1 NGS file formats

The information of biological sequences (nucleotides and amino acids) is stored in various file formats for easy access and analysis. Some of these files are in human-readable (text) format and others are in machine-readable (binary) format.

1.1 AB1 files

First-generation DNA sequencers (i.e. Applied Biosystems) store sequencing information in three files: .ab1, .seq and .phd.1. AB1 files are raw (binary) output files. They are also called as "Chromatogram" or "electropherogram" files. .ab1 file stores the DNA sequence chromatogram and raw data along with some other information. .phd.1 file (Phred file) also called a qual file is a text file containing quality values for each base. ".seq" file is a text file stores sequence in FASTA format.

1.2 PHD.1 files

Phred file or quality file is a simple text file containing each base's Phred quality value (more about quality values in a later section) A typical qual files look like below

```
 >fileName
27 29 31 34 35 36 37 37 38 37 38 38 39 38 36 32 36 36
36 34 35 36 35 36 34 35 34 35 36 36 36 37 36 37 37 34
27 29 31 34 35 36 37 37 38 37 38 38 39 38 36 32 36 36
36 34 35 36 35 36 34 35 34 35 36 36 36 37 36 37 37 34
```

1.3 Fasta files

Fasta file is a text file, consists of a single or multiple sequences. Though it is not mandatory, fasta files generally have an extension of .fa, .fas, fsa, .fna or .fasta. Each sequence has a one-line header, starting with the '>', followed by the nucleotide (or amino acid) sequence. This sequence can be in a single line or span across multiple lines.

```
 >HQ613402.1
TTGAATTTATATCGATCG
ATCGCATCGCGAGCCGAT
CGAATCGCGCGCATCGAT
TAGCCCCGATCGATCGAT
```

1.4 Standard Flowgram Format: SFF

The Standard Flowgram Format(SFF) file is a binary file used to store pyrosequencing (454) data. It is equivalent to the ABI chromatogram files. It contains information about:

- flowgram
- called sequence
- qualities
- recommended quality
- adaptor clipping.

Like the AB1 files, these are binary files and require specialized programs to view or convert them to other formats.

1.5 Fastq files

As the technologies evolved to sequence millions of "short reads", storing the information in multiple files became difficult to manage and also consumes more disk space. To address these difficulties, a new file format, fastq (fast Q) was introduced in 2009. Now, fastq format is the de facto standard of high-throughput sequence file format for next-generation sequencers.
Fastq is a text file (human-readable). It has 4 lines for each sequence entry.

- Header: starts with"@"
- Sequence
- Optional field: starts with "+"
- Qualities

As the sequence and quality correspond to each other, the length of 2nd and 4th lines are always equal.

Depending on the kind of sequencing method, fastq files can be either single-end or paired-end. Single-end reads are sequenced from one direction of the template only, whereas paired-end reads are sequenced from both 5' and 3' ends of a template, giving rise to forward and reverse reads. Paired-end sequences may also have sequence identifiers in the headers ending with "/1" and "/2" for forward and reverse directional reads respectively.

Example fastq file

```
head ~/Sreenu/FQs/goodData-1.fq

@SRR1564803.1 1 length=101
CTGTCTGACTCCCAAGCATCAATACTCTGGCATTCTGAGTTTGATAAGCTTCGCTAACCTCTCGGCCTG...
+SRR1564803.1 1 length=101
CCCFFFFFHHHHHJJJJJJJJJJJJJJJJJJJJJJJJJJIIJJJJJJJJJJJJJJJJJJJJJJIJIHH...
@SRR1564803.2 2 length=101
CCGGTGAGAAACACGCGACGCTGCACCTAAATGCATTTCGGGGAGAACCAGCTATCACGGAGTTTGAGC...
+SRR1564803.2 2 length=101
B@CFDFFFHHHHHJJJJJJJJJJIJJJJJJJJJJJJIJJJJJJHFDDEEDDDDDDDDDDDDDDDDDDDD...
```

Most often, paired-end data stored in two files and they have "R1" and "R2" in their file names. The order of the reads in paired-end files corresponds to their pairing order, i.e. entry 1 in R1 file is sequenced from the same template as entry 1 in R2 file, and so on so forth. Therefore, the total number of sequences should always be equal in R1 and R2 files. If for any reason, any entry is removed from R1, its corresponding entry from R2 should also be removed.

Sometimes paired-end data is saved in interleaved fastq format, storing forward and reverse reads sequentially in a single file. A typical interleaved fastq file looks like below.

```
@B10941:62:000000000-A8CVL:1:2302:15112:1132 1:N:0:10
ATCCTAGGTAGCGACATGATGGCCCCAGGAGATGGGAAGAACCCCCGATAGAGATA
+
CCBCCCBBBGBGFGGGGGGGGG@;FFGGGEG@FF<EE<@FFC,CEGCCGGFF<FGF
@B10941:62:000000000-A8CVL:1:2302:15112:1132 2:N:0:10
TCGGCATGACGCTATCGCATCATTTTACTACGCTAGCATATCGCTTTTAGA
+
DDDCGCGFGGGGGGGGG@;FFGGGEG@FF<EE<@FFC,CEGCCGGFF<FGF
@B10941:63:000000000-A8CVL:1:2302:15112:1132 1:N:0:10
GCGAGGTCGGCATGATGGGGGAATACGAAGCATGGGAAGAAAAGGAGAGATACGAA
+
CCGGGGGGGGAAAACCCGGGGG@;FFGGGEG@FF<EE<@FFC,CEGCCGGGGFFFG
@B10941:63:000000000-A8CVL:1:2302:15112:1132 2:N:0:10
CGCGACGACTCGACGACATACGACACGCGATCGATCGGAAATGAATCGCATCGAC
+
CGGGGGFEGGCGCGAAGGGGG@;FFGGGEG@FF<EE<@FFC,CEAGGGACGGAGA
```

Generally a fastq file is stored with the suffix .fq or .fastq (if compressed, .fq.gz or .fastq.gz)

1.6 Hierarchical Data Formats 5: HDF5

The Hierarchical Data Format Version 5, (HDF5), file formats is a complex format developed to support large, complex and heterogeneous data. HDF5 uses a hierarchical structure to organize data within the file in many different structured ways. It also allows for the embedding of metadata making it self-describing. Moreover, HDF5 is a compressed format. Oxford Nanopore Technologies raw output format FAST5 is a type of HDF5 file format.

1.7 Sequence Alignment/Map (SAM) format

The sequence alignment/map (SAM) format is a tab-delimited text file format developed to store read mapping. Every SAM file has two sections:

1) Header section (optional)
2) Alignment section.

