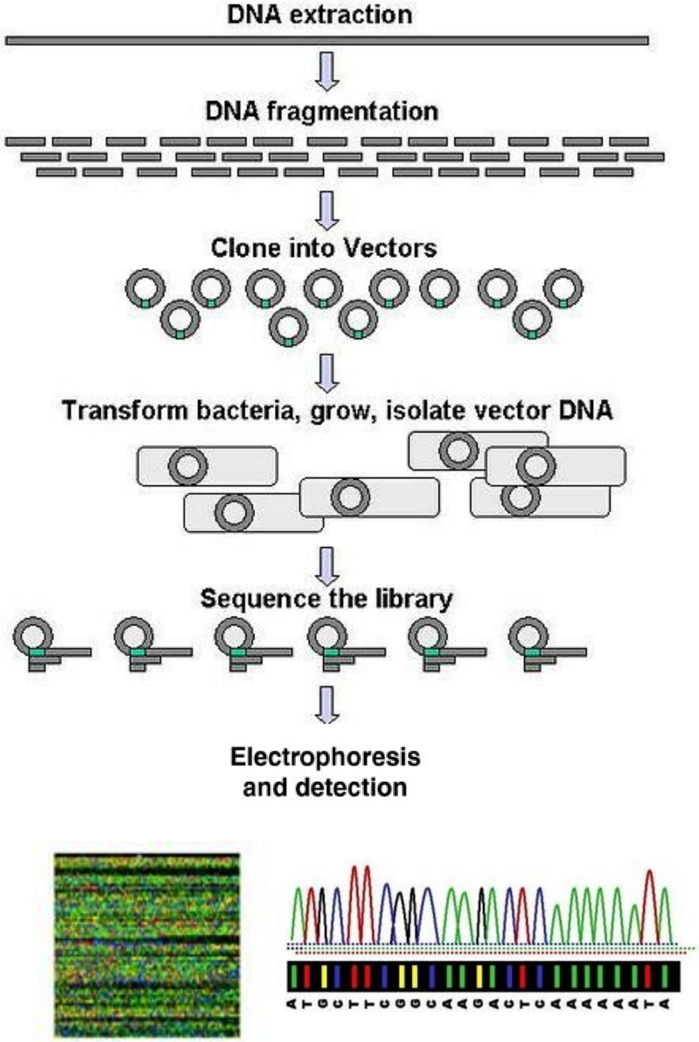
Introduction to NGS data formats, quality check and analytical tools

Valeria Michelacci

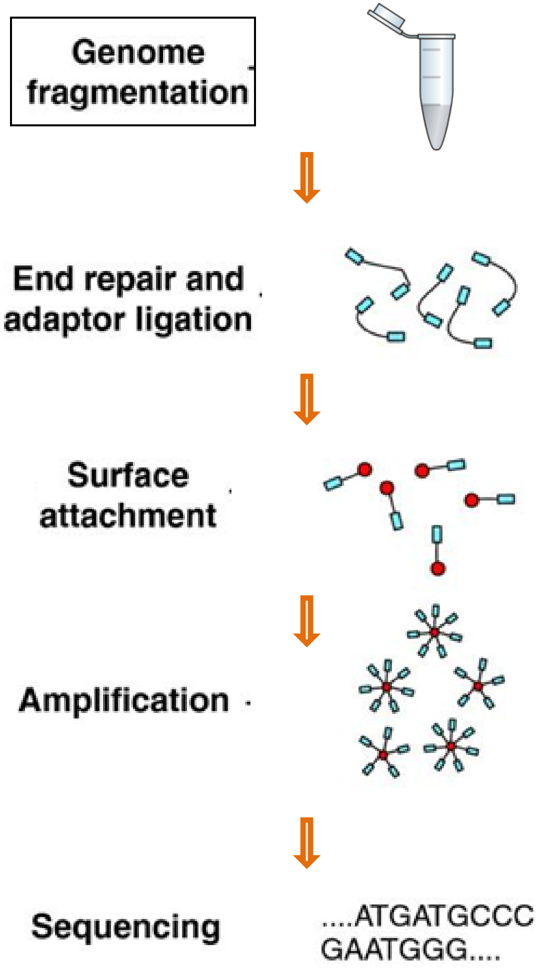
WGS course,
October 2020

Conventional sequencing vs NGS

Conventional

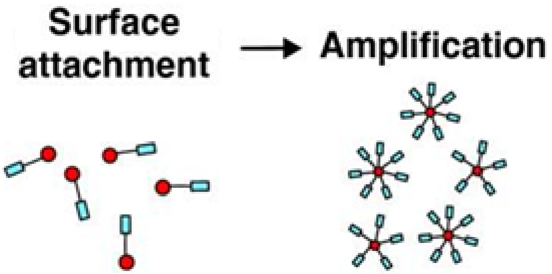


NGS Pipeline



Next generation sequencing

...



...

illumina MiSeq

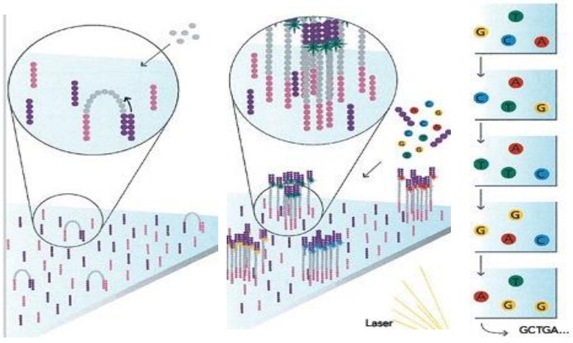
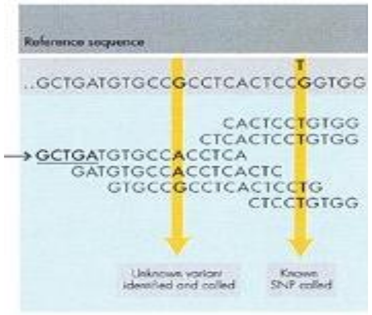
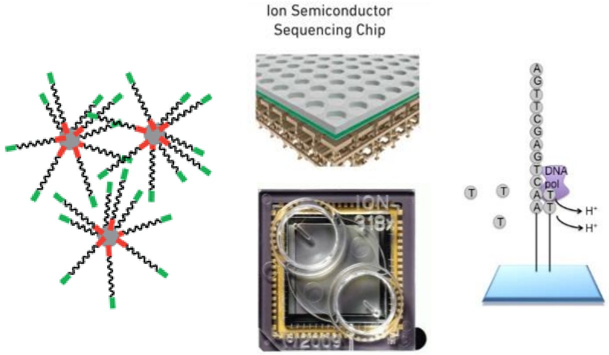


Image capture
Fluoresence detectioin

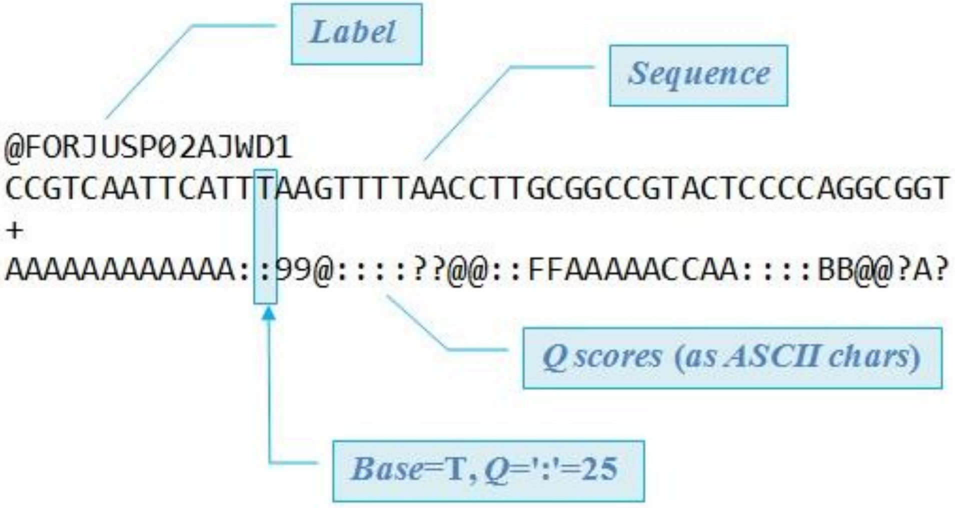


200bp-400bp short reads



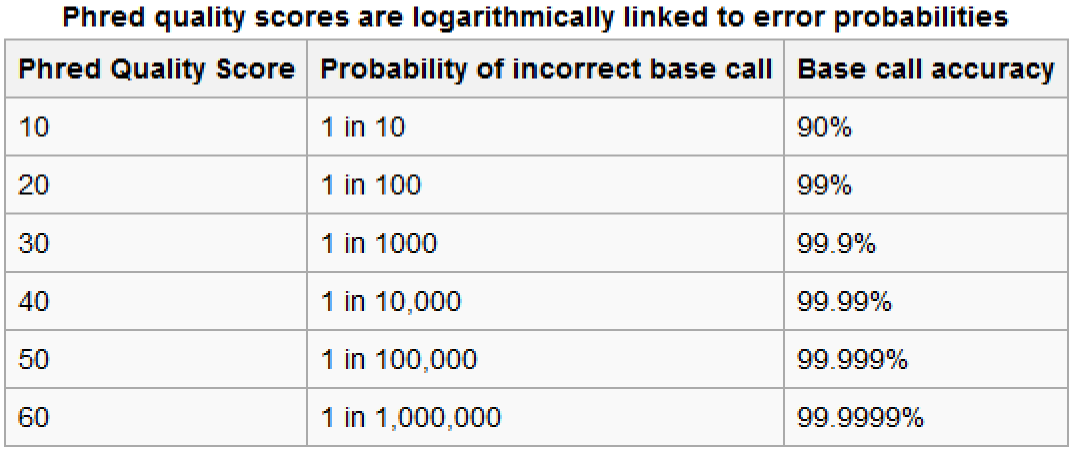
pH variation when incorporating nucleotides in the growing strand

