# Introduction to NGS data formats, quality check and analytical tools

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Bioinformatics training,
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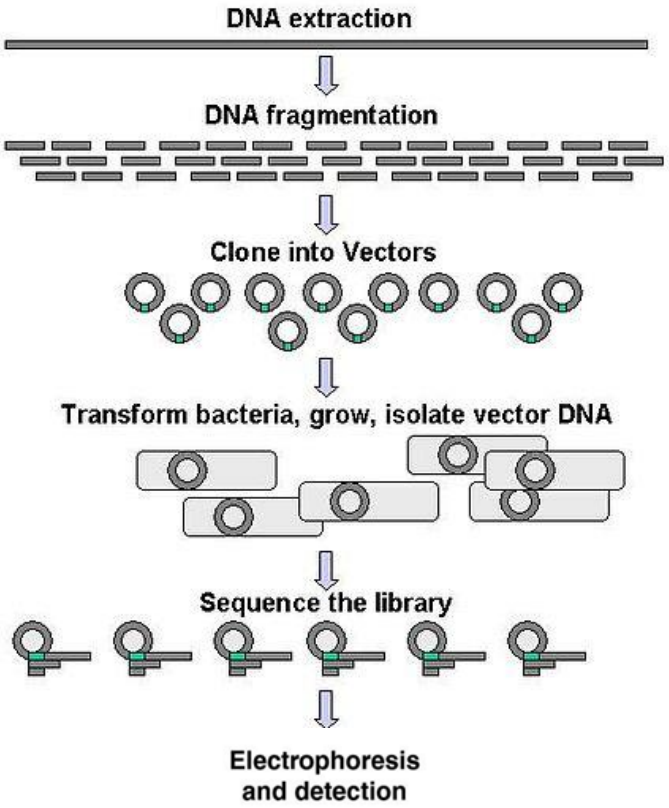
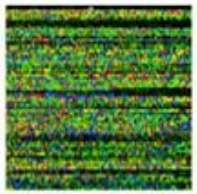
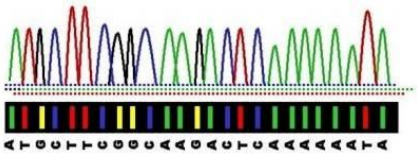
# Conventional sequencing vs NGS

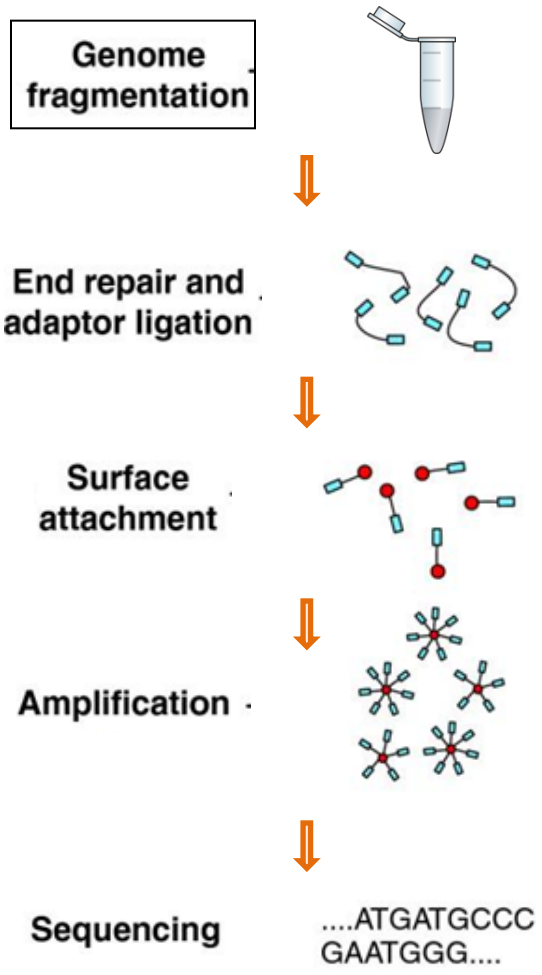
## Conventional



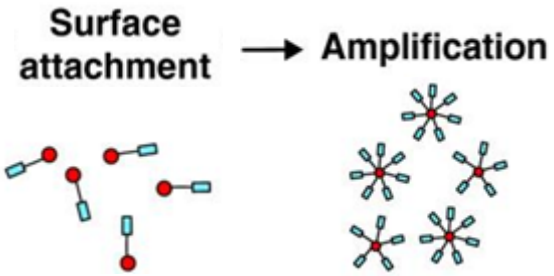





## NGS Pipeline



# Next generation sequencing




...

...

illumina MiSeq

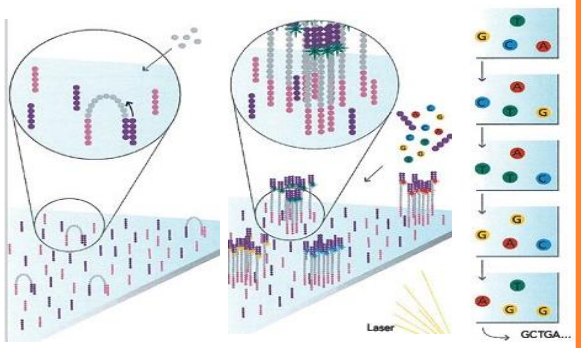
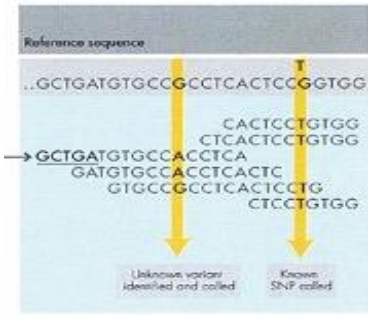
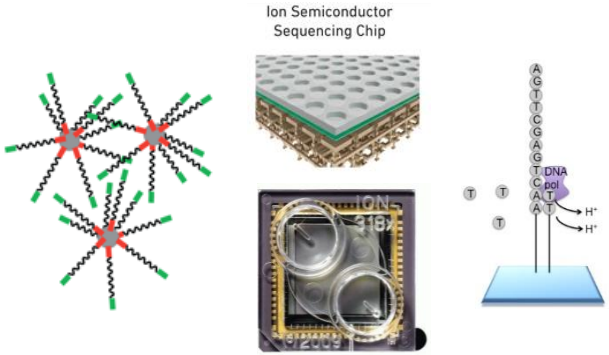



