Quality assessment, read preprocessing and assembly

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UAH - ISCIII

Step in the process

Fastq files

Preprocessing

Assembly

Postprocessing: Genome analysis…

Raw output files format

Illumina

.fastq

454 .sff

SOLiD

.fasta

.qual

Nanopore

FAST

5

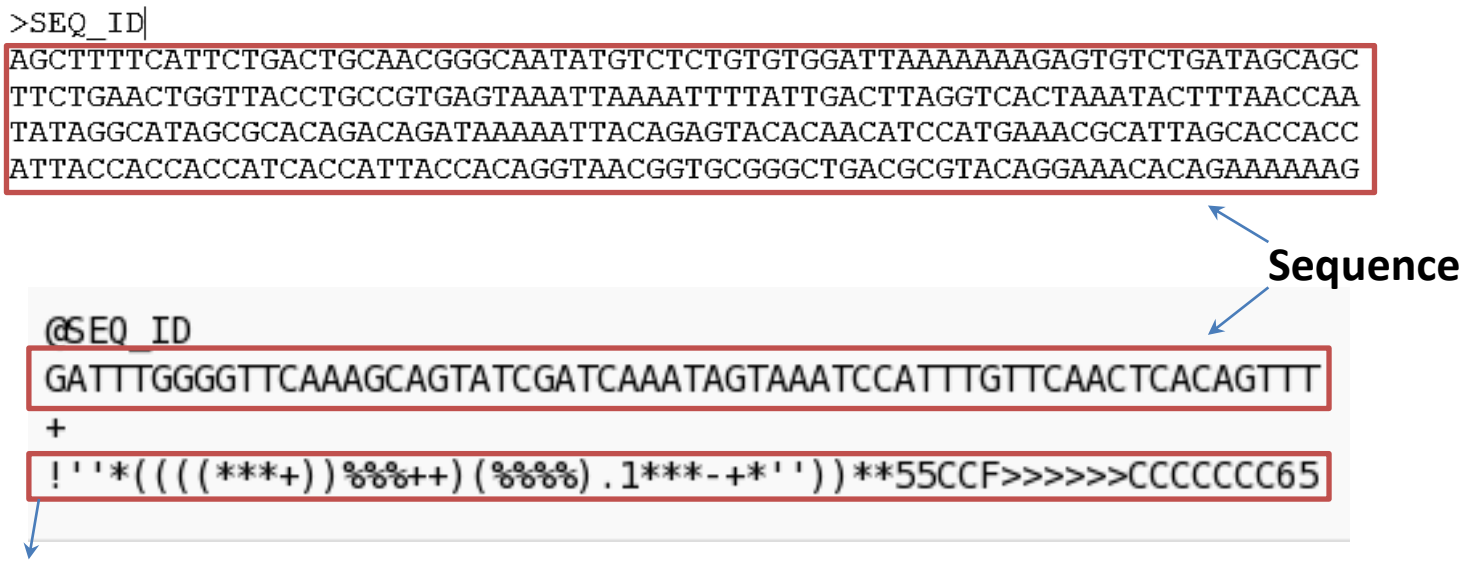
PacBio RSII

Bax.h

5

FASTQ format

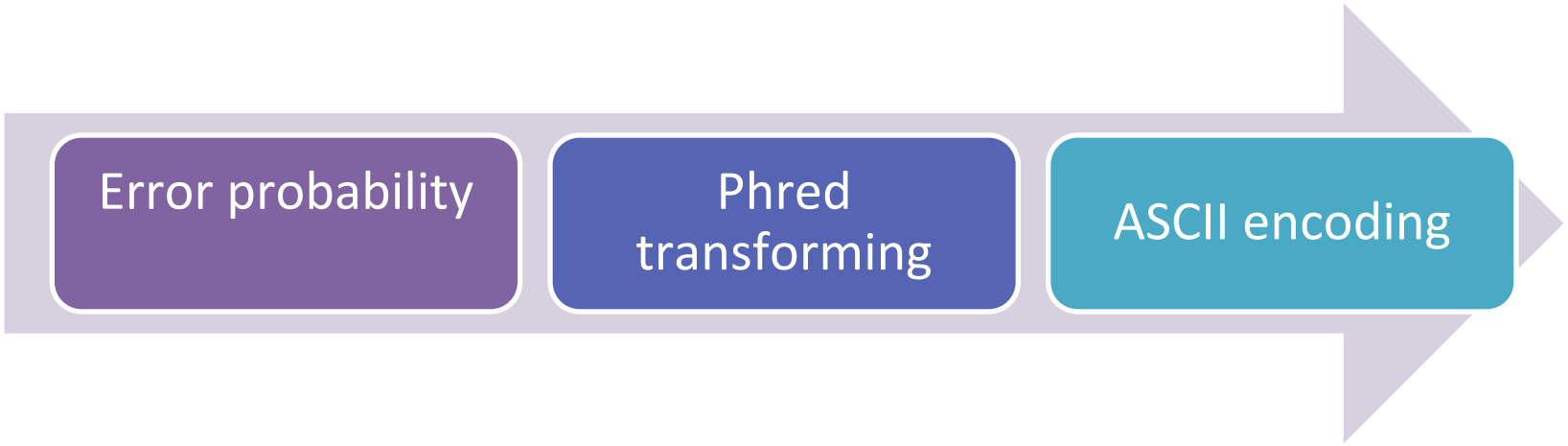
- Is a FASTA file with quality information
- Within HTS, FASTA contain genomes y FASTQ reads



Quality: must be 1 bit

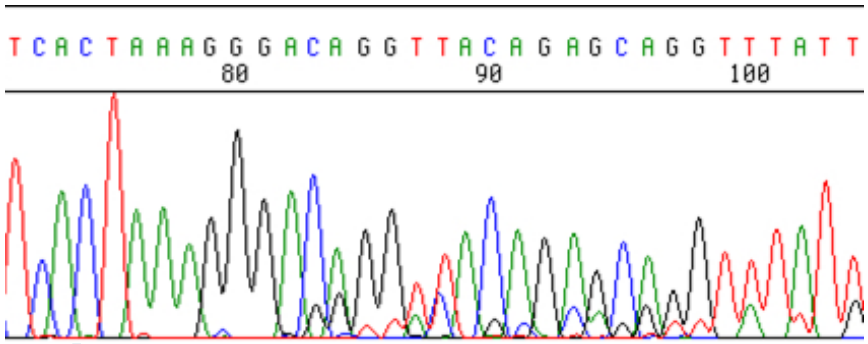
FASTQ format

- Each base has an assigned quality score
  - Sequencing quality scores measure the probability that a base is called incorrectly
- How is it calculated?

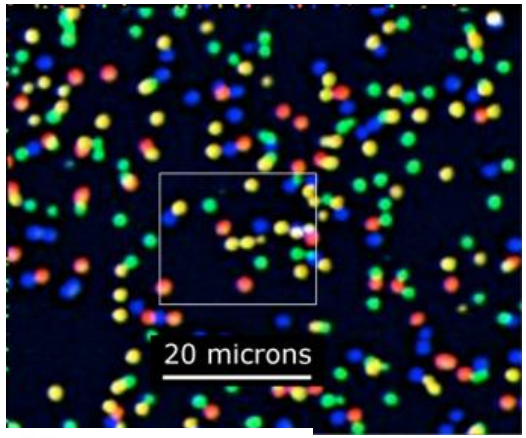


Phred quality and error probability

- **Light intensity** is used to calculate the error probabilities
- Convert error probability into Phred score quality - Ewing B, Green P. (1998)
- Phred originated as an algorithmic approach that considered Sanger sequencing metrics, such as **peak resolution and shape**



wellcome sanger institute



illumina®

Phred quality and error probability

- Convert error probability into Phred score quality - in real time on Illumina platforms
- Q scores are defined as a property that is logarithmically related to the base calling error probabilities (P)
- Phred quality range between 0-40 for Sanger and Illumina 1.8+

$$Q = -10 \log_{10} P$$

Phred Quality Score	Probability of Incorrect Base Call	Base Call Accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1,000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%

Phred quality and error probability

- Convert Phred quality score into ASCII, a compact form, which uses only 1 byte per quality value

ASCII_BASE=33 Illumina, Ion Torrent, PacBio and Sanger

Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII
0	1.00000	33 !	11	0.07943	44 ,	22	0.00631	55 7	33	0.00050	66 B
1	0.79433	34 "	12	0.06310	45 -	23	0.00501	56 8	34	0.00040	67 C
2	0.63096	35 #	13	0.05012	46 .	24	0.00398	57 9	35	0.00032	68 D
3	0.50119	36 $	14	0.03981	47 /	25	0.00316	58 :	36	0.00025	69 E
4	0.39811	37 %	15	0.03162	48 0	26	0.00251	59 ;	37	0.00020	70 F
5	0.31623	38 &	16	0.02512	49 1	27	0.00200	60 <	38	0.00016	71 G
6	0.25119	39 '	17	0.01995	50 2	28	0.00158	61 =	39	0.00013	72 H
7	0.19953	40 (	18	0.01585	51 3	29	0.00126	62 >	40	0.00010	73 I
8	0.15849	41)	19	0.01259	52 4	30	0.00100	63 ?	41	0.00008	74 J
9	0.12589	42 *	20	0.01000	53 5	31	0.00079	64 @	42	0.00006	75 K
10	0.10000	43 +	21	0.00794	54 6	32	0.00063	65 A			

- Phred+33 (Sanger and current Illumina). 0 Phred quality correspond to decimal 33, which is the symbol !

ASCII_BASE=64 Old Illumina

Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII
0	1.00000	64 @	11	0.07943	75 K	22	0.00631	86 V	33	0.00050	97 a
1	0.79433	65 A	12	0.06310	76 L	23	0.00501	87 W	34	0.00040	98 b
2	0.63096	66 B	13	0.05012	77 M	24	0.00398	88 X	35	0.00032	99 c
3	0.50119	67 C	14	0.03981	78 N	25	0.00316	89 Y	36	0.00025	100 d
4	0.39811	68 D	15	0.03162	79 O	26	0.00251	90 Z	37	0.00020	101 e
5	0.31623	69 E	16	0.02512	80 P	27	0.00200	91 [	38	0.00016	102 f
6	0.25119	70 F	17	0.01995	81 Q	28	0.00158	92 \	39	0.00013	103 g
7	0.19953	71 G	18	0.01585	82 R	29	0.00126	93]	40	0.00010	104 h
8	0.15849	72 H	19	0.01259	83 S	30	0.00100	94 ^	41	0.00008	105 i
9	0.12589	73 I	20	0.01000	84 T	31	0.00079	95 _	42	0.00006	106 j
10	0.10000	74 J	21	0.00794	85 U	32	0.00063	96 `			

- Phred+64 (Solexa and Illumina 1.3-1.5)

Phred quality and error probability

- **Phred 33 example**

```
@HWI-ST731_6:1:1101:1322:1938#1@0/1
NTGACAAAGGGCTAATATCCAGAATCTACAAAGAACTTAAACAAATGTATAAGAATAAAAGTATAGTGCTAACAAT
+
#1:BDDADFDFDD@F>BGFIIIB@CFHIHICAGBC9CBCBGGIGCFF??>GGHFHIGGEGI<FECGDE=FHCHEG=
```

P=0.001 → Q=-10*log10(0.001)= 30 → ASCIII 33+30 = 63 → ?

