NGS introduction

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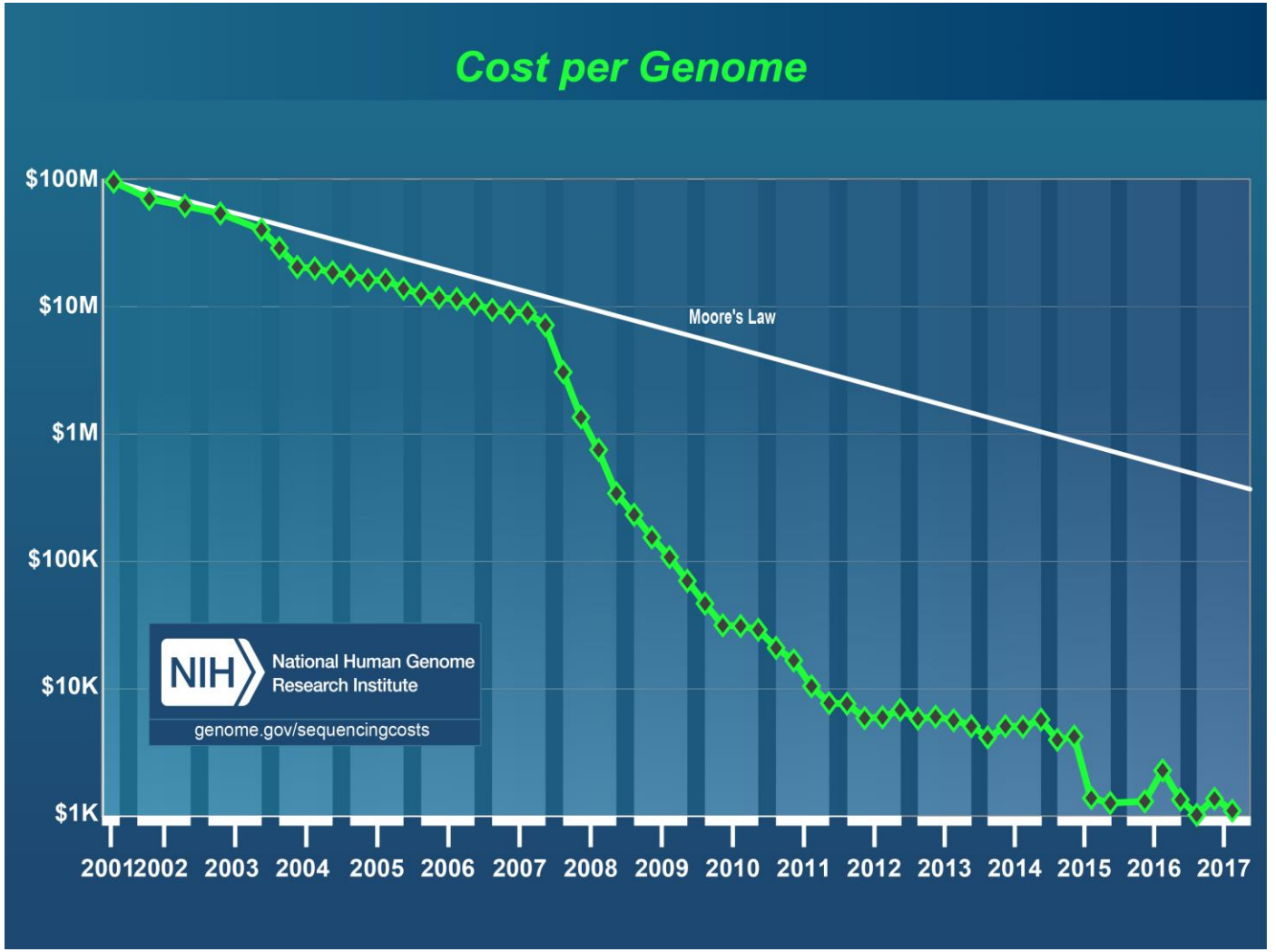
Sequenting costs have decreased massively



History of Illumina sequencing

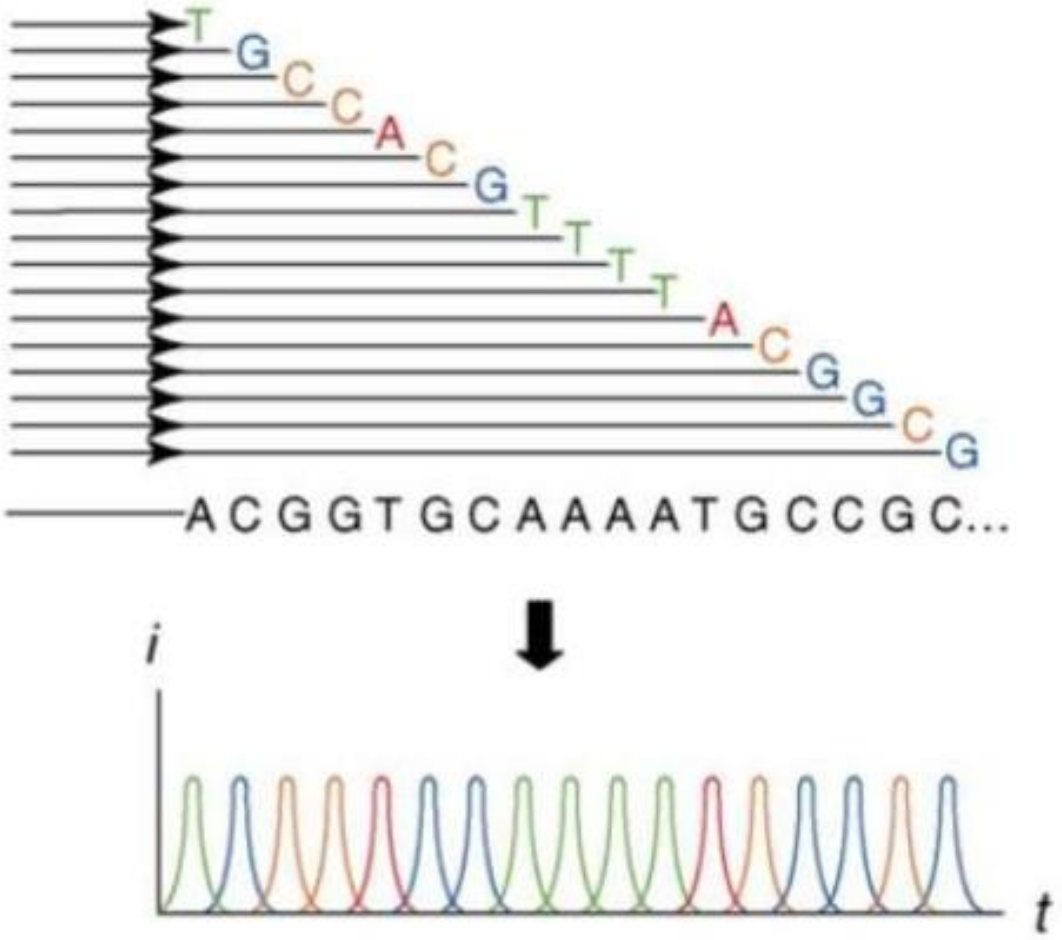
NovaSeq 6000

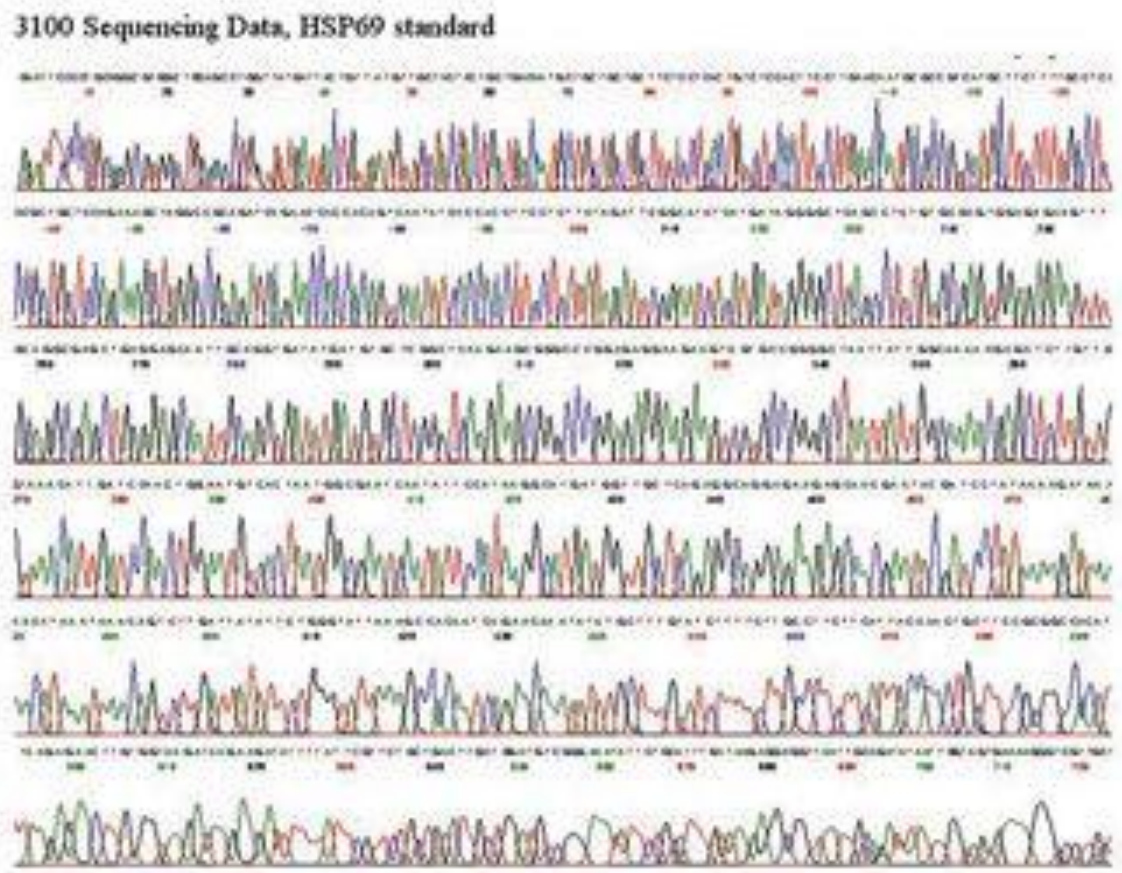
High Throughput Sequencing (=Next Generation Sequencing)

- **Short-read sequencing technologies (2nd generation):**
  - Sequence millions of clonally amplified molecules
  - E.g. Illumina, Ion Torrent, SOLiD

- **Long-read technologies (3rd generation):**
  - Single molecules are sequenced in real-time, fast but expensive and high error rates
  - E.g. PacBio (bought by Illumina last month): ~12kb reads, single molecules are read multiple times to reduce error rate
  - E.g. Oxford Nanopore: up to 900 kb reads, high sequencing error rate (5-15%) and non-random errors, each DNA fragment can only be read 2x

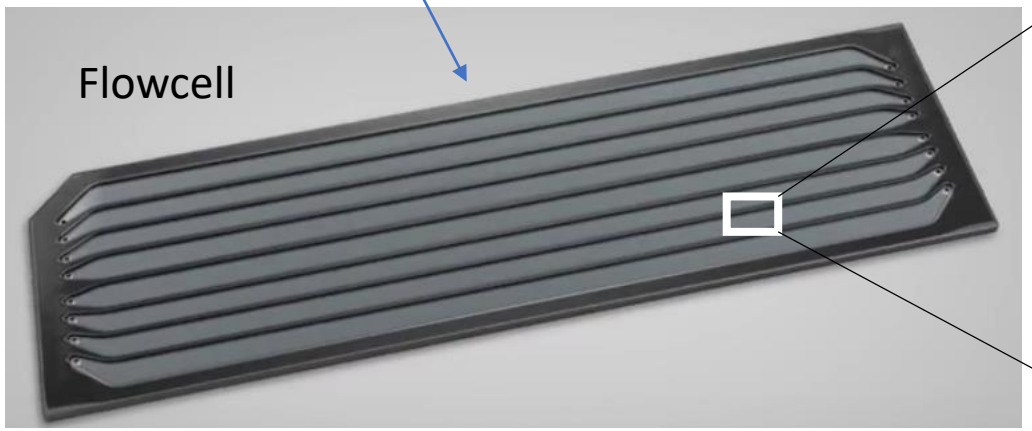
Sanger Sequencing



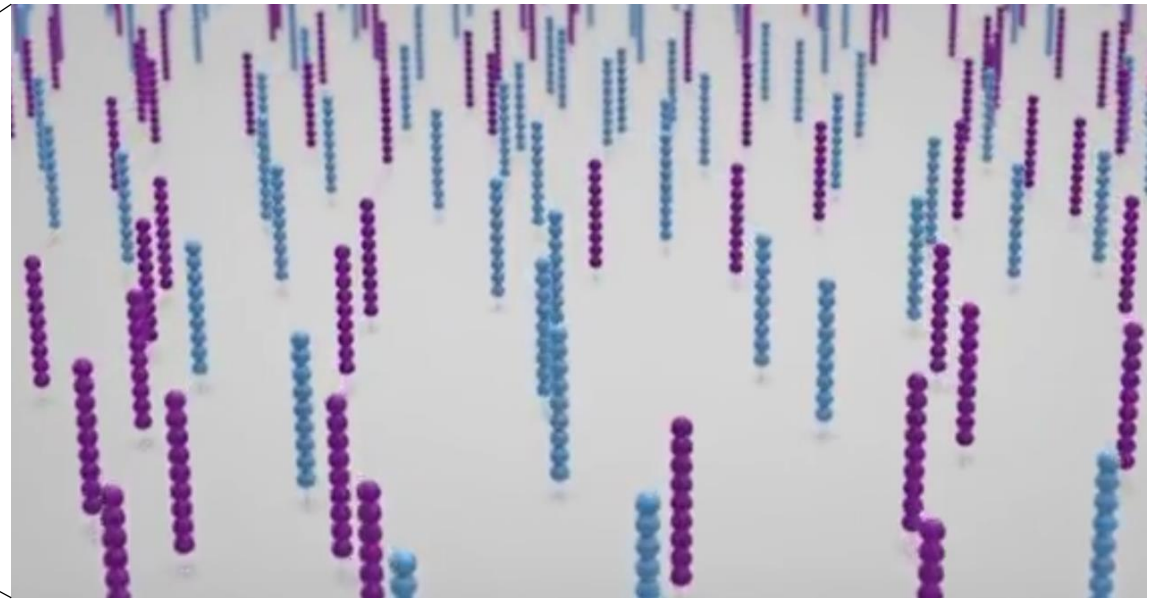


Illumina flowcell: millions of DNA sequences

DNA fragments with Illumina adapters



Each lane contains a dense lawn of Illumina primers



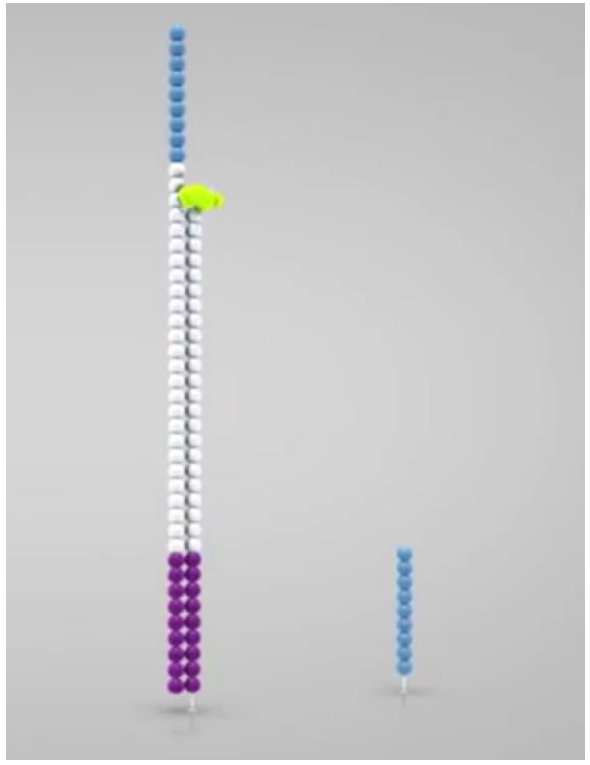
Sequencing by synthesis by Illumina: Read1

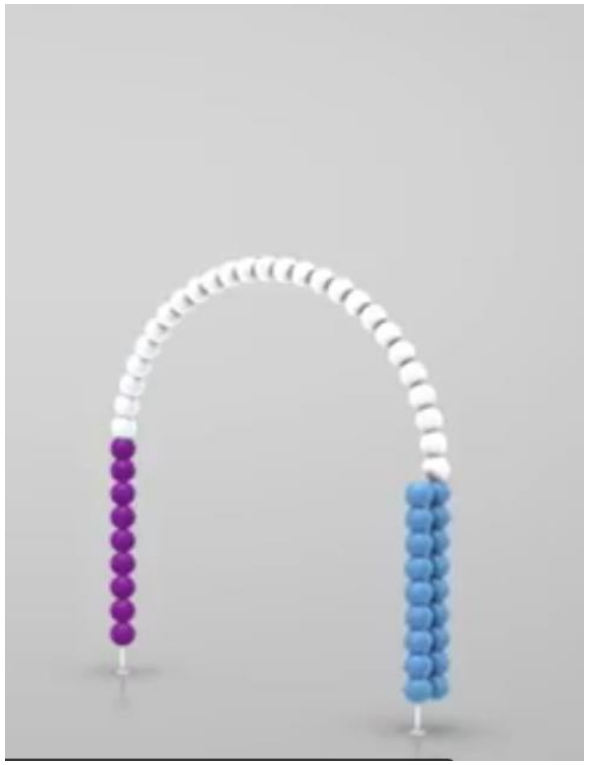
1. DNA fragments bind to the P1 primer
2. polymerase makes it double-stranded
3. Template strand is washed away (denaturated)

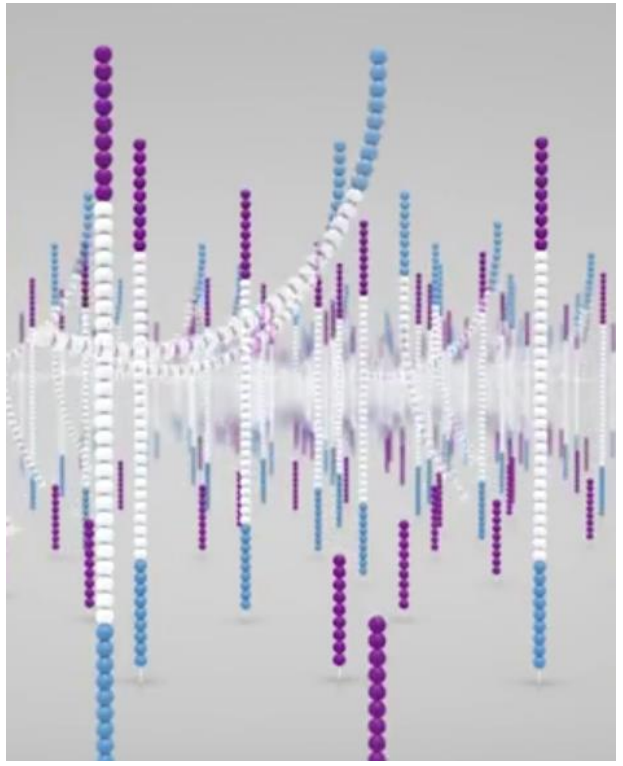
4. DNA strand forms a bridge and binds to the P2 primer
5. Polymerase makes it double-stranded
6. Denaturation -> two single stranded DNA fragments

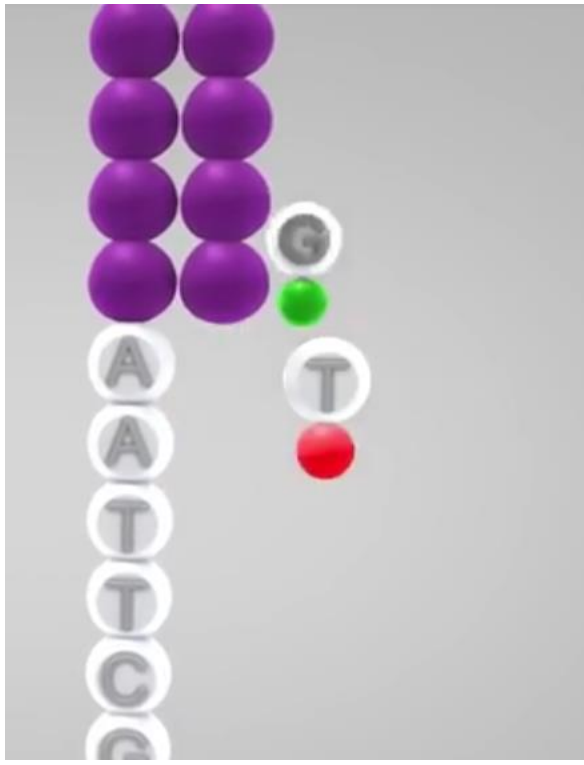
Repeat many times to form clusters of thousands of identical DNA strands
7. The reverse strands are cleaved and washed off

8. Primer annealing
9. Complementary fluorescently tagged nucleotides are incorporated in each cycle
10. Repeat step 9 150x