Image capture
Fluoresence detectioin

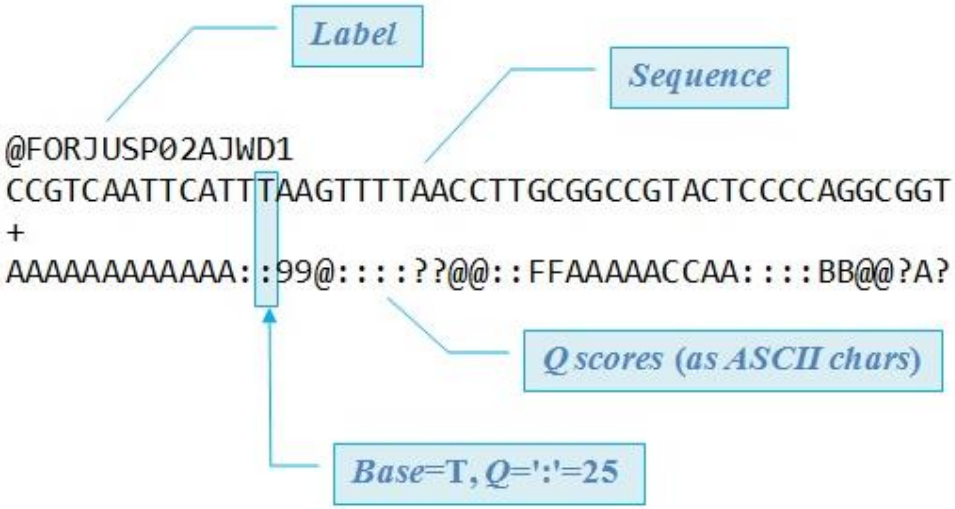



200bp-400bp short reads




pH variation when incorporating nucleotides in the growing strand

# .fastq files




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% |
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| 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
GATTAAAAAAAGAGTGTCTGATAGCAGCTTCTGAACTGGTTACCTGCCGTGAGTAAATTAAAATTTTATTGACTTAGGTCAC
TAAATACTTTAACCAATATAGGCATAGCGCACAGACAGATAAAAAATTACAGAGTACACAACATCCATGAAACGCATTAGCA
CCACCATTACCACCACCATCACCATTACCACAGGTAACGGTGCGGGCTGACGCGTACAGGAAACACAGAAAAAAAGCCCGCA
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
GAAACACAGAAAAAAGCCCGCACCTGACAGTGCGGGCTTTTTTTTCGACCAAAGTAACG
+
=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?9499985555558&4<>2:;661499888...88/56666666$;6/.5:8(..+'++
@X1L6C:01333:03005
GCAATGCCAGGCAGGGCATGTACAGCTACGTACGTCTGAGCATCGATCGATGTACAGCTACGTACGTCTGAGCATCGATCGA
TGTACAGCTACGTACGTCTGAGCATCGATCGATGTACAGCTACG
+
555/55/(///(////(/8:9:<=>?<?@:98A??676<:;;@:5555555554444;=4443333;383338<68>>
68=333118311111111113933644588?==<76992---2+++0/
```

@

@

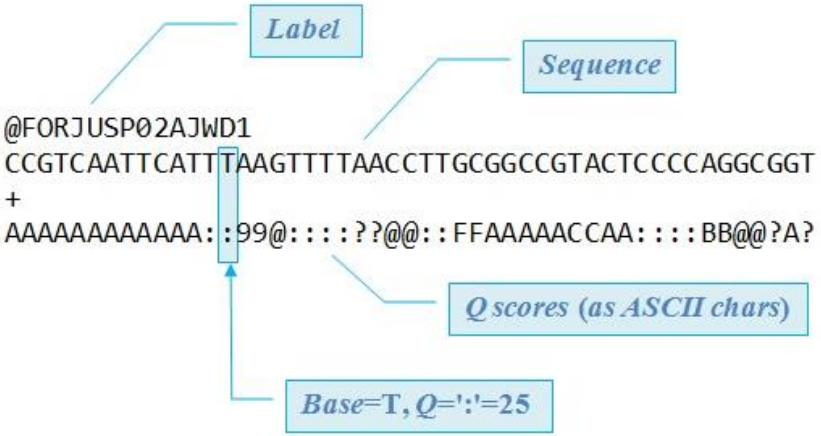
@

@

## …and so on

# Quality check

**Output of NGS sequencers**




**.fastq file**

**Input for quality check**

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

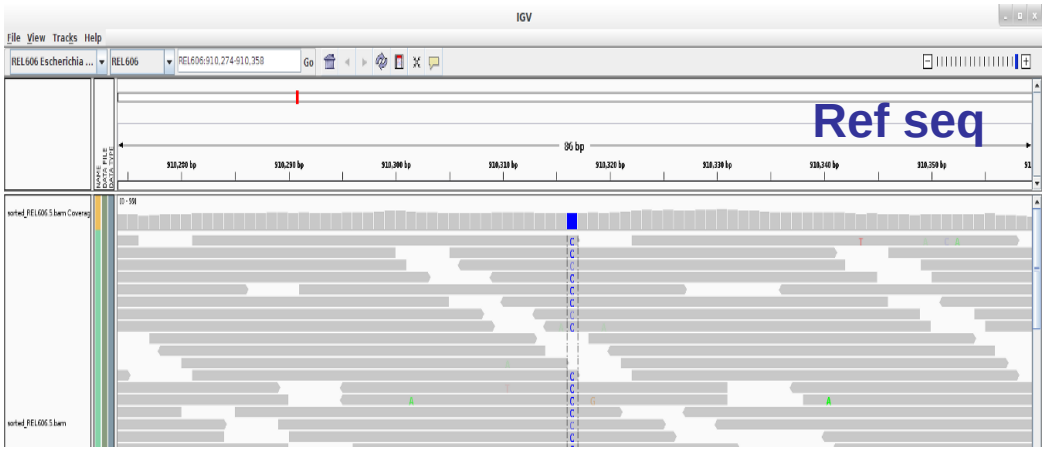
## Coverage assessment:

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

### .fastq file

