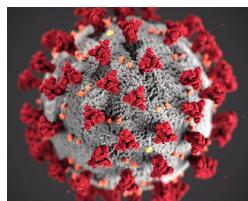


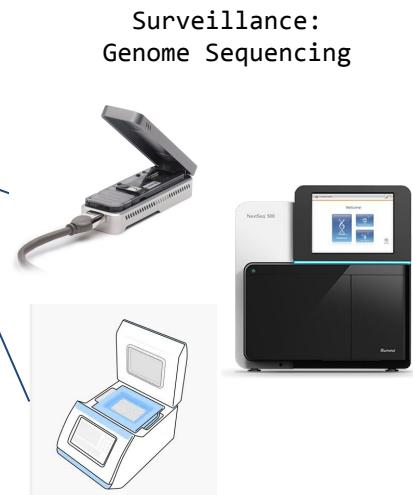
Introduction to viral genome reconstruction using massive sequencing: Sars-cov-2 use case

BU-ISCIII
Bioinformatics Unit, Institute of Health Carlos III
Madrid, Spain

1. Background

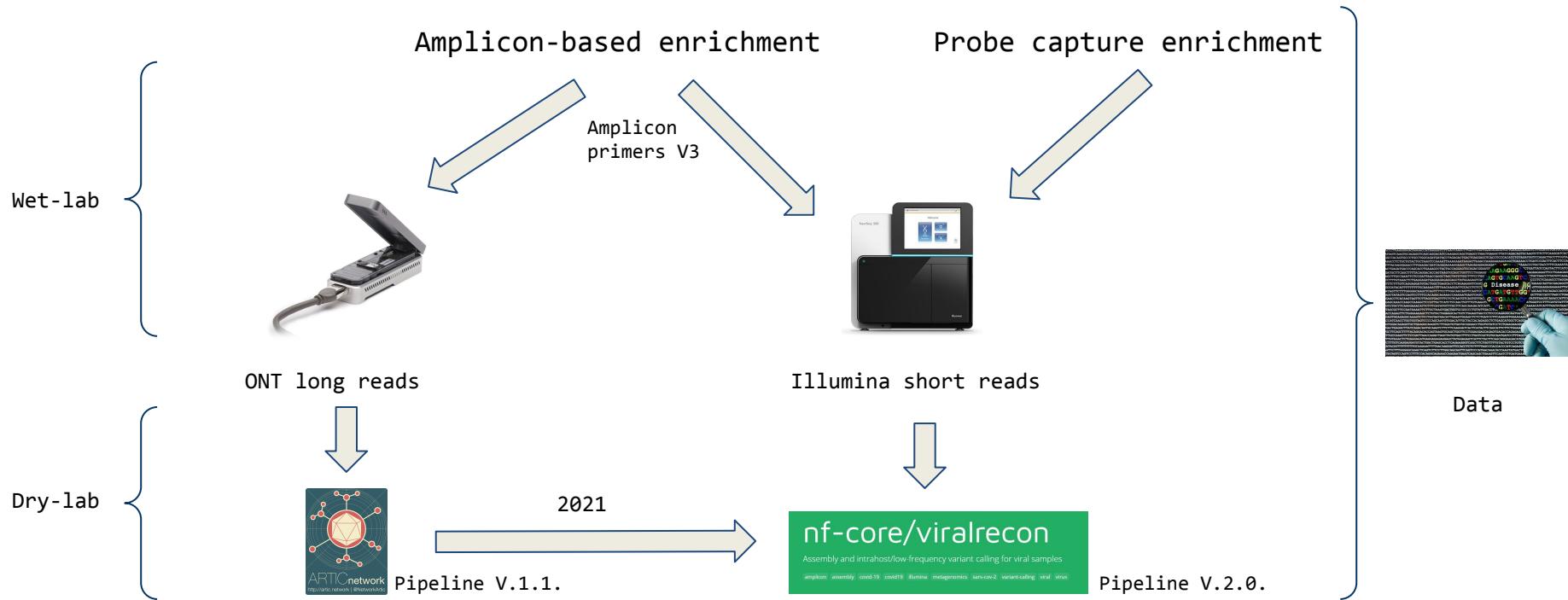


December 2019
SARS-CoV-2



- RT-PCR Primer & Probe efficiency
- Viral variability behaviour
- Correlate viral lineage & severity
- Vaccine development and efficiency surveillance
- Future established disease control (flu)
- Coordinated & rapid response
- Understand immune response to infection

2. Sequencing Approaches



3. History



January 22 2020 Artic Network protocols



https://github.com/BU-ISCIII/SARS_Cov2_consensus-nf



March 18 2020 Our 1st pipeline started



March 30 2020 nf-core collaboration



April 5 2020 1st COVID19 Virtual BioHackathon



<https://github.com/jaleezyy/covid-19-signal>

June 1 2020 viralrecon released v1.0.0



<https://github.com/nodrogluaP/nanostrripper>

June 29 2020 covid-19-signal v1.0.0



<https://github.com/nf-core/viralrecon>

September 3 2020 nanostrripper v1.0.0

May 13 2021 viralrecon v2.0 release

4. Viralrecon

nf-core/ 
viralrecon

<https://github.com/nf-core/viralrecon>



nextflow

Multiple compute infrastructures

Portable

Easy to install

Reproducible

Stable code



The need of standardisation I

Sequencing techniques are starting to be used in clinical diagnosis, and therefore workflows have to assure:

- **Reproducibility**

Results always have to be reproducible

- **Portability**

The analysis workflow must be executable in different platforms

- **Scalability**

The analysis workflow must be able to work with different numbers of samples

Nextflow I

- **Nextflow** is a DSL for parallel and scalable computational pipelines.
- It enables **scalable and reproducible scientific workflows** using software **containers**.
- It **allows the adaptation of pipelines** written in the most common scripting languages.
- Its fluent **DSL** simplifies the implementation and the deployment of complex parallel and reactive workflows on clouds and clusters.

Nextflow II

Fast prototyping

Nextflow allows you to write a computational pipeline by making it simpler to put together many different tasks.

You may reuse your existing scripts and tools and you don't need to learn a new language or API to start using it.

Portable

Nextflow provides an abstraction layer between your pipeline's logic and the execution layer, so that it can be executed on multiple platforms without it changing.

It provides out of the box executors for SGE, LSF, SLURM, PBS and HTCondor batch schedulers and for [Kubernetes](#) and [Amazon AWS](#) cloud platforms.

Continuous checkpoints

All the intermediate results produced during the pipeline execution are automatically tracked.

This allows you to resume its execution, from the last successfully executed step, no matter what the reason was for it stopping.

Reproducibility

Nextflow supports [Docker](#) and [Singularity](#) containers technology.

This, along with the integration of the [GitHub](#) code sharing platform, allows you to write self-contained pipelines, manage versions and to rapidly reproduce any former configuration.

Unified parallelism

Nextflow is based on the *dataflow* programming model which greatly simplifies writing complex distributed pipelines.

Parallelisation is implicitly defined by the processes input and output declarations. The resulting applications are inherently parallel and can scale-up or scale-out, transparently, without having to adapt to a specific platform architecture.

Stream oriented

Nextflow extends the Unix pipes model with a fluent DSL, allowing you to handle complex stream interactions easily.

It promotes a programming approach, based on functional composition, that results in resilient and easily reproducible pipelines.

Nextflow III: nf-core

The nf-core project is a diverse project spread across many groups. It is a community effort to collect a curated set of analysis pipelines built using Nextflow.



nf-core/mag ✓ ★ 42

annotation assembly binning metagenomics

Assembly and binning of metagenomes

Version 1.2.0 Published 3 weeks ago

nf-core/ampliseq ✓ ★ 54

16s amplicon-sequencing metagenomics qiime rrna

16S rRNA amplicon sequencing analysis workflow using QIIME2

Version 1.2.0 Published 4 weeks ago

nf-core/sarek ✓ ★ 93

annotation cancer gatk4 genomics germline pre-processing somatic variant-calling

Analysis pipeline to detect germline or somatic variants (pre-processing, variant calling and annotation) from WGS / targeted sequencing

Version 2.7 Published 1 month ago

nf-core/eager ✓ ★ 45

adna ancient-dna-analysis ancientDNA genome metagenomics pathogen-genomics population-genetics