Entries in the header section always start with "@" and come before alignment section. Each line in the header is tab-delimited and has a two letter header code called TAG. They follow "TAG:VALUE" format. These TAG are:

- HD – The Header Line – 1st line
- SO – Sorting order of alignments (unknown (default), unsorted, queryname and coordinate)
- SQ - Reference sequence dictionary.
- SN - Reference sequence name.
- LN - Reference sequence length.
- PG – Program
- ID – The program ID
- PN – The program name
- VN – Program version number
- CL – The Command actually used to create the SAM file
- RG – Read Group– "a set of reads that were all the product of a single sequencing run on one lane"

In the alignment section, there are 11 mandatory fields. These are

- QNAME: Read Name
- FLAG: Info on if the read is mapped, part of a pair, strand etc
- RNAME: Reference Sequence Name that the read aligns to
- POS: Leftmost position of where this alignment maps to the reference
- MAPQ: Mapping quality of read to reference (phred scale P that mapping is wrong)
- CIGAR: Compact Idiosyncratic Gapped Alignment Report
- RNEXT: Paired Mate Read Name
- PNEXT: Paired Mate Position
- TLEN: Template length/Insert Size (difference in outer co-ordinates of paired reads)
- SEQ: The actual read DNA sequence
- QUAL: ASCII Phred quality scores (+33)
- TAGS: Optional data – Lots of options e.g. MD=String for mismatches

Let us take a close look at FLAG and CIGAR values. FLAG is a sum of alignment bit flags. Below is the table showing what each bit corresponds to.

Bit	Description
0x1	template having multiple segments in sequencing
0x2	each segment properly aligned according to the aligner
0x4	segment unmapped
0x8	next segment in the template unmapped
0x10	SEQ being reverse complemented
0x20	SEQ of the next segment in the template being reversed
0x40	the first segment in the template
0x80	the last segment in the template
0x100	secondary alignment
0x200	not passing quality controls
0x400	PCR or optical duplicate

For example, Flag 91 codes for
64: first in the segment
16: read reverse complemented
8: second read is unmapped
2: each segment is properly aligned
1: paired end reads

This is the only combination that gives the total of 91
You can check flag values using `samtools flag` option. For example: `samtools flag 91` will give you the corresponding flag values.
If you prefer, you can also check online at `https://www.samformat.info/sam-format-flag`.

Concise Idiosyncratic Gapped Alignment Report: CIGAR
CIGAR is a string of alpha-numerics representing the alignment result. For example, take a look at below mapping results

```
Coor     12345678901234  5678901234567890123456789012345
ref      AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT

+r001/1        TTAGATAAAGGATA*CTG
+r002         aaaAGATAA*GGATA
+r003       gcctaAGCTAA
+r004                     ATAGCT..............TCAGC
-r003                           ttagctTAGGC
-r001/2                                      CAGCGCCAT
```

Corresponding SAM file will be

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA   *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC    *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

Where

```
Ref: AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT
R001/1:    TTAGATAAAGGATA*CTG
```

CIGAR values: 8M2I4M1D3M tells us there are

- 8 matches
- 2 insertions
- 4 matches
- 1 deletion
- 3 matches

Below is the list of allowed CIGAR letters and their descriptions

Op	BAM	Description
`M`	0	alignment match (can be a sequence match or mismatch)
`I`	1	insertion to the reference
`D`	2	deletion from the reference
`N`	3	skipped region from the reference
`S`	4	soft clipping (clipped sequences present in SEQ)
`H`	5	hard clipping (clipped sequences NOT present in SEQ)
`P`	6	padding (silent deletion from padded reference)
`=`	7	sequence match
`X`	8	sequence mismatch

1.8 BAM files

Binary Alignment Map (BAM) is a compressed SAM file. It is compressed using BGZF compression method..

1.9 CRAM files

CRAM files are also compressed SAM file, designed by the EBI to reduce the storage space. CRAM files compression is based on the reference the data is aligned to.

Data is compressed using one of the general purpose compressors (gzip, bzip2). CRAM records are compressed using several different encoding strategies. For example, bases are reference compressed by encoding base differences rather than storing the bases themselves. External reference sequences introduce the only external dependency into the CRAM format. When external reference sequences cannot be conveniently used the reference sequences also can be embedded within the CRAM files. However, when embedded reference sequences are used then only those reference sequence regions are preserved in CRAM that has reads aligned against them.

Further reading:
http://samtools.github.io/hts-specs/SAMv1.pdf
https://www.ebi.ac.uk/sites/ebi.ac.uk/files/groups/ena/documents/cram_format_1.0.1.pdf

2 Phred quality scores

The Phred quality score is a nucleotide identification accuracy measure. It is a probability of the base call is incorrect. The quality score calculation takes into account the ambiguity of the signal for the respective base as well as the quality of neighbouring bases. It is developed to help automate DNA sequencing and quality control of the data. In a fastq file Phred qualities are converted to ascii characters (q+33, printable ascii characters start from ascii 33) and stored.

It is calculated as
Phred-Q = -10 $\log_{10}$(P)

If the probability of an error is 0.01 (1 in 100 error), its phred score will be

= -10 X $\log_{10}$(0.01)
= -10 X -2 = 20

ascii character for 53 (20+33) is “5”

Here is the list of phred qualities and their error rates

Phred quality	Q33 code	Error rate	Phred quality	Q33 code	Error rate
0	!	1.00000	21	6	0.00794
1	”	0.79433	22	7	0.00631
2	#	0.63096	23	8	0.00501
3	$	0.50119	24	9	0.00398
4	%	0.39811	25	:	0.00316
5	&	0.31623	26	;	0.00251
6	’	0.25119	27	<	0.00200
7	(	0.19953	28	=	0.00158
8	)	0.15849	29	>	0.00126
9	*	0.12589	30	?	0.00100
10	+	0.10000	31	@	0.00079
11	,	0.07943	32	A	0.00063
12	-	0.06310	33	B	0.00050
13	.	0.05012	34	C	0.00040
14	/	0.03981	35	D	0.00032
15	0	0.03162	36	E	0.00025
16	1	0.02512	37	F	0.00020
17	2	0.01995	38	G	0.00016
18	3	0.01585	39	H	0.00013
19	4	0.01259	40	I	0.00010
20	5	0.01000			

3 NGS data quality control and read cleaning

Quality check of NGS data is a standard practice to do before analysing the data. NGS data is cleaned based on the reads' base qualities, length, and low complexity. Poor quality reads are untrustworthy and can lead to poor alignments, erroneous variant callings and incorrect interpretation of the data.