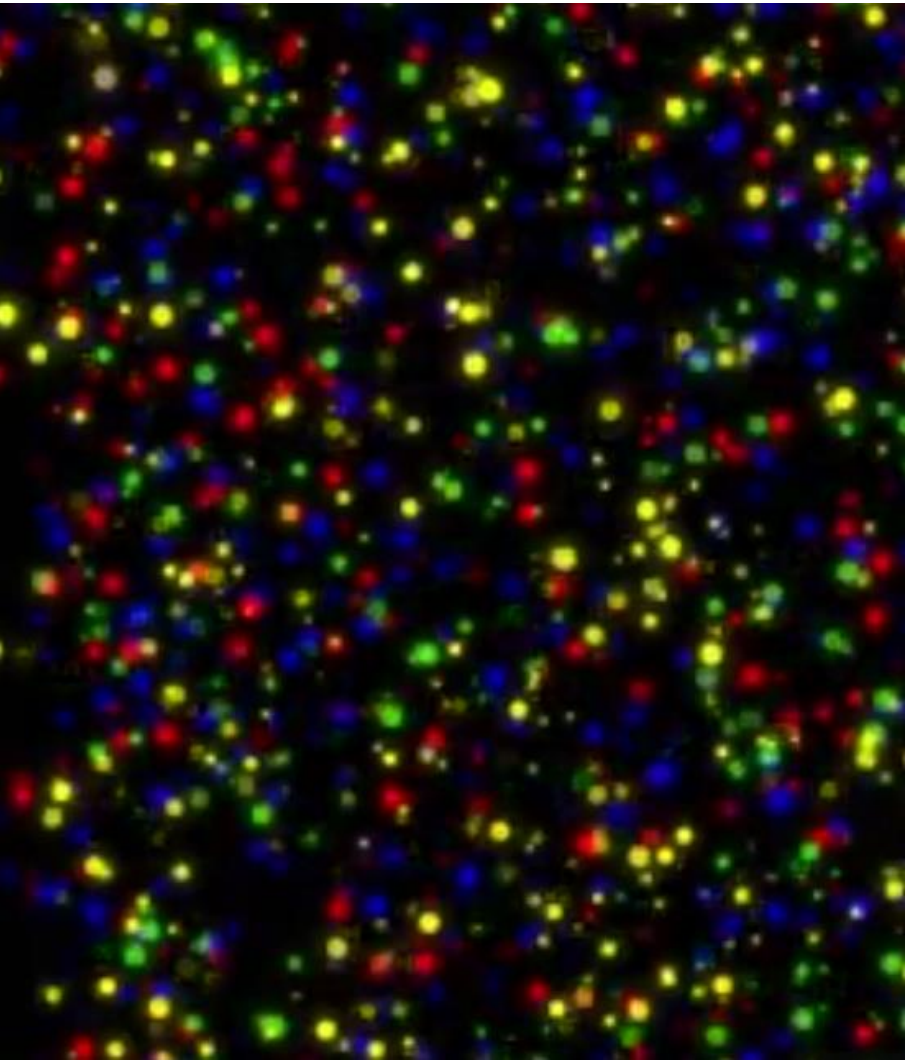


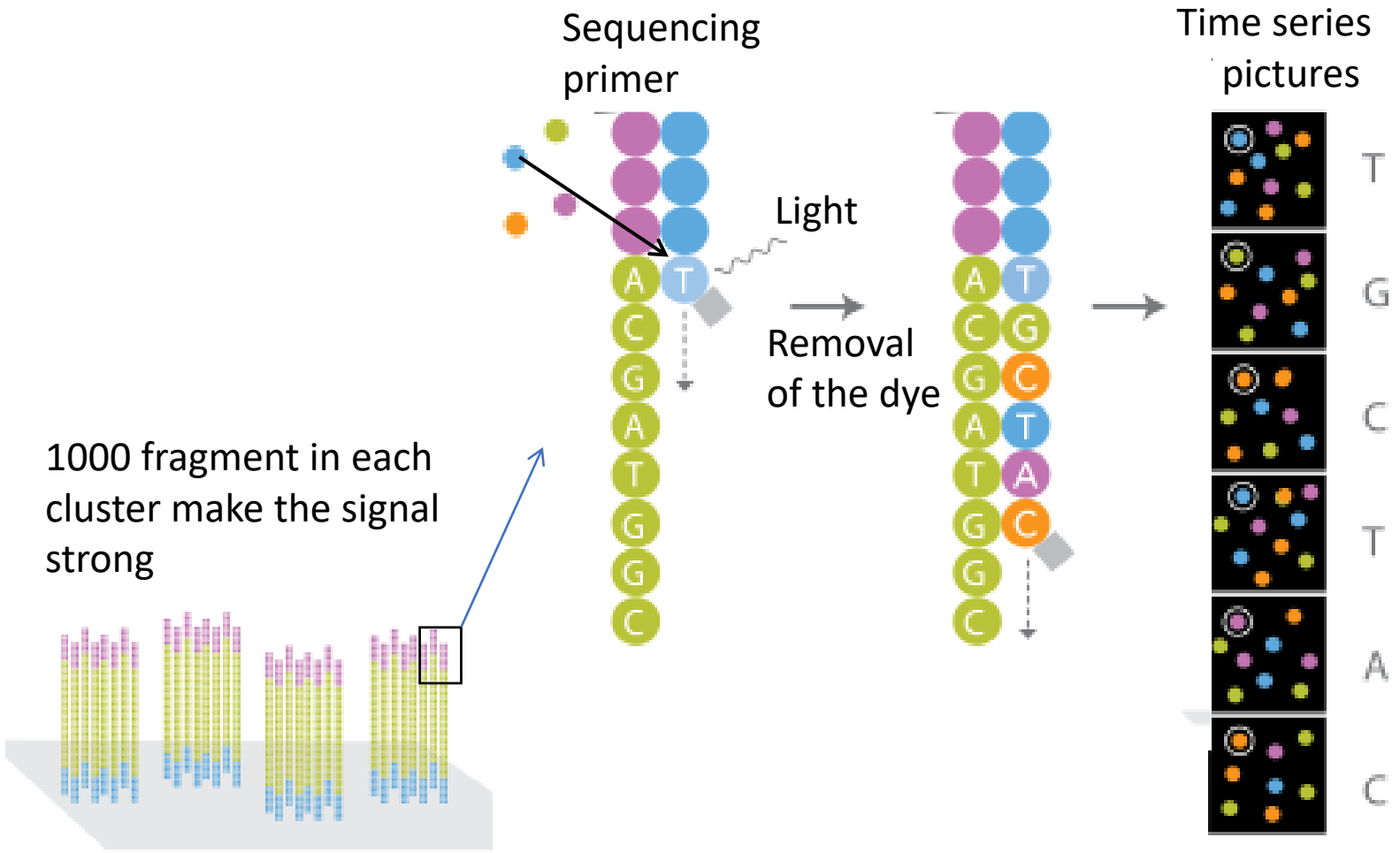






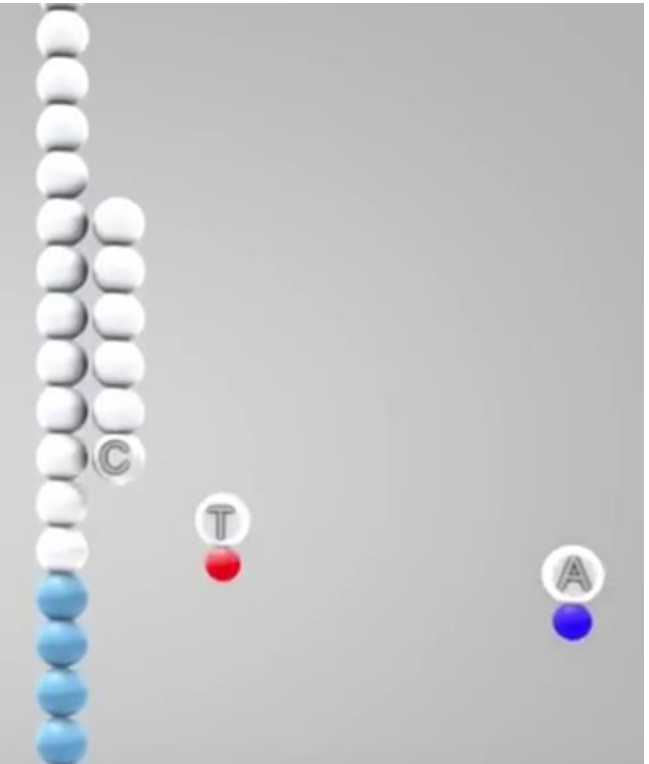
Sequencing by synthesis by Illumina





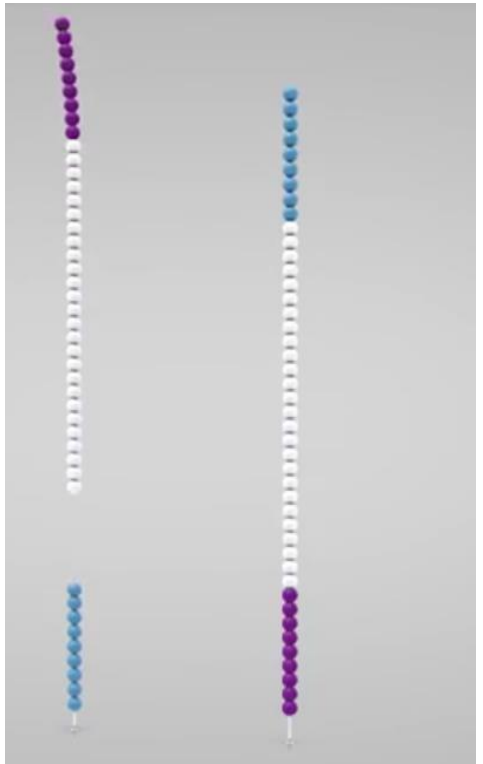
Sequencing by synthesis by Illumina: Read2

11. Denaturation
12. Primer index 1 is added and sequenced
13. The 3’ end is deprotected

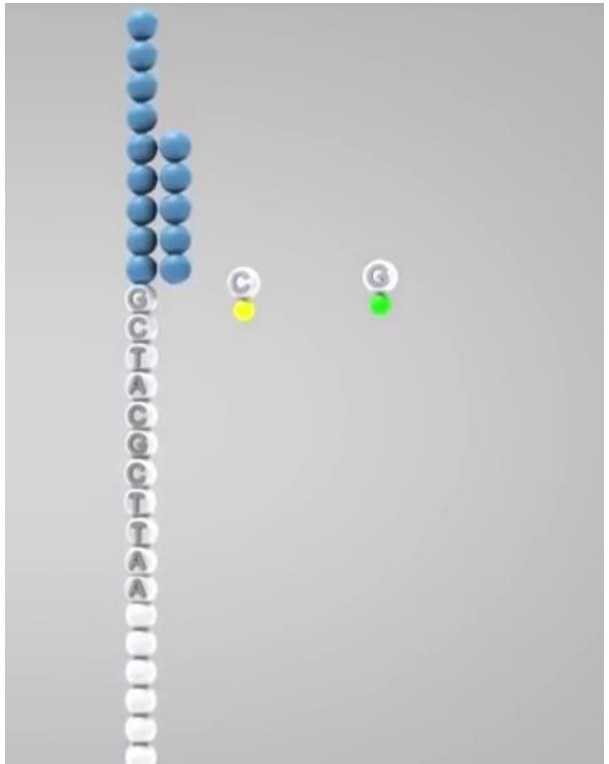


14. The DNA fragment forms a bridge to the reverse primer
15. Sequencing of index 2
16. Polymerase makes bridge double-stranded

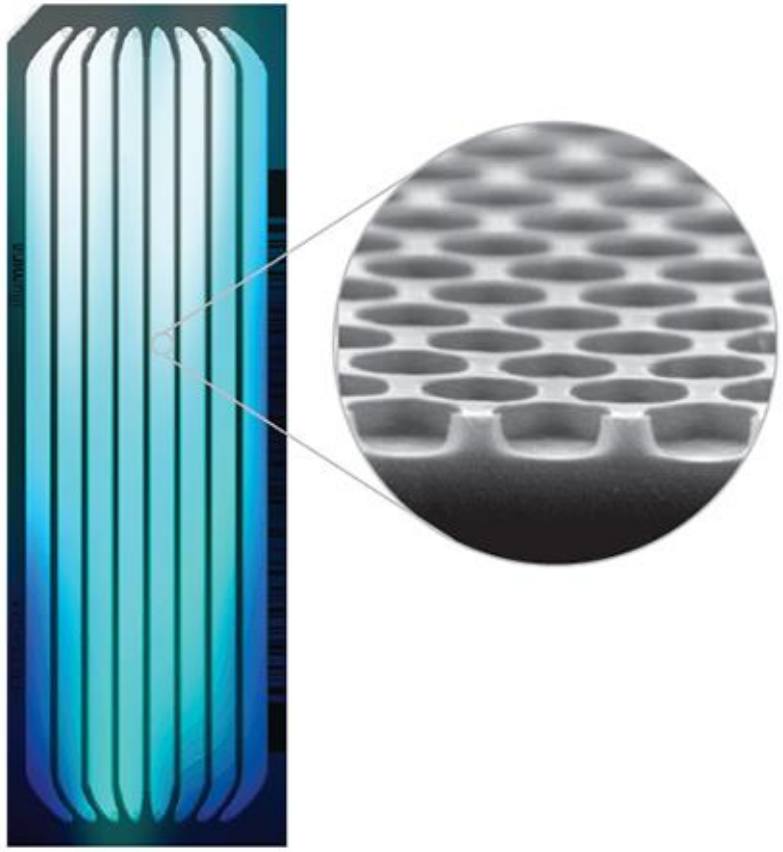
14. Denaturation leads to single-stranded fragments bound to the flowcell
15. Forward strands are cleaved and washed off



16. Second read is sequenced as the first read with sequencing-by-synthesis



Illumina HiSeq4000 and X Ten

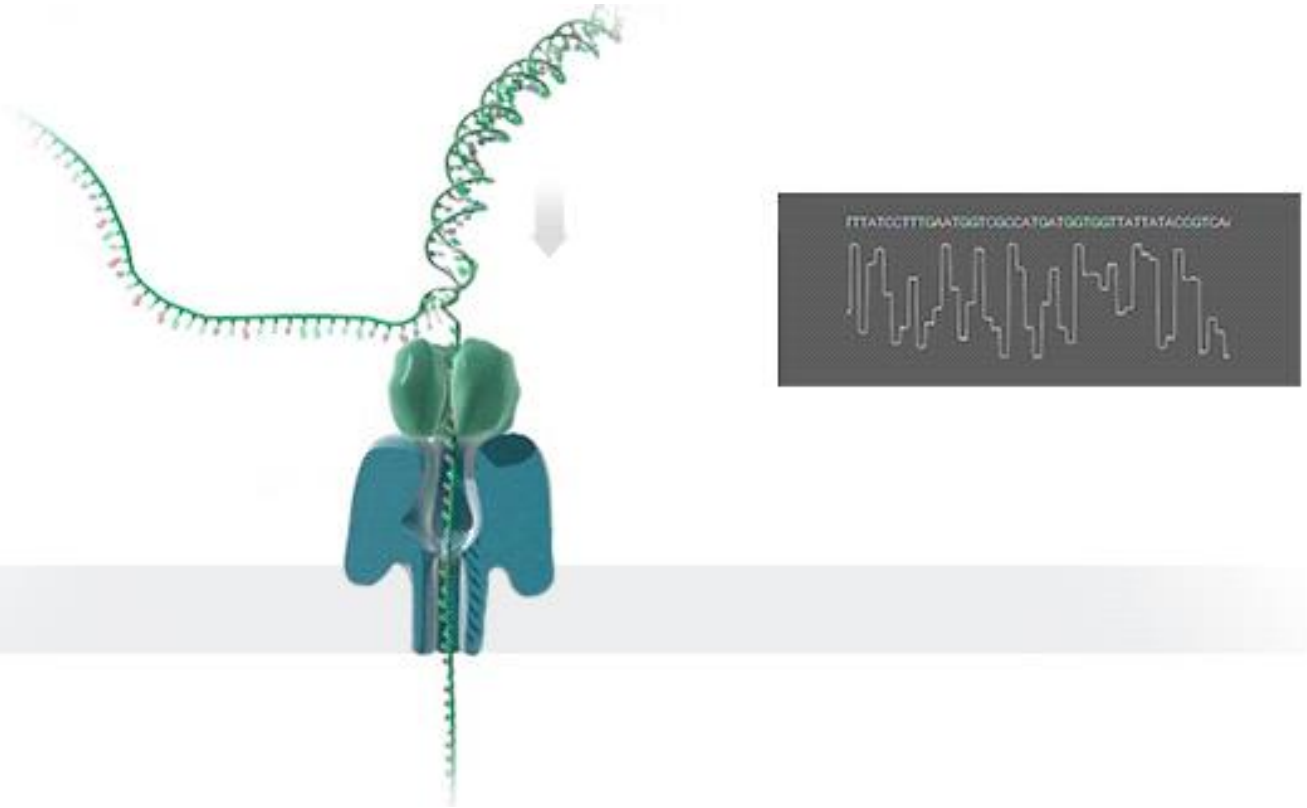


Problem:
Illumina barcode switching
(Index hopping)

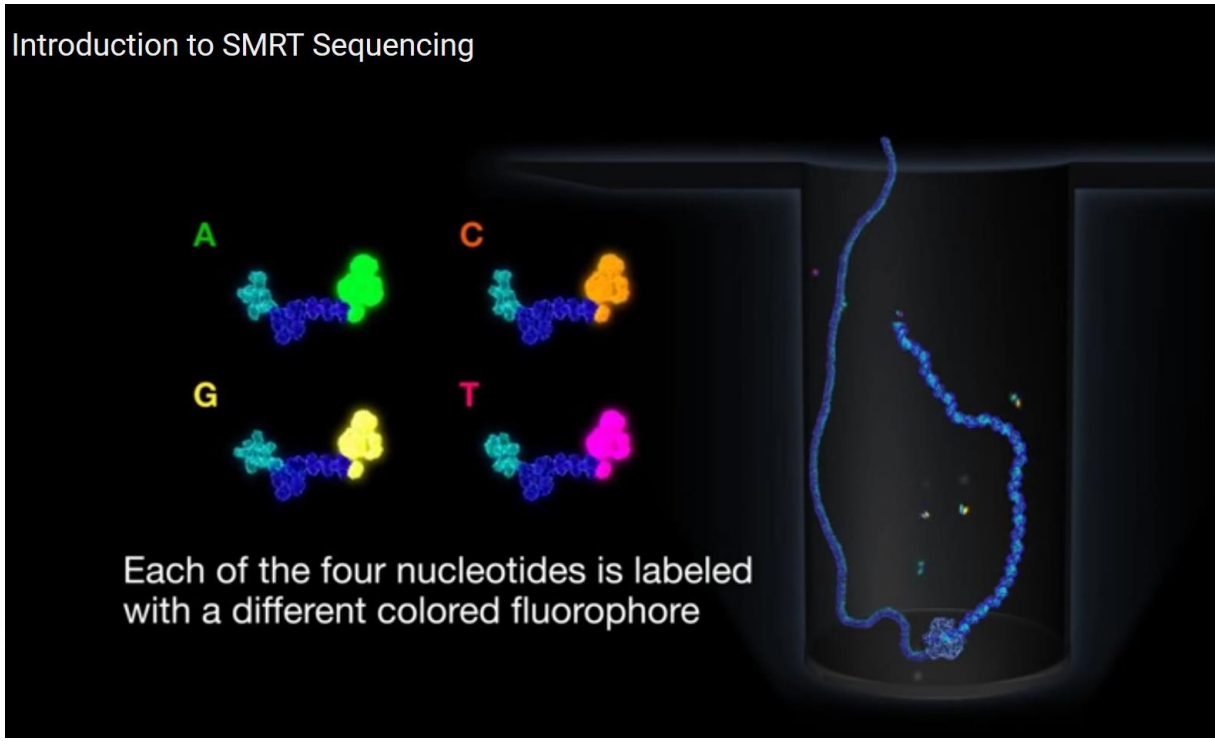
-> use double-indexing
Different P1 and P2 indices