.fastq files



```
@FORJUSP02AJWD1
CCGTCAATTCATTTAAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGT
+
AAAAAAAAAAAA::99@::::??@@::FFAAAAACCAA::::BB@@?A?
```

Each .fastq file covering a 5 Mb genome at 50X weights about **500 MB**



Phred quality scores are logarithmically linked to error probabilities

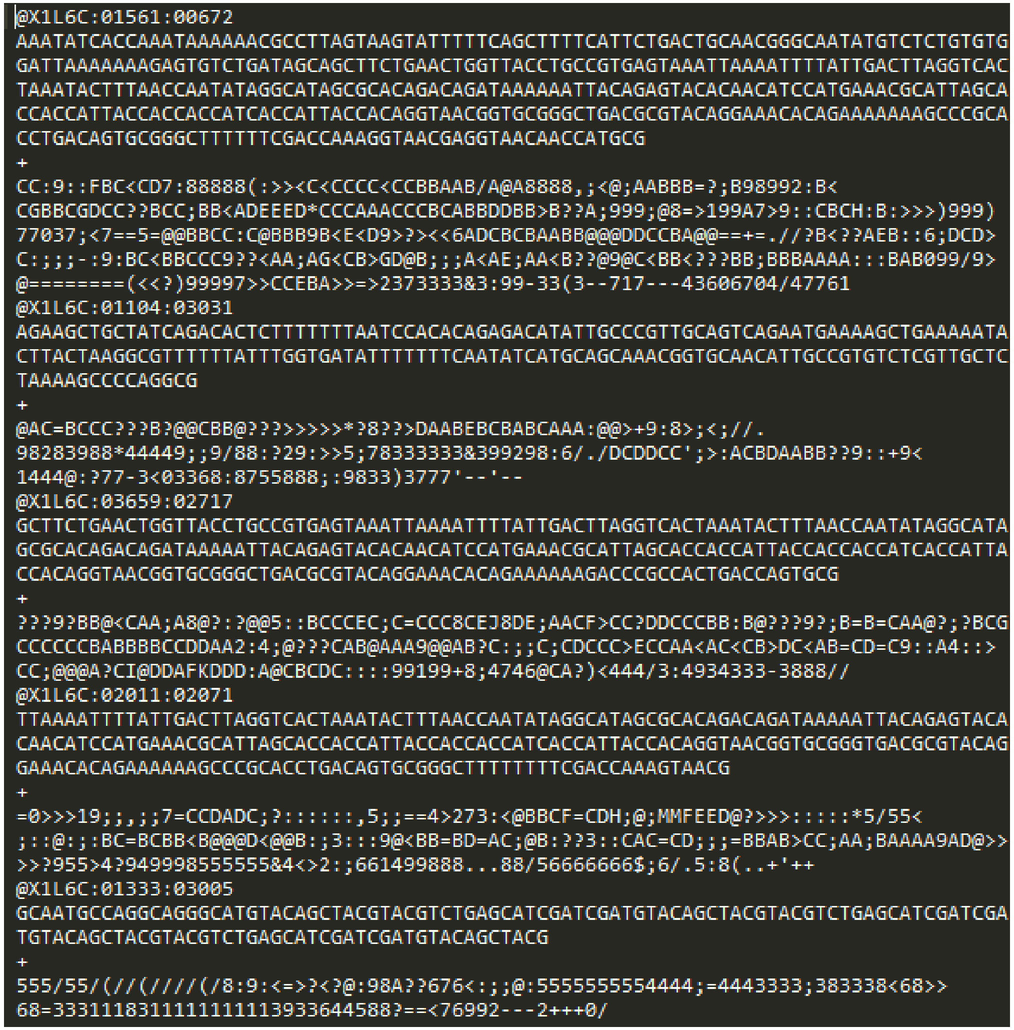
Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

Phred quality score

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

from 0 to 93 using ASCII characters 33 to 126

.fastq files

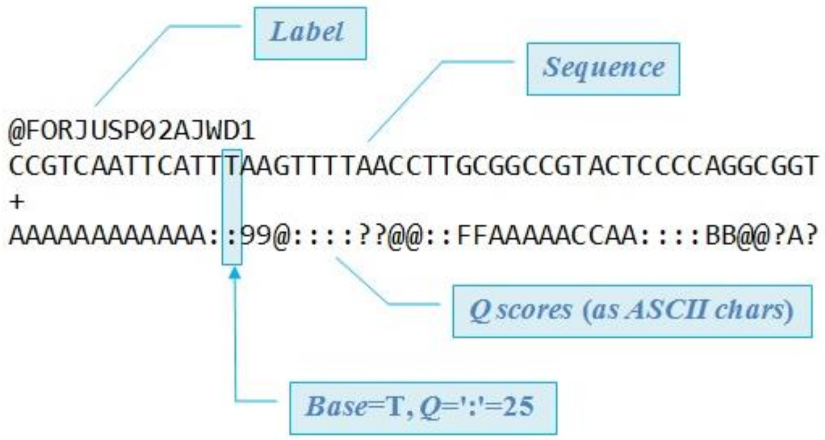


```
@X1L6C:01561:00672
AAATATCACCAAATAAAAAACGCCTTAGTAAGTATTTTTCAGCTTTTCATTCTGACTGCAACGGGCAATATGTCTCTGTGTG
GATTAAAAAAAAGAGTGTCTGATAGCAGCTTCTGAACTGGTTACCTGCCGTGAGTAAATTAAAATTTTATTGACTTAGGTCAC
TAAATACTTTAACCAATATAGGCATAGCGCACAGACAGATAAAAAATTACAGAGTACACAACATCCATGAAACGCATTAGCA
CCACCATTACCACCACCATCACCATTACCACAGGTAACGGTGCGGGCTGACGCGTACAGGAAACACAGAAAAAAAAGCCCGCA
CCTGACAGTGCGGGCTTTTTTCGACCAAAGGTAACGAGGTAACAACCATGCG
+
CC:9::FBC<CD7:88888(:>><C<CCCC<CCBBAAB/A@A8888,;<@;AABBB=?;B98992:B<
CGBBCGDCC??BCC;BB<ADEEED*CCCAAACCCBCABBDDBB>B??A;999;@8=>199A7>9::CBCH:B:>>>)999)
77037;<7==5=@@BBCC:C@BBB9B<E<D9>?><<6ADCBCBAABB@@@DDCCBA@@==+=.//?B<??AEB::6;DCD>
C:;;;-:9:BC<BBCCC9??<AA;AG<CB>GD@B;;;A<AE;AA<B??@9@C<BB<???BB;BBBAAAA:::BAB099/9>
@========(<<?)99997>>CCEBA>>=>2373333&3:99-33(3--717---43606704/47761
@X1L6C:01104:03031
AGAAGCTGCTATCAGACACTCTTTTTTTAATCCACACAGAGACATATTGCCCGTTGCAGTCAGAATGAAAAGCTGAAAAATA
CTTACTAAGGCGTTTTTTATTTGGTGATATTTTTTTCAATATCATGCAGCAAACGGTGCAACATTGCCGTGTCTCGTTGCTC
TAAAAGCCCCAGGCG
+
@AC=BCCC???B?@@CBB@???>>>>>*?8??>DAABEBCBABCAAA:@@>+9:8>;<;//.
98283988*44449;;9/88:?29:>>5;78333333&399298:6/./DCDDCC';>:ACBDAABB??9::+9<
1444@:?77-3<03368:8755888;:9833)3777'--'--
@X1L6C:03659:02717
GCTTCTGAACTGGTTACCTGCCGTGAGTAAATTAAAATTTTATTGACTTAGGTCACTAAATACTTTAACCAATATAGGCATA
GCGCACAGACAGATAAAAATTACAGAGTACACAACATCCATGAAACGCATTAGCACCACCATTACCACCACCATCACCATTA
CCACAGGTAACGGTGCGGGCTGACGCGTACAGGAAACACAGAAAAAAGACCCGCCACTGACCAGTGCG
+
???9?BB@<CAA;A8@?:?@@5::BCCCEC;C=CCC8CEJ8DE;AACF>CC?DDCCCBB:B@???9?;B=B=CAA@?;?BCG
CCCCCCBABBBBCCDDAA2:4;@???CAB@AAA9@@AB?C:;;C;CDCCC>ECCAA<AC<CB>DC<AB=CD=C9::A4::>
CC;@@@A?CI@DDAFKDDD:A@CBCDC::::99199+8;4746@CA?)<444/3:4934333-3888//
@X1L6C:02011:02071
TTAAAATTTTATTGACTTAGGTCACTAAATACTTTAACCAATATAGGCATAGCGCACAGACAGATAAAAATTACAGAGTACA
CAACATCCATGAAACGCATTAGCACCACCATTACCACCACCATCACCATTACCACAGGTAACGGTGCGGGTGACGCGTACAG
GAAACACAGAAAAAAAGCCCGCACCTGACAGTGCGGGCTTTTTTTTCGACCAAAGTAACG
+
=0>>>19;;,;;7=CCDADC;?::::::,5;;==4>273:<@BBCF=CDH;@;MMFEED@?>>>:::::*5/55<
;::@:;:BC=BCBB<B@@@D<@@B:;3:::9@<BB=BD=AC;@B:??3::CAC=CD;;;=BBAB>CC;AA;BAAAA9AD@>>
>>?955>4?949998555555&4<>2:;661499888...88/56666666$;6/.5:8(..+'++
@X1L6C:01333:03005
GCAATGCCAGGCAGGGCATGTACAGCTACGTACGTCTGAGCATCGATCGATGTACAGCTACGTACGTCTGAGCATCGATCGA
TGTACAGCTACGTACGTCTGAGCATCGATCGATGTACAGCTACG
+
555/55/(//(////(/8:9:<=>?<?@:98A??676<:;;@:5555555554444;=4443333;383338<68>>
68=333111831111111113933644588?==<76992---2+++0/
```

…and so on

Quality check

Output of NGS sequencers



```
@FORJUSP02AJWD1
CCGTCAATTCATTTAAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGT
+
AAAAAAAAAAAA::99@::::??@@::FFAAAAACCAA::::BB@@?A?
```

Input for quality check

.fastq file

Sequencing errors would impact every following application

Unreliability of following results (and difficulty to detect the existence of problems!)