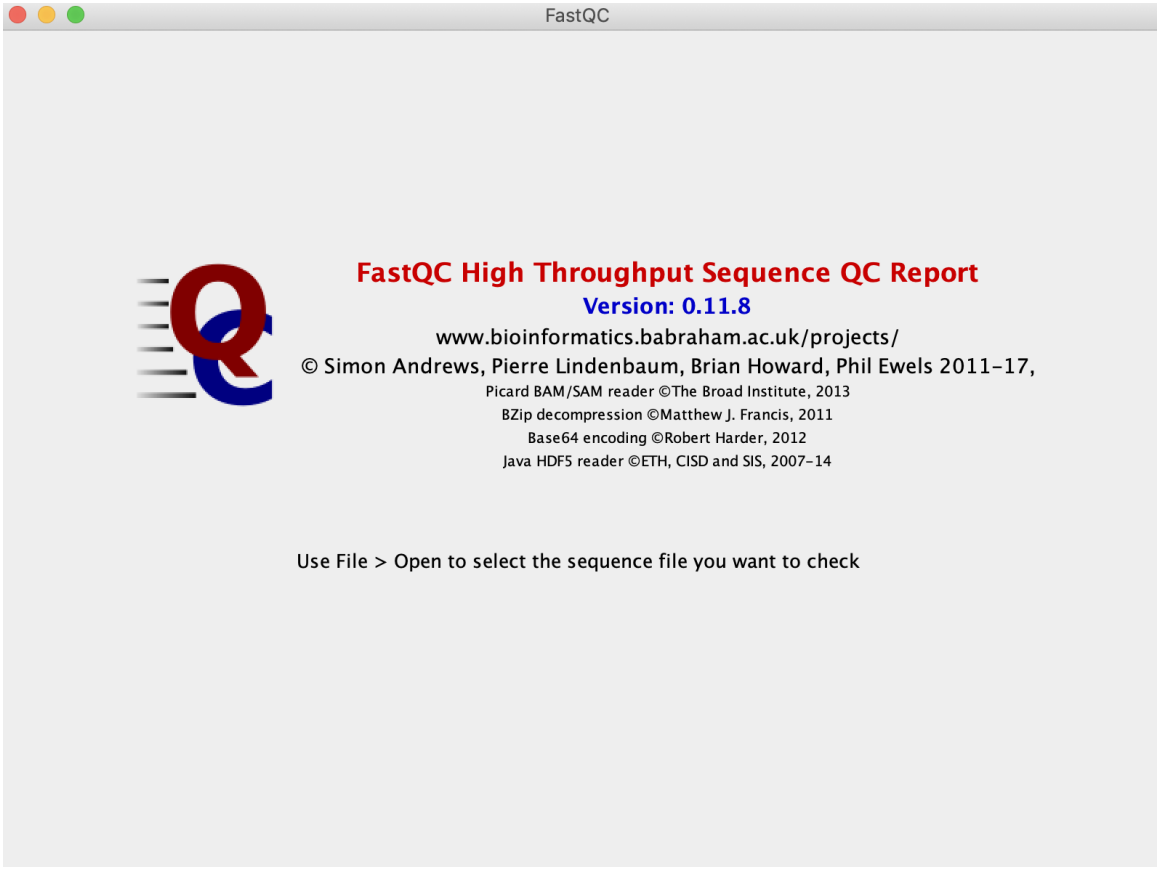
Here are some of the parameters for NGS data quality control.

- Check for primers and adaptors
- Trim low quality ends
- Remove low quality reads
- Remove short sequences
- Remove reads with ambiguous bases (“N”)
- Remove duplicates

In this training, you will be using a program called “fastqc” to visually inspect the NGS data quality. To run fqstqc program, open the terminal and enter

```
fastqc &
```

This will open FastQC program in the background.

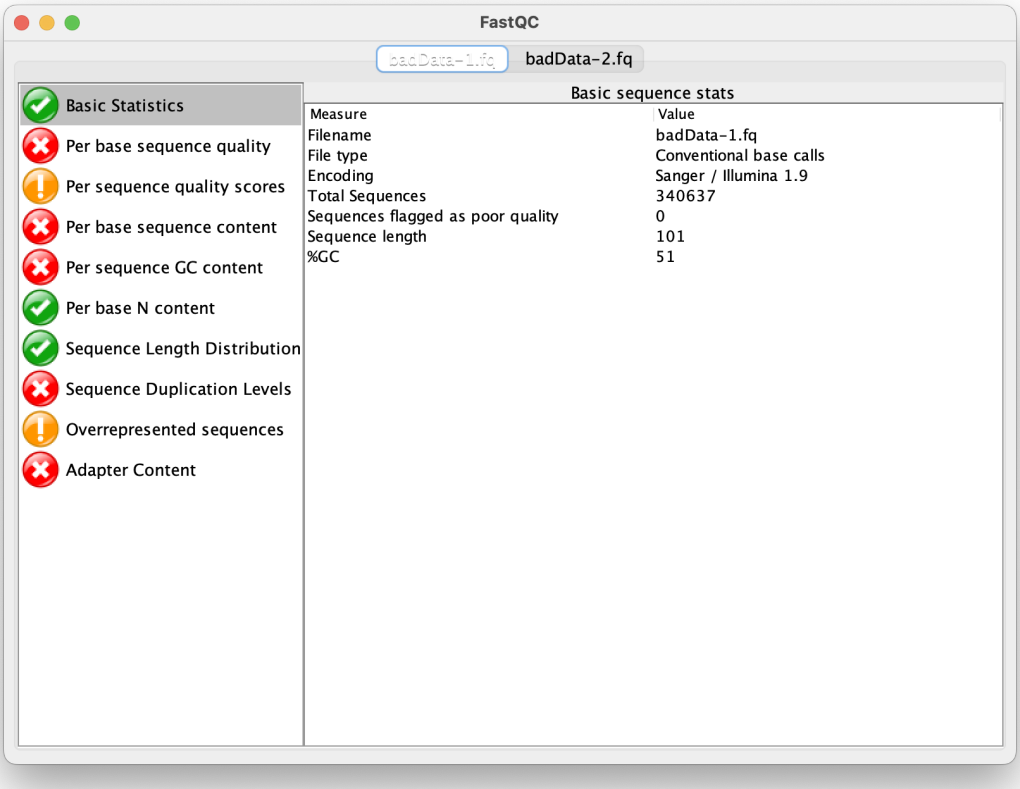


go to File -> Open and navigate to ”Sreenu/FQs/” folder. Holding the control key select

```
badData-1.fq
badData-2.fq
```

It will generate a QC report after checking all the parameters. Here is the summary of all the statistics accroding to developer’s page
(https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)

3.1 Basic Statistics



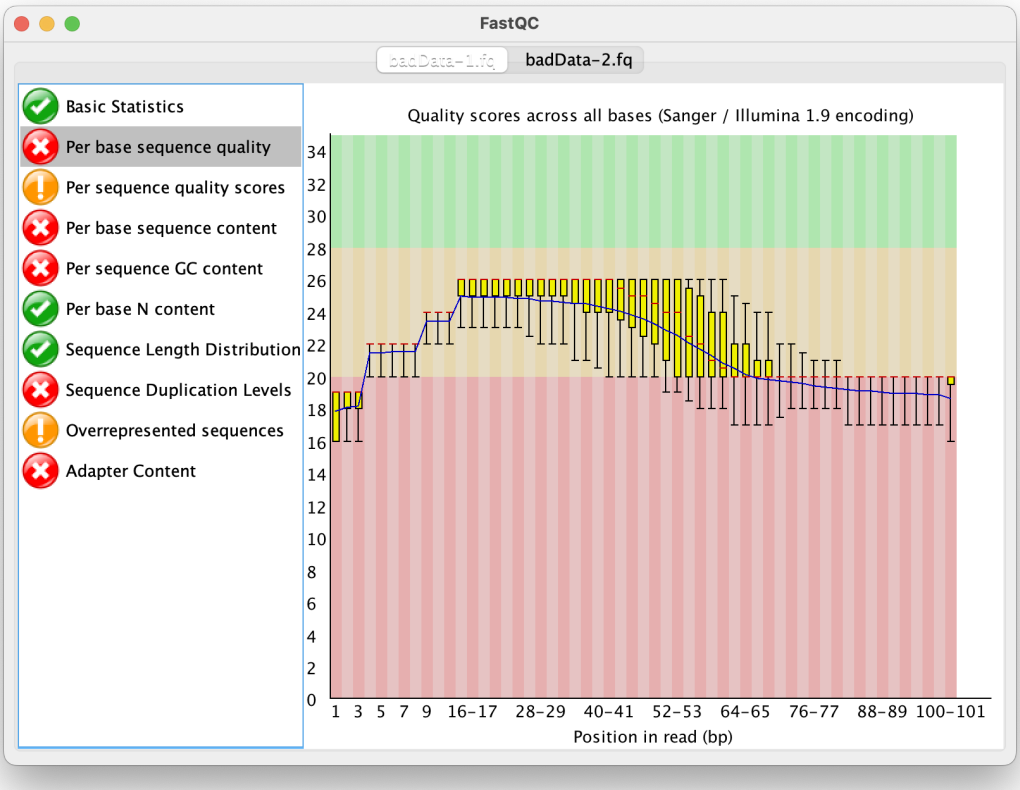
The Basic Statistics module generates some simple composition statistics for the file analysed.

