Quality control of Illumina data

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Quality control of Illumina data

- Primary analysis
- Quality control
- Data pre-processing

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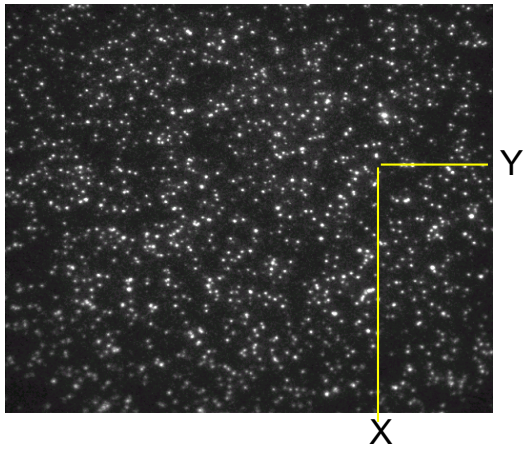
Primary analysis

Images

Image analysis

Intensities



- Determination of cluster position (only for non-patterned flow cells)
- Extraction of intensities for each cluster

Primary analysis

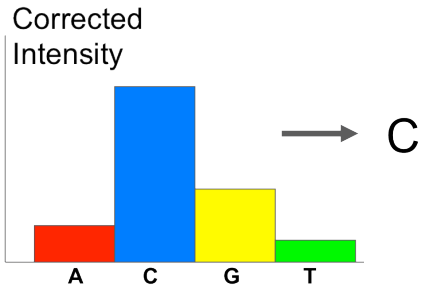
Images

Image analysis

Intensities

Base calling

Sequences + qualities



- Intensity correction
  - Take into account ≠ intensities per molecule for the 4 bases
- Call the base with the maximum intensity
- Determine “Passing filter” clusters
  - Remove clusters that have “too much” intensity corresponding to bases other than the called base

Phred quality scores

- Prediction of the probability of error in base calling

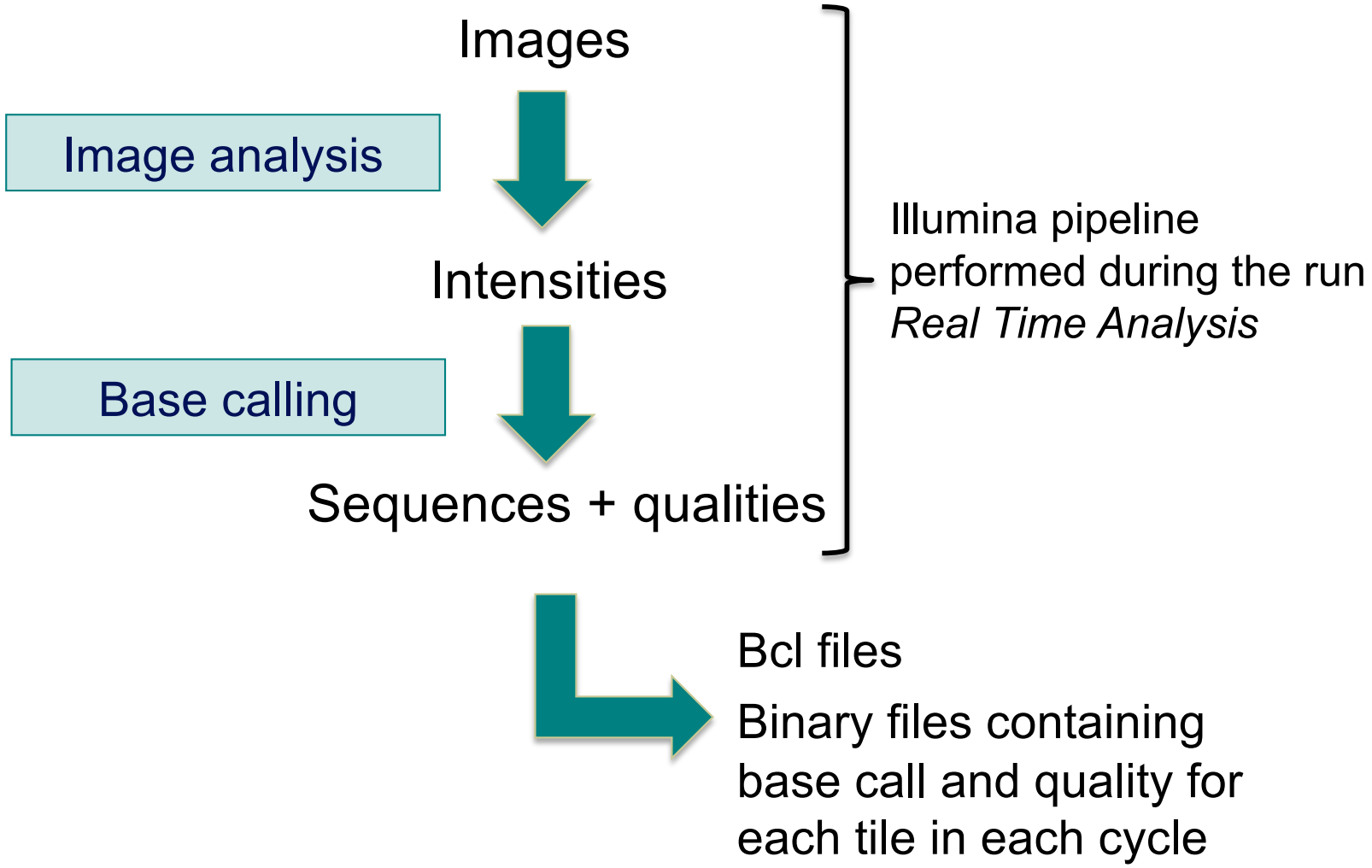
$$Q = -10 \log_{10} P$$

$Q : quality\ score$

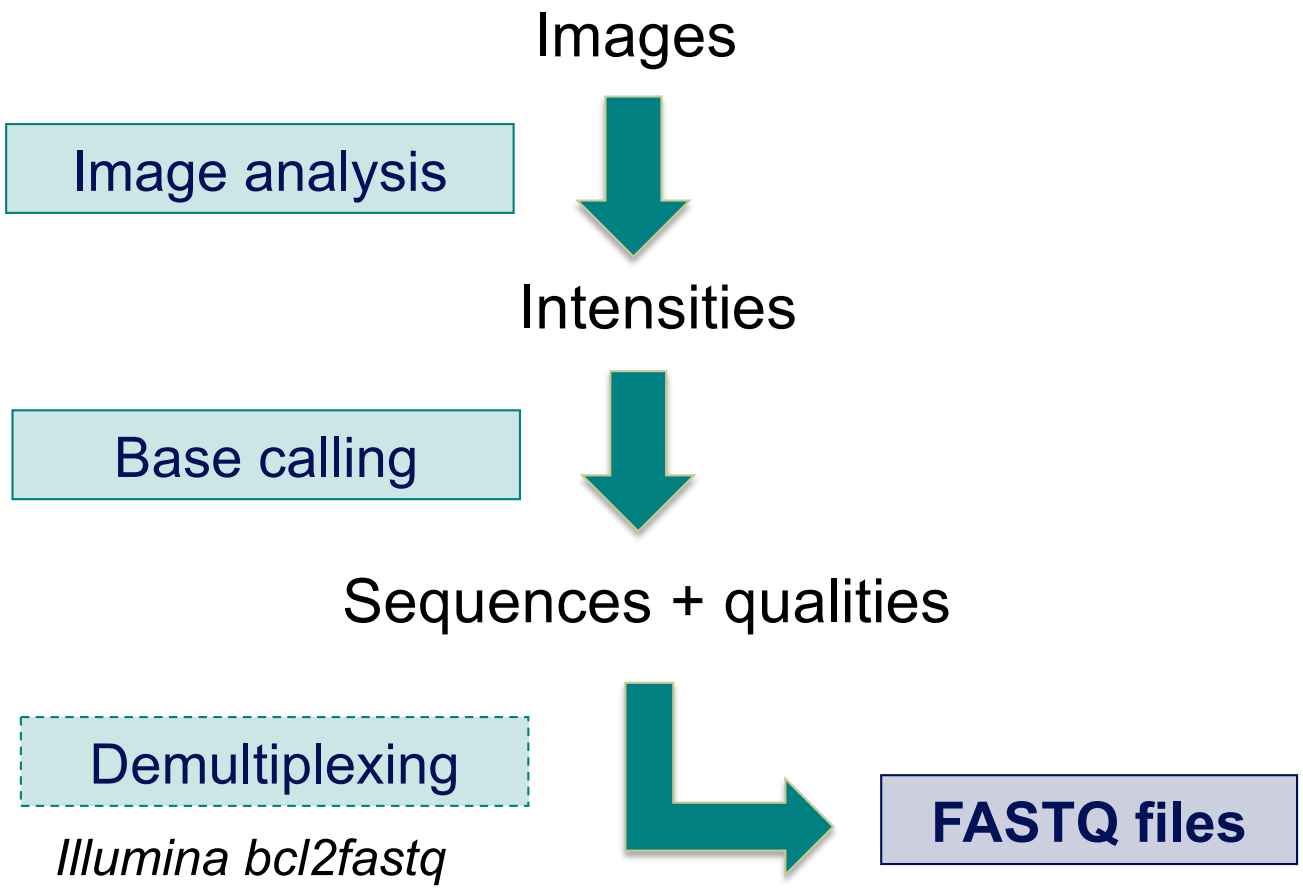
$P : error\ probability$

Quality Score	Error Probability
Q40	0.0001 (1 in 10,000)
Q30	0.001 (1 in 1,000)
Q20	0.01 (1 in 100)
Q10	0.1 (1 in 10)

Primary analysis



Primary analysis



FASTQ file

- Text file containing
  - Sequences
  - Qualities

    Probability that the corresponding base call is incorrect

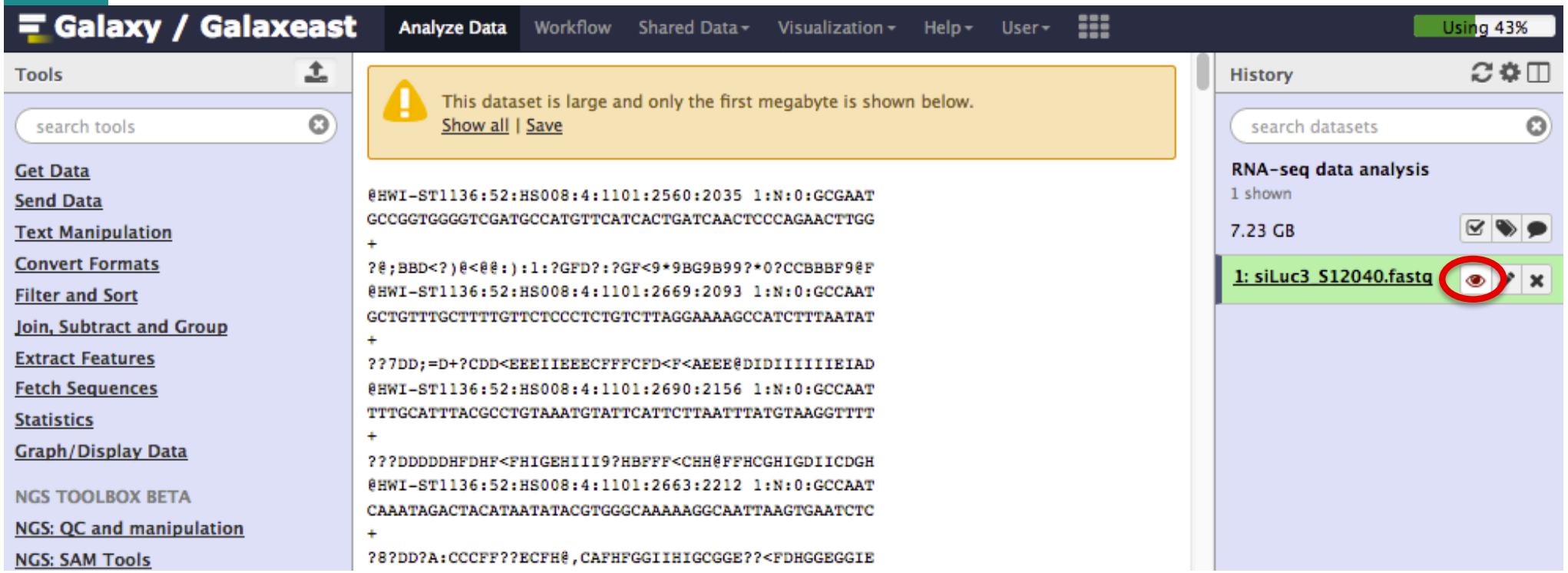
```
@HWI-ST1136:97:HS041:7:1101:1681:2104 1:N:0:ACAGTG
CTTTTTTATTGAATTCTATGATTCTTGTTAGATTTCATAATGGCTGCTTA
+
@@@DBDDDFF8:D?EBAEAH,CF:AF9F+2**9?B?1C<<?9*8D?)9*?
@HWI-ST1136:97:HS041:7:1101:1521:2119 1:N:0:ACAGTG
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
+
@@@?DDDDFAFHHIIHFDAB@B6@B@BBBBBBBBBBBBB359BBBB8BBB
@HWI-ST1136:97:HS041:7:1101:1669:2145 1:N:0:ACAGTG
CTGCTGTTTTCCAAATGTCCGATGTGTGCTATGACTGACAACTACTTTTC
+
@@<1?DDDFDBFDFE>+<CCF>FAG++2+<<F**:?1:C?:8B:9BBBD4
```

4 lines per sequence :

1. @Identifier
2. Sequence
3. \+ optionally followed by same identifier as 1.
4. Quality