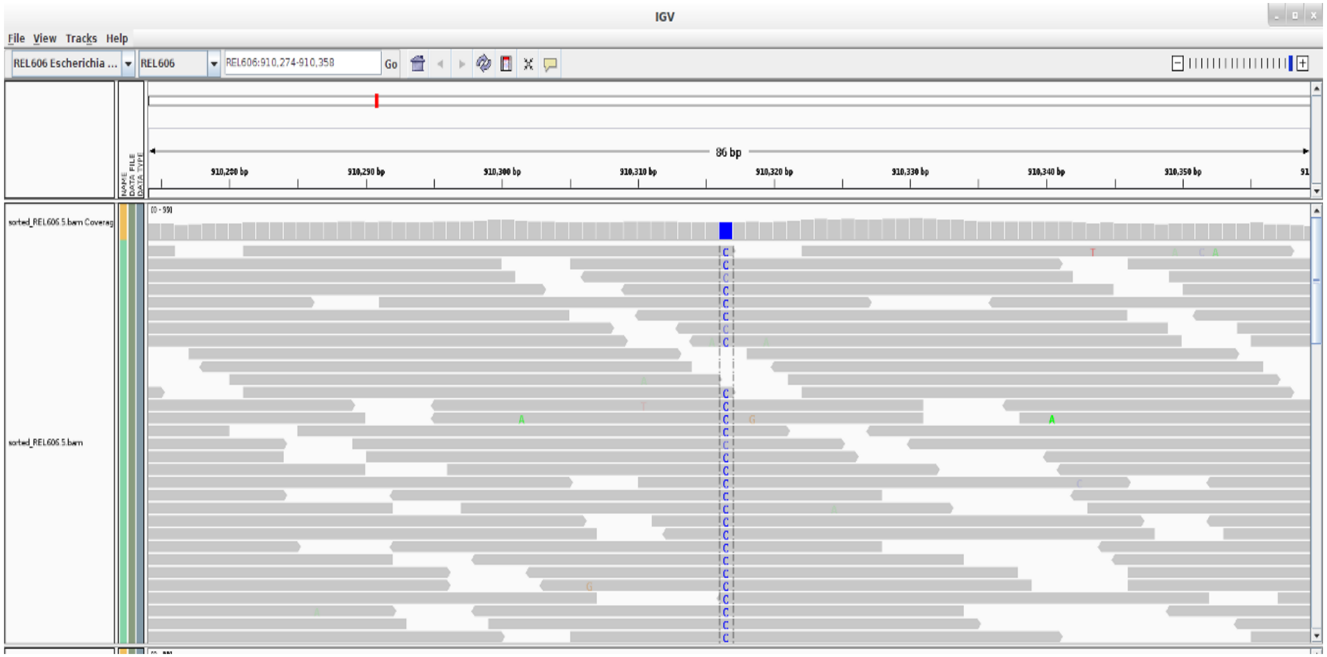
Parameters to control

- Phred score
- GC content distribution over all sequences
- Distribution of undetermined bases (N)
- Distribution of nucleotides
- ★ Length of the reads
- ★ Coverage

Adoption of corrective actions is possible to minimize some of these problems

Coverage (depth)

Reads mapped on a reference genome



Ref seq

COVERAGE

Mapped reads

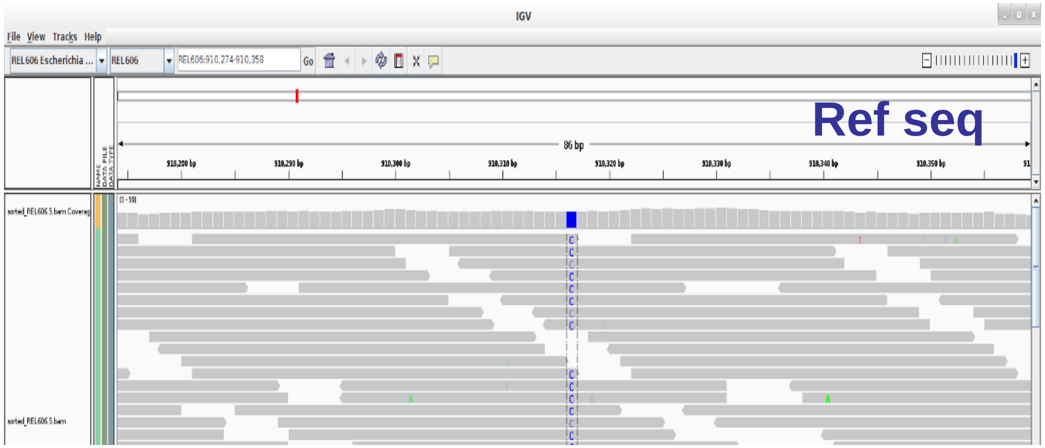
Coverage assessment:

Total length sequenced in Mb / expected genome size (5 Mb)

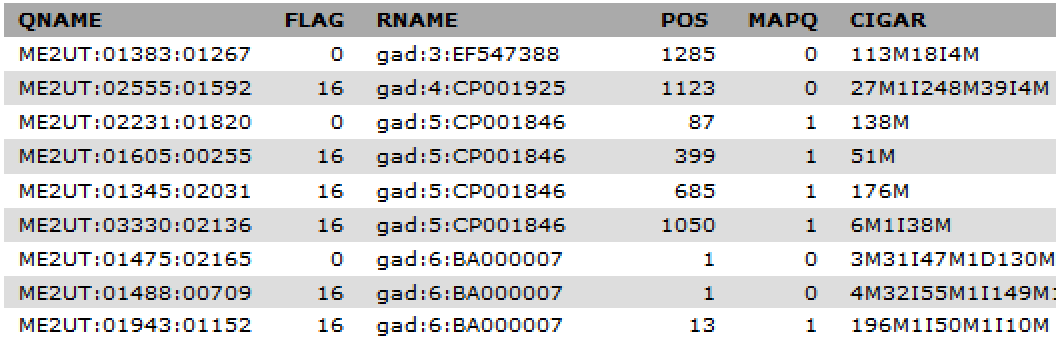
Count of reads mapping on housekeeping genes (e.g. MLST)

Alignment (mapping)

Alignment of the sequencing reads on a reference sequence or on a database of reference sequences



Possibility to directly inspect the **presence/absence of a target sequence** and the presence of **SNPs at interesting** positions by opening the bam file with a graphic viewer (e.g. IGV)



QNAME	FLAG	RNAME	POS	MAPQ	CIGAR
ME2UT:01383:01267	0	gad:3:EF547388	1285	0	113M18I4M
ME2UT:02555:01592	16	gad:4:CP001925	1123	0	27M1I248M39I4M
ME2UT:02231:01820	0	gad:5:CP001846	87	1	138M
ME2UT:01605:00255	16	gad:5:CP001846	399	1	51M
ME2UT:01345:02031	16	gad:5:CP001846	685	1	176M
ME2UT:03330:02136	16	gad:5:CP001846	1050	1	6M1I38M
ME2UT:01475:02165	0	gad:6:BA000007	1	0	3M31I47M1D130M
ME2UT:01488:00709	16	gad:6:BA000007	1	0	4M32I55M1I149M
ME2UT:01943:01152	16	gad:6:BA000007	13	1	196M1I50M1I10M

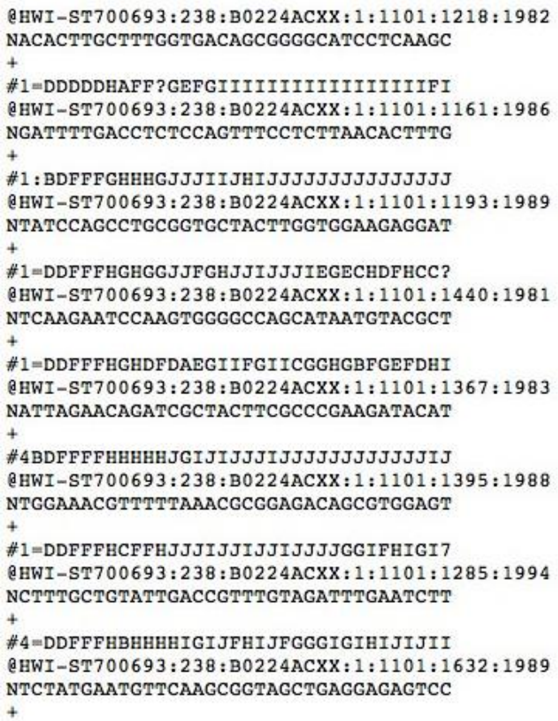
Possibility to convert the output in a sam file (tabular) to extract interesting info and sequences

Assembly

Short sequencing reads

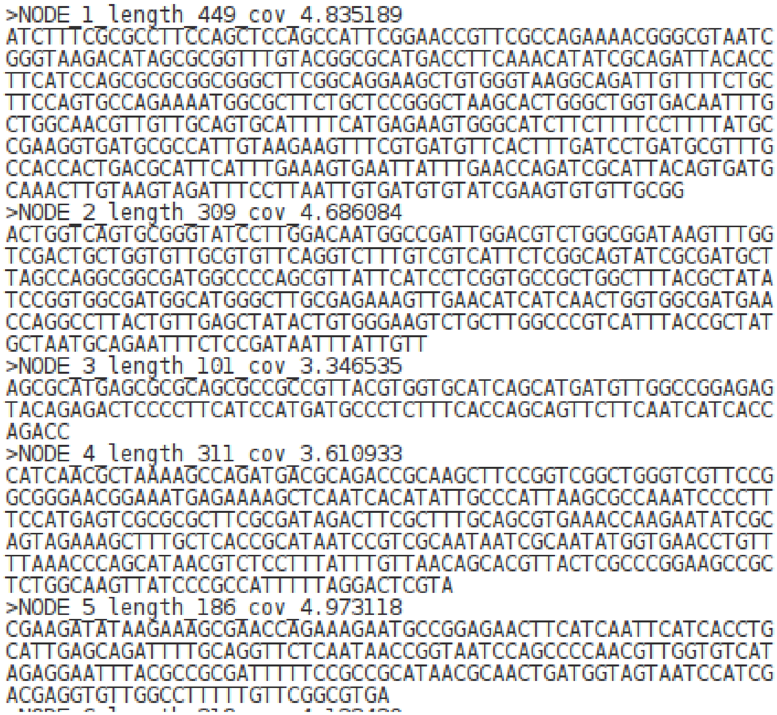
Partially assembled genome (contigs)

