Session 2.1 – Quality assessment and read preprocessing

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Unidades Comunes Científico Técnicas – SGSAFI-ISCIII

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Programa Formación Continua, ISCIII

Step in the process

Preprocessing

Alignment

Postprocessing: Genome analysis…

Raw output files format

Illumina

.fastq

454 .sff

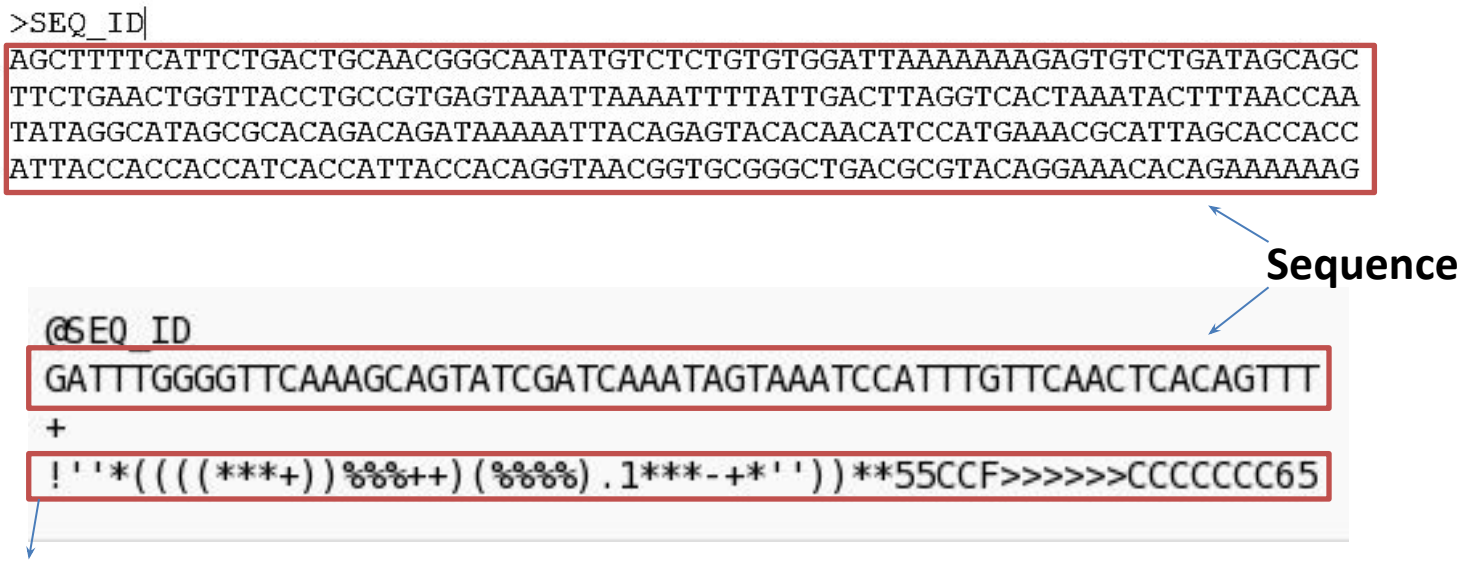
SOLiD
.fasta
.qual

Nanopore
.fast5 or .fastq

PacBio RSII
Bax.h
5

FASTQ format

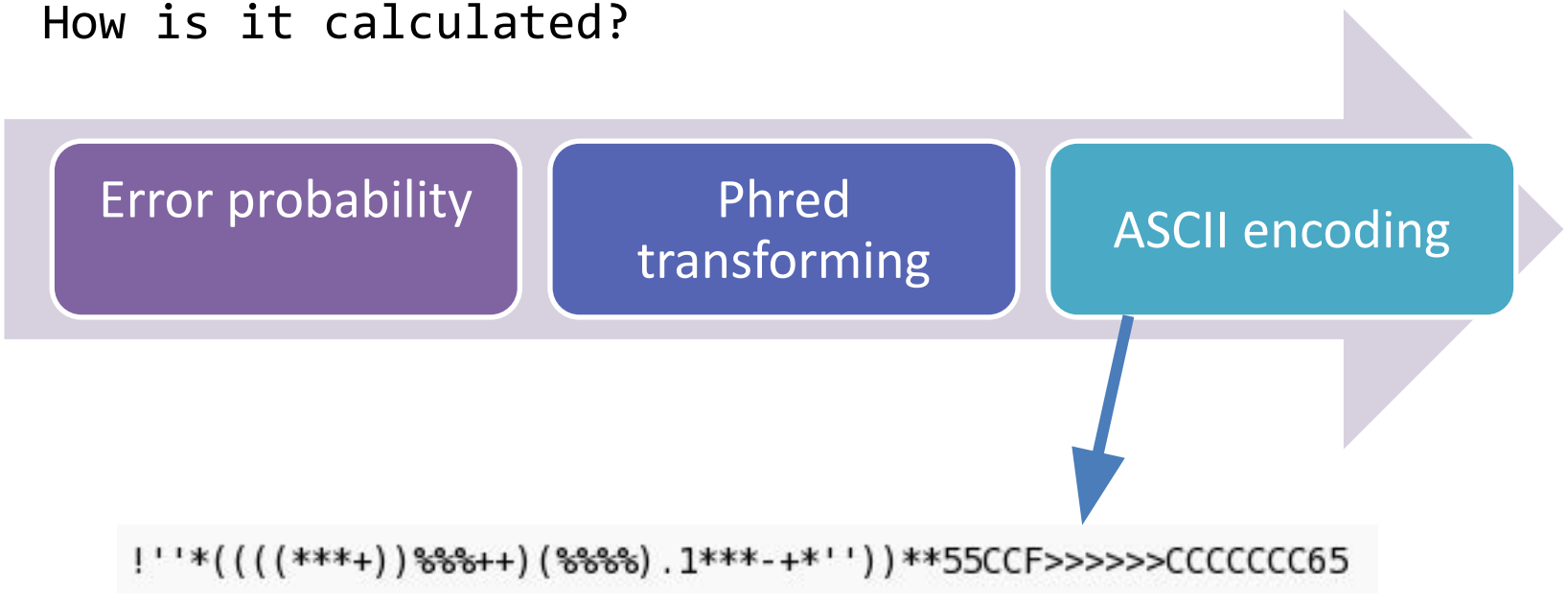
- Is a FASTA file with quality information
- Within HTS, FASTA contain genomes y FASTQ reads



Quality: must be 1 bit

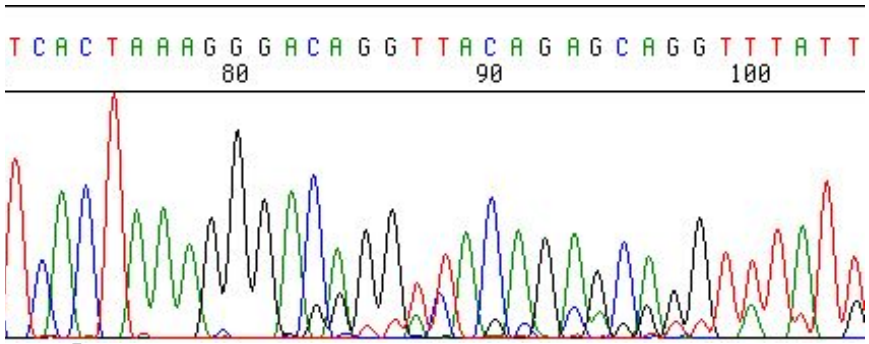
FASTQ format

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

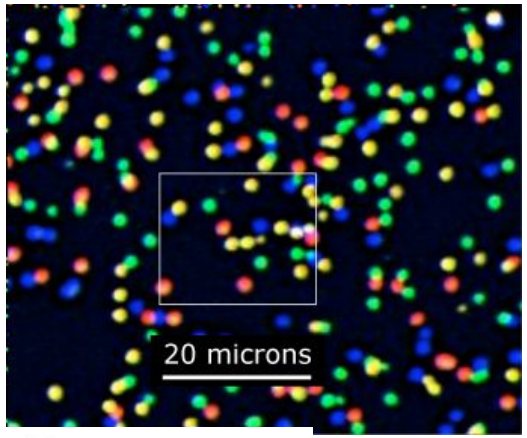


Phred quality and error probability

- **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%

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40	1 in 10,000	99.99%
50	1 in 100,000	99.999%

Error = 1 in 10.000

↓

P = 0.0001

↓

Q = -10*log10(0.0001)

↓

Q = 40

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)

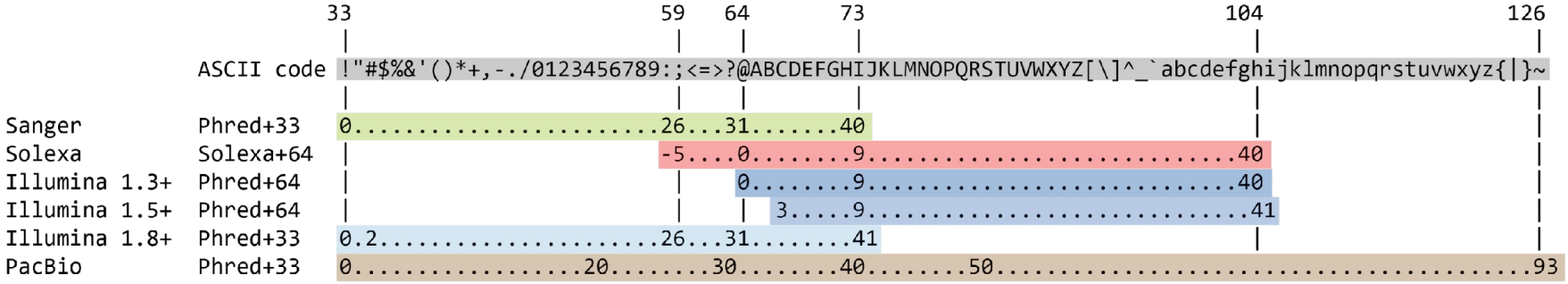
Phred quality and error probability

- **Phred 33 example**

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

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

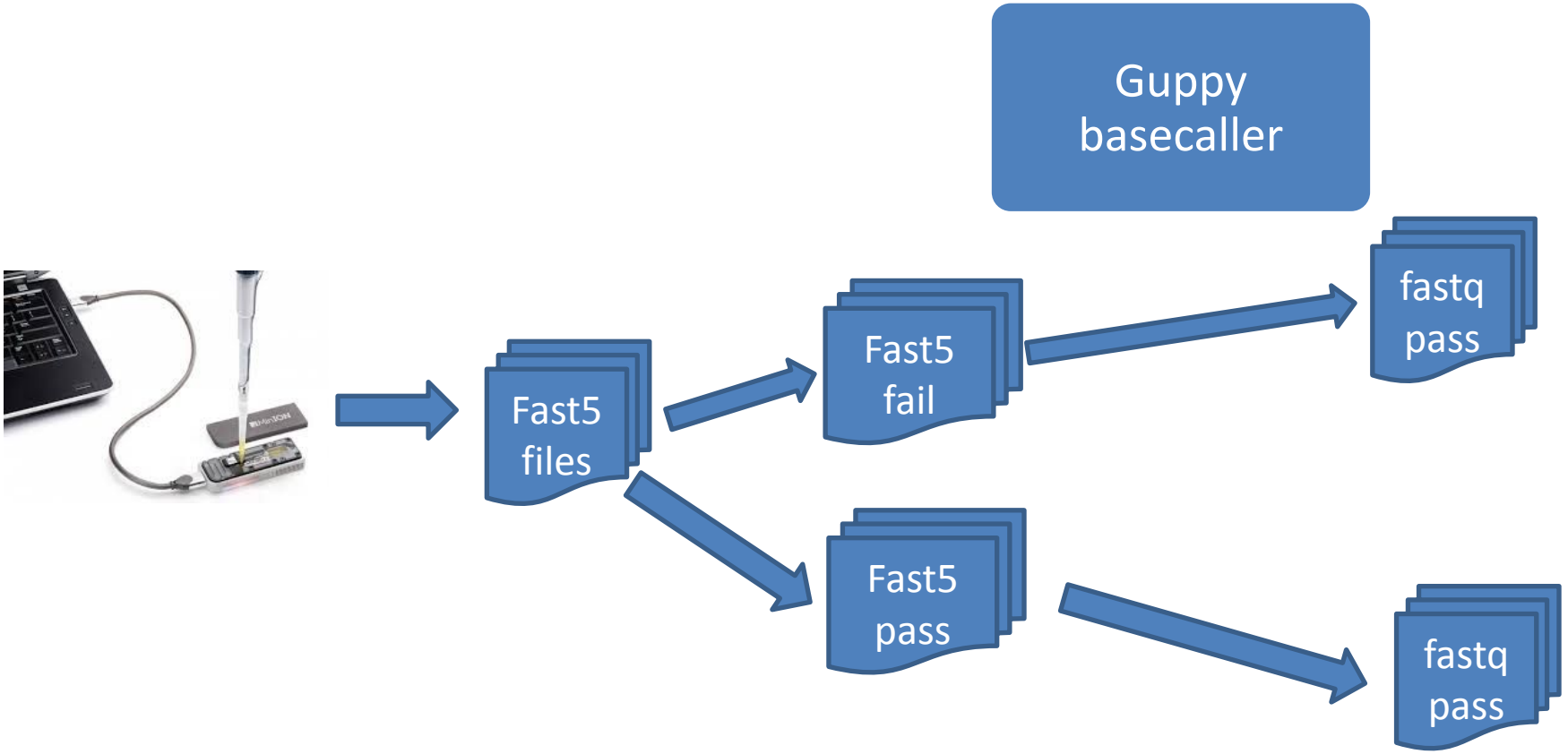
P=0.001 → Q=-10*log10(0.001)= 30 → ASCIII 33+30 = 63 → ?