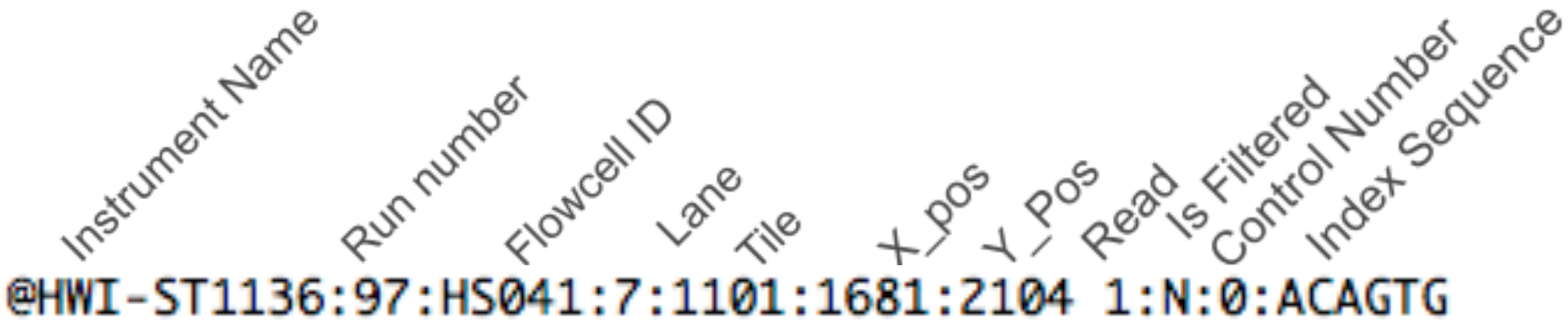
(Cock et al. NAR 2009; 38(1): 1767-1771)

Beginning of siLuc3_S12040.fastq file



Sequence identifier in FASTQ files

- Begins with @
  followed by sequence ID and an optional description
- Illumina sequence identifiers :



- Read :

The member of a pair = 1 or 2 (for paired-end or mate-pair reads)

- Is filtered

Y if the read is bad (the cluster do not pass filter), N otherwise

Recent versions of Illumina pipeline only supply passing filter reads

Quality in FASTQ files

- Phred quality score (Sanger format)
- Encoded in ASCII characters to save space
- 1 ASCII symbol = 1 quality value
- Phred quality scores from 0 to 93
  are encoded using ASCII 33 to 126 :

032	sp	048	0	064	@	080	P	096	`	112	p
033	!	049	1	065	A	081	Q	097	a	113	q
034	"	050	2	066	B	082	R	098	b	114	r
035	#	051	3	067	C	083	S	099	c	115	s
036	$	052	4	068	D	084	T	100	d	116	t
037	%	053	5	069	E	085	U	101	e	117	u
038	&	054	6	070	F	086	V	102	f	118	v
039	'	055	7	071	G	087	W	103	g	119	w
040	(	056	8	072	H	088	X	104	h	120	x
041	)	057	9	073	I	089	Y	105	i	121	y
042	*	058	:	074	J	090	Z	106	j	122	z
043	+	059	;	075	K	091	[	107	k	123	{
044	,	060	<	076	L	092	\	108	l	124	|
045	-	061	=	077	M	093	]	109	m	125	}
046	.	062	>	078	N	094	^	110	n	126	~
047	/	063	?	079	O	095	_	111	o	127	⌂

- Binned in order to save space in the last versions of Illumina software, e.g.
  - 2 < real Q-score < 9 → binned Q-score = 6
  - 10 < real Q-score < 19 → binned Q-score = 15
  - …
  - real Q-score ≥ 40 → binned Q-score = 40

Paired-end FASTQ files

- 2 FASTQ files per sample

XXXX.R1.fastq.gz

```
@HWI-ST1136:163:HS087:7:2310:17264:70630 1:N:0:ATCACG
GTTAGAGGCCAAGGTACAGTGGCCTGTCTTTGTAAATGTGCCTTTATGT
+
CCCFFFFFHHHHHJFHHIJHIJIJJIIJJJJIHHIJIIGIIJIJJJJJI
@HWI-ST1136:163:HS087:7:2310:17415:70636 1:N:0:ATCACG
TGGAGCCTTGGTAACTTTTTGTAGTTTGTATGCGTTTTTGTTTGGTCTC
+
BCCFFFFFHHHHHIJJJJJJJJHIJJJJJIJJJJIIJJJJJIJJJJFGIJ
@HWI-ST1136:163:HS087:7:2310:17337:70637 1:N:0:ATCACG
CTGTTACCCCTCCATTCAGGGGTATGAAGAAGGGCTTCACCTGTAGTTC
+
@CCFFFFFHHHHHJJIJJJJJJJBFHIJJIJJIIIIJIJIJJHHGHIJJJ
```

XXXX.R2.fastq.gz

```
@HWI-ST1136:163:HS087:7:2310:17264:70630 2:N:0:ATCACG
TAATTTTTTGCATCCTGAAAAGTGTTGGAAGTTGGGTTTTCATAGTCAA
+
CCCFFFFFHHHHHJJJIJJJJJICHGIIJIJJIIJIJFHIIJHIJJJIJJ
@HWI-ST1136:163:HS087:7:2310:17415:70636 2:N:0:ATCACG
TGTTCATATGTATGAGATAGATTTGAAAAATCTACTAATTTTTAAAATC
+
CCCFFFFFHHHHHJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJJ
@HWI-ST1136:163:HS087:7:2310:17337:70637 2:N:0:ATCACG
TCCTGACATCAAGCACACTGCTTCTGCATCTATGTGGCACCTAAAACAA
+
CCCFFFFFHHHHHJJIIJJJJJJJJJFIIIJJJJJIJIJIIJIJJJJJJJ
```

Quality control of Illumina data

- Primary analysis
- Quality control
- Data pre-processing

Quality control

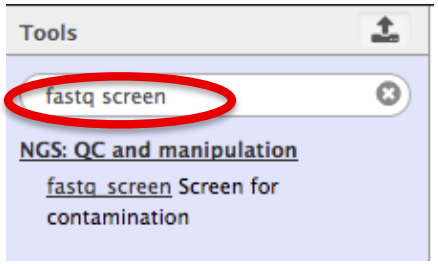
- Why ?
  - Are the data consistent to what is expected ?
  - Are the data suited to answer my biological questions ?
    With what limitations ?
  - ➔ Identify any problems of which you should be aware before doing any further analysis

- What to look for ?
  - Number of reads
  - Base qualities and N calls
  - Base composition relative to reference genome
  - Sequence duplication
  - Presence of adapters
  - Contaminations

Some quality control tools

- FastQC
  - https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
- RSeQC – quality controls specific to RNAseq data
  - http://rseqc.sourceforge.net/
- FastQ Screen – to verify the composition of a library and search for possible contaminations
  - https://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/

FastQ Screen on GalaxEast



fastq_screen Screen for contamination (Galaxy Version 0.4.2) ▾ Options

Job narrative (included in output names as a reminder)

fastq_screen

Only letters, numbers and underscores _ will be retained in this field

Sample this number of reads. Set to 0 or less to use all

500000

Time/precision trade off - fewer reads takes a little less time trading off precision of the estimates.

Single ended or mate-pair ended reads in this library?

Single-end

RNA-Seq FASTQ file

1: siLuc3_S12040.fastq

Nucleotide-space: Must have Sanger-scaled quality values with ASCII offset 33

Installed organism reference sequences to check for alignment to your fastq

1: Installed organism reference sequences to check for alignment to your fastq

Bowtie2 reference genome

hg38

2: Installed organism reference sequences to check for alignment to your fastq

Bowtie2 reference genome

mm10

3: Installed organism reference sequences to check for alignment to your fastq

Bowtie2 reference genome

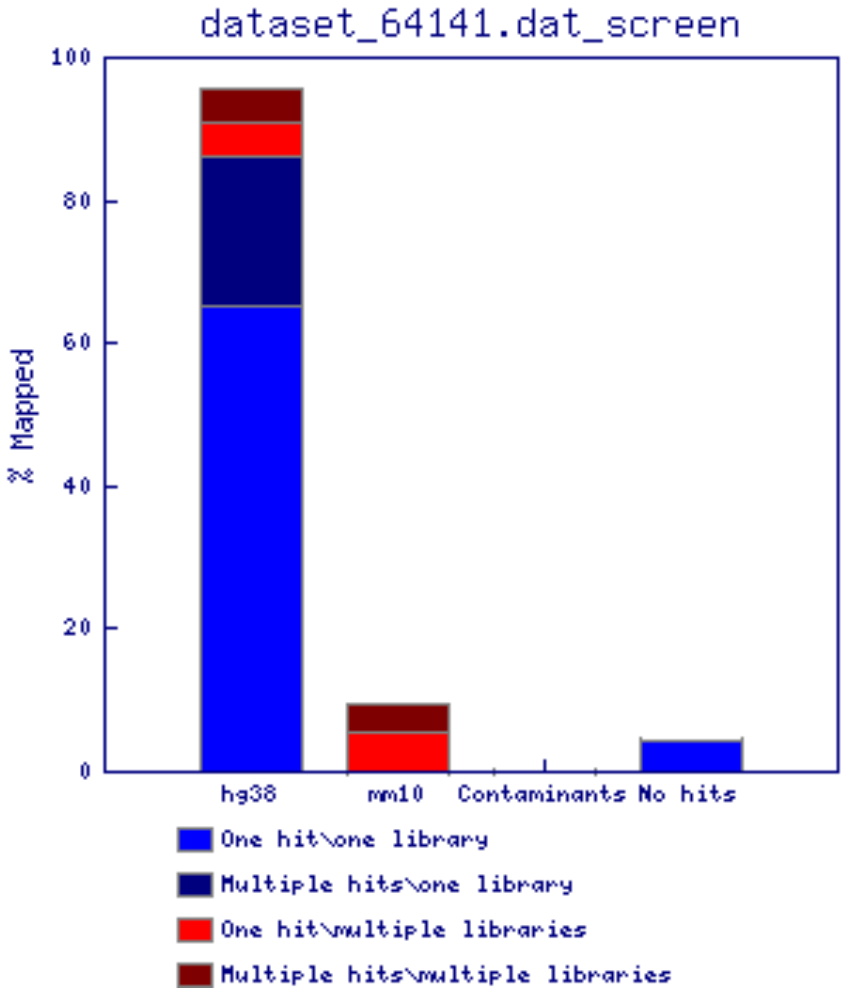
Contaminants

✚ Insert Installed organism reference sequences to check for alignment to your fastq

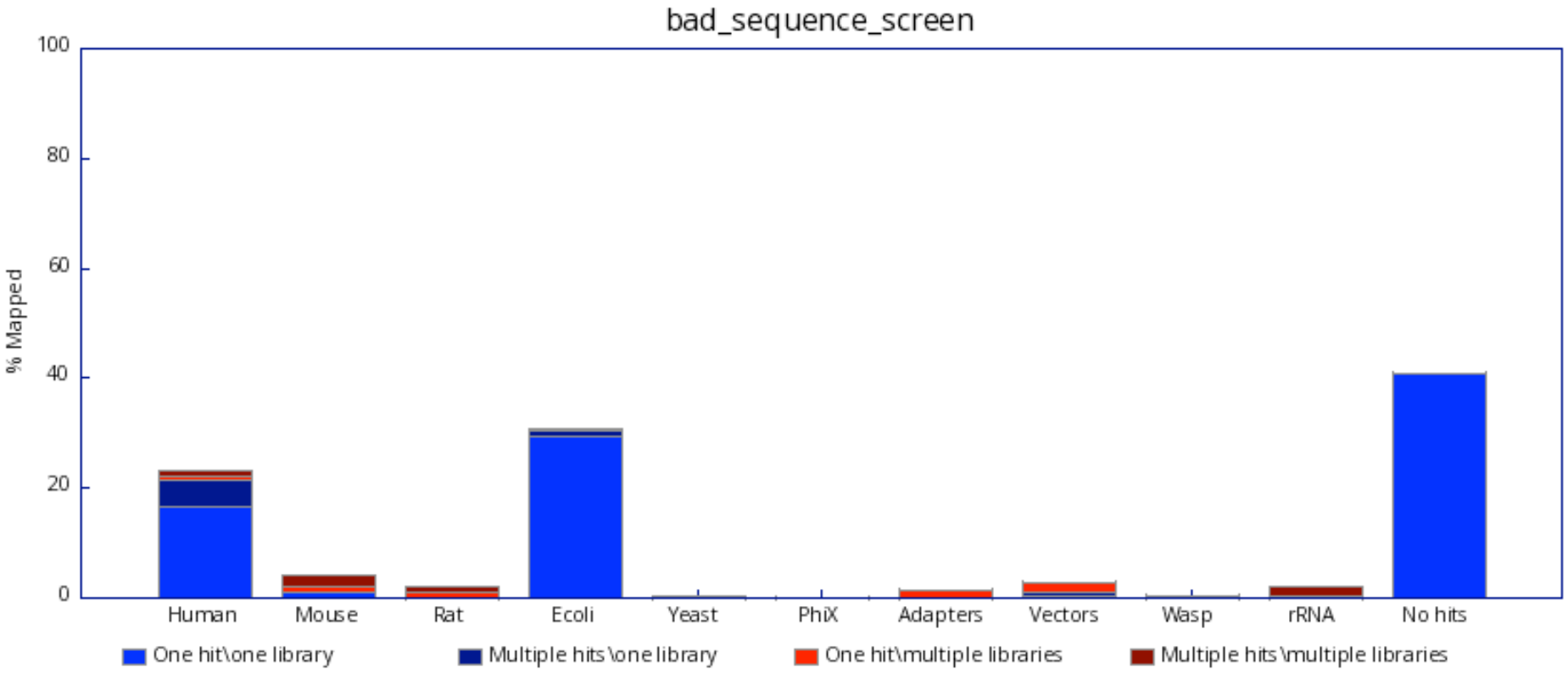
For checking cell culture sequence for contamination, Mycoplasma Genitalium might be a good choice eg

✔ Execute

FastQ Screen result on siLuc3_S12040.fastq



FastQ Screen result on a bad sample



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

FastQC

- Allows quality control of NGS data
  - FASTQ, gzip compressed FASTQ (base or colorspace)
  - SAM, BAM alignment files
- Can be used *via* a graphical interface, in command-line or in Galaxy
- Generates graphs and tables with several quality control analyses

  ➔ Allows a global quality assessment of NGS data and rapid identification of possible problems

Exercise : quality analysis

- Analyse the quality of siLuc3_S12040.fastq file
  - How many reads have been sequenced in this sample ?
  - What do you think about the quality of this sample ?
  - Do you identify bias in these data ?

