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Pré-processando os FASTQ's

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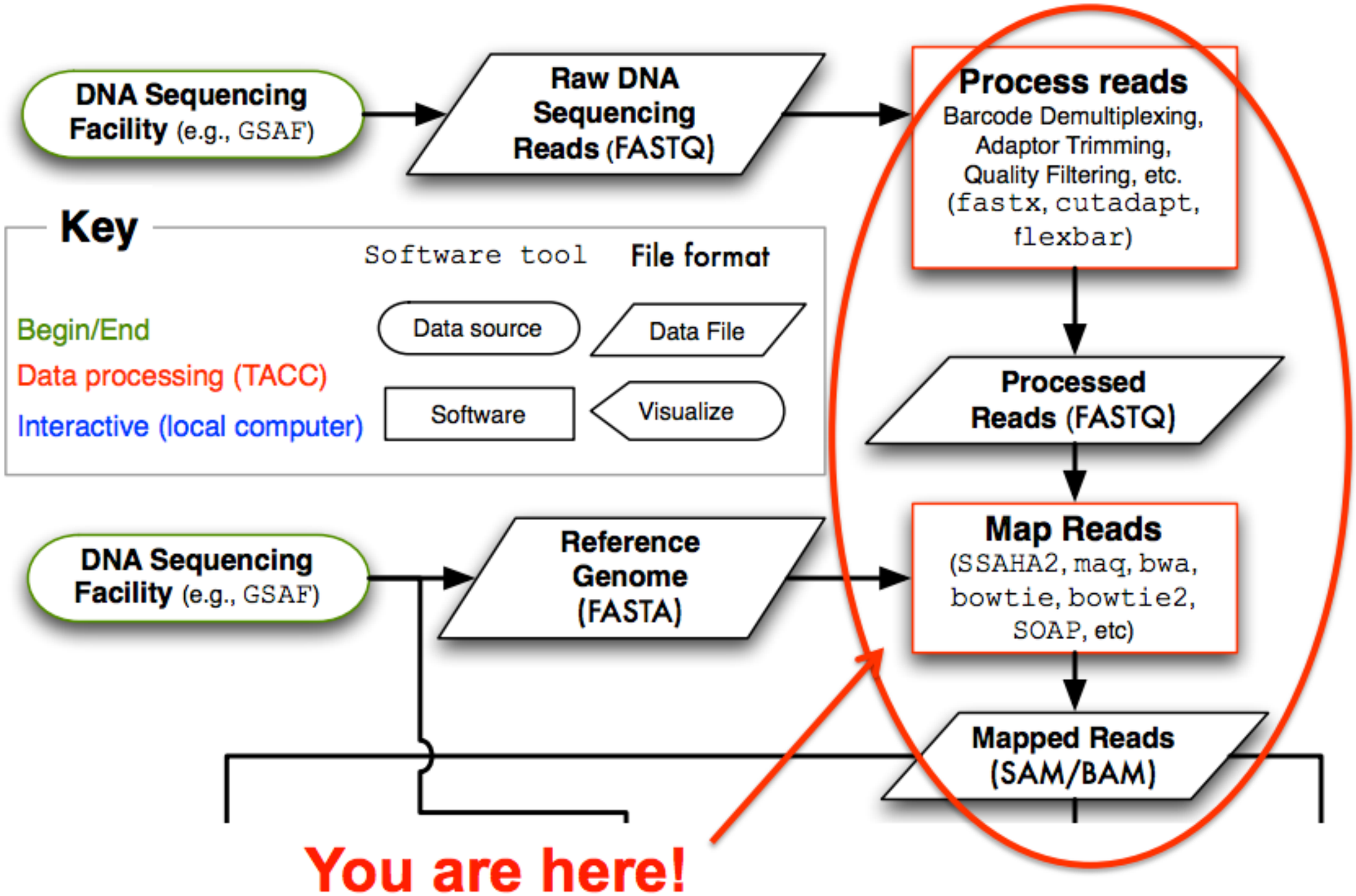
Pipeline



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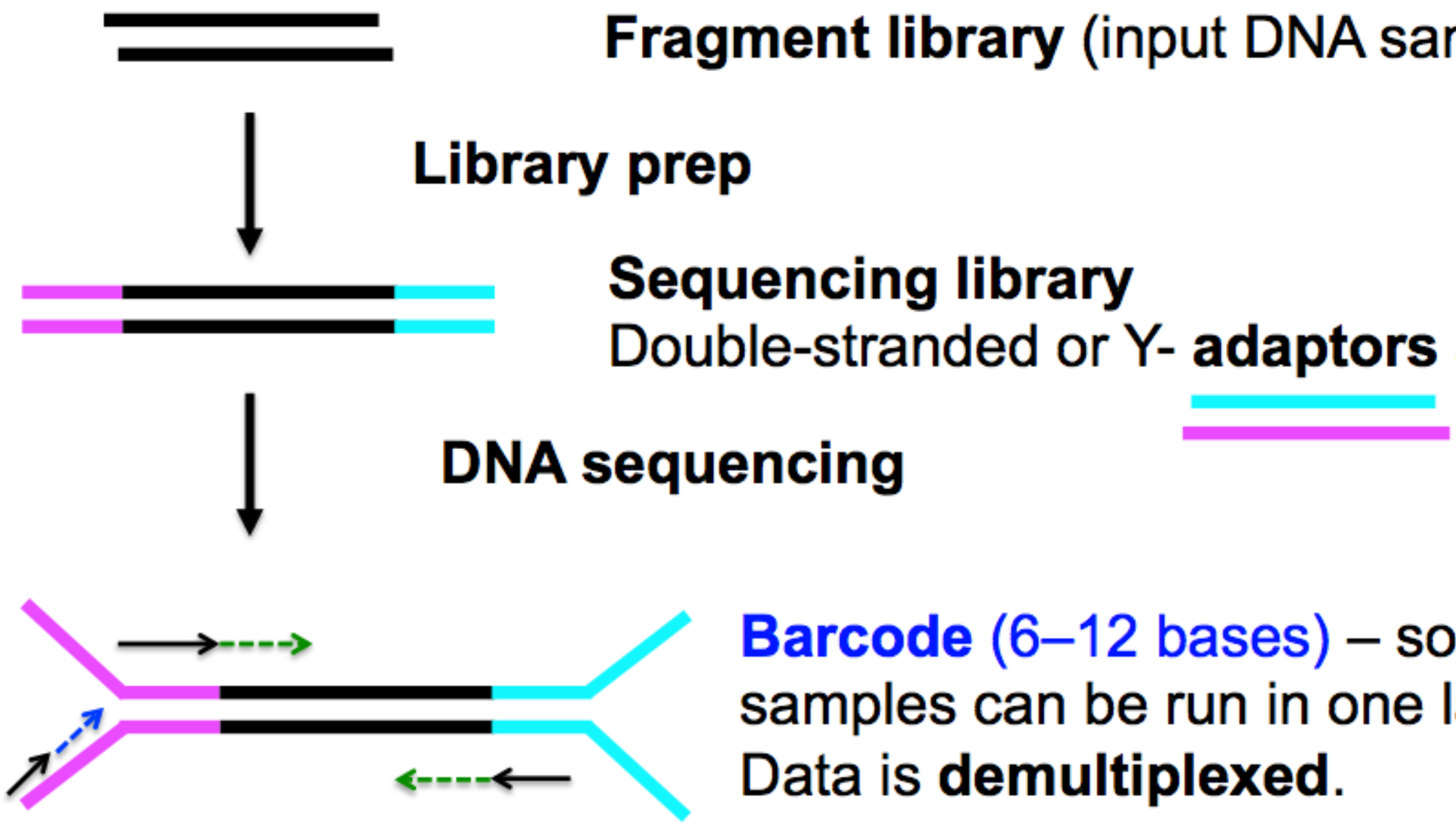
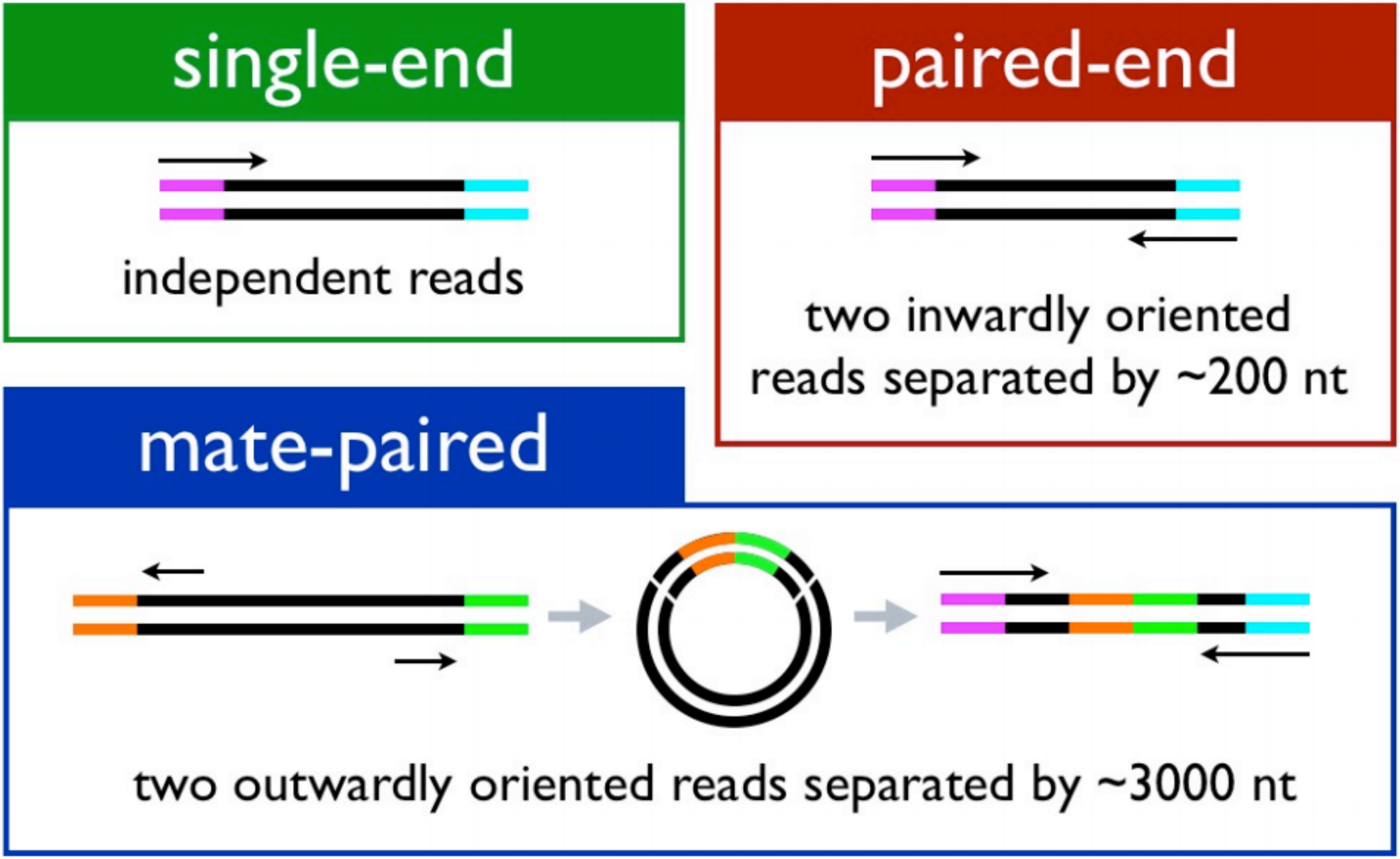
Terminology