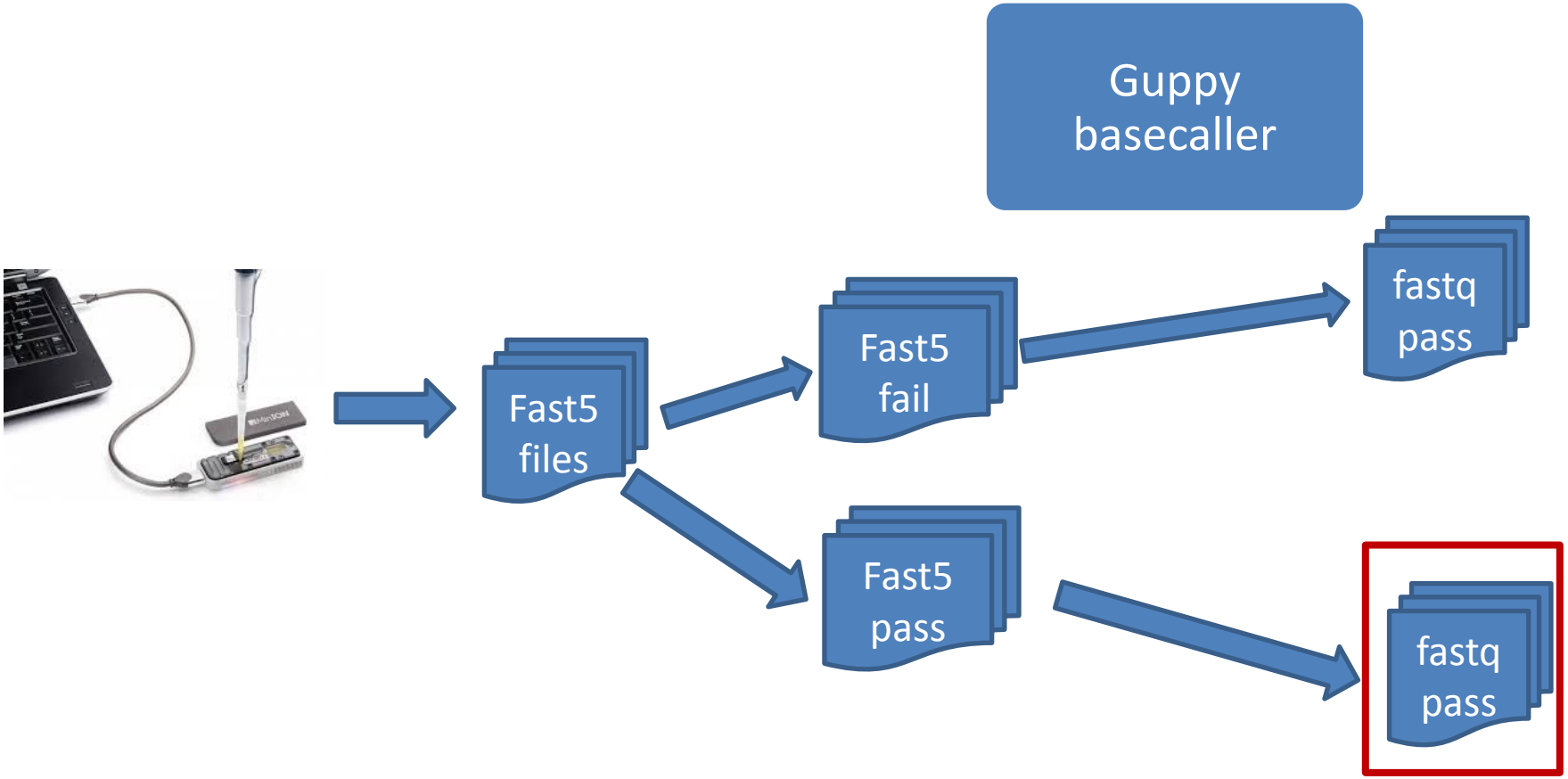
Error rate and Quality in Nanopore

Clara Delahaye, Jacques Nicolas. Nanopore MinION long read sequencer: an overview of its error landscape. 2020. ffhal-03123133f

Error rate and Quality in Nanopore

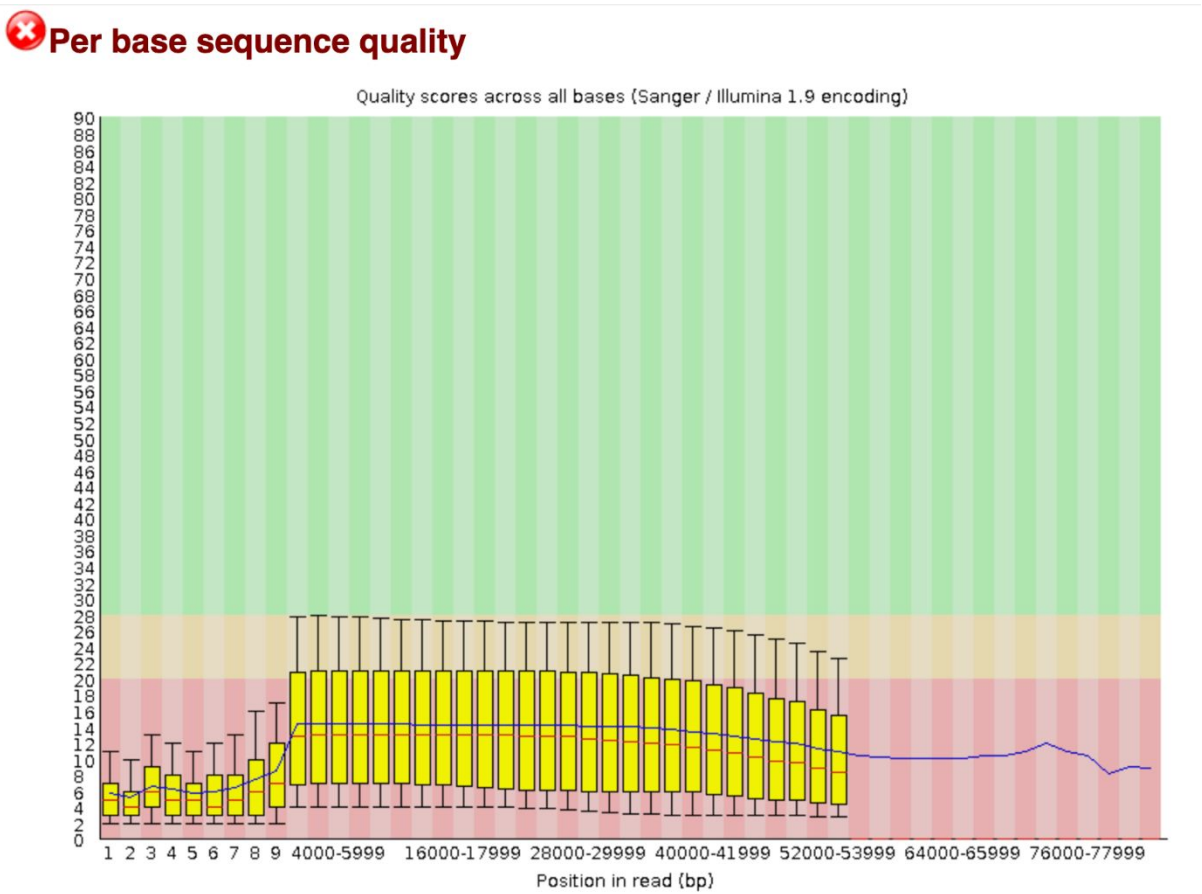


Error rate and Quality in Nanopore



Error rate and Quality in Nanopore

- Nanopore quality score (Q) does not follow Phred score
- To estimate error rate (E) (locally and at read level) E = 0.015Q2 − 1.15 + 24



FASTQ format

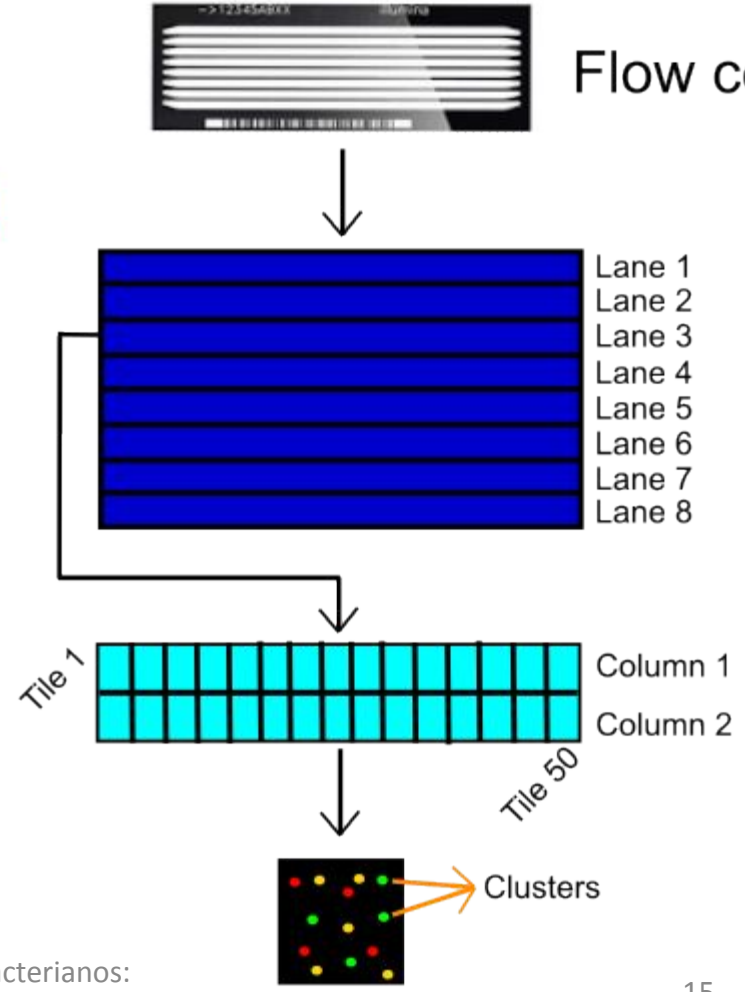
Illumina read header

Sequence Header +Sequence ID

a b c d e f g h i j k

@HWI-ST486:166:C06K9ACXX:7:1101:1443:1995 1:N:0:ACAGTG

a. unique instrument name
b. run id
c. flowcell id
d. flowcell lane
e. tile number within the flowcell lane
f. x-coordinate of the cluster within the tile
g. y-coordinate of the cluster within the tile
h. the member of a pair, 1 or 2 (paired-end or mate-pair reads only)
i. Y if the read fails filter (read is bad), N otherwise
j. 0 when no control bits are on
k. index sequence



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h. the member of a pair, 1 or 2 (paired-end or mate-pair reads only)
i. Y if the read fails filter (read is bad), N otherwise
j. 0 when no control bits are on
k. index sequence

```
@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
+
IIIIIHIHIIGGEGG#AACA@?=?BHHIIIIIHHIHIIIHIHIHHGIHIHGHGIGIHGEGGGGHG@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):

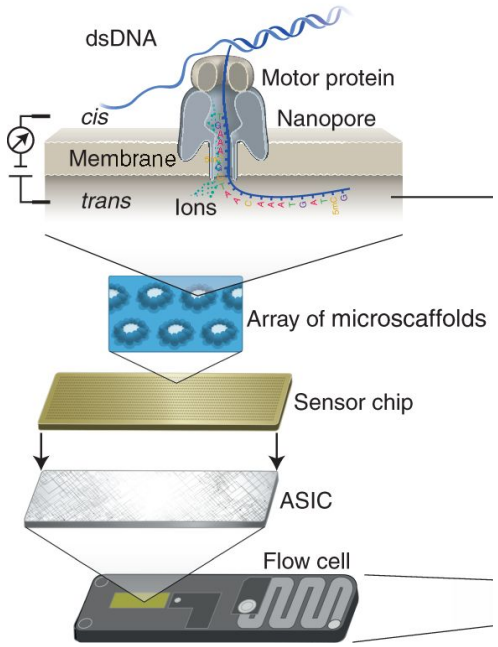
- "!"#$%" lowest quality
- "FGHI" highest quality

FASTQ format

Nanopore read header