P=0.0001 → Q=-10*log10(0.001)= 40 → ASCIII 33+40 = 73 → I

```
Quality encoding: !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHI
                  |         |         |         |         |
   Quality score: 0........10........20........30........40
```

FASTQ format

Illumina read header

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

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

```
@HWUSI-EAS1752R:21:FC64JUKAAXX:3:1:2458:1027 1:N:0:ACAGTG
AGAAAAAACCTTGGANGGAAAAAAATCAGACATTTTCTAGAGGTGGAAGGCAAACTGAACAAAGAAATAATTCACA
+
DGGGEDHHHHGGGFE#CBACBCA<?HHHHBHHHHHHHHDHHHHEHEFEGGGGGG/GGDDDGHFHGFCHFHHEHEH8
@HWUSI-EAS1752R:21:FC64JUKAAXX:3:1:3082:1029 1:N:0:ACAGTG
GGTAATACAGACTGANATGATCAAAGGCATGCTGGAAACAAACCTATTAAAGATAAGCTTGGATCAAGCTTTCATT
+
B:B:?BB/:=55177#55877<775EDD>E=B?BBBBGGGDDAG@G>GGGGGG@)EEEEBEG>GGGGGGGAAA?<D
@HWUSI-EAS1752R:21:FC64JUKAAXX:3:1:3185:1033 1:N:0:ACAGTG
TCTGGGACATTGCTCNTGGCTGGGAGTCACCTGTCTGGGACATTGCTCAGGGCTGGGAGACACGTGTTGGAGGGAC
+
BC??A66;)74781<#7??;452.27'64(8,851DDG8GB?##############################
@HWUSI-EAS1752R:21:FC64JUKAAXX:3:1:3268:1033 1:N:0:ACAGTG
ATTCAAATTAGAAGANAGTTGATCGTTCTTCATGATGCCCAAAAATTTCACTGAGAAAACCCTTTTTTAAGCCCAC
+
IIIIIIIIIIFFFFE#ABACFEEFFIIGIIIFIHE@BIIIIIIIIIHHIIFIIF>HHIHIFGDIIIIIIGFHIEGH
@HWUSI-EAS1752R:21:FC64JUKAAXX:3:1:3400:1035 1:N:0:ACAGTG
TCCTGCTTTAGGAGANTCCTCATGCTCTGACAGGATGCTCTCTATGTGAGTTGAGCTGGTCTTCTCACTTTTATAG
+
IIIIIHIHIIGGGEGG#AACA@?=?BHHIIIIIHHIHIIIIHIHIHHGIHIHGHGIGIHGEGGGGHG@EFGGCEFAB
@HWUSI-EAS1752R:21:FC64JUKAAXX:3:1:3962:1033 1:N:0:ACAGTG
CCACCAACACAGTCTNCACCTTCTGTTGCTGGTGATAGATTTTTGCACCTTTCCATCCTCCAGGTTTCAAAATAGC
+
HHFHHDHDHH>C?CA#EEEE>?A?>HHDGHEGBGBCEEEEGHHF8HEHEEHECH,=>>==EAEE>BEBBAEAACAB
@HWUSI-EAS1752R:21:FC64JUKAAXX:3:1:4491:1028 1:N:0:ACAGTG
AGAGAGAGAGAGAGANAGAGGACTCTGGAGATGCCGAAGCACAAGCCTGCAAGAGTCCCAGCAAAGAAAATAAAAA
+
GADGGEGGEGBBB?B#@=@@72;64GGGFGB>GGGBDG<DBGB<DA??/?##########################
```

ASCII-coded (0-40):

- "!"#$%" lowest quality
- "FGHI" highest quality

Sequencing quality assessment

- To asses 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**

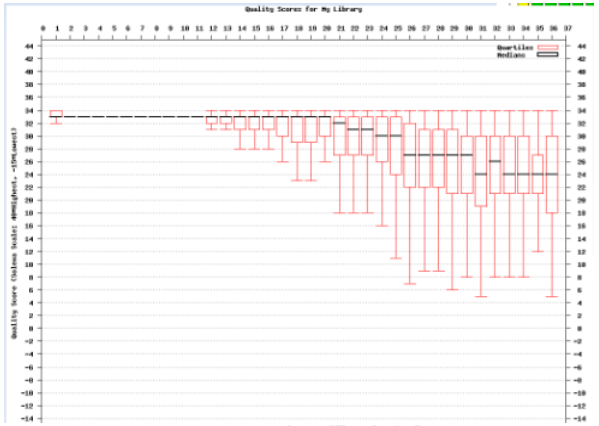
Sequencing quality assessment: Artifacts

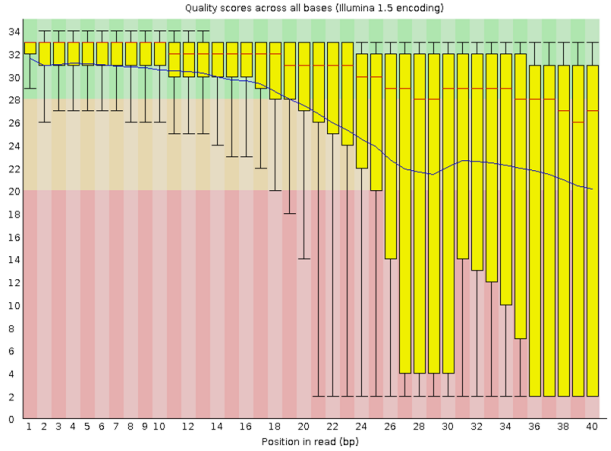
HTS methods are bounded by their technical and theoretical limitations and sequencing errors cannot be completely eliminated (Hadigol M, Khiabanian H. 2018)

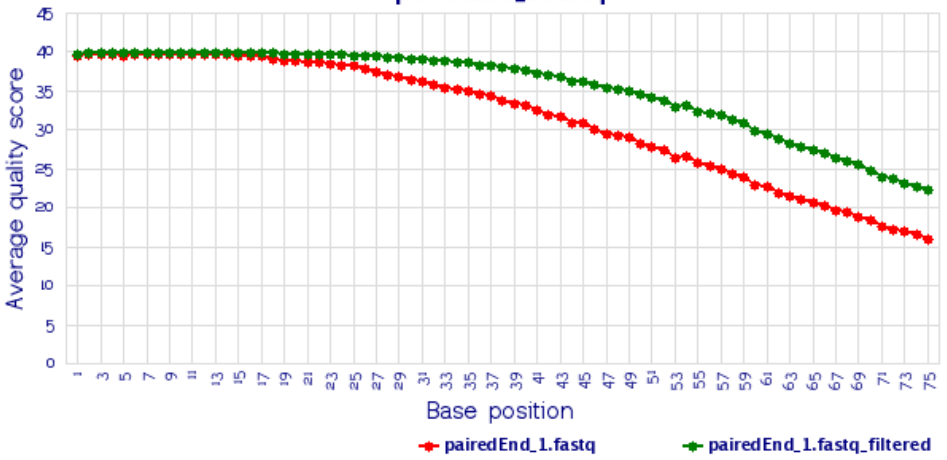
- **Artifacts in library preparation**
  - Remaining adapters
  - High rate of duplicates
  - GC regions bias
  - Polymerase error rate
  - DNA damage during breakdown
- **Artifacts during secuencing**
  - Low quality in sequence ends(Phasing: cluster loose sync)
  - Complication in certain regions:
    - Repetitions
    - Homopolymers
    - High CG content

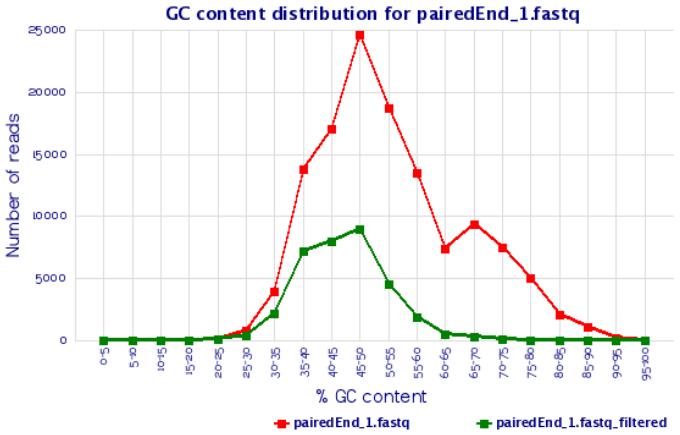
Sequencing quality assessment

- FastQC, fastx-toolkit, sfftools, NGSQCToolkit, etc…



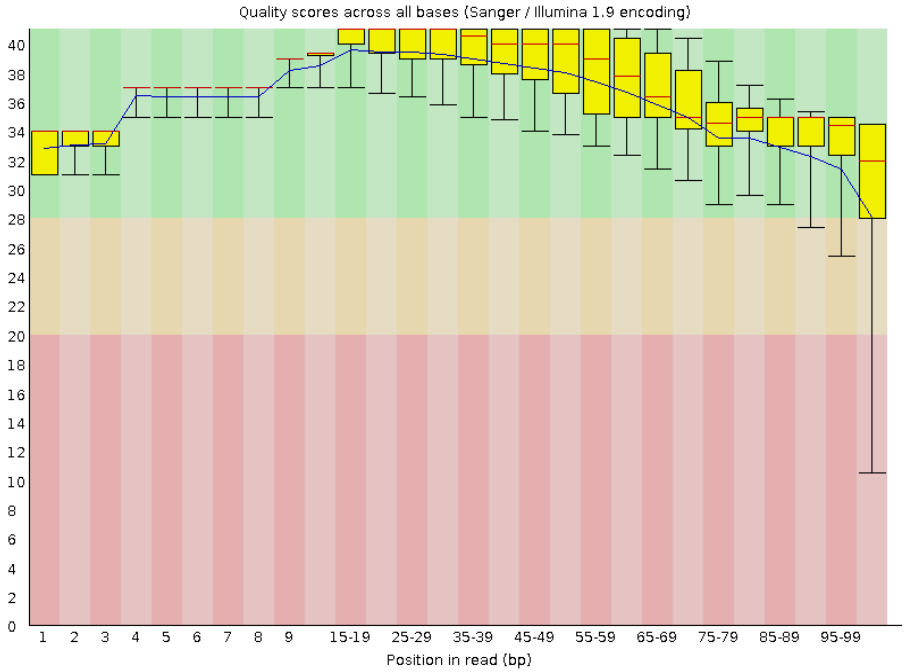




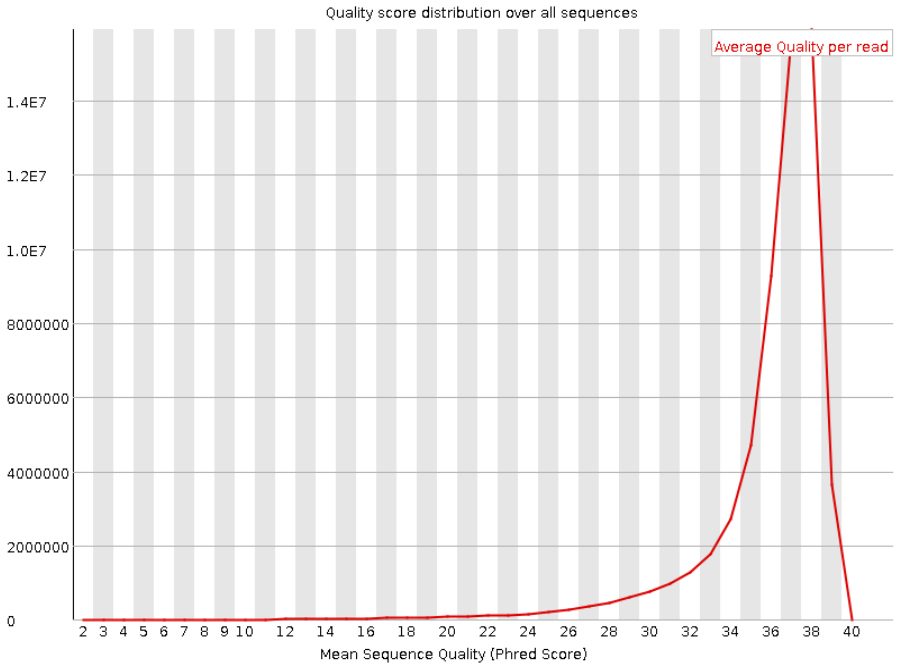


Sequencing quality assessment: FastQC

Per base sequence quality



Per sequence quality scores



https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

FastQC: Basic Statistics

- Self defined overall stats
  - Encoding: Phred33 or Phred64

Basic Statistics

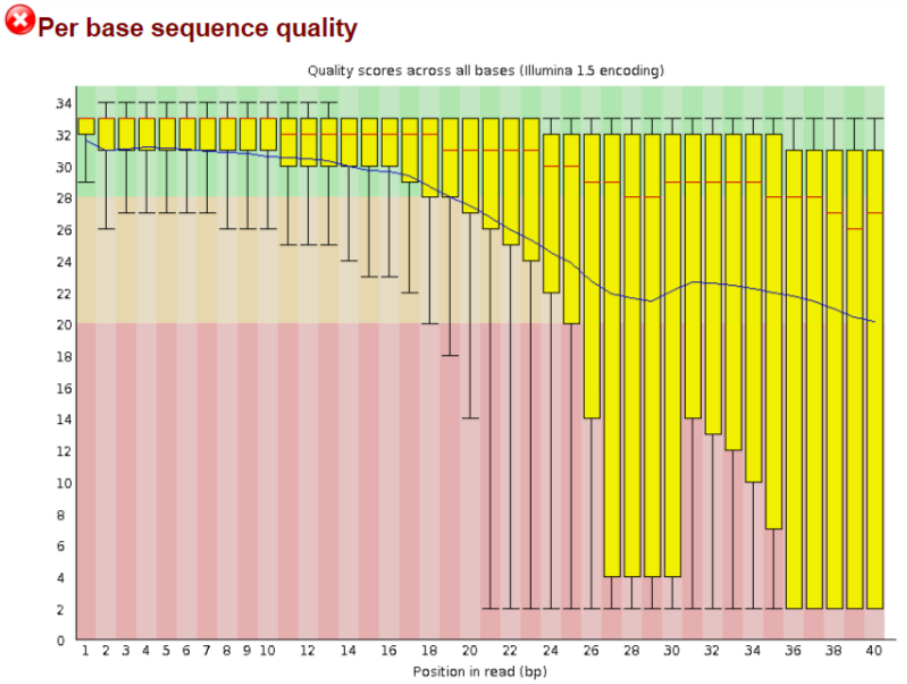
Measure	Value
Filename	bad_sequence.txt
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	395288
Sequences flagged as poor quality	0
Sequence length	40
%GC	47