A fully reproducible and state-of-the-art ancient DNA analysis pipeline

Version 2.3.1 Published 2 months ago

nf-core/cageseq ✓ ★ 3

cage cage-seq cageseq-data gene-expression rna

nf-core/rnaseq ✓ ★ 298

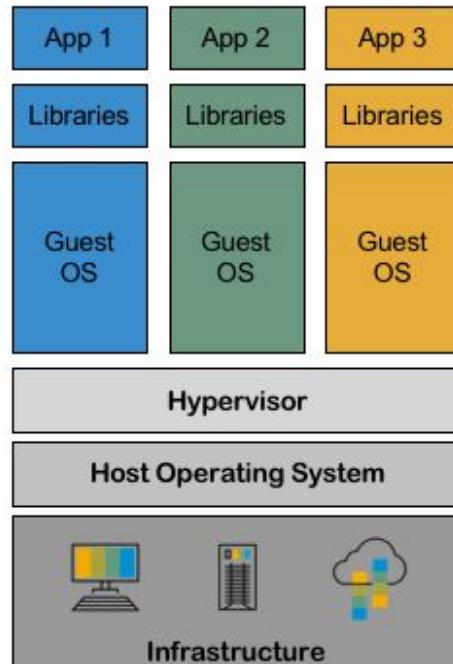
rna rnaseq

Containers I

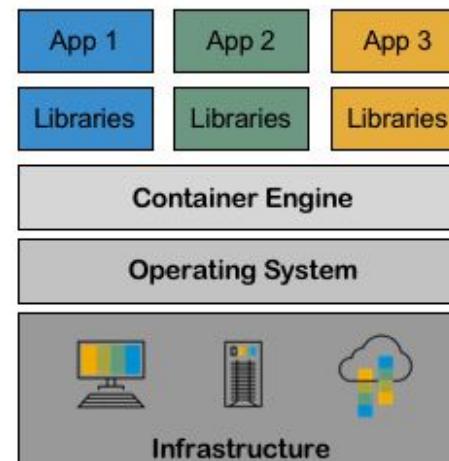
Linux containers is a generic term for an implementation of operating system-level virtualization for the Linux operating system.

Containers allow us to **port** pipelines and **replicate** their exact execution environments across different hardware.

Singularity I



Hypervisor virtualization



Container virtualization

Singularity II

Singularity is a free, cross-platform and open-source computer program that performs operating-system-level virtualization.

One of the main uses of Singularity is to bring containers and **reproducibility to scientific computing** and the HPC world.

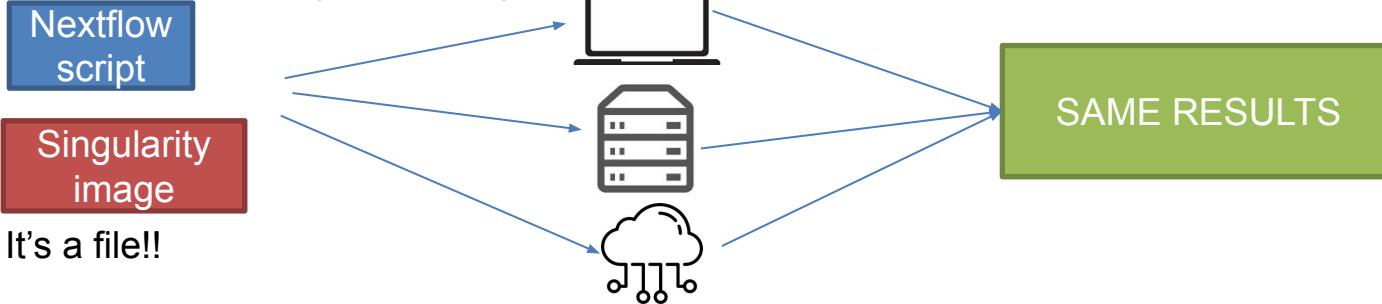
While Docker is broadly used, Singularity is **fully compatible with Docker**, plus Singularity does **not require root permissions** to be executed.

And now is when you think...

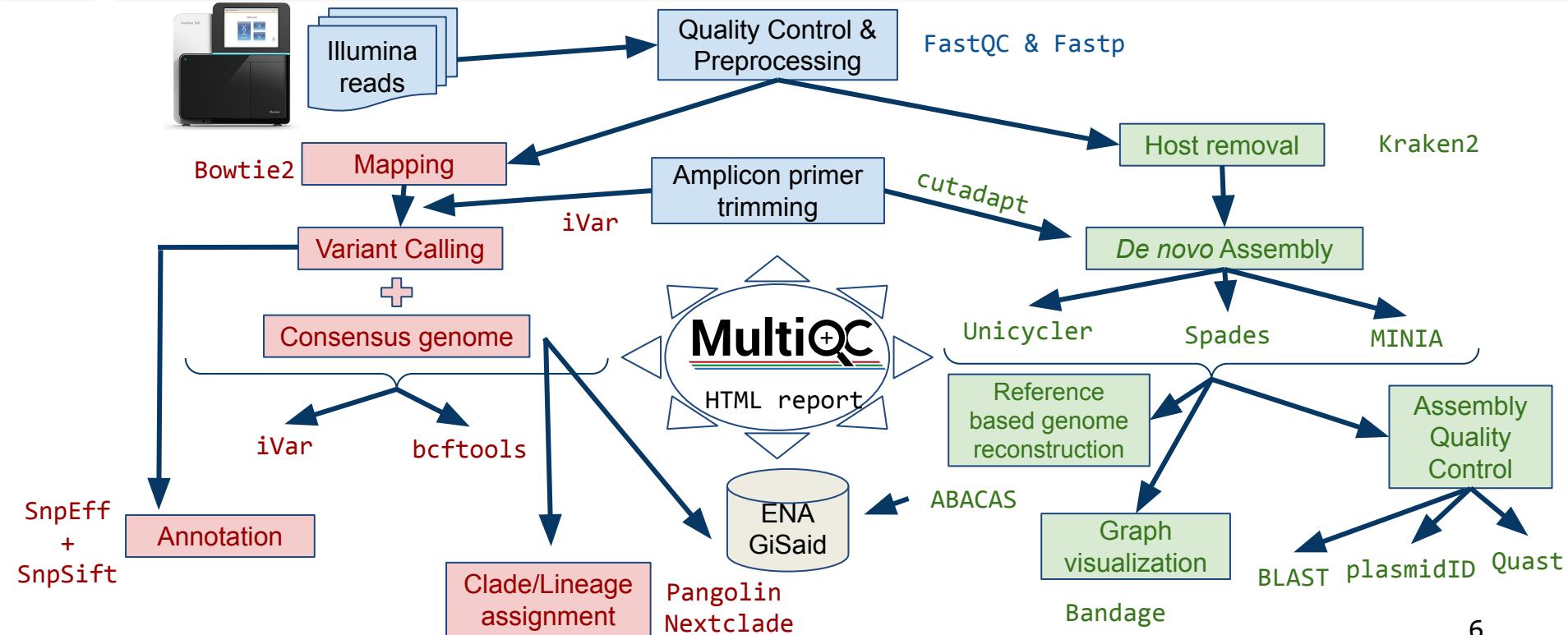
**How all this hard to understand
stuff can make my life
easier???**

How all this hard to understand stuff can make my life easier?

- Reproducibility is imperative in clinical bioinformatics.
- Once this is all set you can just:
 - Run your pipeline ANYWHERE getting same results.
 - With ANYWHERE I mean your laptop, your home pc, your computation cluster, just one Amazon server you rent for some time...
 - You don't need to change ANYTHING in your code, just some config.
 - You don't need to install ANYTHING anywhere EVERYTHING is installed and in the same versión in your shiny c



4. Viralrecon for Illumina reads



4. Viralrecon ISCIII



GENOMICS UNIT

Sequencing



192

48h



UTIC - CPD *utic*

Infrastructure
Data management
Storage



2h



320 cores
4TB RAM



BIOINFORMATICS UNIT

>gtex_BU_ISCIII

Data processing
Consensus genome sequence
Variants / Lineage Report

nf-core/ viralrecon



<https://github.com/nf-core/viralrecon>

30min



Input data

Which input format do we have from massive sequencing?

FASTQ format

- Is a FASTA file with quality information
- Within HTS, FASTA contain genomes y FASTQ reads

>SEQ_ID

```
AGCTTTCTTGACTGCAACGGGAAATATGTCTCTGTGTGGATTAAAAAAAGAGTGTCTGATAGCAGC  
TTCTGAAGTGGTACCTGCCGTGAGTAAATTAAATTATTGACTTAGGTCACTAAATACTTAAACCAA  
TATAGGCATAGCGCACAGACAGATAAAAATTACAGAGTACACAACATCCATGAAACGCTTAGCACCACC  
ATTACCAACCACCATCACCATTACCAACAGGTAAACGGTGCAGCTGACCGTACAGGAAACACAGAAAAAG
```

@SEQ_ID

```
GATTTGGGTTCAAAGCAGTATCGATCAAATGTAATCCATTGTTCAACTCACAGTTT
```

+

```
! ''*( (( (**+) %%++ ) %% . 1***-+*'') )**55CCF>>>>CCCCCCC65
```

Sequence

Quality: must be 1 bit

Let's run the pipeline!!

