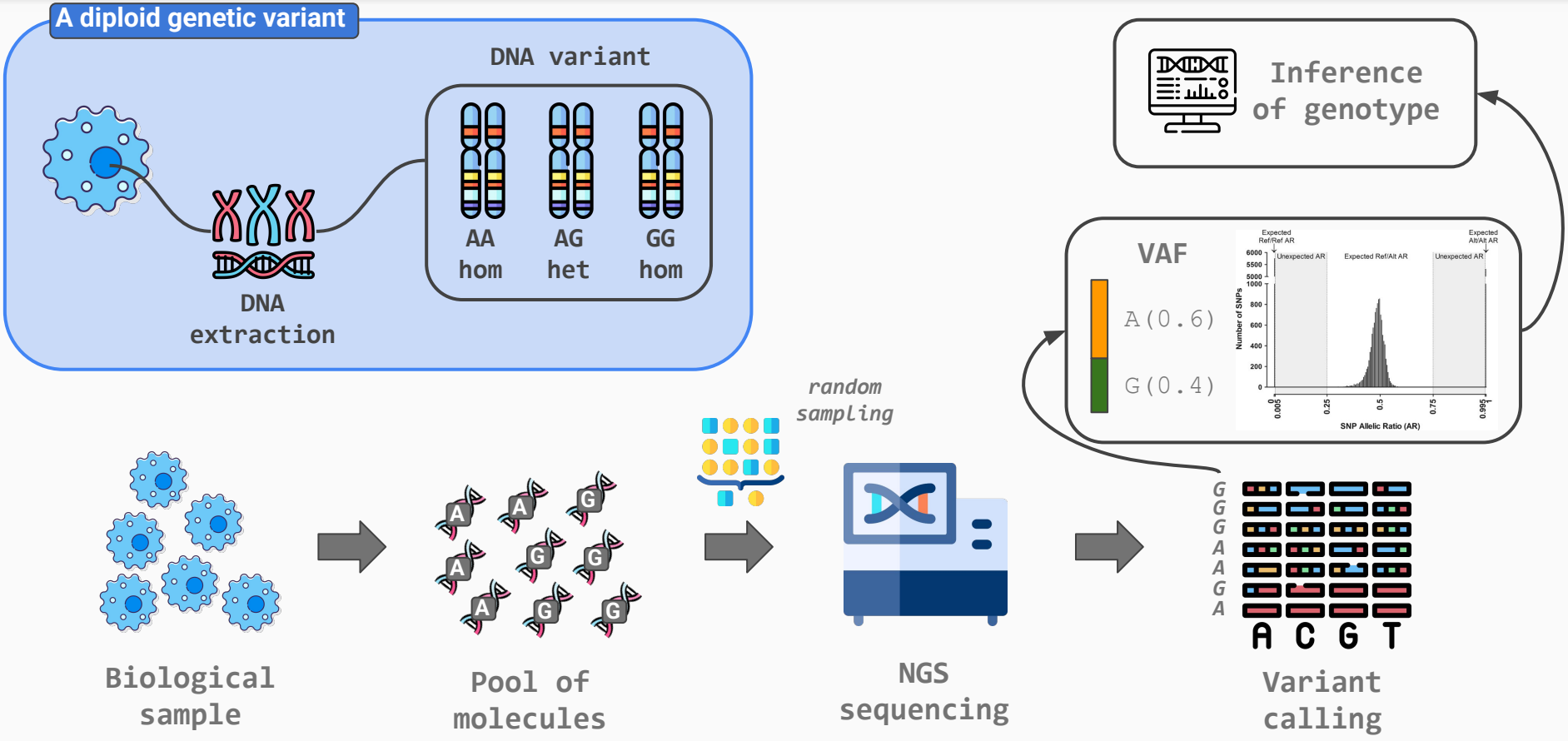
# Introduction

NGS basic concepts and short-reads QC

# Variant calling from NGS - what is this?



# NGS basic concepts

# NGS workflow

Essential steps in NGS data generation for short and long-reads

# NGS Sequencing technologies

## Next-Generation Sequencing

Massive parallel sequencing of DNA fragments

### 2nd generation

Short fragments (50-300bp)