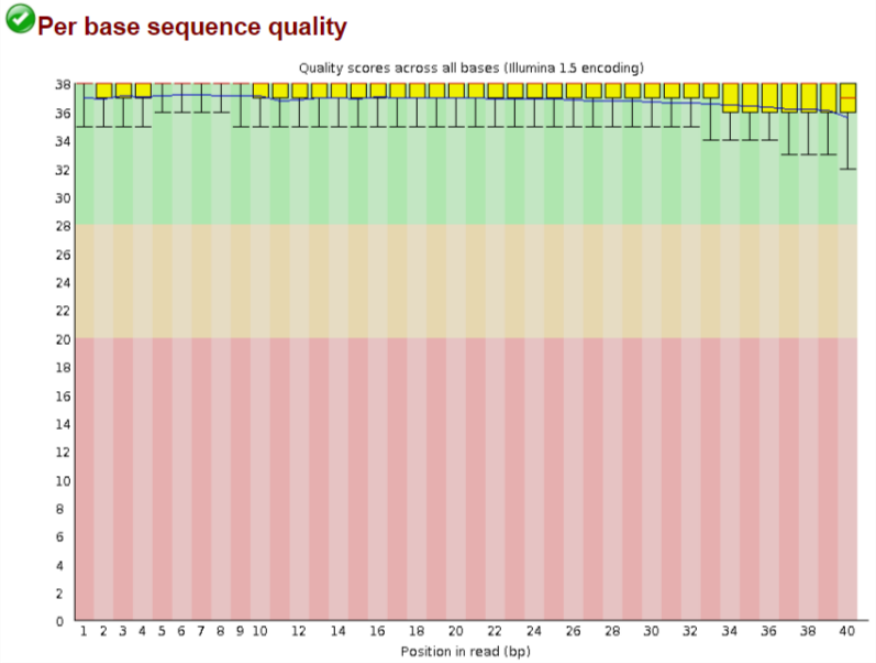
Basic Statistics

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

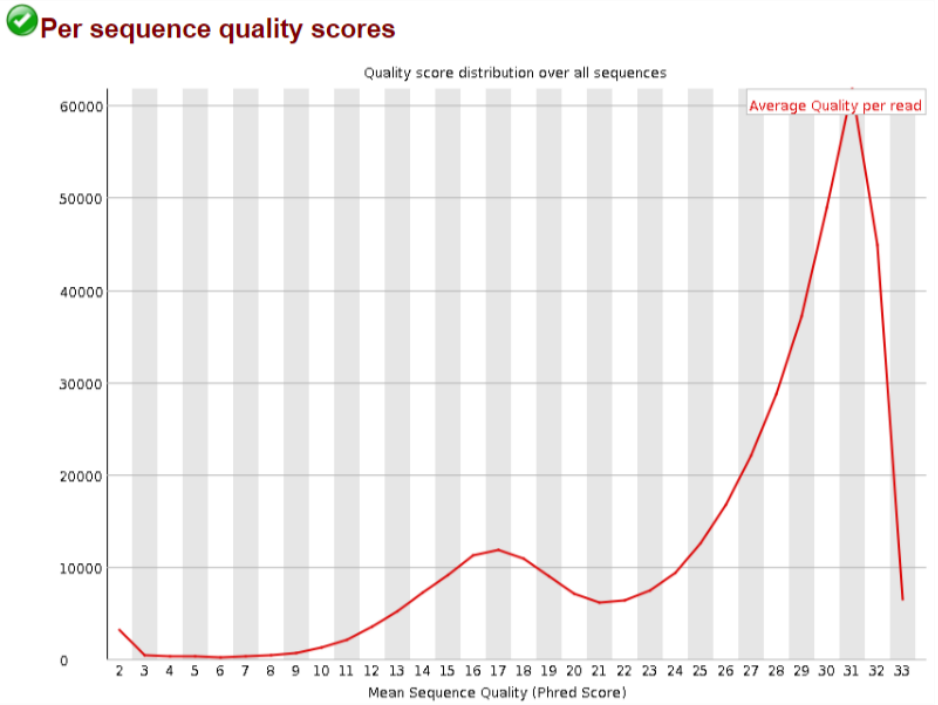
- Overview of the range of quality values across all bases at each position in the FastQ file
- **Median**, **inter-quartile range** **(25-75%), 10-90% points,** **mean quality**

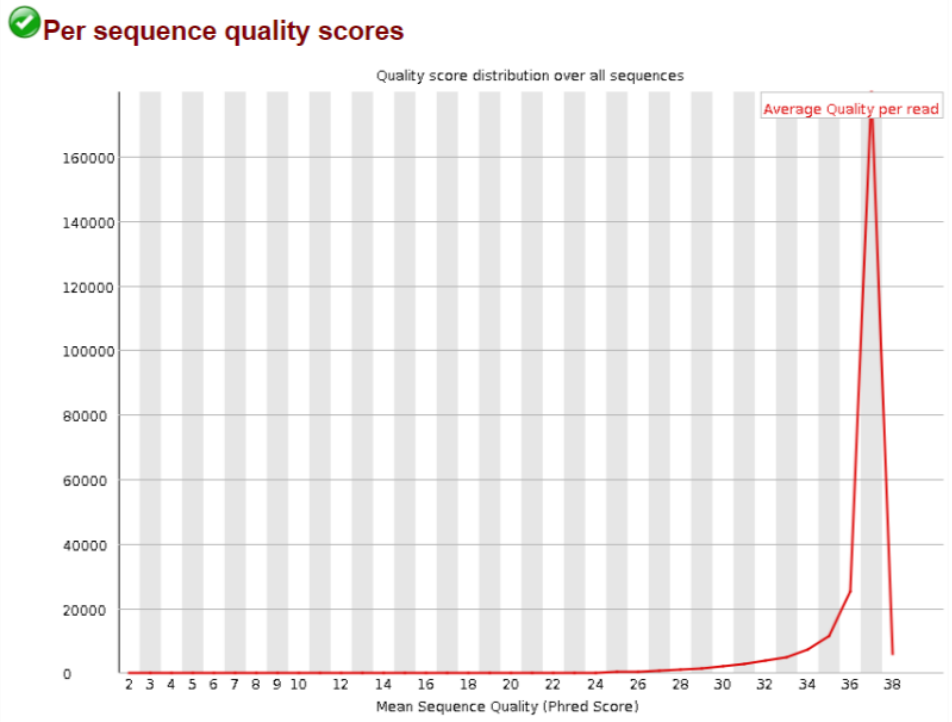




FastQC: Per sequence quality score

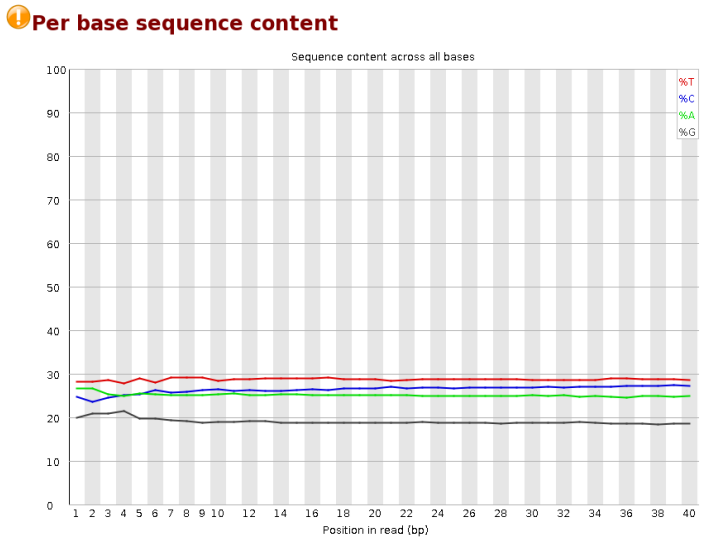
- Number of sequences with the same mean quality

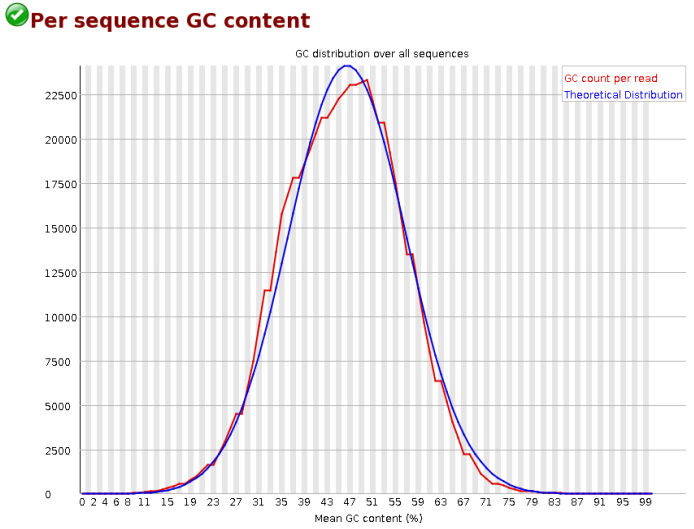




FastQC: Nucleotide related errors

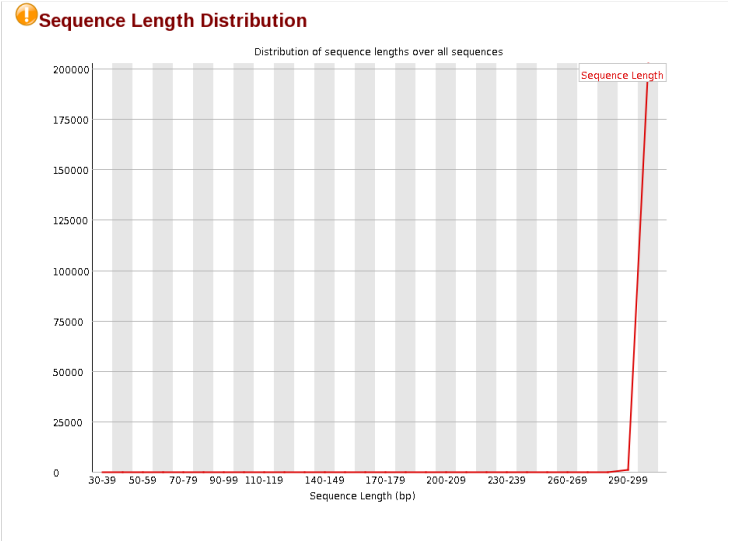
- How expected nucleotide distribution deviates from expected
  - Per base sequence content
  - Per base GC content
  - Per sequence GC content
  - Per base N content

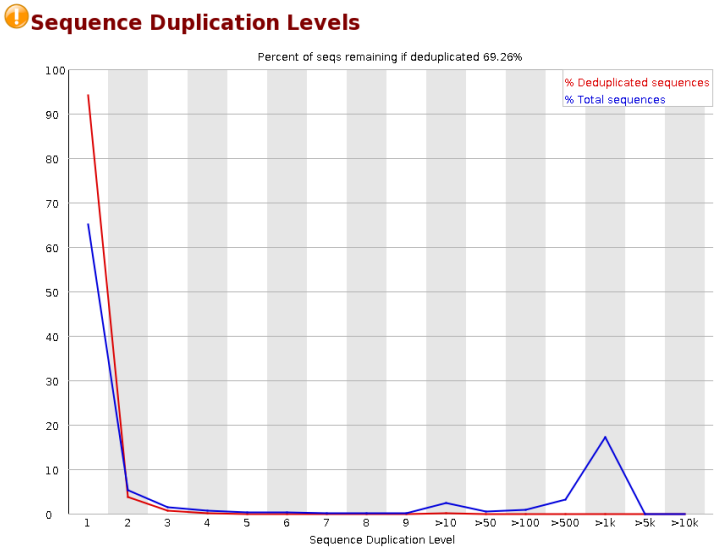




FastQC: Sequence related errors

- How expected nucleotide distribution deviates from expected
  - Sequence Length Distribution - Fragments
  - Sequence Duplication Levels
  - Overrepresented sequences
  - Adapter Content