Sequencing quality assessment

- To assess quality, software uses **Phred per-base quality score** is used
- Is the **first quality control step** after sequencing. There should be one after every step of the analysis
- After quality assessment user can know how **reliable** are their datasets
- QC will determine the next **filtering** step
- Filtering decisions will **impact** directly in **further analysis**
- Many other steps also use this quality as variable in their **algorithms**

FastQC: Basic Statistics

- Self defined overall stats
 - Encoding: Phred33 or Phred64



Basic Statistics

Measure	Value
Filename	bad_sequence.txt
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	395288
Sequences flagged as poor quality	0
Sequence length	40
%GC	47

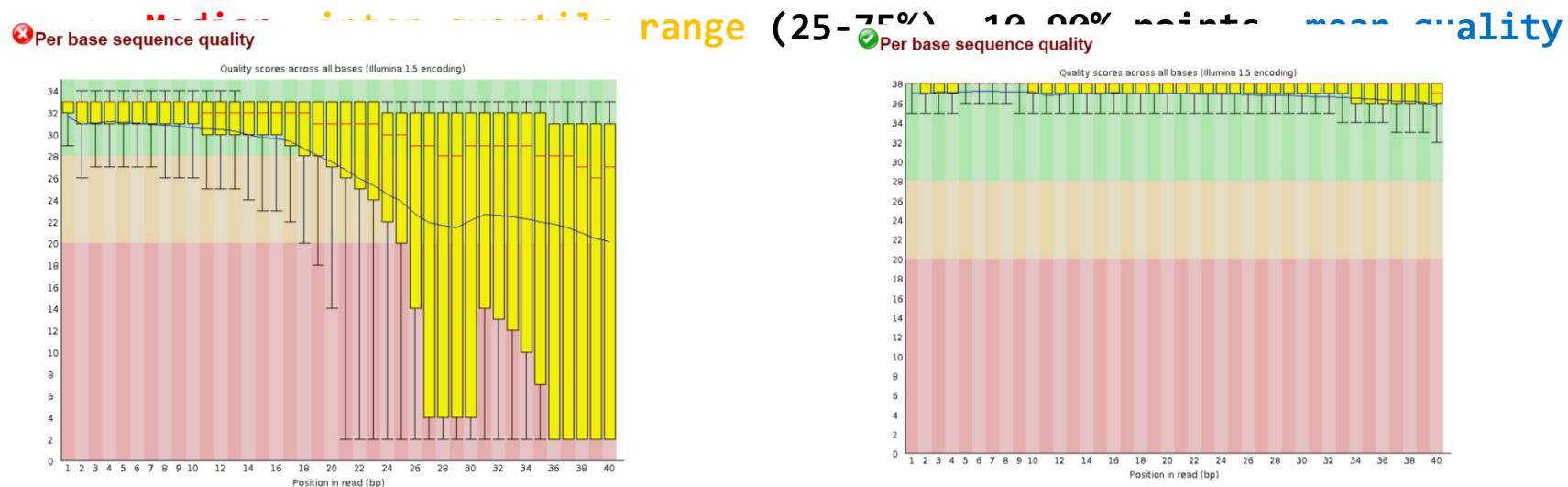


Basic Statistics

Measure	Value
Filename	good_sequence_short.txt
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	250000
Sequences flagged as poor quality	0
Sequence length	40
%GC	45

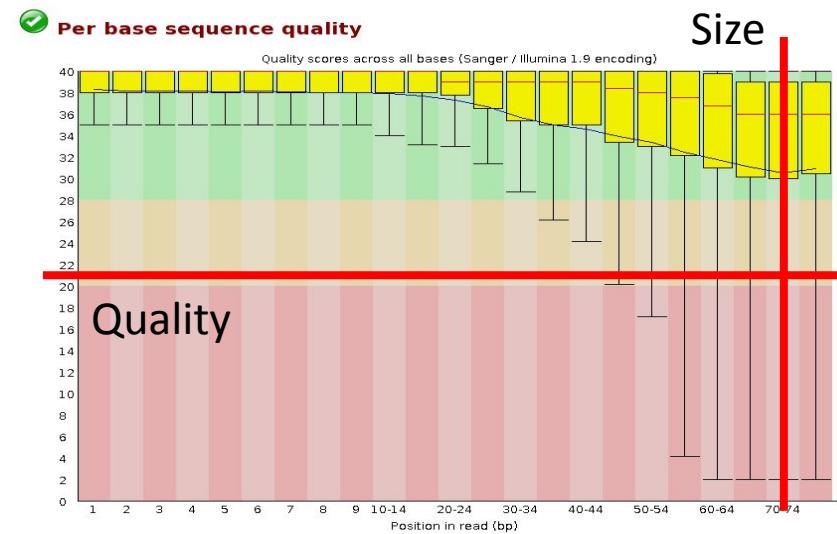
FastQC: Per base sequence quality

- Overview of the range of quality values across all bases at each position in the FastQ file



Sequence filtering

- Remove residual adapters
 - Depending on used library
- Filtering parameters
 - Quality filtering
 - Overall mean quality
 - Local mean quality
 - Sequence end
 - Sliding window
 - Size filtering
 - Overall sequence size
 - Remaining sequence size after filtering



Quality control results

Mapping

BOWTIE2

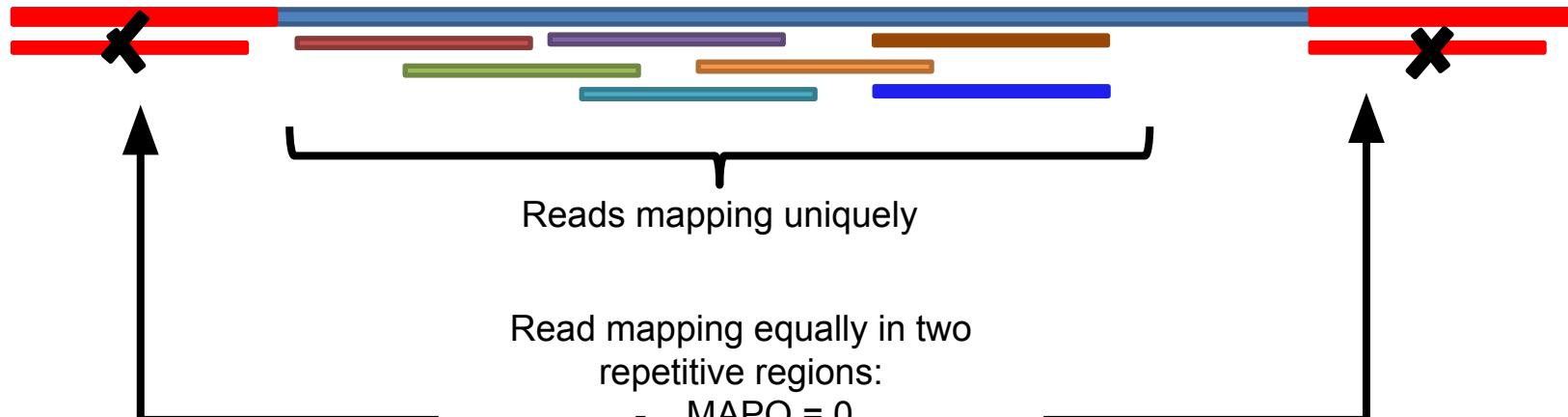


Mapping

BOWTIE2

Mapping software looks for the best match for each read in the genome.
Paired-end reads help the mapper to find the perfect spot!

Reference genome



Depends on parameters!!

Input data

Which output format do we have from mapping step?