Long read sequencing technologies

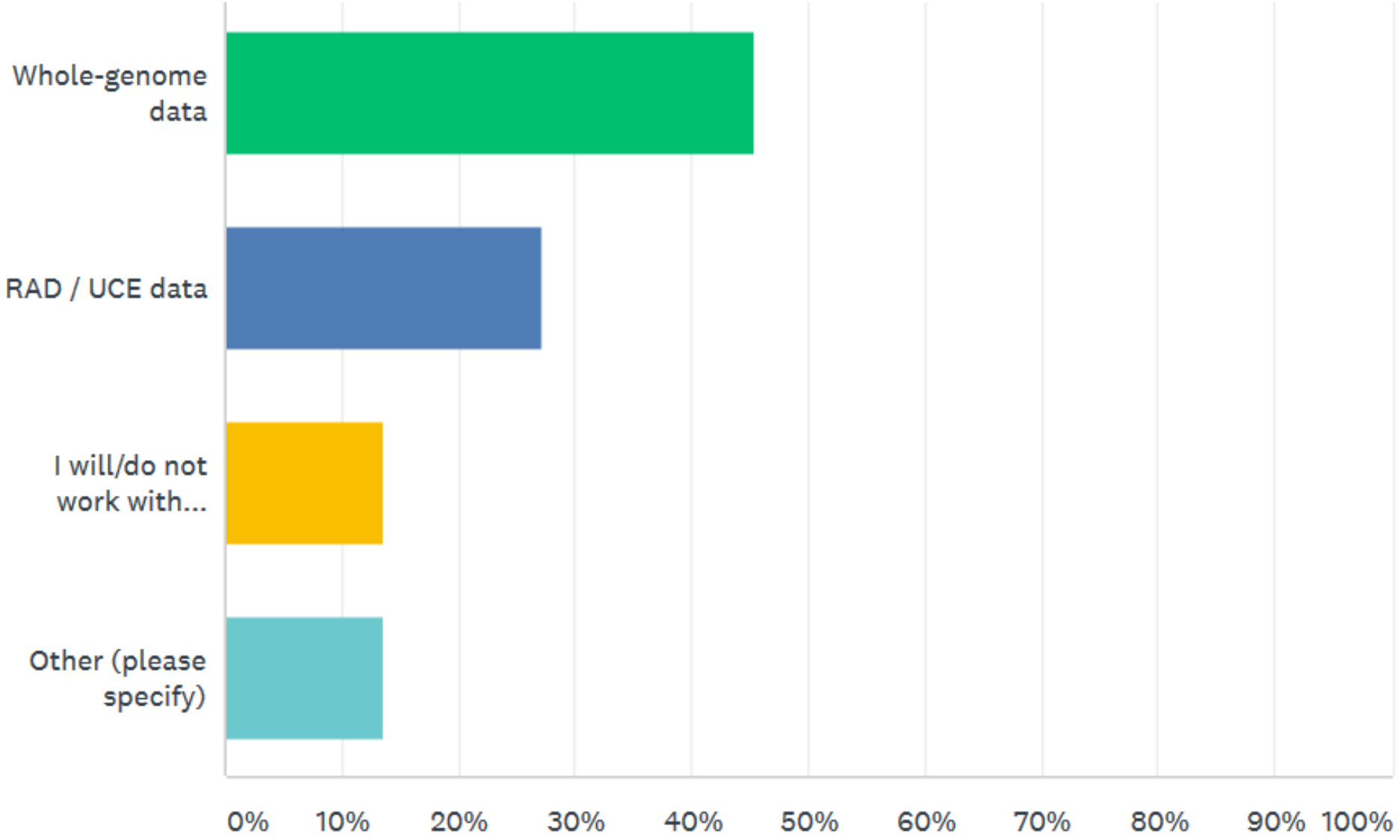
Nanopore



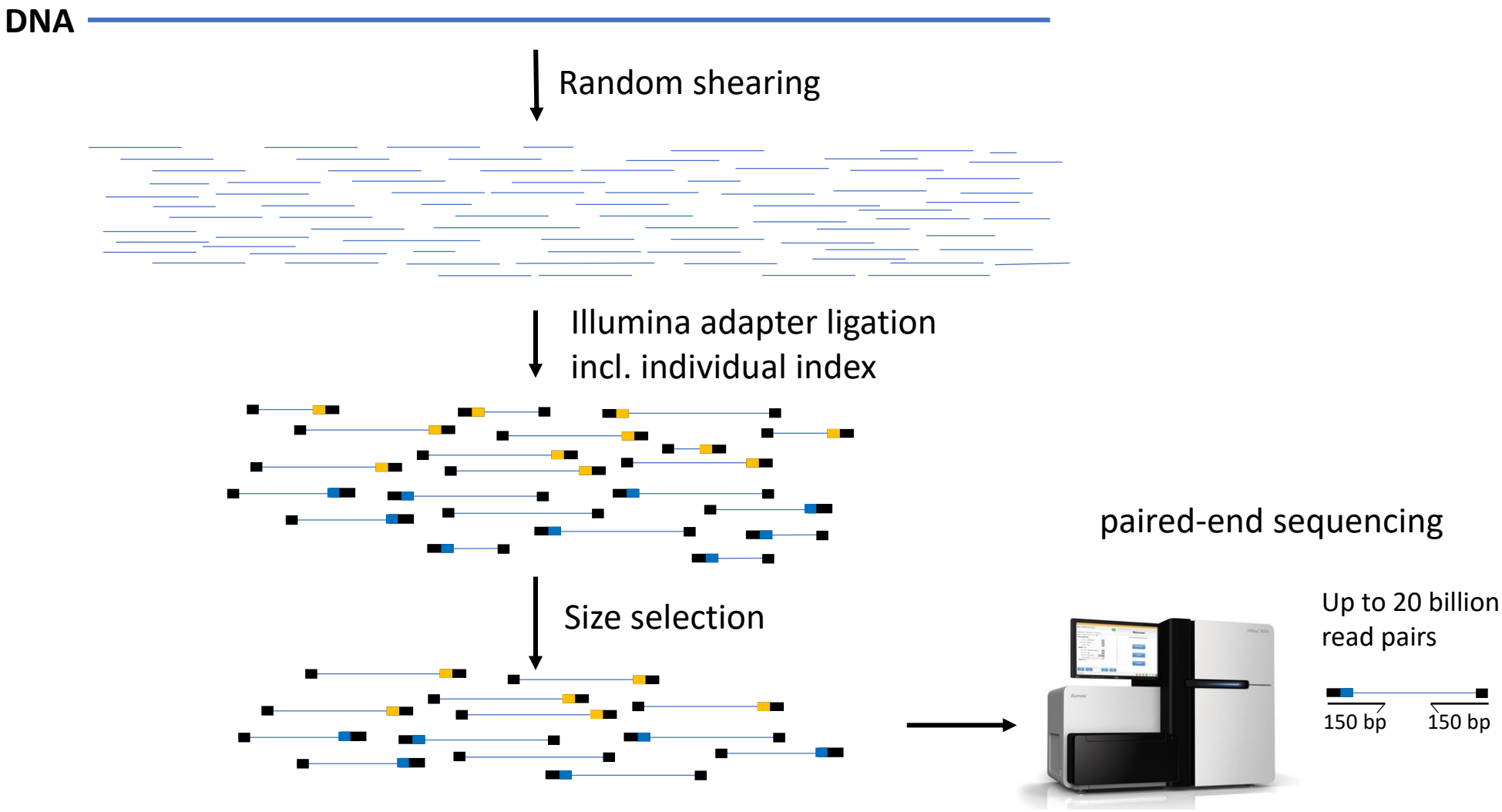
PacBio



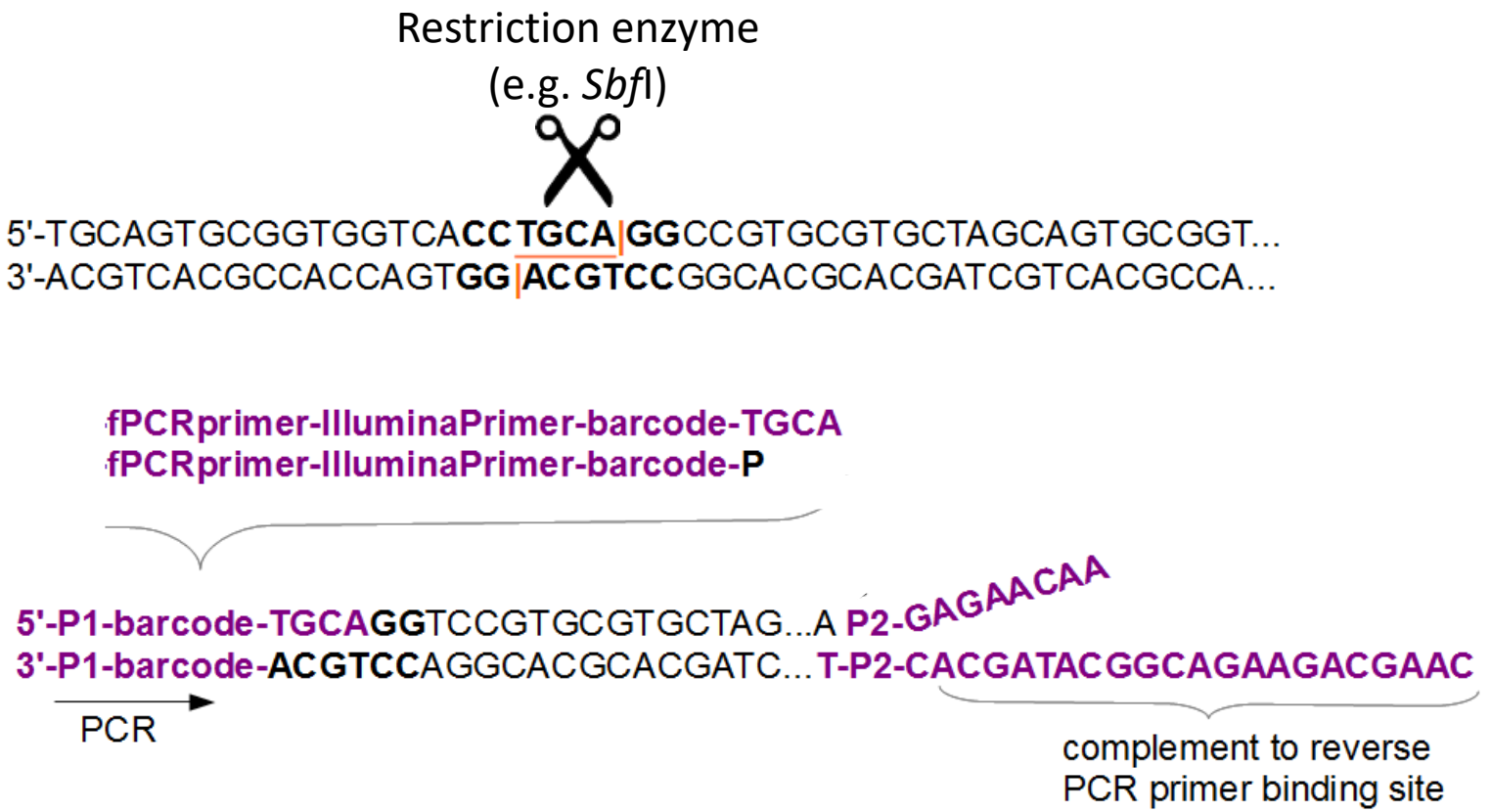
DNA preparation methods



Whole-genome sequencing (shotgun sequencing)

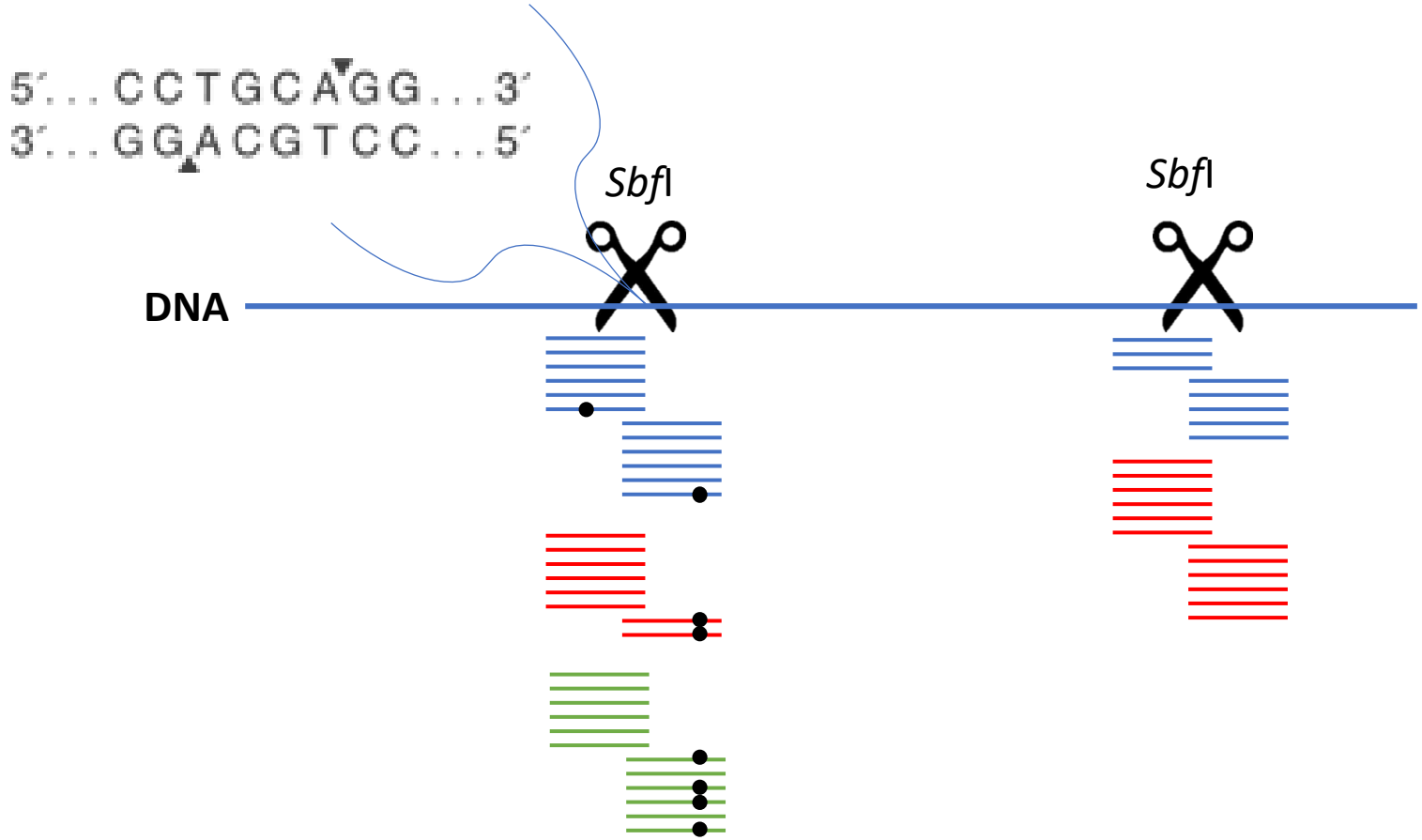


RAD sequencing
Restriction **A**ssociated **D**NA **sequencing**



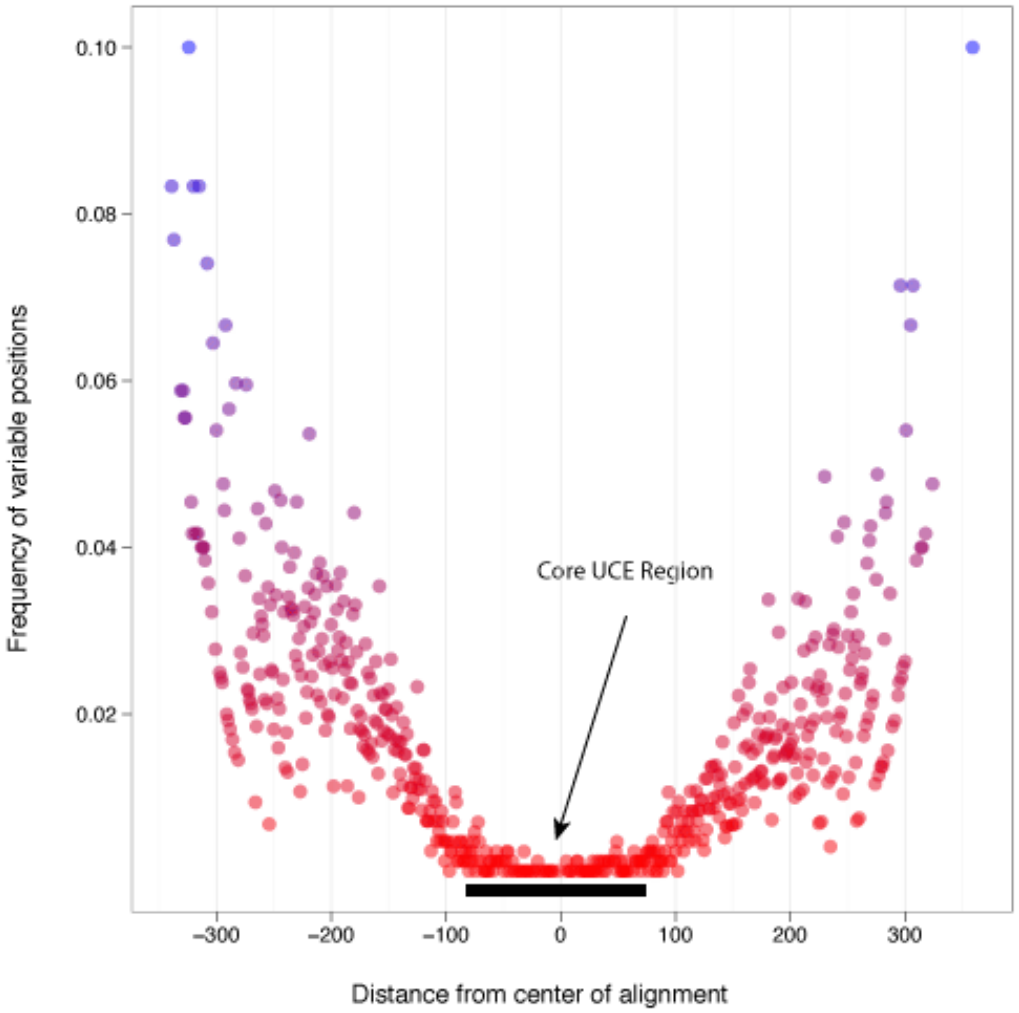
RAD sequencing

Restriction **A**ssociated **D**NA **sequencing**



Other «reduced-representation» techniques

- **ddRAD sequencing** (double-digest RAD
  restriction enzyme and size selection in
- **GBS** (genotyping by sequencing): no she
  and PCR and sequencing select short fra
- **UCE**: Selection of DNA fragments throug
  based on ultraconserved elements (con
  taxa)



Other «reduced-representation» techniques

- **CRoPs/ddRAD sequencing** (double-digest RAD sequencing): uses second restriction enzyme and size selection instead of shearing
- **GBS** (genotyping by sequencing): no shearing, just one restriction site and PCR and sequencing select short fragments
- **UCE**: Selection of DNA fragments through sequence capture with baits based on ultraconserved elements (conserved across highly divergent taxa)
- **Transcriptome sequencing**: RNAseq, only coding regions of the genome, easy to annotate, depth informative on expression
- **Targeted resequencing:** Sequence capture or amplicons

Trade-offs: Splitting reads (i.e. costs) among:

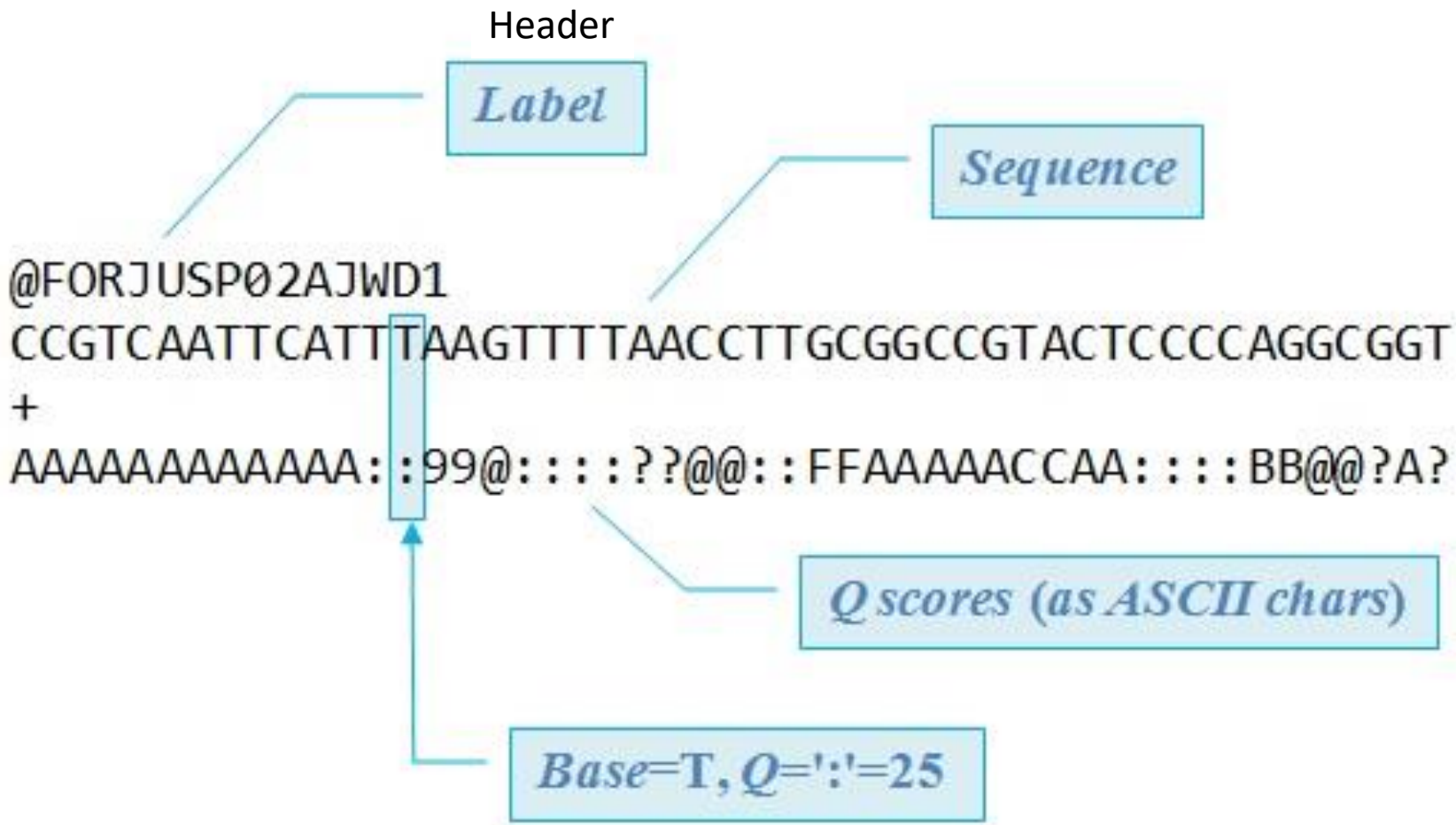
- Number of sites to sequence
- Number of samples
- Depth of coverage

- Example: 1 Hiseq2500 flow cell (about 1000 Euro) ~250 mio read pairs of 125 bp each -> 75 Gb data
  - 5 whole-genomes of a species with 1 Gb genome size at 15x coverage
  - 50 whole-genomes of a species with 500 Mb genome size at 3x coverage
  - 30 Mbp sequenced for 100 samples with a reduced-representation technique at a sequencing depth of 25

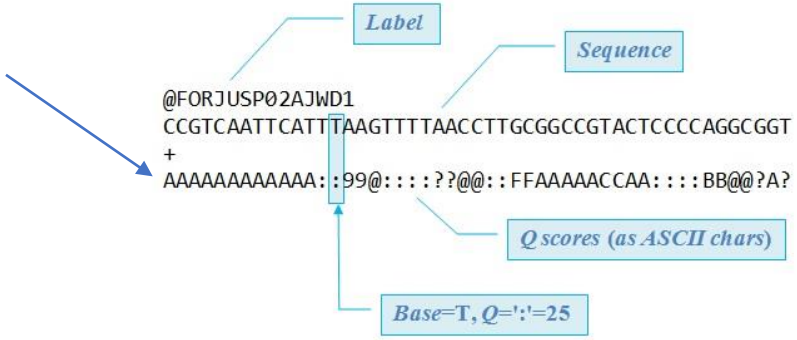
Considerations in choosing the library preparation and sequencing techniques

- Research question and planned analyses
- Genome size
- Availability & quality of reference genome (no ref genome -> not wgs)
- Available budget
- Number of samples to sequence (tradeoff with sequencing depth)
- Amounts of DNA available
- Sequencing depth aimed at
- Divergence between samples
- Heterozygosity of samples
- Phase required
- Accuracy of each single position (if high needed, avoid PCR-based methods)
- Importance of annotations
- Neutral dataset or specific regions wanted

Fastq format



Quality scores



$$\text{Phred} = -10 \log_{10} p$$

p = Probability call is incorrect

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 10000	99.99%

ASCII encoding

40:@	90:Z	141:a
41:A	91:[	142:b
42:B	92:\	143:c
43:C	93:]	144:d
44:D	94:^	145:e
45:E	95:_	146:f
… :…	… :…	… :…

Read header

```
@EAS139:136:FC706VJ:2:2104:15343:197393 1:Y:18:ATCACG
```

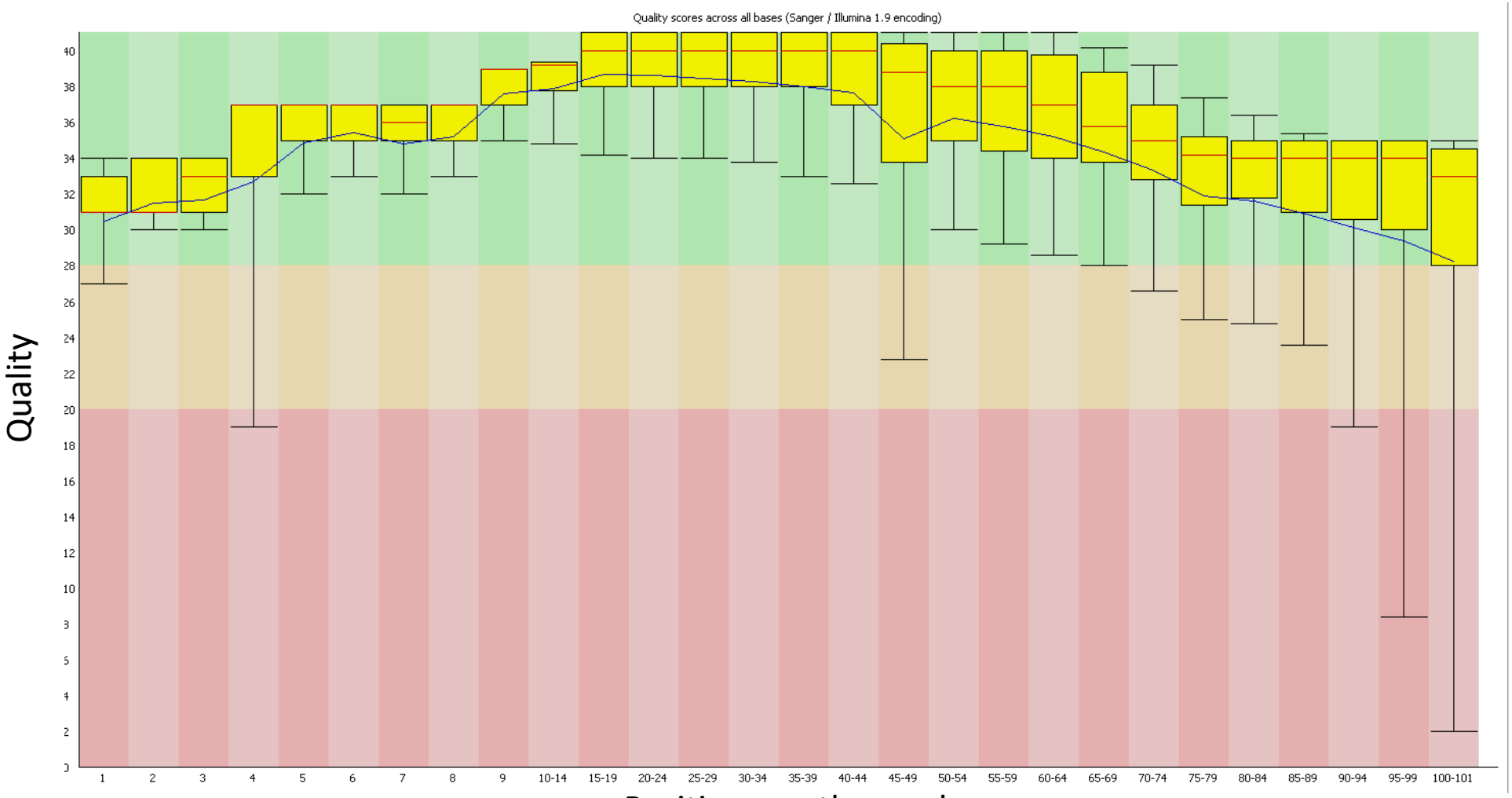
EAS139	the unique instrument name
136	the run id
FC706VJ	the flowcell id
2	flowcell lane
2104	tile number within the flowcell lane
15343	'x'-coordinate of the cluster within the tile
197393	'y'-coordinate of the cluster within the tile
1	the member of a pair, 1 or 2 *(paired-end or mate-pair reads only)*
Y	Y if the read is filtered, N otherwise
18	0 when none of the control bits are on, otherwise it is an even number
ATCACG	index sequence

FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)