.fastq file



```
@HWI-ST700693:238:B0224ACXX:1:1101:1218:1982
NACACTTGCTTTGGTGACAGCGGGGCATCCTCAAGC
+
#1=DDDDDHAFF?GEFGIIIIIIIIIIIIIIIIIIFI
@HWI-ST700693:238:B0224ACXX:1:1101:1161:1986
NGATTTTGACCTCTCCAGTTTCCTCTTAACACTTTG
+
#1:BDFFFGHHHGJJJIIJHIJJJJJJJJJJJJJJJ
@HWI-ST700693:238:B0224ACXX:1:1101:1193:1989
NTATCCAGCCTGCGGTGCTACTTGGTGGAAGAGGAT
+
#1=DDFFFHGHGGJJFGHJJIJJJIEGECHDFHCC?
@HWI-ST700693:238:B0224ACXX:1:1101:1440:1981
NTCAAGAATCCAAGTGGGGCCAGCATAATGTACGCT
+
#1=DDFFFHGHDFDAEGIIFGIICGGHGBFGEFDHI
@HWI-ST700693:238:B0224ACXX:1:1101:1367:1983
NATTAGAACAGATCGCTACTTCGCCCGAAGATACAT
+
#4BDFFFFHHHHHJGIJIJJJIJJJJJJJJJJJJIJ
@HWI-ST700693:238:B0224ACXX:1:1101:1395:1988
NTGGAAACGTTTTTAAACGCGGAGACAGCGTGGAGT
+
#1=DDFFFHCFFHJJJIJJIJJIJJJJGGIFHIGI7
@HWI-ST700693:238:B0224ACXX:1:1101:1285:1994
NCTTTGCTGTATTGACCGTTTGTAGATTTGAATCTT
+
#4=DDFFFHBHHHHIGIJFHIJFGGGIGIHIJIJII
@HWI-ST700693:238:B0224ACXX:1:1101:1632:1989
NTCTATGAATGTTCAAGCGGTAGCTGAGGAGAGTCC
+
```

.fasta file



```
>NODE_1_length_449_cov_4.835189
ATCTTTCGCGCCTTCCAGCTCCAGCCATTCGGAACCGTTCGCCAGAAAACGGGCGTAATC
GGGTAAGACATAGCGCGGTTTGTACGGCGCATGACCTTCAAACATATCGCAGATTACACC
TTCATCCAGCGCGCGGCGGGCTTCGGCAGGAAGCTGTGGGTAAGGCAGATTGTTTTCTGC
TTCCAGTGCCAGAAAATGGCGCTTCTGCTCCGGGCTAAGCACTGGGCTGGTGACAATTTG
CTGGCAACGTTGTTGCAGTGCATTTTCATGAGAAGTGGGCATCTTCTTTTCCTTTTATGC
CGAAGGTGATGCGCCATTGTAAGAAGTTTCGTGATGTTCACTTTGATCCTGATGCGTTTG
CCACCACTGACGCATTCATTTGAAAGTGAATTATTTGAACCAGATCGCATTACAGTGATG
CAAACTTGTAAGTAGATTTCCTTAATTGTGATGTGTATCGAAGTGTGTTGCGG
>NODE_2_length_309_cov_4.686084
ACTGGTCAGTGCGGGTATCCTTGGACAATGGCCGATTGGACGTCTGGCGGATAAGTTTGG
TCGACTGCTGGTGTTGCGTGTTCAGGTCTTTGTCGTCATTCTCGGCAGTATCGCGATGCT
TAGCCAGGCGGCGATGGCCCCAGCGTTATTCATCCTCGGTGCCGCTGGCTTTACGCTATA
TCCGGTGGCGATGGCATGGGCTTGCGAGAAAGTTGAACATCATCAACTGGTGGCGATGAA
CCAGGCCTTACTGTTGAGCTATACTGTGGGAAGTCTGCTTGGCCCGTCATTTACCGCTAT
GCTAATGCAGAATTTCTCCGATAATTTATTGTT
>NODE_3_length_101_cov_3.346535
AGCGCATGAGCGCGCAGCGCCGCCGTTACGTGGTGCATCAGCATGATGTTGGCCGGAGAG
TACAGAGACTCCCCTTCATCCATGATGCCCTCTTTCACCAGCAGTTCTTCAATCATCACC
AGACC
>NODE_4_length_311_cov_3.610933
CATCAACGCTAAAAGCCAGATGACGCAGACCGCAAGCTTCCGGTCGGCTGGGTCGTTCCG
GCGGGAACGGAAATGAGAAAAGCTCAATCACATATTGCCCATTAAGCGCCAAATCCCCTT
TCCATGAGTCGCGCGCTTCGCGATAGACTTCGCTTTGCAGCGTGAAACCAAGAATATCGC
AGTAGAAAGCTTTGCTCACCGCATAATCCGTCGCAATAATCGCAATATGGTGAACCTGTT
TTAAACCCAGCATAACGTCTCCTTTATTTGTTAACAGCACGTTACTCGCCCGGAAGCCGC
TCTGGCAAGTTATCCCGCCATTTTTAGGACTCGTA
>NODE_5_length_186_cov_4.973118
CGAAGATATAAGAAAGCGAACCAGAAAGAATGCCGGAGAACTTCATCAATTCATCACCTG
CATTGAGCAGATTTTGCAGGTTCTCAATAACCGGTAATCCAGCCCCAACGTTGGTGTCAT
AGAGGAATTTACGCCGCGATTTTTCCGCCGCATAACGCAACTGATGGTAGTAATCCATCG
ACGAGGTGTTGGCCTTTTTGTTCGGCGTGA
```

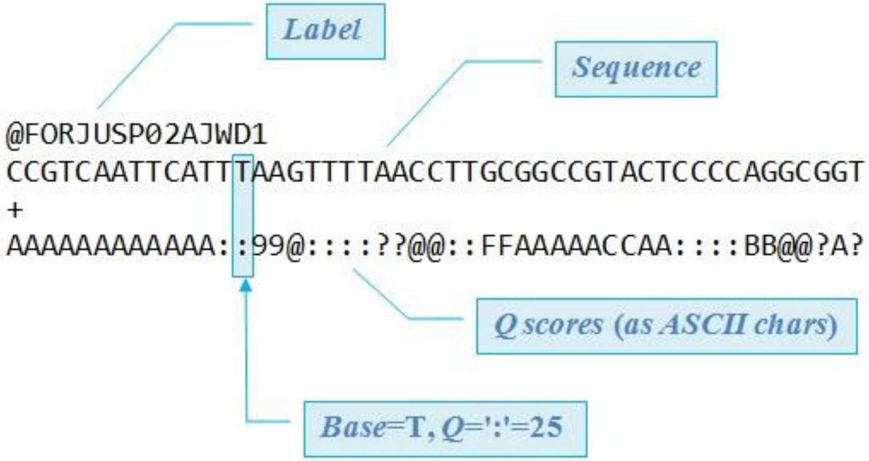
FastqSize ≈ GenomeSize x Coverage x 2

At least 0.5 GB per genome

FastaSize for *E. coli* contigs

~5.5 MB

What should be trimmed out?



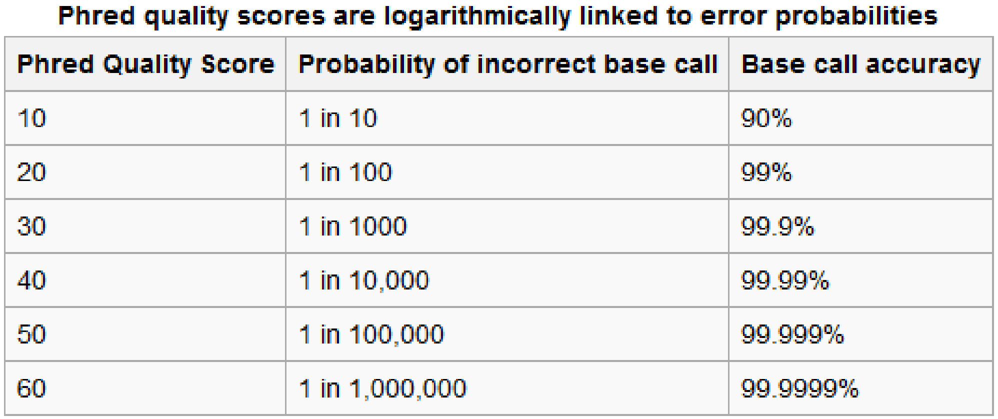
Label

Sequence

```
@FORJUSP02AJWD1
CCGTCAATTCATTTAAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGT
+
AAAAAAAAAAAA::99@::::??@@::FFAAAAACCAA::::BB@@?A?
```

Q scores (as ASCII chars)

Base=T, Q=':'=25



Phred quality scores are logarithmically linked to error probabilities

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
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30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
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Low quality positions