```
@d76be4fb-11a9-47e7-90be-c4f15591e0d9
runid=ba02134f00f2059e7b2dc248113c02f76577b101 read=11 ch=142
start_time=2019-06-27T11:09:03Z flow_cell_id=FAH59799 protocol_group_id=k6963
sample_id=k6963
```

1. @read identifier
2. run-id
3. read-id
4. channel
5. start_time
6. flow_cell_id
7. protocol_group_id
8. sample_id



FASTQ format

Nanopore read header

@d76be4fb-11a9-47e7-90be-c4f15591e0d9
runid=ba02134f00f2059e7b2dc248113c02f76577b101 read=11 ch=142
start_time=2019-06-27T11:09:03Z flow_cell_id=FAH59799 protocol_group_id=k6963
sample_id=k6963

1. @read identifier
2. run-id
3. read-id
4. channel
5. start_time
6. flow_cell_id
7. protocol_group_id
8. sample_id

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**

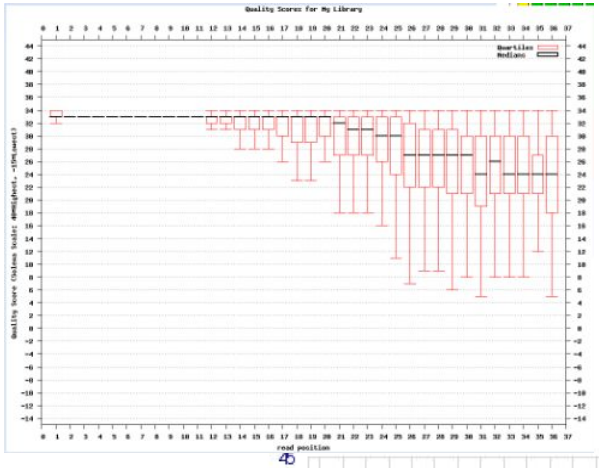
Sequencing quality assessment: Artifacts

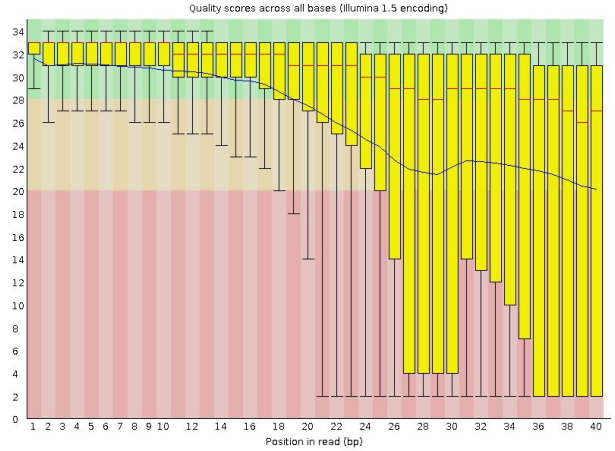
HTS methods are bounded by their technical and theoretical limitations and sequencing errors cannot be completely eliminated (Hadigol M, Khiabanian H. 2018)

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

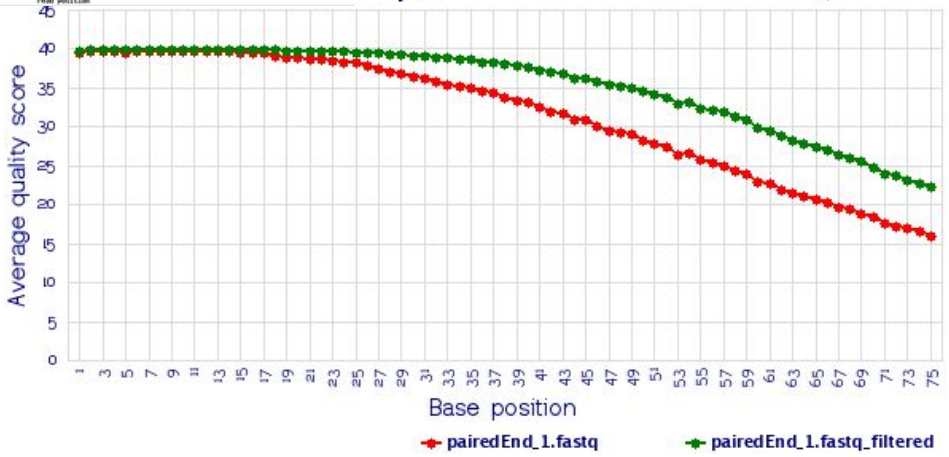
Sequencing quality assessment

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



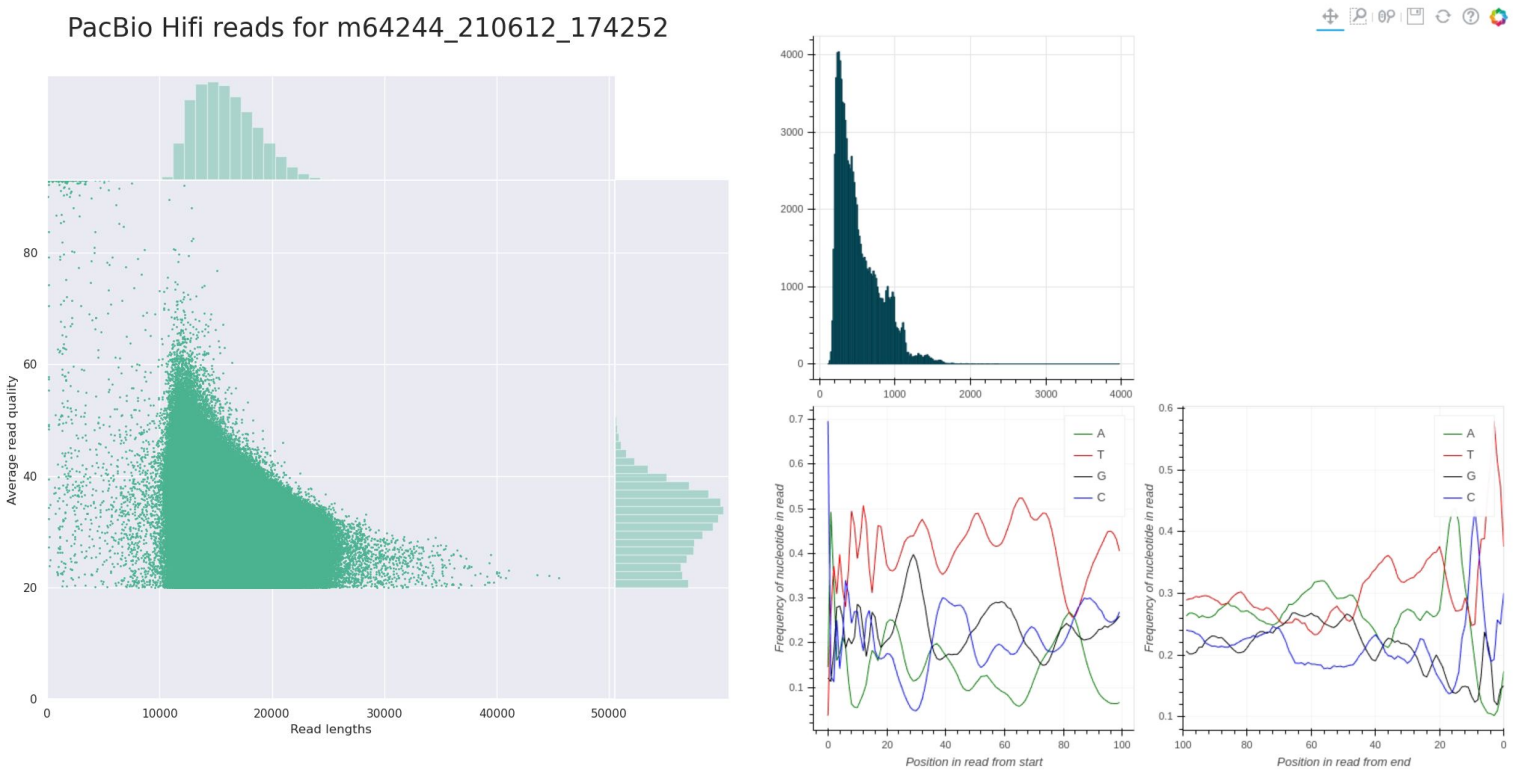


pair



Sequencing quality assessment

- Long reads: Nanoplot, PycoQC, NanoQC, etc.



Sequencing quality assessment: fastp

- **Fastp**

fastp report

Summary

General

fastp version:	0.20.1 (https://github.com/OpenGene/fastp)
sequencing:	paired end (149 cycles + 149 cycles)
mean length before filtering:	116bp, 116bp
mean length after filtering:	117bp, 117bp
duplication rate:	1.704150%