Summary

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

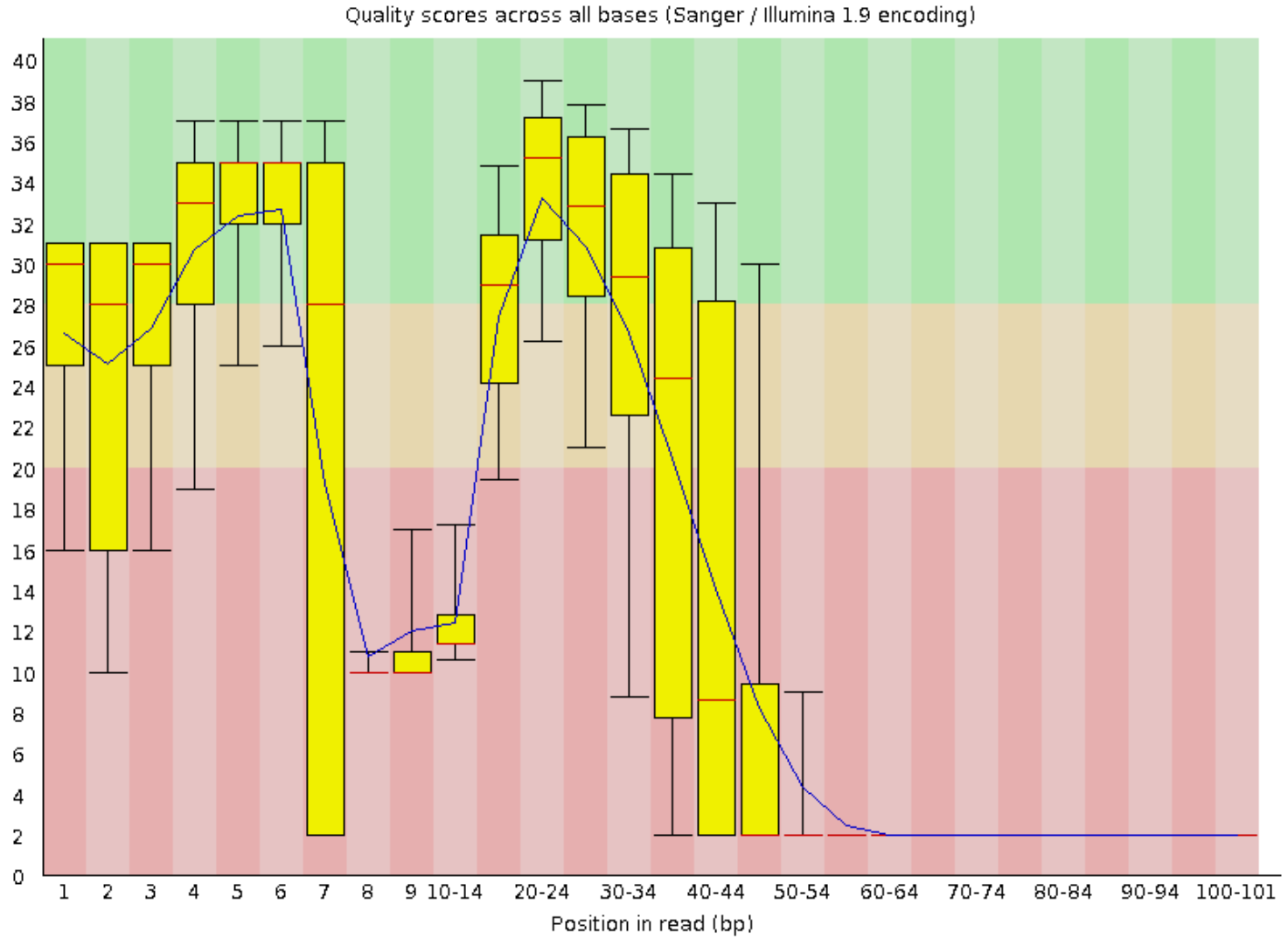
FastQC: Quality across bases (good example)



>Q30
Error prob. < 0.001

<Q20
Error prob. < 0.01

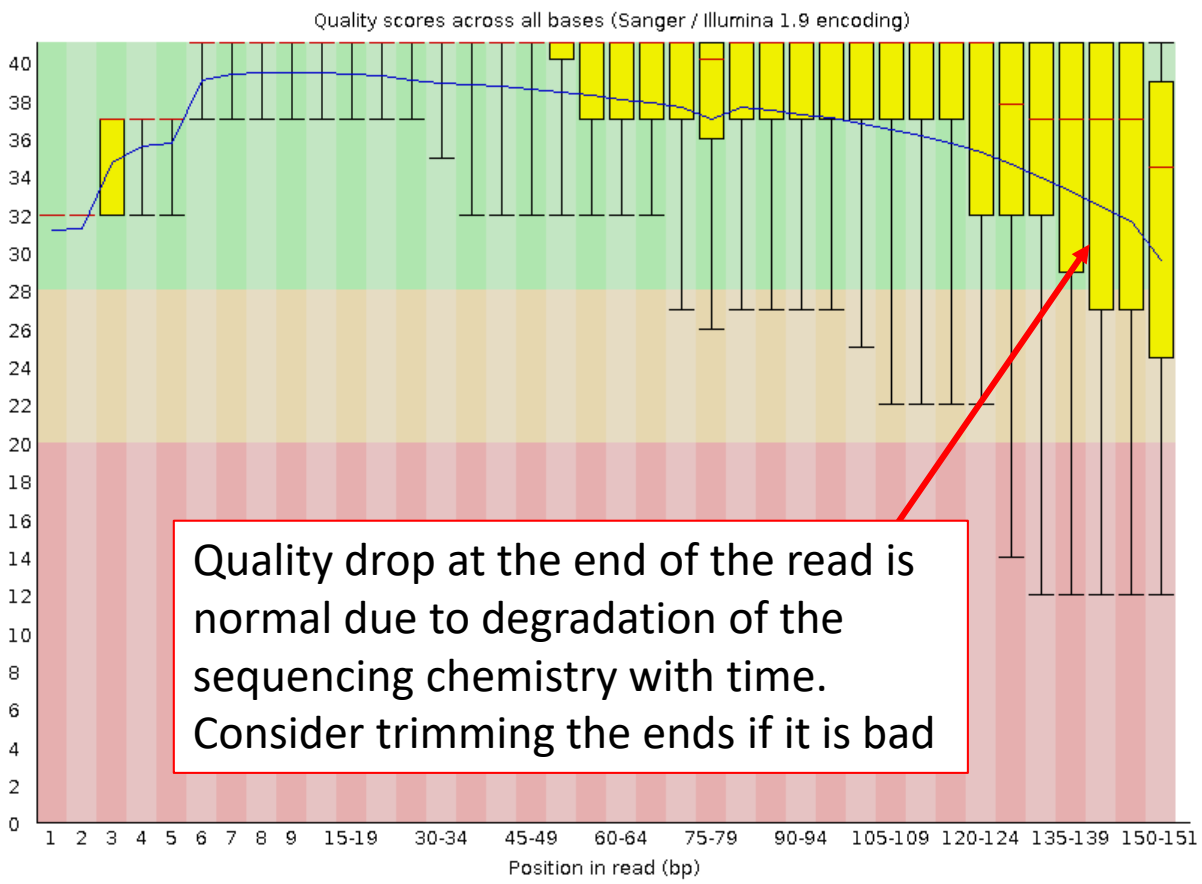
FastQC: Quality across bases (bad example)



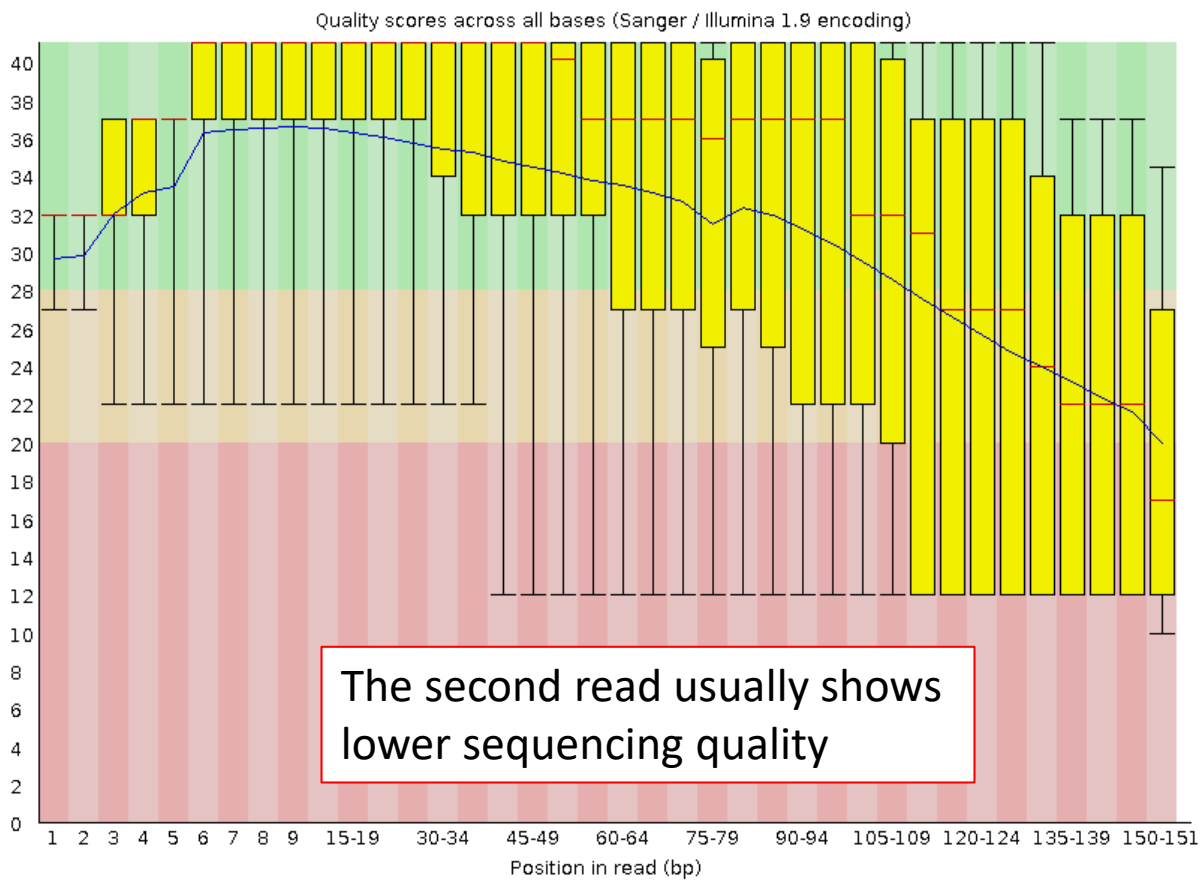
Let’s have a look at the first few sequences and check the sequencing quality with fastqc

Quality scores across bases:
Whole genome sequencing (PCR free library prep)

forward

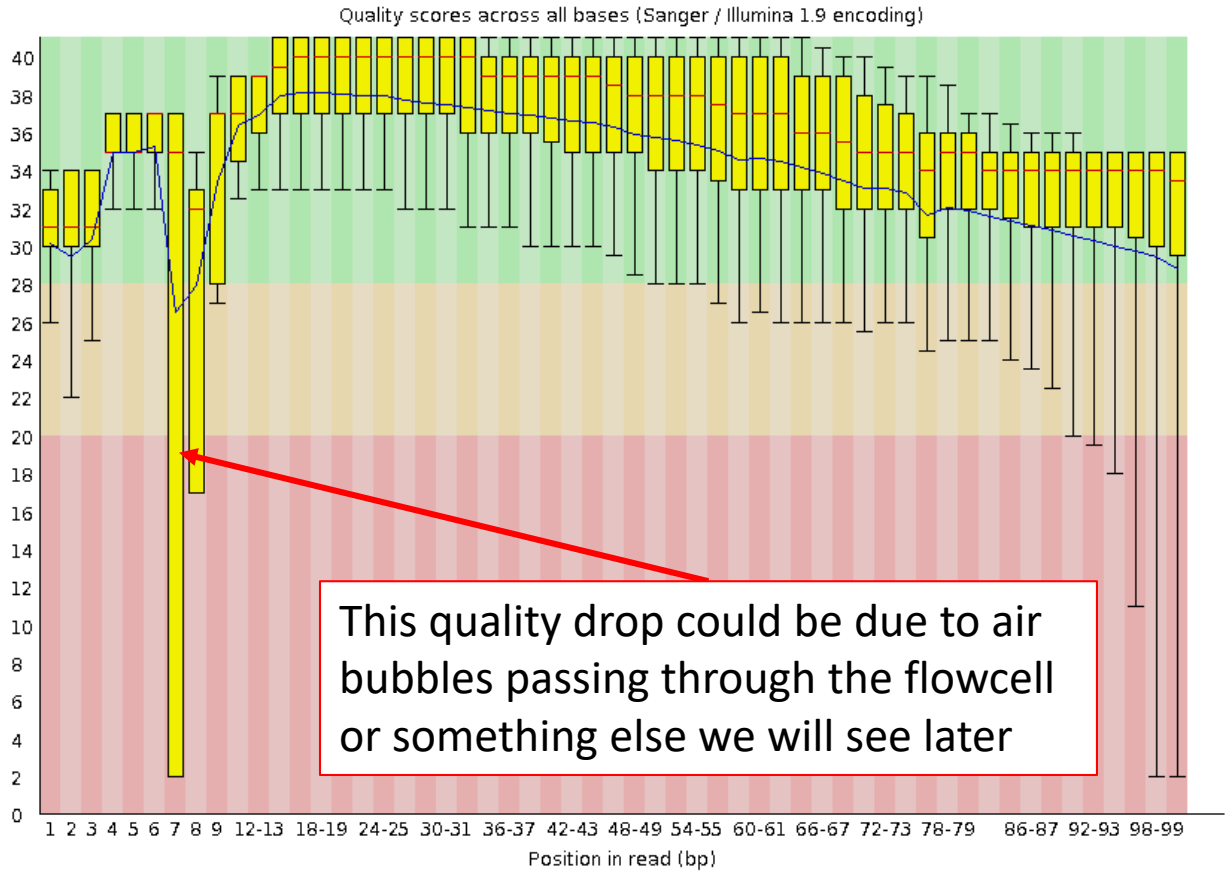


reverse

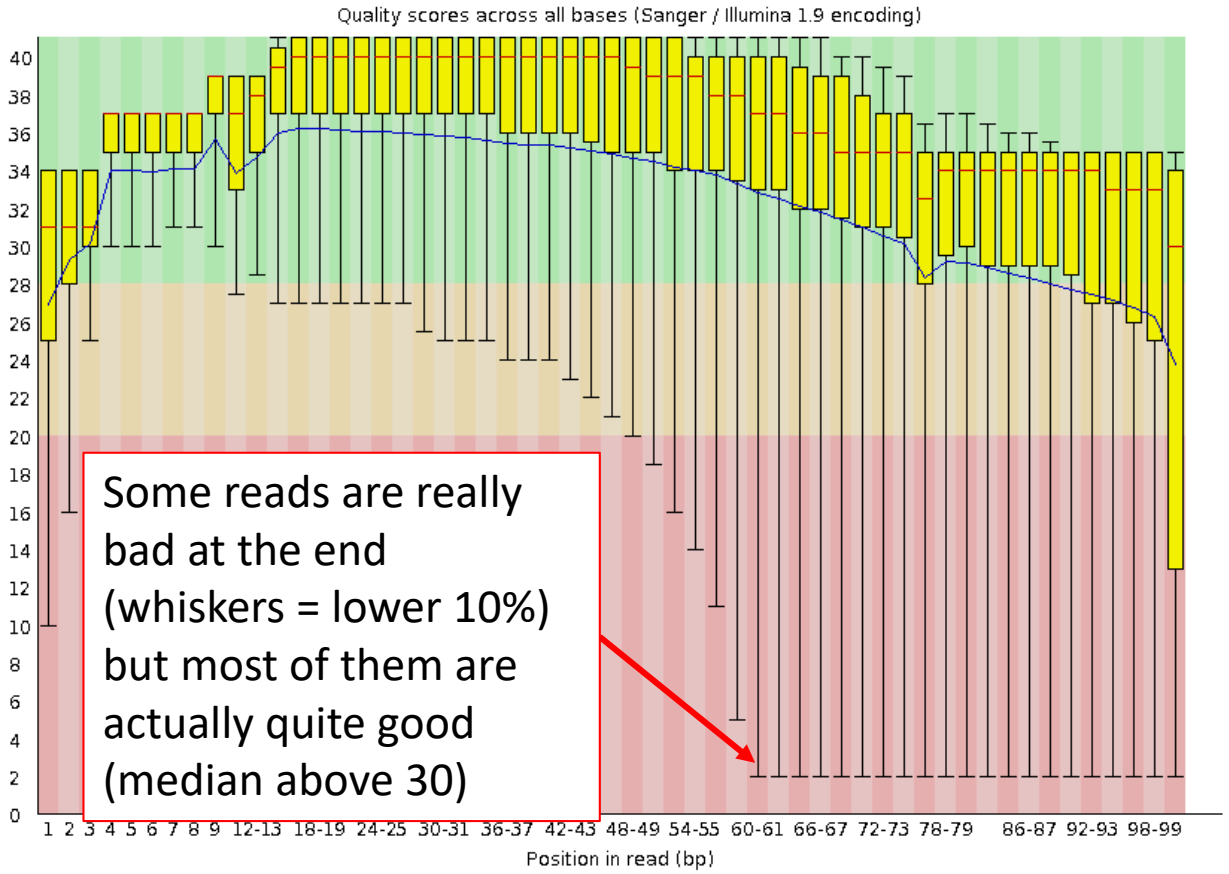


Quality scores across bases: RAD datasets

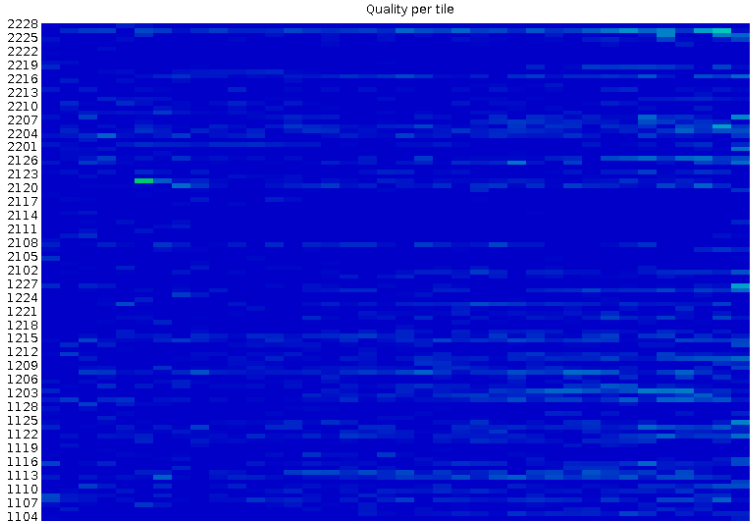
RAD1



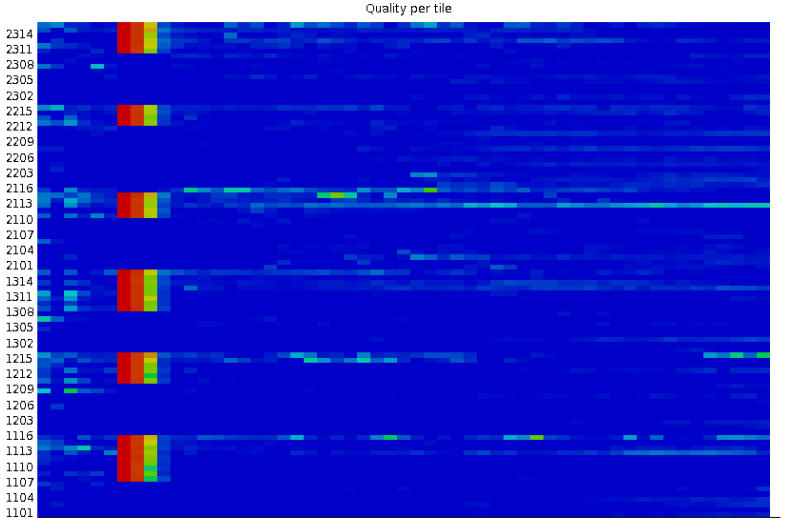
RAD2



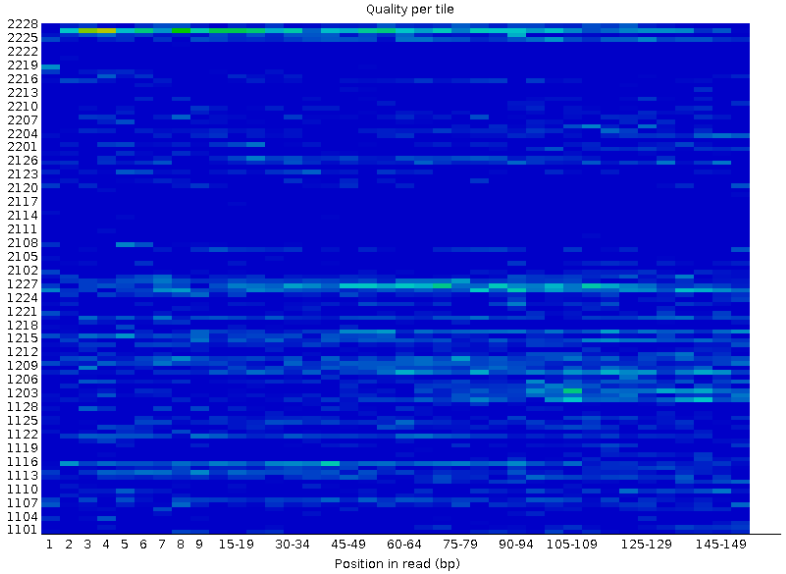
Per tile sequencing quality



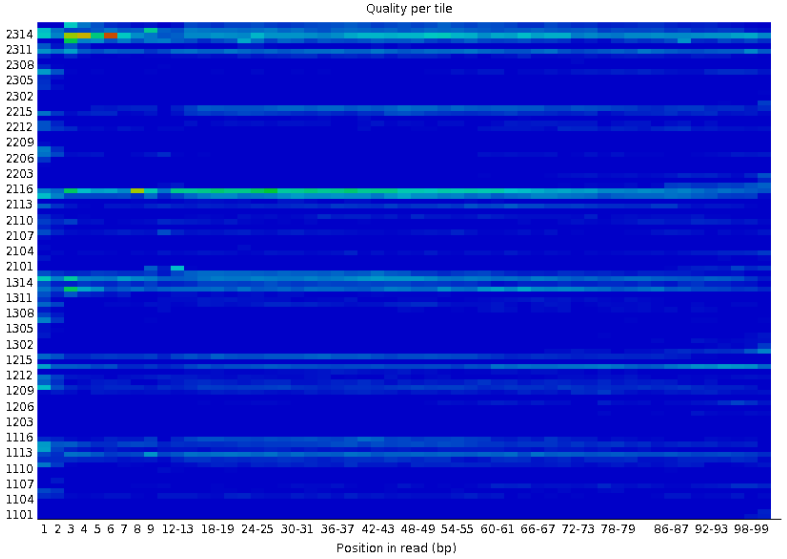
Forward reads wgs



RAD1

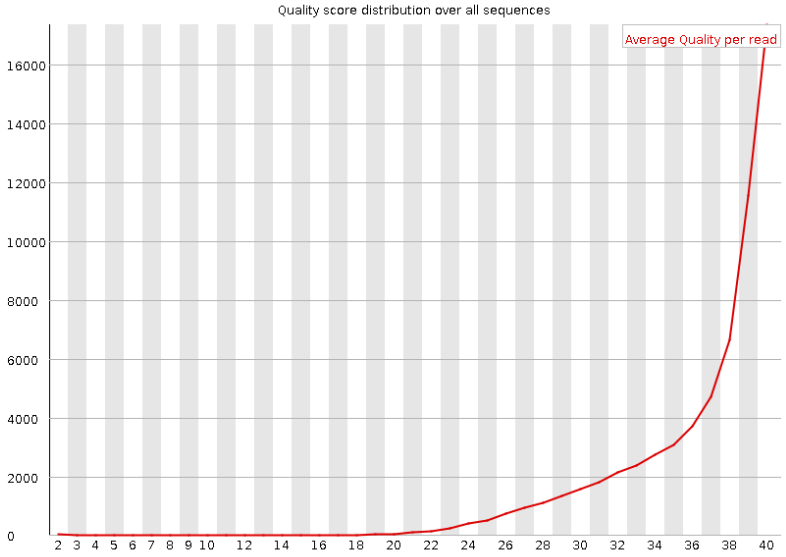


Reverse reads wgs

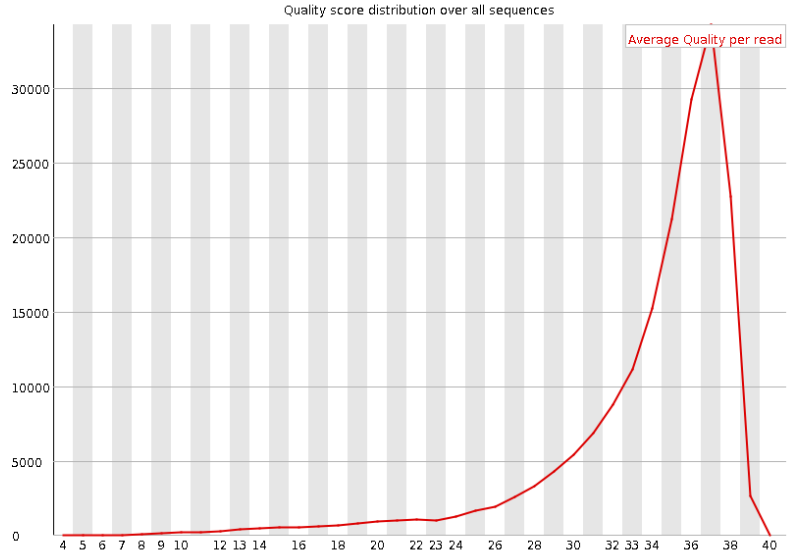


RAD2

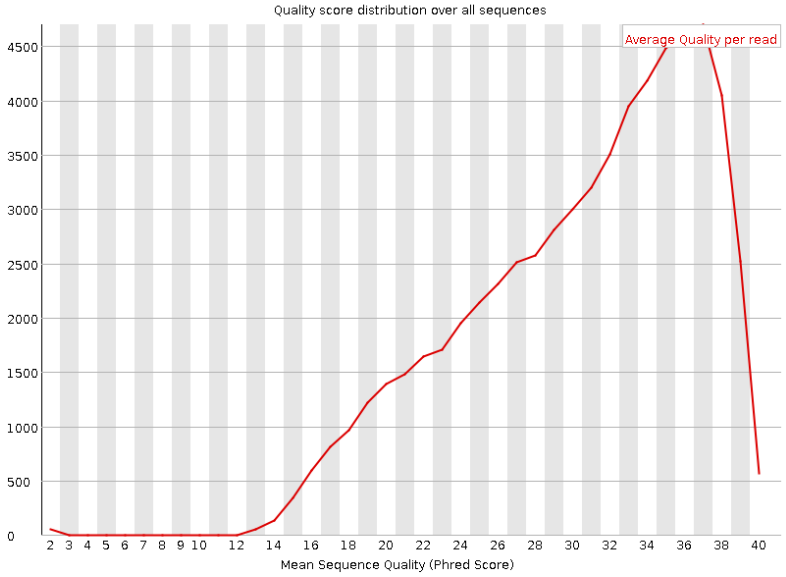
Quality score distribution over all sequences