```
@HWI-ST700693:238:B0224ACXX:1:1101:1218:1982
NACACTTGCTTTGGTGACAGCGGGGCATCCTCAAGC
+
#1=DDDDDHAFF?GEFGIIIIIIIIIIIIIIIIIFI
@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
+
#4BDFFFFFHHHHHJGIJIJJJIJJJJJJJJJJJJIJ
@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
+
```

FastqSize ≈ GenomeSize x Coverage x 2

**At least 0.5 GB per genome**

## Partially assembled genome (contigs)

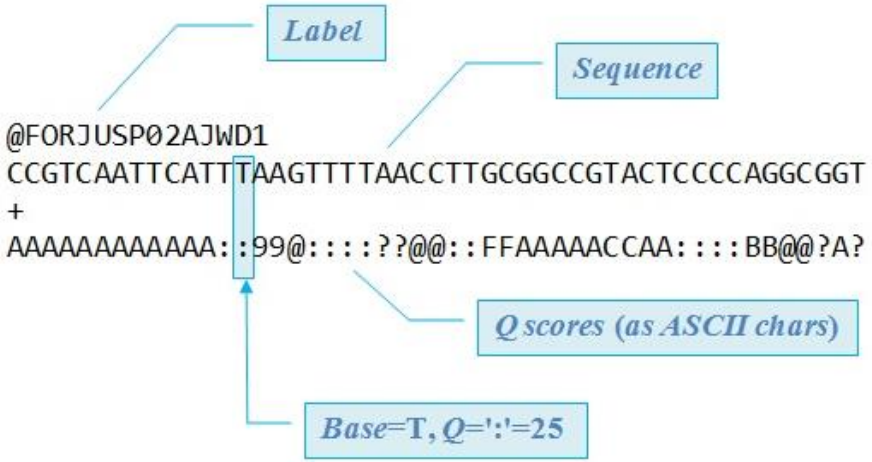
### .fasta file