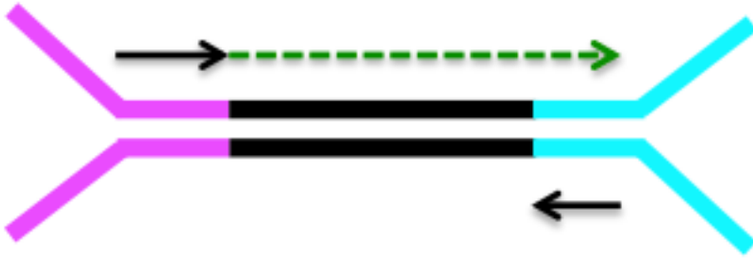
Types of Illumina fragment libraries



Read Sequences Quality Control

Garbage in = garbage out

- Contaminated with other samples?
- Sample barcodes removed?
- Adaptor sequences trimmed?
  - RNAseq, MiSeq data
- Trim ends of reads with poor quality?
  - *de novo* Assembly
- Know your data
  - Paired reads? Relative orientations?
  - Technology specific concerns?
    - Indels with 454



Read Sequences

FASTQ Format

```
@HWI-EAS216_91209:1:2:454:192#0/1
GTTGATGAATTTCTCCAGCGCGAATTTGTGGGCT
+HWI-EAS216_91209:1:2:454:192#0/1
B@BBBBBB@BBBBAAAA>@AABA?BBBAAB??>A?
```

Line 1: @read name

Line 2: called base sequence

Line 3: +read name (optional after +)

Line 4: base quality scores

FASTQ format

Standard Format for NGS data

Conversion can be done from sff, fasta + qual, . . .

Extension of the Fasta format

Text-based formats (easy to use!)

If not compressed, it can be huge
http://en.wikipedia.org/wiki/FASTQ_format

Decipher base quality scores

http://www.asciitable.com/

```
Quality character   !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHI
                    |         |         |         |         |
ASCII Value         33        43        53        63        73
Base Quality (Q)    0         10        20        30        40
```

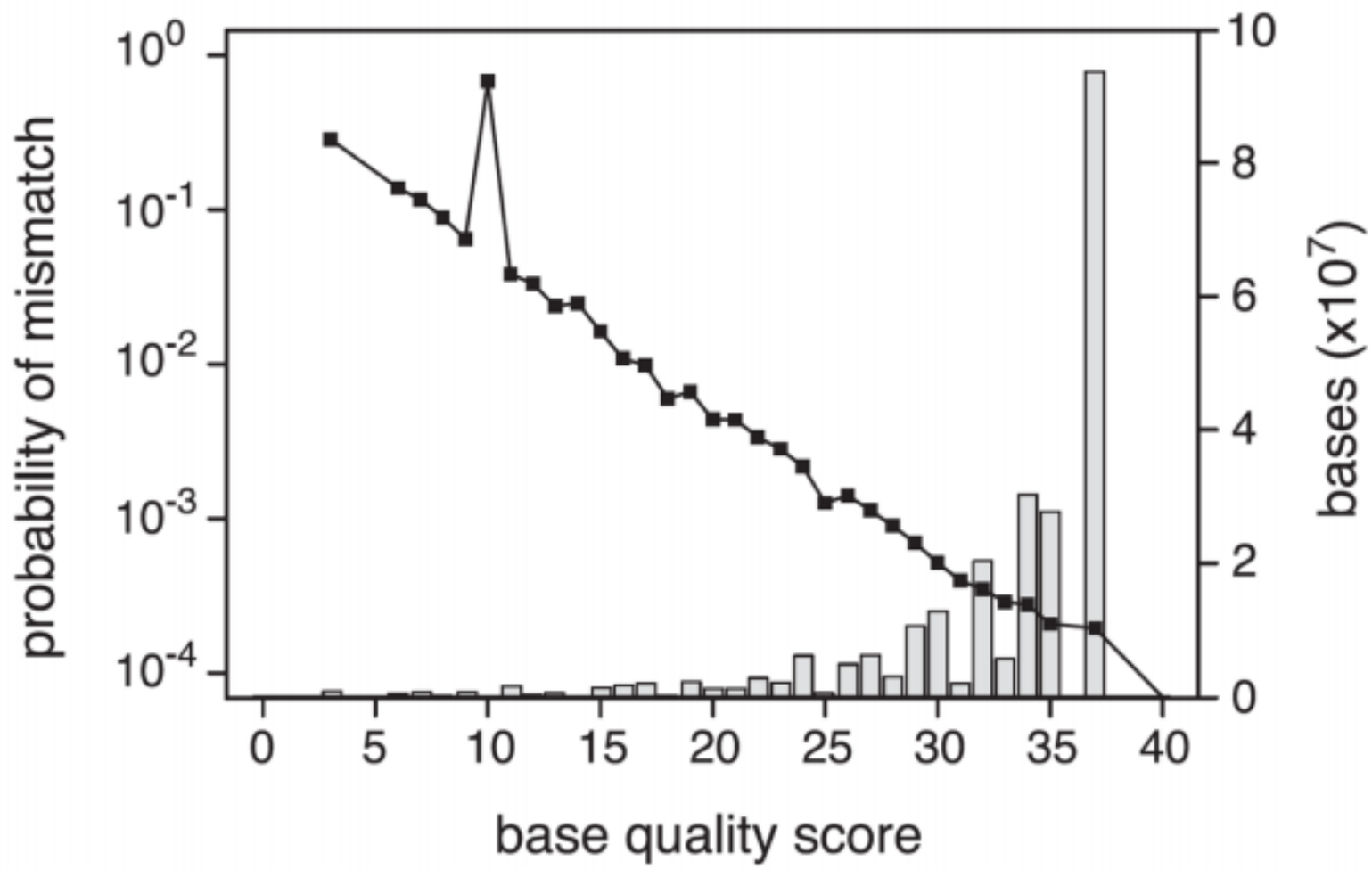
$$\text{Probability of Error} = 10^{-Q/10}$$

(This is a **Phred** score, also used for other types of qualities.)

* Very low quality scores can mean something special – Illumina Q ≤ 3 means something like: "I'm lost, you might want to stop believing sequencing cycles from here on out."

* In older FASTQ files, the formula and ASCII offset might differ. Consult: http://en.wikipedia.org/wiki/FASTQ_format

Example of Illumina Data



- Most bases have high qualities (Q>30).
- Overall qualities are well calibrated*.