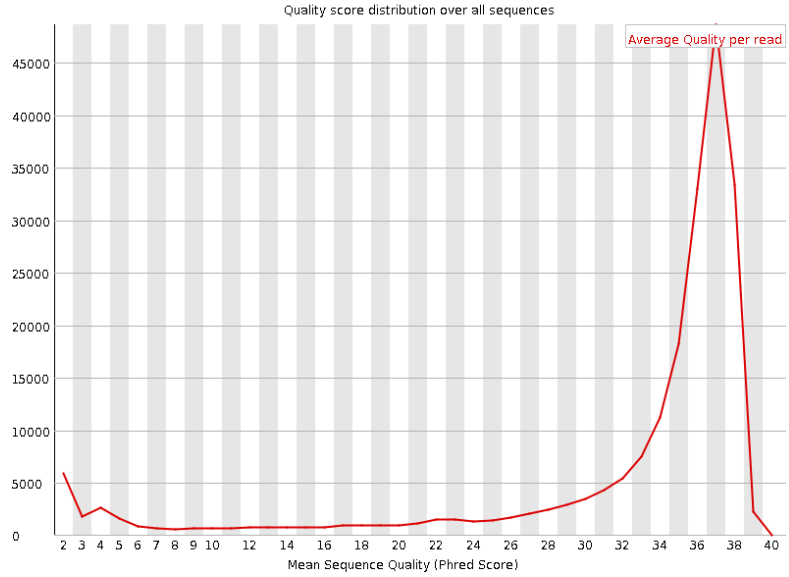
Forward reads wgs



RAD1



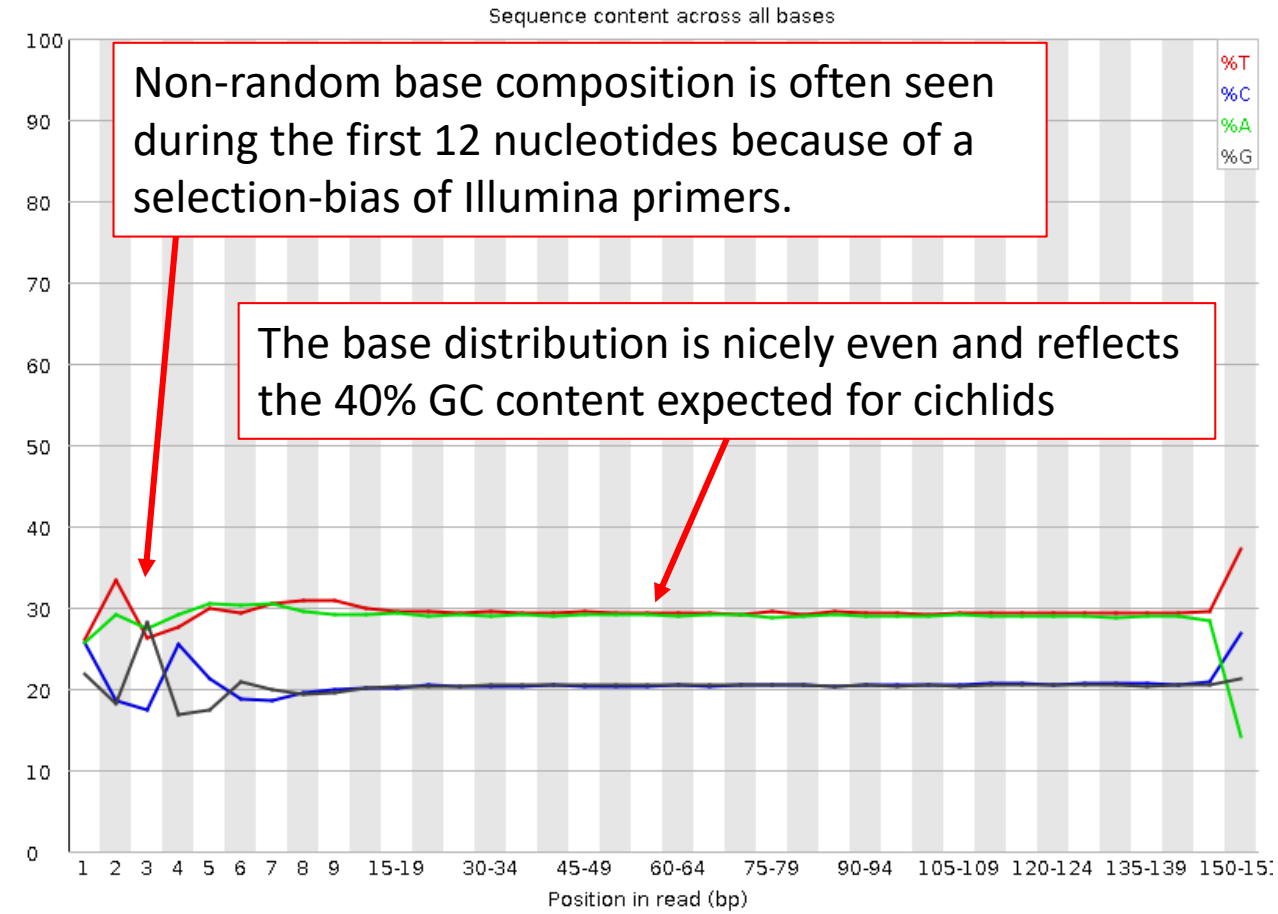
Reverse reads wgs



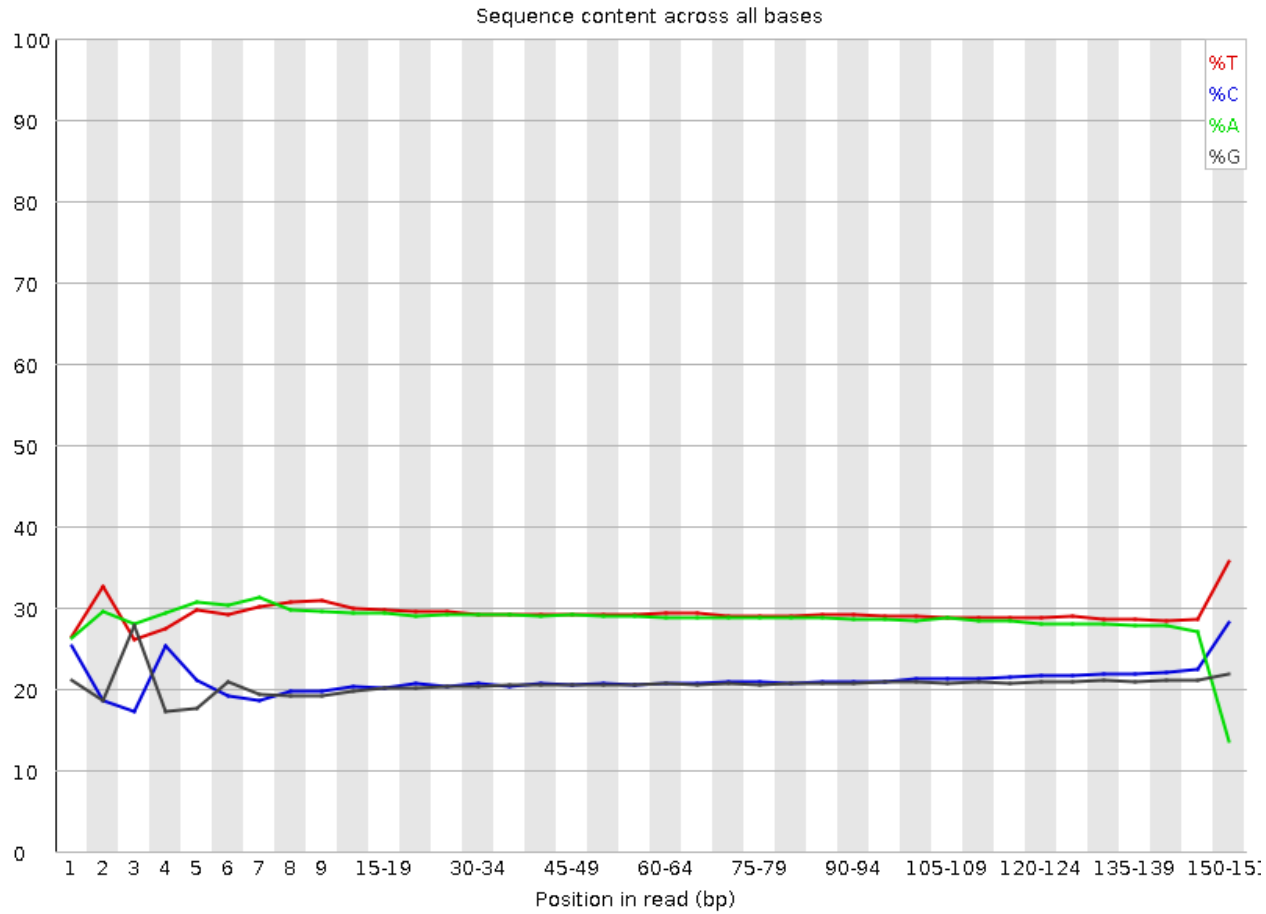
RAD2

Per base sequence content

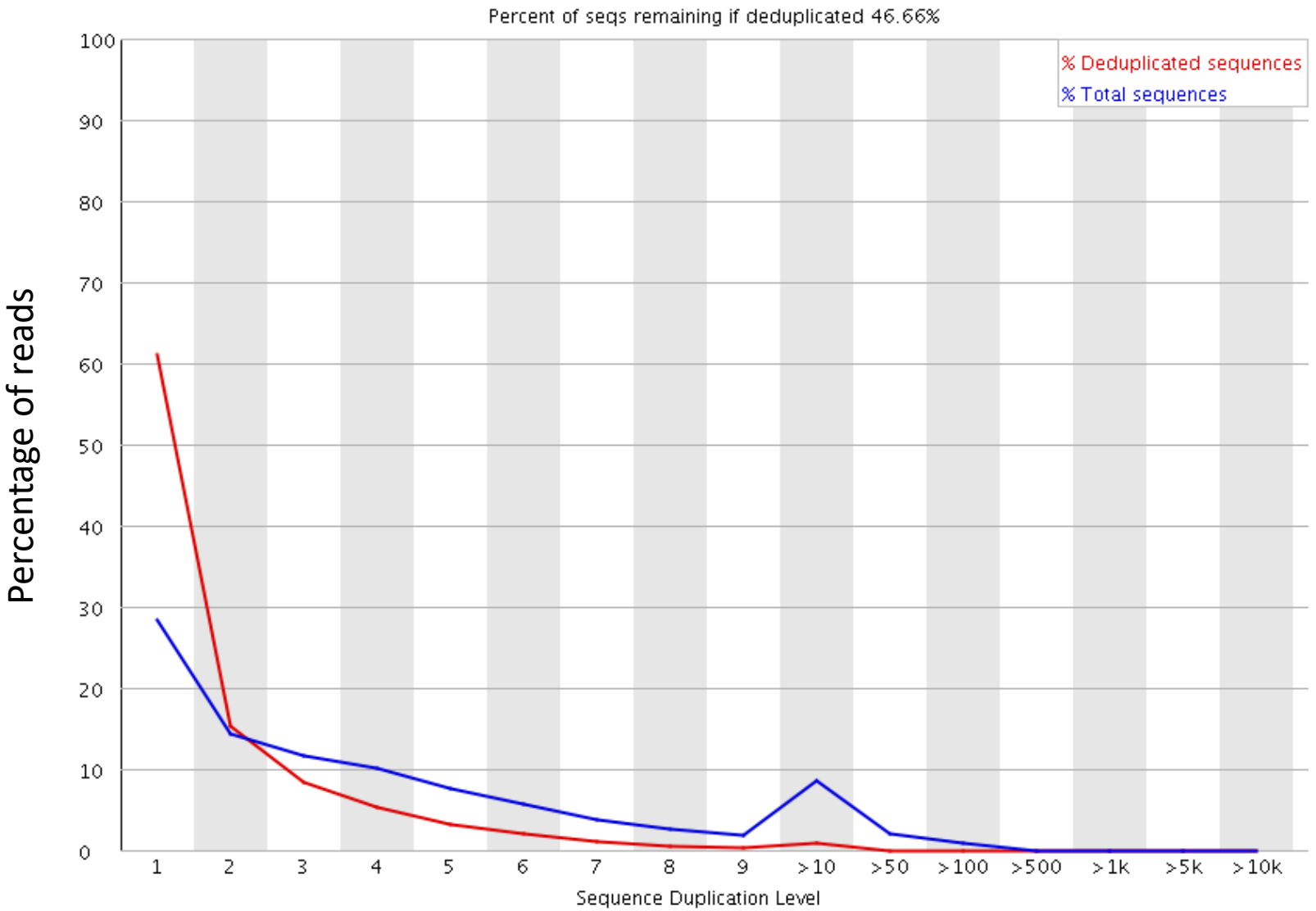
forward



reverse



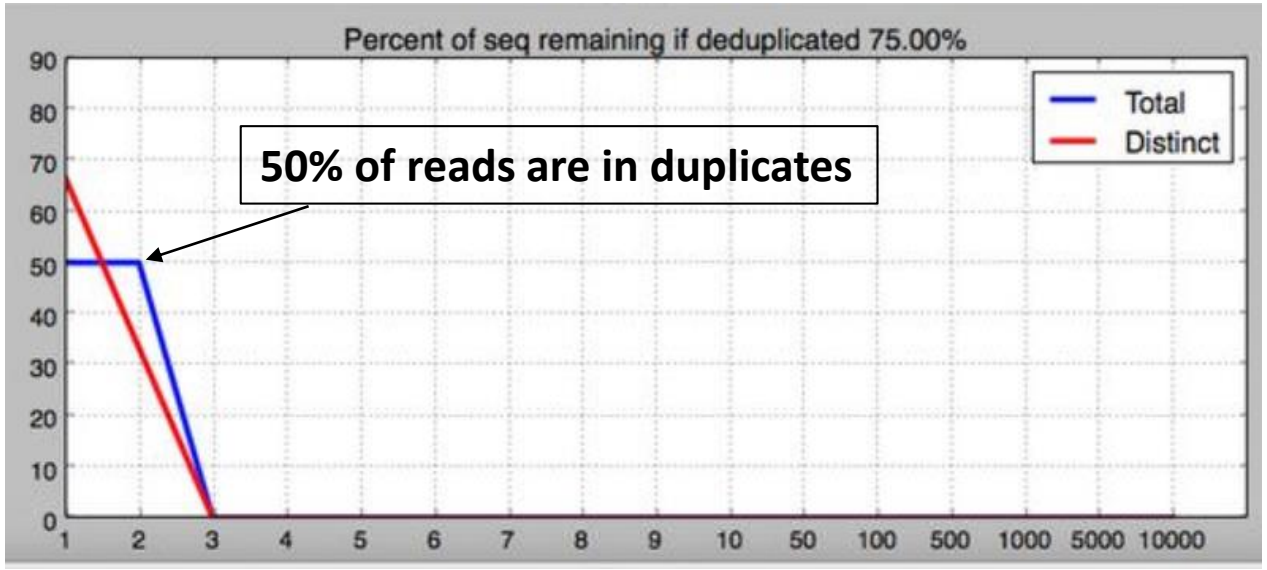
Sequence duplication level



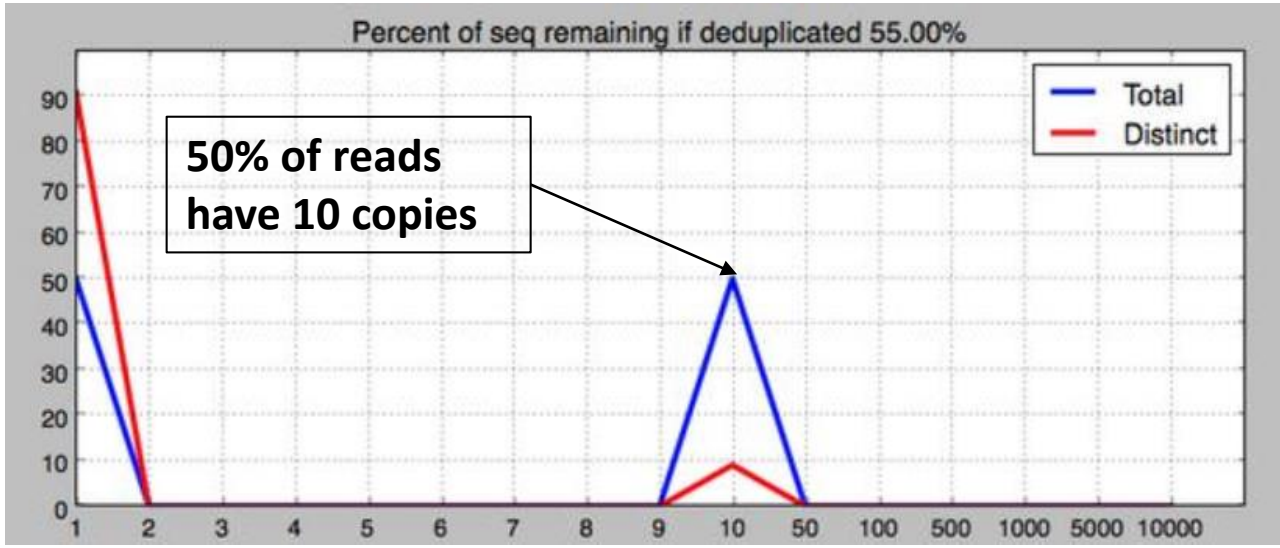
Duplication level:
= Percentage of reads that have x copies

Sequence duplication level

Example 1: 20 reads total
10 unique sequences + 5 sequences each present twice



Example 2: 20 reads total
10 unique sequences + 1 sequence present 10x



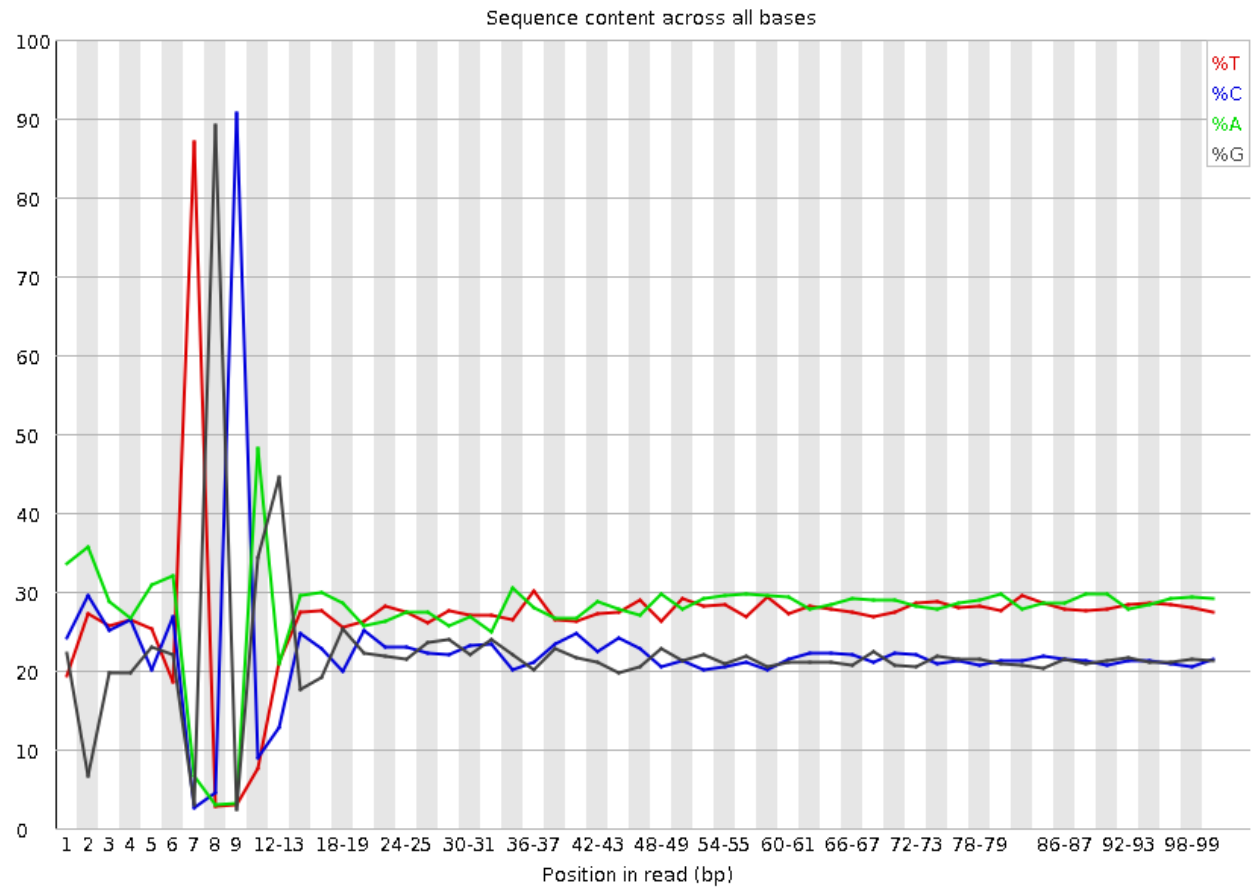
Deduplicated sequences (=number of distinct copies)
15 distinct sequences are distributed as 10 singletons and 5 duplicates, 10/15=66% and 5/15=33% is the slope of the red line. Thus 15/20=75% remaining after deduplication (distinct reads).

Deduplicated sequences (=number of distinct copies)
11 total groups where 10/11=91% are singletons and 1/11=9% of the groups form at duplication rate of 10x. Therefore, 11/20 = 55% distinct reads.