FastQC results

FastQC Report

ven. 28 mai 2021
siLuc3_S12040.fastq

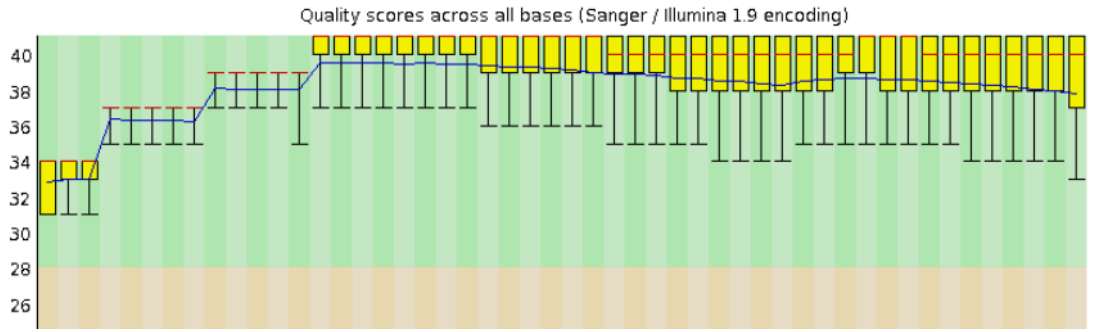
Summary

- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content
- Kmer Content

Basic Statistics

Measure	Value
Filename	siLuc3_S12040.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	50079515
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Per base sequence quality



History

search datasets

INSERM training 2106 RNAseq
3 shown, 7 deleted

9.57 GB

3: FastQC on data 1: Raw Data

2: FastQC on data 1: Webpage

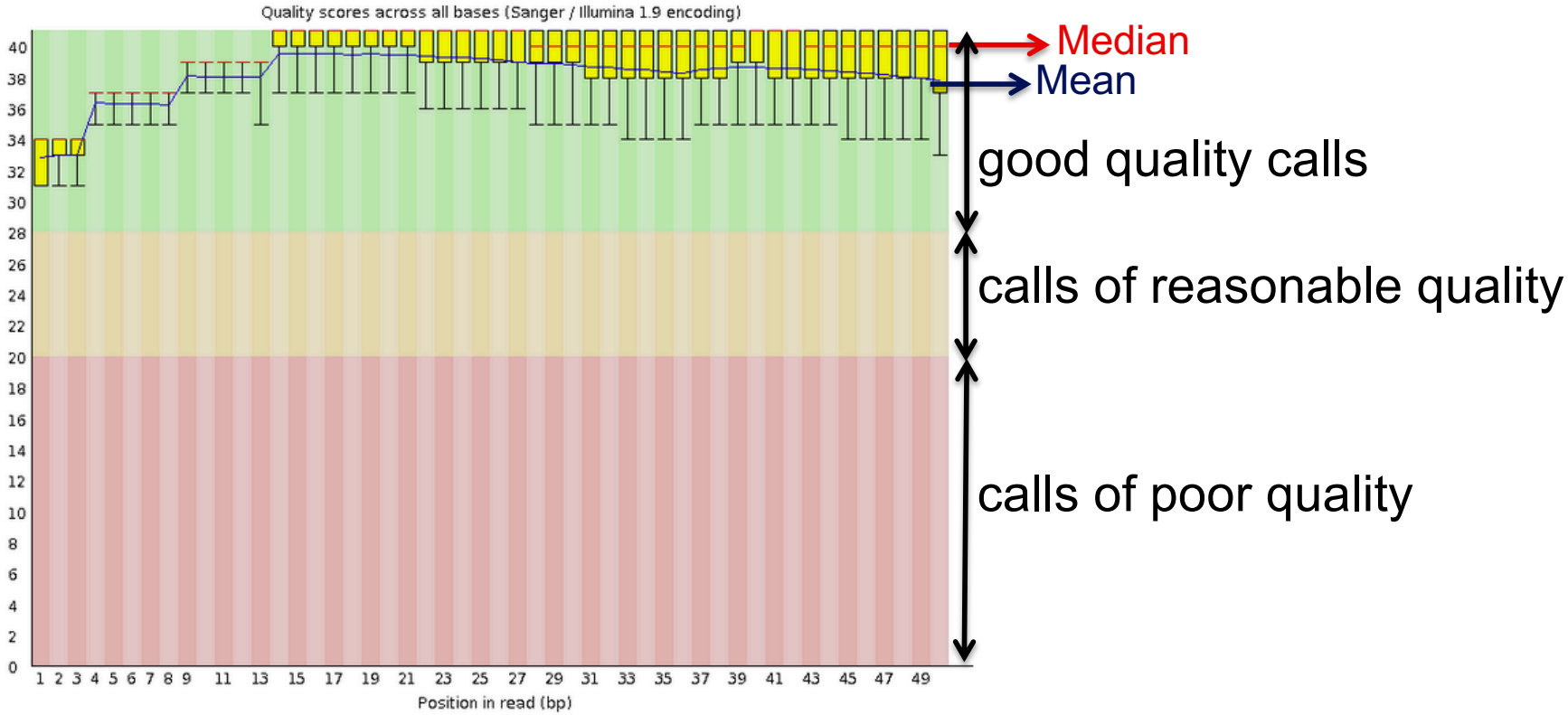
1: siLuc3_S12040.fastq

Basic Statistics

Measure	Value
Filename	siLuc3_S12040.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	50079515
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

- **File type** : Base calls or colorspace data
- **Encoding** : Which ASCII encoding of quality values was found in this file
- **Total Sequences**: A count of the total number of sequences in the file
- **Sequences flagged as poor quality** : Sequences flagged will be removed from all analyses. The total sequences count above will not include these filtered sequences
- **Sequence length**: Length of the shortest and longest sequence
  If all sequences have the same length only one value is reported
- **%GC**: The overall %GC of all bases in all sequences

Per base sequence quality



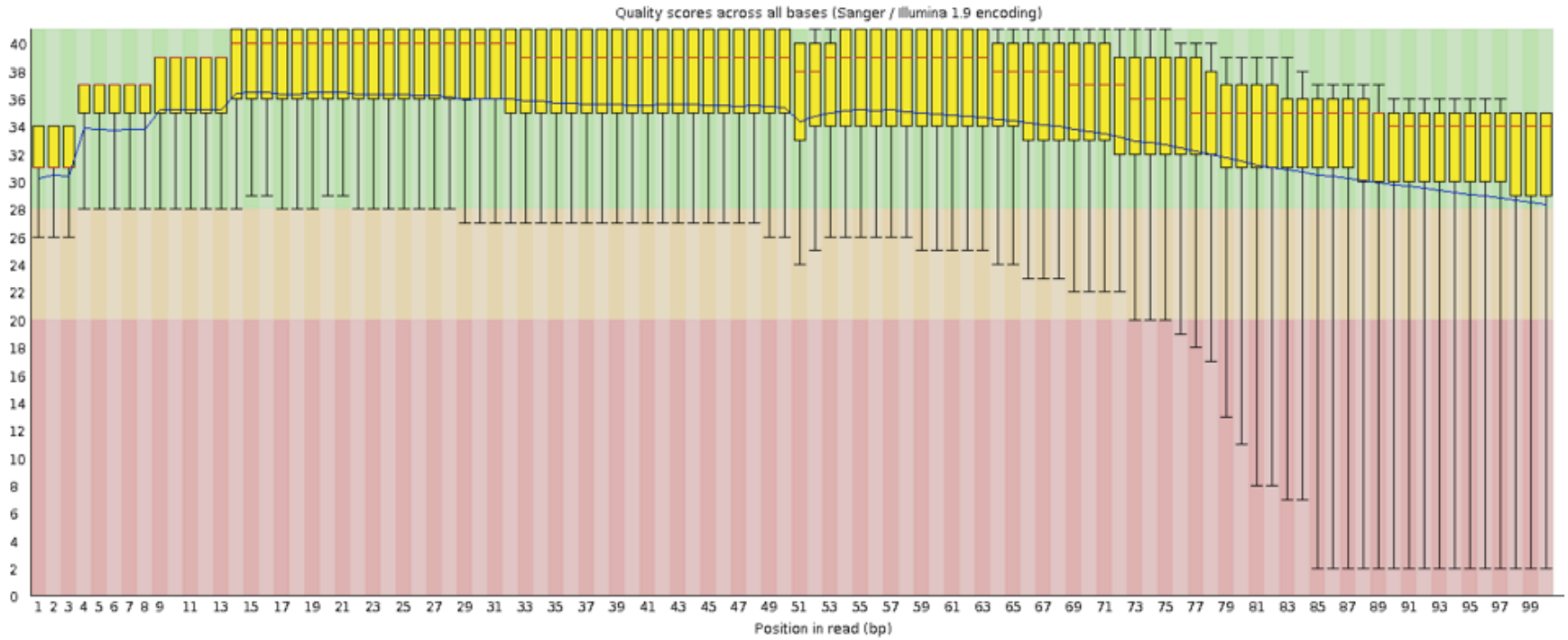
- Yellow boxes : inter-quartile range (25-75%)
- Upper and lower whiskers : 10% and 90%

➔ **Sample of good quality**

Per base sequence quality on another sample

- The quality of calls decreases as the run progress

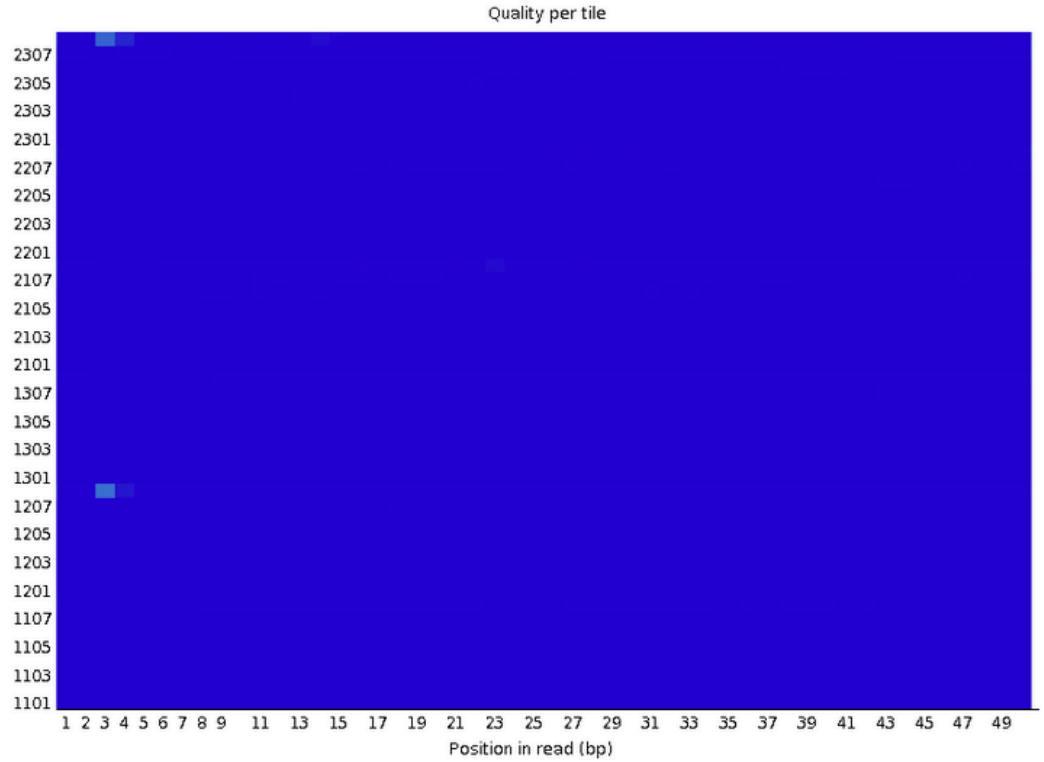
  e.g. 2nd read of a 2x100bp run :