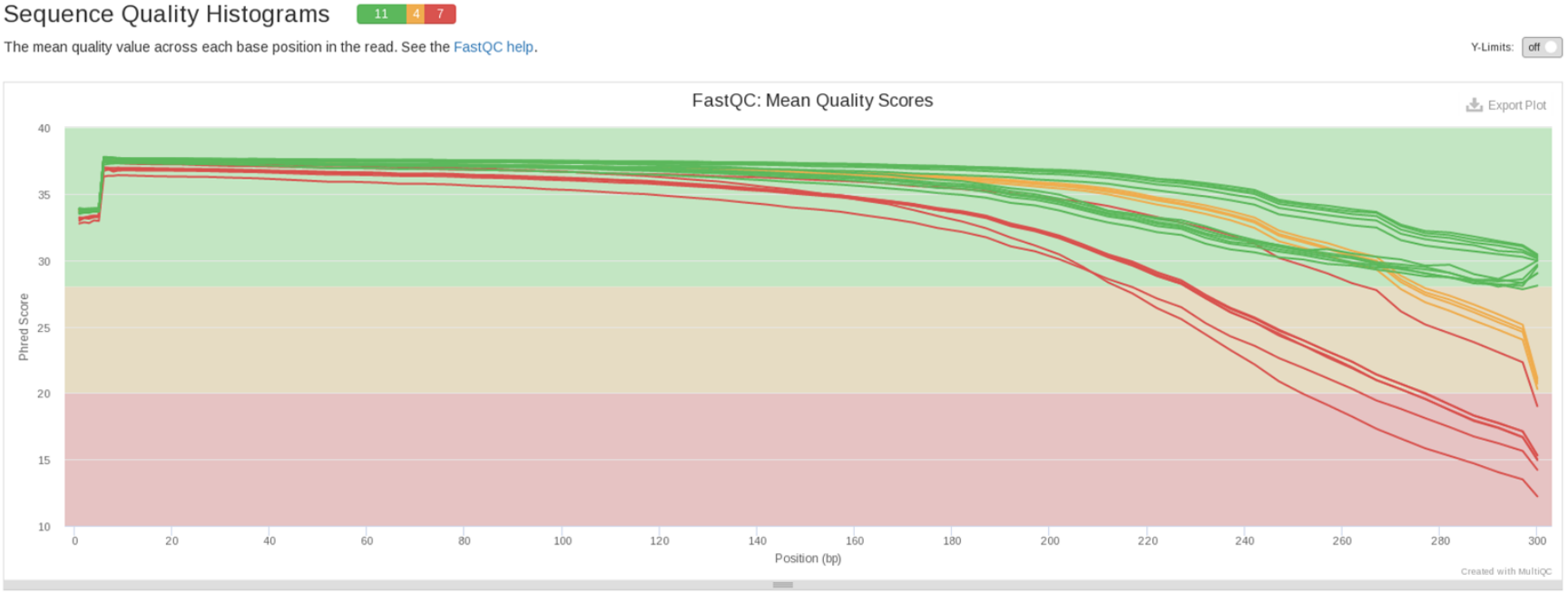


Sequence filtering

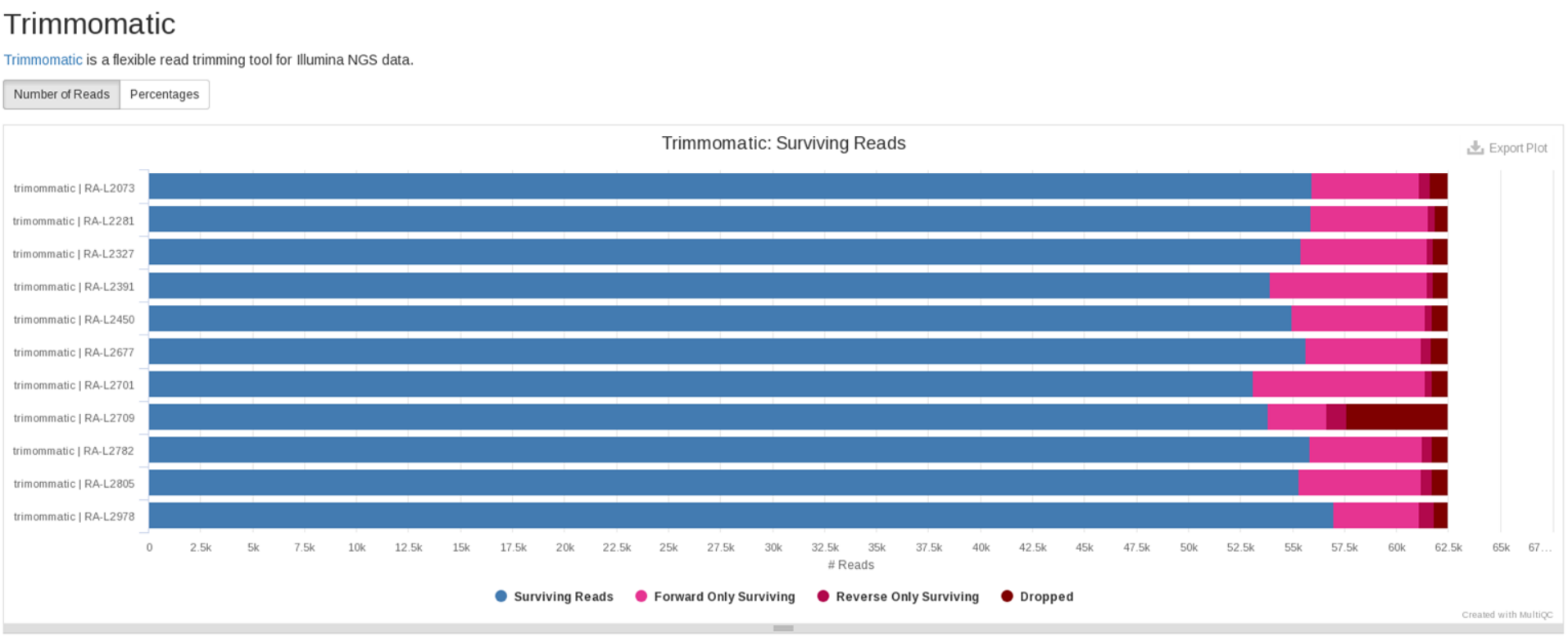
- **Remove residual adapters**
  - Depending on used library

- **Filtering parameters**
  - Quality filtering
    - Overall mean quality
    - Local mean quality
      - Sequence end
      - Sliding window
  - Size filtering
    - Overall sequence size
    - Remaining sequence size after filtering

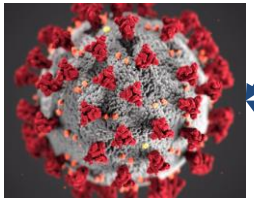
Sequence filtering: stats with MultiQC



Sequence filtering: stats with MultiQC



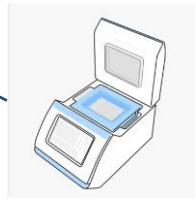
1. Background



December 2019

SARS-Cov-2

Surveillance:
Genome Sequencing



Detection:
RT-PCR

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

nf-core

Amplicon-based enrichment

Probe capture enrichment

Amplicon primers V1

Wet-lab

ONT long reads

Illumina short reads

Data

Dry-lab

2020

2020

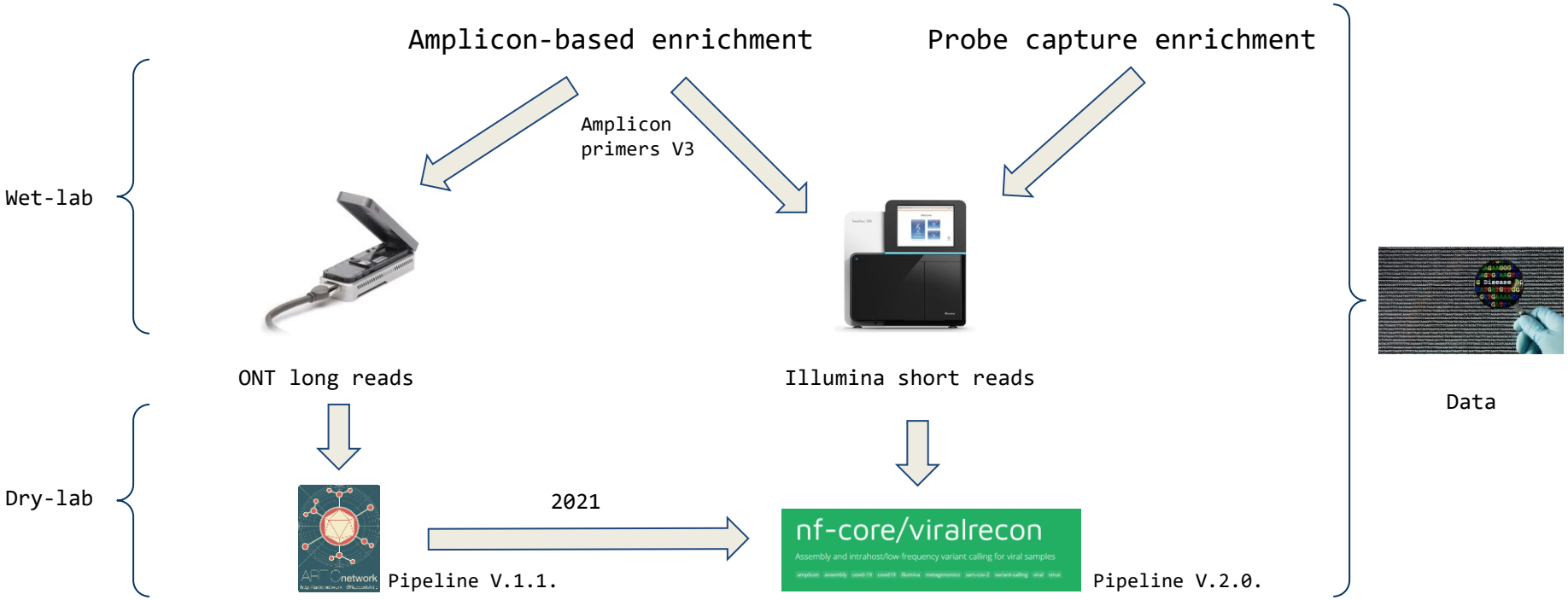
Pipeline V.1.0.

nf-core/viralrecon

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

Pipeline V.1.0.