Insert size peak:	95
Detected read1 adapter:	CACCTAAGTTGGCGTATACGCGTAATATATCTGGGTTTTCTACAAAATCATACCAGTCCT
Detected read2 adapter:	CACCTAAGTTGGCGTATACGCGTAATATATCTGGGTTTTCTACAAAATCATACCAGTCCT

Before filtering

total reads:	1.296756 M
total bases:	151.424921 M
Q20 bases:	143.112834 M (94.510754%)
Q30 bases:	137.905419 M (91.071812%)
GC content:	40.410939%

After filtering

total reads:	854.250000 K
total bases:	100.537720 M
Q20 bases:	99.598139 M (99.065444%)
Q30 bases:	97.968091 M (97.444115%)
GC content:	39.665634%

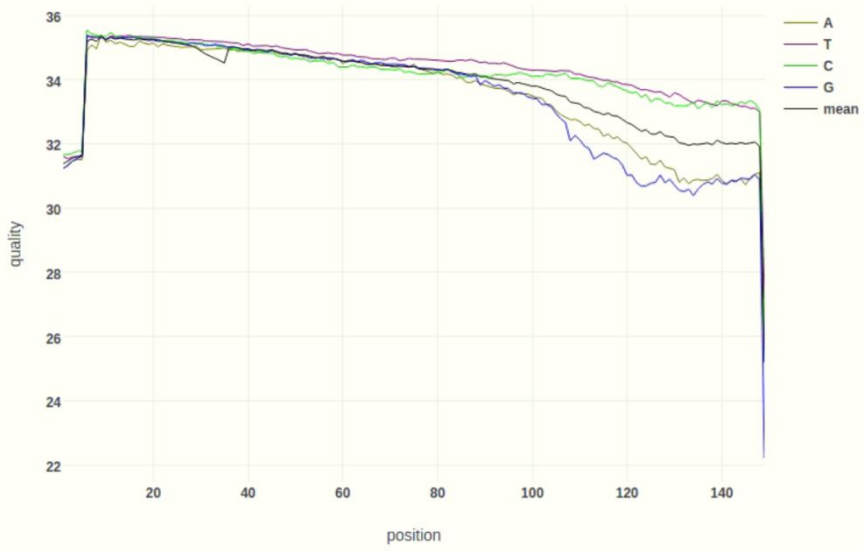
Filtering result

reads passed filters:	854.250000 K (65.875924%)
reads with low quality:	352.272000 K (27.165635%)
reads with too many N:	84 (0.006478%)
reads too short:	90.150000 K (6.951963%)

Before filtering

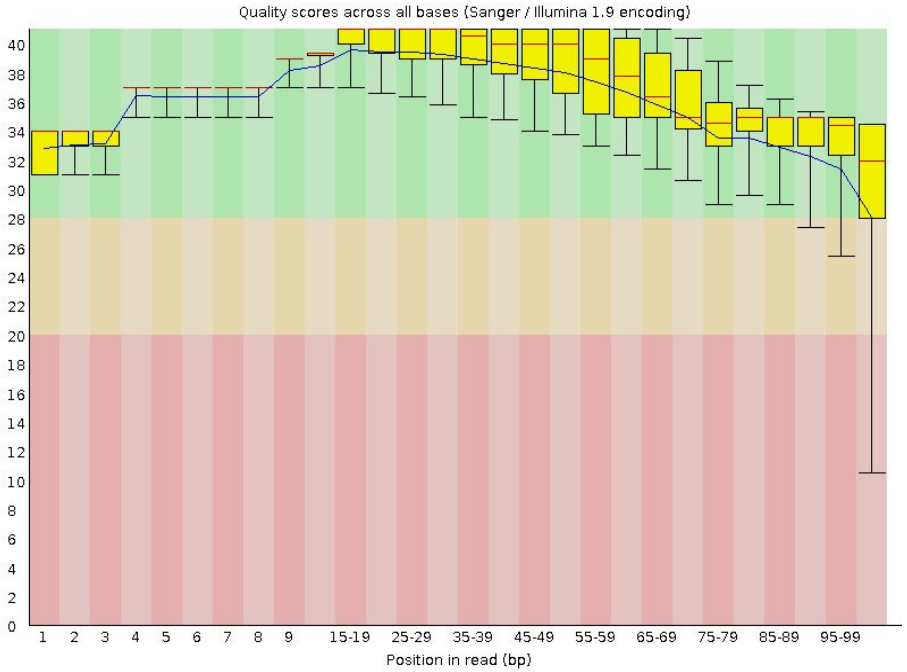
Before filtering: read1: quality

Value of each position will be shown on mouse over.

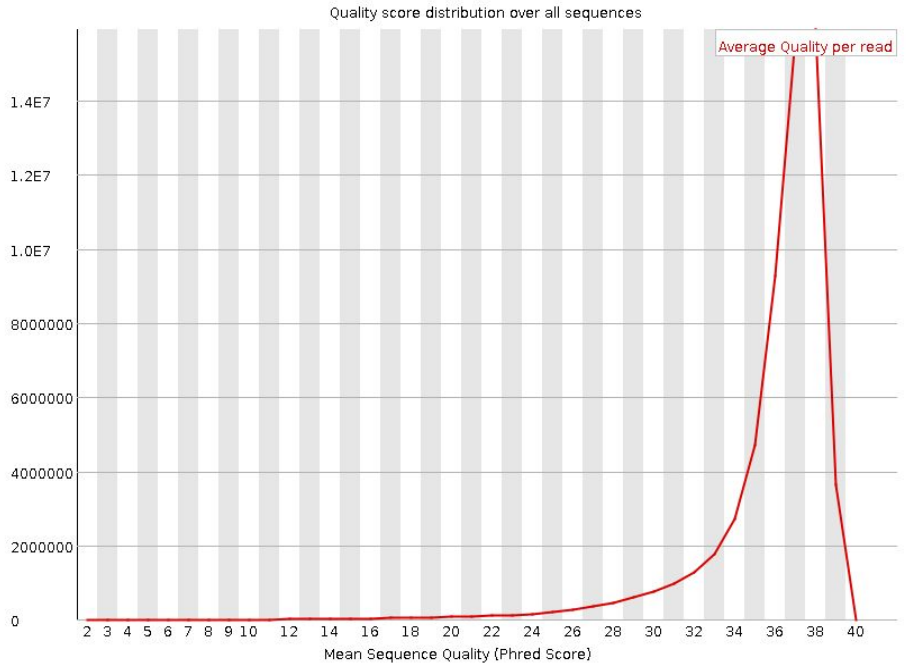


Sequencing quality assessment: FastQC

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

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Measure	Value
Filename	good_sequence_short.txt
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	250000
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Sequence length	40
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FASTQ format

Illumina read header

Sequence Header +Sequence ID

a b c d e f g h i j k

@HWI-ST486:166:C06K9ACXX:7:1101:1443:1995 1:N:0:ACAGTG

a. **unique instrument name**
b. run id
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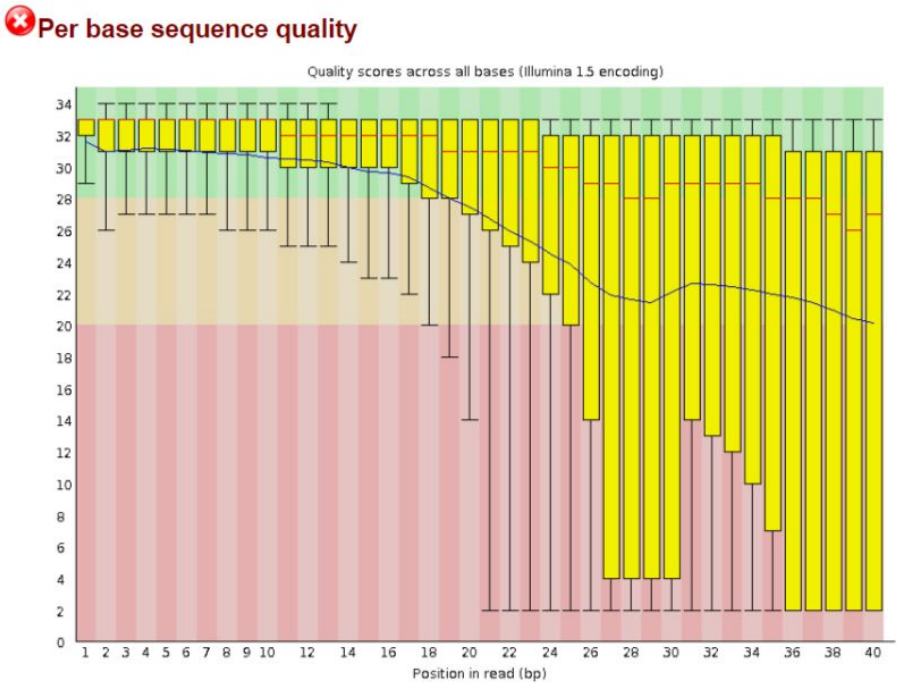