➔In such cases reads can be trimmed

Per tile sequence quality

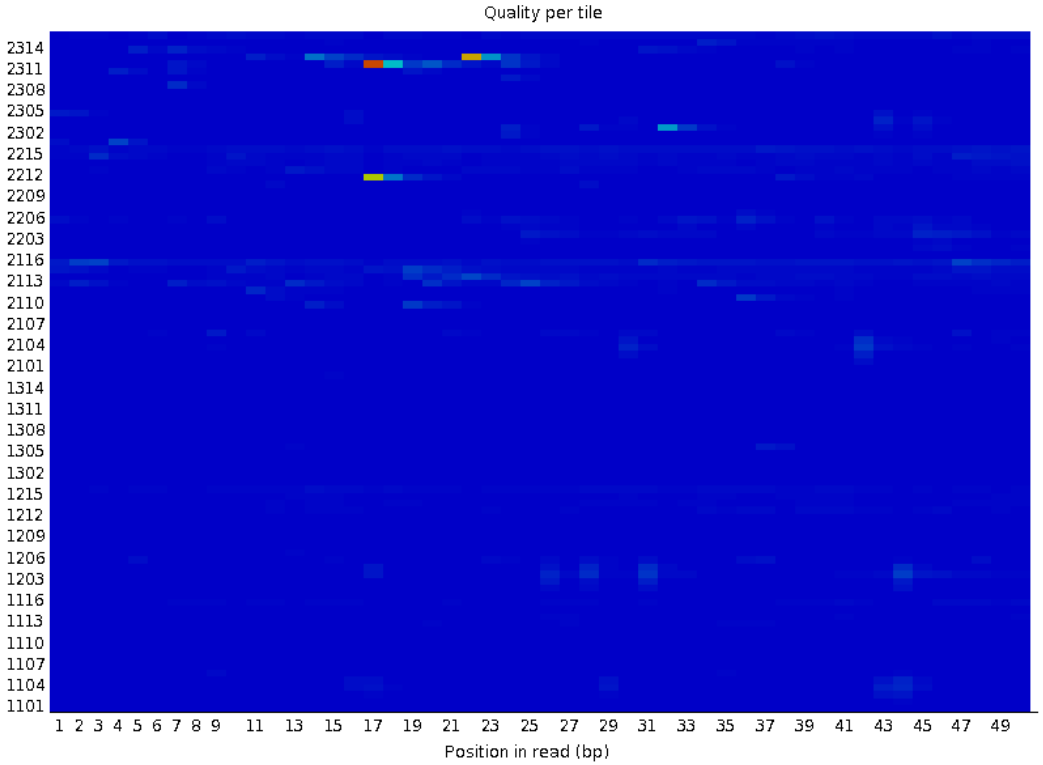
Quality scores from each tile across all bases :
show the deviation from the average quality for each tile



→ To see if there was a loss in quality associated with only one part of the flowcell

→ No poor quality tile for this sample

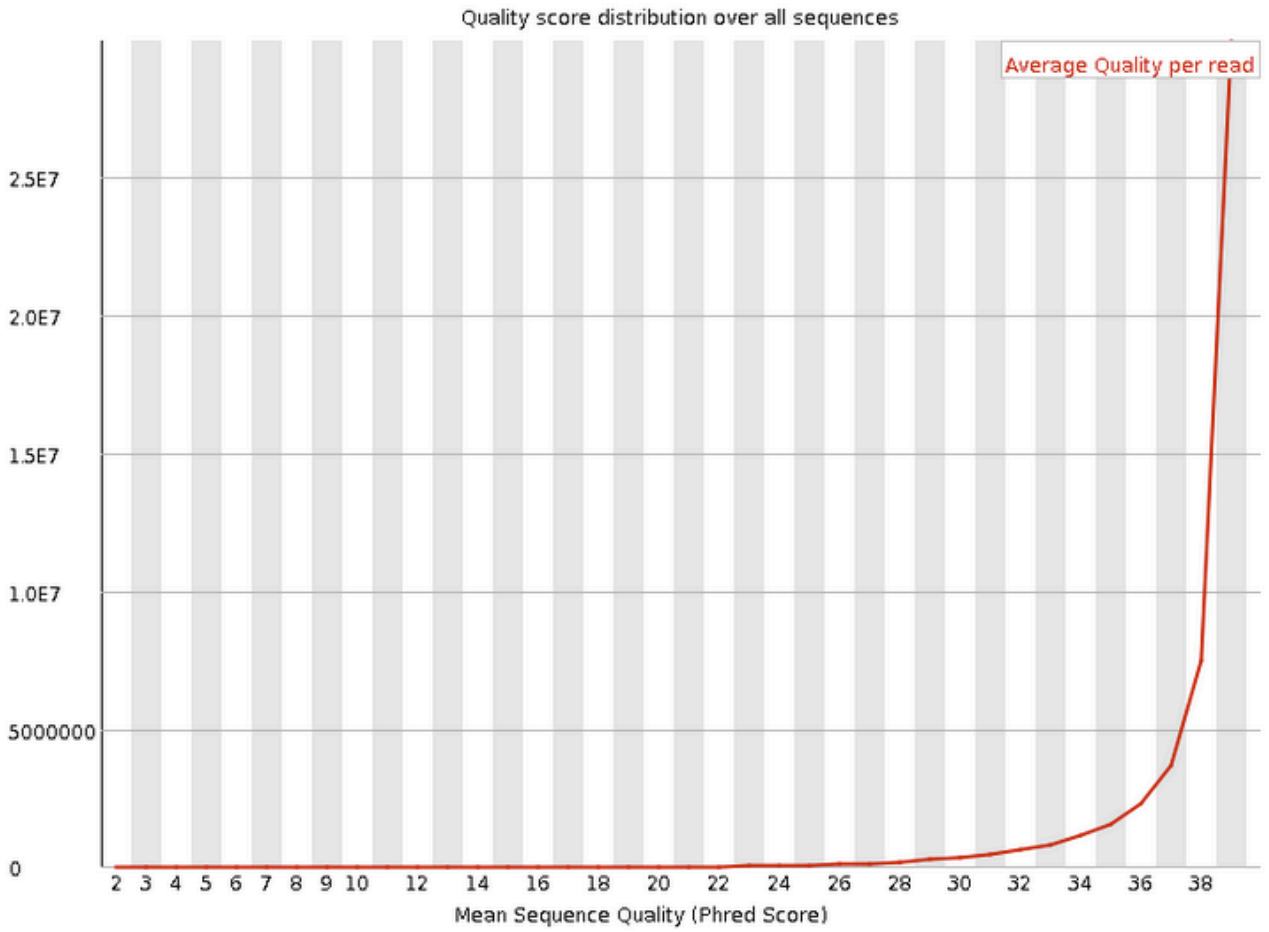
Per tile sequence quality on another sample



- Colours on a cold to hot scale
- Cold colours : positions where the quality was at or above the average for that base in the run
- Hotter colours : a tile had worse qualities than other tiles for that base

→ A good plot should be blue all over

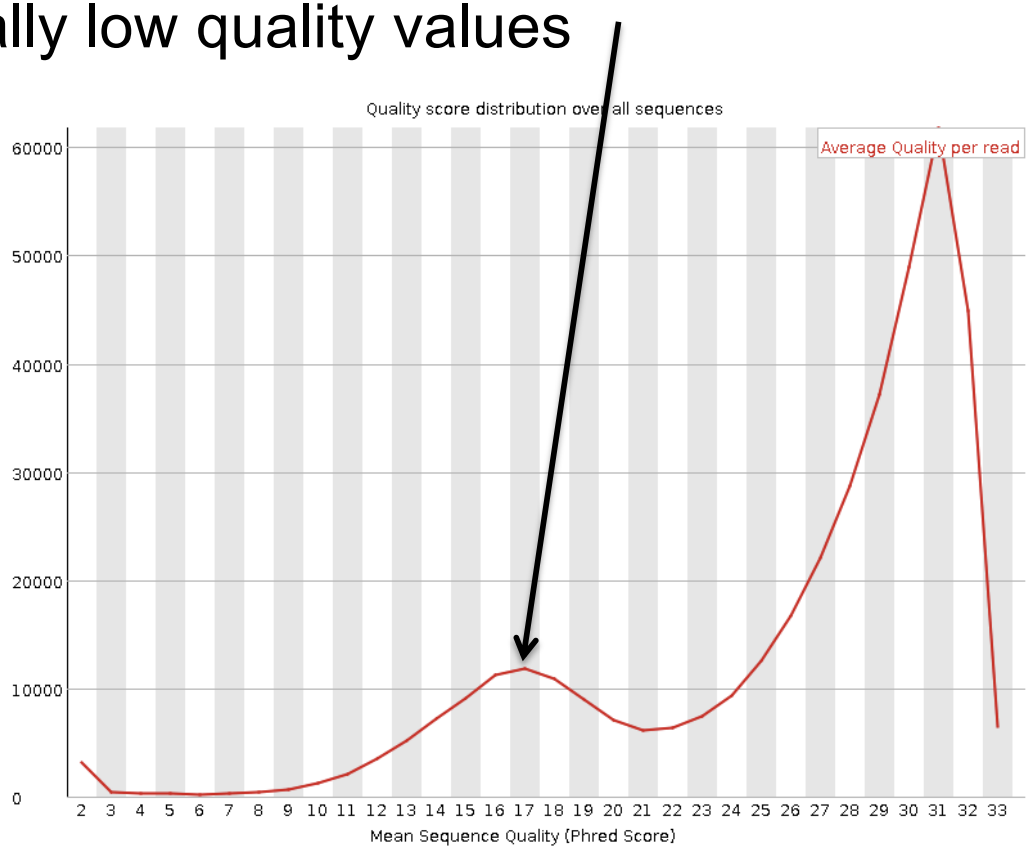
Per sequence quality scores



➔ **Good quality of all sequences**

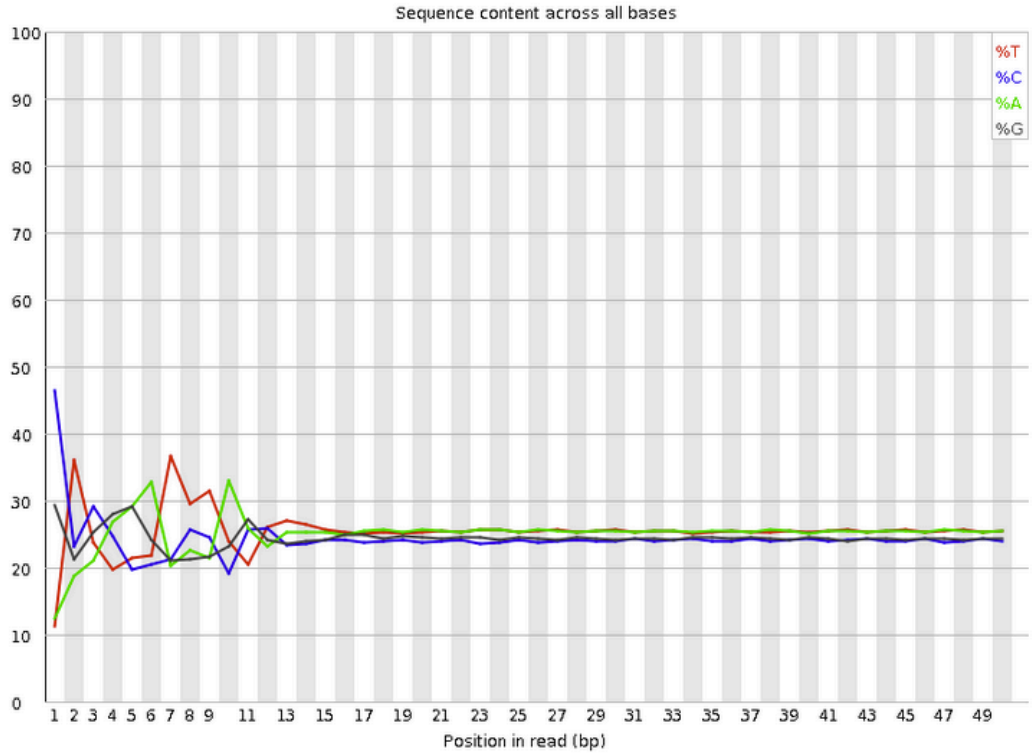
Per sequence quality score on another sample

- Allows you to see if a subset of your sequences have universally low quality values



➔ these should represent only a small percentage of the total sequences

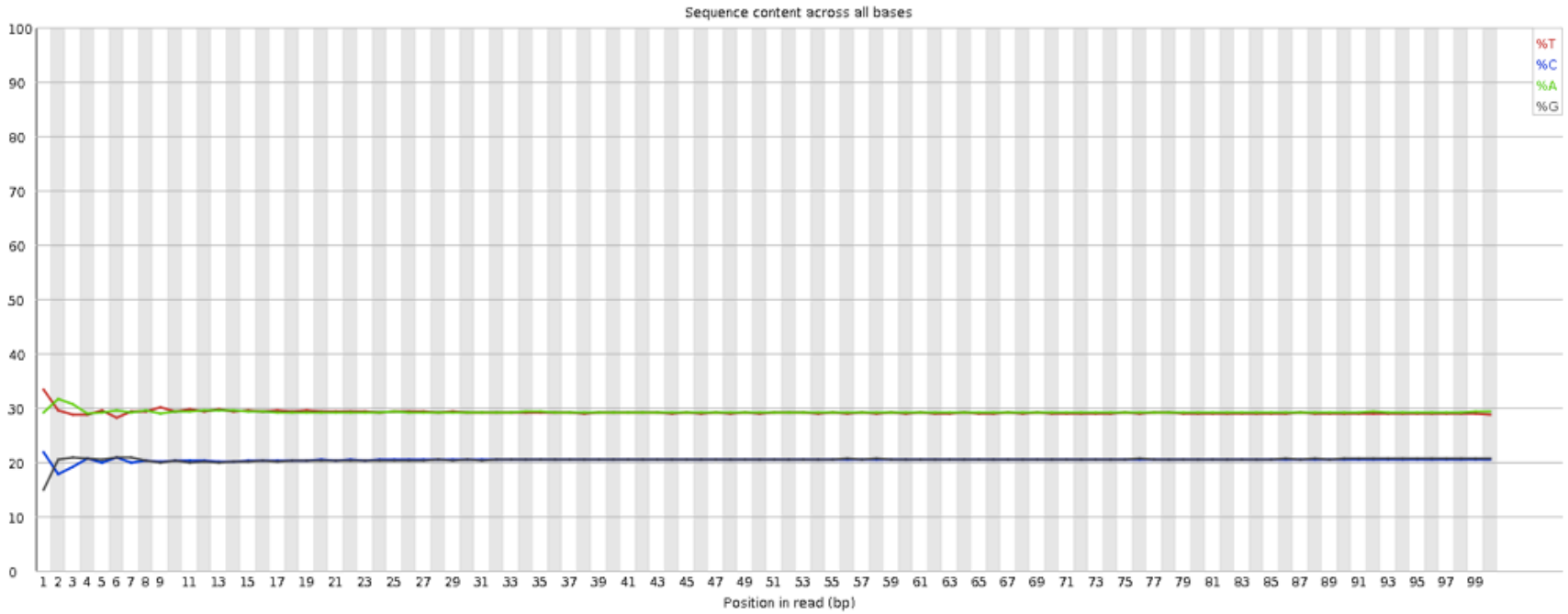
Per base sequence content



- **Known bias in the repartition of the first nt in RNA-seq libraries**
  - Because random primers used during RT are “not so random”
  - “Reproducible bias” ➔ Comparative analyses OK
  - c.f. Hansen et al. 2010;38(12):e131.
    Li et al. Genome Biology 2010;11(5):R50.