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

```

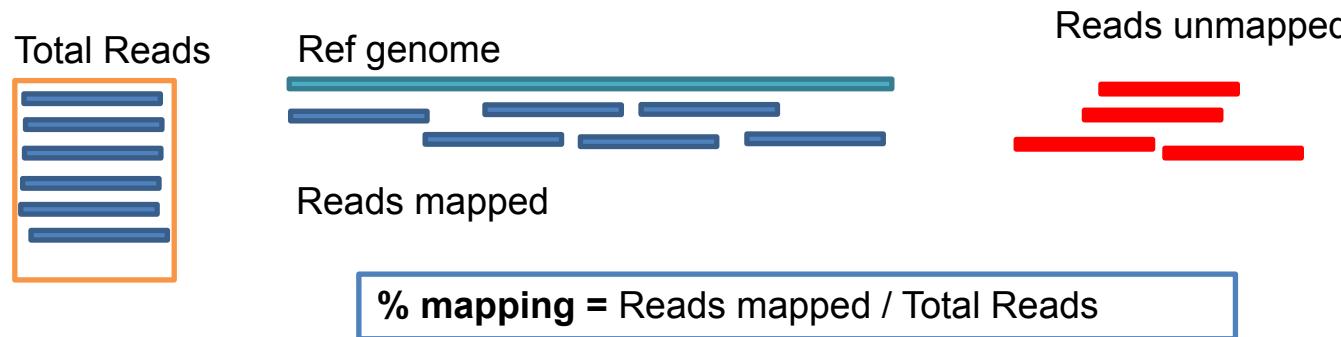
@HD VN:1.5 SO:coordinate
@SQ SN:ref LN:45
r001 99 ref 7 30 8M2I4M1D3M = 37 39 TTAGATAAAGGATACTG *
r002 0 ref 9 30 3S6M1P1I4M * 0 0 AAAAGATAAGGATA *
r003 0 ref 9 30 5S6M * 0 0 GCCTAACGCTAA * SA:Z:ref,29,-,6H5M,17,0;
r004 0 ref 16 30 6M14N5M * 0 0 ATAGCTTCAGC *
r003 2064 ref 29 17 6H5M * 0 0 TAGGC * SA:Z:ref,9,+,5S6M,30,1;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

```

Mapping quality control

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

Picard
Samtools

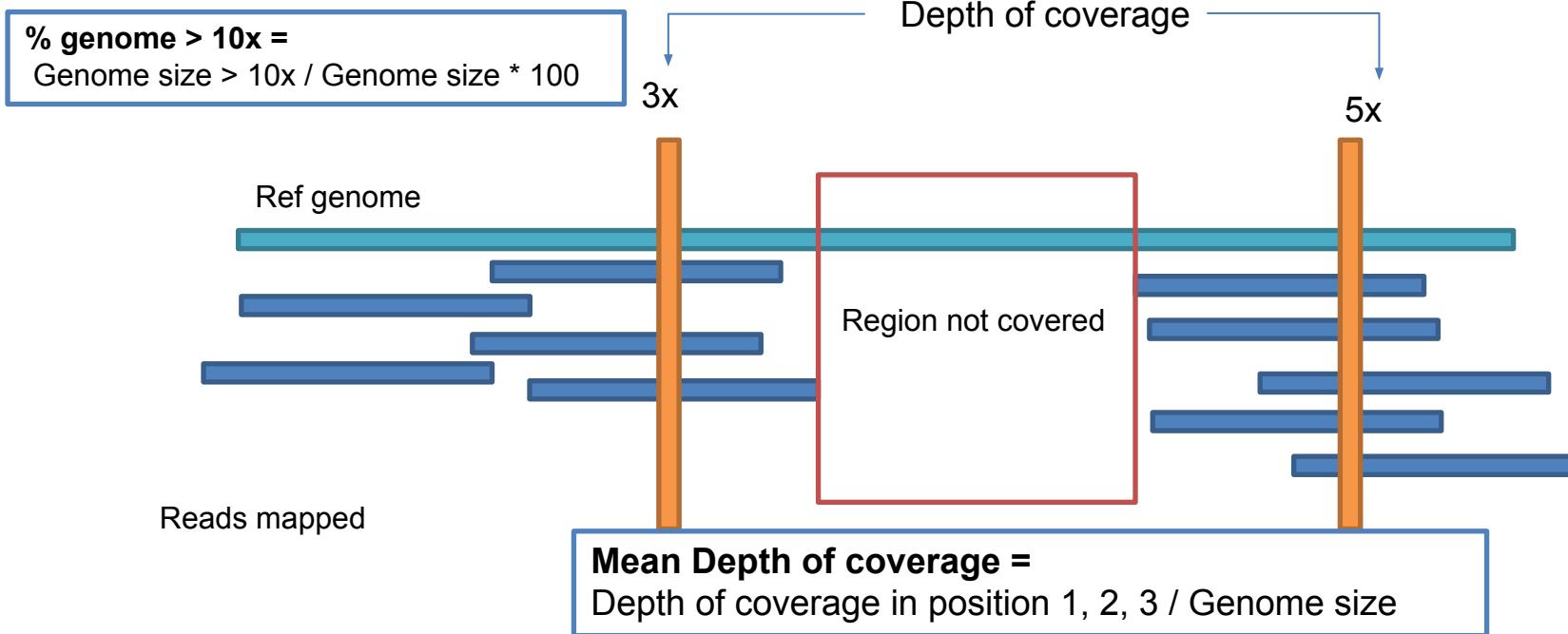


Mandatory parameter for microbial genomics!! It indicates us how many reads we have from our organism of interest. In human genomics this is almost always 99.99% unless something terrible happens. Not here!!!

Mapping quality control

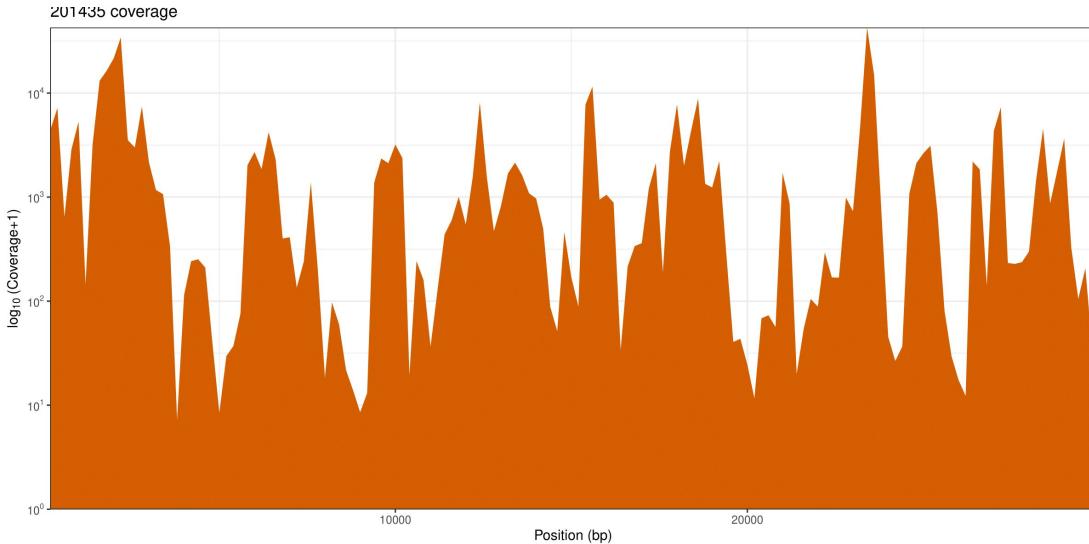
- **% genome > 10x:** percentage of genome covered with more than 10 reads.
- **Mean Depth of coverage:** mean of reads covering a genome position.

Picard
Samtools

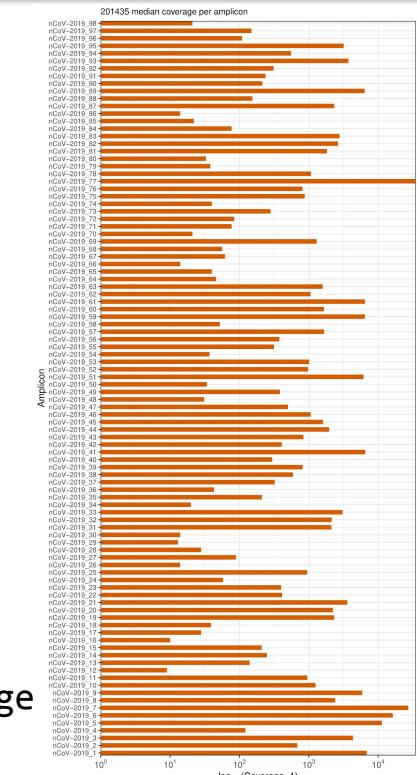


Amplicon QC results

Genome coverage



Amplicon coverage



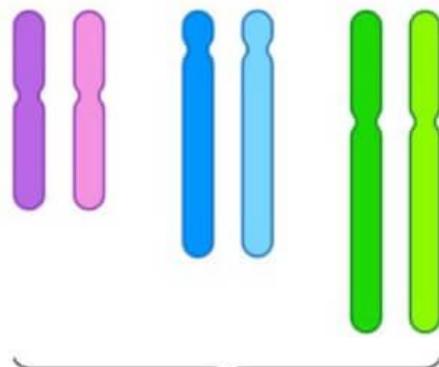
Mapping results

Diploid vs Haploid

Differences between Diploid and Haploid

Diploid ($2n$)

Two copies of each chromosome



Three pairs of homologous chromosomes
(of maternal and paternal origin)

