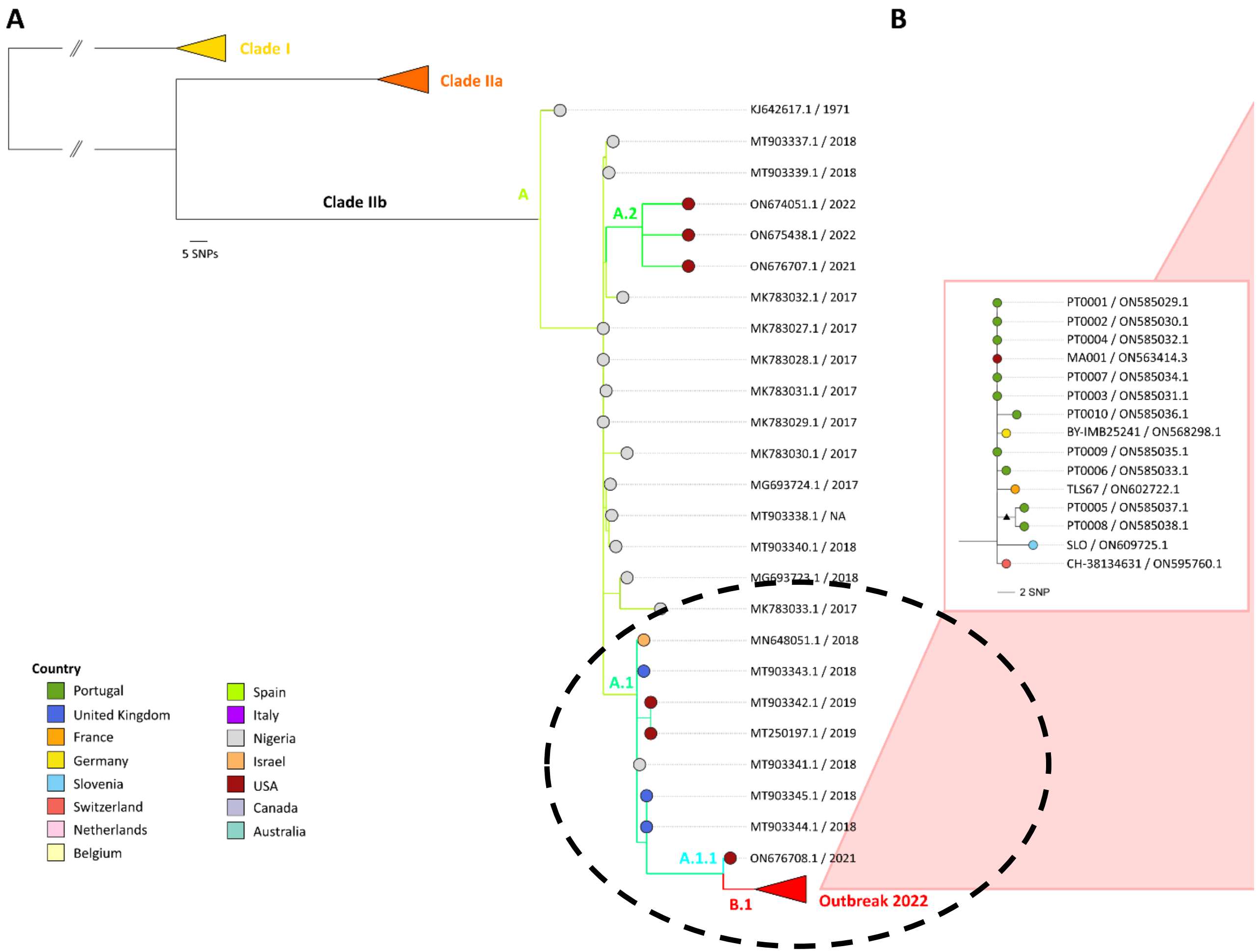
Monkeypox Genome Reports

May, 23rd, 2022

https://virological.org/t/multi-country-outbreak-of-monkeypox-virus-genetic-divergence-and-first-signs-of-microevolution/806

Mpox in Portugal

Emergence of a novel viral threat



- Shotgun metagenomics allowed the **rapid reconstruction and phylogenomic characterization of the first mpox outbreak genome sequences**, showing that this mpox belongs to (current) clade IIb (within the former "West African" clade)
- **All outbreak 2022 mpox strains tightly cluster together**, suggesting that the ongoing outbreak has a single origin.
- **Excess of accumulated mutations**, unexpected for a poxvirus
- **NGS is contributing to monitor in "real-time" the evolution of an outbreak-related pathogen.**

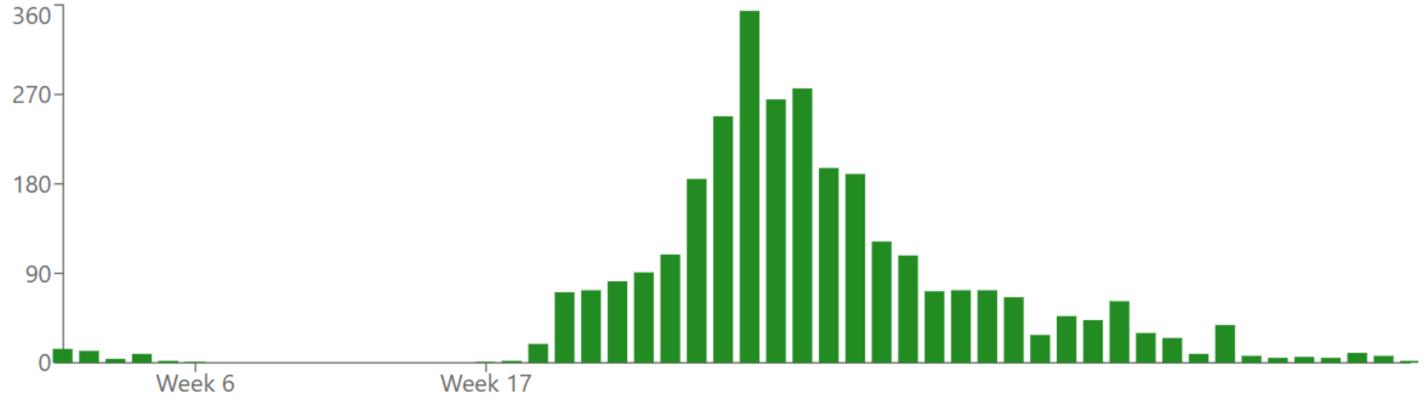
Isidro J, Borges V, Pinto M et al., (2022), Nat Med; 28(8):1569-1572.

mpoxSPECTRUM

https://mpox.genspectrum.org/

Sequences over time

Weekly bars | Monthly bars



Geographic distribution

Country	Number sequences ↓
USA	3125
Germany	831
Portugal	511
United Kingdom	311
Belgium	274
Colombia	222
Canada	141
Spain	57
Slovenia	42

5637 sequences (NCBI GenBank) in 2022

Recent development of multiplexed PCR amplicon schemas for WGS allowed scaling up mpox genomic data activities:

e.g.,

Chantal Vogels and colleagues (USA)

https://www.protocols.io/view/monkeypox-virus-multiplexed-pcr-amplicon-sequencin-5qpvob1nbl4o/v1

Matthijs Welkers and colleagues (The Netherlands)

https://www.protocols.io/view/monkeypox-virus-whole-genome-sequencing-using-comb-ccc7sszn.html