```
>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
AGCGCATGAGCGCGCAGCGCCGCGTTACGTGGTGCATCAGCATGATGTTGGCCGGAGAG
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
```

FastaSize for *E. coli* contigs

**~5.5 MB**

# What should be trimmed out?

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Low quality positions

Adaptors and barcodes

Very short sequencing reads

# What should be trimmed out?

**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**

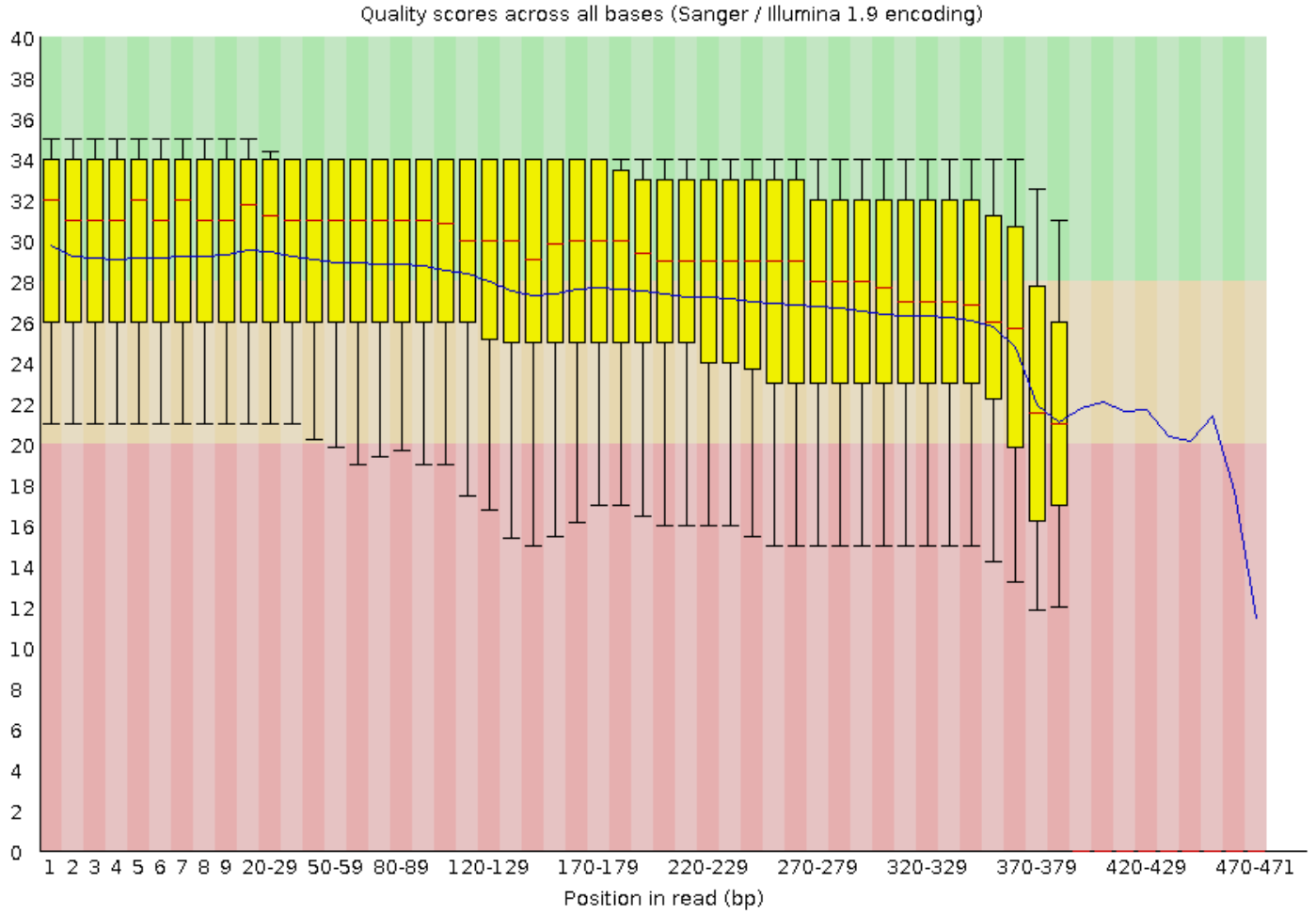
-1

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

Execute

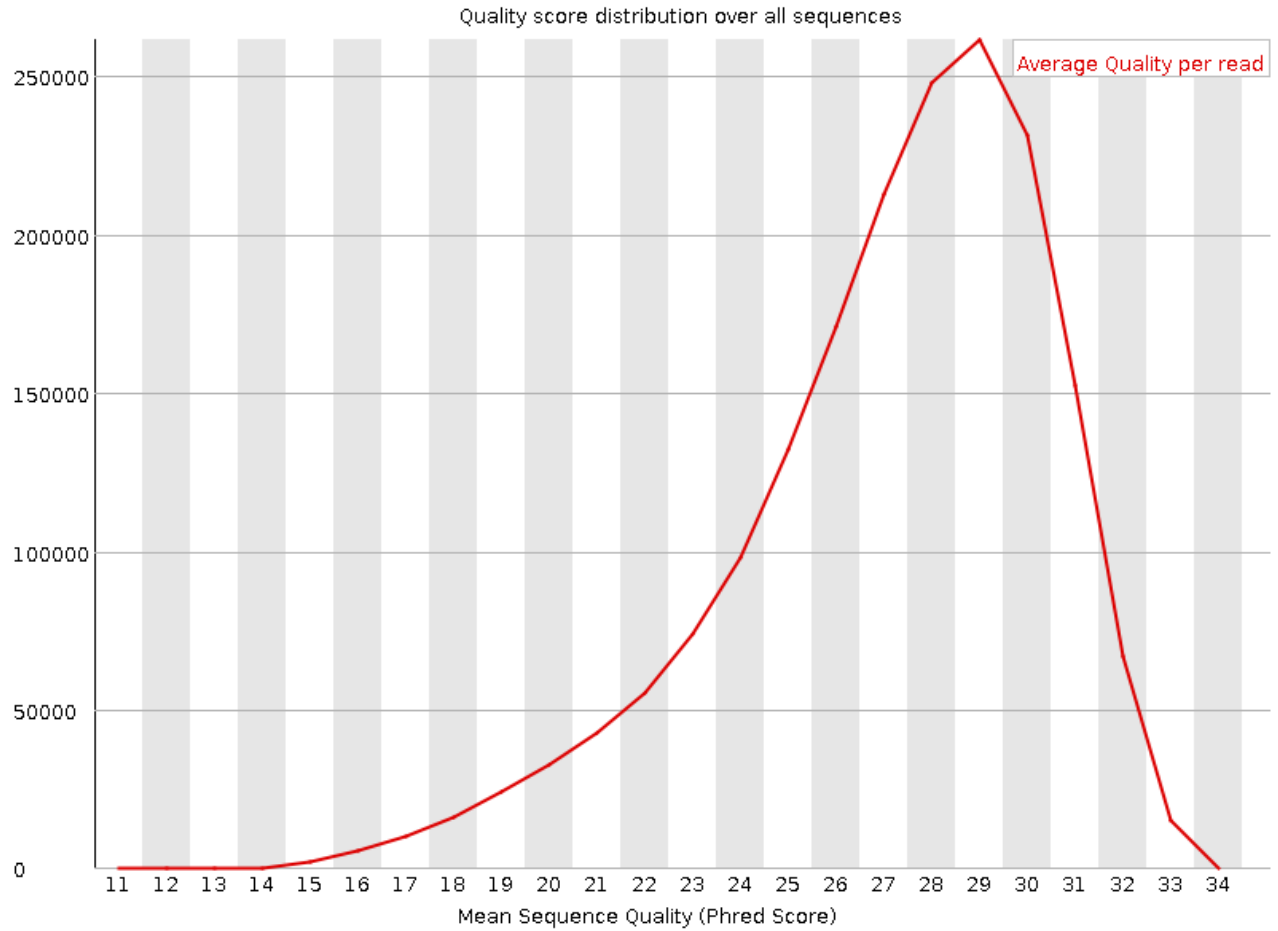