- Filename: The original filename of the file which was analysed
- File type: Says whether the file appeared to contain actual base calls or colorspace data which had to be converted to base calls
- Encoding: Says which ASCII encoding of quality values was found in this file.
- Total Sequences: A count of the total number of sequences processed. There are two values reported, actual and estimated. At the moment these will always be the same. In the future it may be possible to analyse just a subset of sequences and estimate the total number, to speed up the analysis, but since we have found that problematic sequences are not evenly distributed through a file we have disabled this for now.
- Filtered Sequences: If running in Casava mode sequences flagged to be filtered will be removed from all analyses. The number of such sequences removed will be reported here. The total

sequences count above will not include these filtered sequences and will the number of sequences actually used for the rest of the analysis.

- Sequence Length: Provides the length of the shortest and longest sequence in the set. If all sequences are the same length only one value is reported.
- %GC: The overall %GC of all bases in all sequences

3.2 Per base sequence quality



This view shows an overview of the range of quality values across all bases at each position in the FastQ file. For each position a BoxWhisker type plot is drawn. The elements of the plot are as follows:

- The central red line is the median value
- The yellow box represents the inter-quartile range (25-75

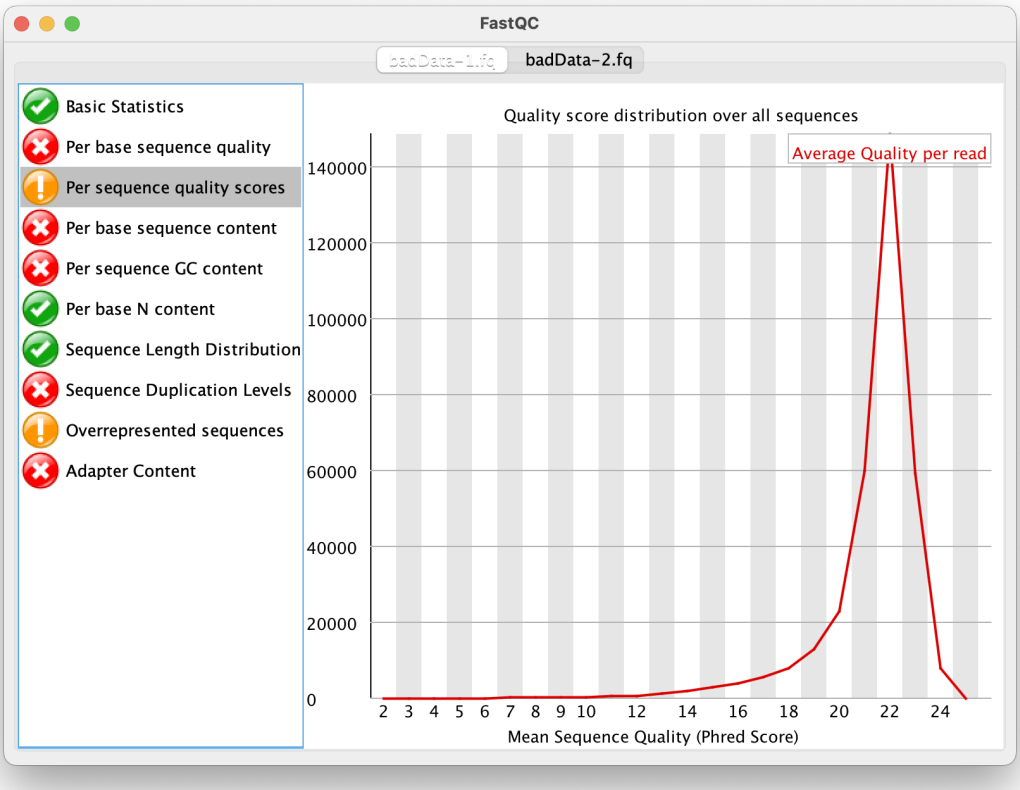
- The upper and lower whiskers represent the 10
- The blue line represents the mean quality

The y-axis on the graph shows the quality scores. The higher the score the better the base call. The background of the graph divides the y axis into very good quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). The quality of calls on most platforms will degrade as the run progresses, so it is common to see base calls falling into the orange area towards the end of a read.

It should be mentioned that there are number of different ways to encode a quality score in a FastQ file. FastQC attempts to automatically determine which encoding method was used, but in some very limited datasets it is possible that it will guess this incorrectly (ironically only when your data is universally very good!). The title of the graph will describe the encoding FastQC thinks your file used.

Results from this module will not be displayed if your input is a BAM/SAM file in which quality scores have not been recorded.