Haploid (n)

One copy of each chromosome



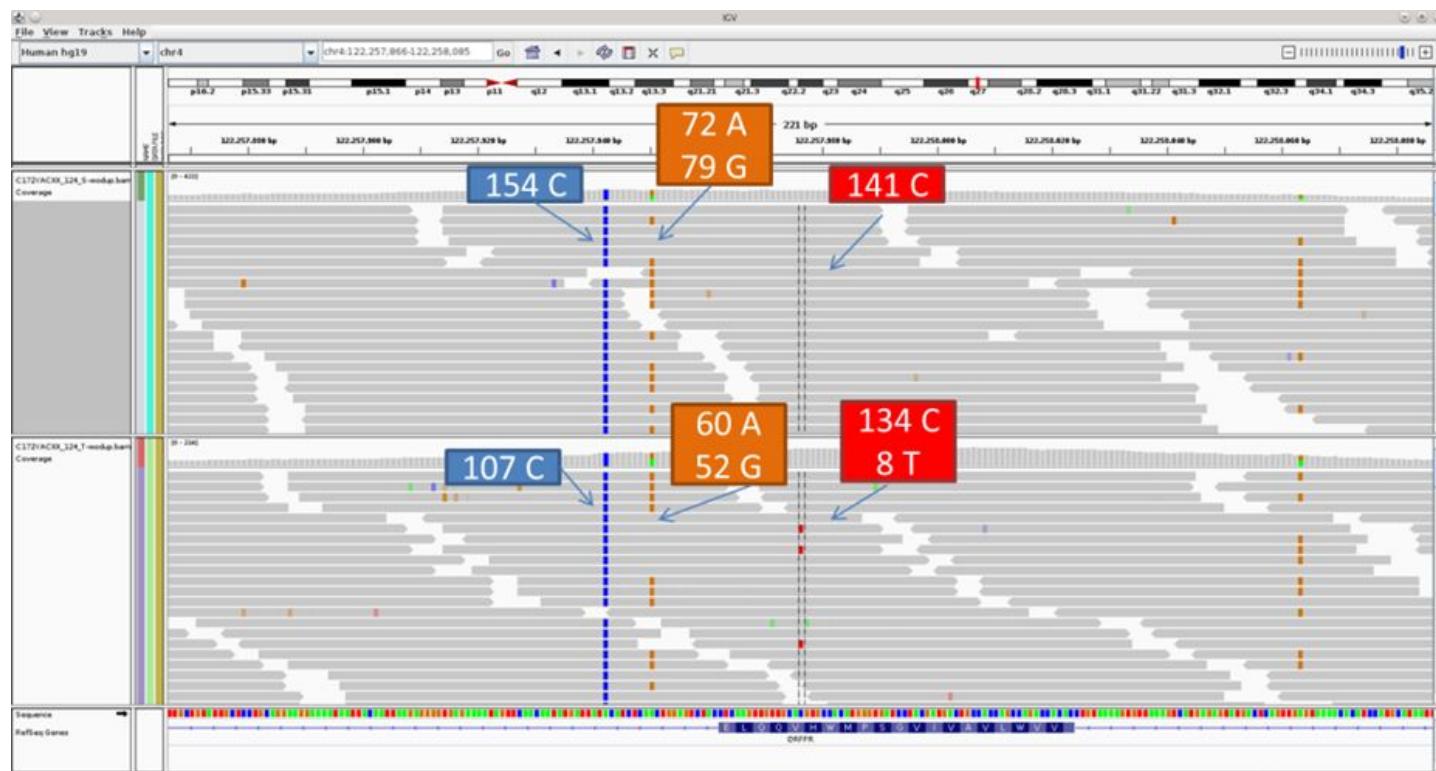
Three non-homologous chromosomes



Diploid vs Haploid

How do we see this differences on massive sequencing?

Diploid vs Haploid

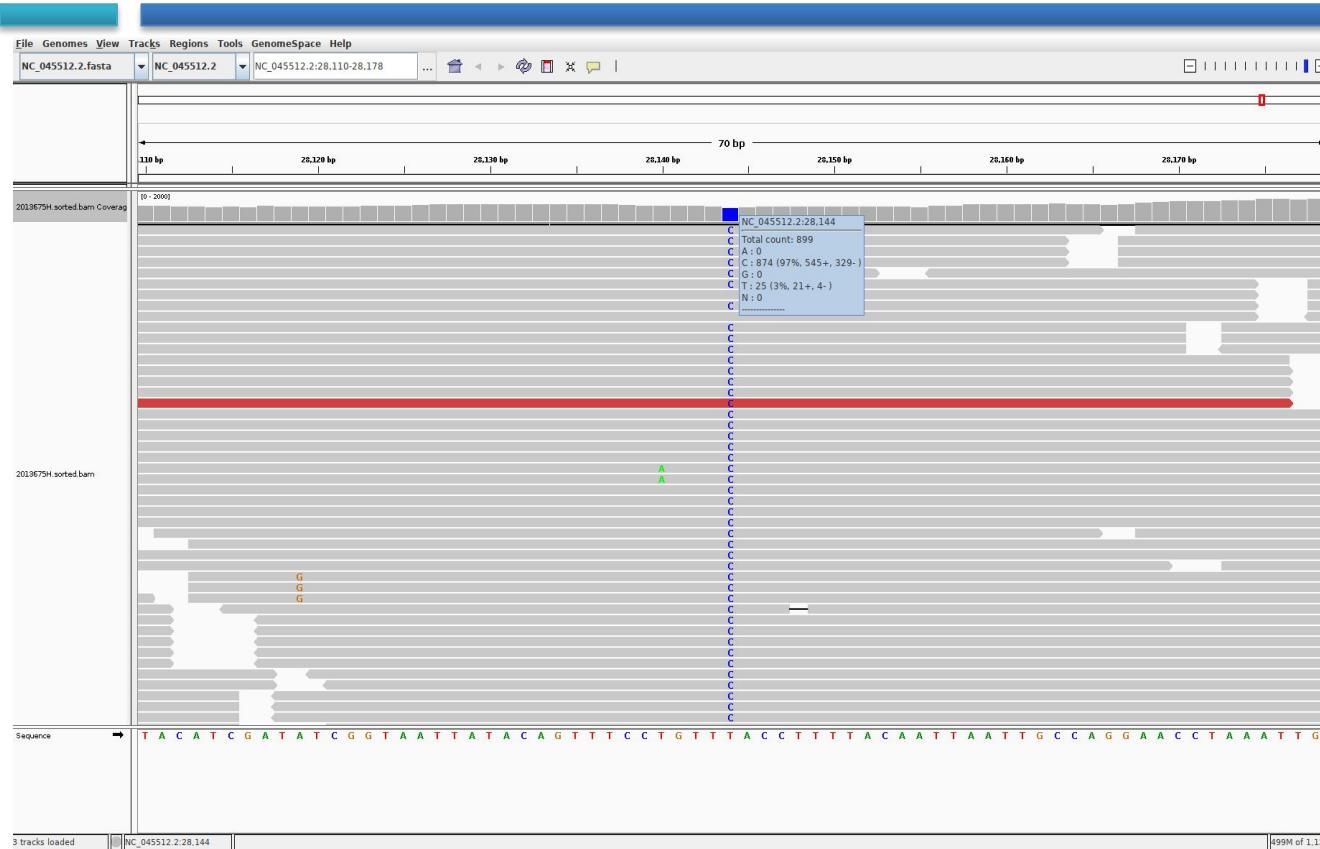


We must say variant callers which type of genotypes we are looking for.

Diploid: 1/1,
0/1 or 1/1

Haploid:1 or 0.

Variant calling



Variant callers walk through each alignment column independently and evaluate if there is a variant.