*Needs DNA fragmentation*




### 3rd generation

Long fragments (10-100kb or more)

*DNA molecules are directly used for sequencing*


# NGS short reads - library preparation

Template DNA

Fragmentation

Adapters

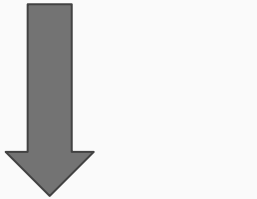
Clonal amplification

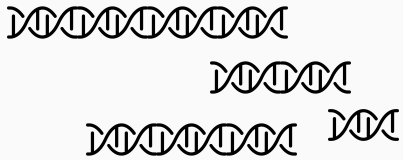
Sequencing

## Long reads


UV light shearing

Enzymatic cut





Variable length fragments

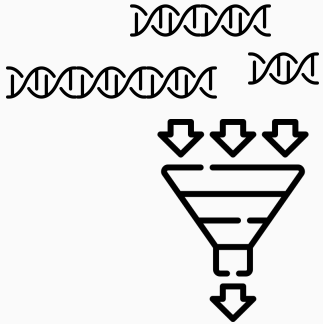
## Short reads

Enzymatic cut

Sonication

Nebulization


Targeted amplification



Size selection

Uniform size small fragments

# NGS short reads - library preparation

Template DNA

Fragmentation

**Adapters**

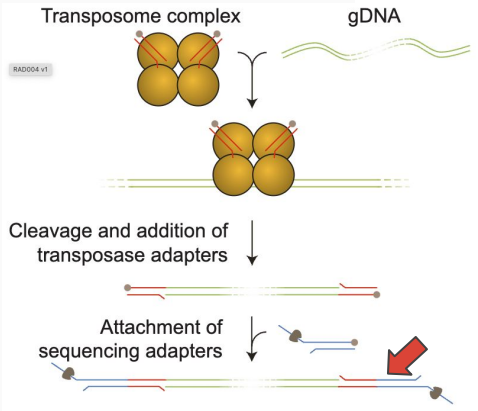
Clonal amplification

Sequencing

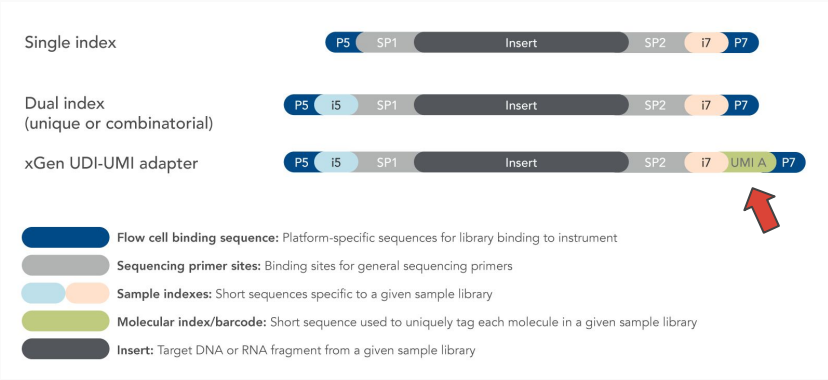
## Long reads

PacBio


Oxford NANOPORE Technologies




## Short reads



# NGS short reads - library preparation

Template DNA

Fragmentation

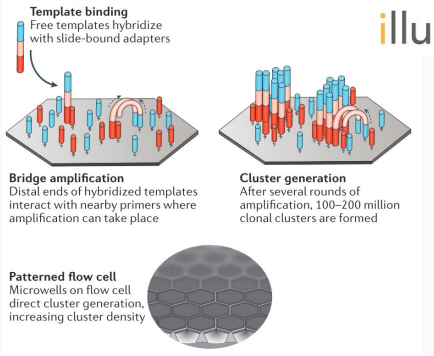