Adaptors and barcodes

Very short sequencing reads

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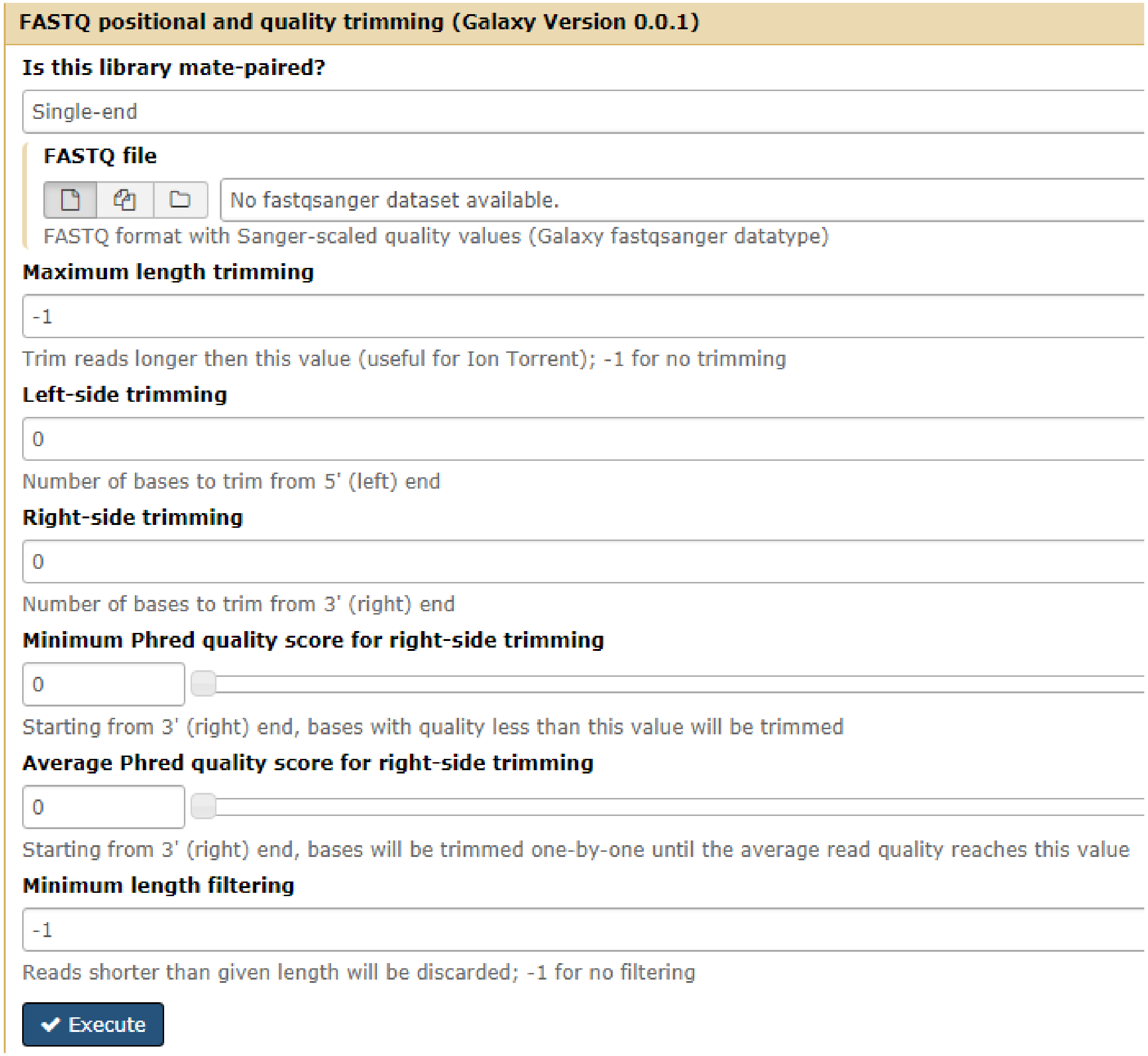


FASTQ positional and quality trimming (Galaxy Version 0.0.1)

Is this library mate-paired?

Single-end

FASTQ file

No fastqsanger dataset available.

FASTQ format with Sanger-scaled quality values (Galaxy fastqsanger datatype)

★ Maximum length trimming

-1

Trim reads longer then this value (useful for Ion Torrent); -1 for no trimming

★ Left-side trimming

0

Number of bases to trim from 5' (left) end

Right-side trimming

0

Number of bases to trim from 3' (right) end

★ Minimum Phred quality score for right-side trimming

0

Starting from 3' (right) end, bases with quality less than this value will be trimmed

★ Average Phred quality score for right-side trimming

0

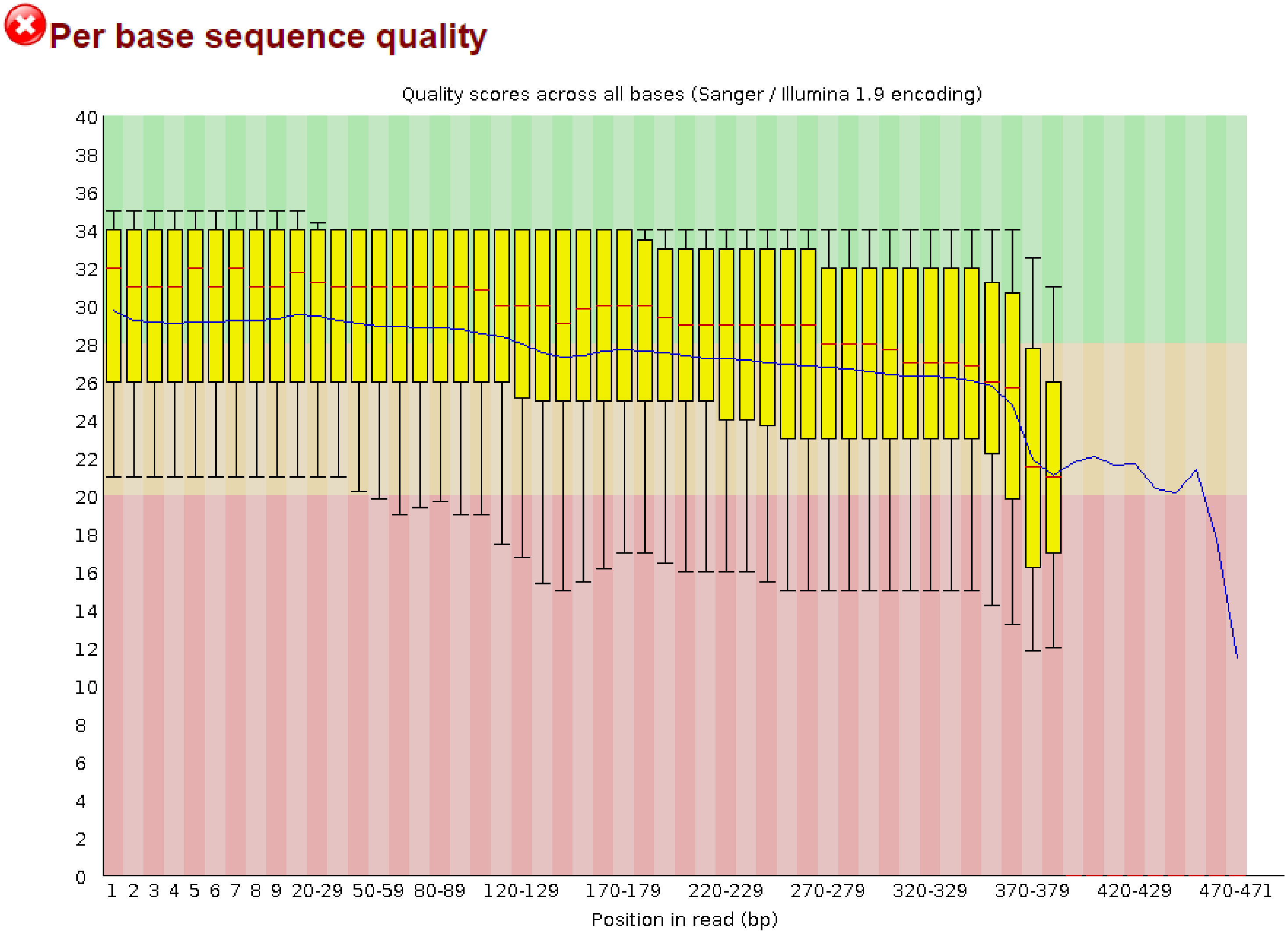
Starting from 3' (right) end, bases will be trimmed one-by-one until the average read quality reaches this value

★ Minimum length filtering

-1

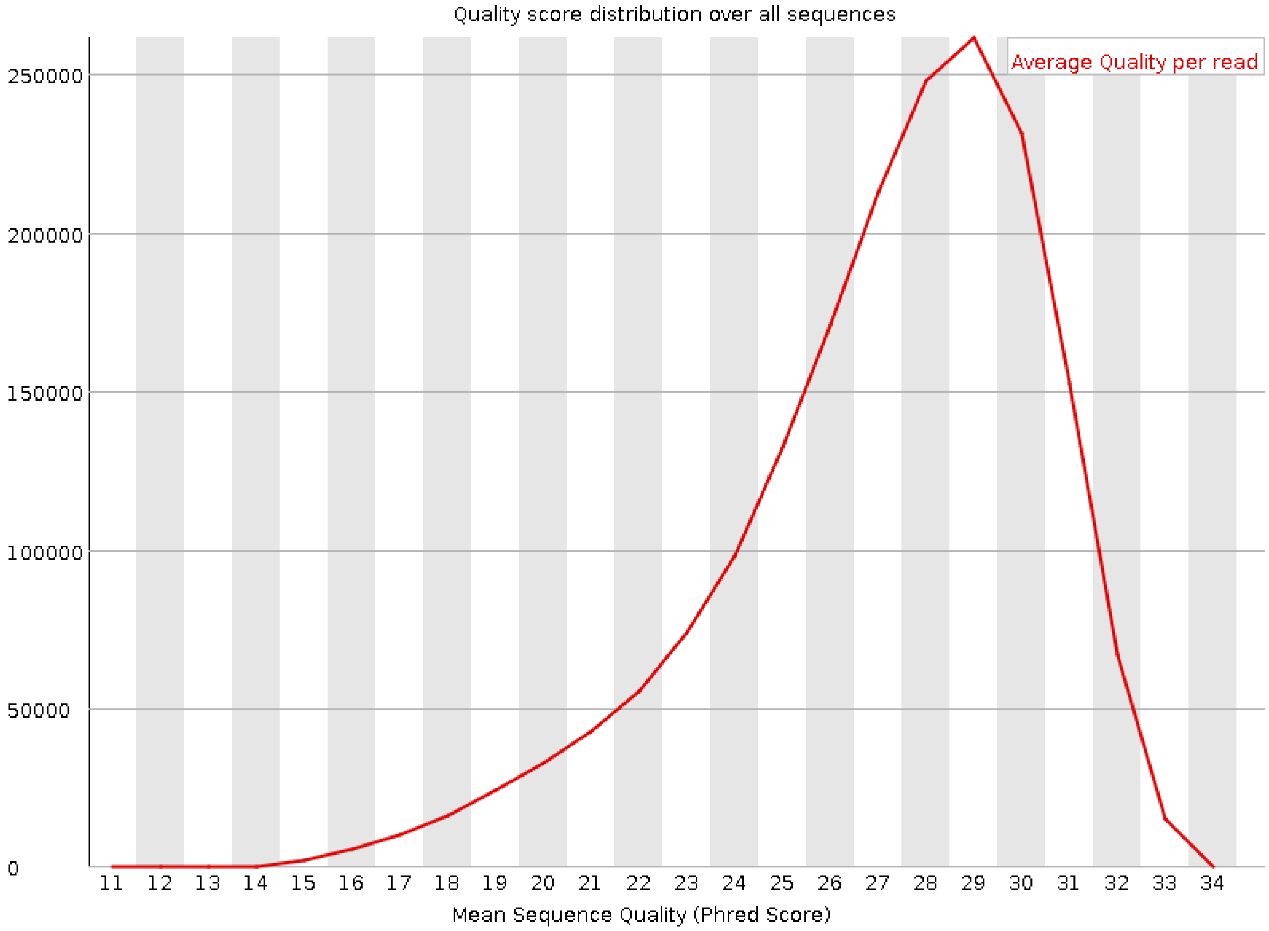
Reads shorter than given length will be discarded; -1 for no filtering