2. Sequencing Approaches



3. History

nf-core

January 22 2020 Artic Network protocols

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

March 18 2020 Our 1st pipeline started

nf-core

March 30 2020 nf-core collaboration

BIOHACKATHON

April 5 2020 1st COVID19 Virtual BioHackathon

nf-core/viralrecon

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

June 1 2020 viralrecon released v1.0.0

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

June 29 2020 covid-19-signal v1.0.0

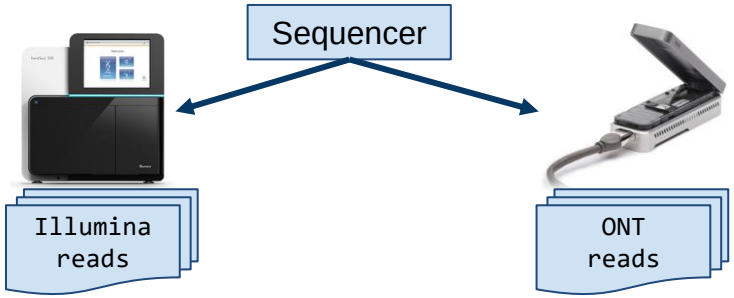
https://github.com/nodrogluap/nanostripper

September 3 2020 nanostripper v1.0.0

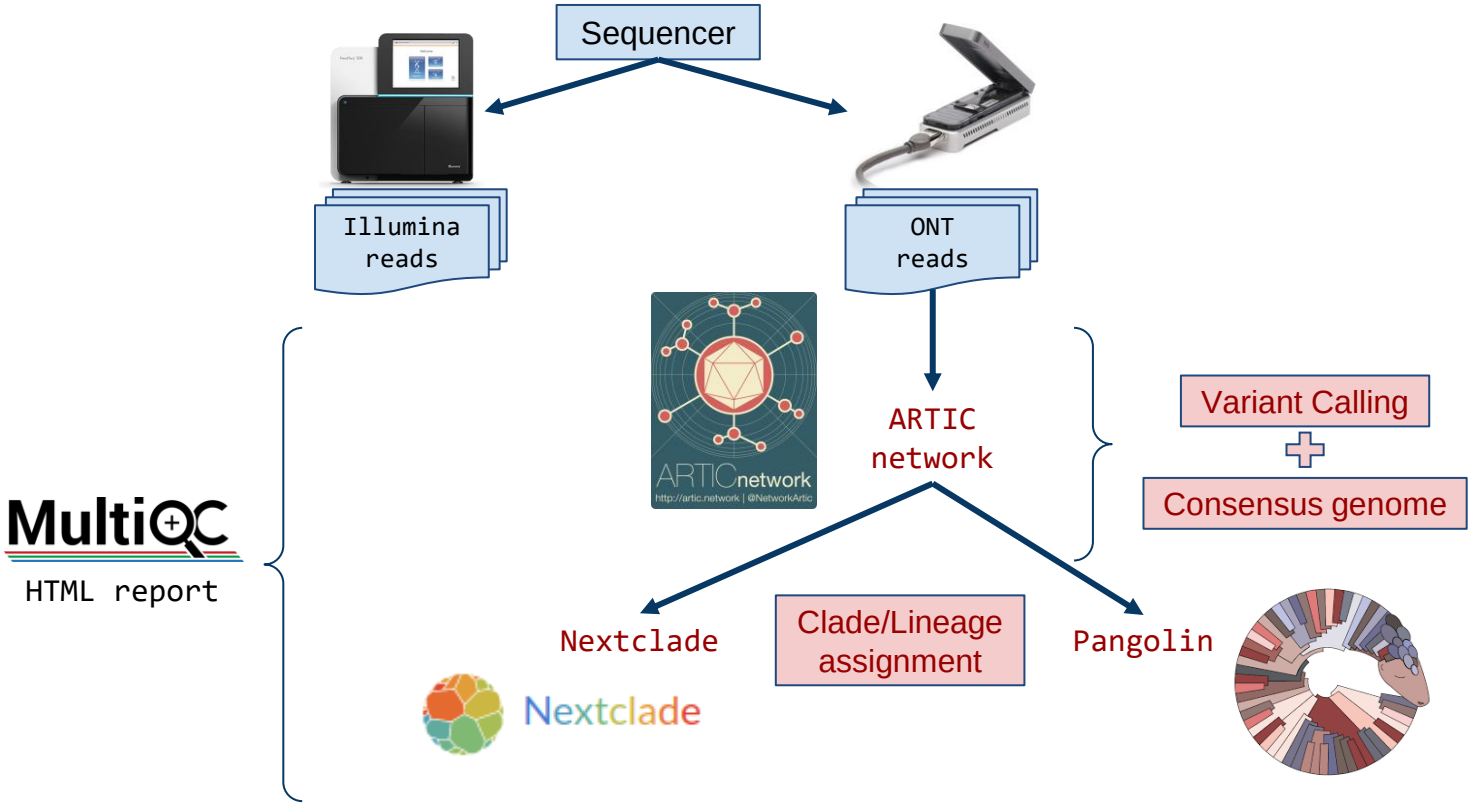
https://github.com/nf-core/viralrecon

May 13 2021 viralrecon v2.0 release

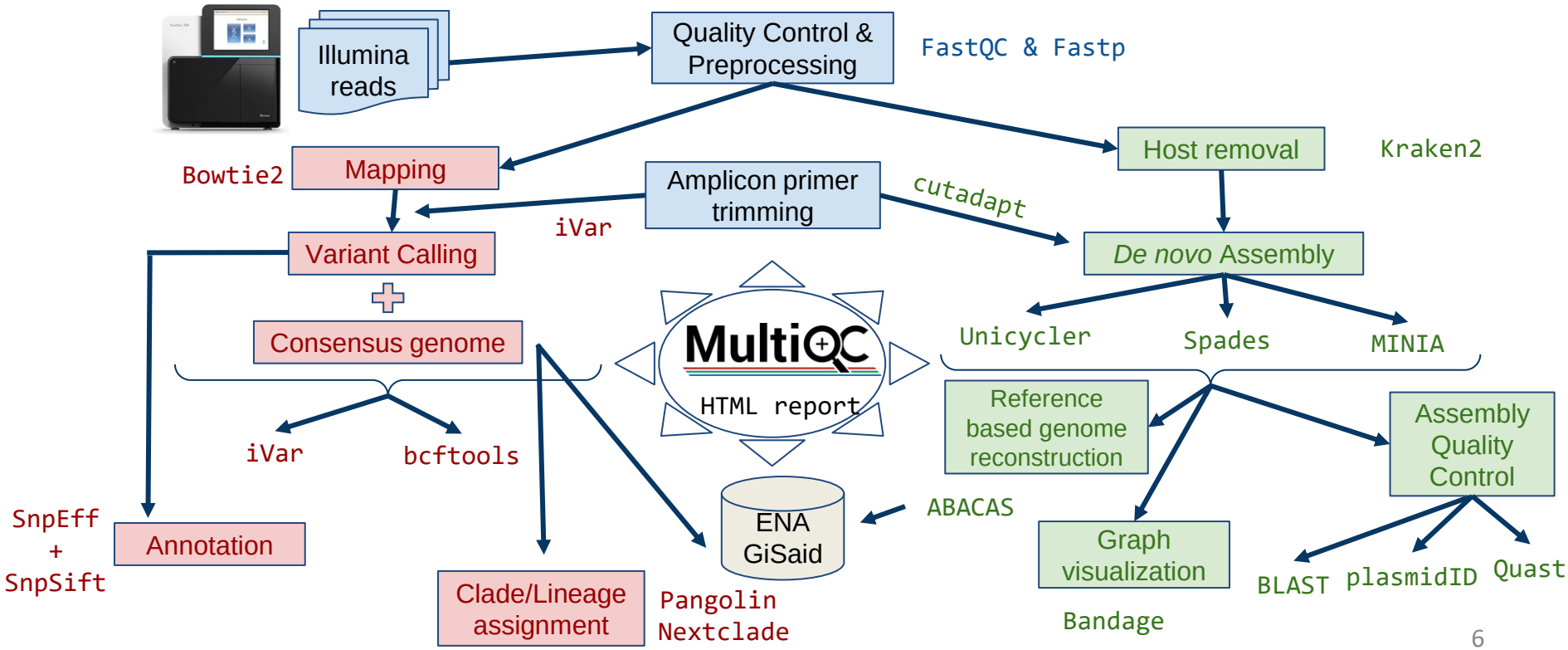
4. Viralrecon



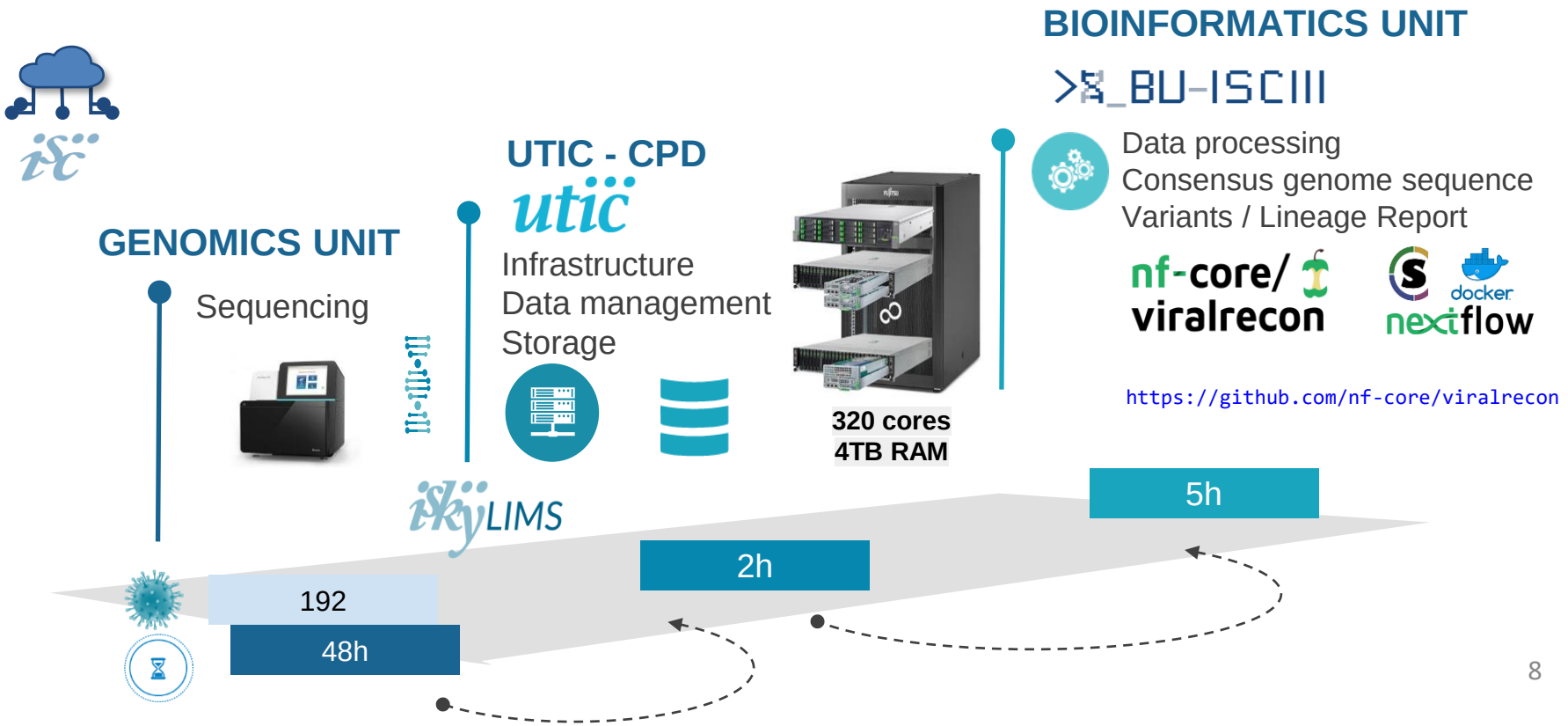
4. Viralrecon for ONT reads



4. Viralrecon for Illumina reads



4. Viralrecon ISCIII nf-core

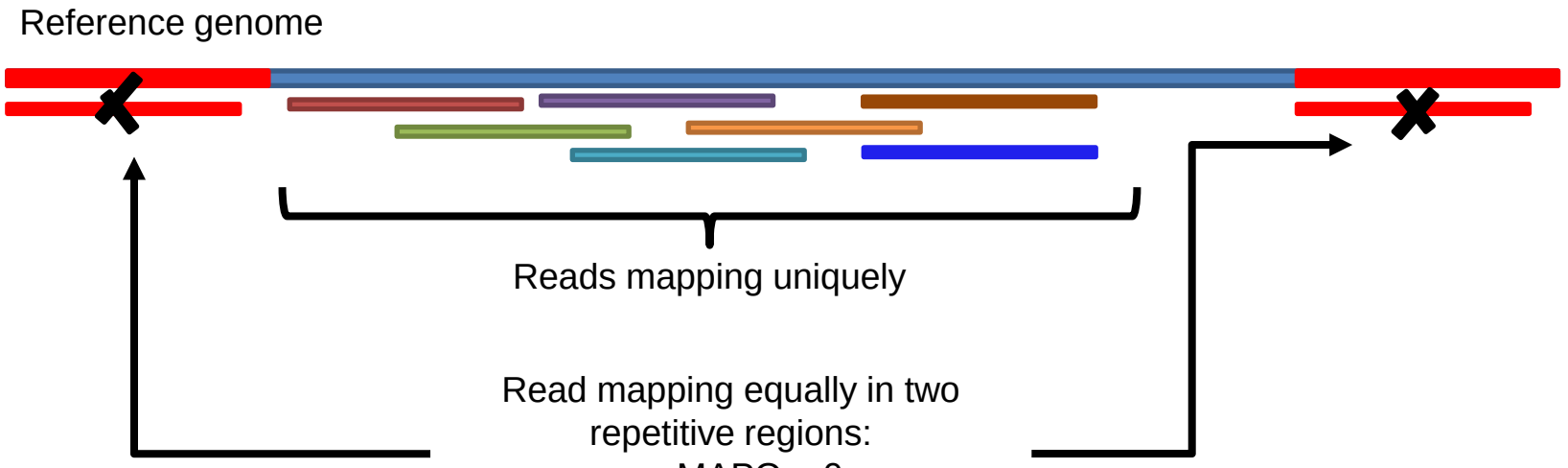


Mapping

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!