Adapters

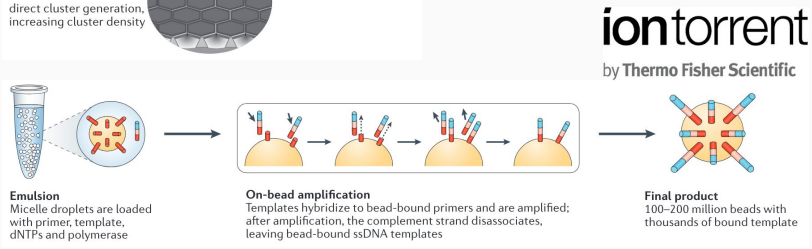
**Clonal amplification**

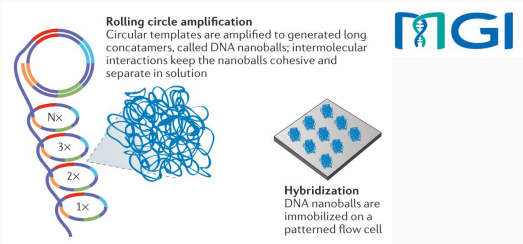
Sequencing

## Long reads


## Short reads




*Goodwin et al., 2016*

# NGS short reads - library preparation

Template DNA

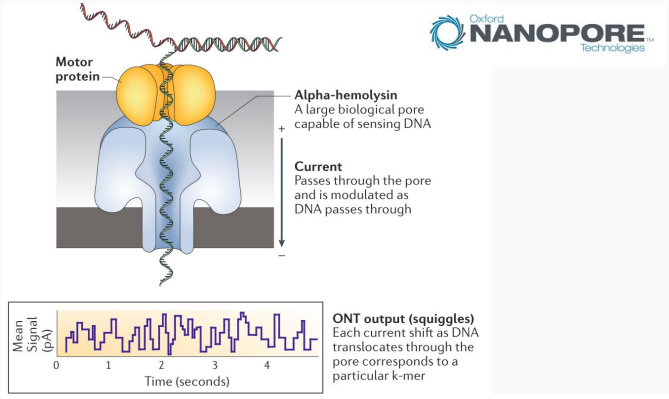
Fragmentation

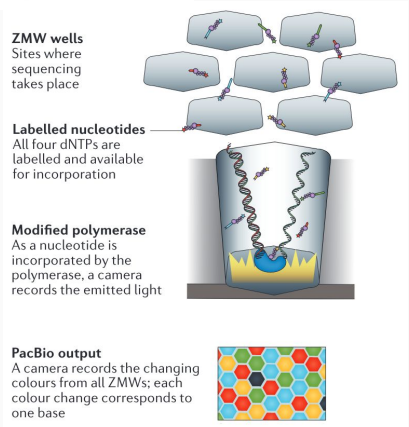
Adapters

Clonal amplification

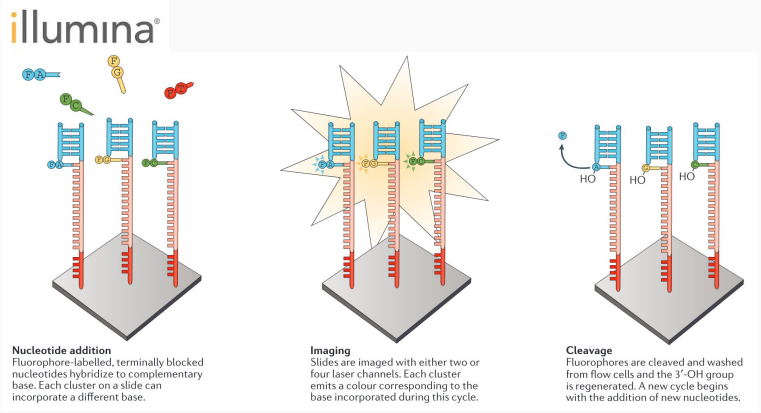
**Sequencing**

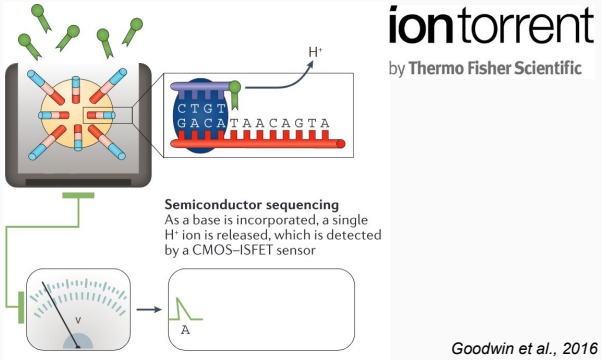