FastQC – quality check of aw data



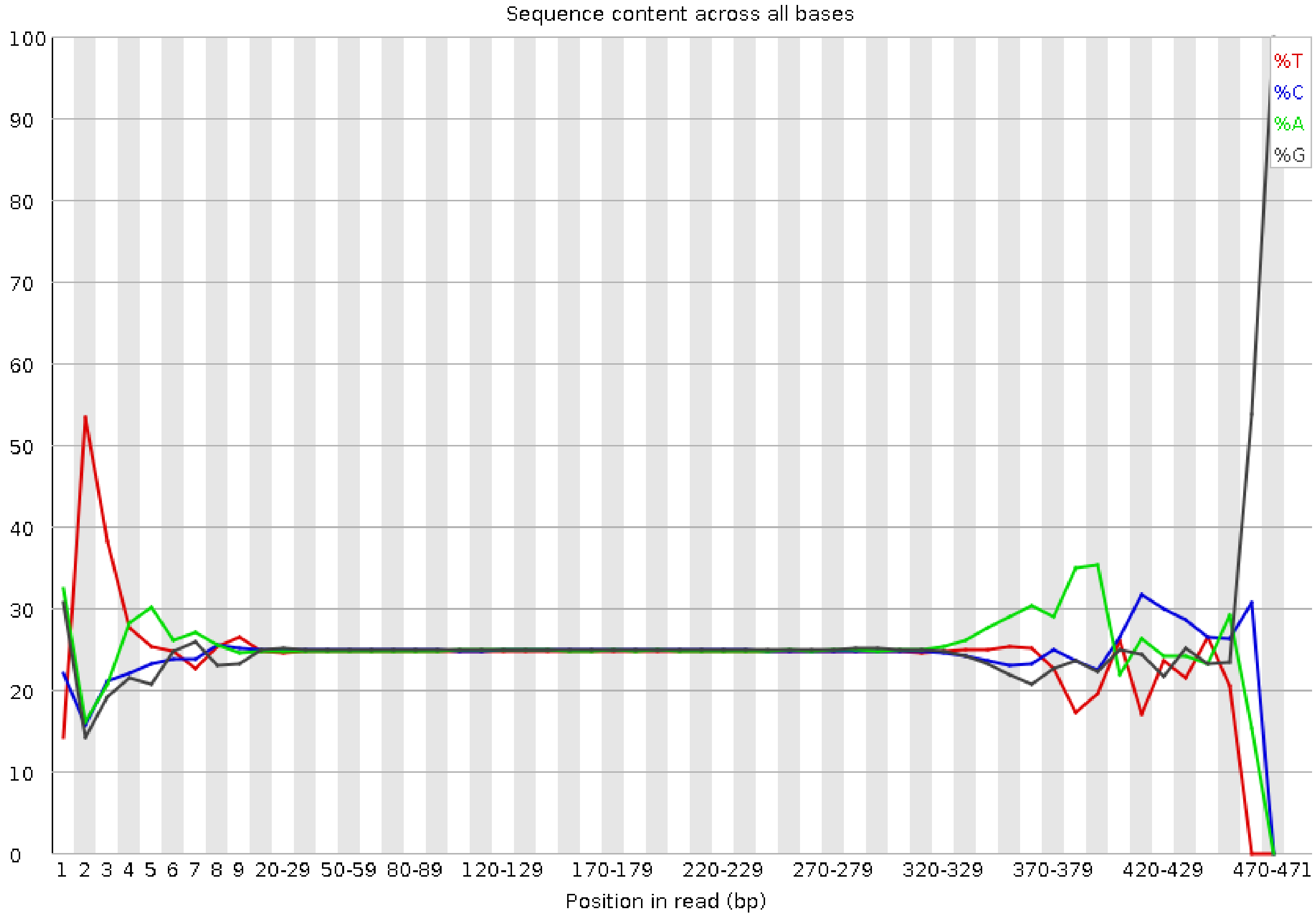
FastQC – quality check of aw data

✓ Per sequence quality scores



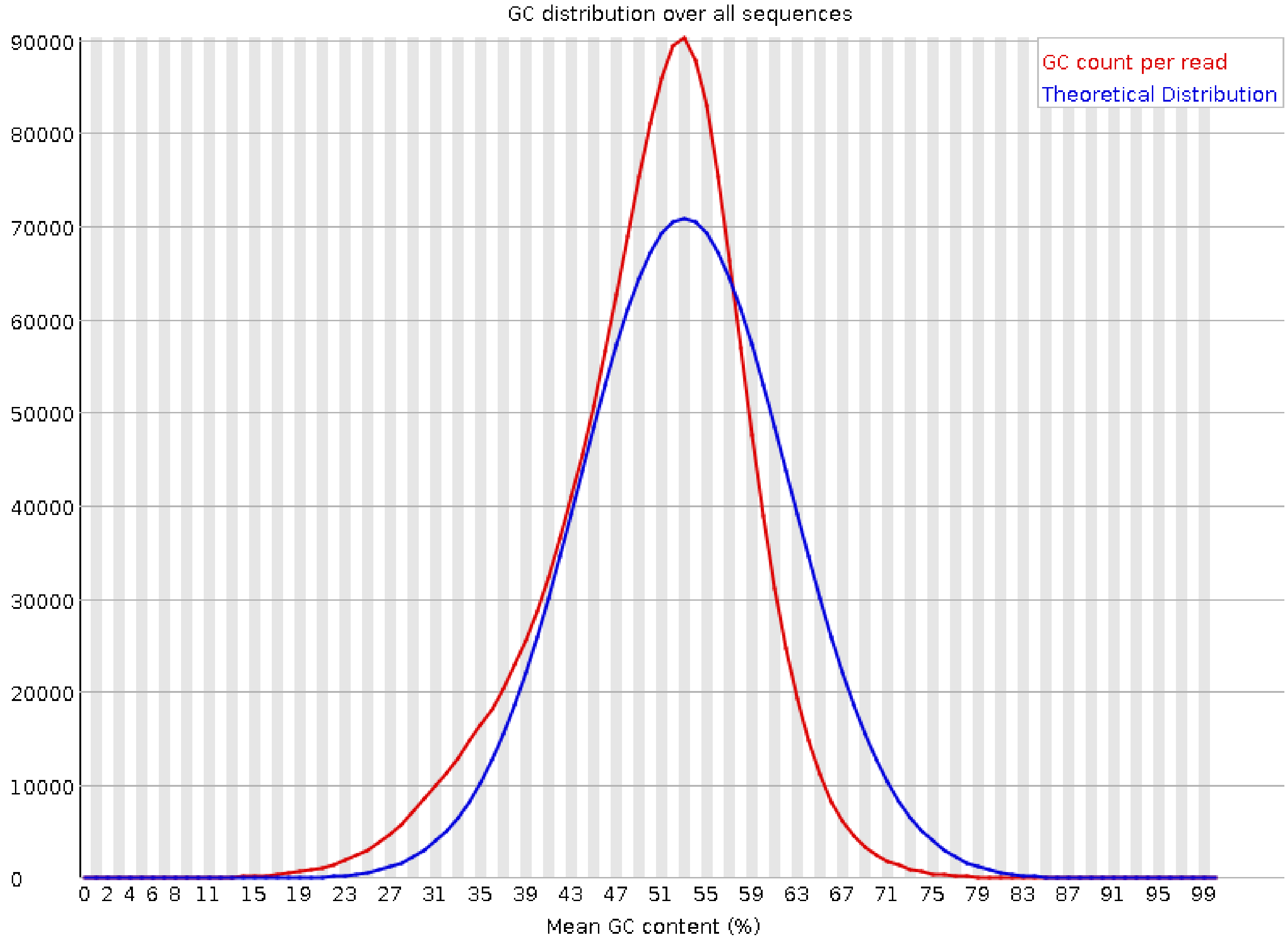
FastQC – quality check of raw data

Per base sequence content



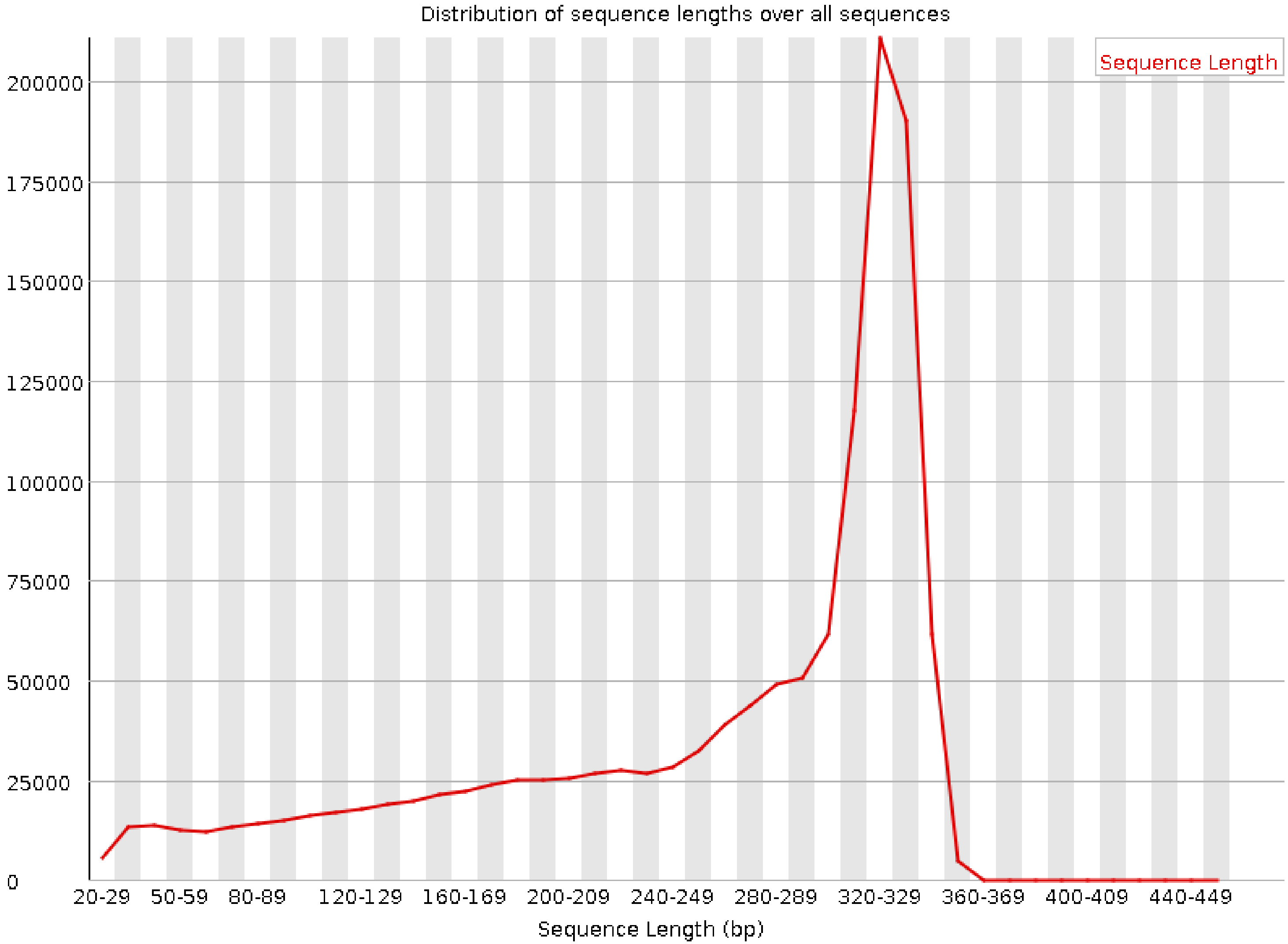
FastQC – quality check of aw data

Per sequence GC content



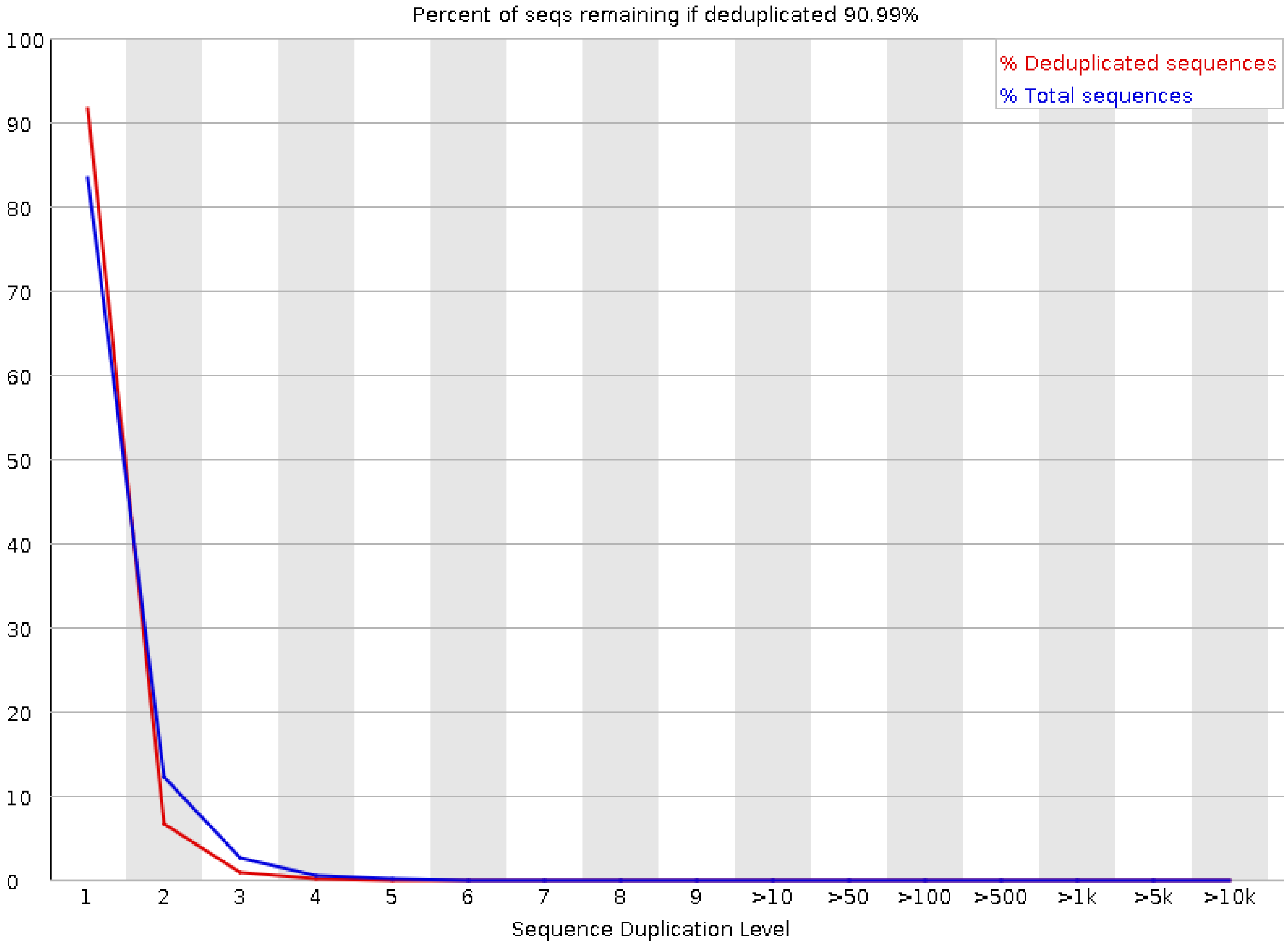