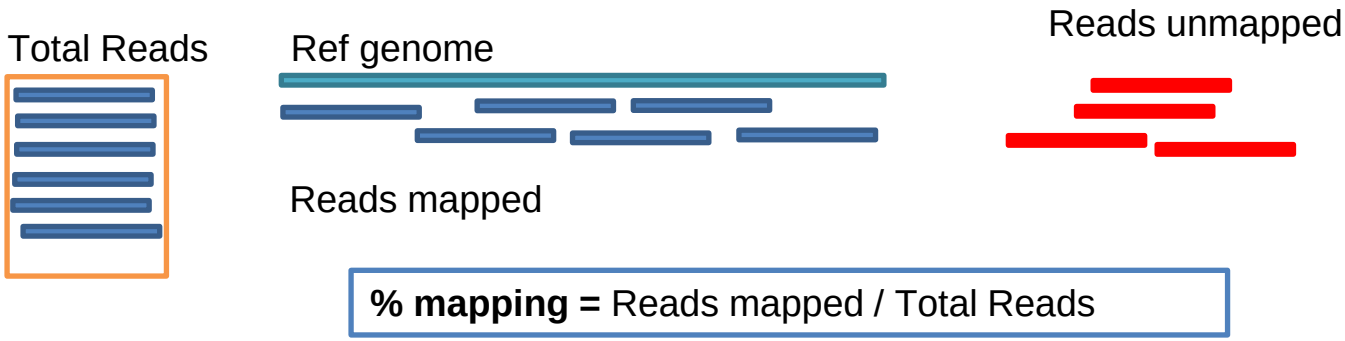


Depends on parameters!!

Mapping quality control

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

Picard
Samtools



% mapping = Reads mapped / Total Reads

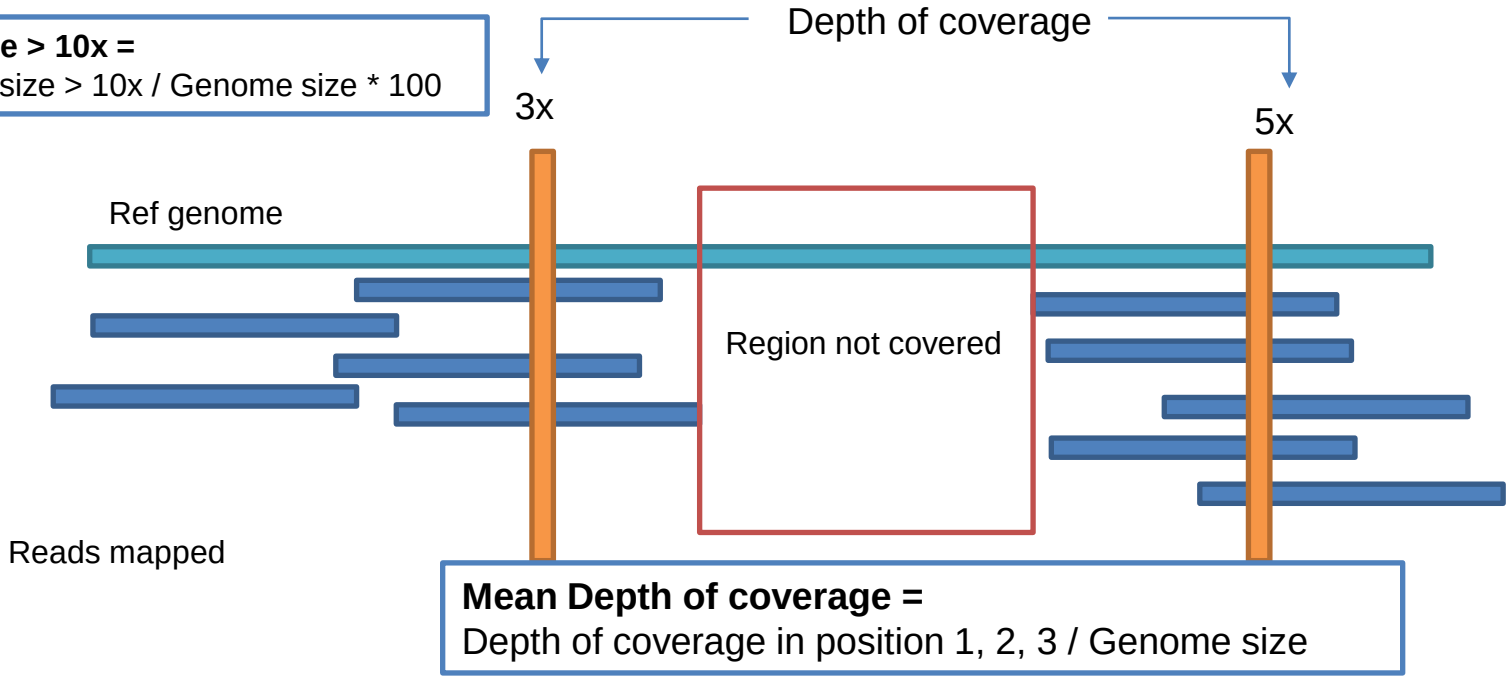
Mandatory parameter for microbial genomics!! It indicates us how many reads we have from our organism of interest. In human genomics this is almost always 99.99% unless something terrible happens. Not here!!!

Mapping quality control

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

Picard
Samtools

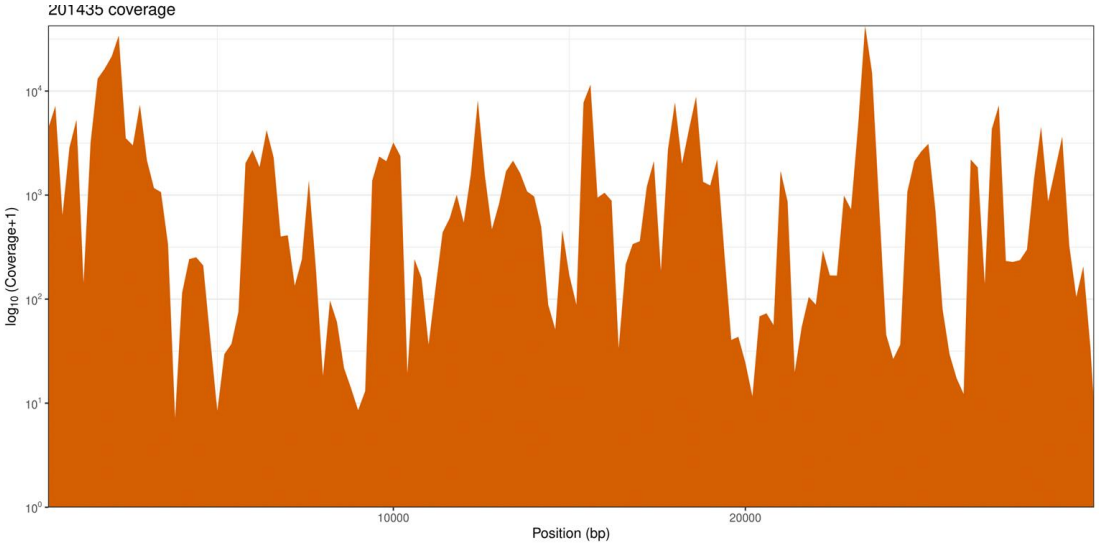
% genome > 10x =
Genome size > 10x / Genome size * 100



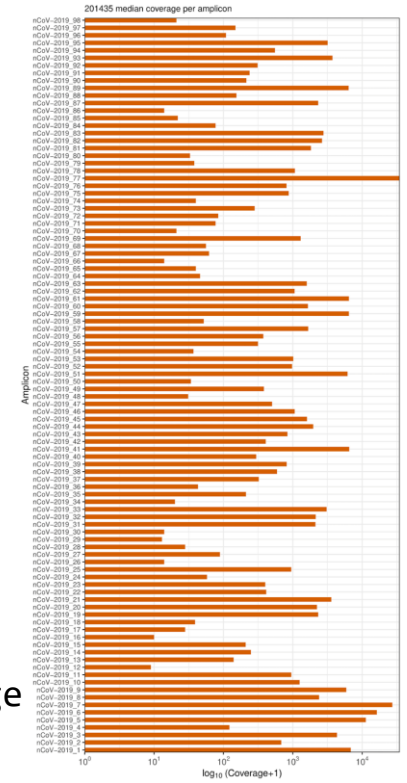
Mean Depth of coverage =
Depth of coverage in position 1, 2, 3 / Genome size