## LONG READS




## SHORT READS



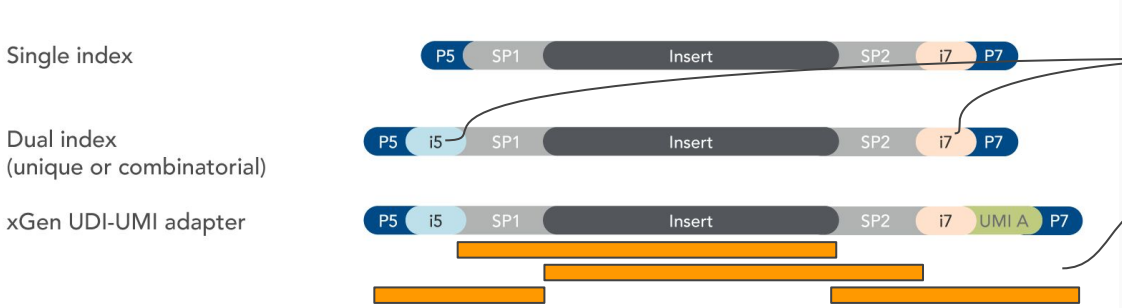

Goodwin et al., 2016

# NGS reads structure

Understand the structure of sequencing libraries and the resulting data

- adapters
- read configuration
- UMIs

# NGS data - understand library structure

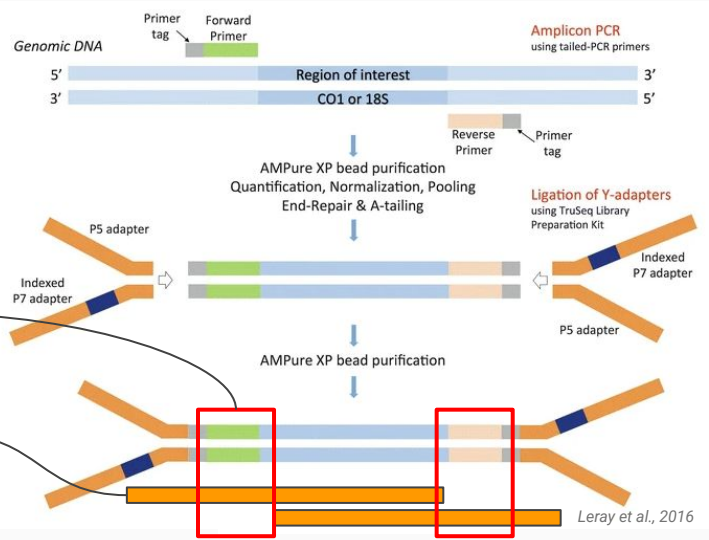



Library barcode(s) for multiplexing

Example of sequencing reads

## Standard library preparation

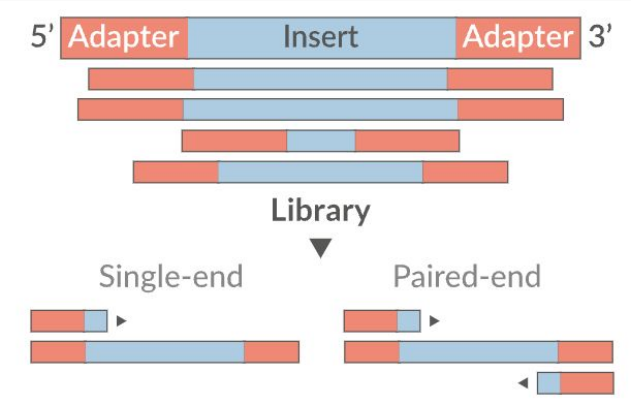