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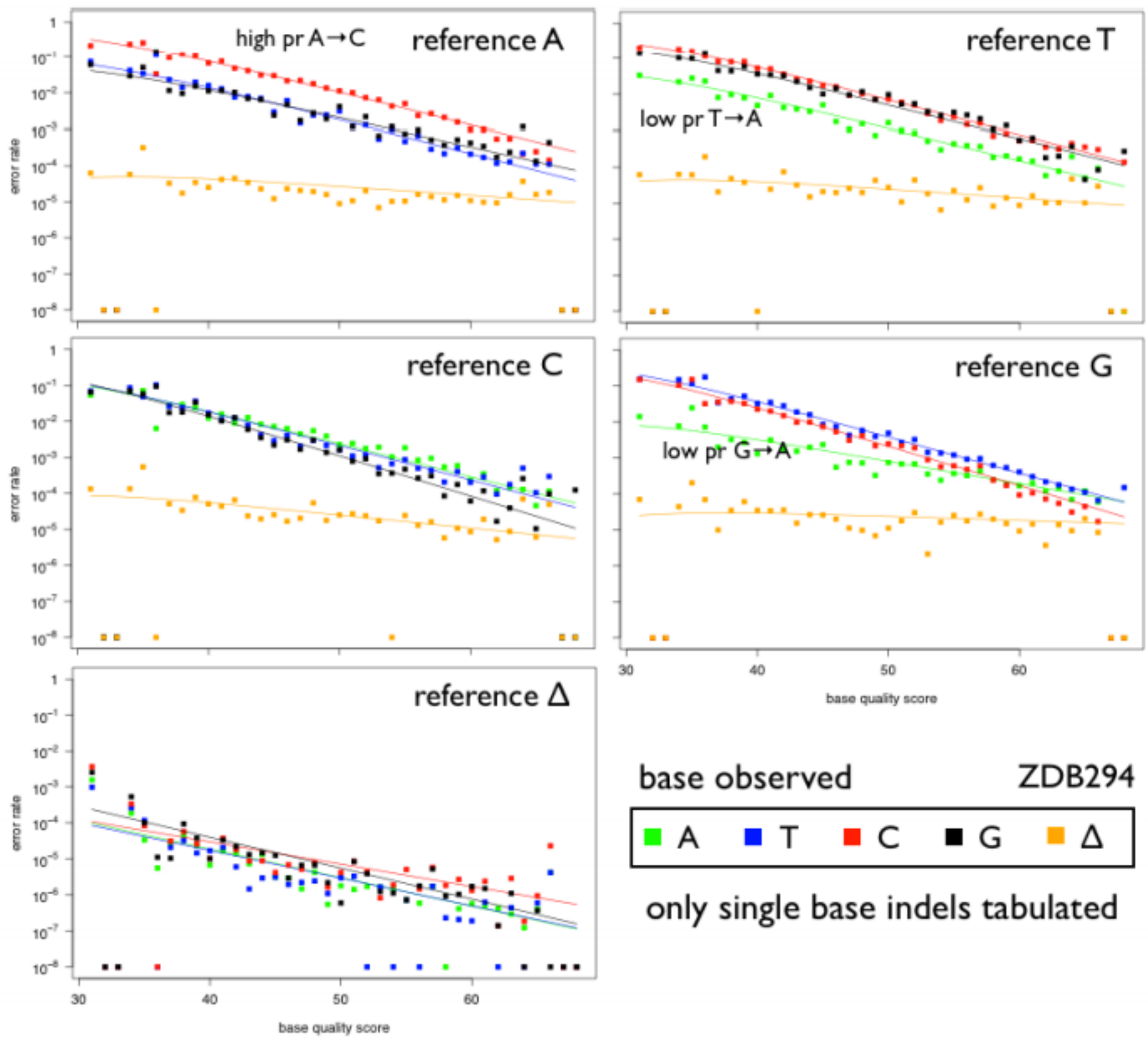
Example of Illumina Data



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FASTQC

Quality Assurance tool for FASTQ sequences

FastQC website:
http://www.bioinformatics.babraham.ac.uk

FastQC report documentation:
http://www.bioinformatics.babraham.ac.uk/projects/fastqc/Help/3%20Analysis%20Modules/

Good Illumina dataset:
http://www.bioinformatics.babraham.ac.uk/projects/fastqc/good_sequence_short_fastqc/fastqc_report.html

Bad Illumina dataset:
http://www.bioinformatics.babraham.ac.uk/projects/fastqc/bad_sequence_fastqc/fastqc_report.html

FASTQC

- Report basic statistics on your data
- Identify issues with your data

Basic Statistics

Measure	Value
Filename	tmp.fastq
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	250000
Filtered Sequences	0
Sequence length	101
%GC	51

FastQC Report

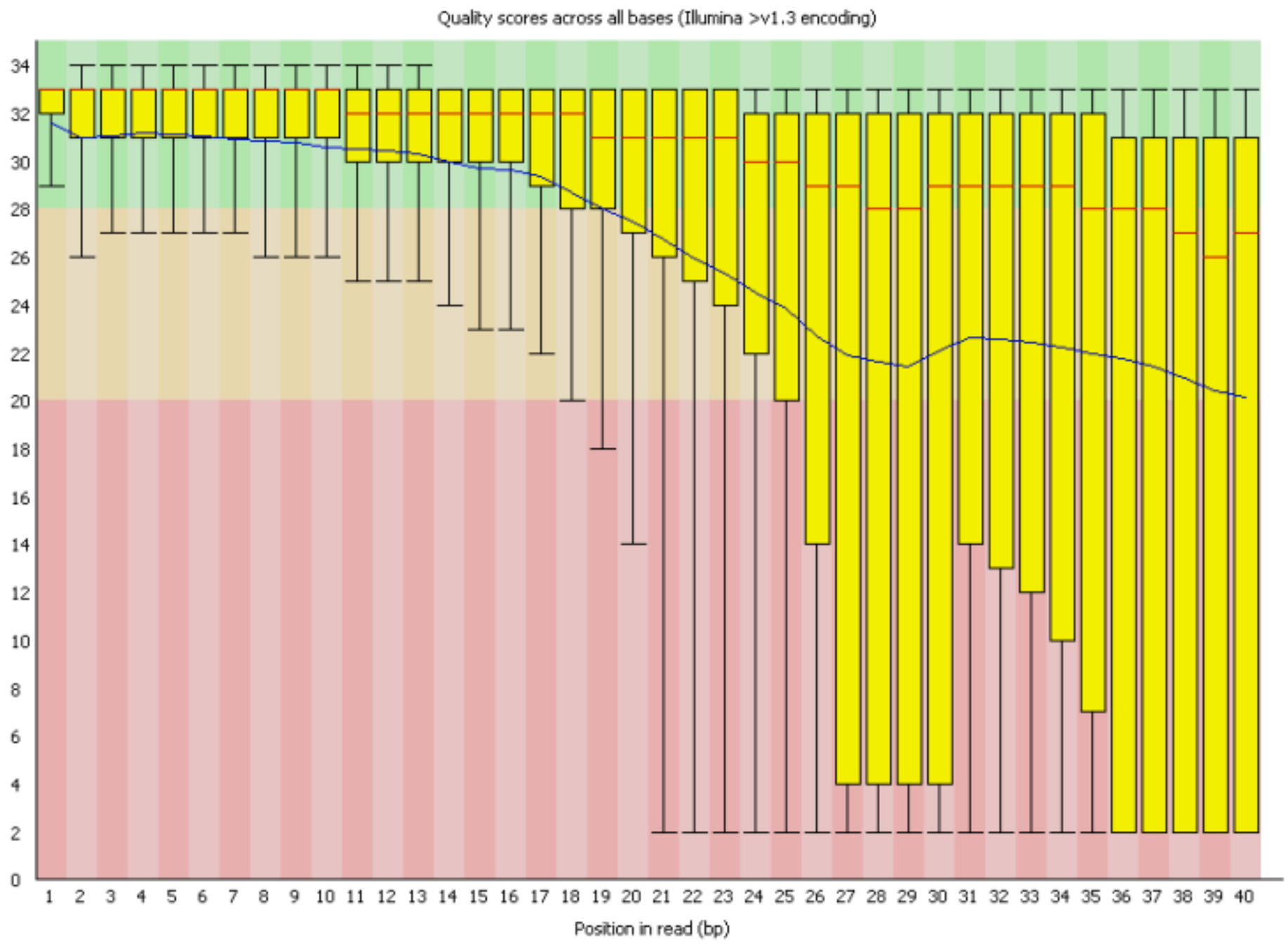
Summary

- 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

Useful reports

- Should I trim low quality bases?
  - Per-base sequence quality Report
    - based on *all* sequences
- Do I need to remove adapter sequences?
  - Overrepresented sequences Report
    - based on *1st 200,000* sequences
- How complex is my library?
  - Sequence duplication levels Report
    - estimate based on *1st 200,000* sequences