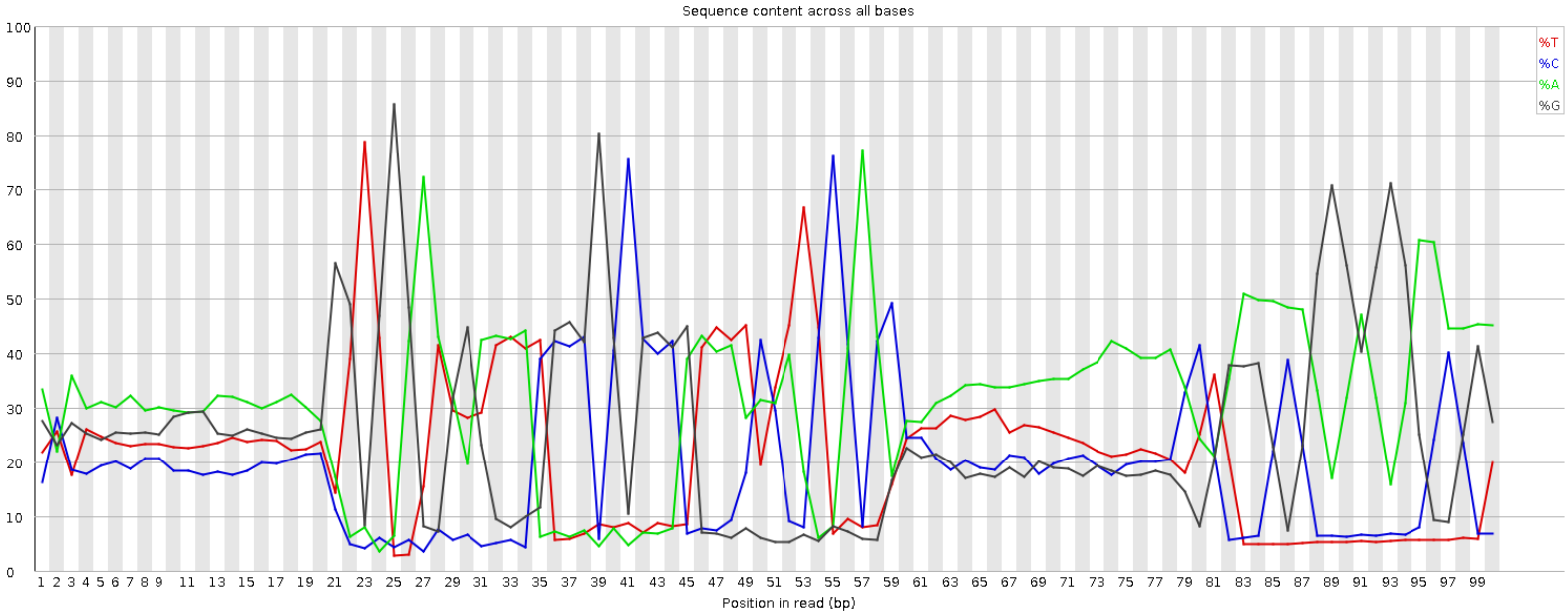
Per base sequence content on other samples

- 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
- Example for a DNAseq sample :



Per base sequence content on other samples

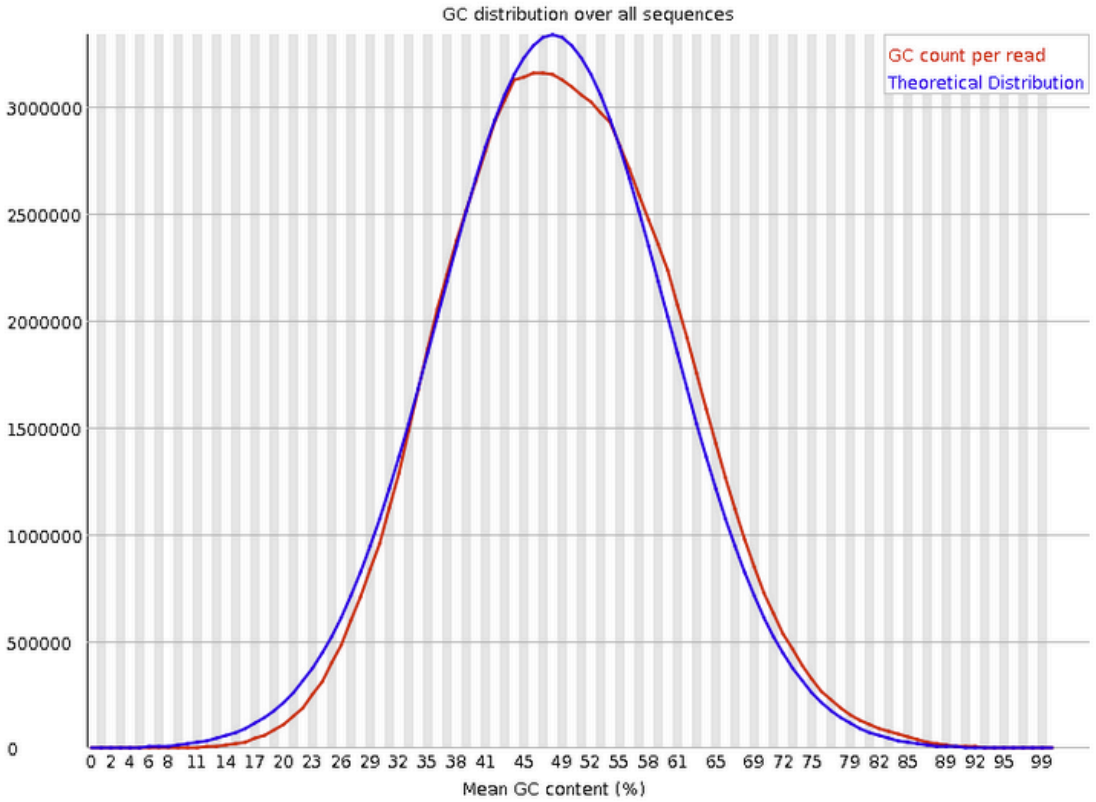
- Strong biases which change in different bases
  - Usually indicates an overrepresented sequence, e.g. adapters :



- Bias which is consistent across all bases
  - indicates that the original library was sequence biased
  - or that there was a systematic problem during sequencing

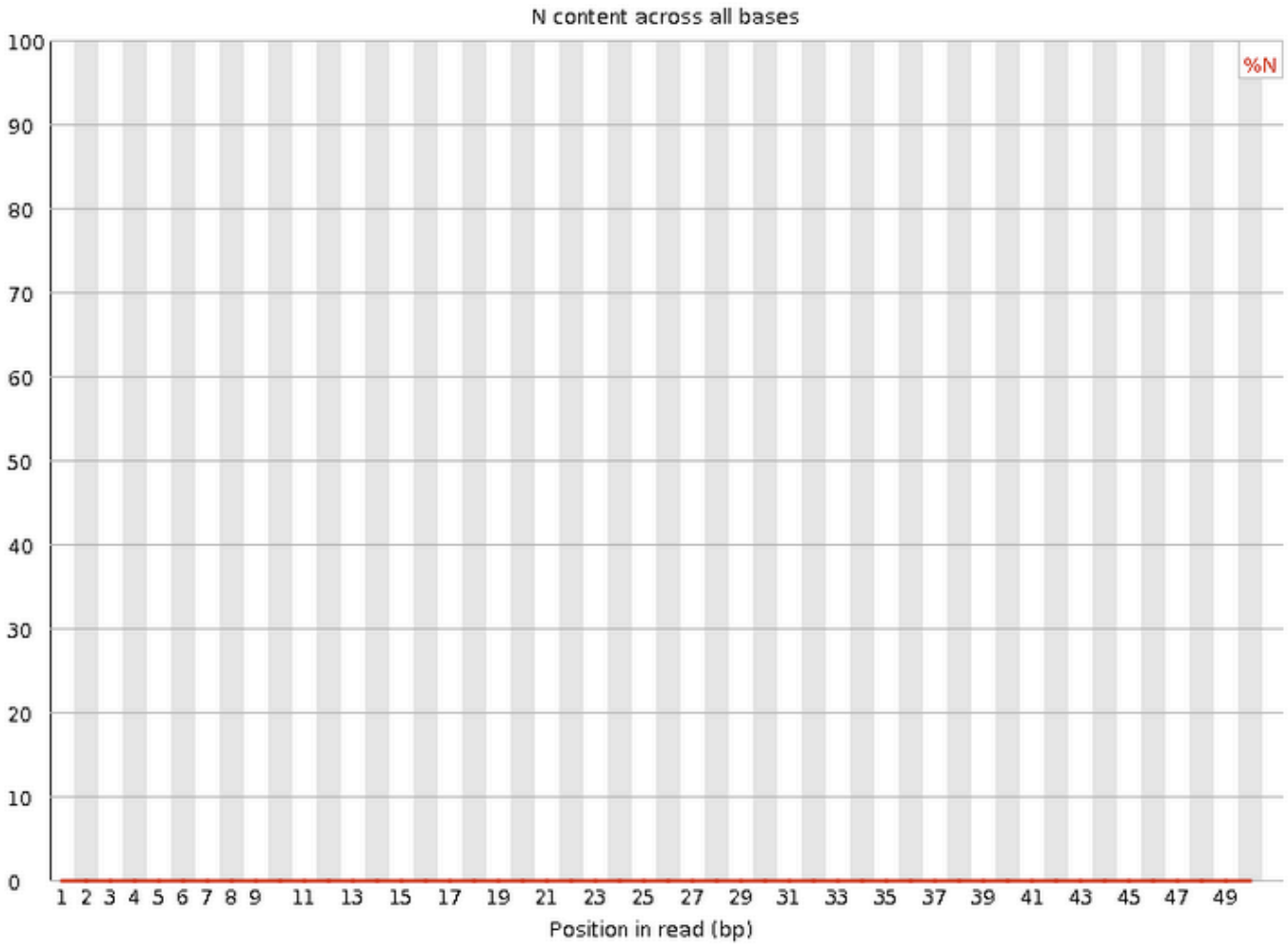
Per sequence GC content

- Compares GC content of all sequences to a modelled normal distribution of GC content (mode calculated from the data and used to build the reference distribution)