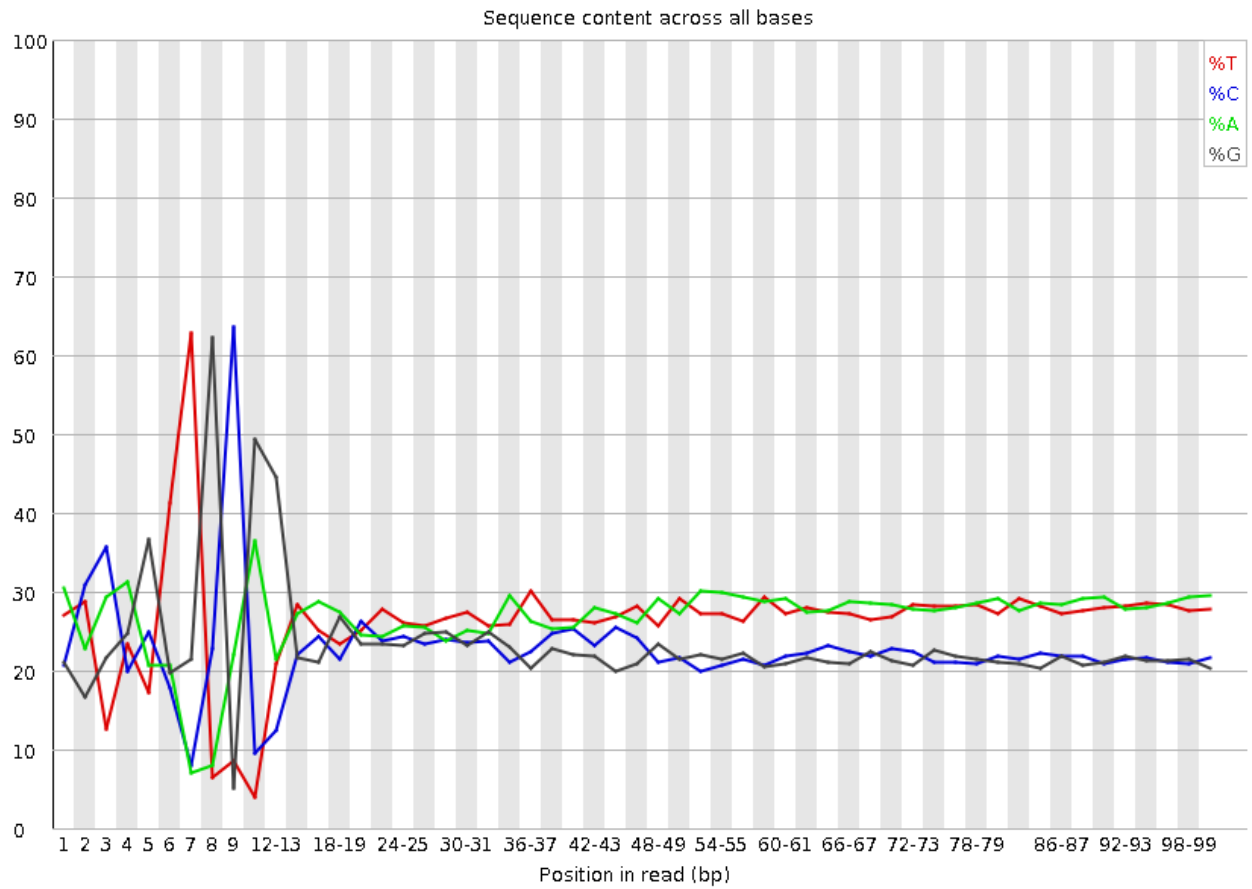
Examples and plots from https://www.biostars.org/p/107402/

Per base sequence content

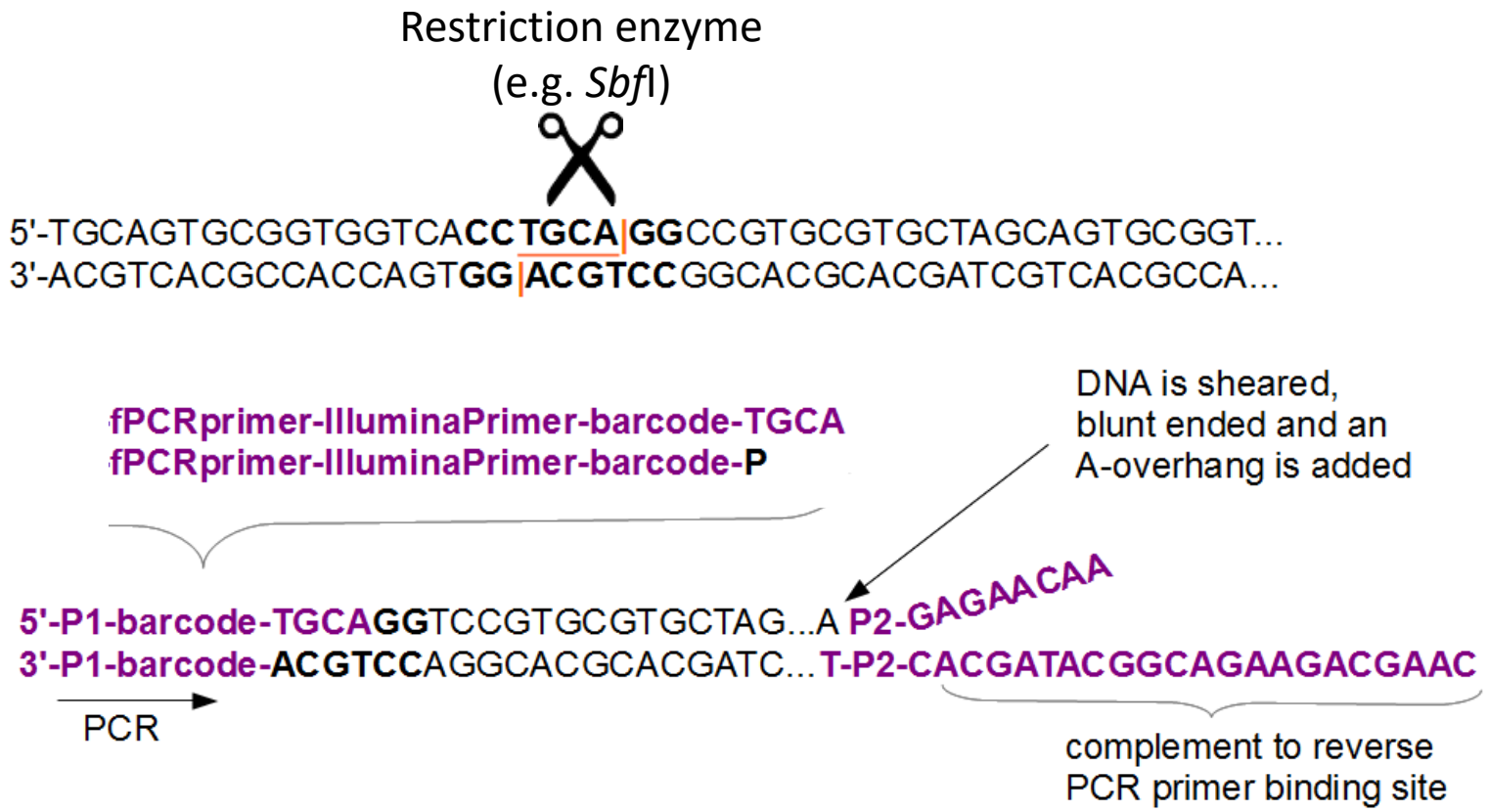
RAD1



RAD2



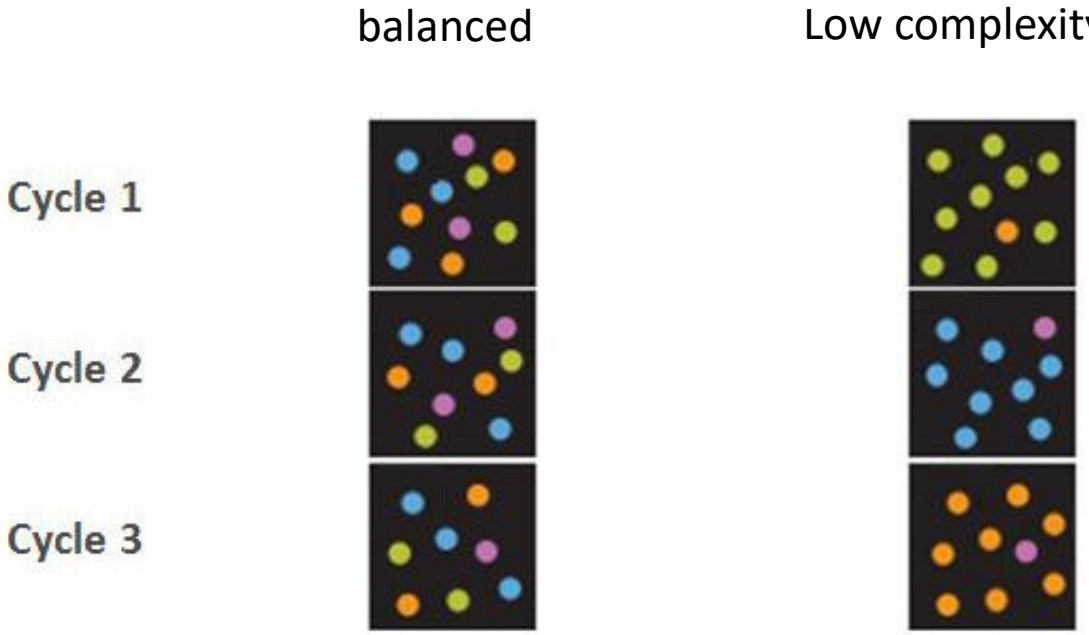
RAD/GBS



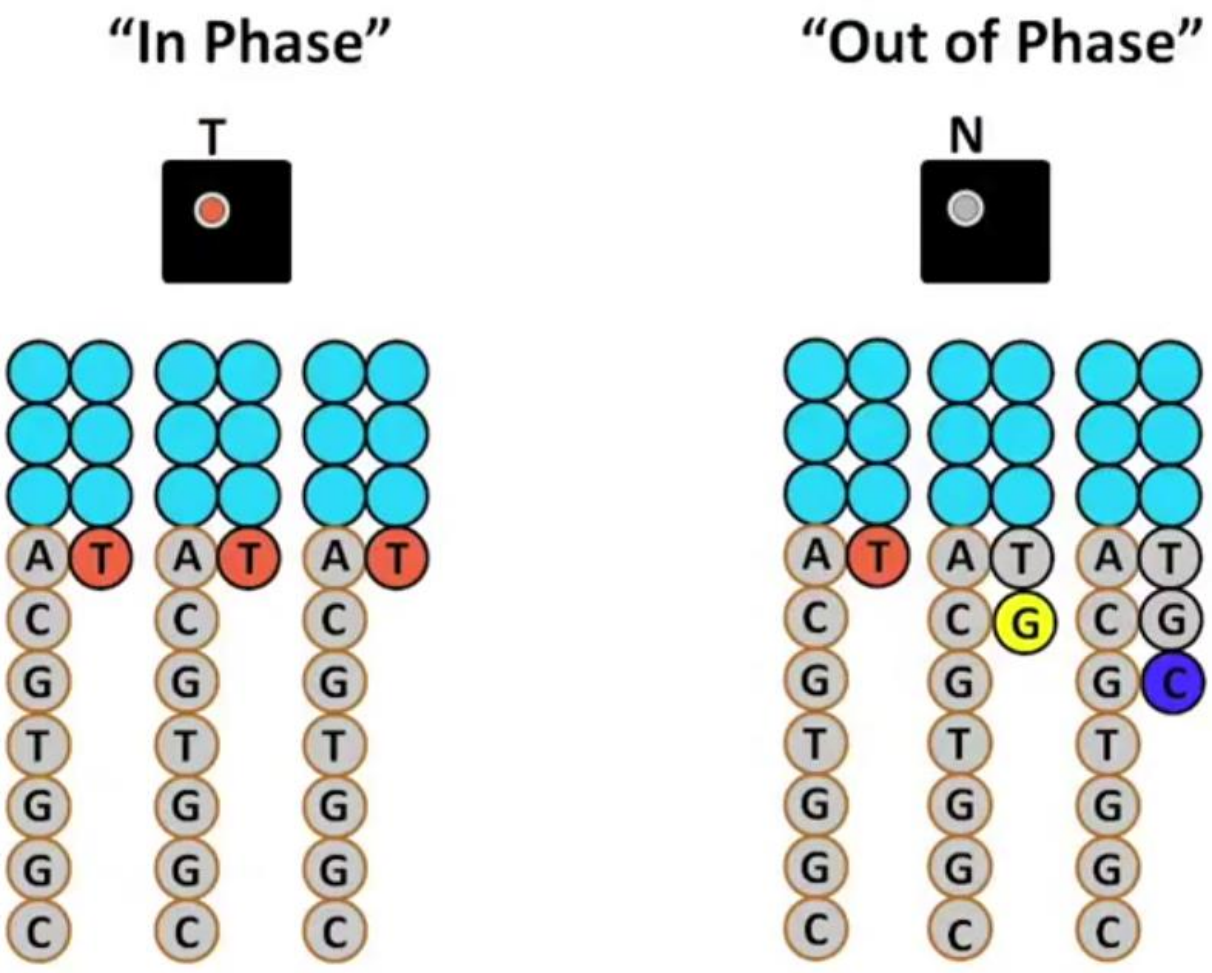
Each read: will start with the barcode, then the restriction site, then a variable sequence

Issues with cluster identification

Due to low complexity at the beginning of the sequence,
Illumina cannot distinguish if a signal comes from one or two clusters



Phasing issues

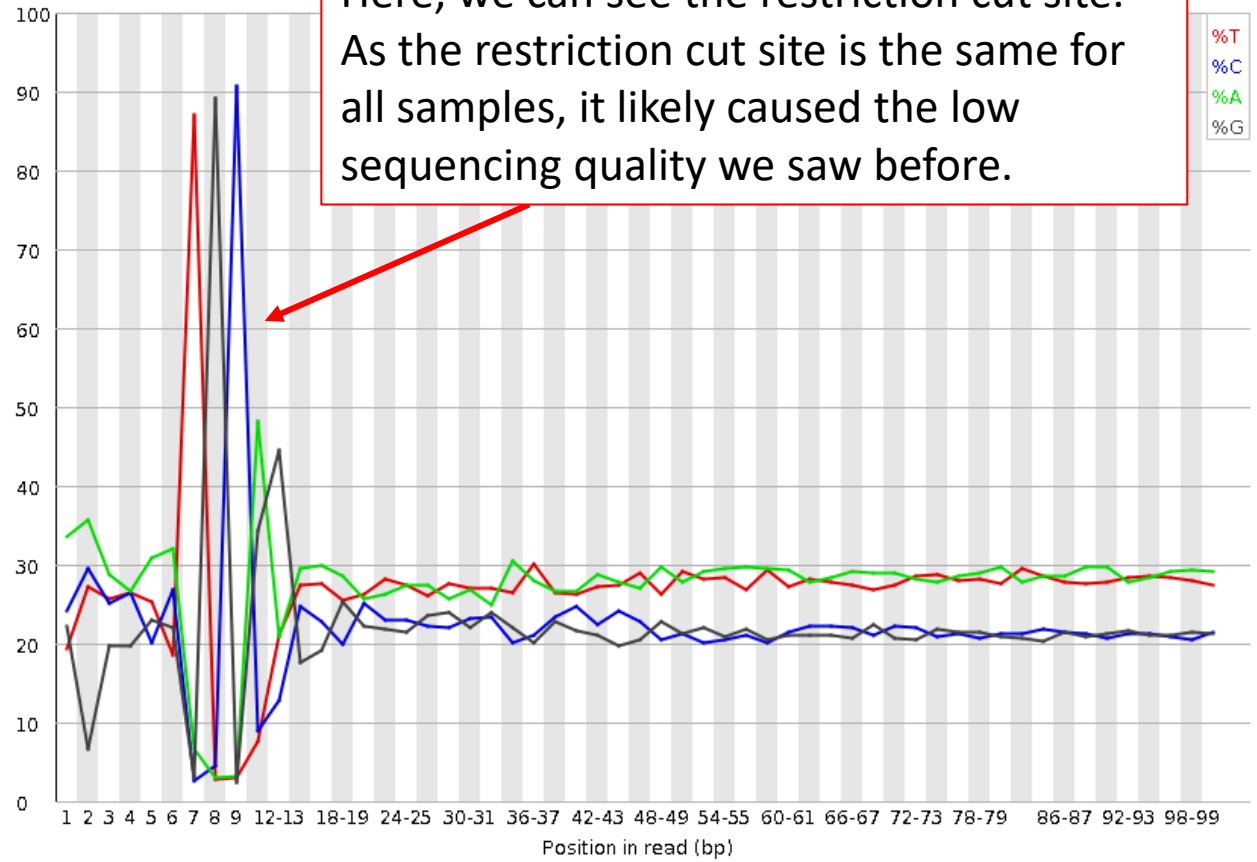


The first 12 nucleotides are also used for «phasing», i.e. correcting for reads that are out of phase. The algorithm expects random nucleotide distribution!

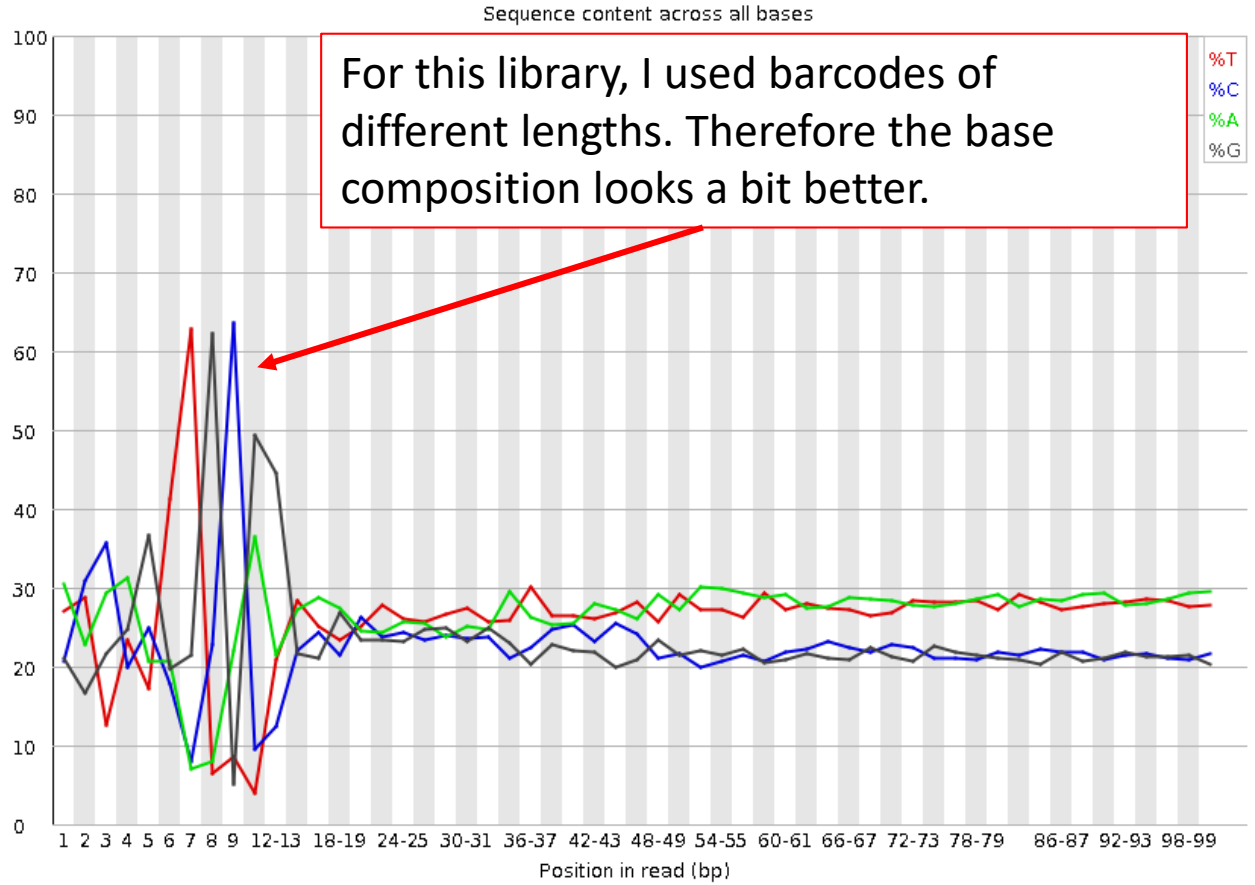
-> Barcodes of the same length may lead to low quality overall

Per base sequence content

Here, we can see the restriction cut site. As the restriction cut site is the same for all samples, it likely caused the low sequencing quality we saw before.



For this library, I used barcodes of different lengths. Therefore the base composition looks a bit better.

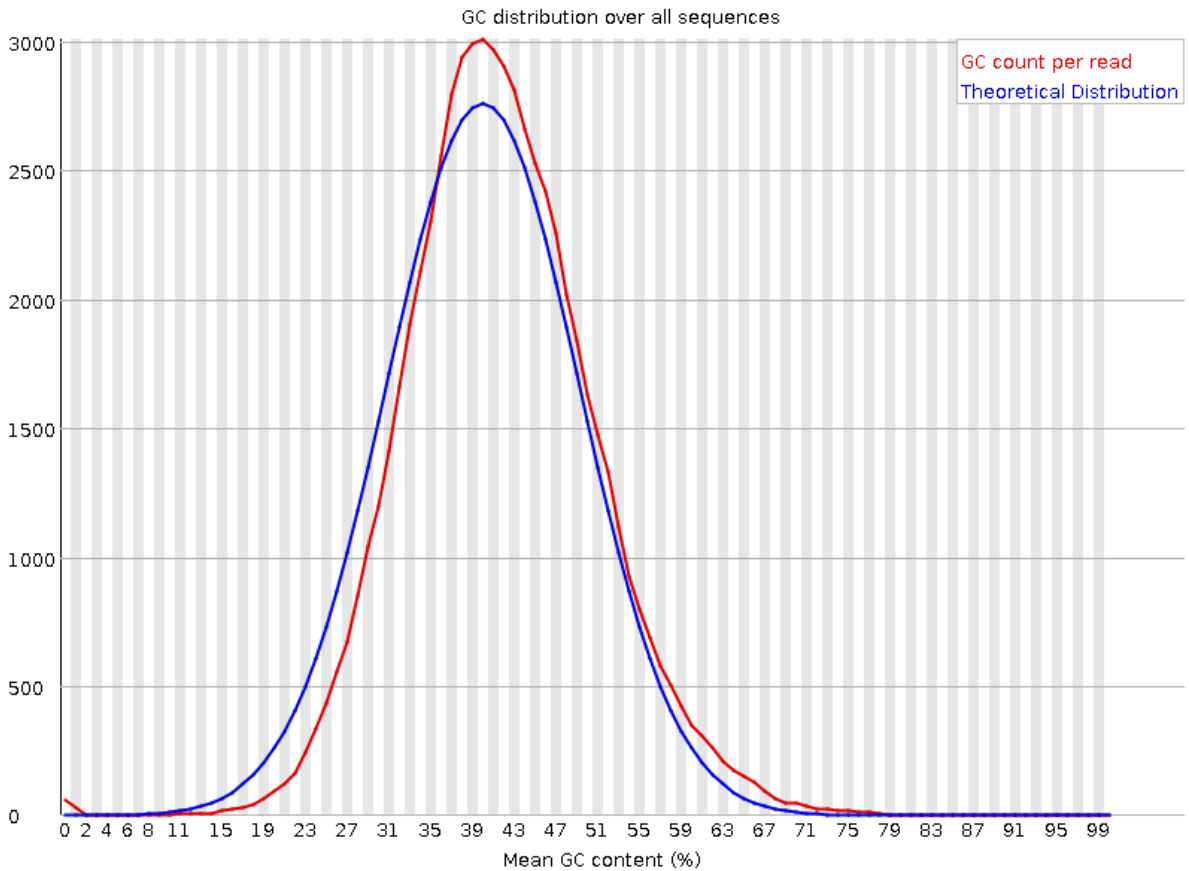


How to minimize the problem

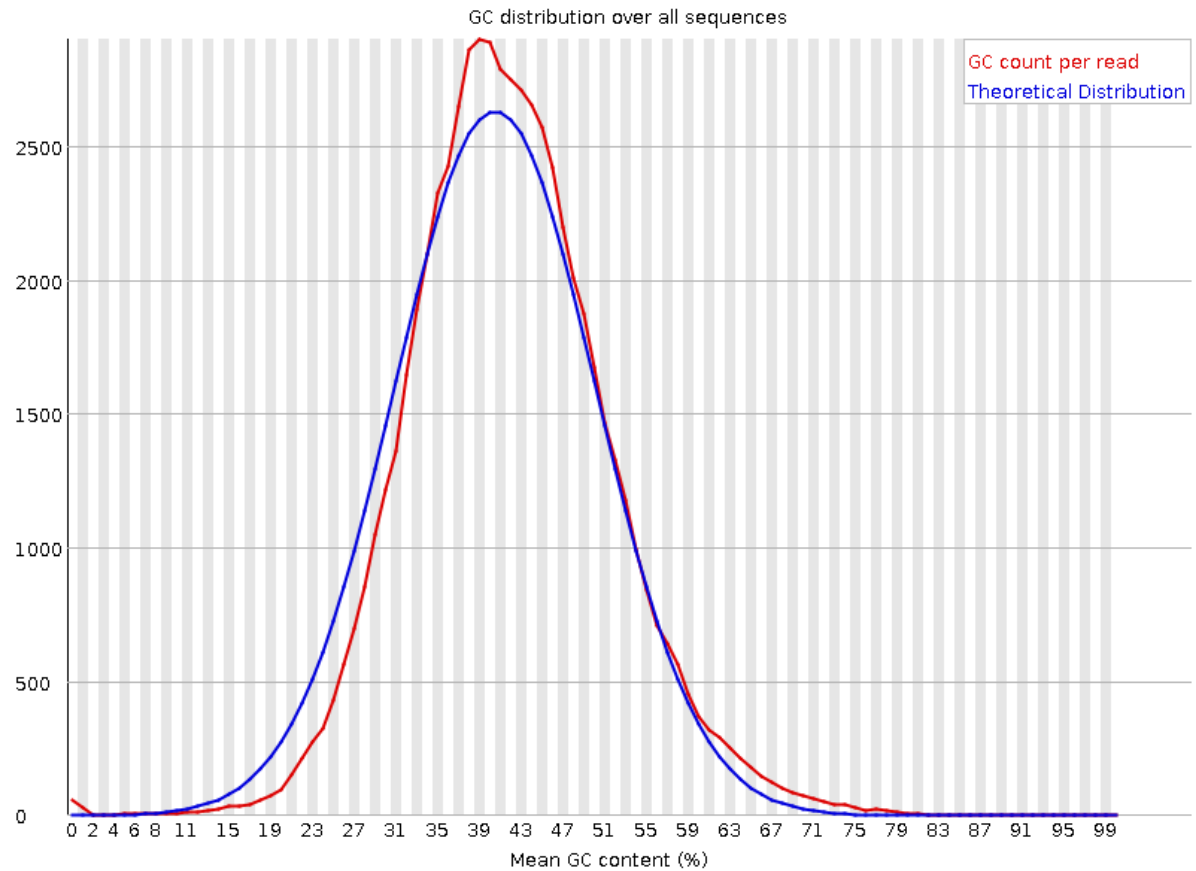
- Use barcodes of different lengths to shift the restriction enzyme cut site
- Add PhiX virus DNA to the RAD libraries to increase the complexity of reads (‘spiking’)
- Reduce loading concentrations of Illumina plates
- Potentially: filter out bad reads