# FastQC – quality check of aw data

## Per base sequence quality



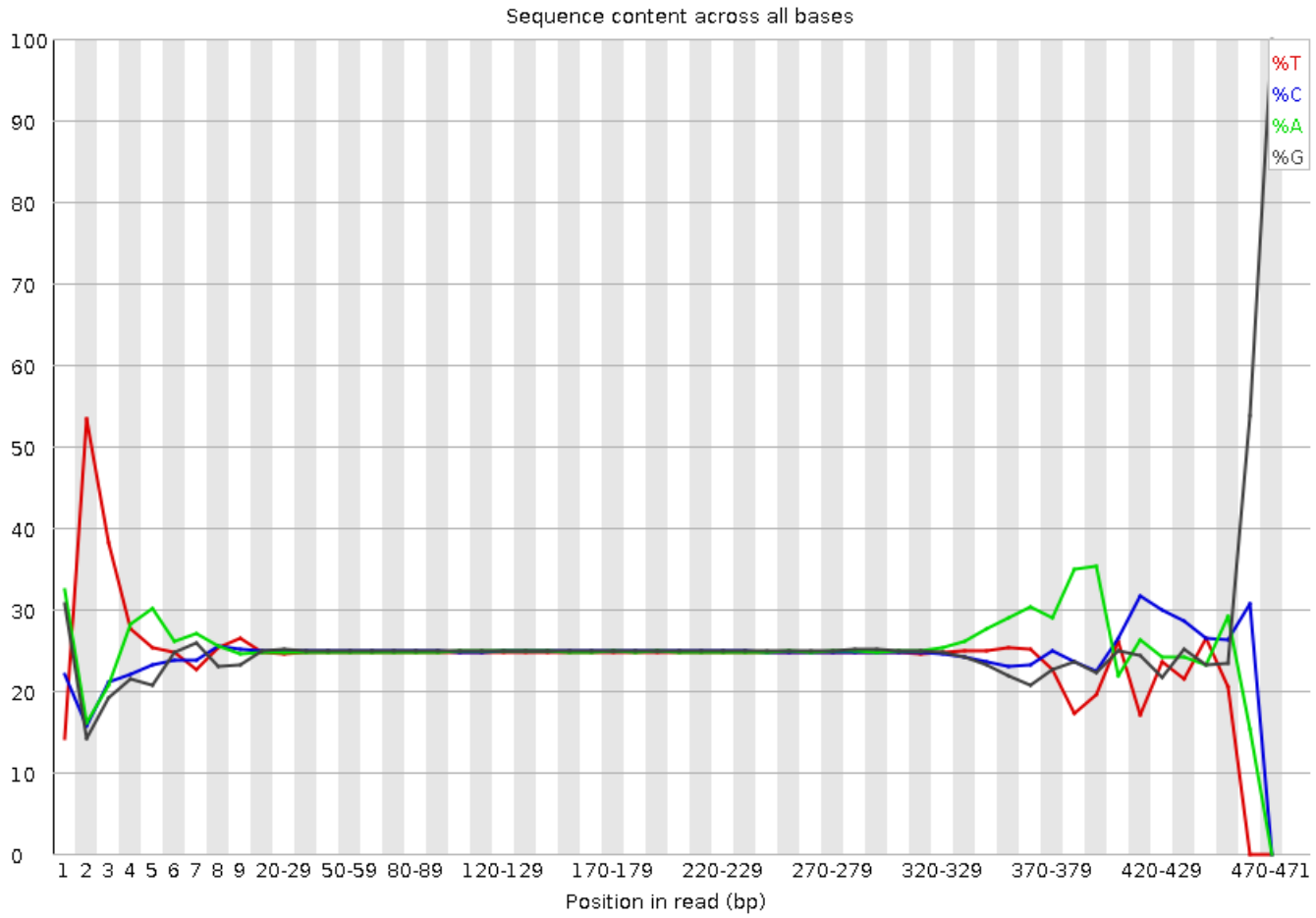
# FastQC – quality check of aw data

## Per sequence quality scores



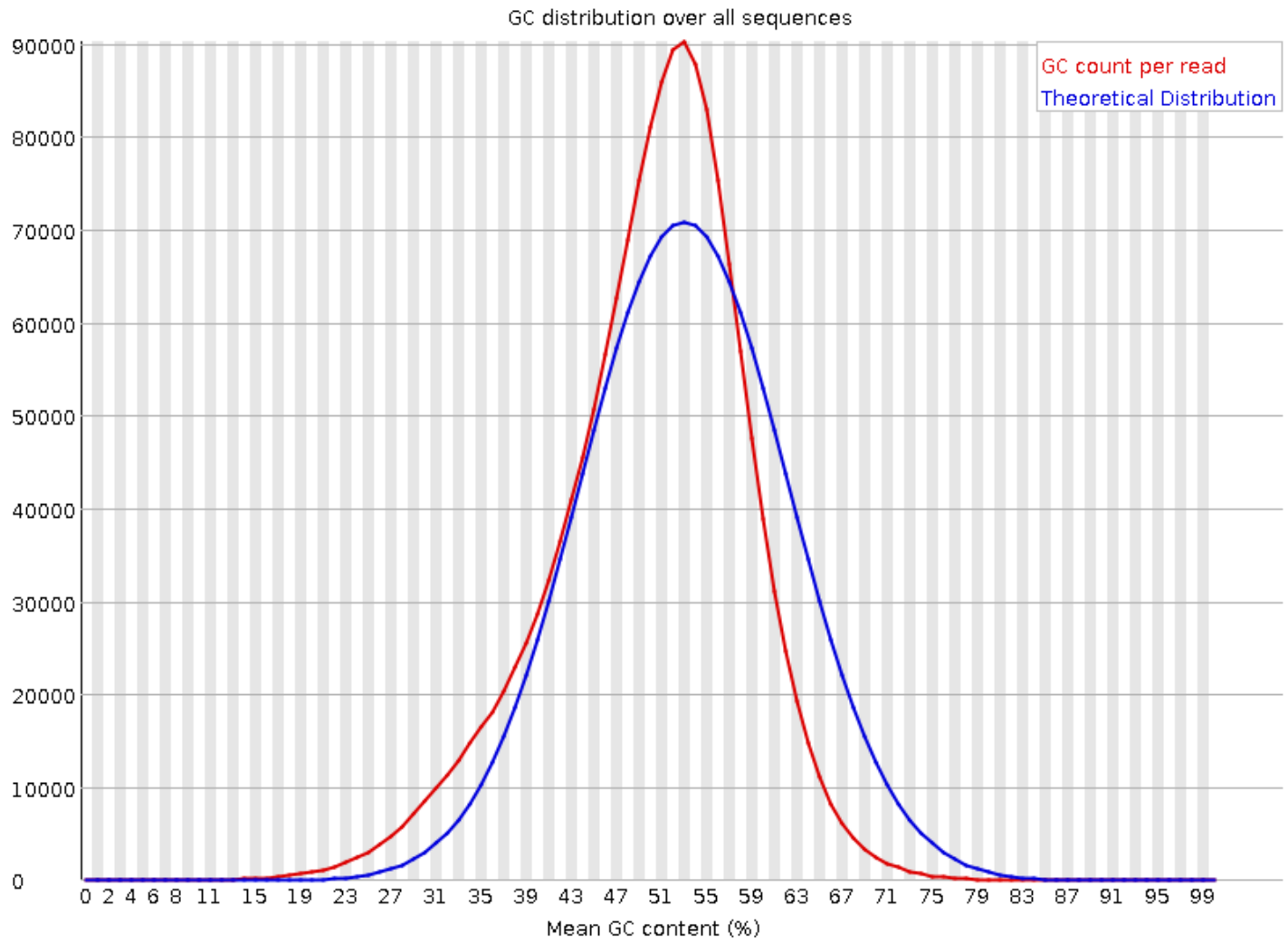
# FastQC – quality check of raw data

**Per base sequence content**



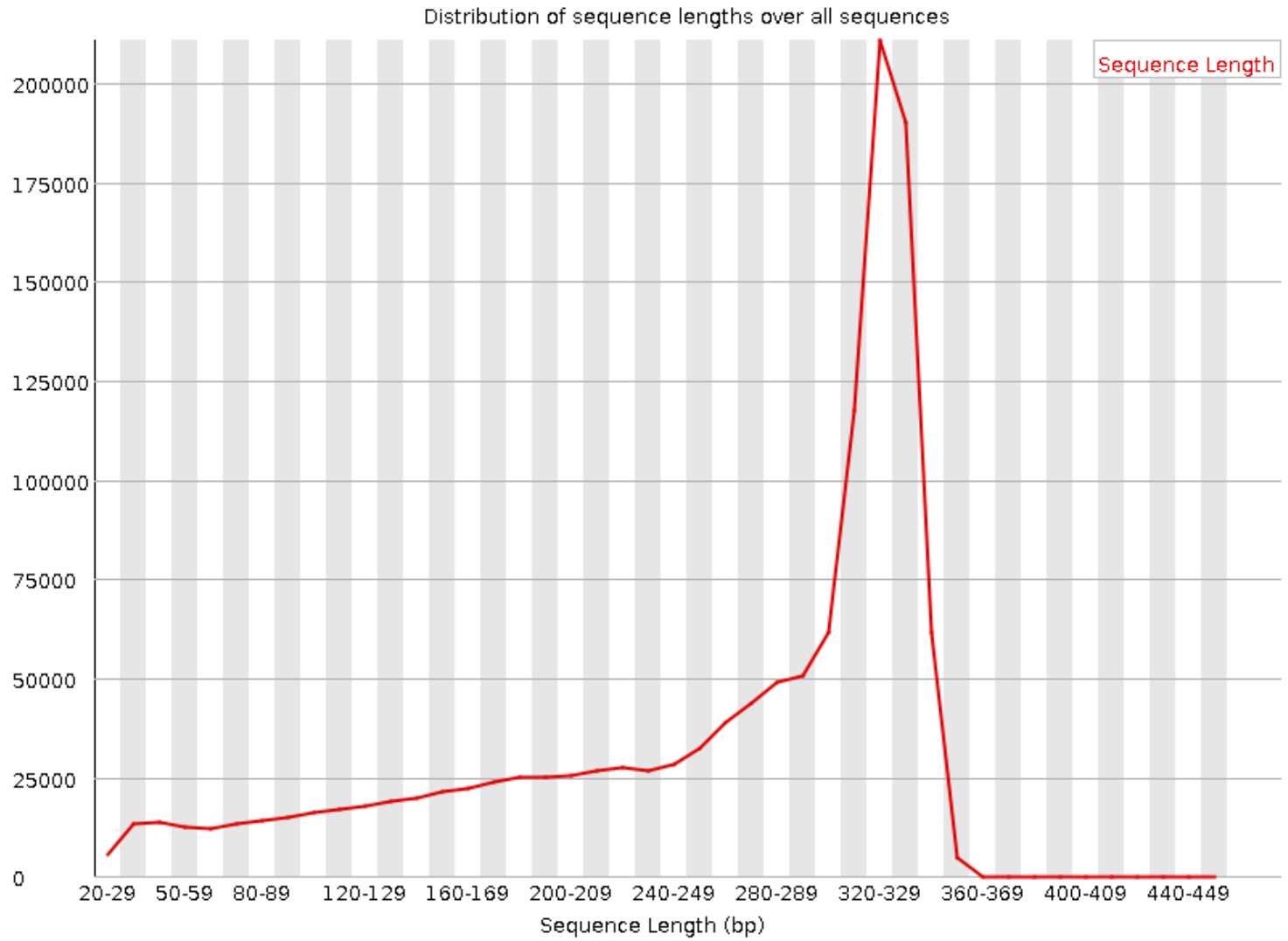