3.3 Per Sequence Quality Scores

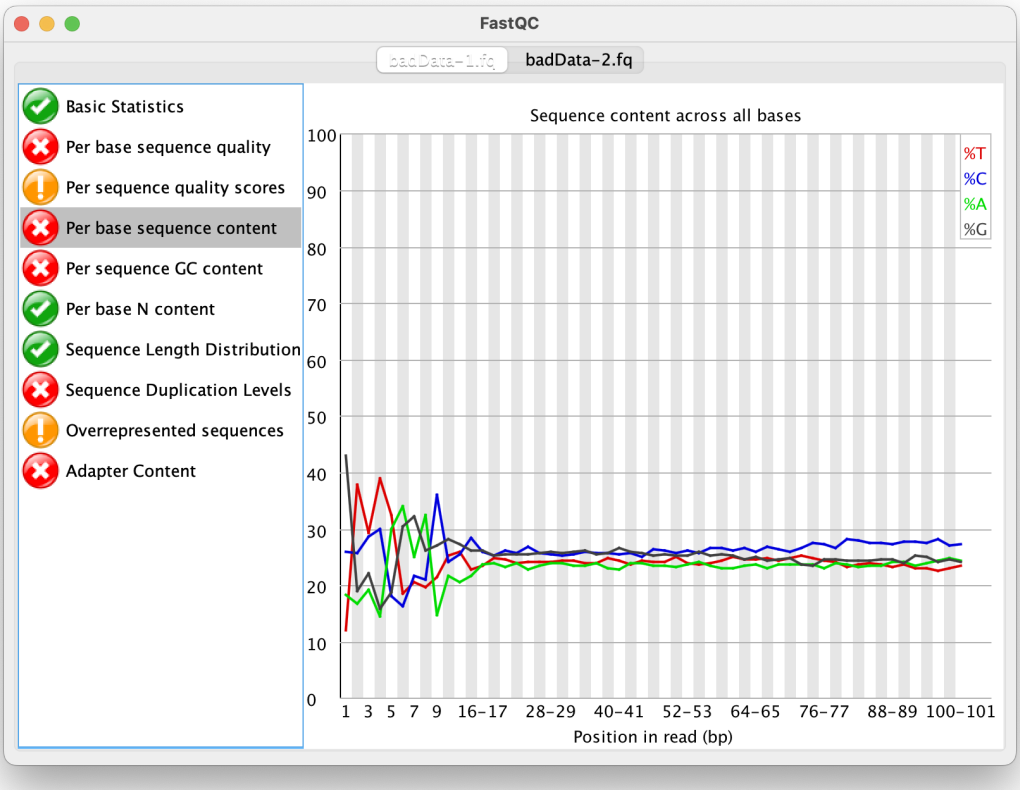


The per sequence quality score report allows you to see if a subset of your sequences have universally low quality values. It is often the case that a subset of sequences will have universally poor quality, often because they are poorly imaged (on the edge of the field of view etc), however these should represent only a small percentage of the total sequences.

If a significant proportion of the sequences in a run have overall low quality then this could indicate some kind of systematic problem - possibly with just part of the run (for example one end of a flowcell).

Results from this module will not be displayed if your input is a BAM/SAM file in which quality scores have not been recorded.

3.4 Per Base Sequence Content

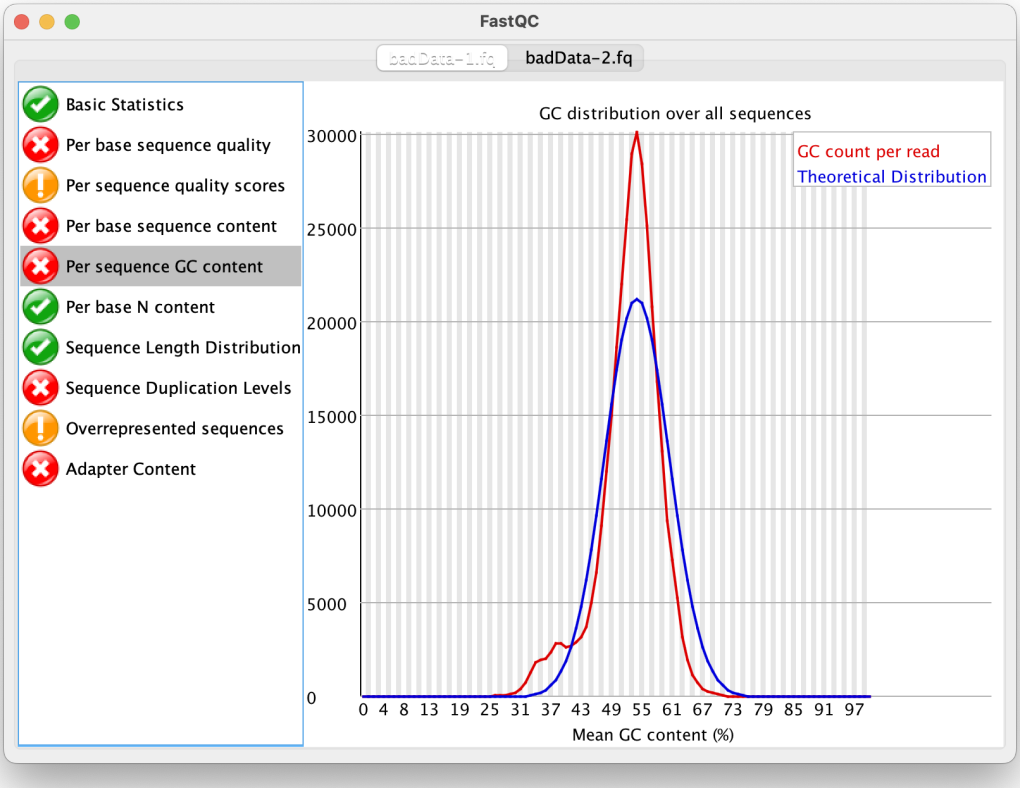


Per Base Sequence Content plots out the proportion of each base position in a file for which each of the four normal DNA bases has been called.

In a random library you would expect that there would be little to no difference between the different bases of a sequence run, so the lines in this plot should run parallel with each other. The relative amount of each base should reflect the overall amount of these bases in your genome, but in any case they should not be hugely imbalanced from each other.

It's worth noting that some types of library will always produce biased sequence composition, normally at the start of the read. Libraries produced by priming using random hexamers (including nearly all RNA-Seq libraries) and those which were fragmented using transposases inherit an intrinsic bias in the positions at which reads start. This bias does not concern an absolute sequence, but instead provides enrichement of a number of different K-mers at the 5' end of the reads. Whilst this is a true technical bias, it isn't something which can be corrected by trimming and in most cases doesn't seem to adversely affect the downstream analysis. It will however produce a warning or error in this module.

3.5 Per Sequence GC Content

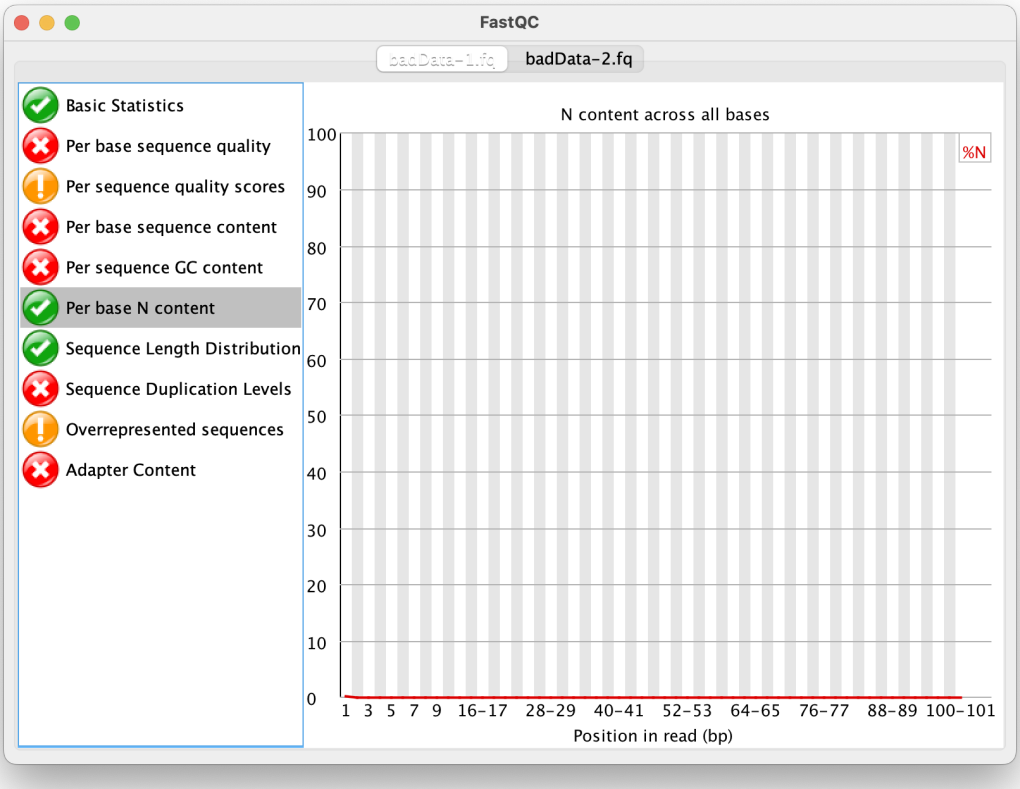


This module measures the GC content across the whole length of each sequence in a file and compares it to a modelled normal distribution of GC content.