GC distribution over all sequences

forward

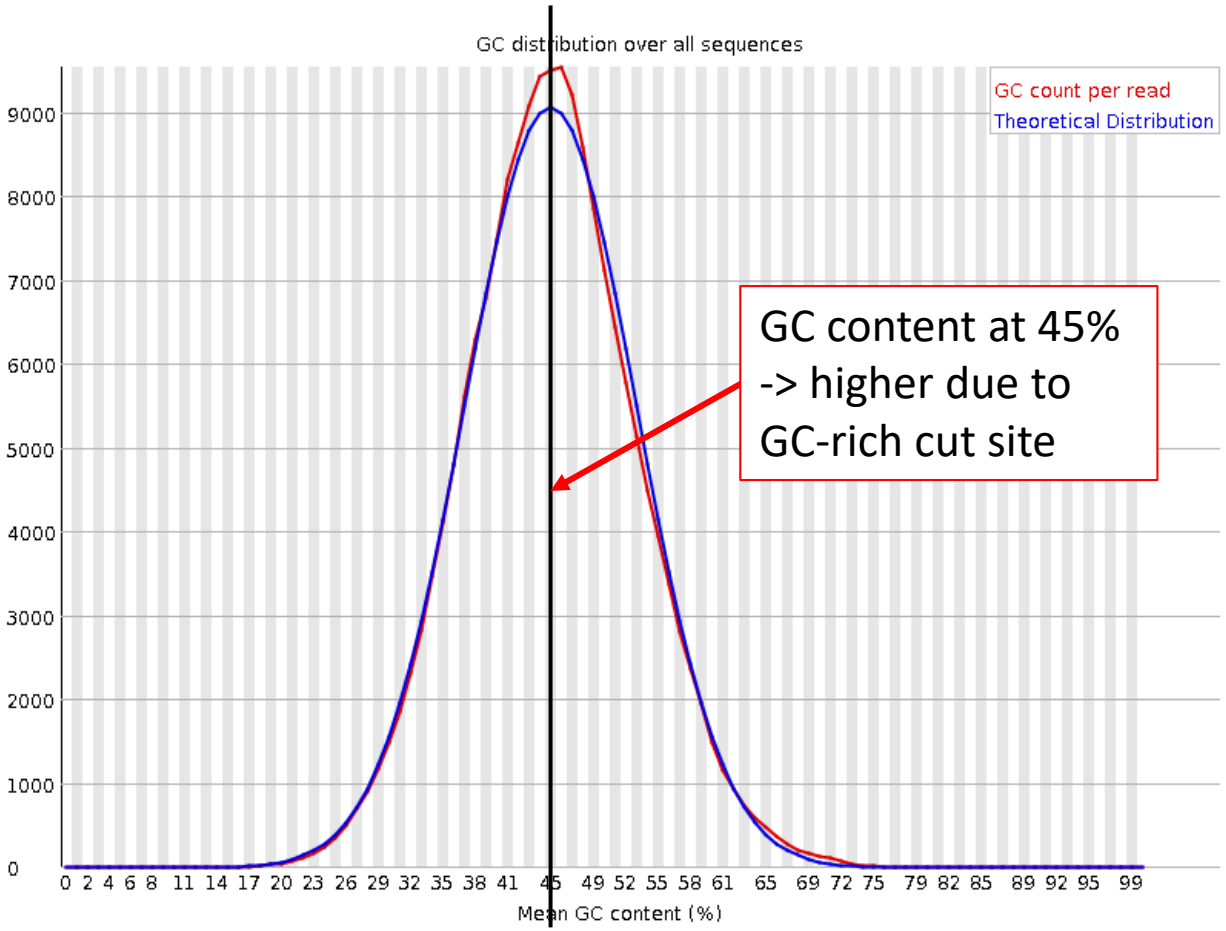


reverse

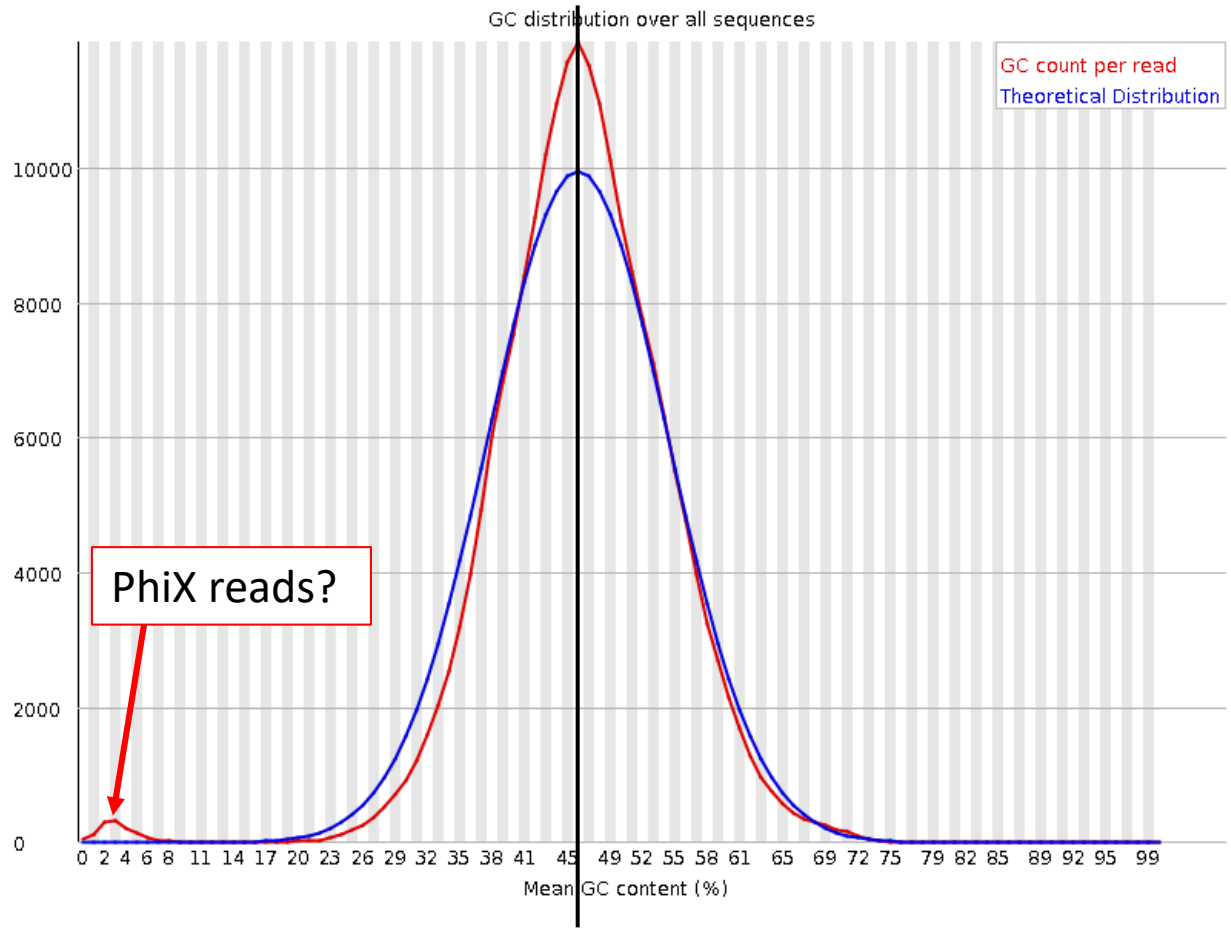


GC distribution over all sequences

RAD1

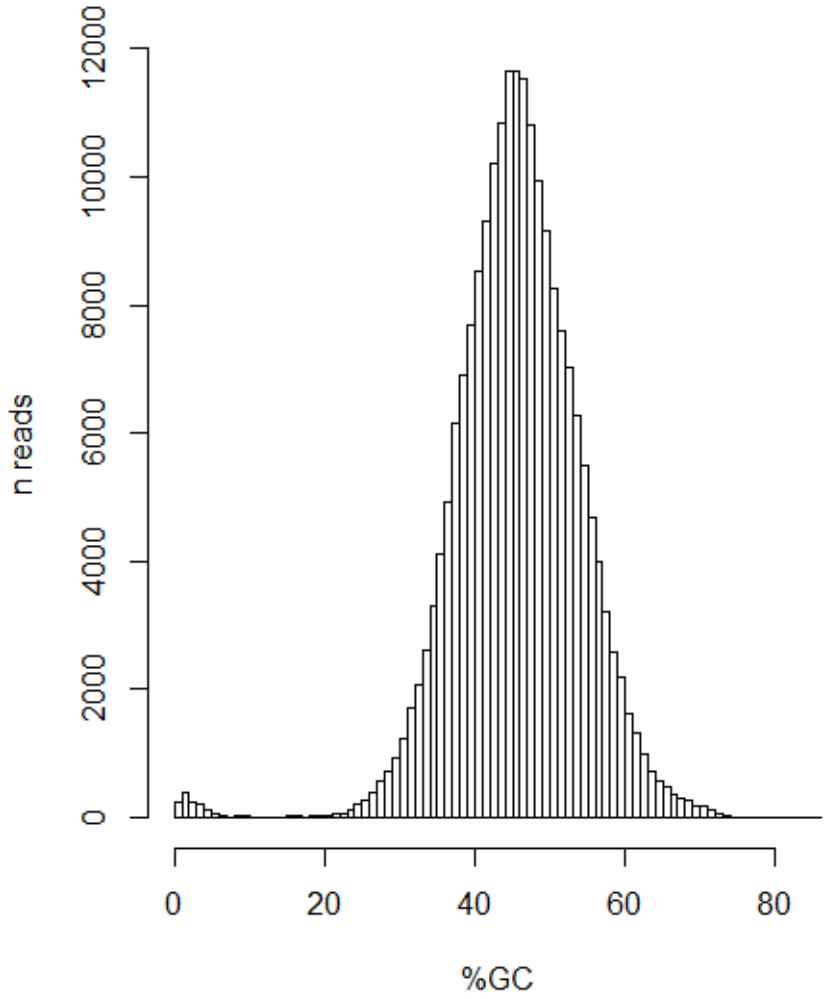


RAD2

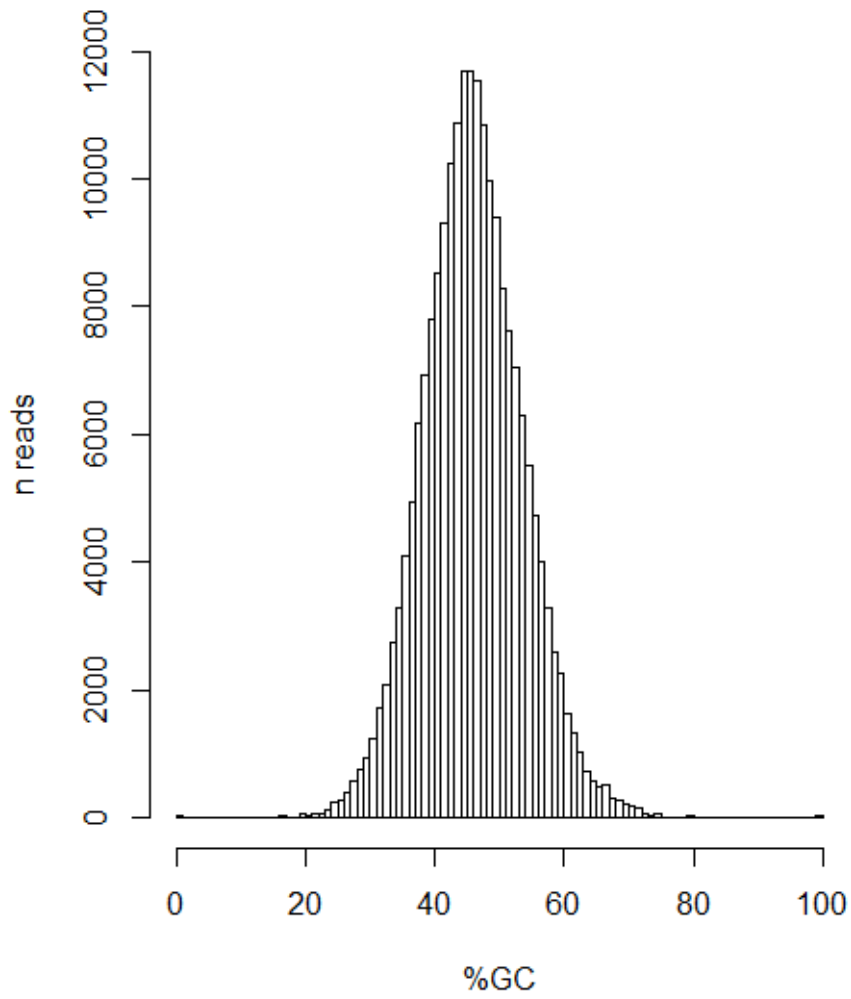


GC distribution over all sequences

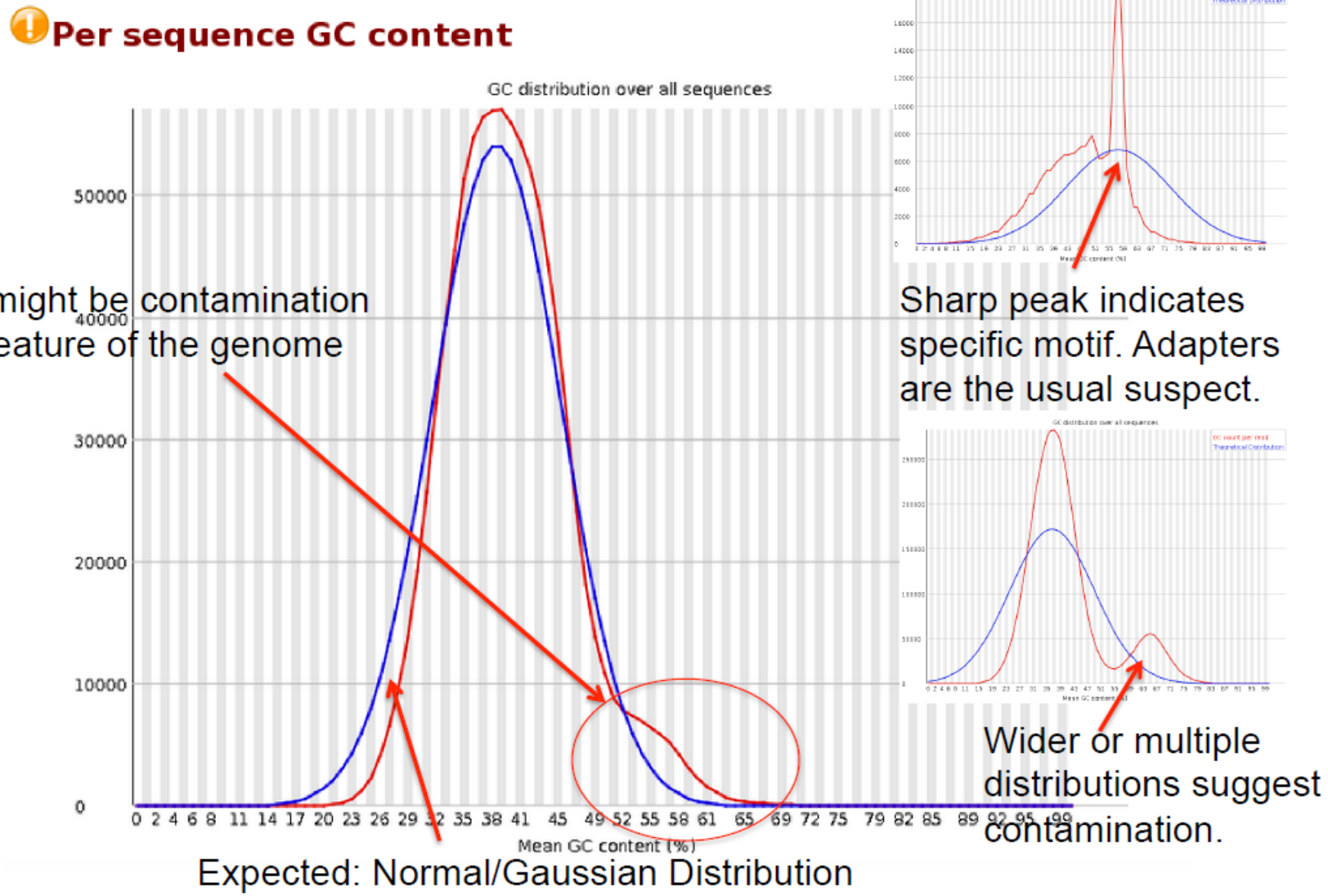
GC/(total sites)



Excluding Ns from total count



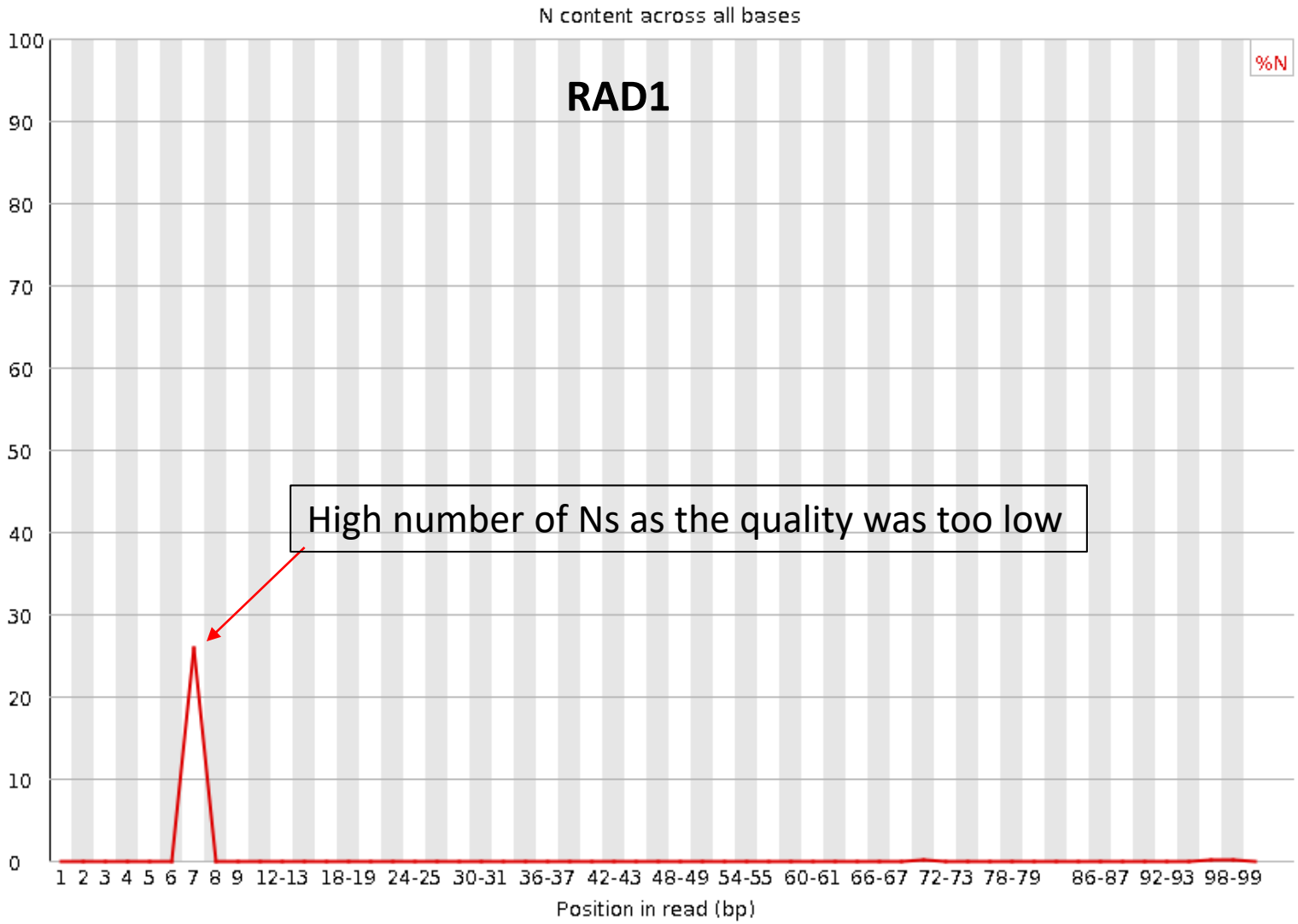
Fastqc: Per sequence GC content



Sharp peak indicates specific motif. Adapters are the usual suspect.