# FastQC – quality check of aw data

## Per sequence GC content



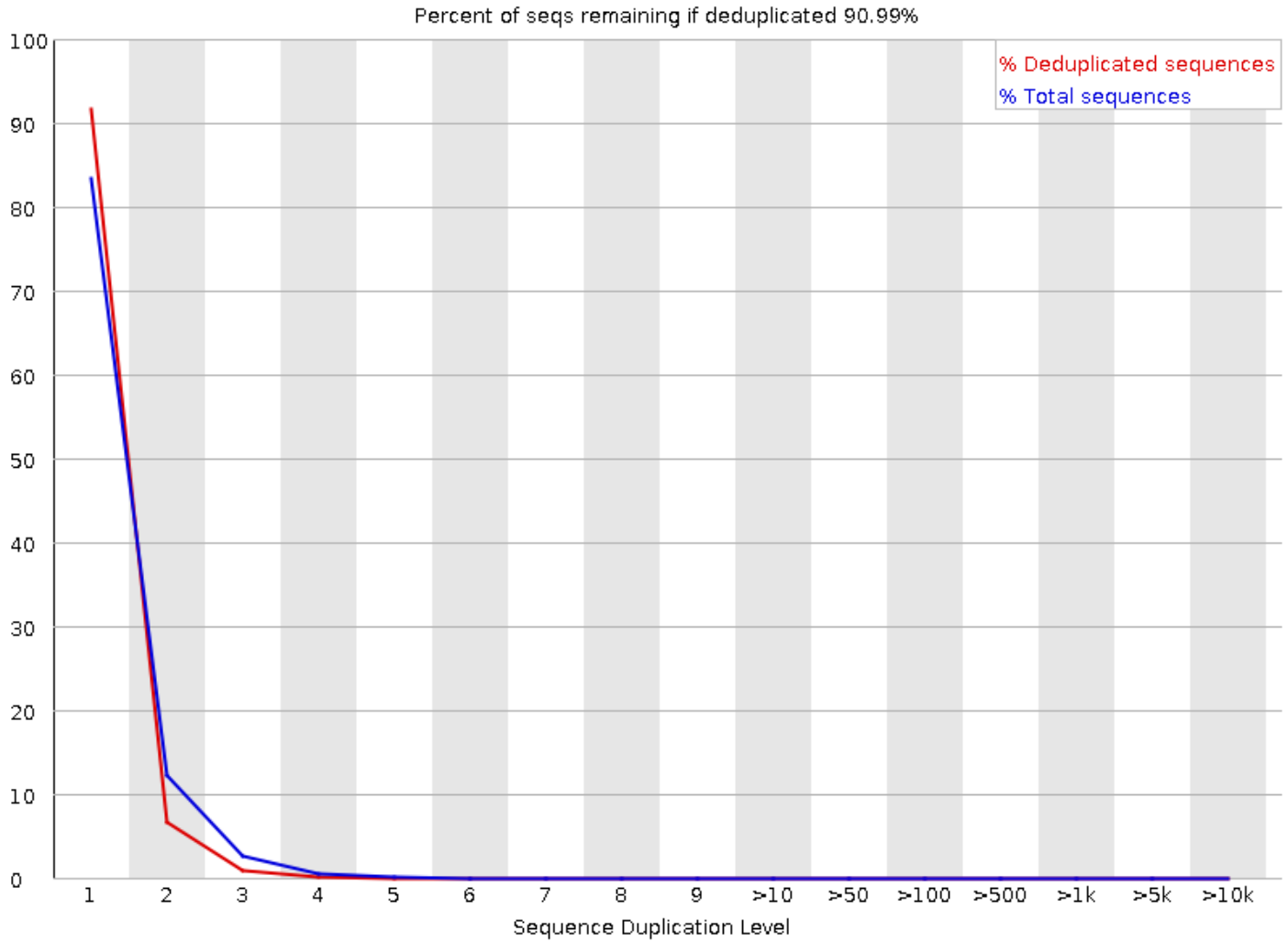
# FastQC – quality check of aw data

## Sequence Length Distribution



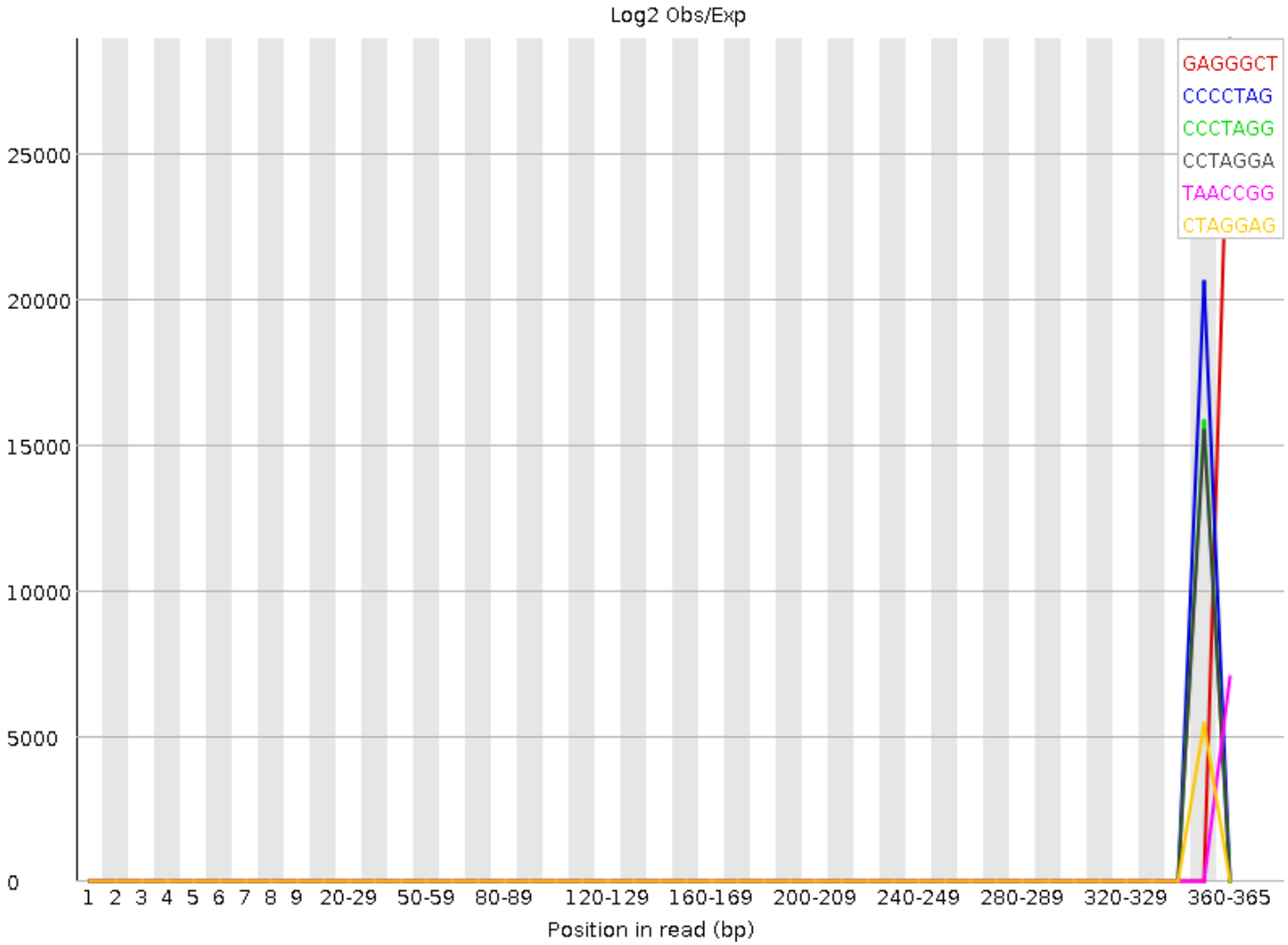
# FastQC – quality check of aw data

## Sequence