Per Base Sequencing Quality



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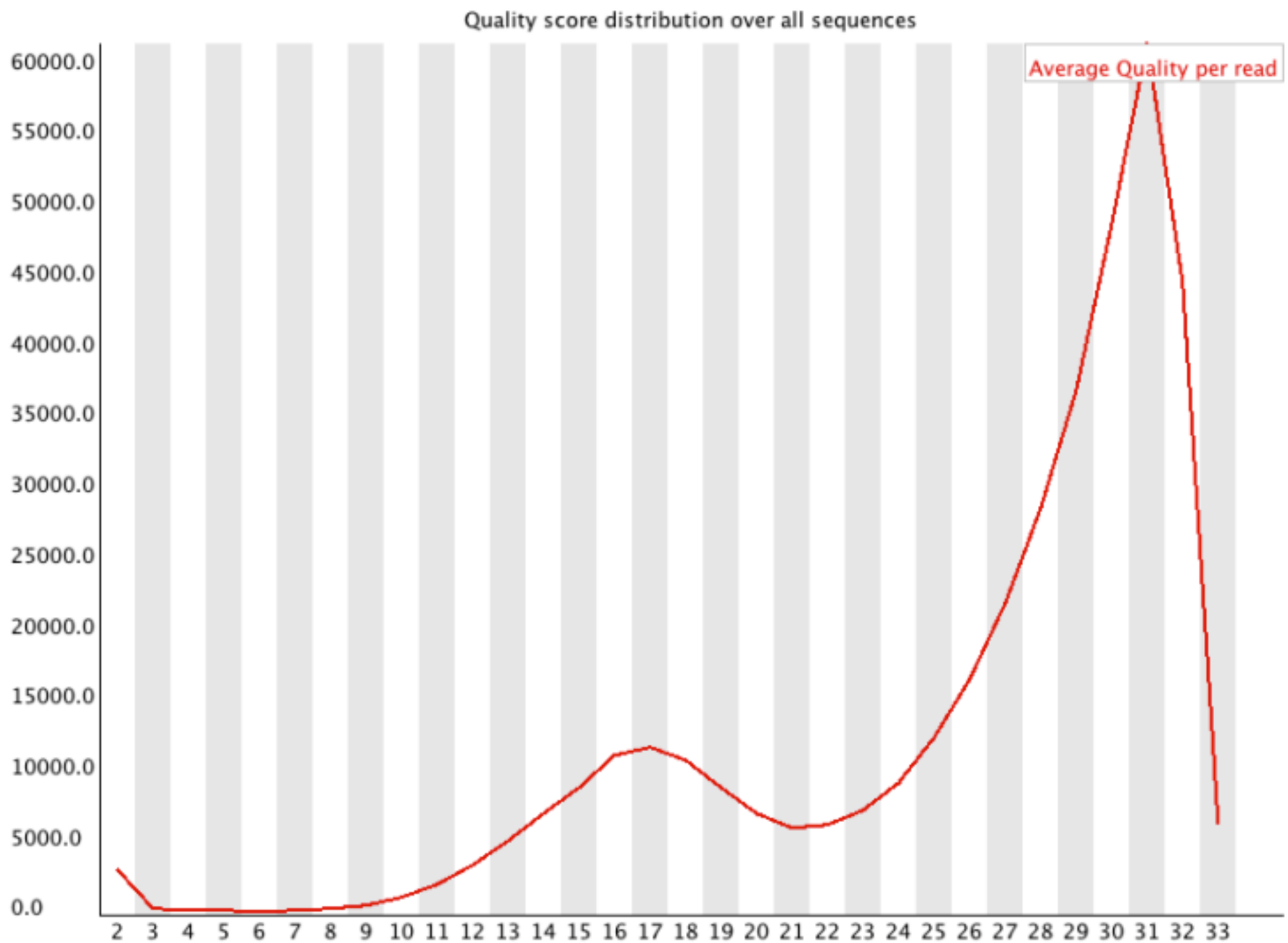
Per Sequencing Quality