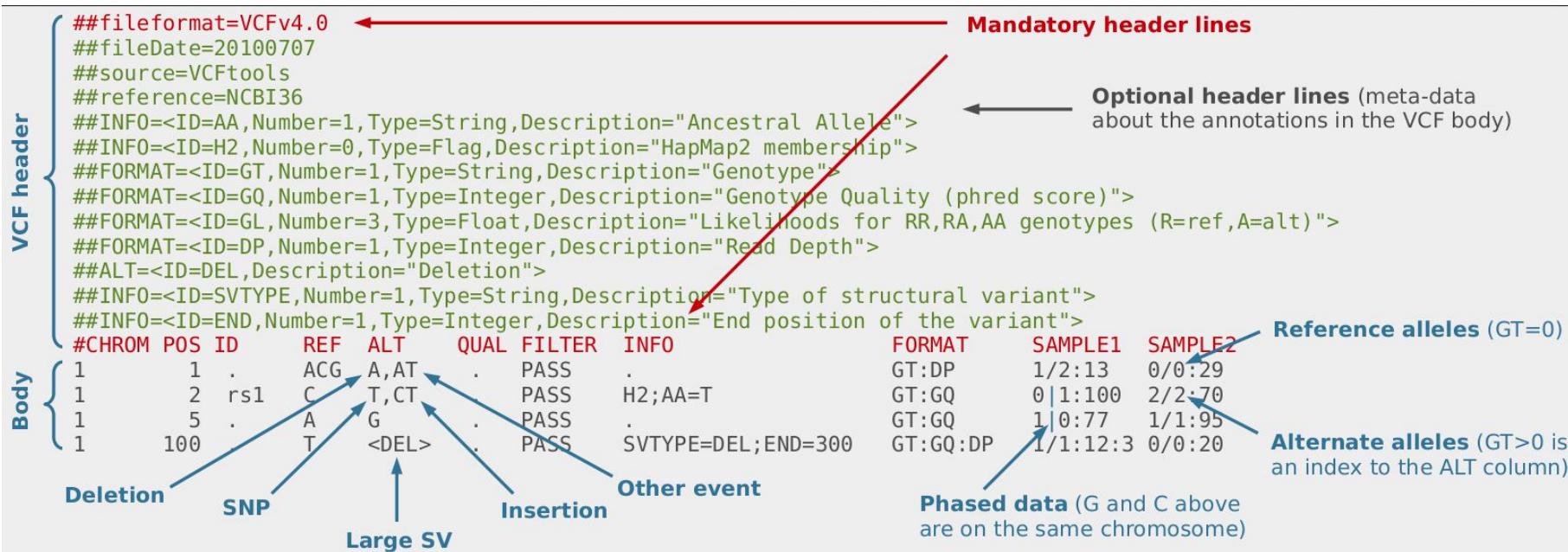
A variant is called in haploid genomes when it's supported by at least 90% of the reads.

VARSCAN2
IVAR

Input data

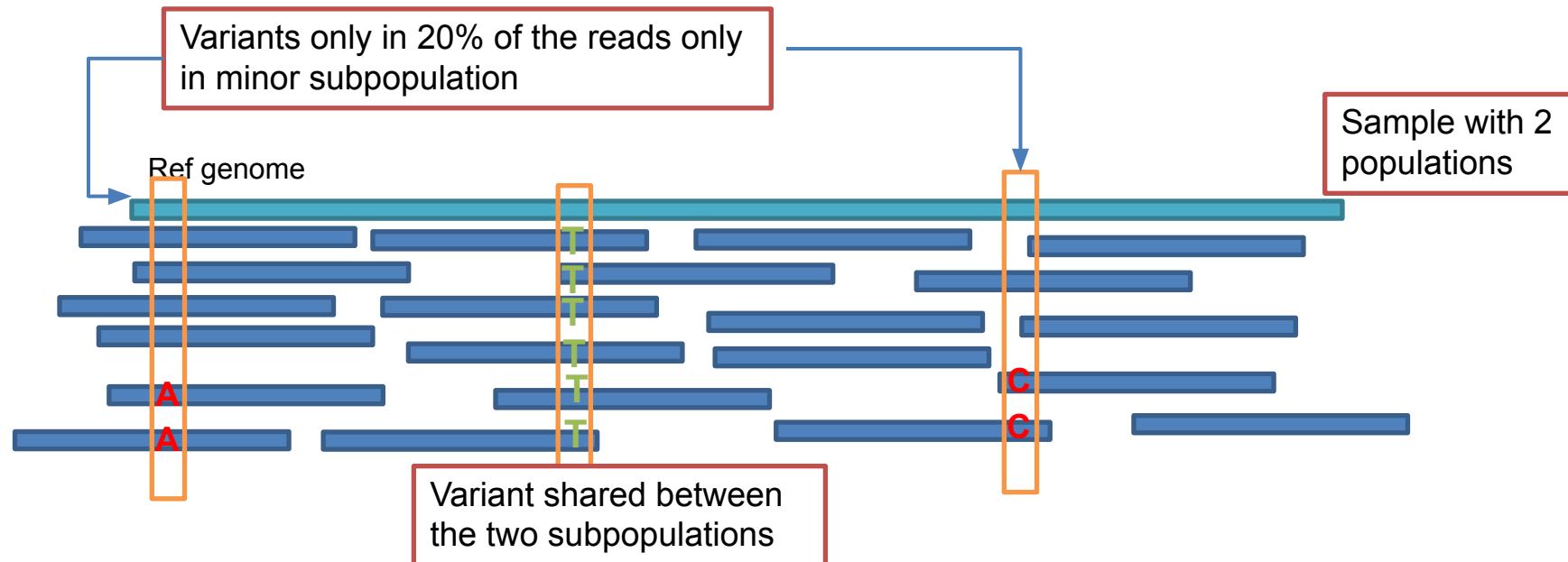
Which output format do we have from variant calling step?

VCF format



Viral subpopulation - Quasispecies

- Just as in clonal subpopulations in tumor samples, we can have viral subpopulations called quasispecies in viral samples.
- We detect them using the alternative allele frequency.



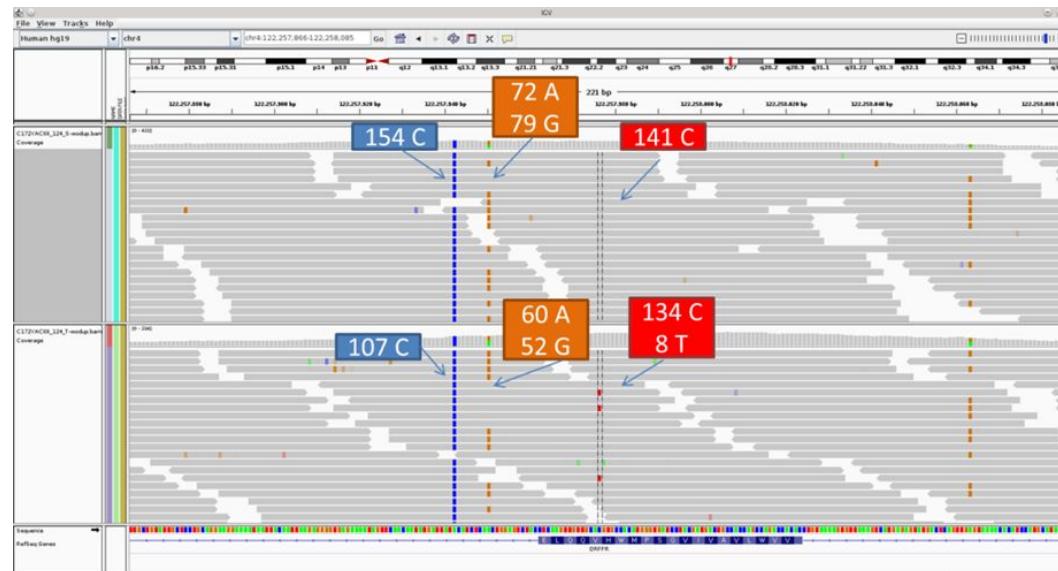
Population Allele frequency vs Sample Allele frequency

- **Population allele frequency:** probability of finding an allele in the population. Number of individuals carrying an allele vs total of individuals in the population.



Population Allele frequency vs Sample Allele frequency

- **Alternate/Base allele frequency:** number of reads supporting the alternate allele vs total of reads.



Variant calling results

Consensus genome

- Select variants: > 90% allele frequency
- Include variants in reference genome.
- Mask low frequency positions: <10x.

Ref genome



Variants > 90%

265 T
1050 G
10233 A

Consensus genome



Mask low depth of coverage regions



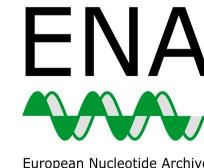
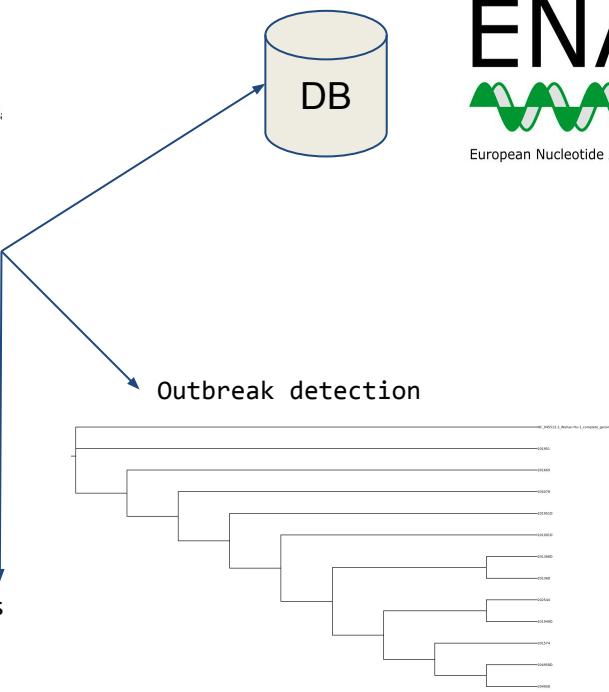
Consensus and lineage results

And...after all this?