➔ **Observed GC distribution similar to the theoretical one**

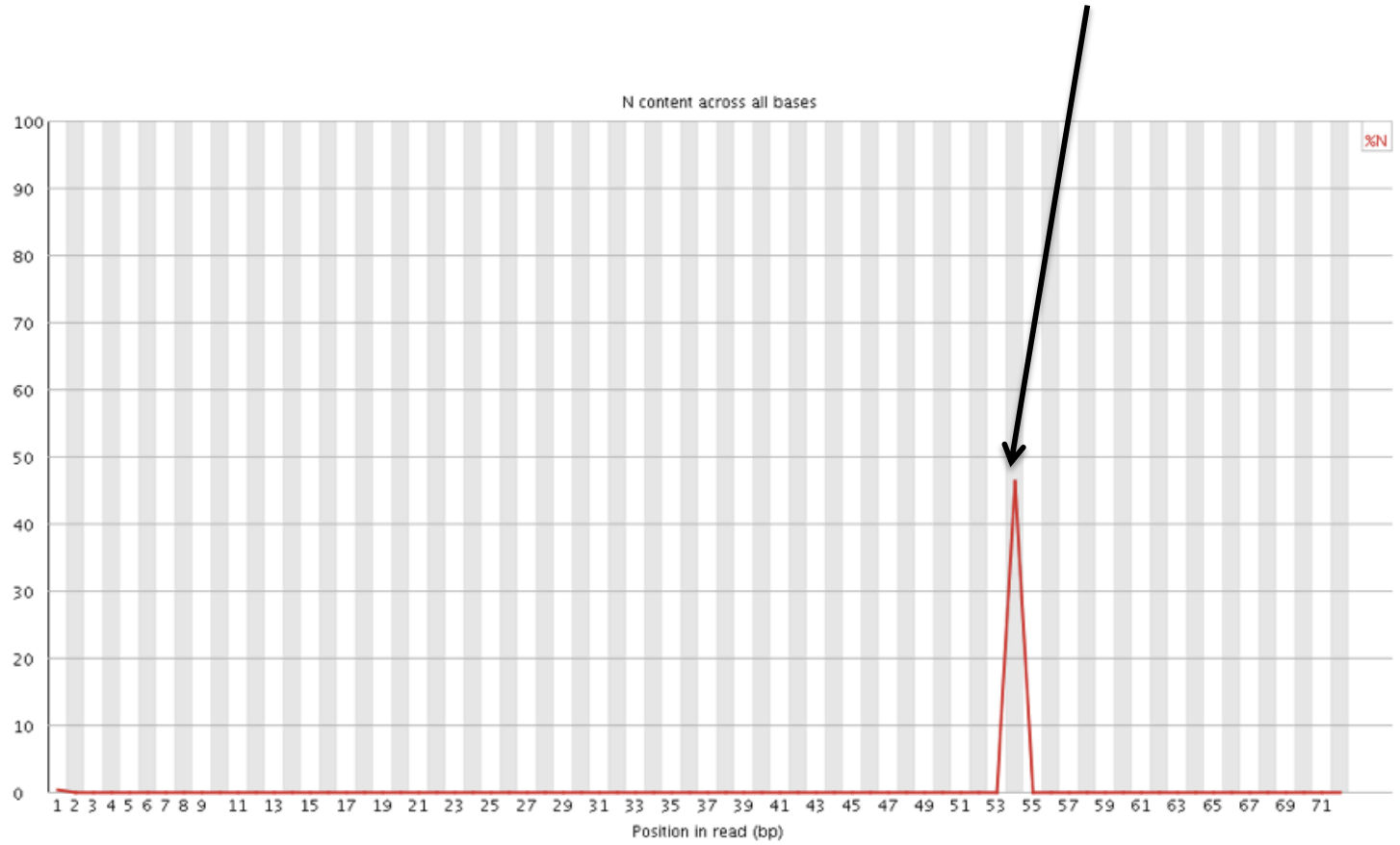
Per base N content



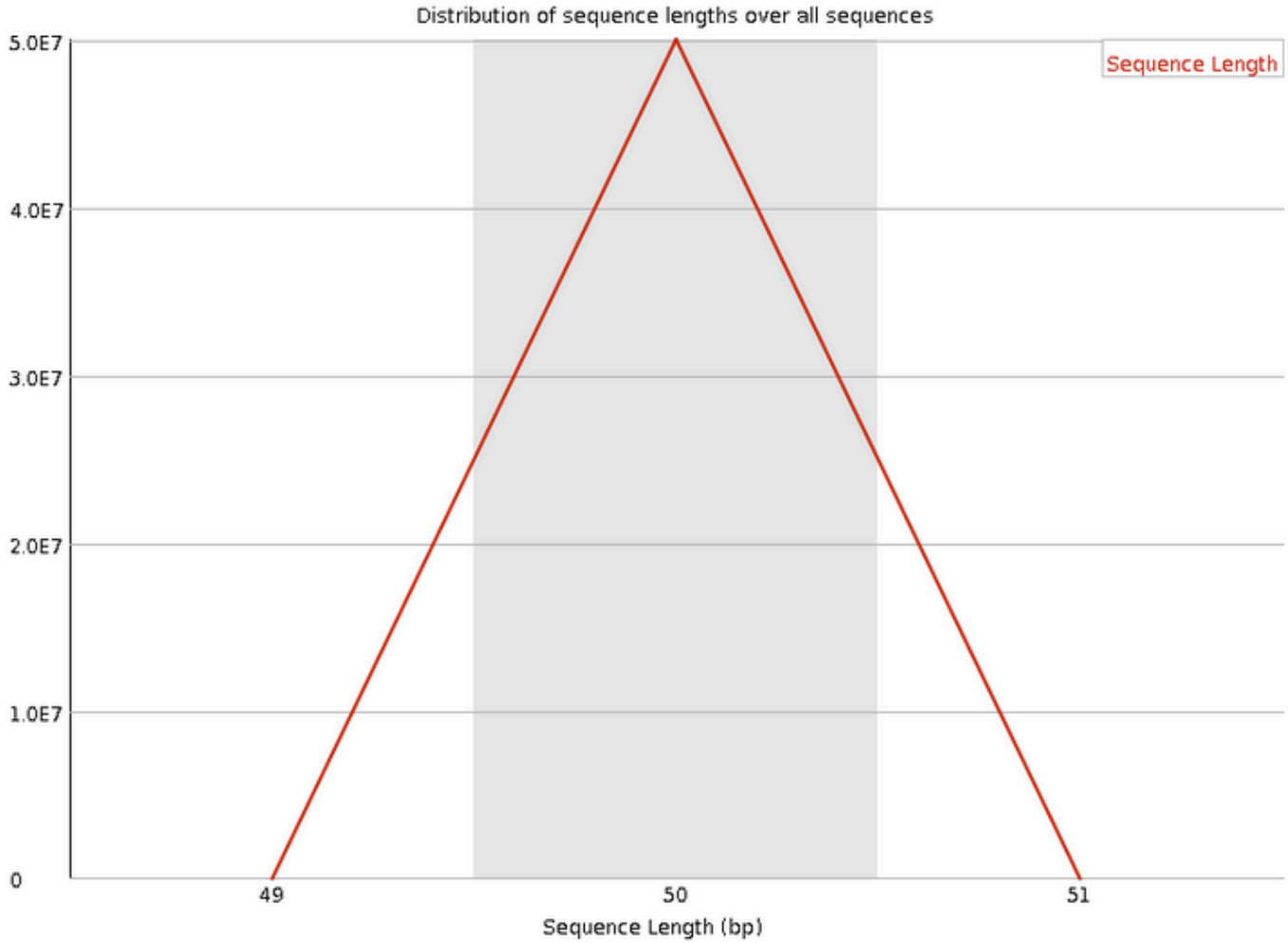
➔ **Very low N content**

Per base N content on another sample

- Can be used to detect bubbles (“Bottom Middle Swath”)



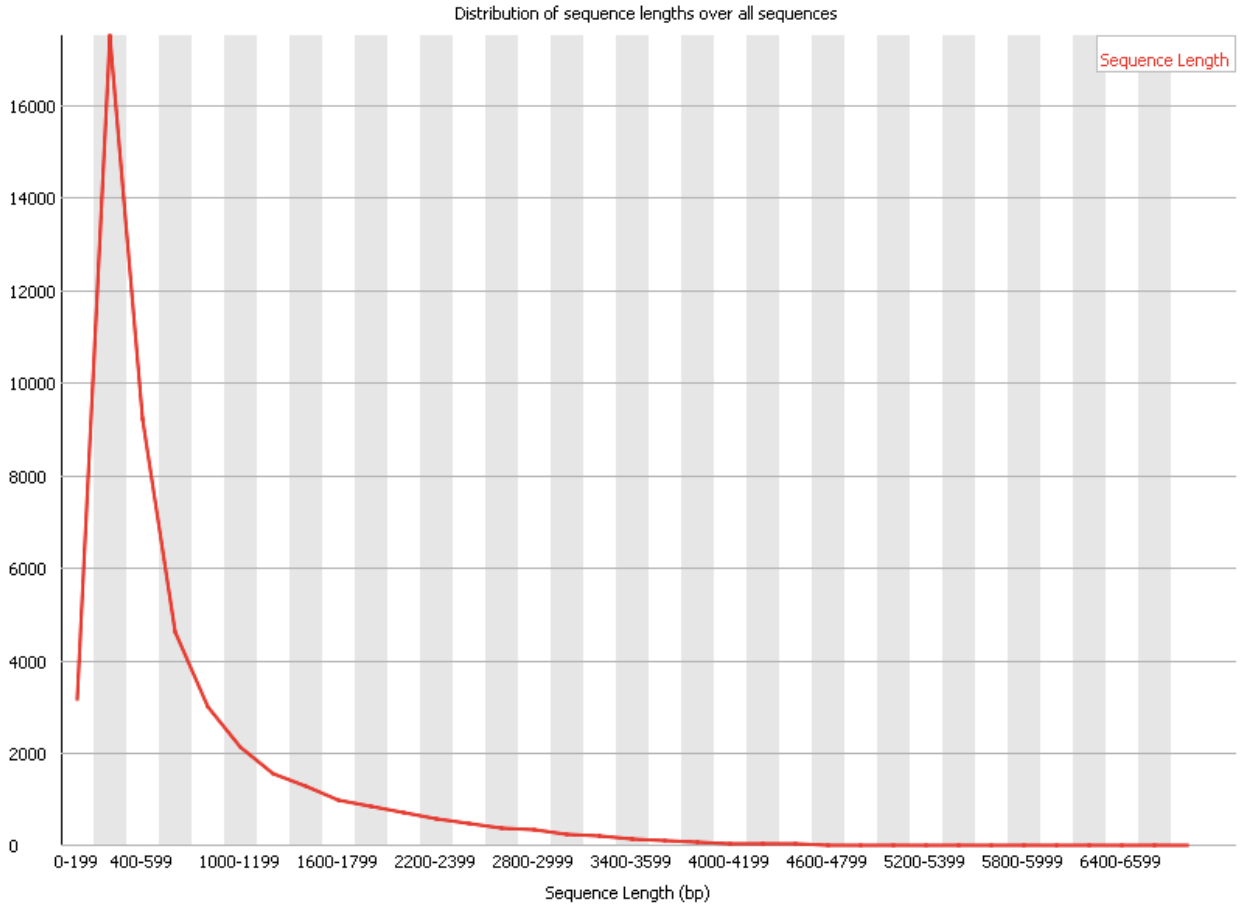
Sequence length distribution



➔ **All sequences = 50bp reads**

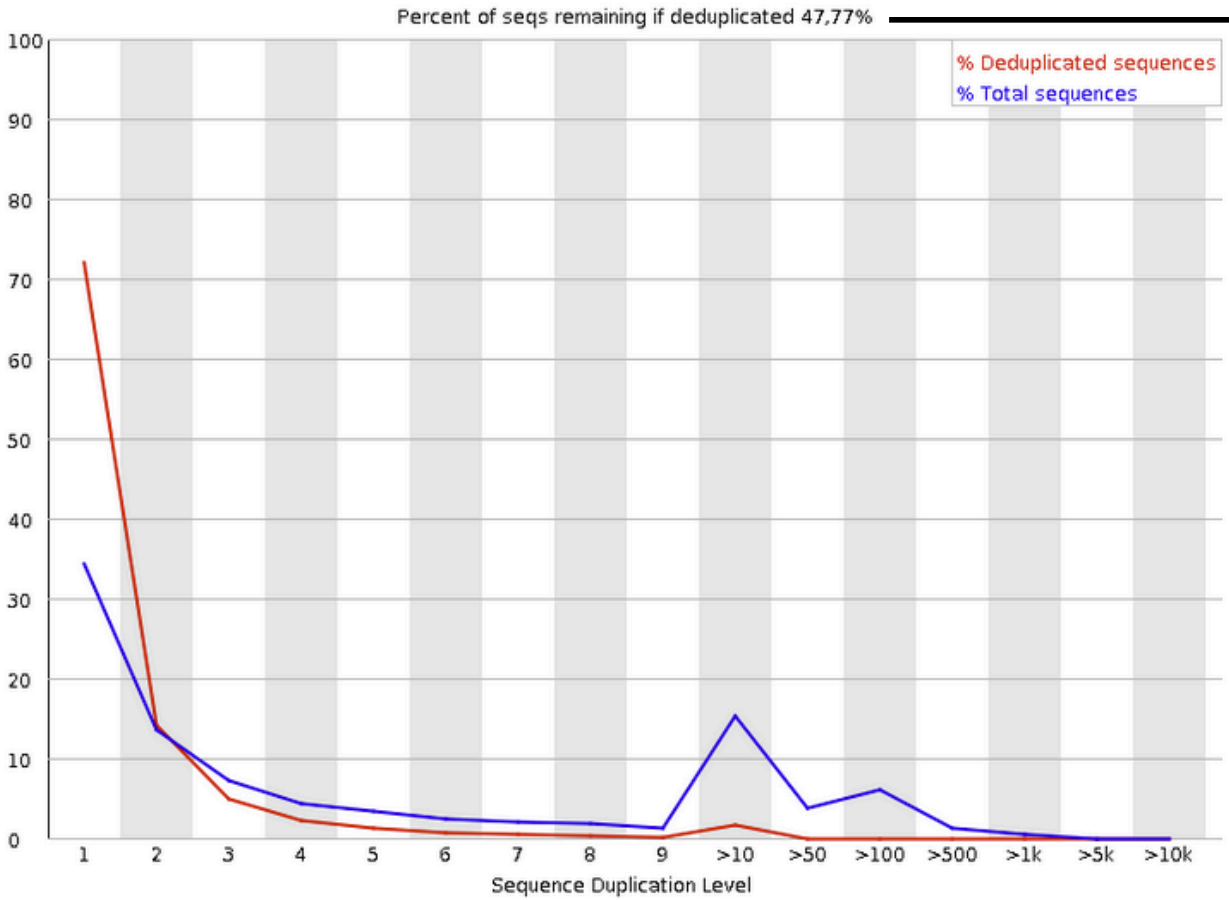
Sequence length distribution on another sample

- Useful when different sequence lengths in the file



Sequence duplication levels

- Relative number of sequences with different degrees of duplication



$$\frac{\text{Nb distinct reads}}{\text{Nb total reads}} \times 100$$

(100,000 first reads
>75bp reads → truncated to 50bp)