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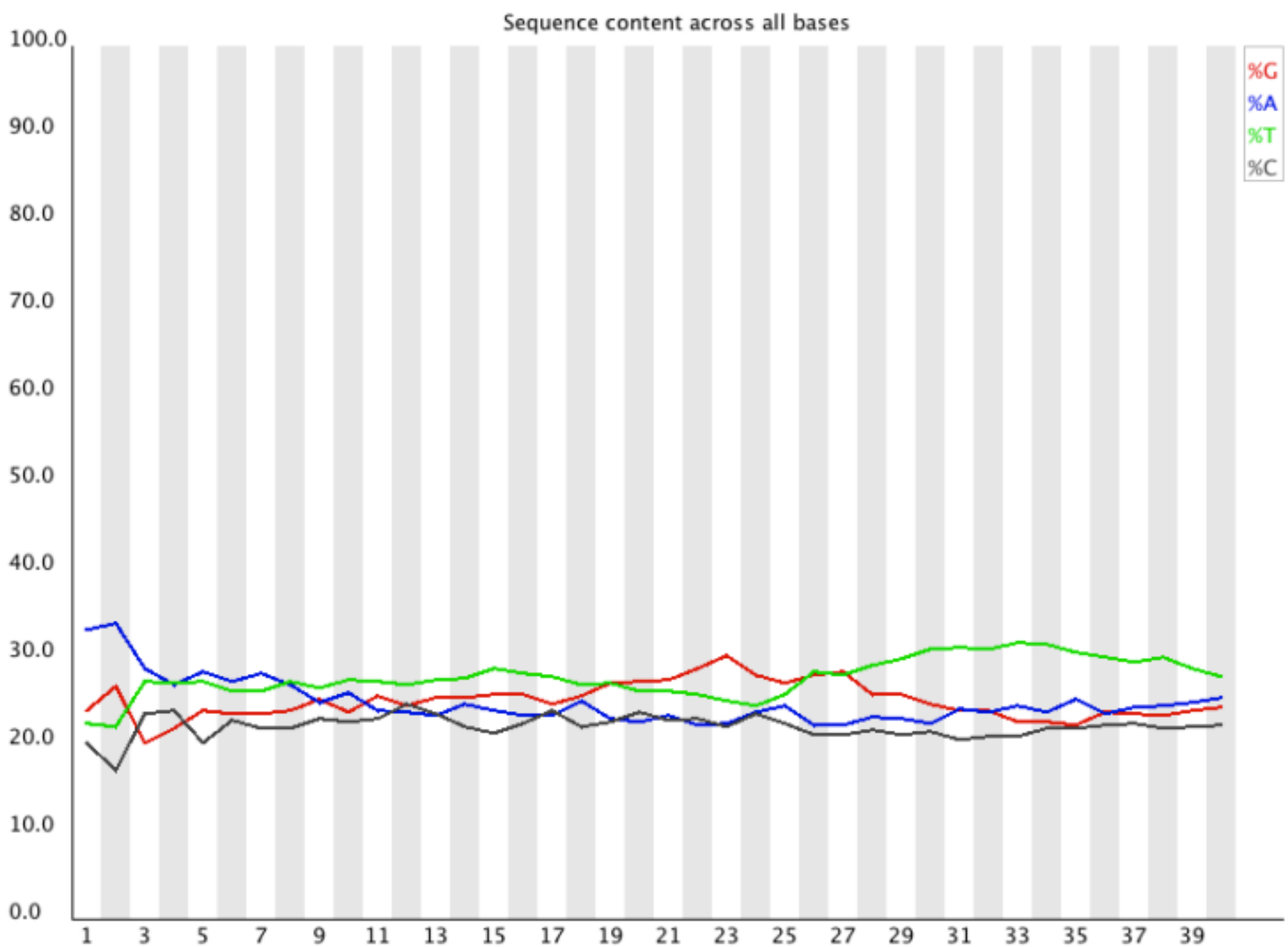
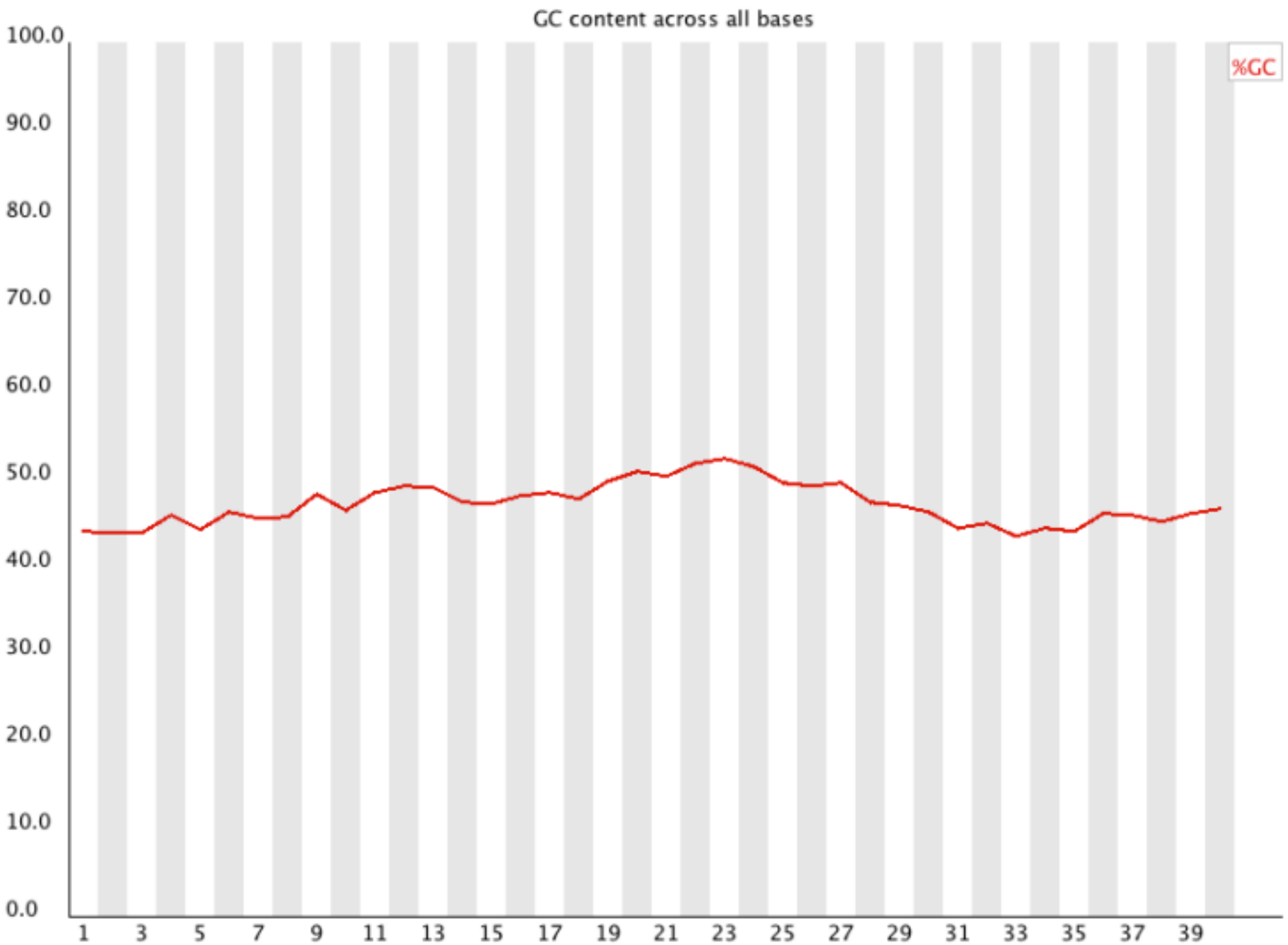
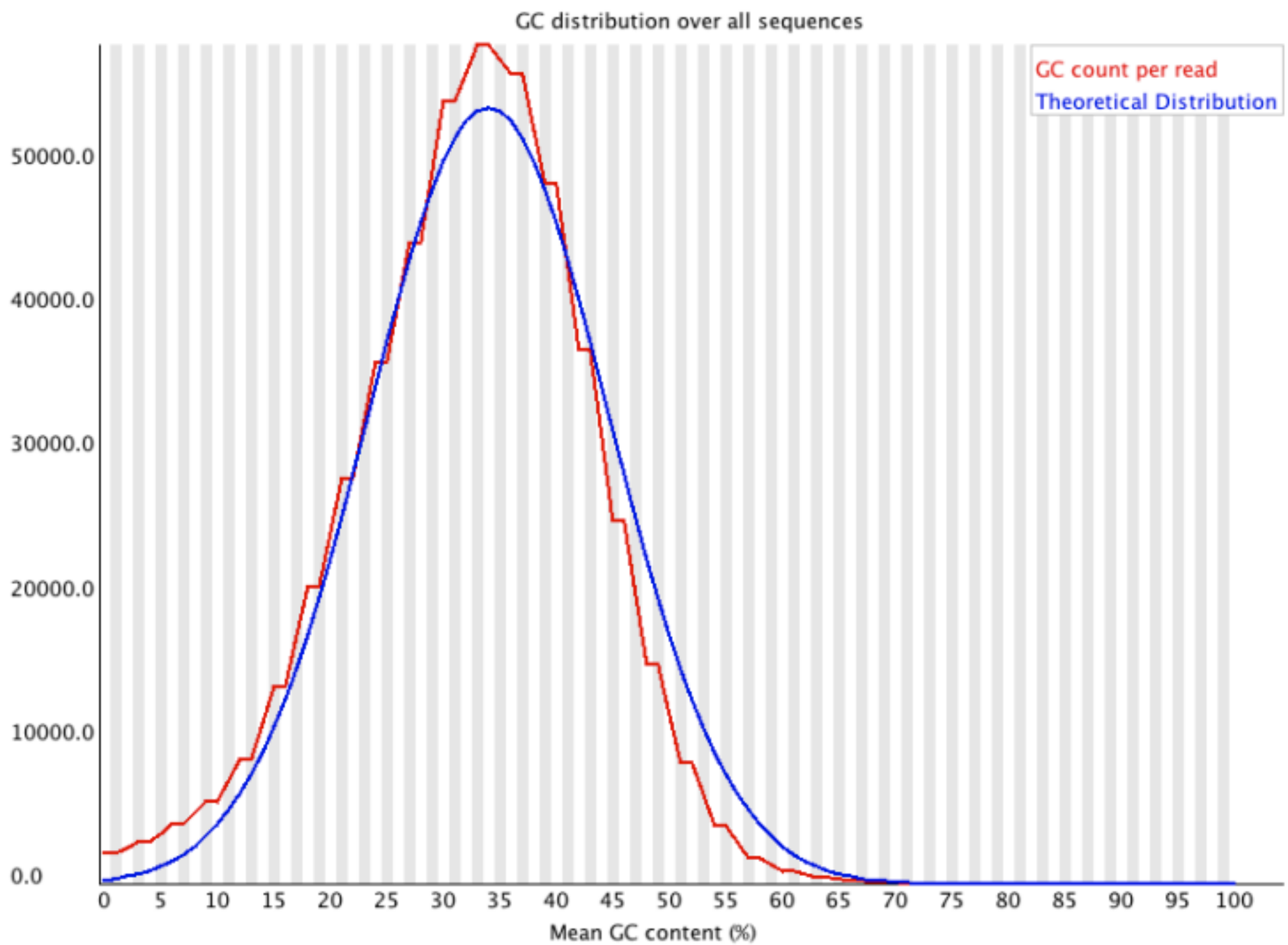
Per Base Sequencing content