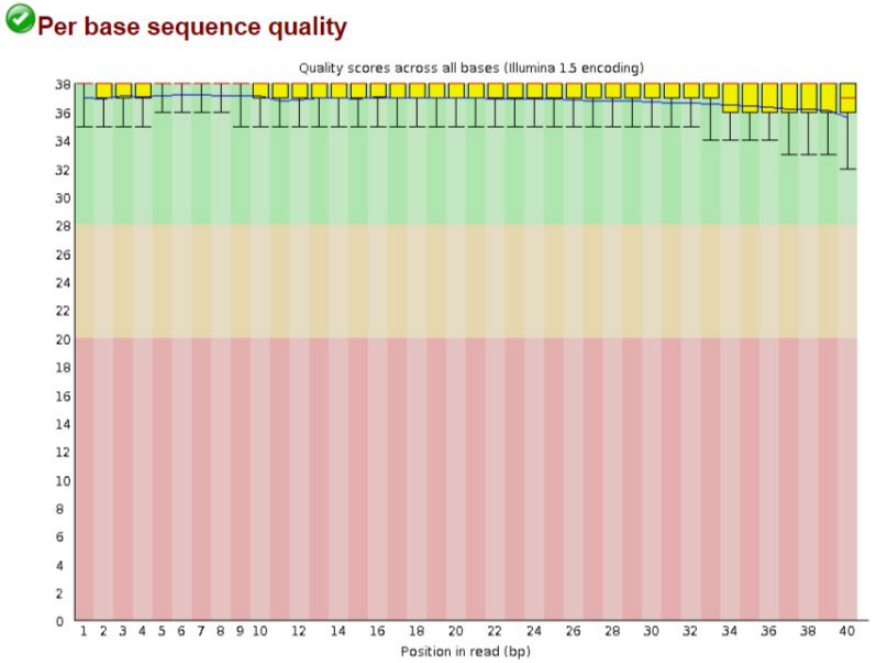
FastQC: Per base sequence quality

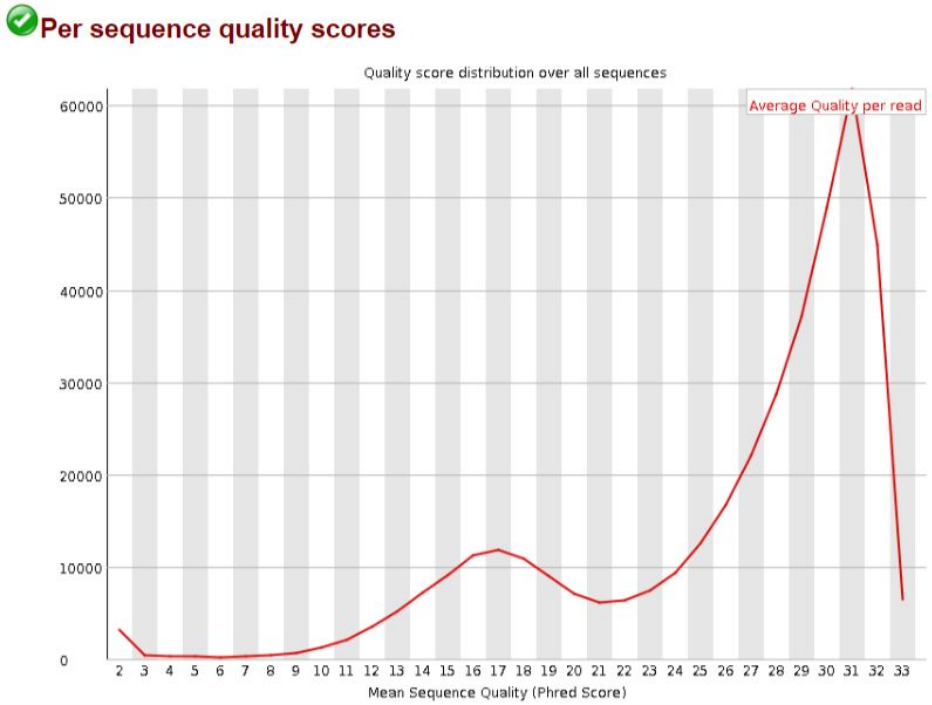
- Overview of the range of quality values across all bases at each position in the FastQ file
- **Median**, **inter-quartile range** **(25-75%), 10-90% points,** **mean quality**

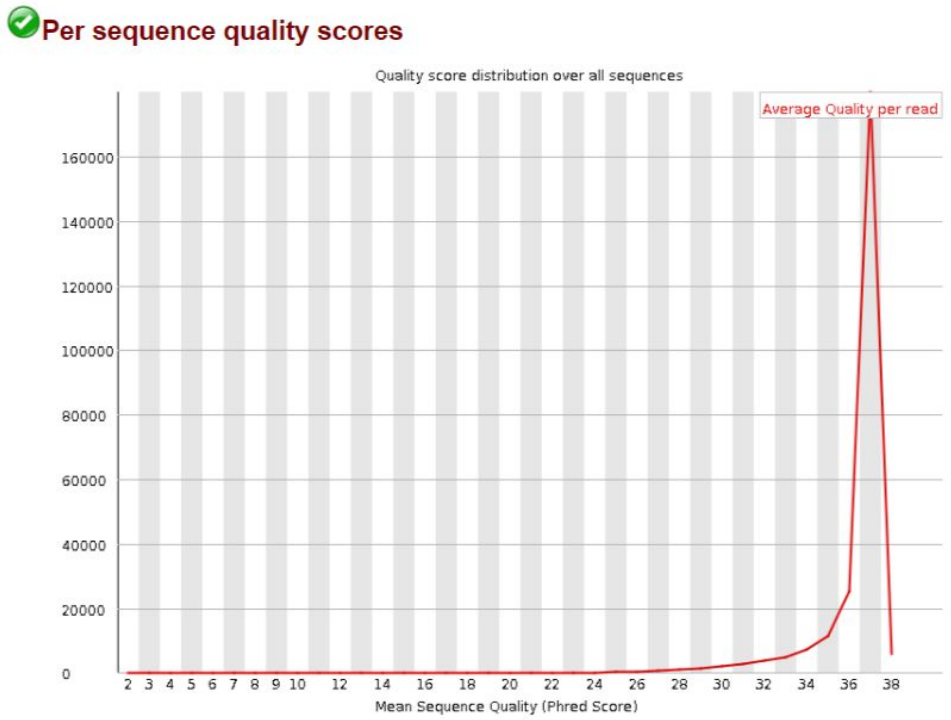




FastQC: Per sequence quality score

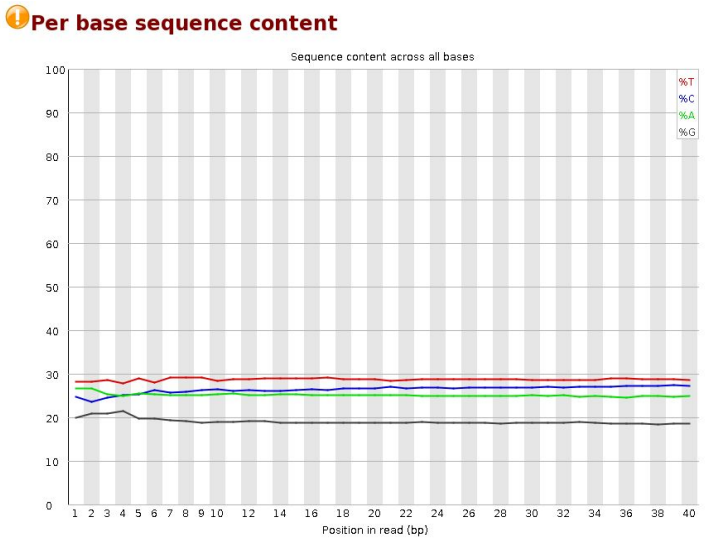
- Number of sequences with the same mean quality

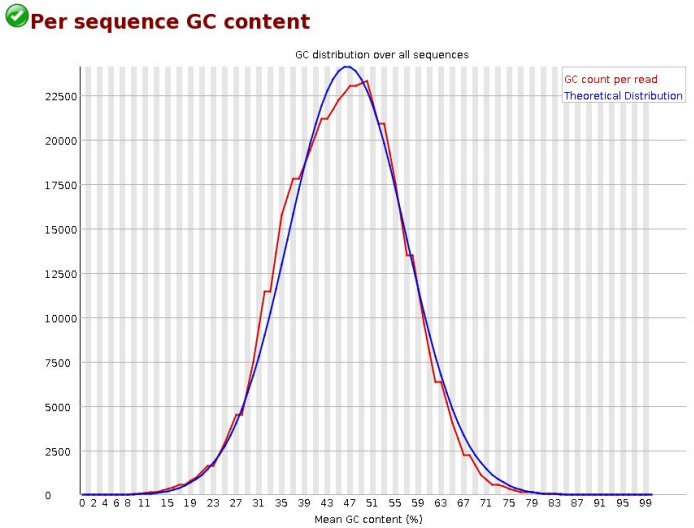




FastQC: Nucleotide related errors

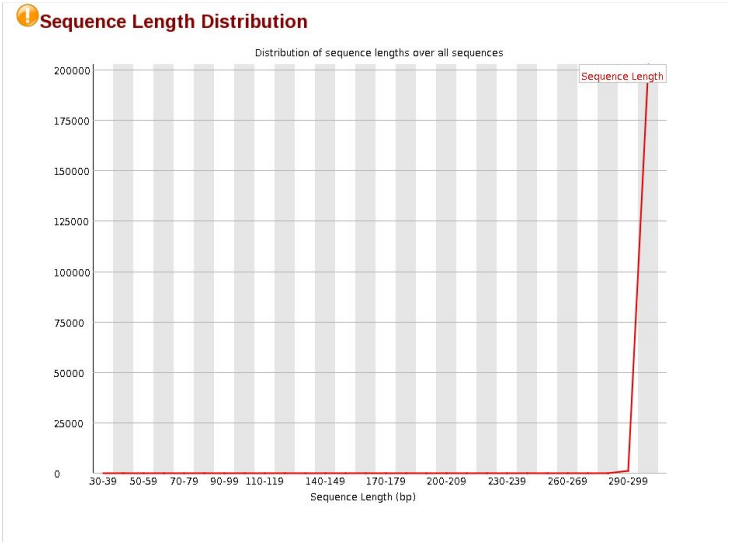
- How expected nucleotide distribution deviates from expected
  - Per base sequence content
  - Per base GC content
  - Per sequence GC content
  - Per base N content

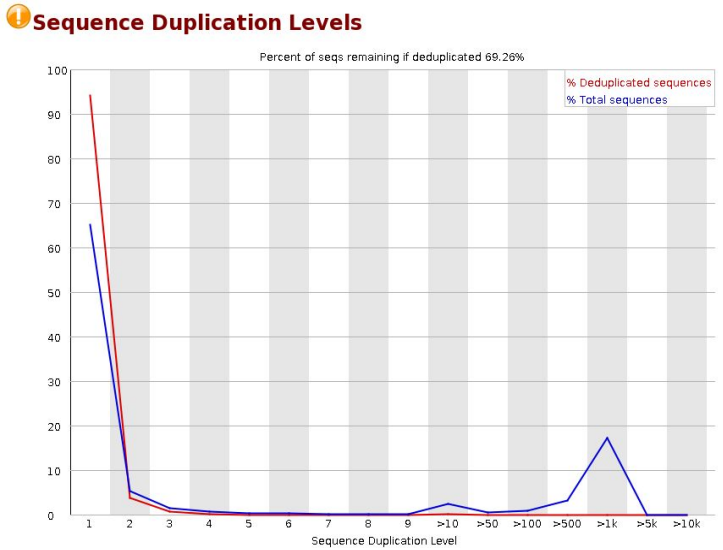




FastQC: Sequence related errors

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





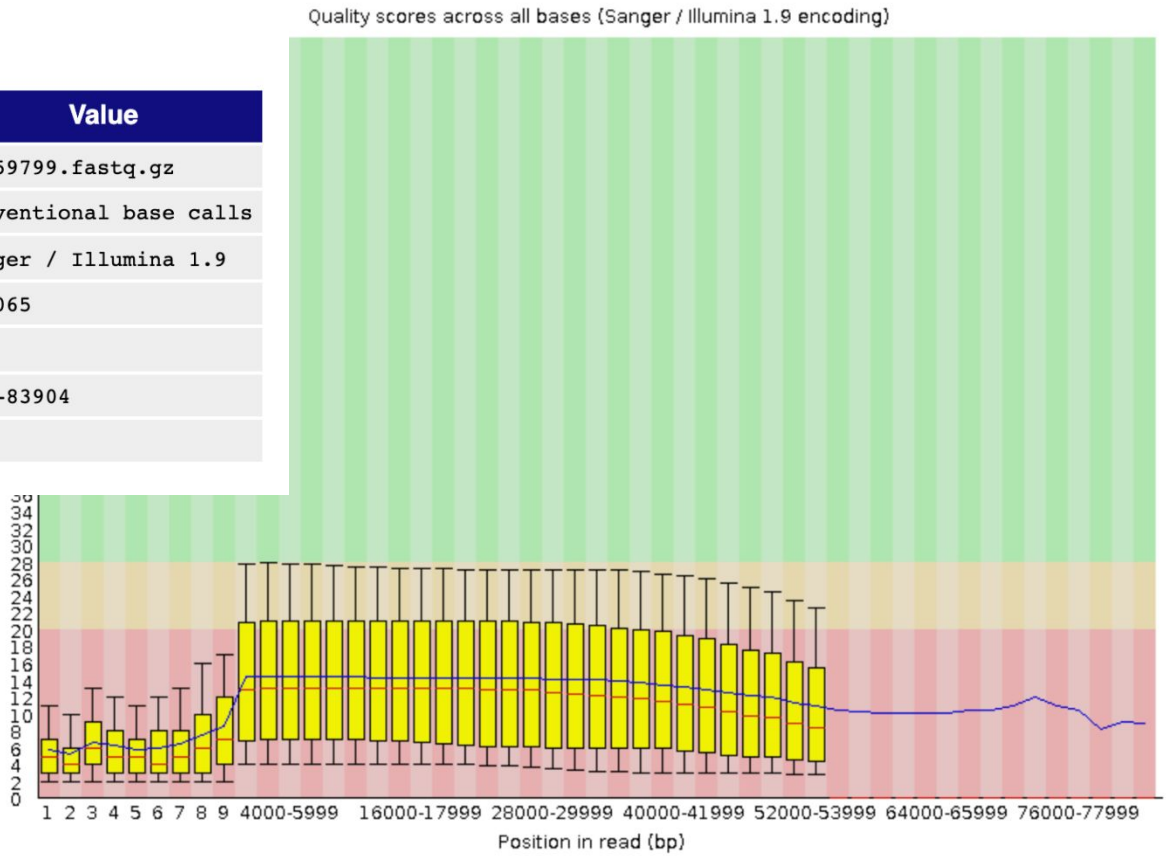
FastQC: Per base sequence quality

- **Nanopore**

Basic Statistics

Measure	Value
Filename	FAH59799.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	468065
Sequences flagged as poor quality	0
Sequence length	116-83904
%GC	54

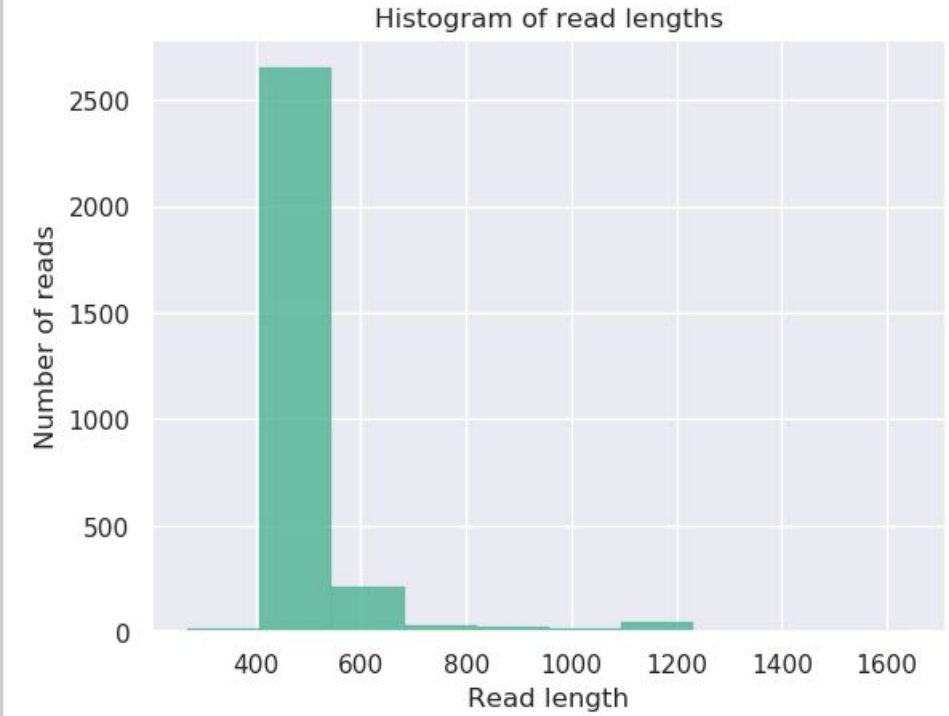
Per base sequence quality



Sequencing quality assessment: NanoPlot

- **NanoPlot**

Histogram of read lengths



NanoPlot report

Summary statistics

feature	
General summary	
Mean read length	537.5
Mean read quality	13.9
Median read length	516.0
Median read quality	14.0
Number of reads	3,000.0
Read length N50	517.0
Total bases	1,612,409.0
Number, percentage and megabases of reads above quality cutoffs	