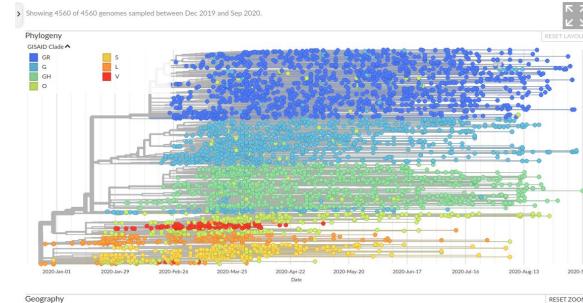
```
>NC_045512.2 Severe acute respiratory syndrome coronavirus 2 isolate Wuhan complete genome
ATTAAAGGTTTATACCTTC CAGGTAAACAAAC CAACCAACTTGCATCTTGATCTTGAGATCTGTTCTTAA
CGAACTTTAAATCTGTGGCTGTCACTGGCTGAGCTTAGTGCACTCACGAGTATAATTAAAC
TAATTACTGTGCTTGACAGGACACGAGTAACTCGTCTATCTTGCAAGGCTGCTTACGGTTCTGCGTCCGTG
TTGCAGCCGATCATCAGCACATCTAGGTTCTGCCGGTGTGACCGAAAGGTAAGATGGAGAGCTTGTG
CCTGGTTAACAGGAAAACACAGTCCAACCTAGTTGGCTGTGTTACAGGTTGGCAGCTGCTGTGAC
GTGGGTTGGAGACTCGTGTGGAGGGAGTCTTACAGAGGCACGTCACATCTTAAAGATGGCAGTGTG
CTTAGTGAAGGTTGAAAAGGGGTTTGCTTCACTTAAAGGCTTATGTGTCATCAAACAGTTGGAT
GCTGCAACTGCACTCATGGTCATGTTATGTTGAGCTGGTACGAAACTCGAAGGGCATTCAGTGGTC
GTAGTGTGAGACACTTGTGCTTGTCTTGTCTTGTGCTTGTGCTTGTGCTTGTGCTTGTGCTTGTG
TCTTGTGAGACACTTGTGCTTGTCTTGTGCTTGTGCTTGTGCTTGTGCTTGTGCTTGTGCTTGTG
TCTTGTGAGACTCATGGTGAAGCTTAAAGGAGCTTAAAGGAGCTTAAAGGAGCTTAAAGGAGCTTAAAGG
GCGCAGACGCTTGGCACTGATCTTATGAAAGATTTCAGAAAAACTGGCAACACTAACATAGCAGTTGG
TTCAGCTTGGTACCCCTCTTGAGTCATTAAGAACCTTCTAGCAGCTGCTGTTAAAGCTTCTGCACTTGG
TCCGAAACACTGGACTTTATGAGACTAAGAGGGGTGTTAGTACTGCTGCCGTGAAAGAT6AGCATGAAATTG
CTTGGTACCGGAAAGCTCTGAAAGAGCTTGAATTTCAGACACCTTGGAAATTAAATTGGAAAAGGA
ATTGACACCTTCAATGGGAATGTCAAATTGTTGATTTCCCTTAAATTCCATAATCAAGACTTCAA
CCAAGGGTTGAAAAGAAAAGCTTGTGCTTATGGGTAGAATTGATCTGCTATCCAGTGTGCTAC
```

.fasta consensus genome

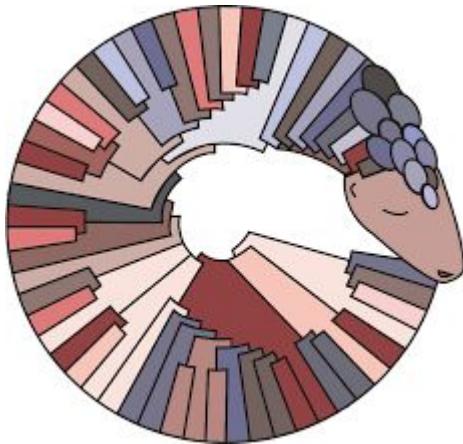
Other analyses



Clade classification



Linage assignment: Pangolin



pangolin assigns lineages to query sequences as described in Rambaut et al 2020.

<https://cov-lineages.org/>

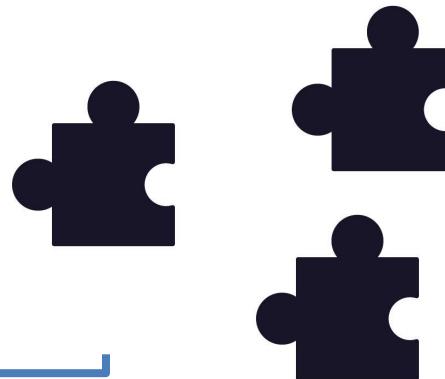
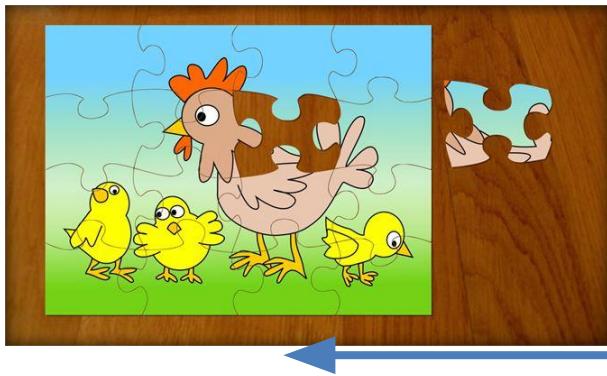
Assembly concepts

Assembly

Reconstruct a representation of the original DNA from shorter DNA sequences or small fragments known as reads

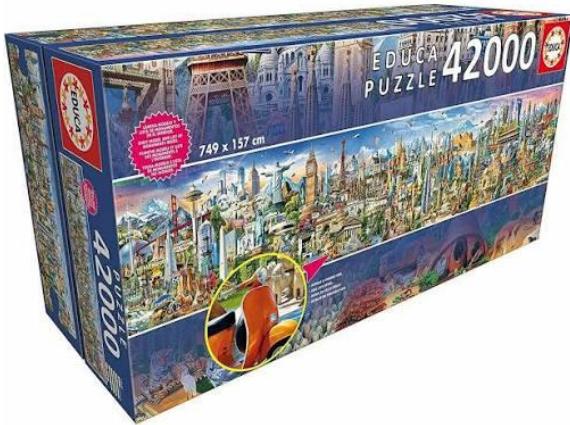
- ***De novo***: with no previous knowledge of the genome to be assembled. It overlap the end of the end of each read in order to create a longer sequence.
- ***Assembly with reference***: A similar but not identical genome guides the assembly process. Map reads over supplied genome.

Assembly



The bigger the pieces the easier to reconstruct the puzzle!

Assembly



Obviously this a little bit
more complicated....