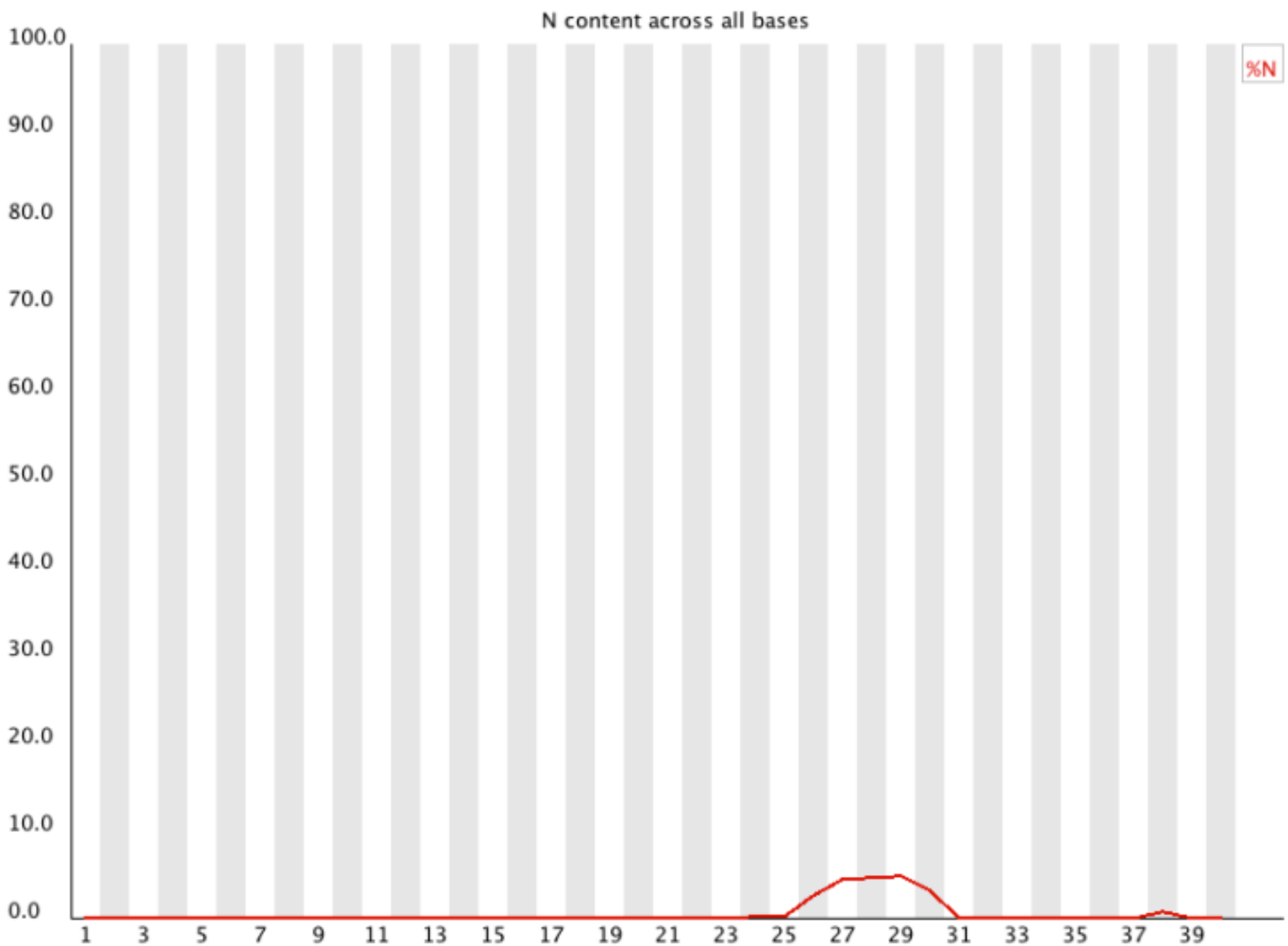
Per Base GC Content



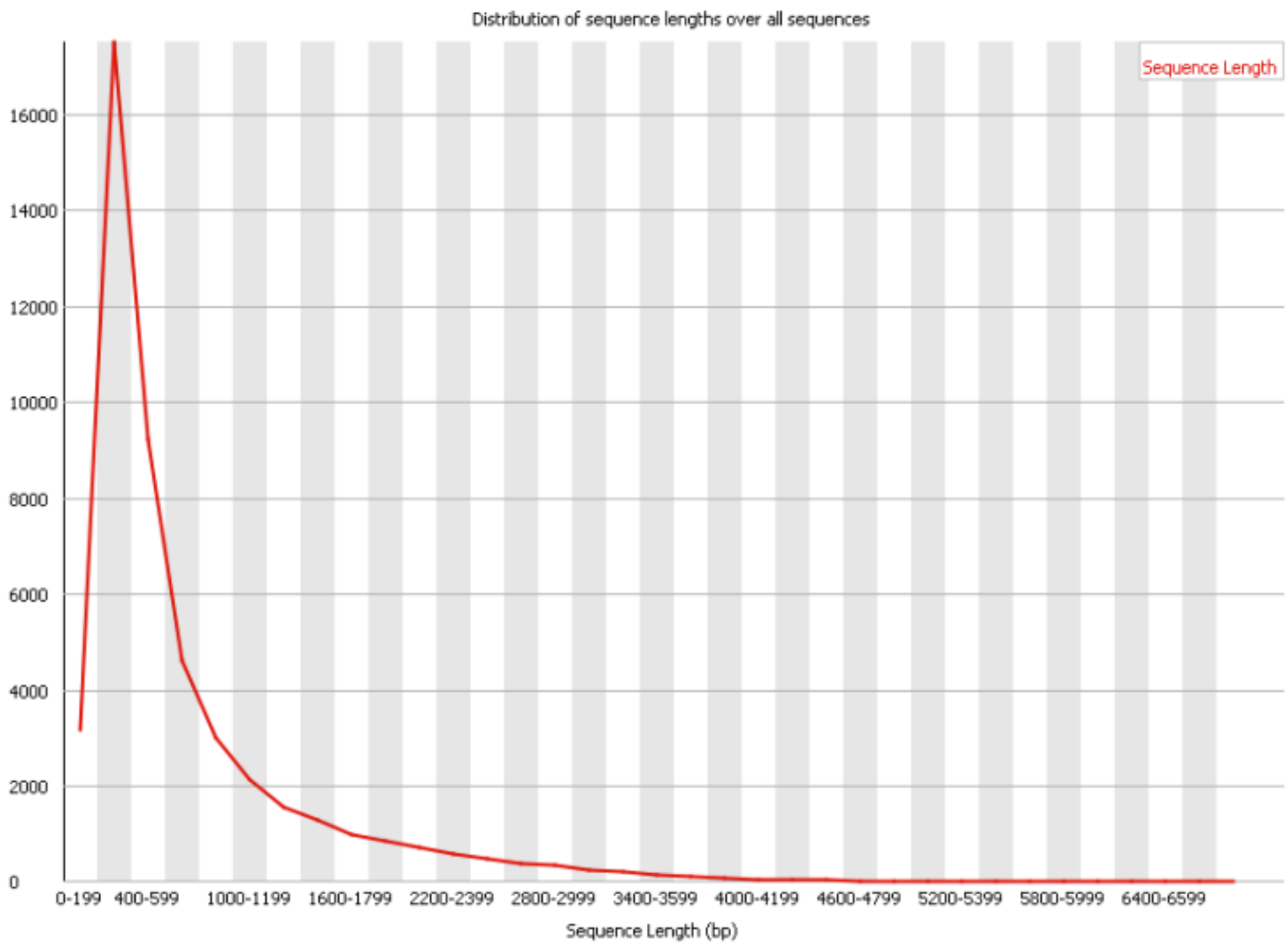
Per Sequencing Nucleotide Content



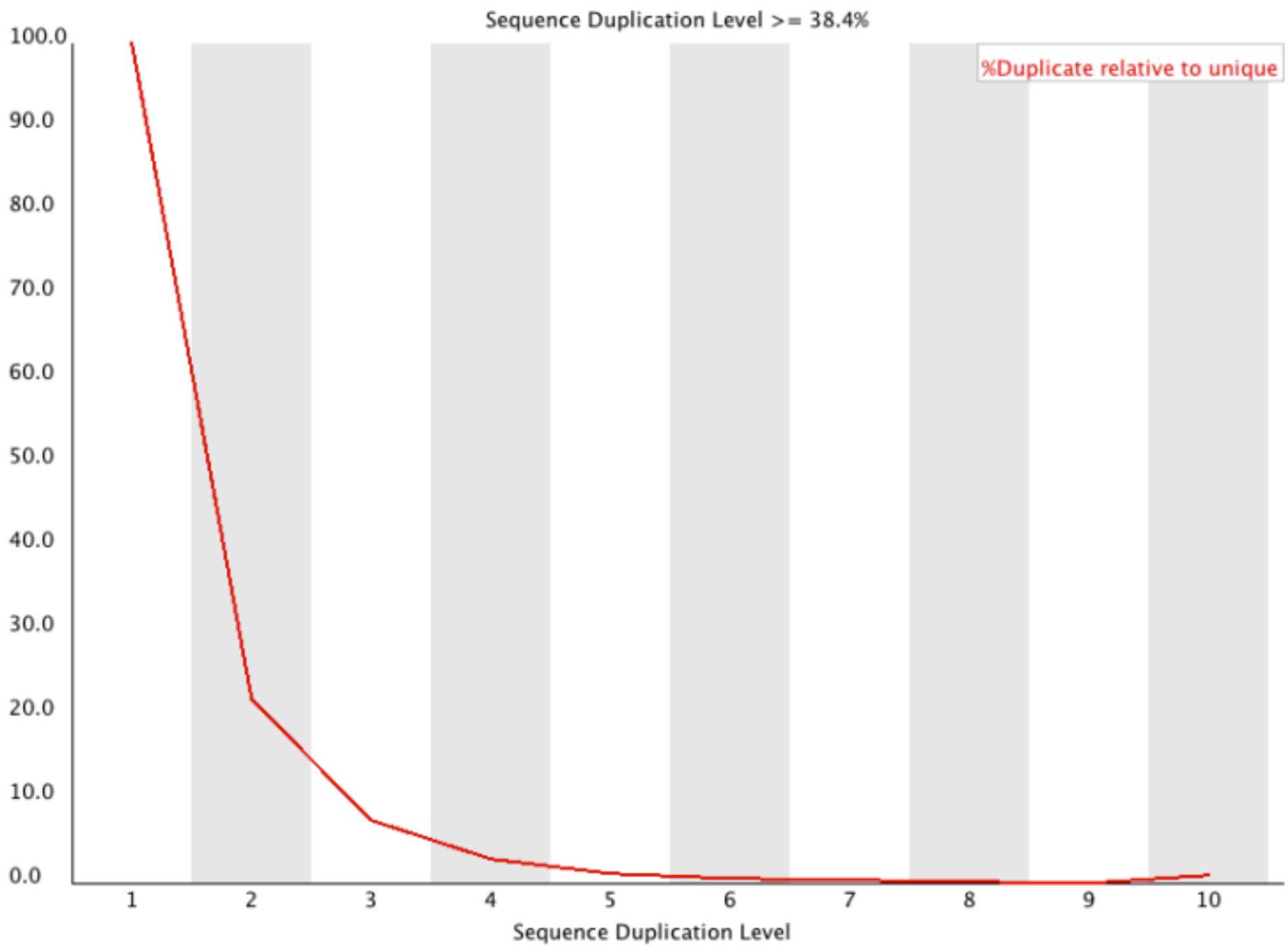
Per Base N content



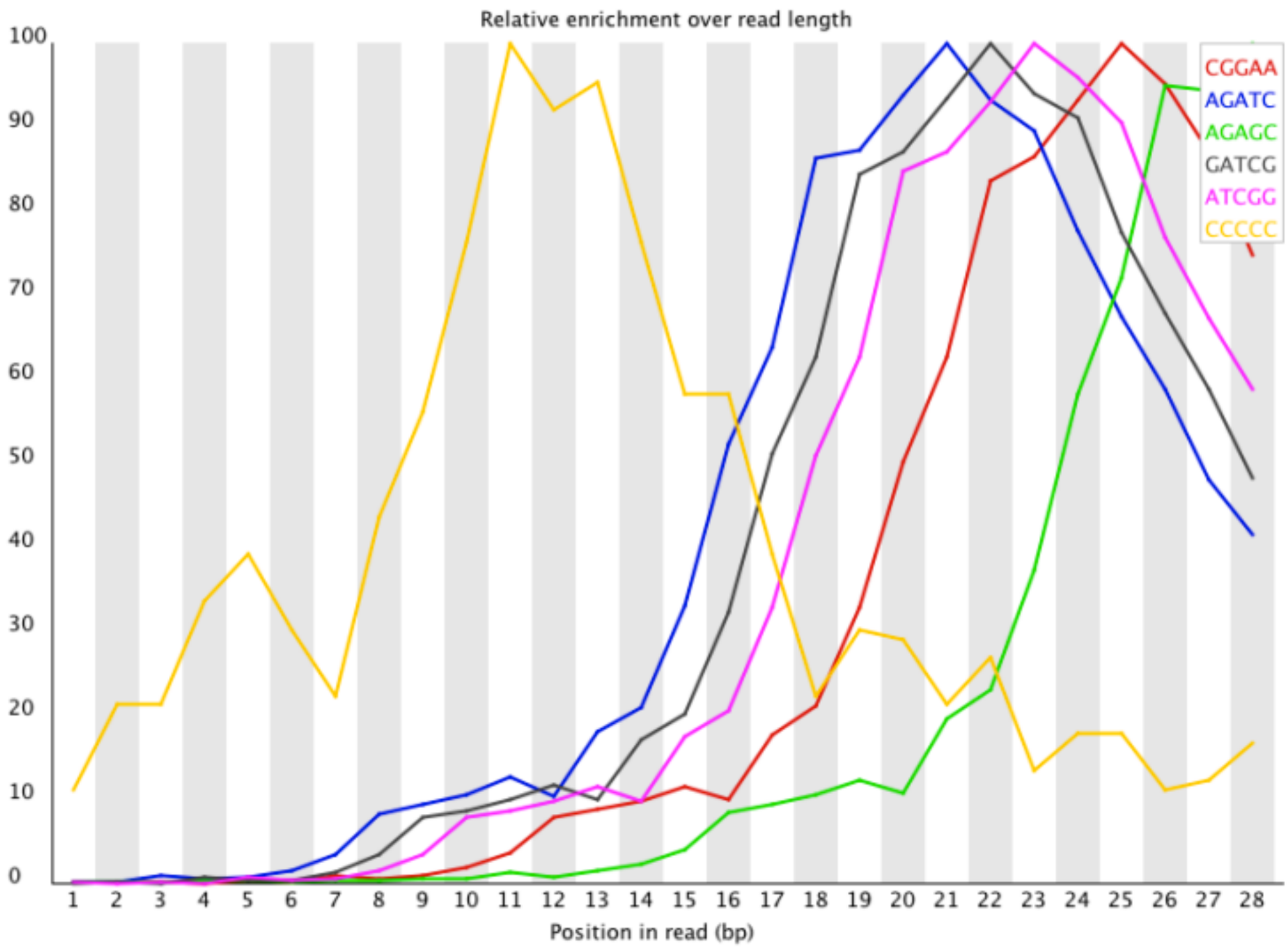
Sequence Length Distribution



Duplicate Sequences Distribution



Overrepresented K-mers



Overrepresented K-mers

- What is a *k*-mer?
- Create a sliding window of size *k*, move it over all your reads and count occurrence of *k*-mers