>Q5	3000 (100.0%) 1.6Mb
>Q7	3000 (100.0%) 1.6Mb
>Q10	2865 (95.5%) 1.5Mb
>Q12	2461 (82.0%) 1.3Mb
>Q15	905 (30.2%) 0.5Mb
Top 5 highest mean basecall quality scores and their read lengths	
1	21.3 (504)
2	20.2 (517)
3	20.1 (509)
4	20.0 (526)
5	19.9 (530)
Top 5 longest reads and their mean basecall quality score	
1	1643 (13.5)
2	1641 (16.7)
3	1533 (12.5)
4	1427 (13.2)
5	1383 (15.0)

Sequencing quality assessment: NanoPlot

- **NanoPlot**

NanoPlot report

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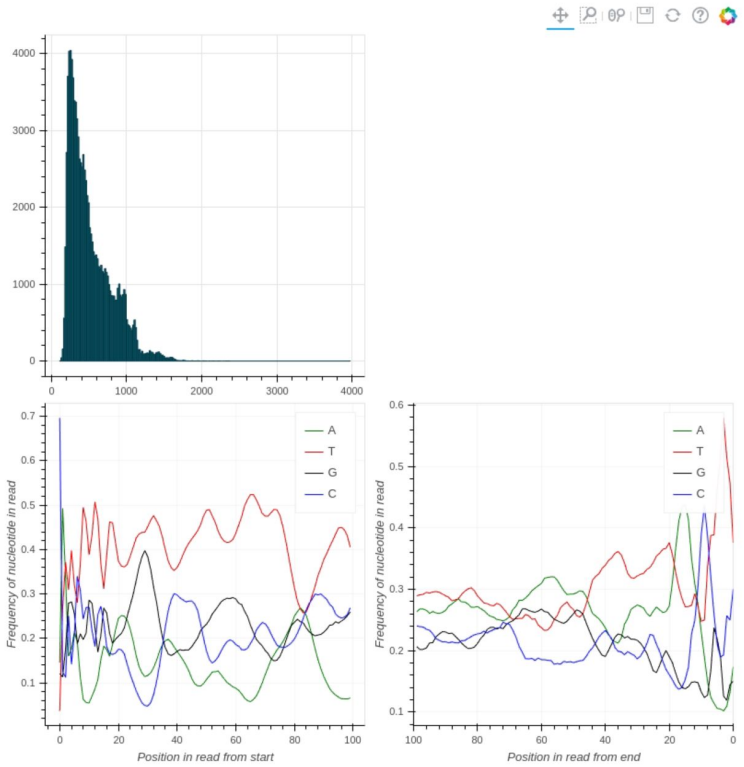
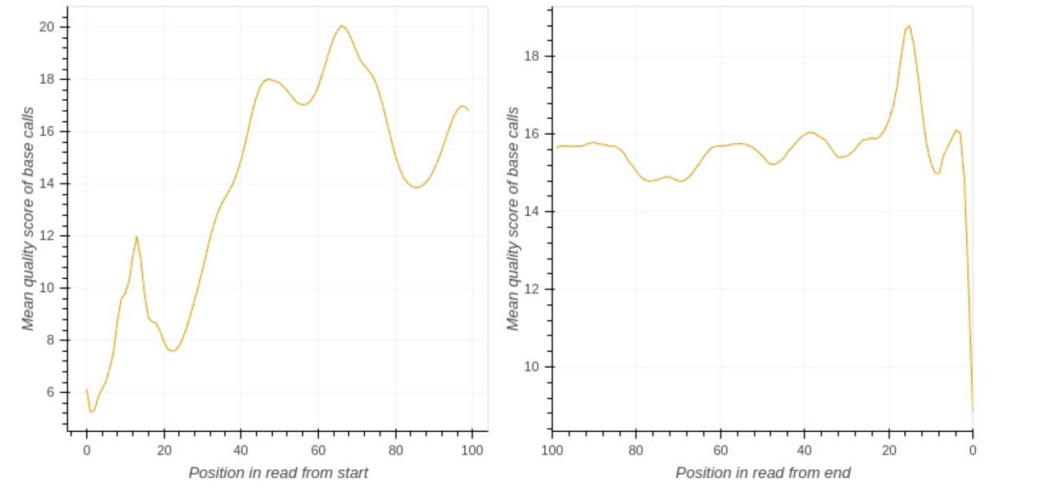
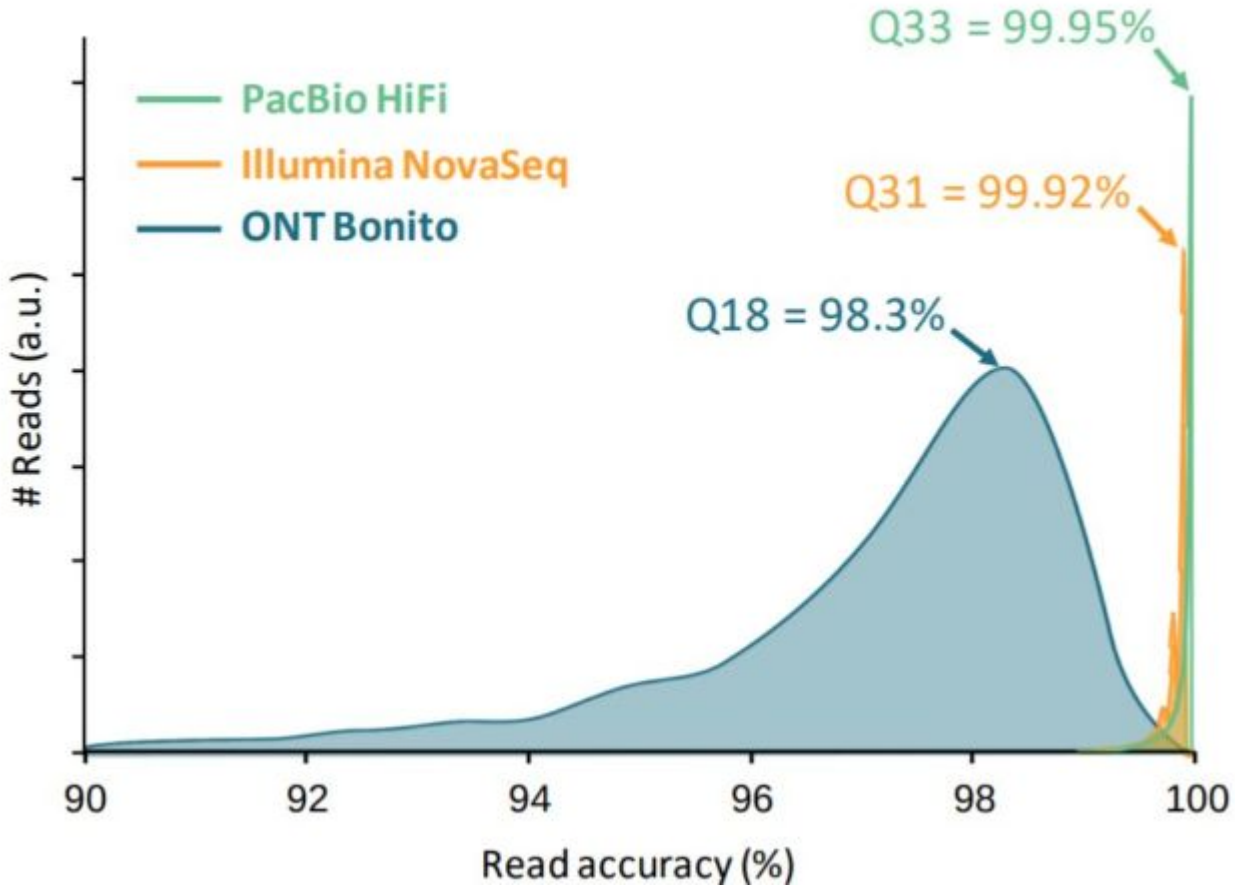
Sequencing quality assessment: NanoPlot

- **NanoQC**



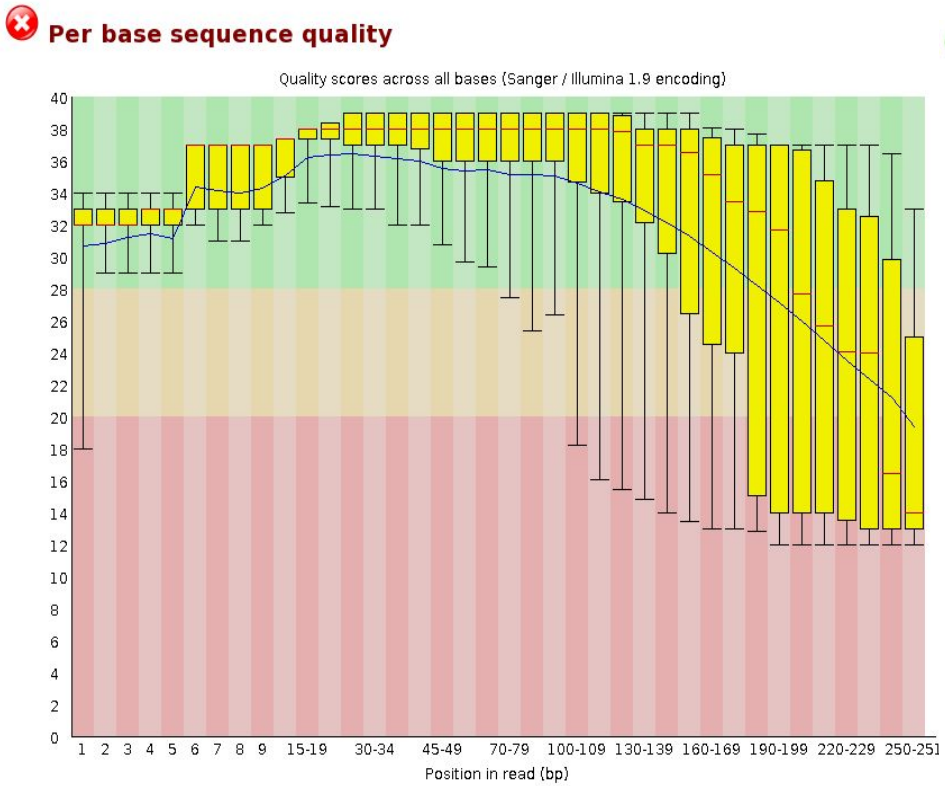


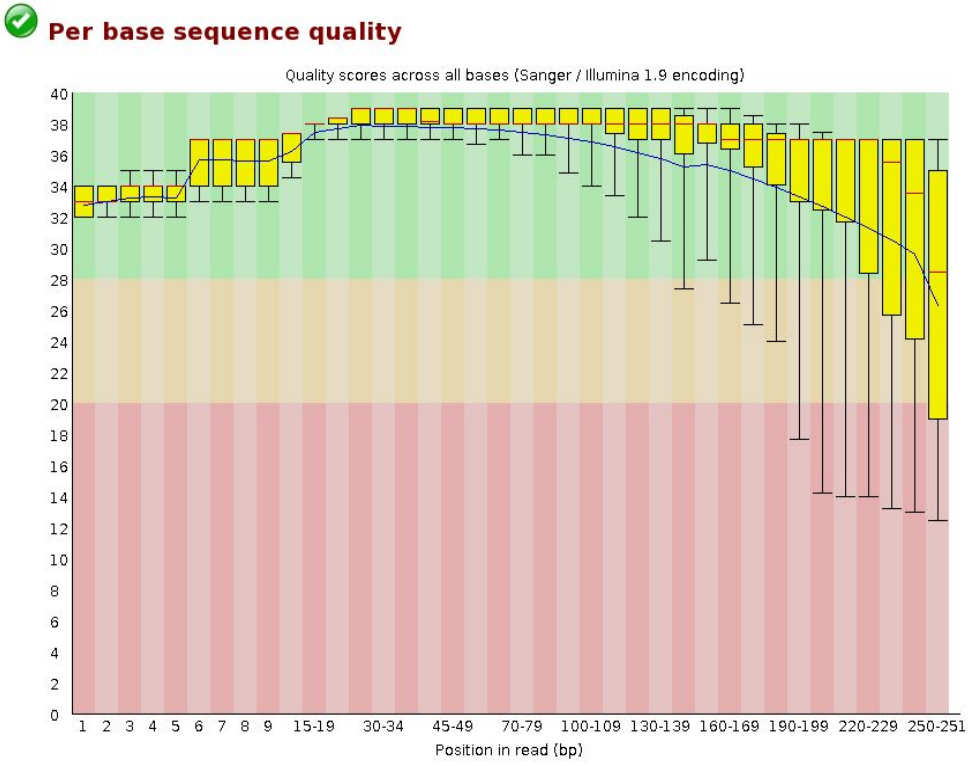
Sequencing quality assessment



FastQC: Per base sequence quality

- **Miseq asymmetry**





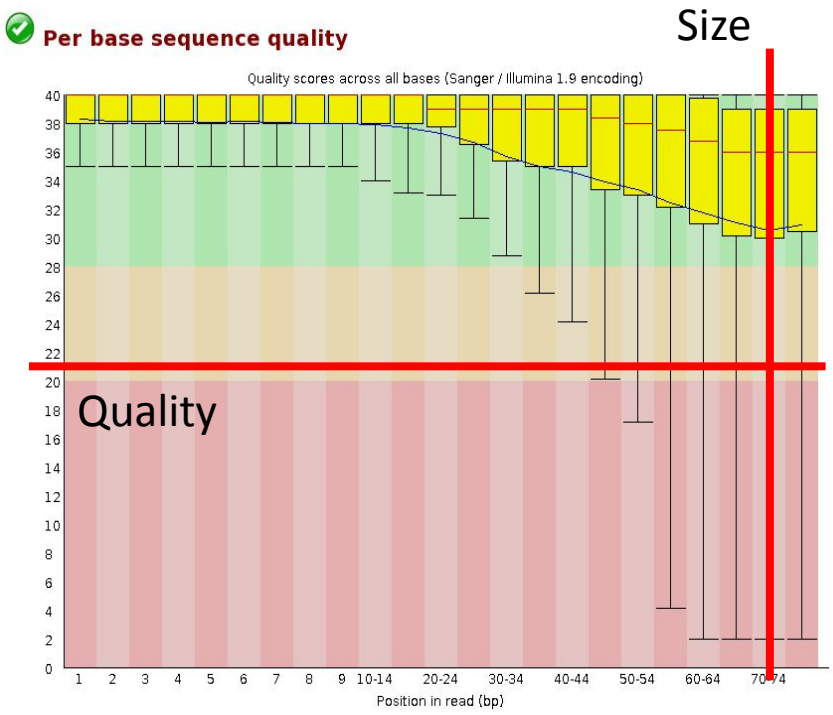
Sequencing quality assessment: Bias

- **Small/micro RNA**: In small RNA libraries, we typically have a relatively small set of unique, short sequences. Small RNA libraries are not randomly sheared before adding sequencing adapters to their ends: all the reads for specific classes of microRNAs will be identical. It will result in:
  - Extremely biased per base sequence content
  - Extremely narrow distribution of GC content
  - Very high sequence duplication levels
  - Abundance of overrepresented sequences
  - Read-through into adapters
- **Amplicon:** Amplicon libraries are prepared by PCR amplification of a specific target. For example, the V4 hypervariable region of the bacterial 16S rRNA gene. All reads from this type of library are expected to be nearly identical. It will result in:
  - Extremely biased per base sequence content
  - Extremely narrow distribution of GC content
  - Very high sequence duplication levels
  - Abundance of overrepresented sequences
- **Bisulfite or Methylation sequencing:** With Bisulfite or methylation sequencing, the majority of the cytosine (C) bases are converted to thymine (T). It will result in:
  - Biased per base sequence content
  - Biased per sequence GC content
- **Adapter dimer contamination**: Any library type may contain a very small percentage of adapter dimer (i.e. no insert) fragments. They are more likely to be found in amplicon libraries constructed entirely by PCR (by formation of PCR primer-dimers) than in DNA-Seq or RNA-Seq libraries constructed by adapter ligation. If a sufficient fraction of the library is adapter dimer it will become noticeable in the FastQC report:
  - Drop in per base sequence quality after base 60
  - Possible bi-modal distribution of per sequence quality scores
  - Distinct pattern observed in per bases sequence content up to base 60
  - Spike in per sequence GC content
  - Overrepresented sequence matching adapter
  - Adapter content > 0% starting at base 1

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



Sequencing quality filtering

- Illumina:
  - **Fastp**
  - **Trimmomatic**
  - Trim galore!
- Nanopore:
  - Nanofilt
  - nanoq

Sequencing quality filtering: fastp

- **Fastp**

fastp report

Summary

General

fastp version:	0.20.1 (https://github.com/OpenGene/fastp)
sequencing:	paired end (149 cycles + 149 cycles)