Duplication Levels



# FastQC – quality check of raw data

## Kmer Content



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-1

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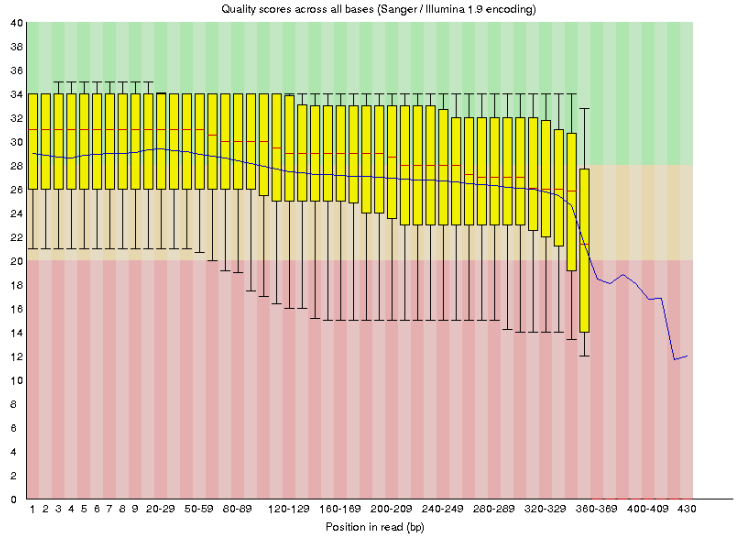
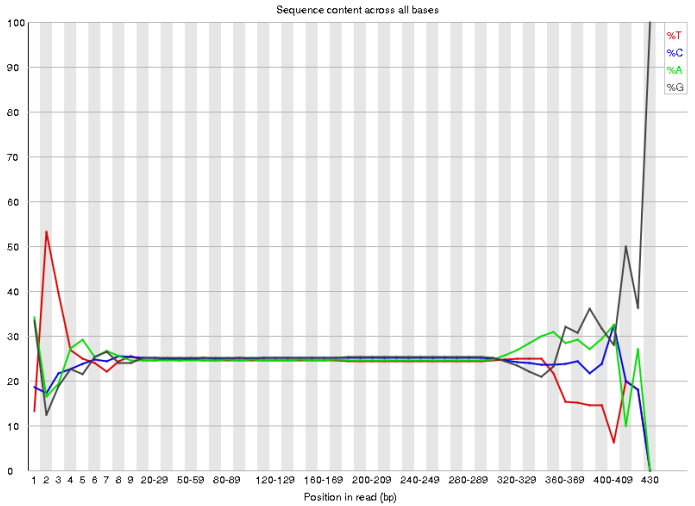
# Before trimming




# After trimming