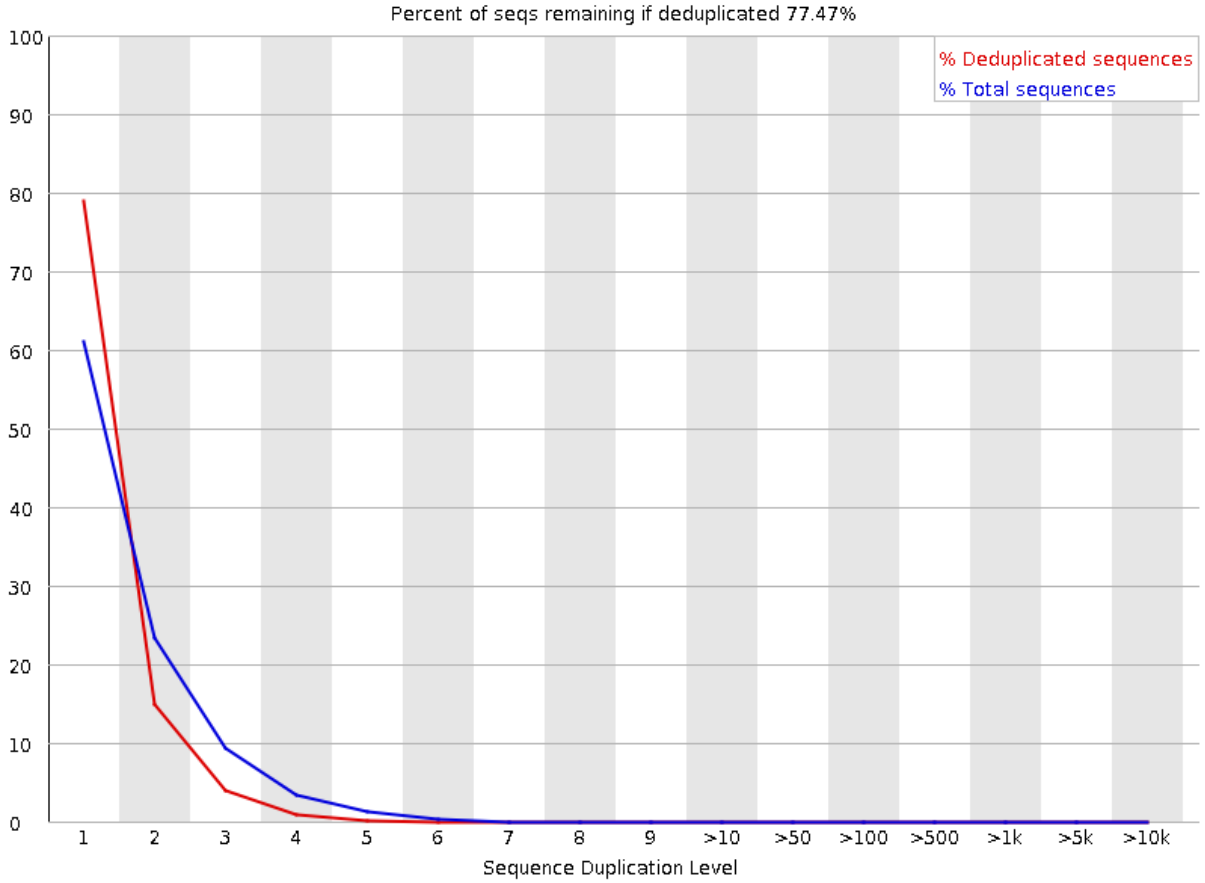
Count of all the sequences that are duplicated at a given rate (% of full sequence set)

Number of distinct sequences that are duplicated at a given rate (% of deduplicated set)

➔ OK for an RNA-seq sample :
Abundant mRNAs could lead to duplicated sequences

Sequence duplication levels on other samples

- Example for a DNA-seq sample



- A high level of duplication may indicate an enrichment bias, e.g. PCR over amplification

Overrepresented sequences

- Lists all sequences representing more than 0.1% of the total

No overrepresented sequences

➔ **No sequences representing > 0.1% of the total**

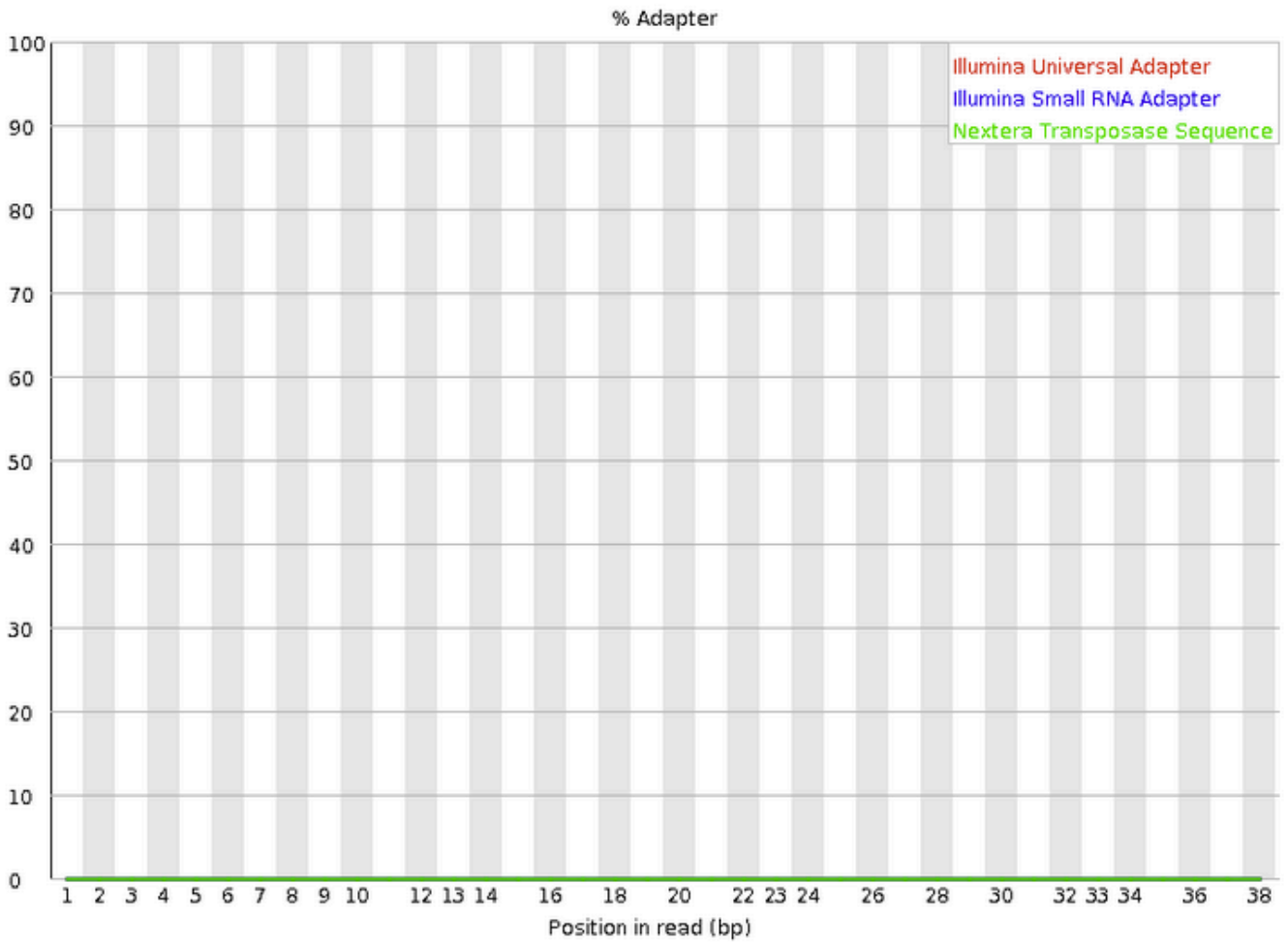
Overrepresented sequences on another sample

- For each overrepresented sequence, FastQC will look for matches in a database of common contaminants

  ➔ report the best hit, e.g. :

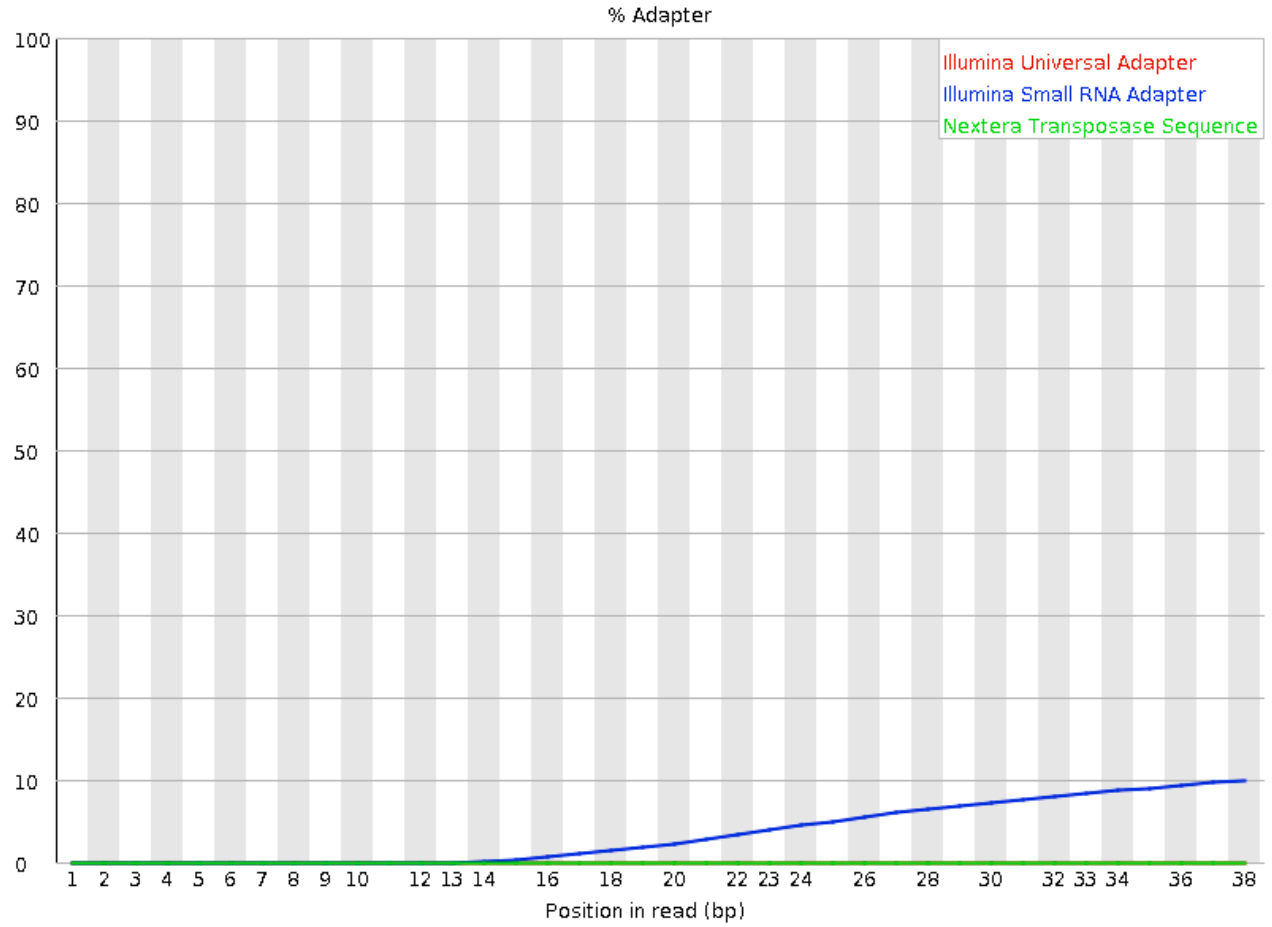
Sequence	Count	Percentage	Possible Source
AGATCGGAAGAGCACACTTCTGAACTCCAGTCACCGATGTATCTCGTATG	113163	0.614990735439532	TruSeq Adapter, Index 2 (97% over 49bp)
AGATCGGAAGAGCACACGTCTGAACTCAAGTCACCGATGTATCTCGTATG	41889	0.22764814397662272	TruSeq Adapter, Index 2 (97% over 49bp)
AGATCGGAAGAGCACACCTCTGAACTCCAGTCACCGATGTATCTCGTATG	39078	0.21237160520228368	TruSeq Adapter, Index 2 (97% over 49bp)