Amplicon QC results

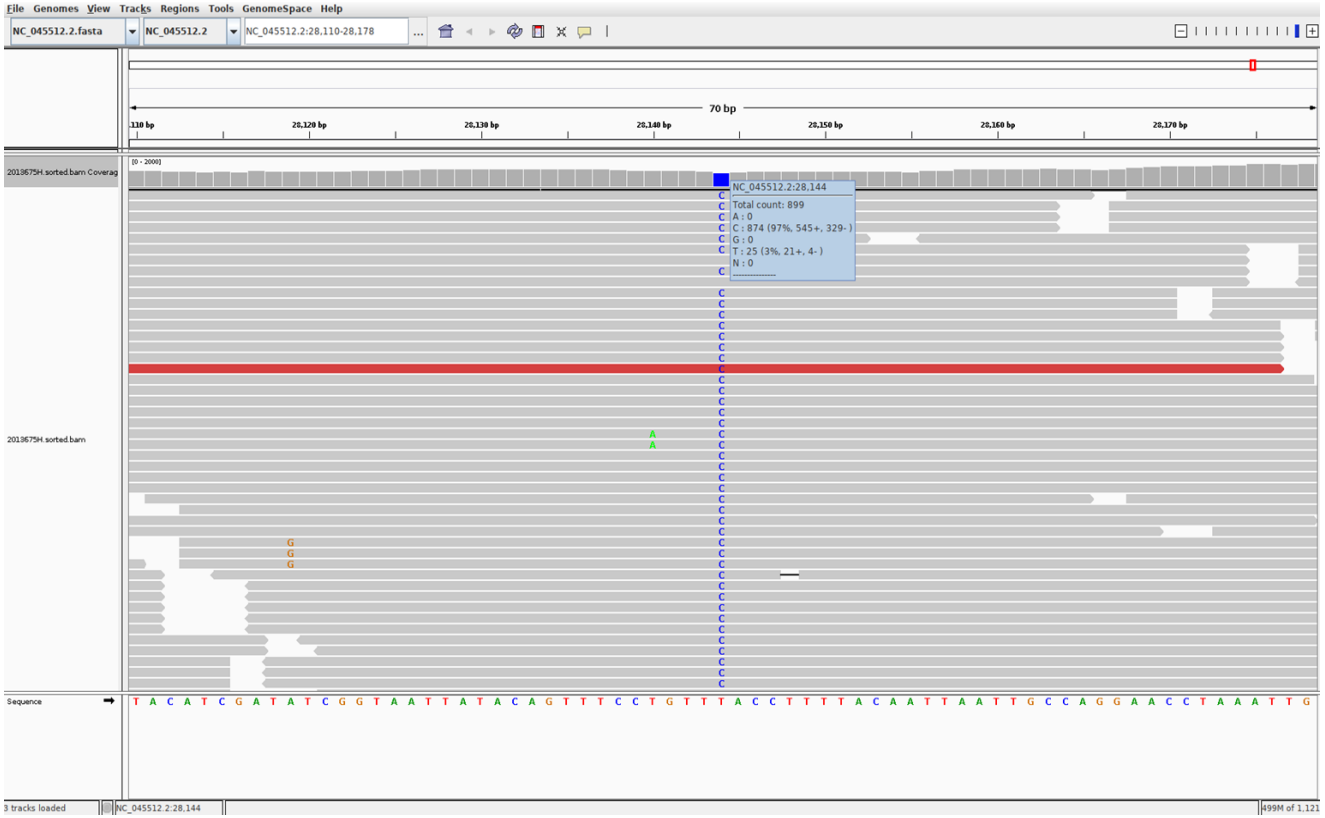
Genome coverage



Amplicon coverage



Variant calling



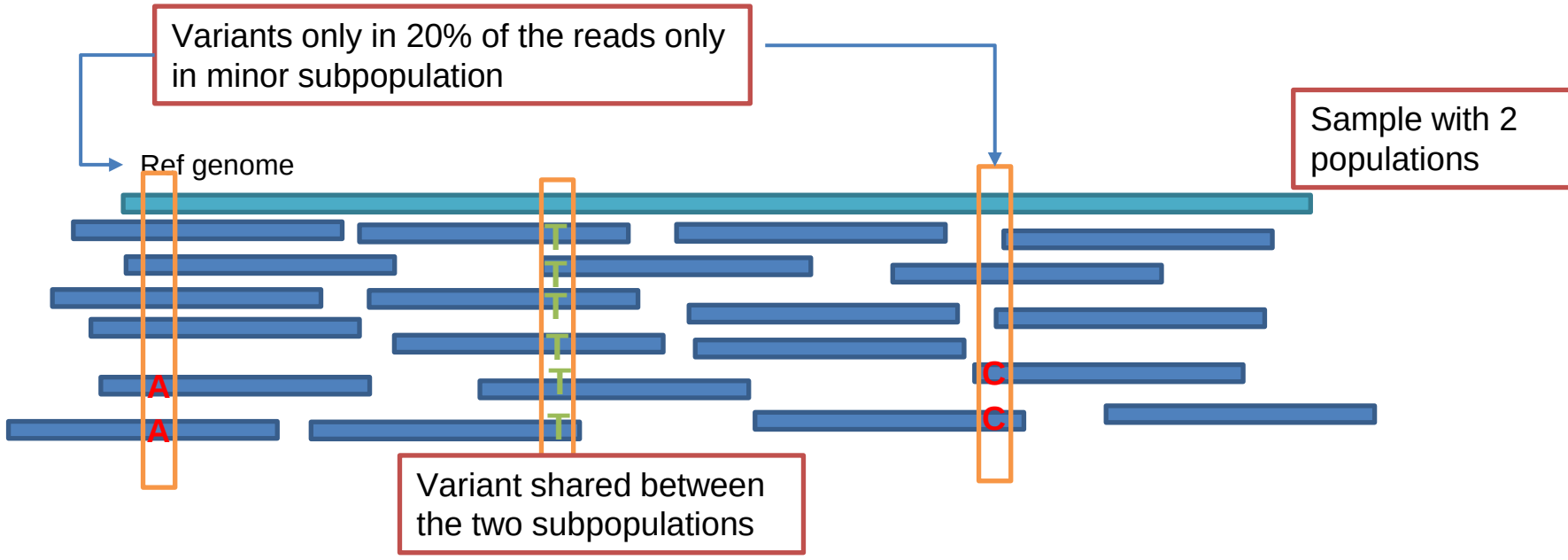
Variant callers walk through each alignment column independently and evaluate if there is a variant.

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

VARSCAN2
IVAR

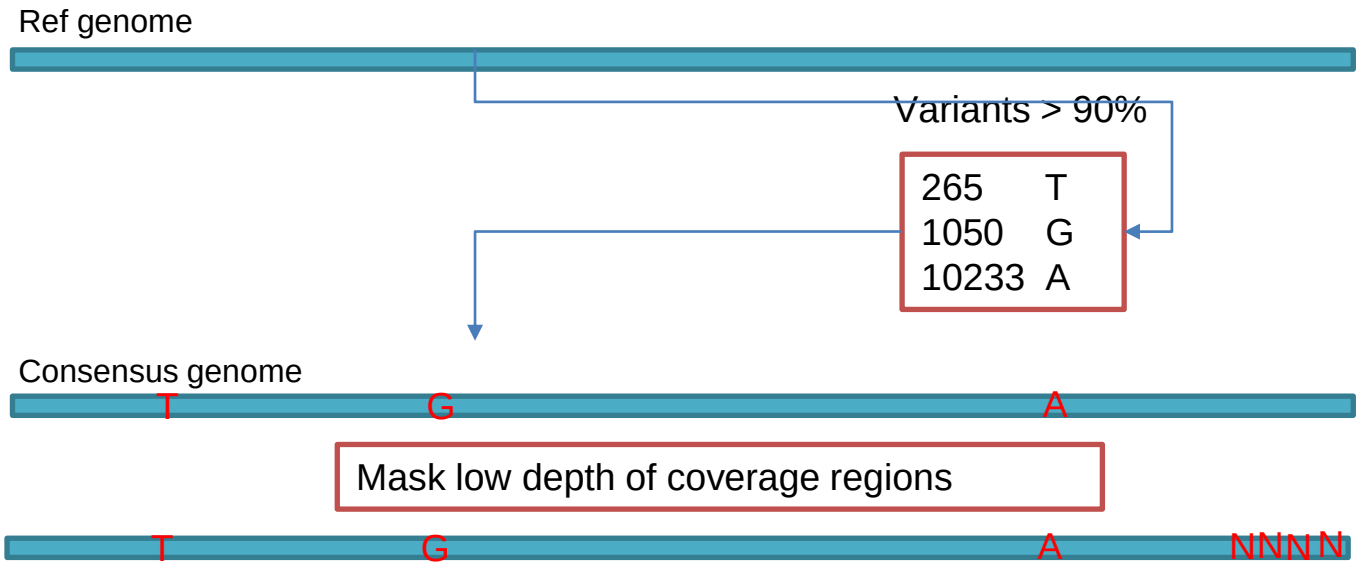
Viral subpopulation - Quasispecies

- 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.

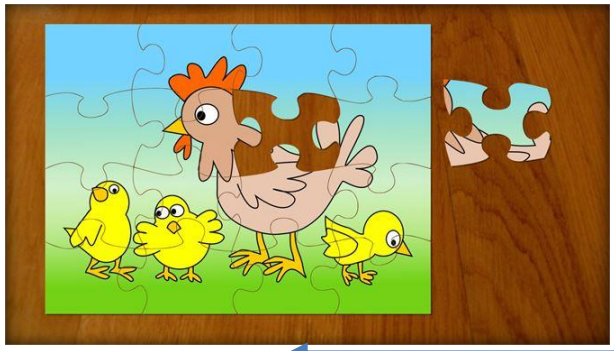


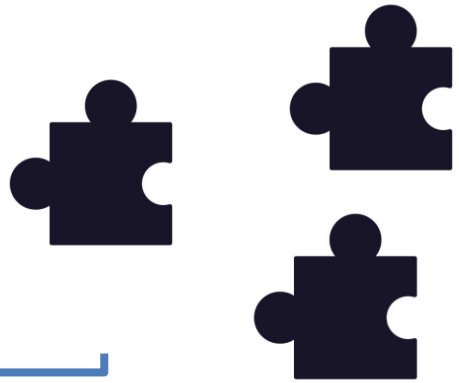
Consensus genome

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



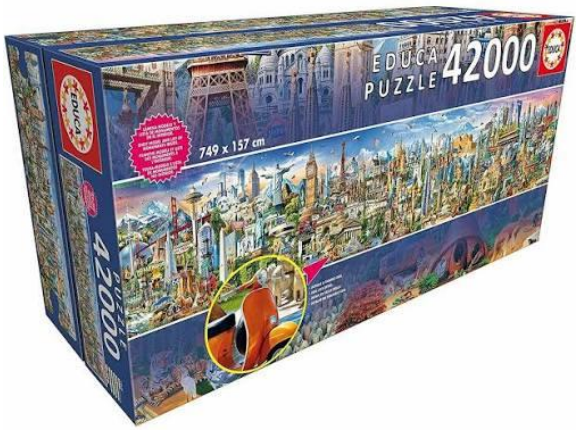
Assembly





The bigger the pieces the easier to reconstruct the puzzle!

Assembly



Obviously this a little bit more complicated….

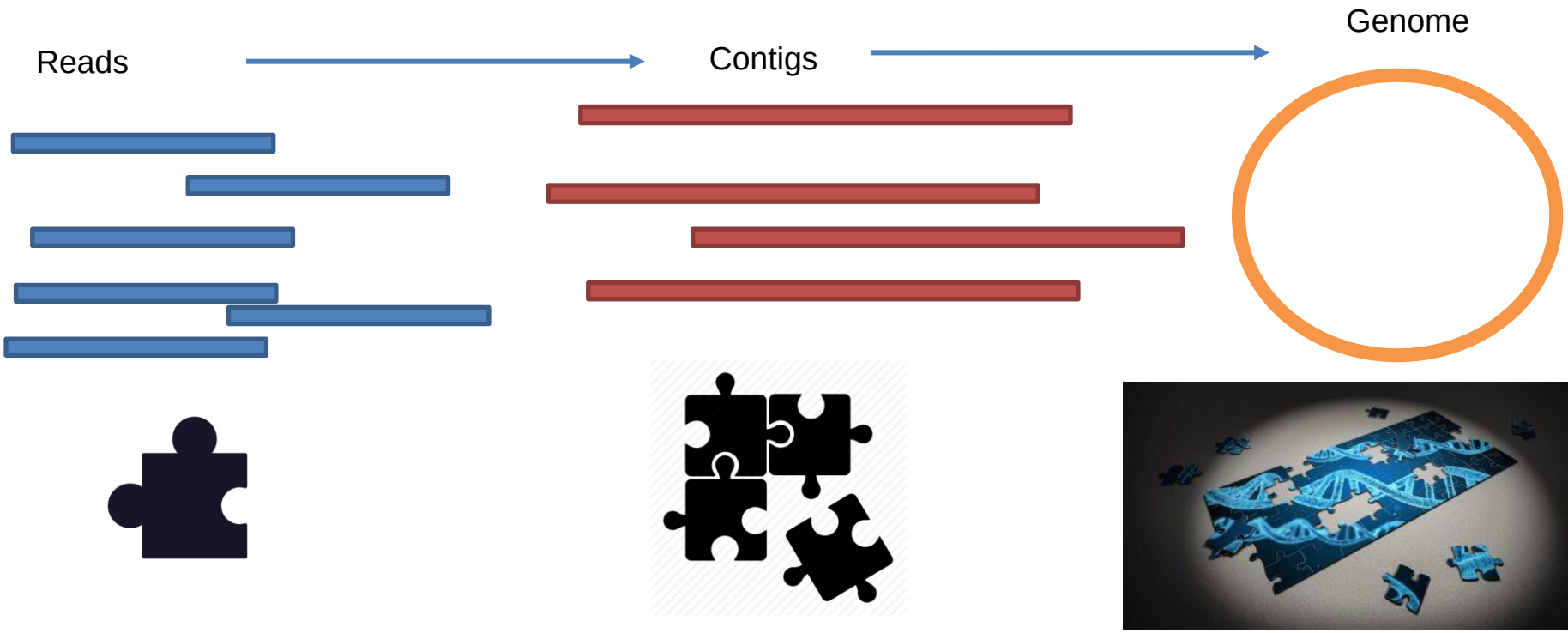
We have LOADS of really little pieces and a big puzzle in proportion

Assembly

Obviously this a little bit more complicated….