FastQC – quality check of aw data

Sequence Length Distribution



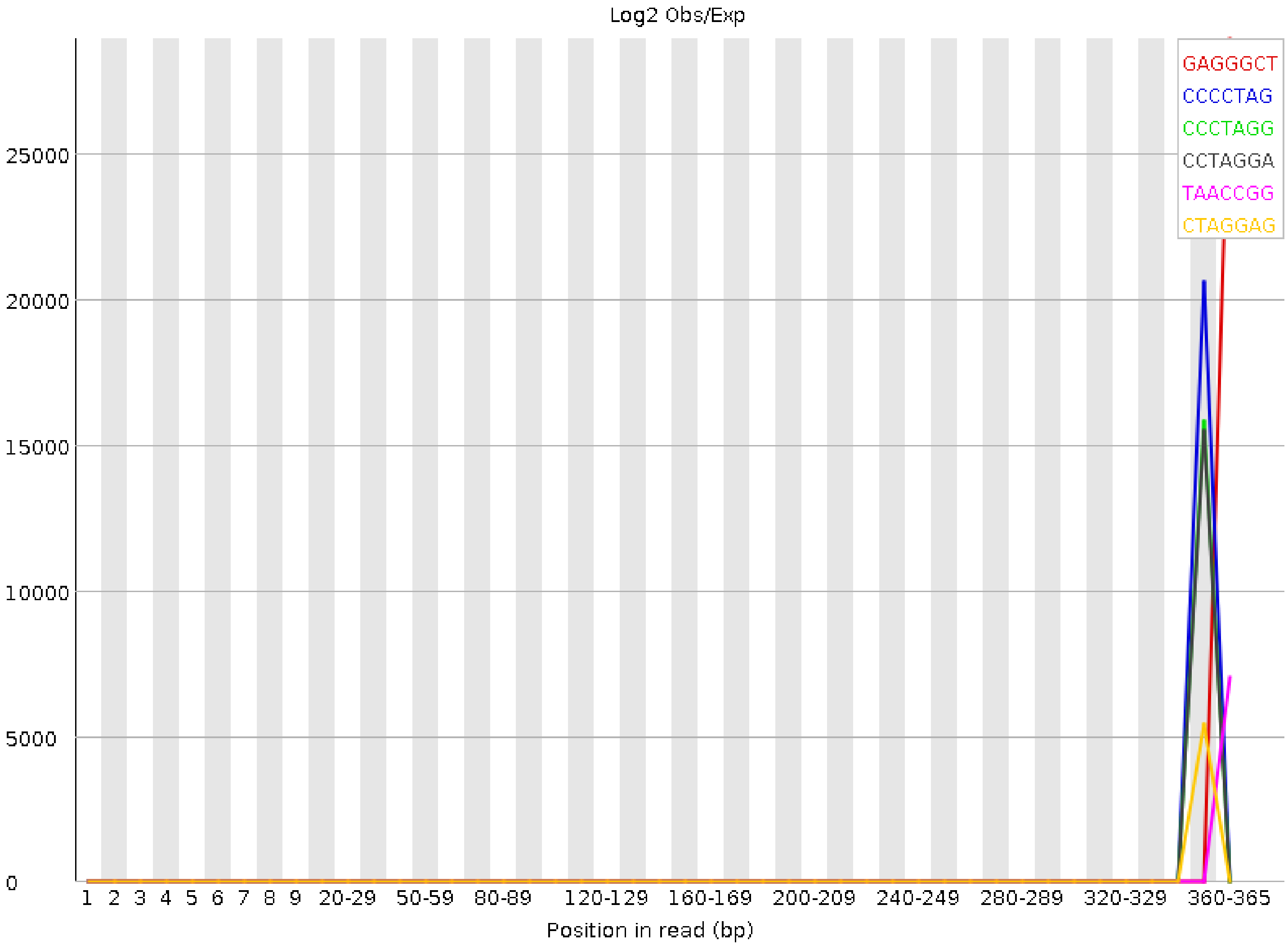
FastQC – quality check of aw data

✓ Sequence Duplication Levels

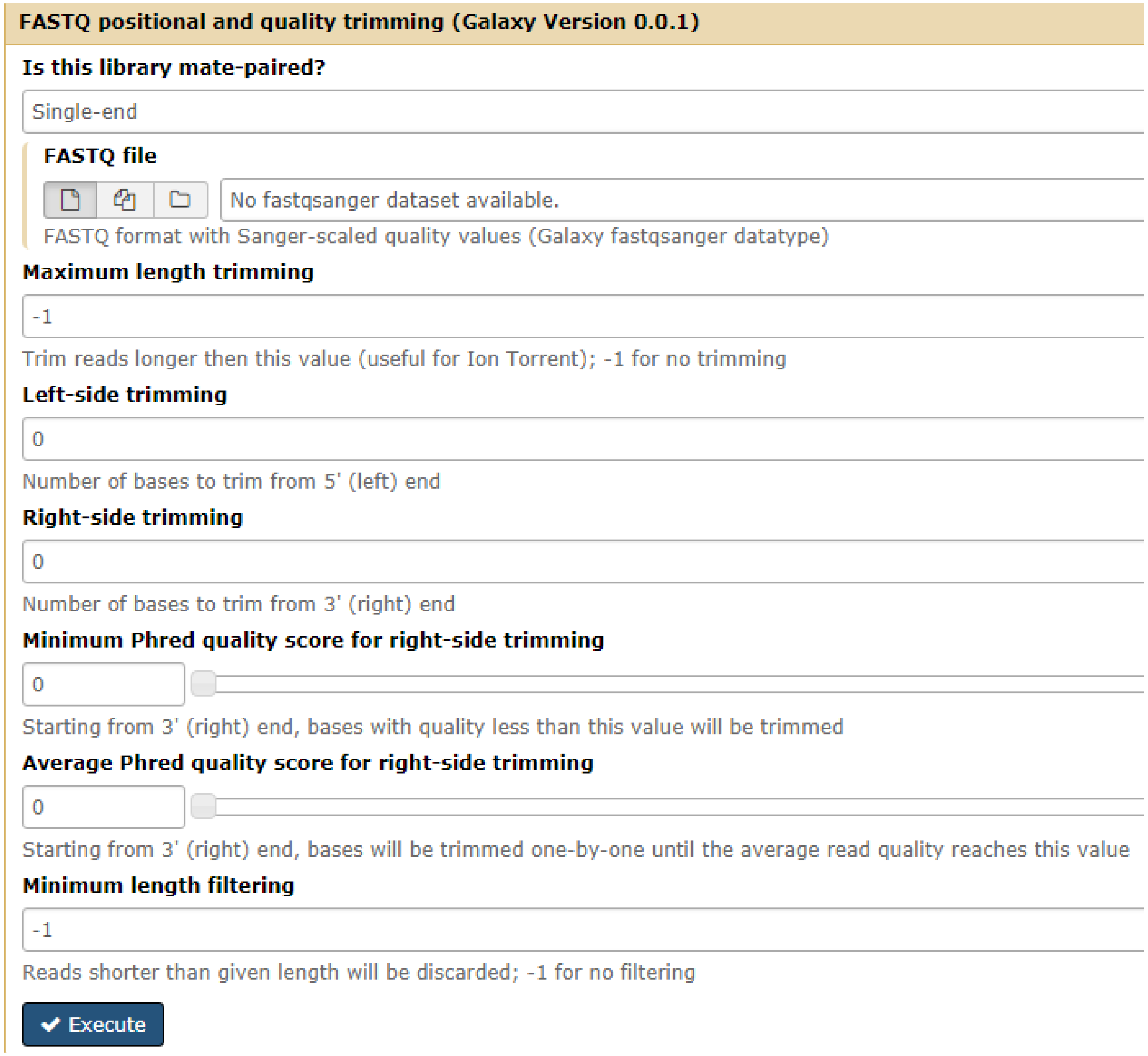


FastQC – quality check of raw data

Kmer Content



What should be trimmed out?



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★ Minimum length filtering