Adapter content



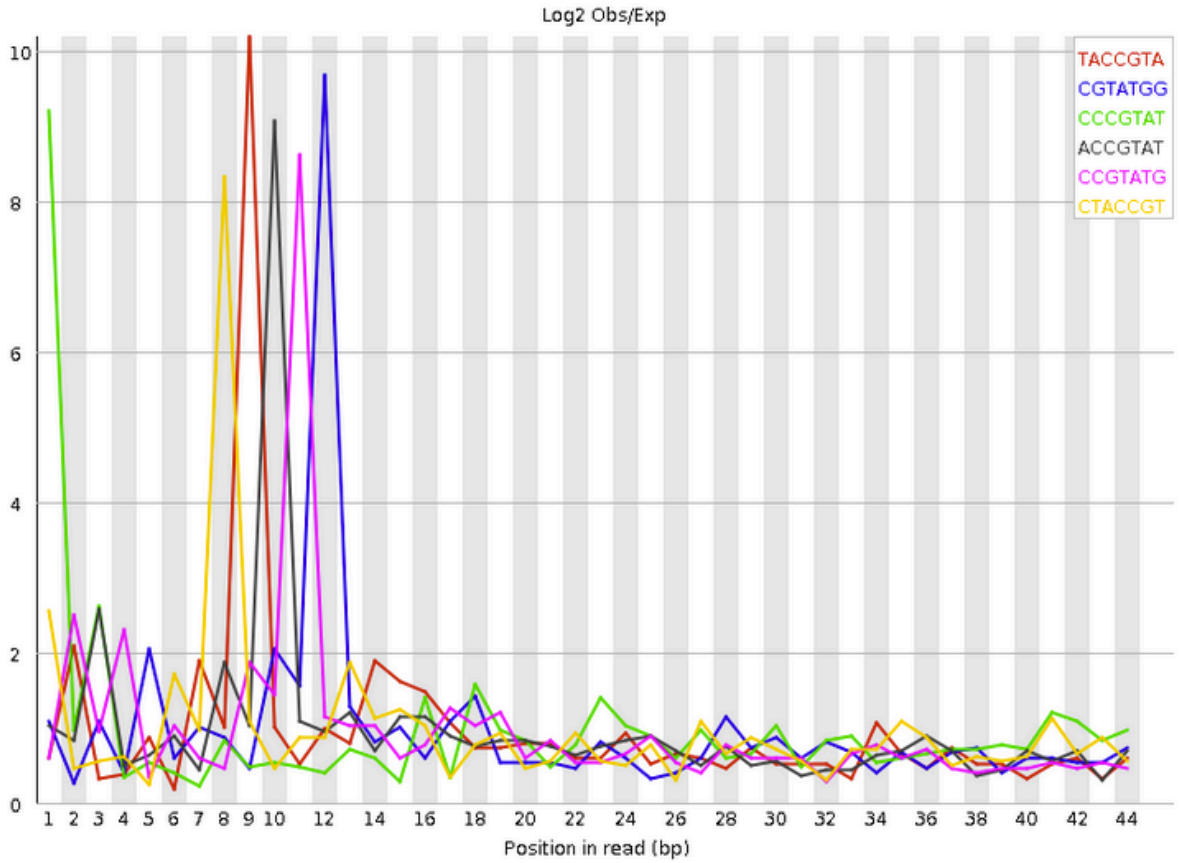
➔ **No adapters**

Adapter content on another sample



➔ Reads have to be trimmed before analysis

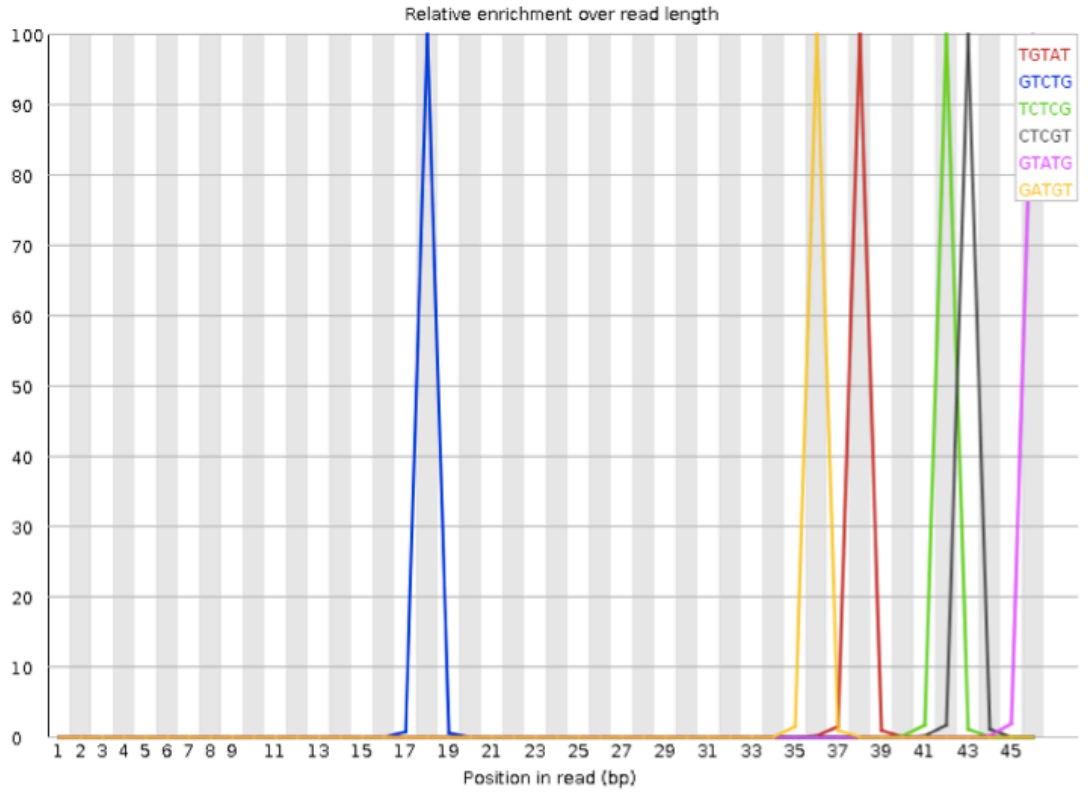
K-mer content



➔ **Bias in the repartition of the first nucleotides in RNA-seq libraries (as in the “per base sequence content” graph)**

K-mer content on other samples

- Presence of overrepresented sequences, e.g. adapters



Quality control of Illumina data

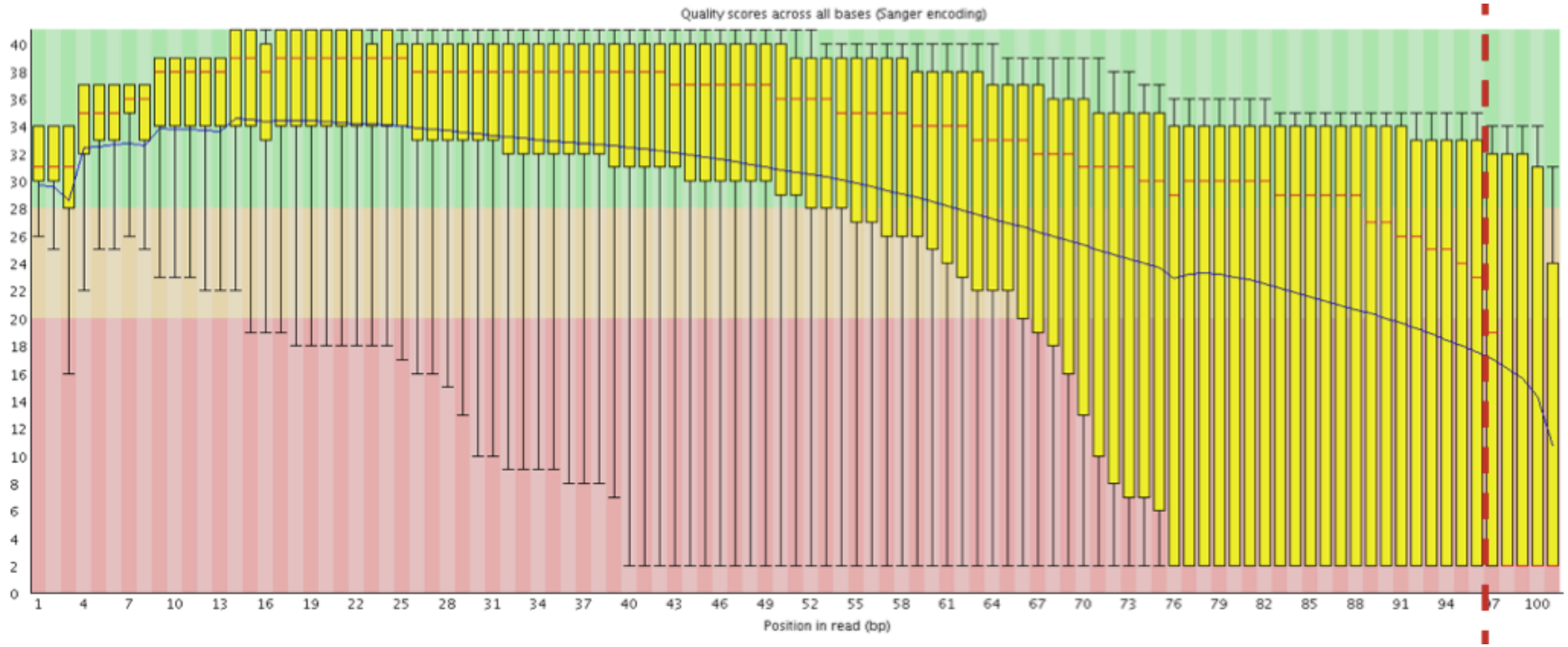
- Primary analysis
- Quality control
- Data pre-processing

Data pre-processing

- Why ?
  - Remove bad quality/contaminant data
  - Improve confidence of downstream analysis
- Needed ?
  - Depend on what type of data and what type of analysis you want to perform on your data
    - e.g. small RNA-seq : adapters removal required
    - e.g. assembly : cleaned data required
    - e.g. variant calling : has to be performed only on good quality reads / part of reads
- Example of tools
  - Galaxy : e.g. http://www.galaxeast.fr/ section NGS: QC and manipulation
  - Cutadapt : https://cutadapt.readthedocs.io/

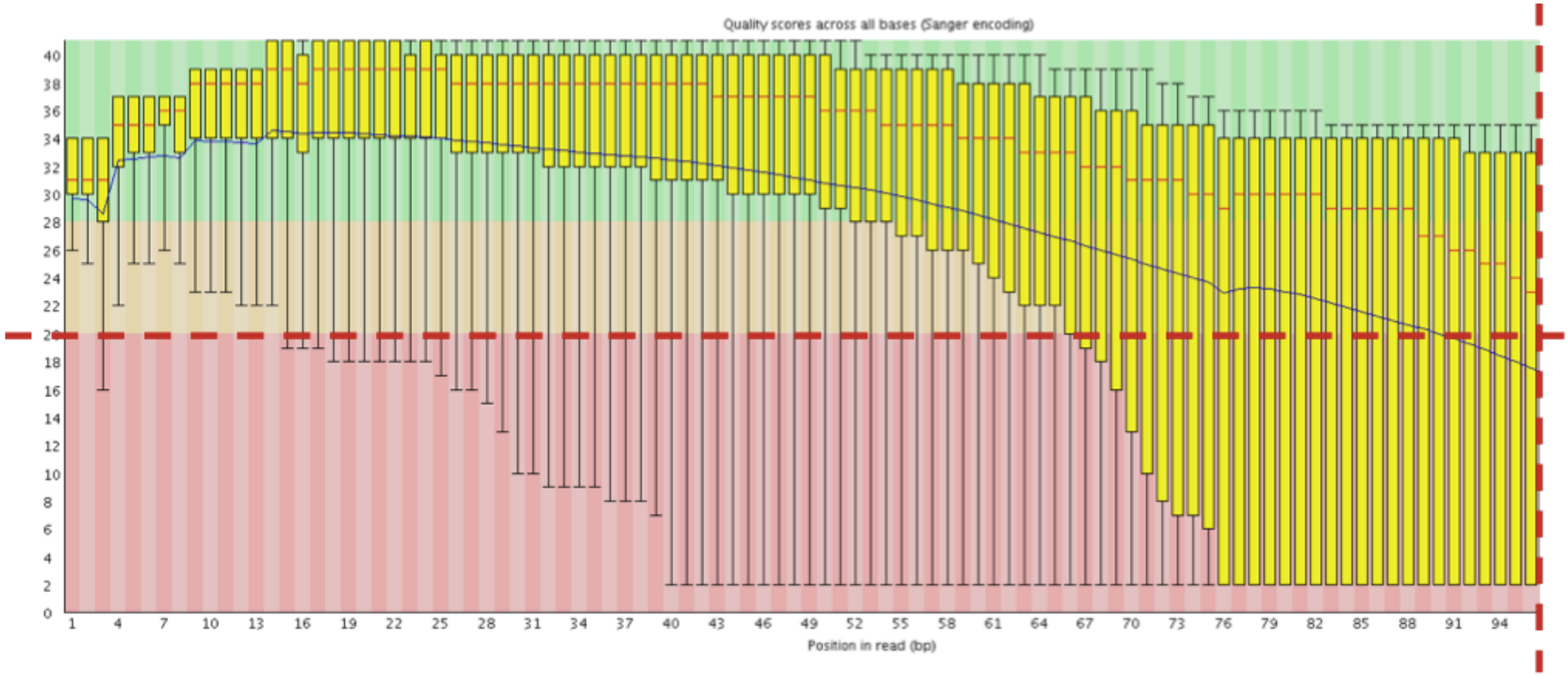
Trimming

- Remove low quality bases from the sequence end
- e.g. trim reads when the median base quality falls bellow 20



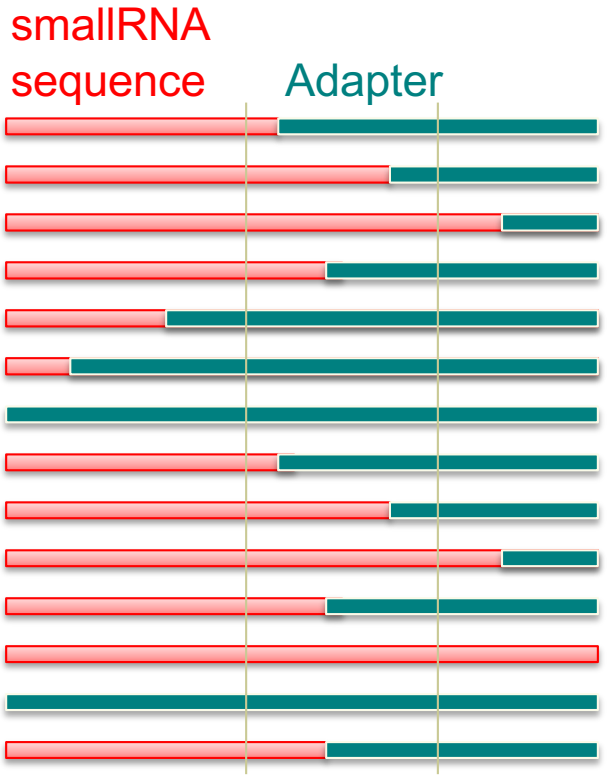
Filtering low quality reads

- Keep only reads with a sufficient quality
- e.g. retain only reads with an average base quality score ≥ 20



Removing/clipping adapter sequences

- e.g. small RNA-seq library
  - Remove adapter sequences
  - Remove too-short sequences
  - Remove too-long sequences
  - Clip adapters



Removing contaminants

Possibly :

- Sequences used during library preparation
  - e.g. Spikes
- Sequences from other organisms
  - e.g. Xenografts
- rRNA sequences