- We can use this to correct sequencing errors!

→

DNA: ACGTGTAACGTGACGTTGGA

g. *k*=5

```
ACGTG
 CGTGT
  GTGTA
```

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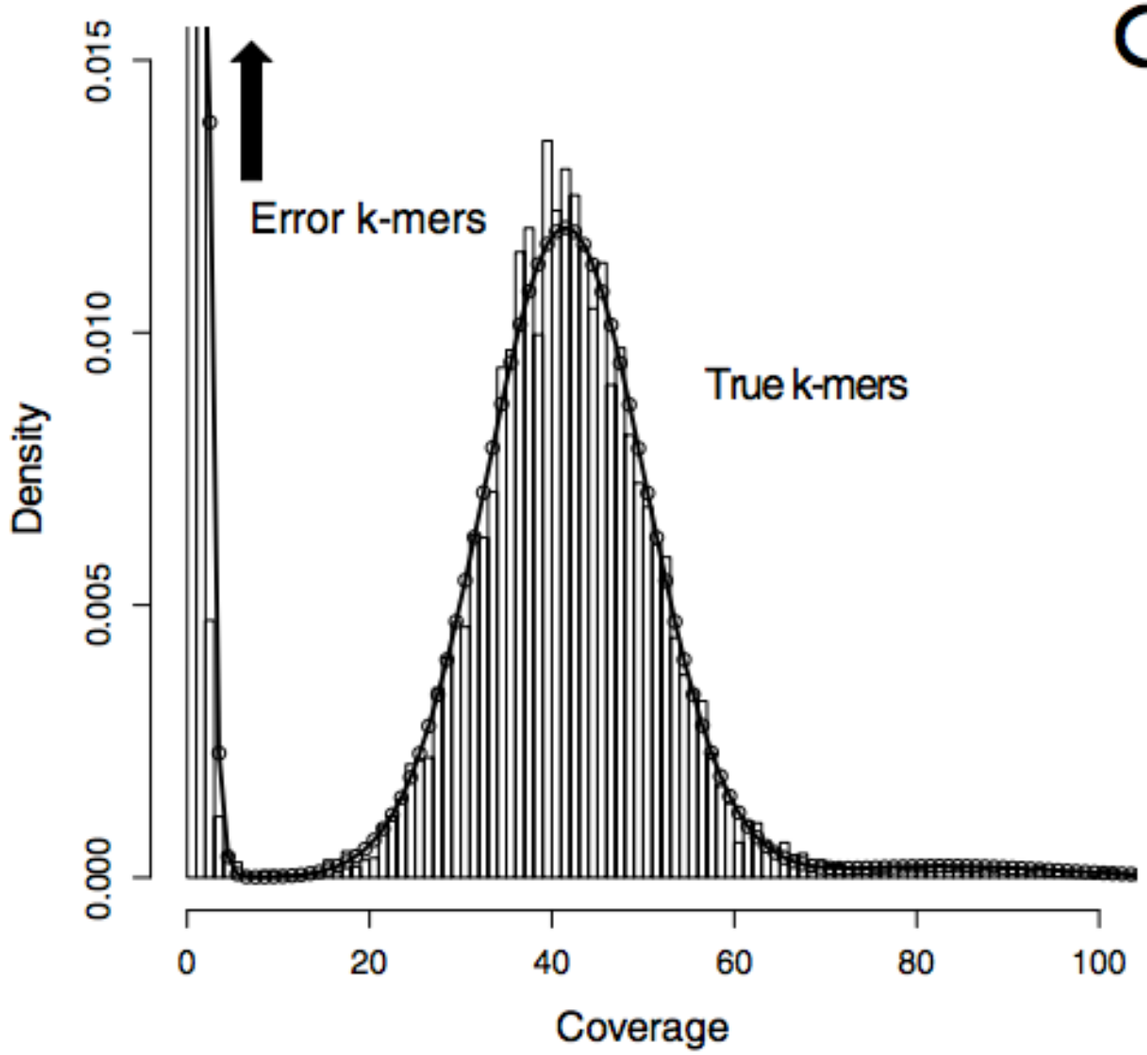
Overrepresented K-mers



Concept: Rare *k*-mers are seq. errors
Need >15X coverage

ACGTGGTT**G**CCCTTAAA
ACGTGGTT**A**CCCTTAAA
ACGTGGTT**A**CCCTTAAA
ACGTGGTT**A**CCCTTAAA
ACGTGGTT**A**CCCTTAAA
ACGTGGTT**A**CCCTTAAA
ACGTGGTT**A**CCCTTAAA
ACGTGGTT**A**CCCTTAAA
ACGTGGTT**A**CCCTTAAA