In a normal random library you would expect to see a roughly normal distribution of GC content where the central peak corresponds to the overall GC content of the underlying genome. Since we don't know the the GC content of the genome the modal GC content is calculated from the observed data and used to build a reference distribution.

An unusually shaped distribution could indicate a contaminated library or some other kinds of biased subset. A normal distribution which is shifted indicates some systematic bias which is independent of base position. If there is a systematic bias which creates a shifted normal distribution then this won't be flagged as an error by the module since it doesn't know what your genome's GC content should be.

3.6 Per Base N Content

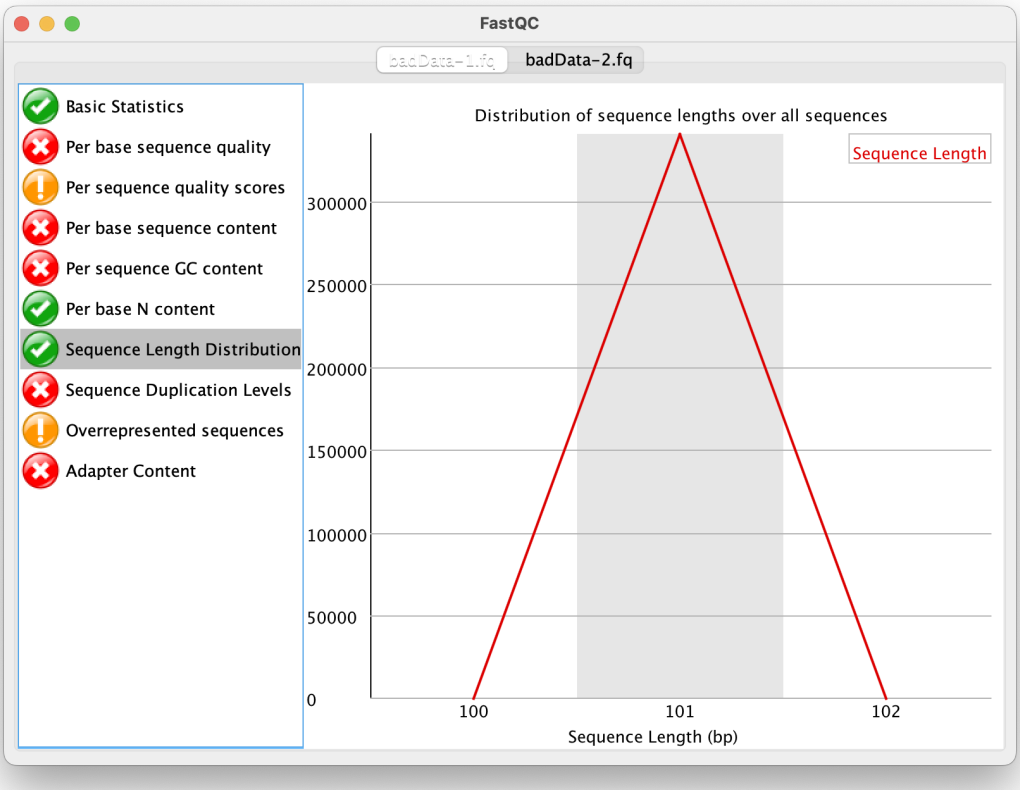


If a sequencer is unable to make a base call with sufficient confidence then it will normally substitute an N rather than a conventional base] call

This module plots out the percentage of base calls at each position for which an N was called.

It's not unusual to see a very low proportion of Ns appearing in a sequence, especially nearer the end of a sequence. However, if this proportion rises above a few percent it suggests that the analysis pipeline was unable to interpret the data well enough to make valid base calls.

3.7 Sequence Length Distribution

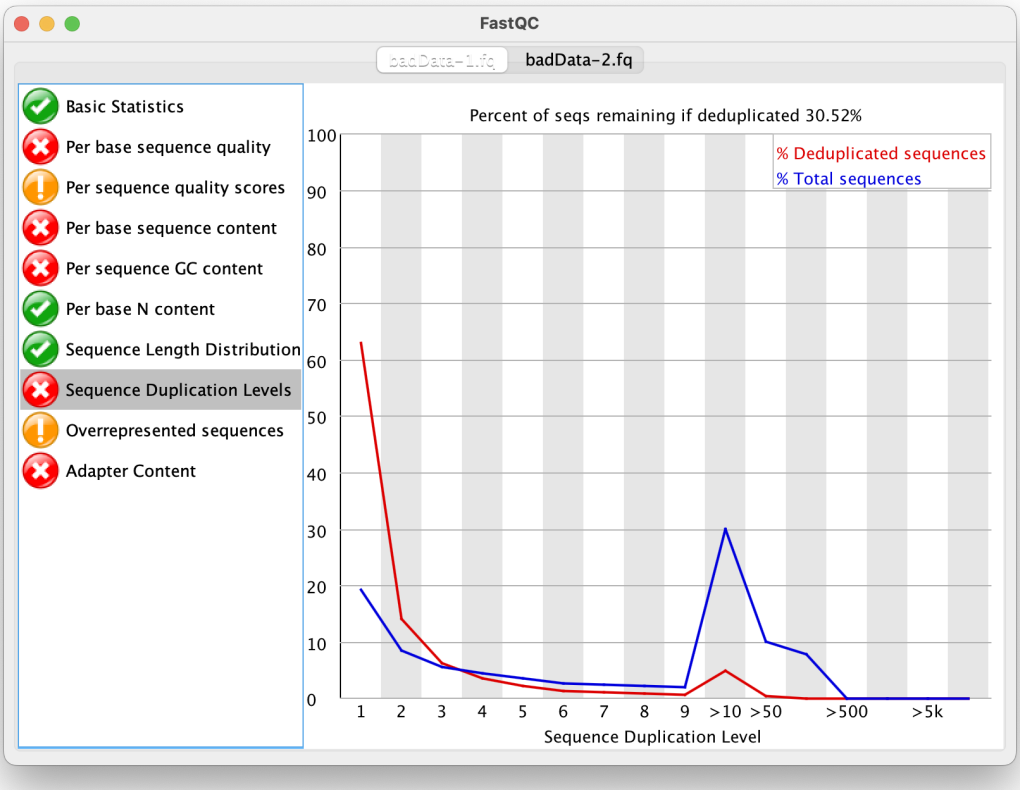


Some high throughput sequencers generate sequence fragments of uniform length, but others can contain reads of wildly varying lengths. Even within uniform length libraries some pipelines will trim sequences to remove poor quality base calls from the end.