We have LOADS of really
little pieces and a big puzzle
in proportion

Assembly

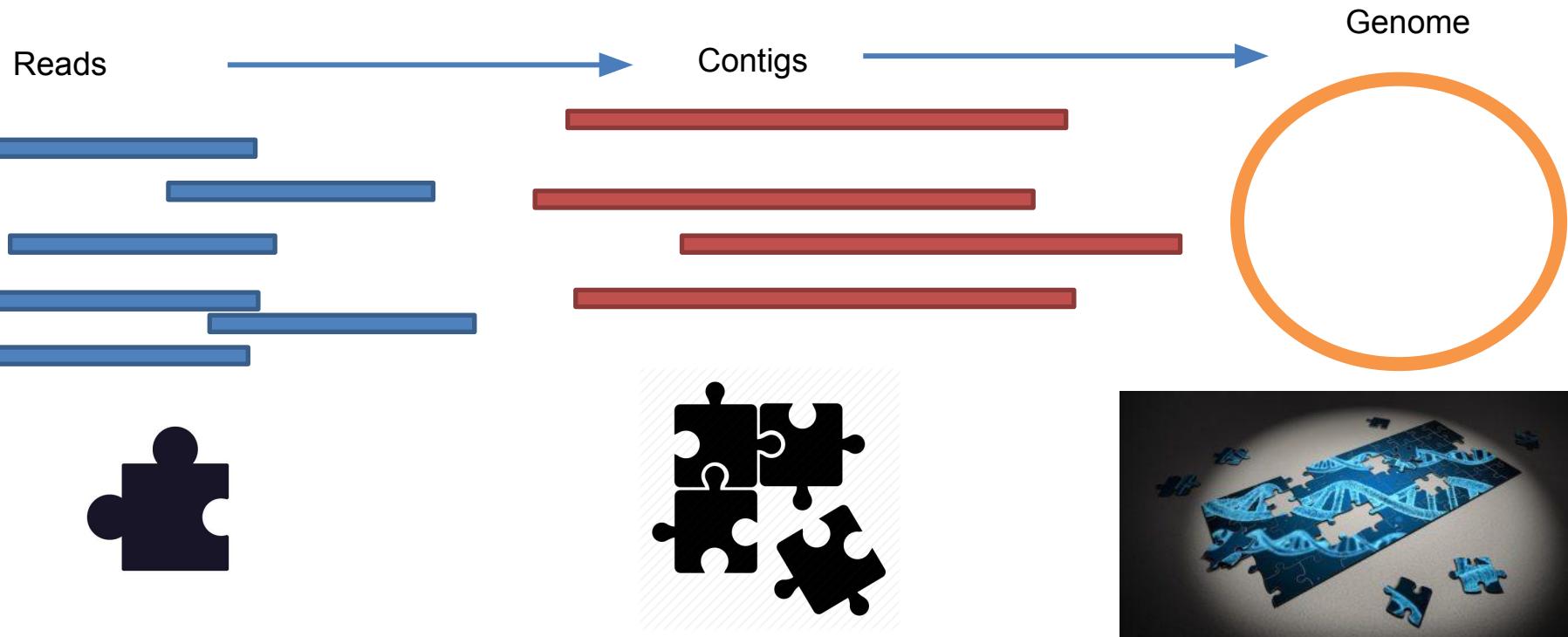


Obviously this a little bit
more complicated....



Actually we don't even have
the box image most of the
time

Assembly



Let's run the assembly
steps

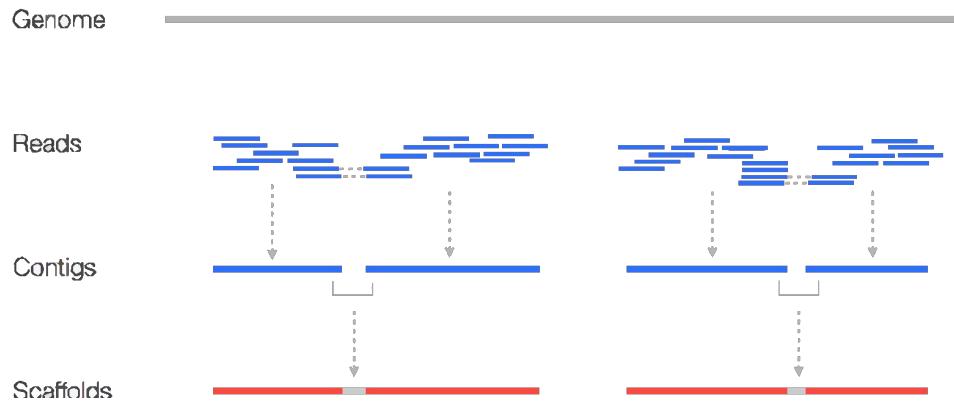
Assembly

Reconstruct a representation of the original DNA from shorter DNA sequences or small fragments known as reads

- ***De novo***: with no previous knowledge of the genome to be assembled. It overlap the end of the end of each read in order to create a longer sequence.
- ***Assembly with reference***: A similar but not identical genome guides the assembly process. Map reads over supplied genome.

Assembly: contig y scaffold

- **Contig:** continuous sequence made up of overlapping shorter sequences
- **Scaffold:** two or more contigs located and rearranged according to spatial information(pair-end, mate pair, reference)



<https://www.biostars.org/p/253222/>

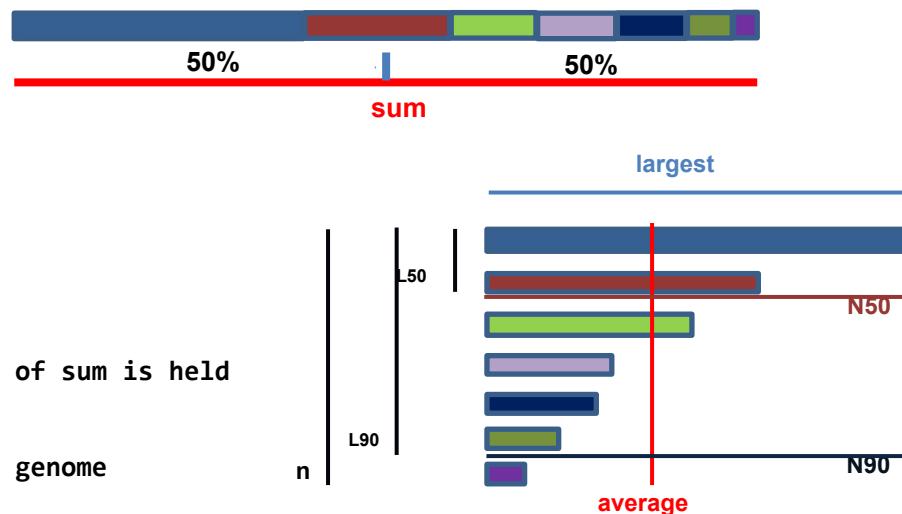
Assembly polishing and quality control

- Abacas sort contigs according to reference genome.
- Scaffolding
- Visualization

Assembly results

Assembly polishing and quality control

- sum = total bases number
- n = contigs number
- average = average contig length
- largest = largest contig
- N50 = length of the shortest contig where 50% of sum is held
- L50 = number of contigs which have 50% of the genome
- N90 = length of the shortest contig where 90% of sum is held.
- L90 = number of contigs which have 90% of the genome



MultiQC and summary stats

Conclusions

You can find
viralrecon in:

<https://github.com/nf-core/viralrecon>



[nf-core / viralrecon](https://github.com/nf-core/viralrecon)
forked from drpatelh/nf-core-viralrecon

[Code](#) [Issues 6](#) [Pull requests 1](#) [Actions](#) [Security](#) [Insights](#)

This branch is 1109 commits ahead of drpatelh:master.

File	Description	Last Commit
.github	Merge branch 'dev' into dev	3 months ago
assets	Minor fixes	3 months ago
bin	Update code	3 months ago
conf	Update time	4 months ago
docs	Update docs	3 months ago
.gitattributes	initial template build from nf-core/tools, version 1.9	6 months ago
.gitignore	initial template build from nf-core/tools, version 1.9	6 months ago
CHANGELOG.md	Merge branch 'dev' into dev	3 months ago
CITATIONS.md	Add mosdepth	3 months ago
CODE_OF_CONDUCT.md	initial template build from nf-core/tools, version 1.9	6 months ago
Dockerfile	Bump versions	3 months ago
LICENSE	initial template build from nf-core/tools, version 1.9	6 months ago
README.md	Fix markdownlint	3 months ago
environment.yml	Add biostings	3 months ago
main.nf	Fix naming	3 months ago
nextflow.config	Add --min_mapped_reads param and do some cool stuff with it	3 months ago

[Clone with SSH](#) [Use HTTPS](#)
Use a password protected SSH key.
`git@github.com:nf-core/viralrecon.git`

[Download ZIP](#)

About
Assembly and intrahost/low-frequency variant calling for viral samples

Tags
nf-co.re/viralrecon

viral metagenomics amplicon assembly variant-calling illumina pipeline workflow nextflow nf-core covid-19 covid19 virus sars-cov-2

Readme

License
MIT License

Releases 2
[nf-core/viralrecon v1.1.0 - S... \[Latest\]](#)
on Jun 23

+ 1 release

Packages
No packages published
Publish your first package

Languages


Language	Usage (%)
Nextflow	70.7%
Python	20.1%
R	7.3%
HTML	1.5%
Dockerfile	0.4%

Thanks for your attention!

Thanks to all my colleges in BU-ISCIII



Thanks to Genomics Unit and the
Reference Laboratory Labs in
CNM



Sarai Varona
Luis Chapado
Pablo Mata
Jaime Ozáez
Emilia Arjona
Isabel Cuesta



(Find us in <https://github.com/BU-ISCIII>)

And special thanks to the nf-core community (<https://nf-co.re/>)

Harshil Patel

(<https://github.com/drpatelh>)

Bioinformatics & Biostatistics Group at The Francis Crick Institute, London, now in Seqera Labs