-1

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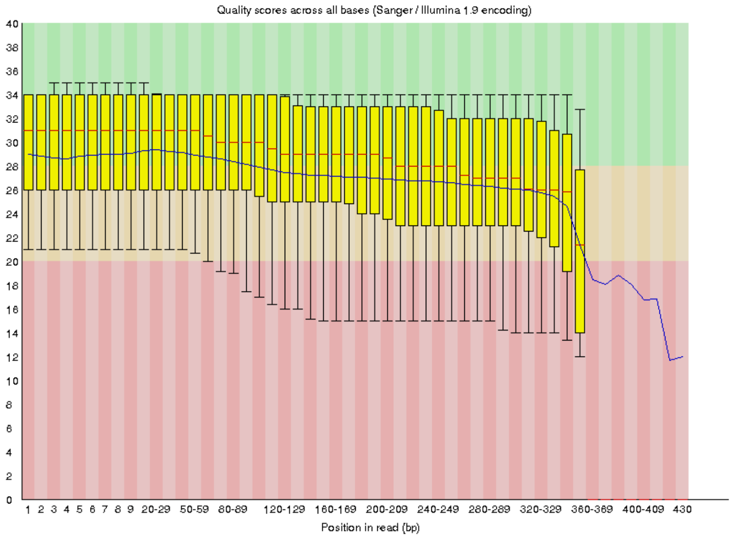
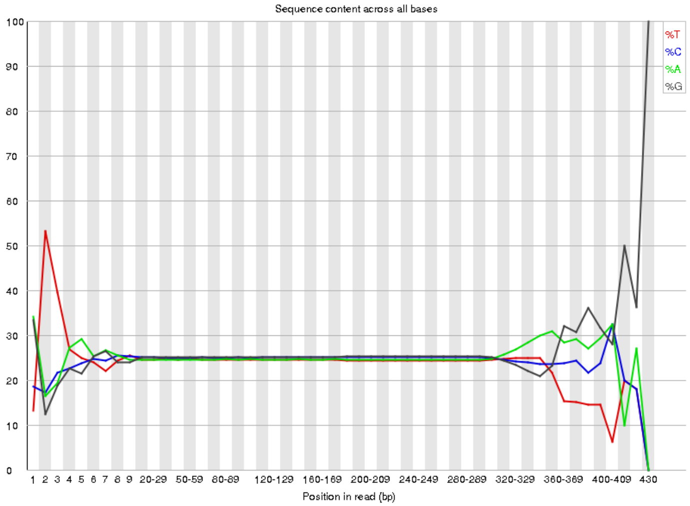
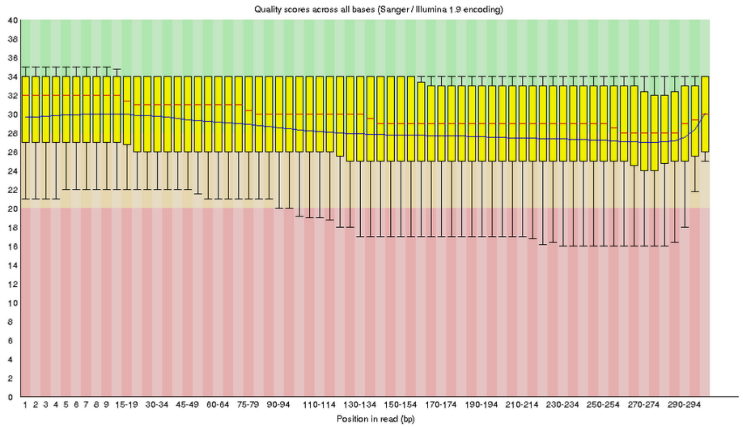
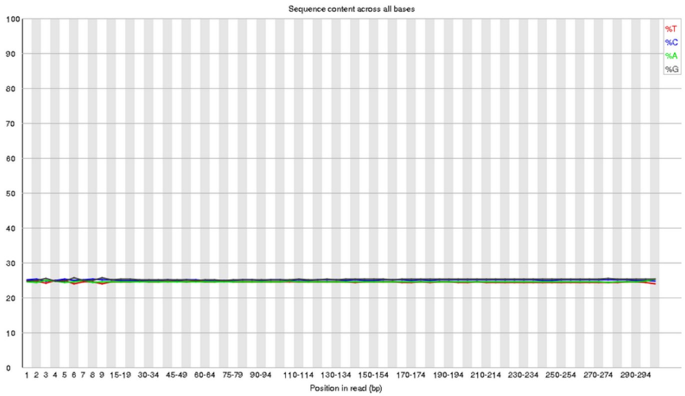
Before trimming





After trimming


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)


Sequence content across all bases
%T
%C
%A
%G
Position in read (bp)