This module generates a graph showing the distribution of fragment sizes in the file which was analysed.

In many cases this will produce a simple graph showing a peak only at one size, but for variable length FastQ files this will show the relative amounts of each different size of sequence fragment.

3.8 Sequence Duplication Levels



In a diverse library most sequences will occur only once in the final set. A low level of duplication may indicate a very high level of coverage of the target sequence, but a high level of duplication is more likely to indicate some kind of enrichment bias (eg PCR over amplification).

This module counts the degree of duplication for every sequence in a library and creates a plot showing the relative number of sequences with different degrees of duplication.

To cut down on the memory requirements for this module only sequences which first appear in the first 100,000 sequences in each file are analysed, but this should be enough to get a good impression for the duplication levels in the whole file. Each sequence is tracked to the end of the file to give a representative count of the overall duplication level. To cut down on the amount of information in the final plot any sequences with more than 10 duplicates are placed into grouped bins to give a clear impression of the overall duplication level without having to show each individual duplication value.

Because the duplication detection requires an exact sequence match over the whole length of the

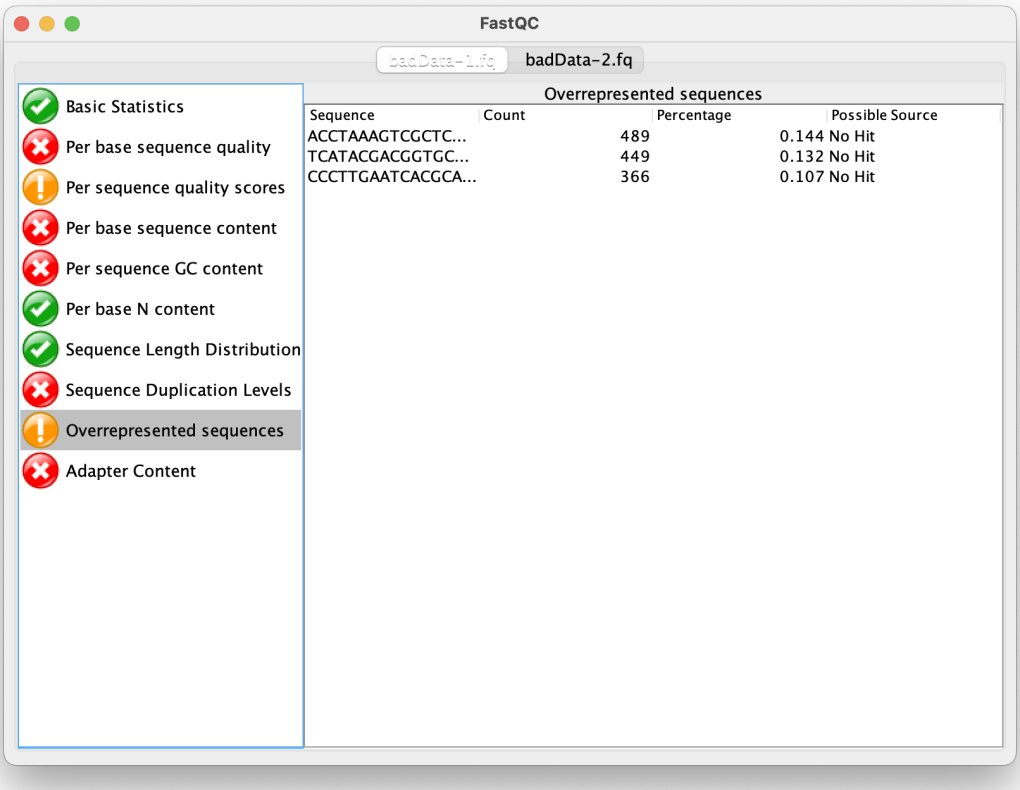
sequence, any reads over 75bp in length are truncated to 50bp for the purposes of this analysis. Even so, longer reads are more likely to contain sequencing errors which will artificially increase the observed diversity and will tend to underrepresent highly duplicated sequences.

The plot shows the proportion of the library which is made up of sequences in each of the different duplication level bins. There are two lines on the plot. The blue line takes the full sequence set and shows how its duplication levels are distributed. In the red plot the sequences are de-duplicated and the proportions shown are the proportions of the deduplicated set which come from different duplication levels in the original data.

In a properly diverse library most sequences should fall into the far left of the plot in both the red and blue lines. A general level of enrichment, indicating broad oversequencing in the library will tend to flatten the lines, lowering the low end and generally raising other categories. More specific enrichments of subsets, or the presence of low complexity contaminants will tend to produce spikes towards the right of the plot. These high duplication peaks will most often appear in the blue trace as they make up a high proportion of the original library, but usually disappear in the red trace as they make up an insignificant proportion of the deduplicated set. If peaks persist in the blue trace then this suggests that there are a large number of different highly duplicated sequences which might indicate either a contaminant set or a very severe technical duplication.

The module also calculates an expected overall loss of sequence were the library to be deduplicated. This headline figure is shown at the top of the plot and gives a reasonable impression of the potential overall level of loss.

3.9 Overrepresented Sequences



A normal high-throughput library will contain a diverse set of sequences, with no individual sequence making up a tiny fraction of the whole. Finding that a single sequence is very overrepresented in the set either means that it is highly biologically significant, or indicates that the library is contaminated, or not as diverse as you expected.

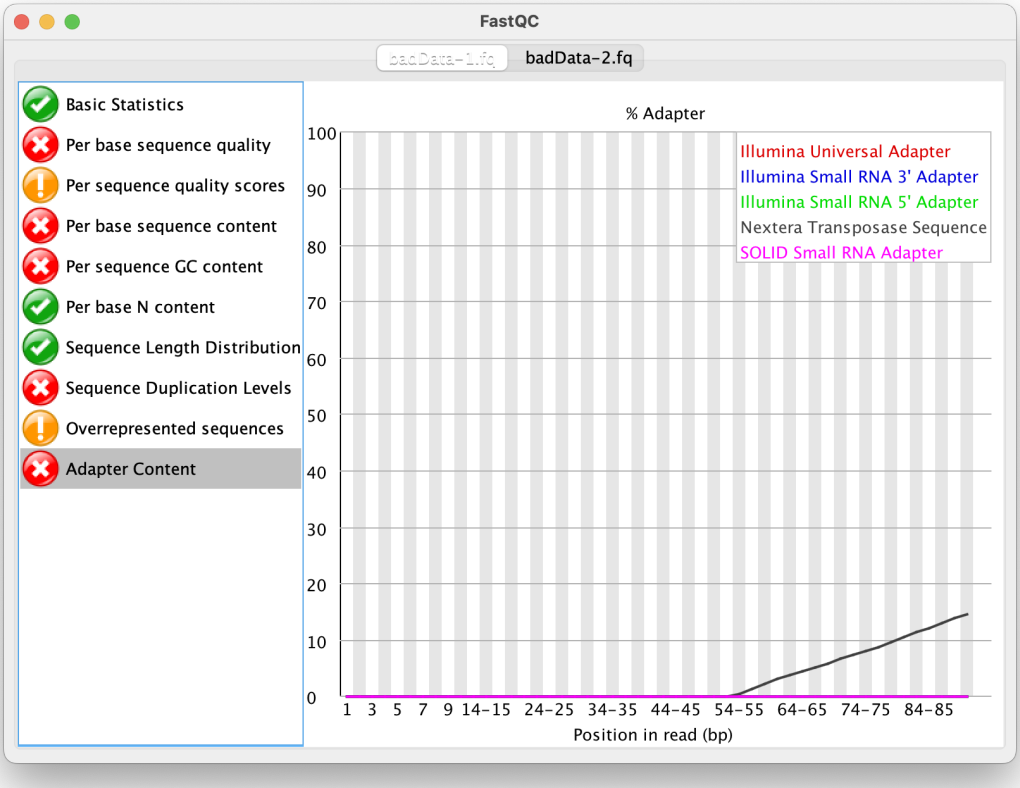
This module lists all of the sequence which make up more than 0.1