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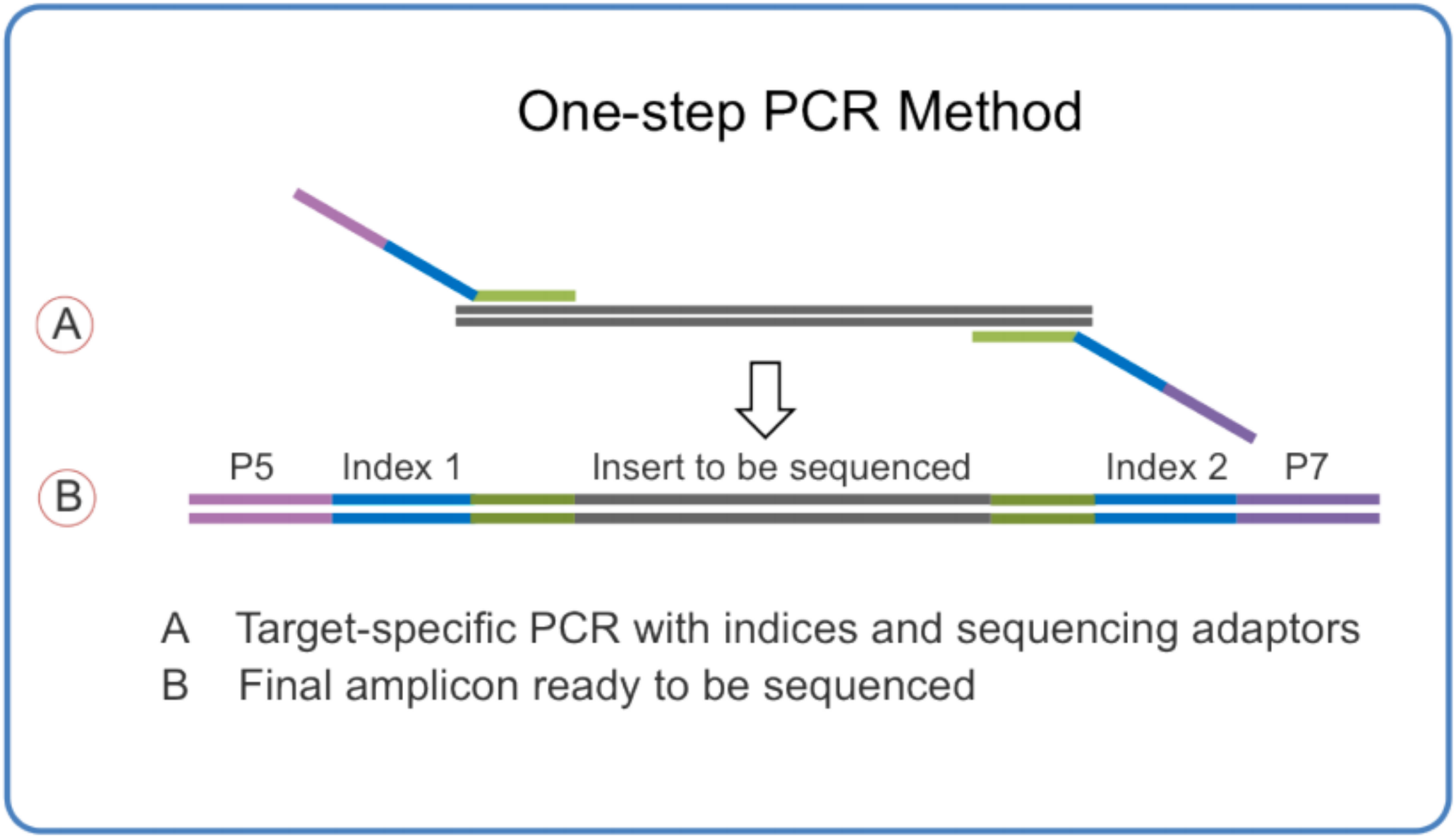
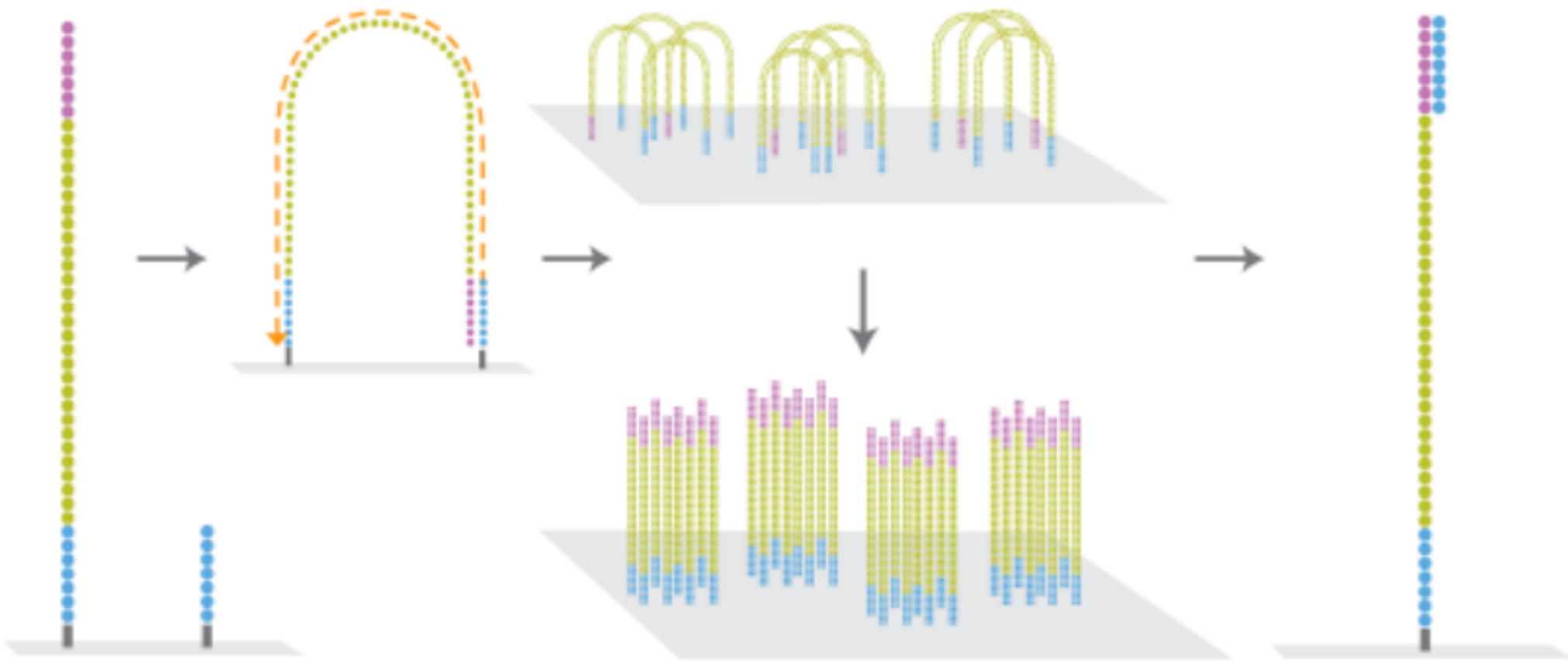
Sequencing Process: PCR primers



PCR primers



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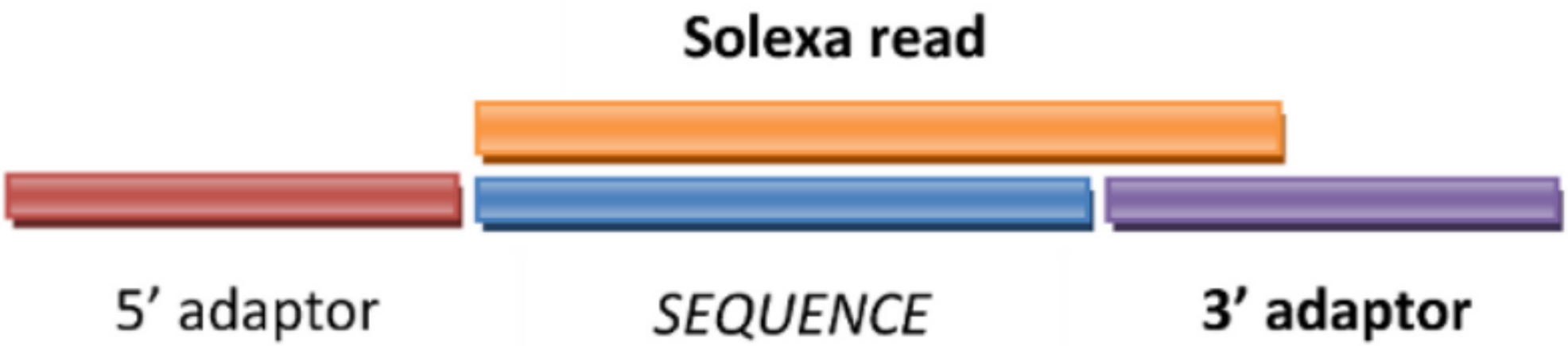
NGS adaptors & Cutadpt



NGS adaptors & Cutadpt

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATATCGTATGC	1547768	38.192098035156306	TruSeq Adapter, Index 1 (98% over 50bp)
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGC	146635	3.61830603513262	TruSeq Adapter, Index 1 (100% over 50bp)
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCAAGATATCGTATGC	6639	0.16382128255358863	TruSeq Adapter, Index 1 (97% over 41bp)
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATTTCGTATGC	6462	0.15945370204267054	TruSeq Adapter, Index 1 (98% over 50bp)
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATTACGATATCGTATGC	5433	0.1340625136486891	TruSeq Adapter, Index 1 (97% over 41bp)
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATAACGATATCGTATGC	5147	0.1270052931621209	TruSeq Adapter, Index 1 (97% over 41bp)
GATCGGAAGAGCACACGTCTGAACTCCAGTCACACCACGATATCGTATGC	4703	0.11604932849066535	TruSeq Adapter, Index 1 (97% over 41bp)

Very important if your DNA fragment is shorter than read length

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Coverage

- Coverage/depth is how many times that your data covers the genome (on average)

$$C = N \times \frac{L}{G}$$

- Example:
  - N: Number of reads: 5 mill
  - L: Read length: 100
  - G: Genome size: 5 Mbases
  - C = 5*100/5 = 100X
  - On average there are 100 reads covering each position in the genome

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