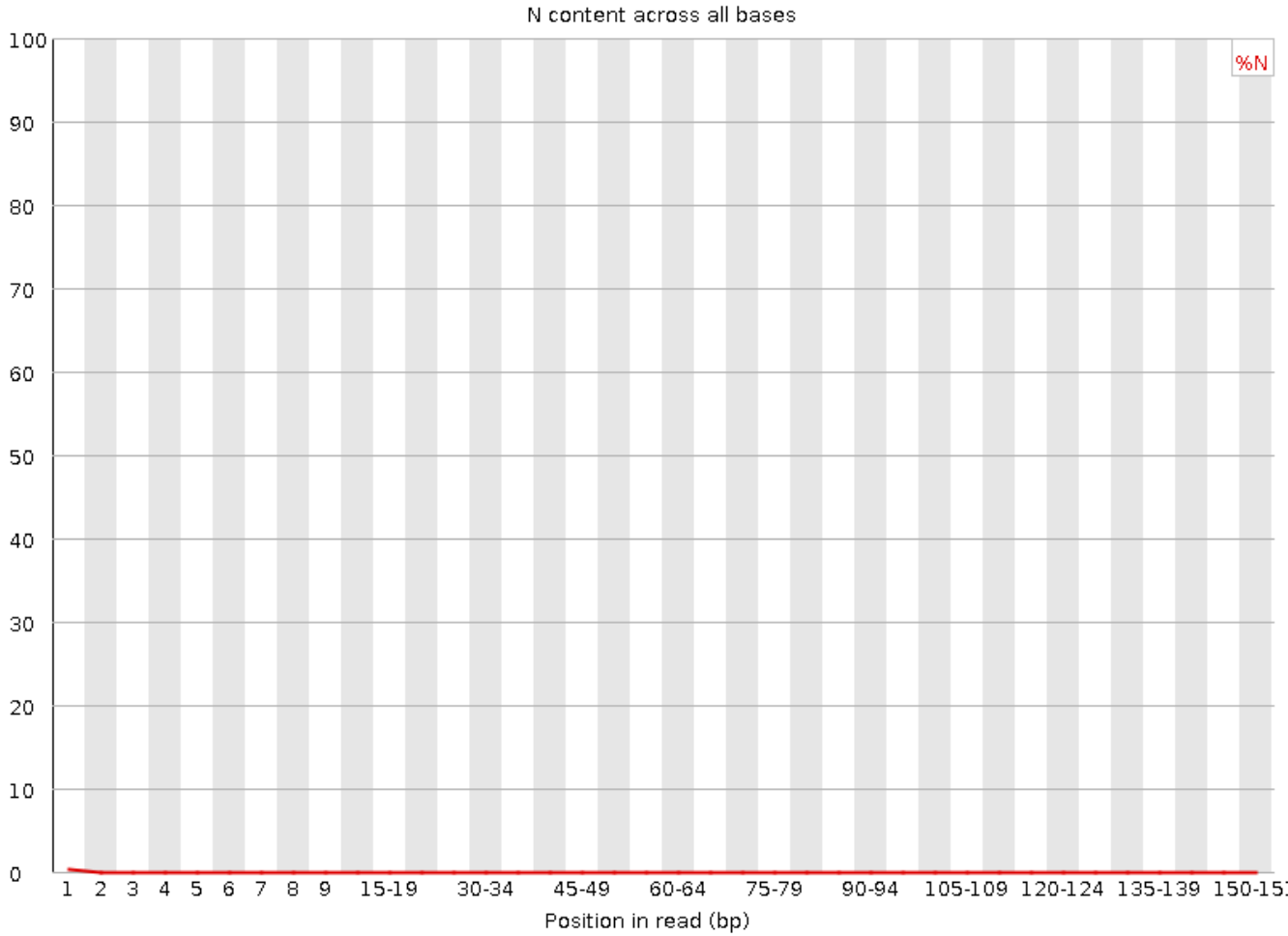
Wider or multiple distributions suggest contamination.

Per base N content

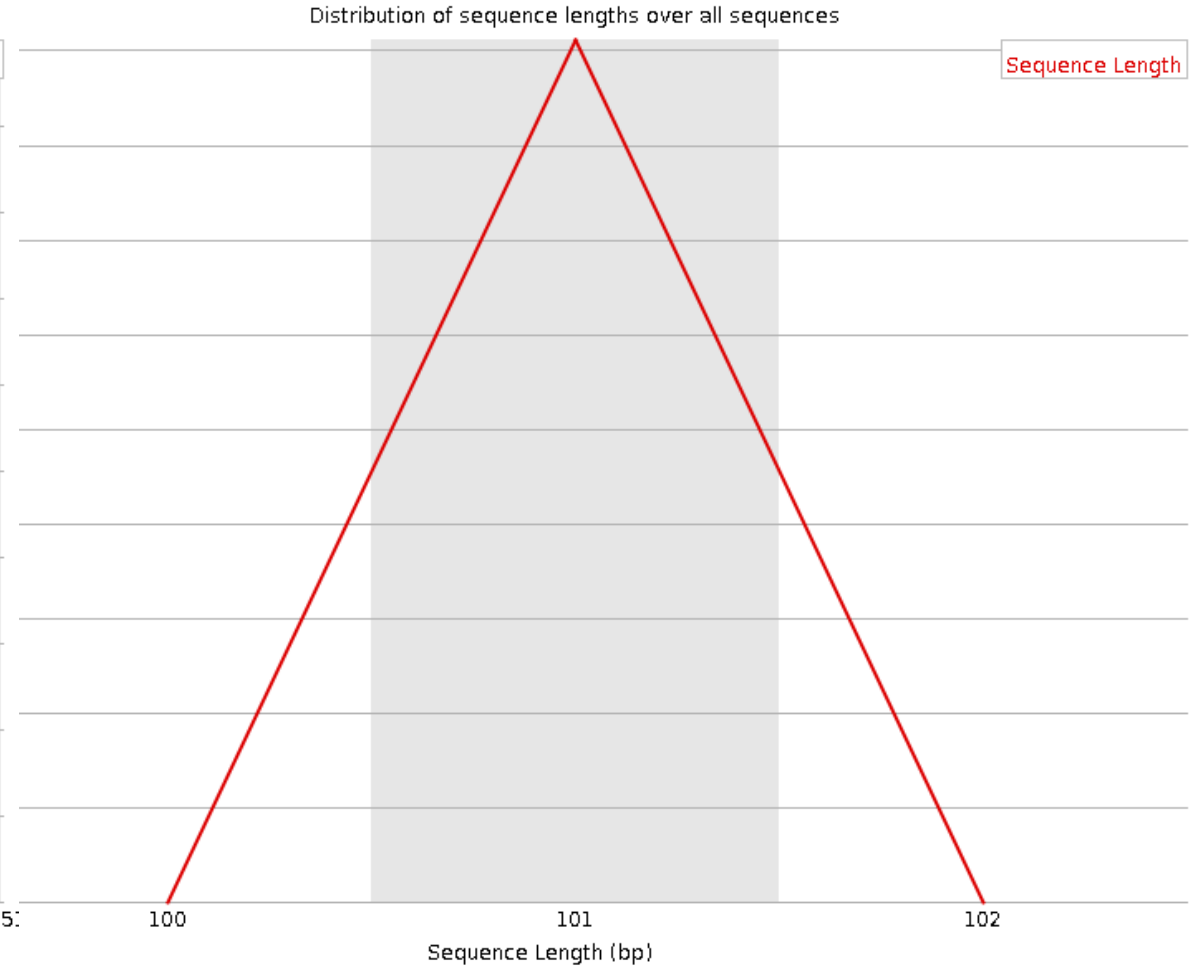


Sequence Length Distribution

wgs

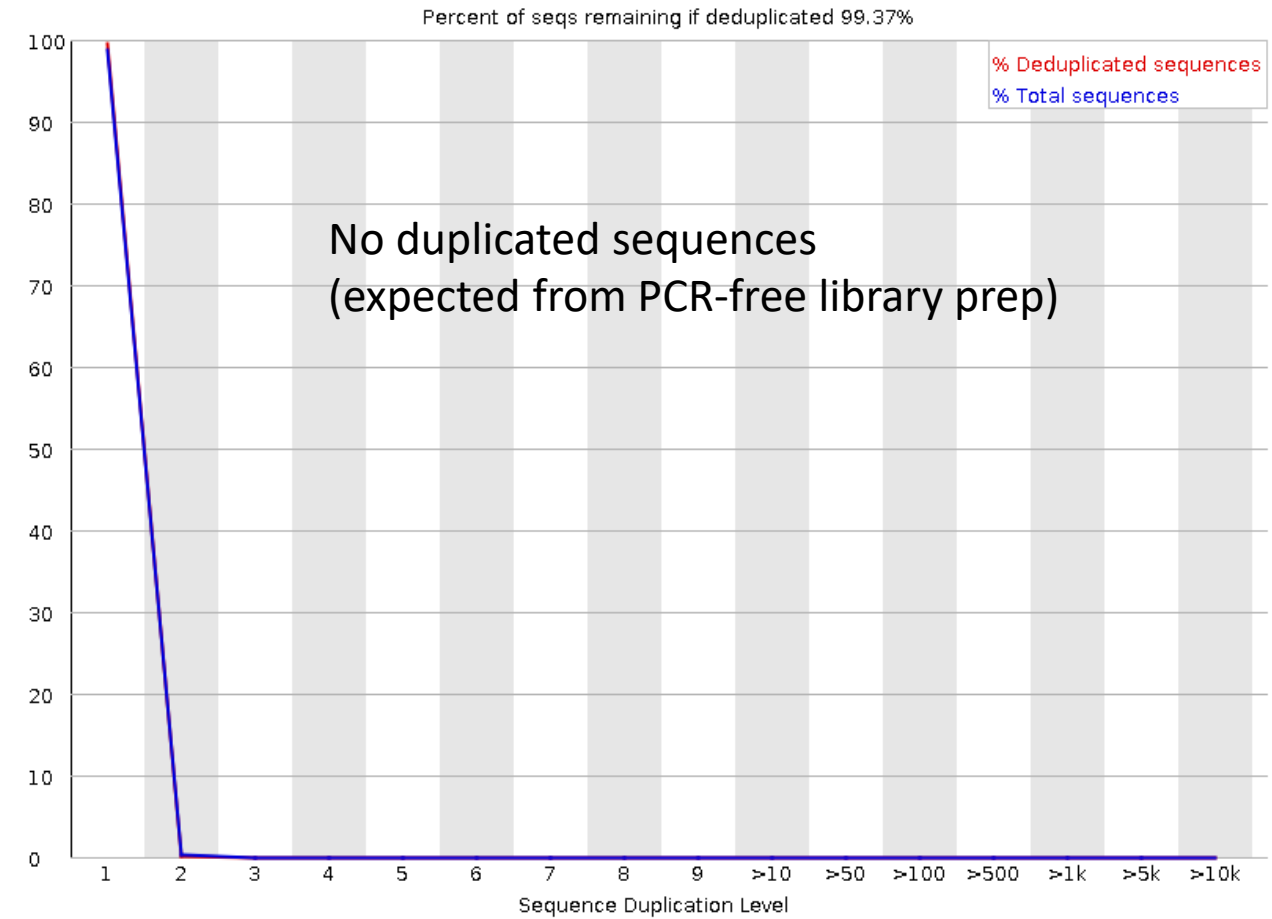


RAD

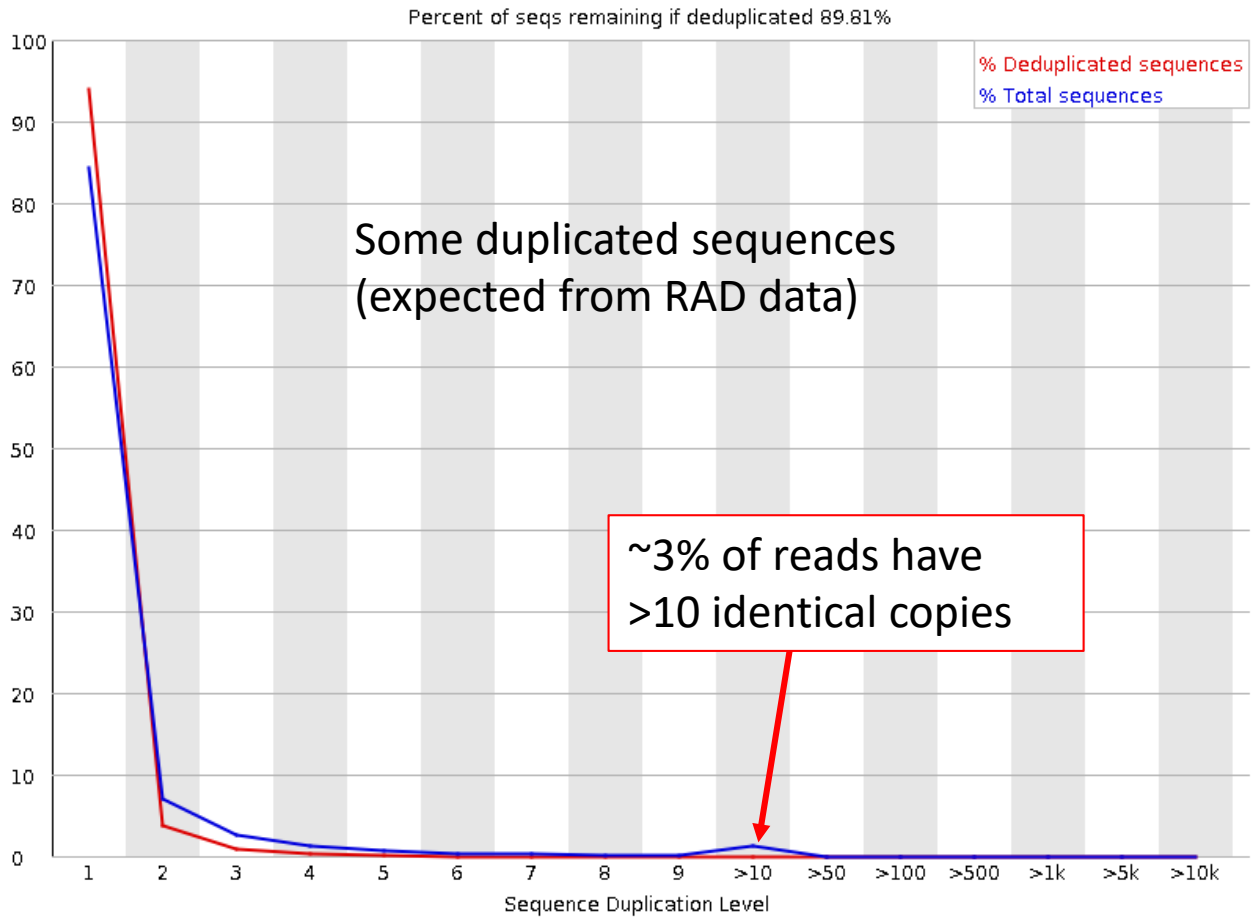


Sequence duplication level

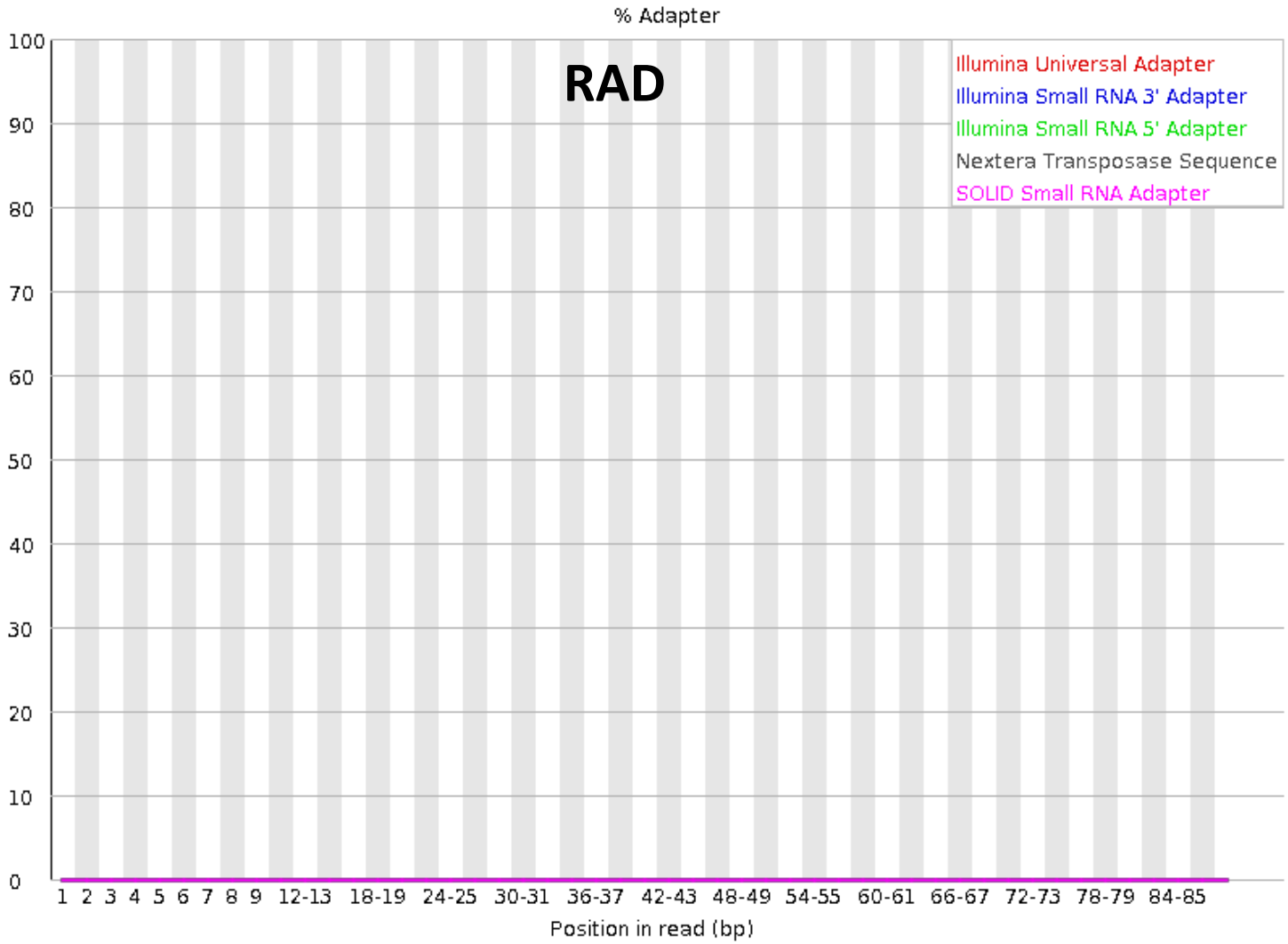
wgs



RAD



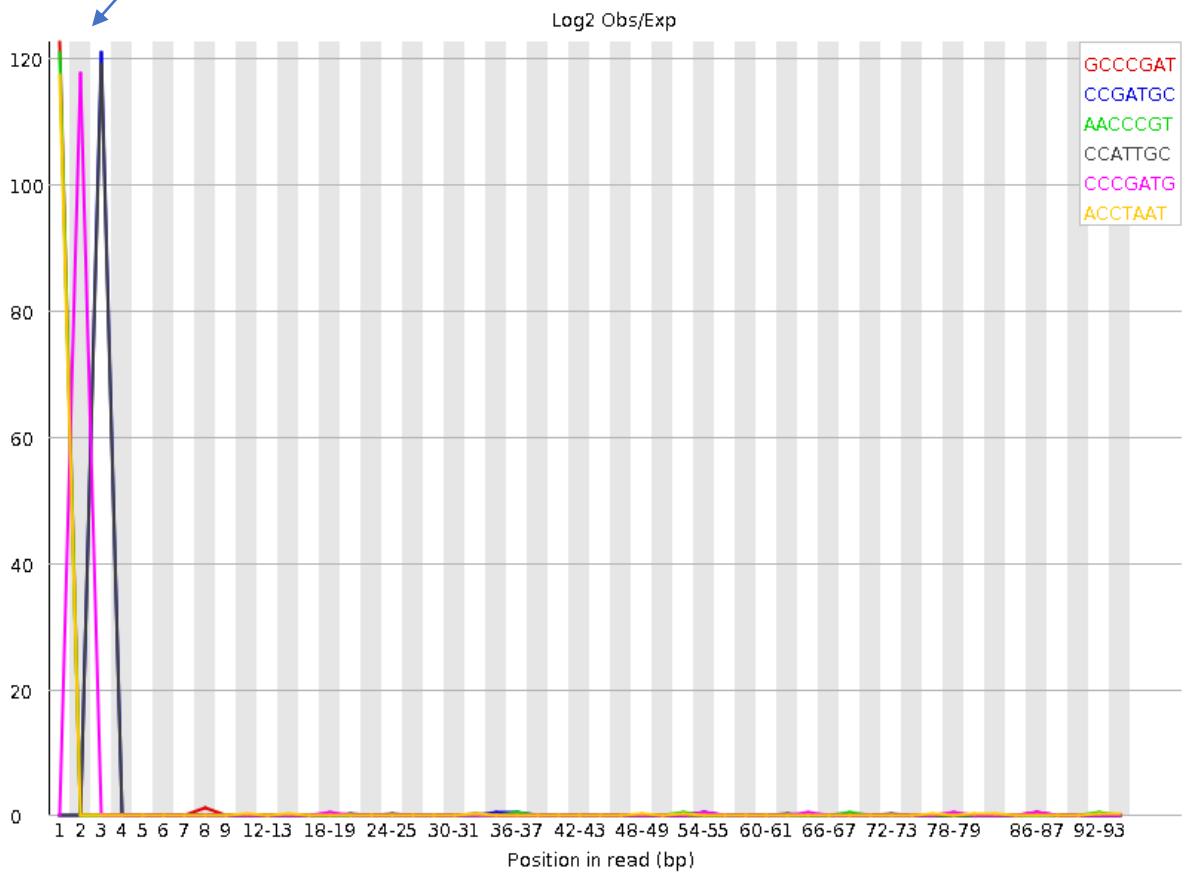
Adapter content



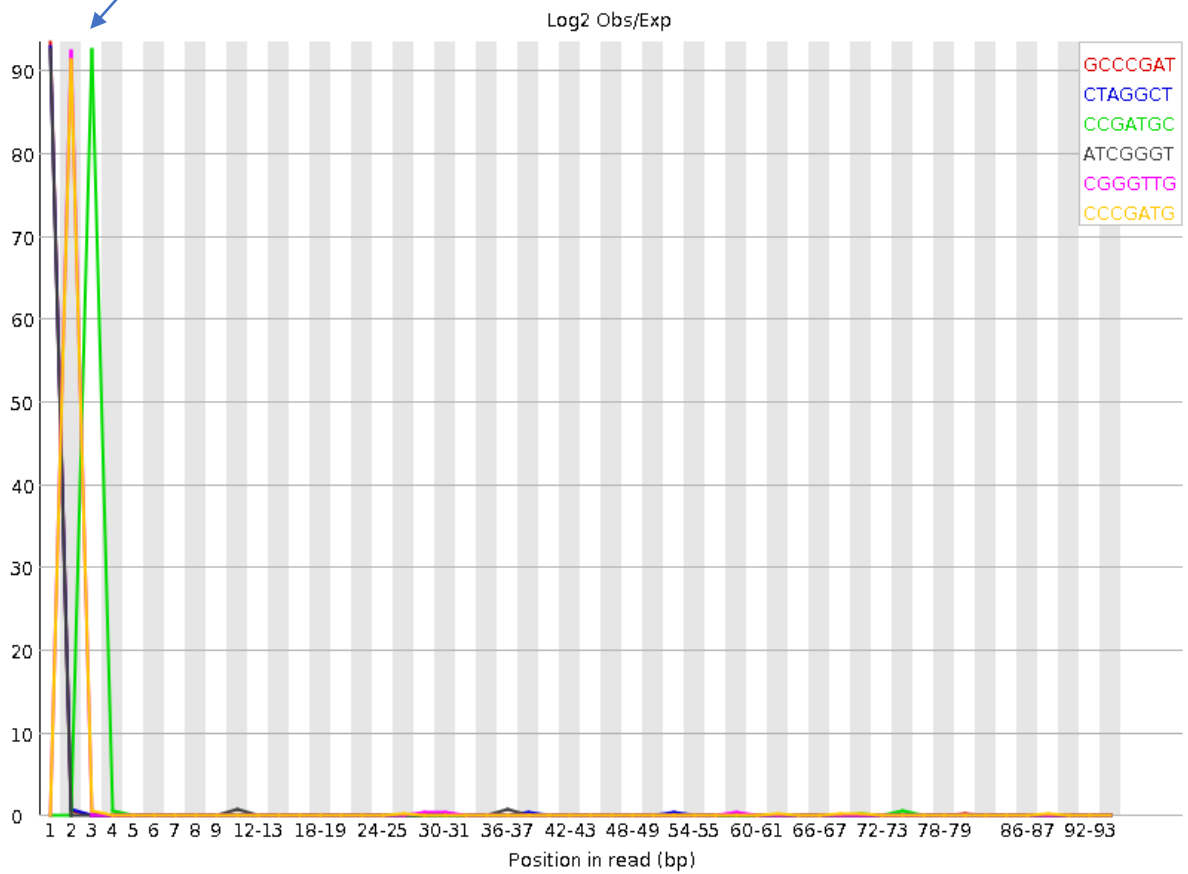
In wgs datasets not even shown

Kmer content

Most common barcodes + cut site



Most common barcodes + cut site



GBS

How many SNPs will I get?

Species	Genome Size (Mb)	Enzyme	Sample Size	No. SNPs
Maize	2,600	*Ape*KI	33,000	1,200K
Rice	400	*Ape*KI	850	60K
Grape	500	*Ape*KI	1000	200K
Willow*	460	*Ape*KI	459	23K
Pine*	16,000	*Ape*KI	12	63K
Vole*	3,400	*Pst*I	283	53K
Fox*	2,400	*Eco*T22I	48	16K
Cow	3,000	*Pst*I	48	64K
Verticilliflorum (fungus isolates)	40	*Ape*KI	2	10K

*No reference genome. UNEAK analysis pipeline used for analysis. To avoid homology/paralogy issues this pipeline calls SNPs very conservatively.