For each overrepresented sequence the program will look for matches in a database of common contaminants and will report the best hit it finds. Hits must be at least 20bp in length and have no more than 1 mismatch. Finding a hit doesn't necessarily mean that this is the source of the contamination, but may point you in the right direction. It's also worth pointing out that many adapter sequences are very similar to each other so you may get a hit reported which isn't technically correct, but which has very similar sequence to the actual match.

Because the duplication detection requires an exact sequence match over the whole length of the sequence any reads over 75bp in length are truncated to 50bp for the purposes of this analysis.

Even so, longer reads are more likely to contain sequencing errors which will artificially increase the observed diversity and will tend to underrepresent highly duplicated sequences.

3.10 Adapter Content



The Kmer Content module will do a generic analysis of all of the Kmers in your library to find those which do not have even coverage through the length of your reads. This can find a number of different sources of bias in the library which can include the presence of read-through adapter sequences building up on the end of your sequences.

You can however find that the presence of any overrepresented sequences in your library (such as adapter dimers) will cause the Kmer plot to be dominated by the Kmers these sequences contain, and that it's not always easy to see if there are other biases present in which you might be interested.

One obvious class of sequences which you might want to analyse are adapter sequences. It is useful to know if your library contains a significant amount of adapter in order to be able to assess whether

you need to adapter trim or not. Although the Kmer analysis can theoretically spot this kind of contamination it isn't always clear. This module therefore does a specific search for a set of separately defined Kmers and will give you a view of the total proportion of your library which contain these Kmers. A results trace will always be generated for all of the sequences present in the adapter config file so you can see the adapter content of your library, even if it's low.

The plot itself shows a cumulative percentage count of the proportion of your library which has seen each of the adapter sequences at each position. Once a sequence has been seen in a read it is counted as being present right through to the end of the read so the percentages you see will only increase as the read length goes on.

Now open the goodData-1.fq and goodData-2.fq and compare the results.

As we can see, in the bad data the quality of the reads drops at 3' ends and needs trimming of these regions.

We use "trim_galore" to remove adaptors, to trim low quality reads and to remove short sequences. Internally it uses another program called cutadapt. It will work on compressed and uncompressed fastq files. To run it

```
 cd ~/Sreenu/FQs

trim_galore -q 15 --length 60 --paired badData-1.fq badData-2.fq
```

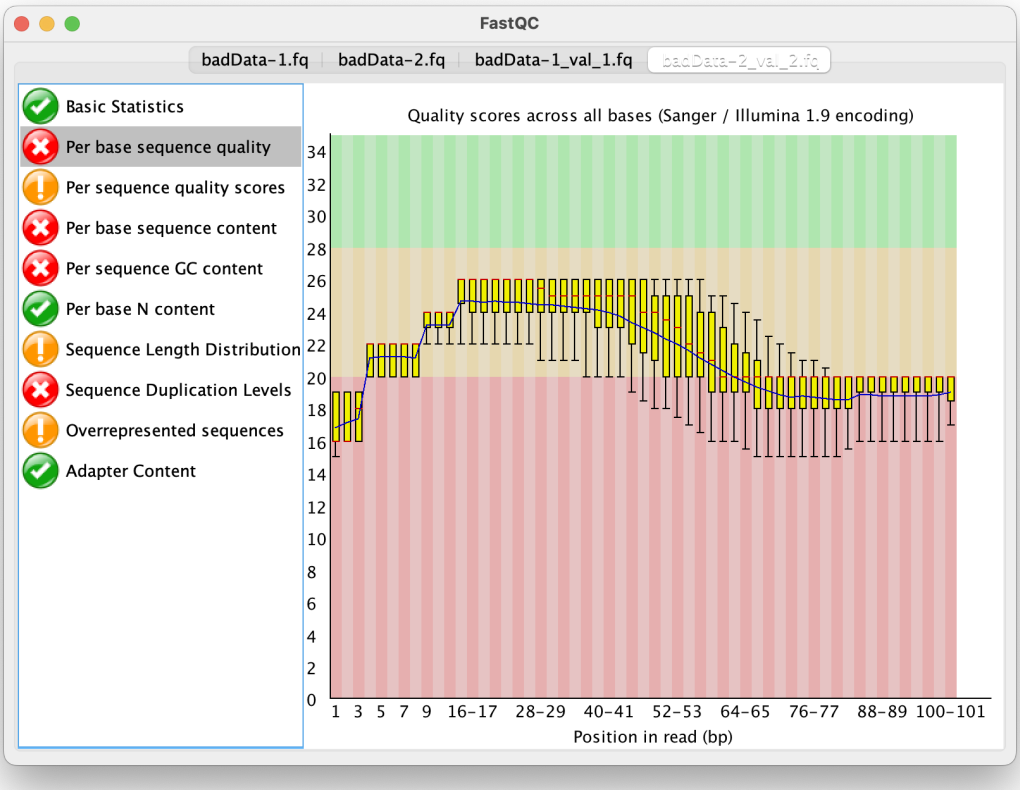
This trims reads with phred quality less than 15 and length shorter than 60bp. By default, it also detects commonly used adaptors and removes them if present. If special adaptor sequences are used in sequencing, provide the adaptor's sequence with "-a" option to remove.

After trimming, for each fastq file it generates summary files (badData-1.fq_trimming_report.txt and badData-2.fq_trimming_report.txt) and validated reads files (badData-1_val_1.fq and badData-2_val_2.fq)*.

(* These file names may vary based on the program version)

Recheck the cleaned data using fastqc. Did you see any change in the quality after trimming (do you see any difference at all?)

Here is the per base sequence quality



There is some improvement on the per base quality, though it is not a through cleaning. It is a good practice to clean the data before we use it for any downstream analysis.

Now use "prinseq" and clean data. Check if you see any differences.

```
prinseq-lite.pl -fastq badData-1.fq -fastq2 badData-2.fq -out_format 3 -min_len 60 -min_qual_score
15
```

Here, files with `prinseq_good` will store all the reads that pass the quality filter and `prinseq_bad` will store all the failed entries. These file names will be appeended with random strings to not to overwrite.