mean length before filtering:	116bp, 116bp
mean length after filtering:	117bp, 117bp
duplication rate:	1.704150%
Insert size peak:	95
Detected read1 adapter:	CACCTAAGTTGGCGTATACGCGTAATATATCTGGGTTTTCTACAAAATCATACCAGTCCT
Detected read2 adapter:	CACCTAAGTTGGCGTATACGCGTAATATATCTGGGTTTTCTACAAAATCATACCAGTCCT

Before filtering

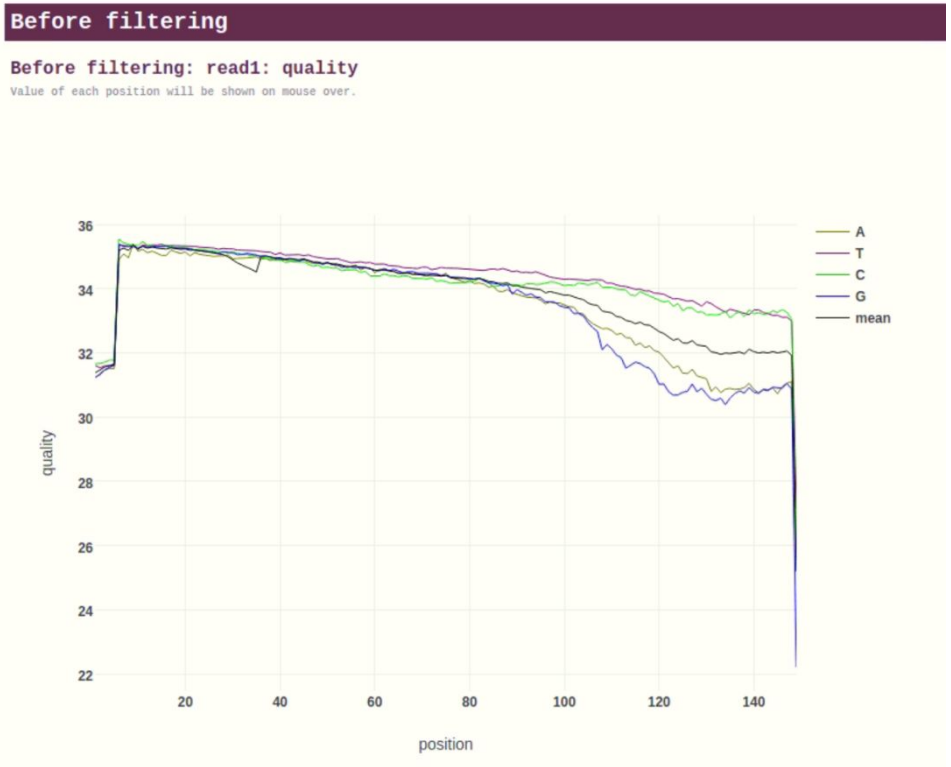
total reads:	1.296756 M
total bases:	151.424921 M
Q20 bases:	143.112834 M (94.510754%)
Q30 bases:	137.905419 M (91.071812%)
GC content:	40.410939%

After filtering

total reads:	854.250000 K
total bases:	100.537720 M
Q20 bases:	99.598139 M (99.065444%)
Q30 bases:	97.968091 M (97.444115%)
GC content:	39.665634%

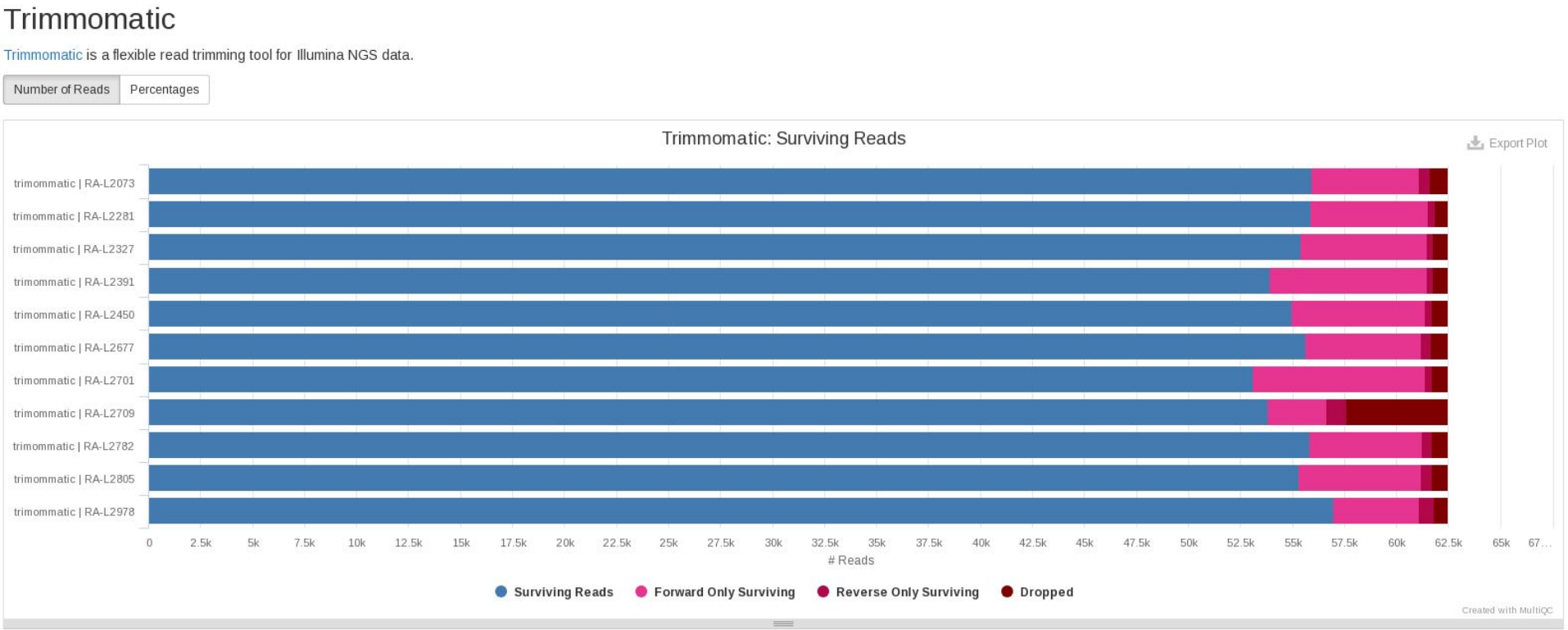
Filtering result

reads passed filters:	854.250000 K (65.875924%)
reads with low quality:	352.272000 K (27.165635%)
reads with too many N:	84 (0.006478%)
reads too short:	90.150000 K (6.951963%)



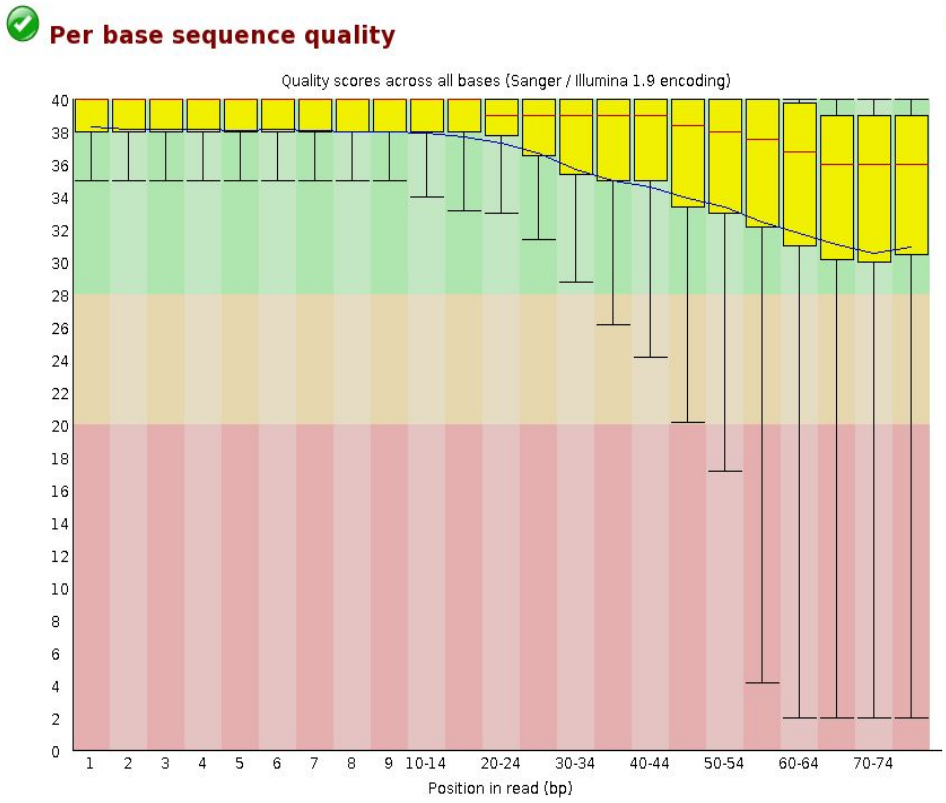
Sequencing quality filtering: Trimmomatic

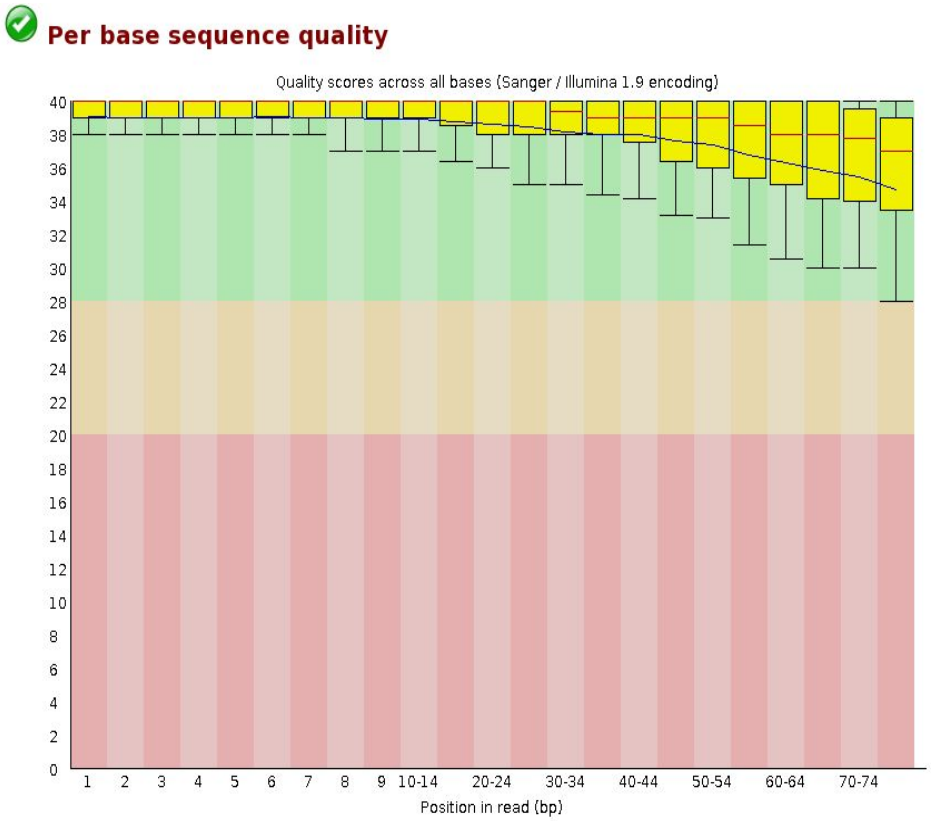
- **Trimmomatic**



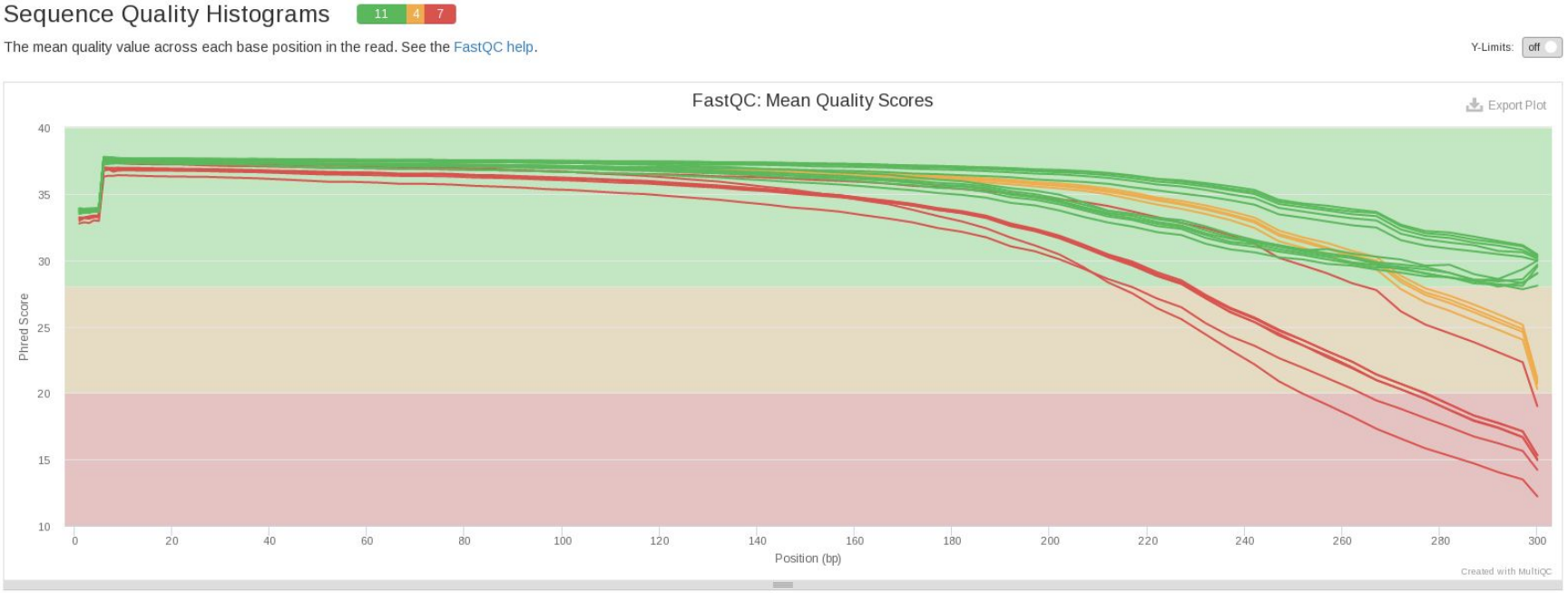
Sequence filtering

- **Example of quality filtering**

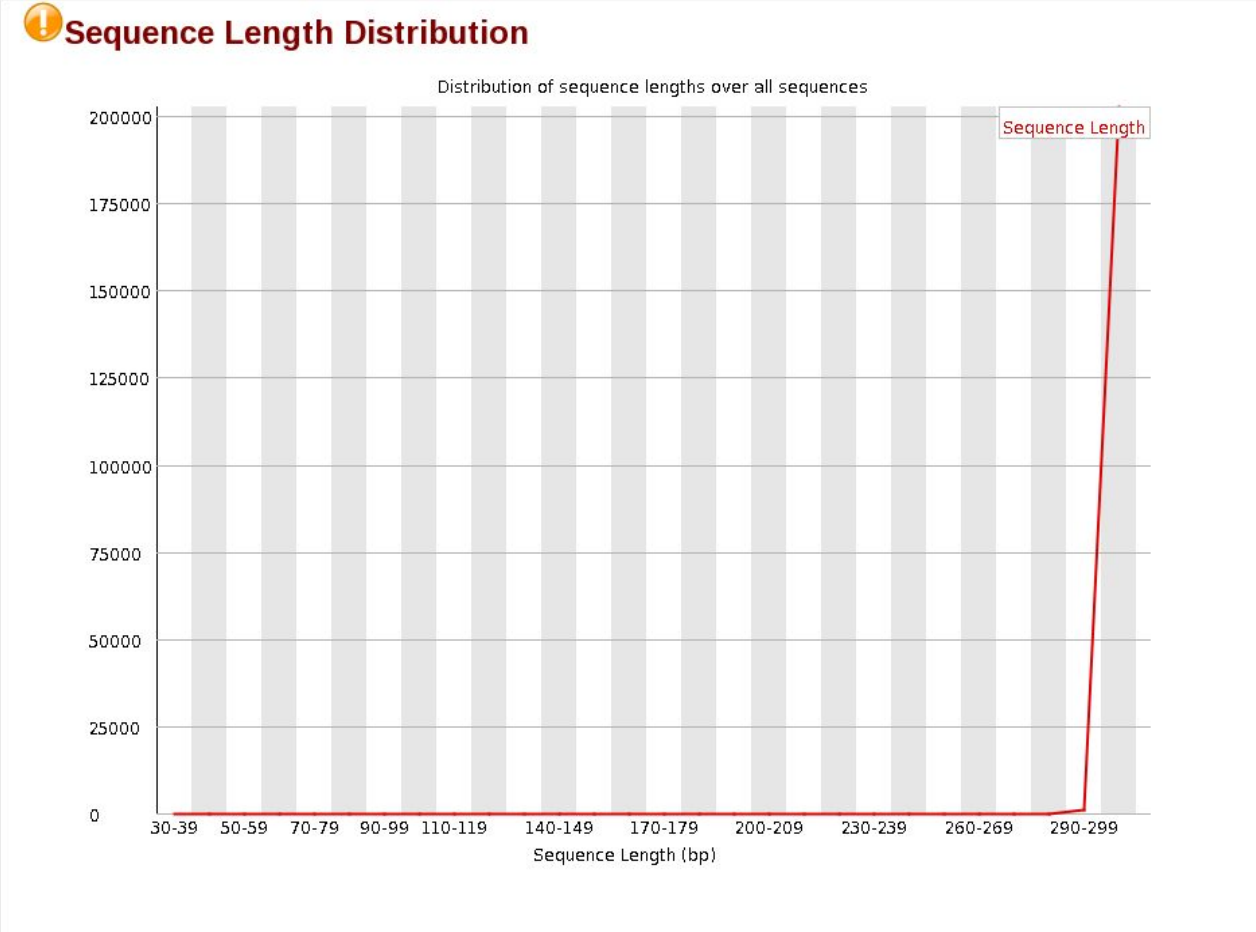




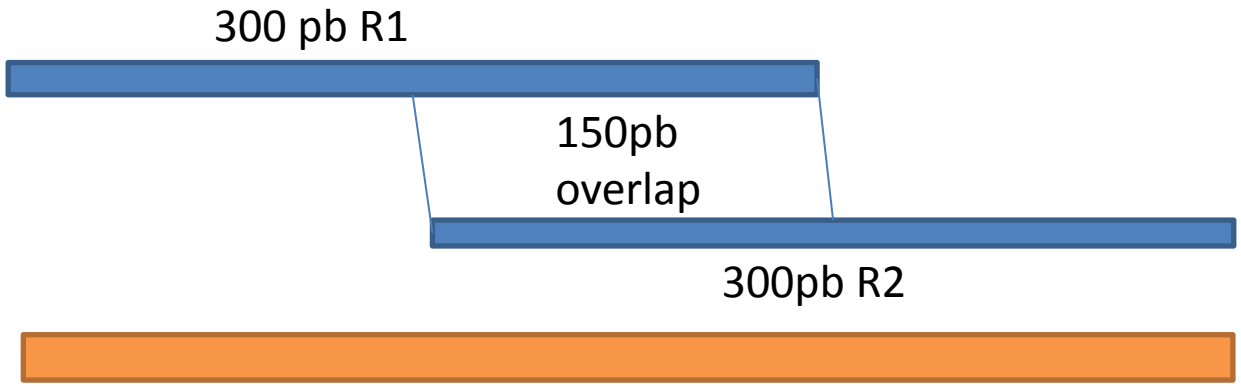
Sequence filtering: stats with MultiQC



Quality filtering in metagenomic samples

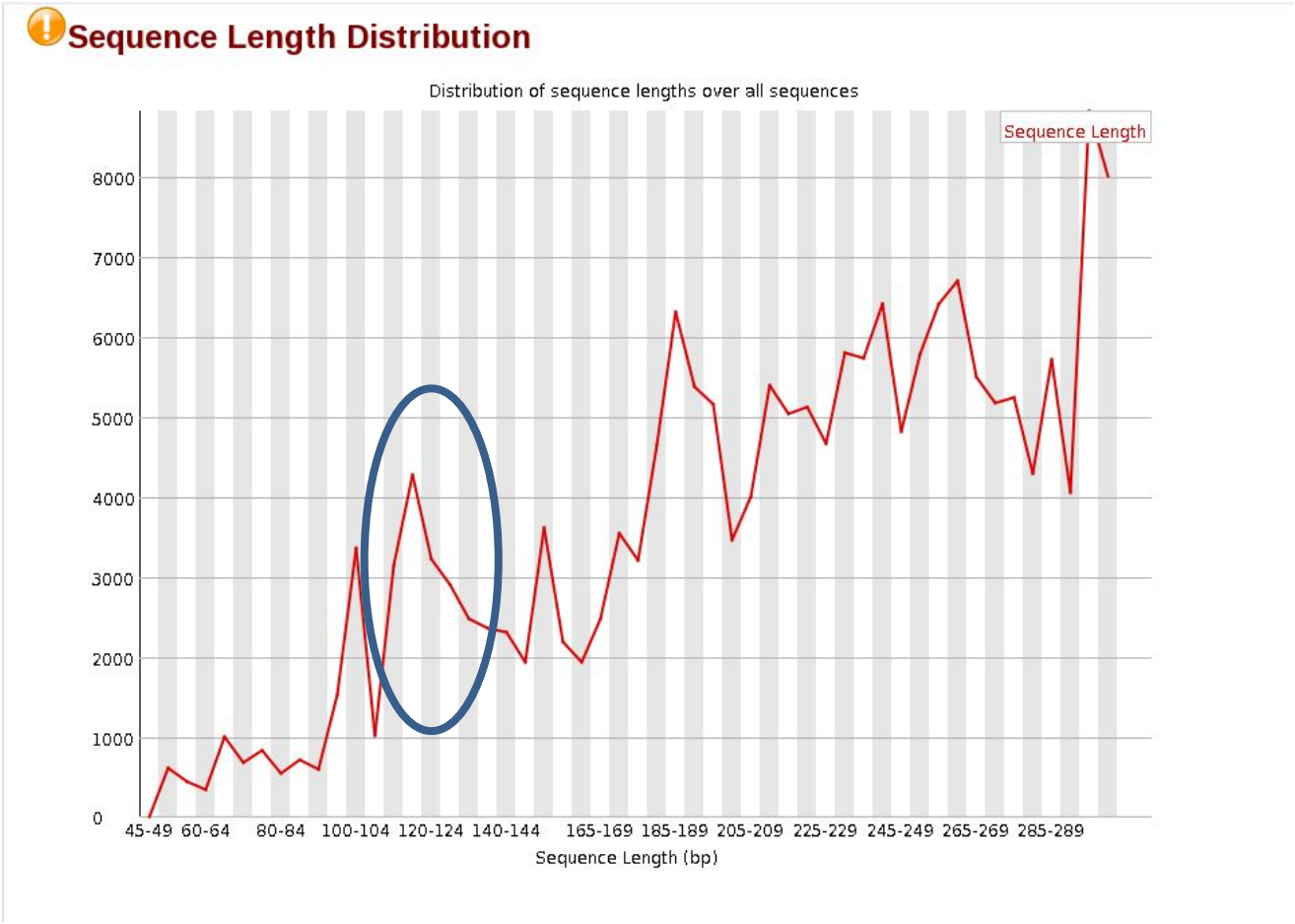


Quality filtering in metagenomic samples



450 aprox. pb
fragment
V3-V4 16S region

Quality filtering in metagenomic samples



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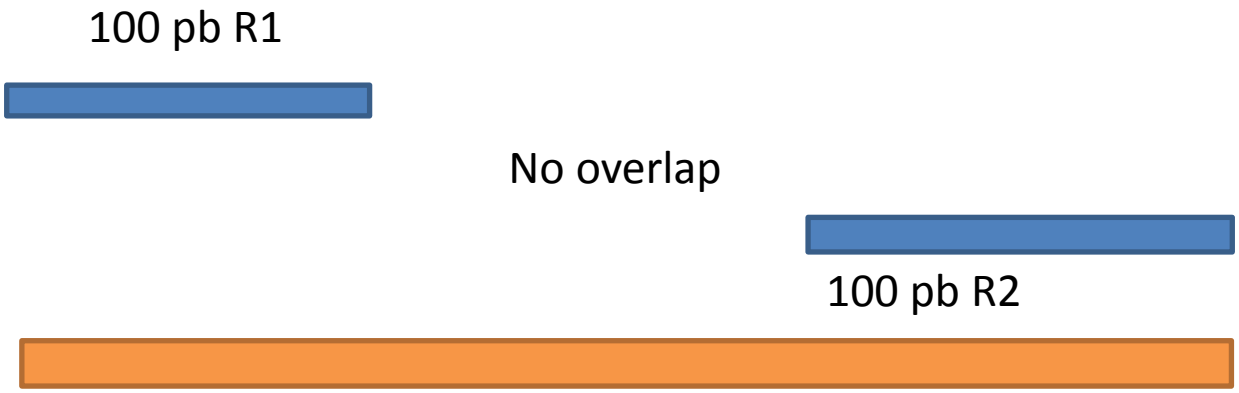

Questions?