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

Assembly



Assembly

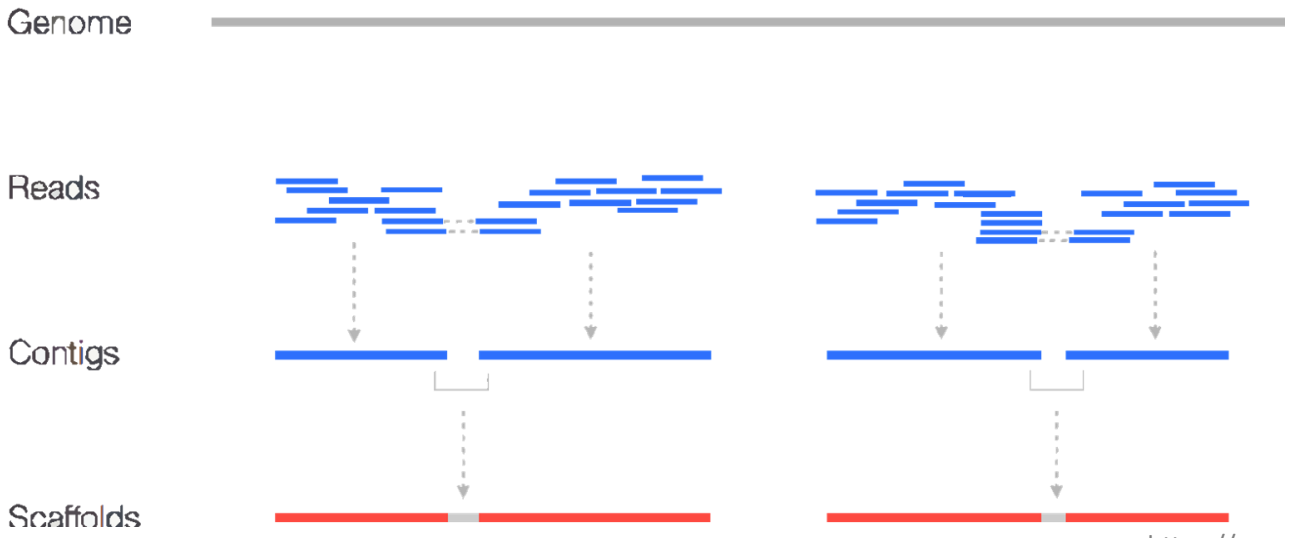
Reconstruct a representation of the original DNA from shorter DNA sequences or small fragments known as reads

- ***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 créate 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

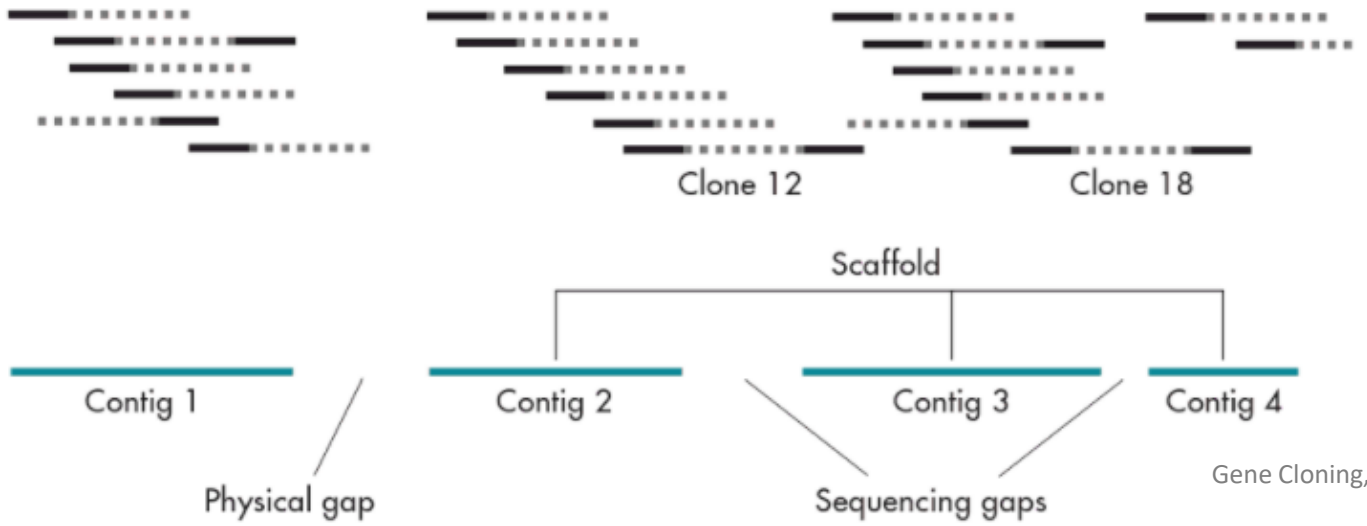
- **Contig:** continuous sequence made up of overlaping shorter sequences
- **Scaffold:** two or more contigs located and rearranged according to spatial information(pair-end, mate pair, reference)



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

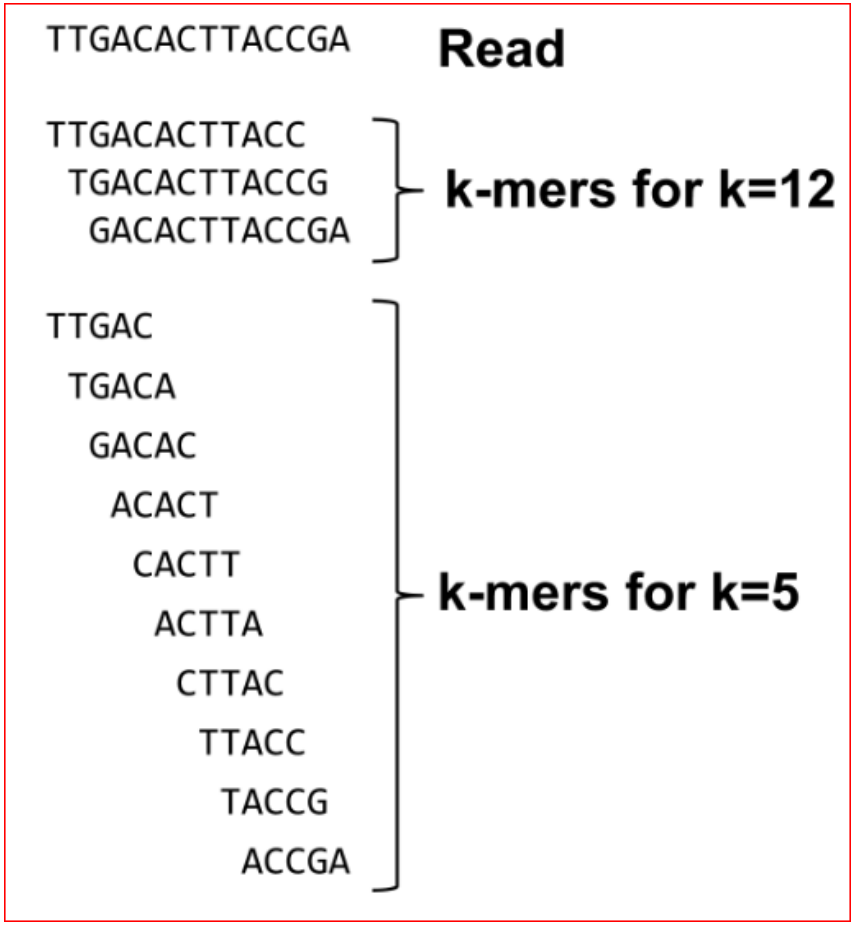
Assembly: gaps

- **Sequencing gaps:** Position and orientation known by spatial information
- **Physical gaps:** No information about adjacent contigs



Gene Cloning, Lodge *et al*.

Algorithms: DBG



Algorithms: DBG

Example #1:

HAPPI PINE INESS APPIN

All 4-mers:

HAPP PINE INES APPI

APPI NESS PPIN

Unique 4-mers:

HAPP APPI PINE PPIN INES NESS

Algorithms: DBG

Example #1:

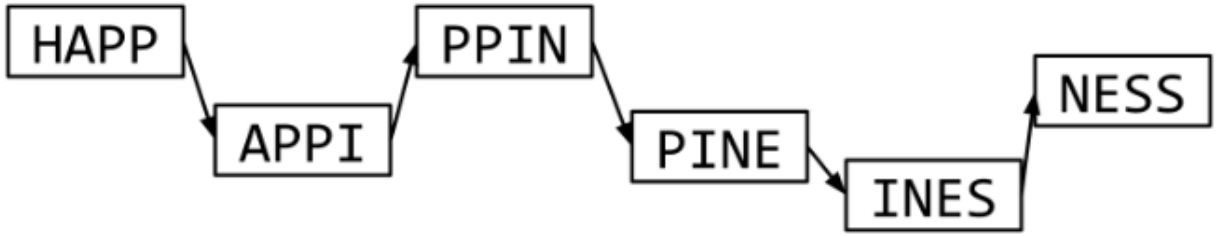
HAPPI PINE INESS APPIN

k = 4 k-mers:

HAPP APPI

PINE PPIN

INES NESS



Algorithms: DBG

Example #1:

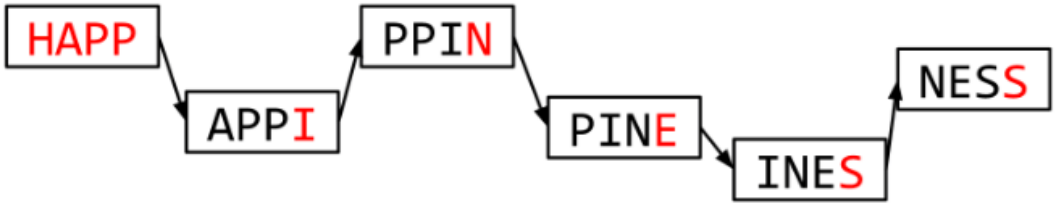
HAPPI PINE INESS APPIN

k = 4 k-mers:

HAPP APPI

PINE PPIN

INES NESS



HAPPINESS

Easy!

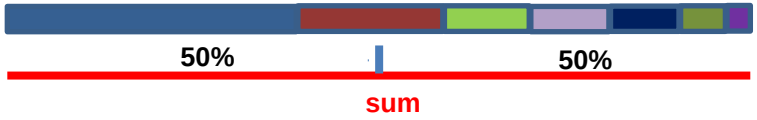
Algorithms: DBG

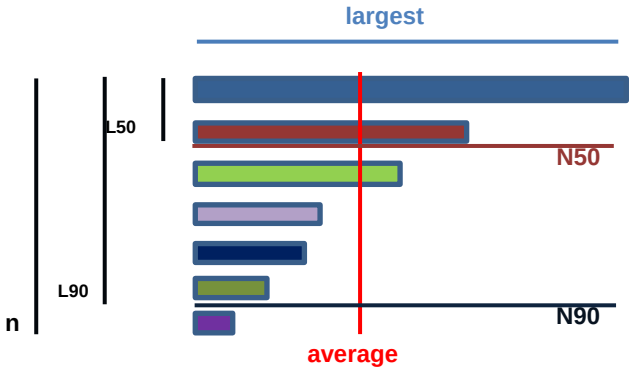
- Lower k
  - More connections
  - Less chance of resolving small repeats
  - Higher k-mer coverage
- Higher k
  - Less connections
  - More chance of resolving small repeats
  - Lower k-mer coverage

Optimum value for k will balance these effects.

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





Assembly: Evaluation - Quast

- Assembly evaluation: Quast, *Gurevich et al., Bioinformatics 2013, 29:8*

Worst Median Best ☑ Show heatmap

Genome statistics	RA_L2073_paired_assembly	RA_L2391_paired_assembly	RA_L2677_paired_assembly	RA_L2978_paired_assembly	RA_L2281_paired_assembly	RA_L2450_paired_assembly	RA_L2701_paired_assembly
Genome fraction (%)	81.079	88.828	84.92	90.172	85.733	88.172	92.463
Duplication ratio	1	1	1.001	1.001	1.001	1	1
# genomic features	1736 + 824 part	2113 + 600 part	1881 + 768 part	2157 + 611 part	1992 + 637 part	2073 + 643 part	2368 + 412 part
Largest alignment	16 612	33 033	21 336	25 068	29 638	30 305	40 471
Total aligned length	2 405 510	2 635 297	2 519 300	2 675 166	2 543 440	2 615 874	2 743 222
NGA50	3176	6162	4234	5948	5104	5358	9519
LGA50	267	151	219	153	166	166	96
Misassemblies							
# misassemblies	23	1	14	2	17	12	4
Misassembled contigs length	84 193	9611	45 868	6390	111 490	72 879	37 962
Mismatches							
# mismatches per 100 kbp	17	18.78	15	16.71	341.39	15.75	13.49
# indels per 100 kbp	1.21	1.25	1.87	1.94	7.27	1.45	0.87
# N's per 100 kbp	0	0	0	0	0	0	0
Statistics without reference							
# contigs	748	546	684	569	569	584	392
Largest contig	16 612	33 033	21 336	25 068	30 915	30 305	40 471
Total length	2 440 656	2 676 227	2 562 578	2 714 287	2 629 607	2 618 624	2 787 129
Total length (>= 1000 bp)	2 439 127	2 676 227	2 559 569	2 714 287	2 628 029	2 615 105	2 785 415
Total length (>= 10000 bp)	257 236	739 181	320 638	811 392	700 516	658 319	1 419 641
Total length (>= 50000 bp)	0	0	0	0	0	0	0

Extended report

Assembly: Evaluation - Quast

- Assembly evaluation: Quast, *Gurevich et al., Bioinformatics 2013, 29:8*

Thanks for your attention!

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