## Amplicon targeted library



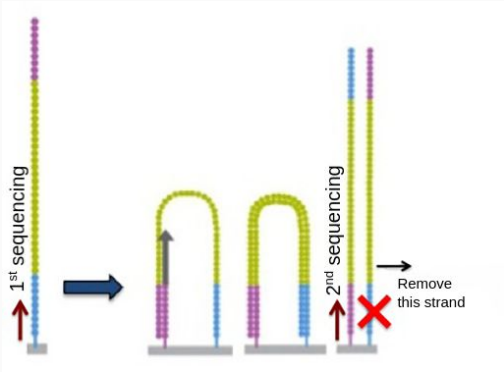

Read contains the PCR primer

Example of sequencing reads

# NGS data - single end and paired-end short reads

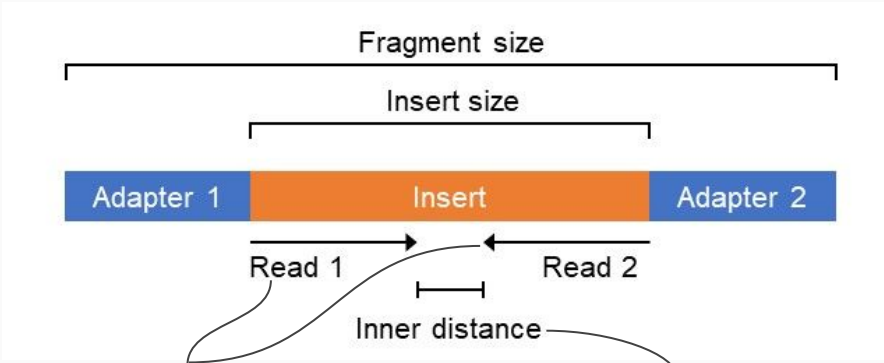



Illumina paired-end sequencing

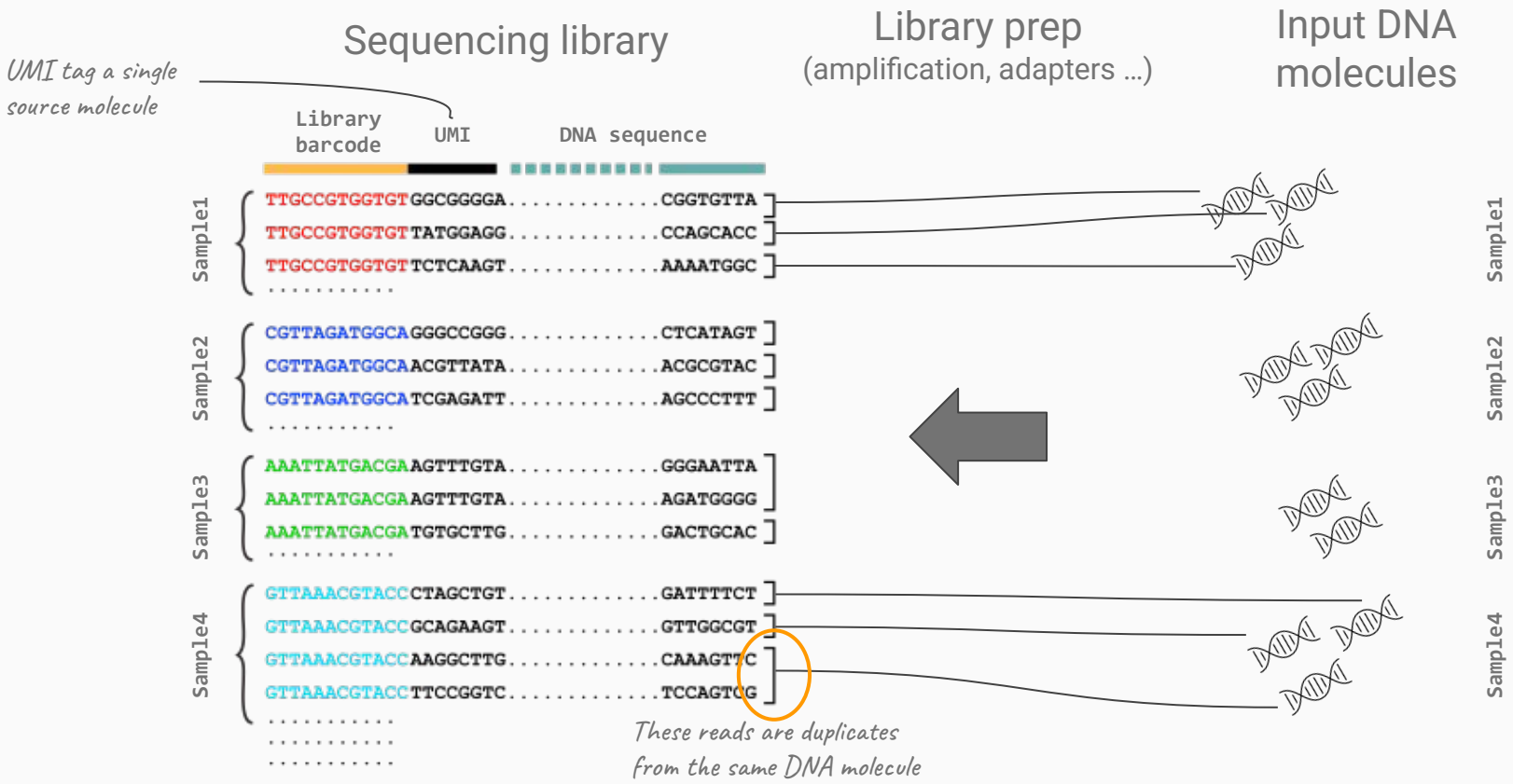



In **single end protocol**, each DNA molecule is sequenced once starting from a specific end.

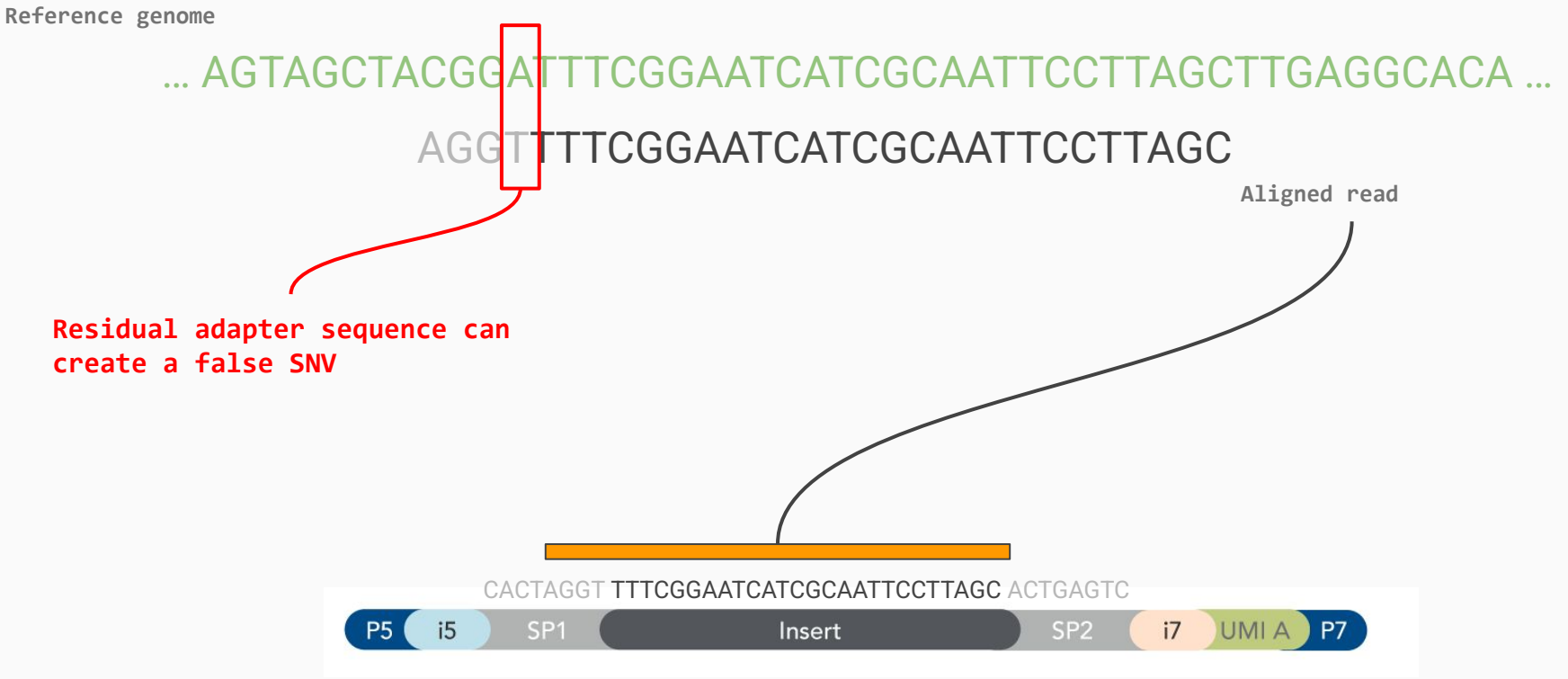
In **paired-end protocol**, it is sequenced twice starting from opposing ends



# NGS data - understand UMIs



# NGS data - library structure impacts downstream analysis



# NGS data - library structure impacts downstream analysis


- A sequencing read derives from multiple molecular manipulations and contains specific elements besides the template DNA
- Knowing the elements and structure of sequencing reads is crucial for proper downstream processing
- Fail to remove adapters parts can results in increased error rate in variant calling
- When a library is generated by targeted amplification, no variant can be detected in the target primers sequences.

# Data formats

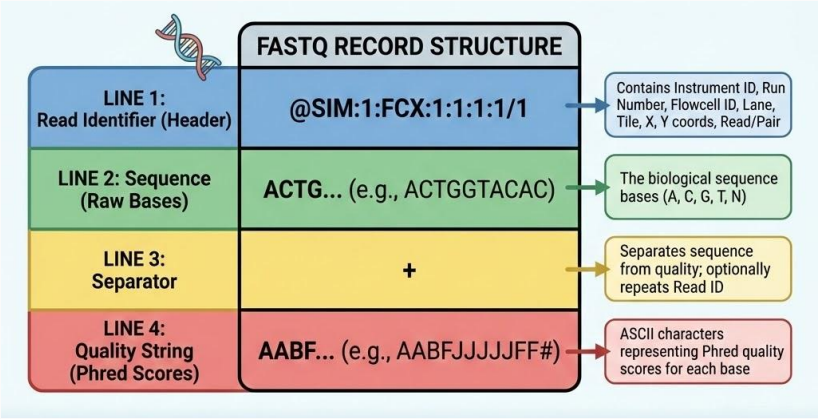
# Main file formats

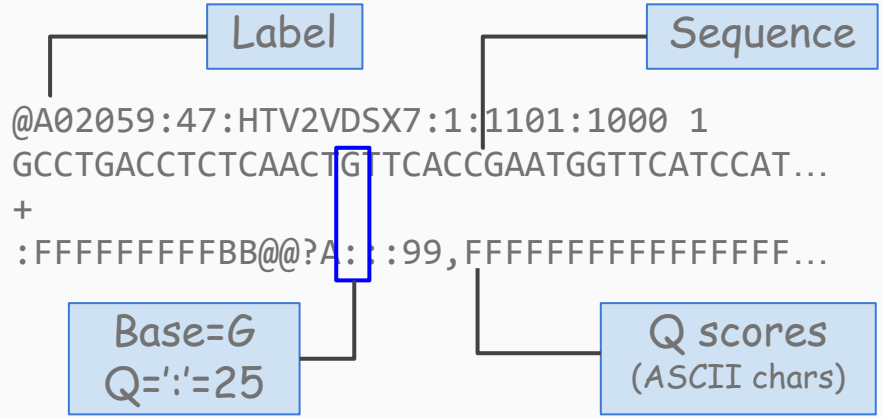
- FASTQ: sequences

# Sequences - FASTQ files

Sequence and base quality of each read

```
@A02059:47:HTV2VDSX7:1:1101:2284:1000 1:N:0:TGTAATCGAC+NGCGGTGATC
GCCTGACCTCTCCATCAACTGTTCACCGAATGGTTCATCCATGTTGGGTTTTGCCTAAATCACTTTACATCATTAGAGTTTGAA
+
:FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF:FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF
@A02059:47:HTV2VDSX7:1:1101:4743:1000 1:N:0:TGTAATCGAC+NGCGGTGATC
GGCCTCGGCCAGGCACGGTGGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCAGGCAGATCACCTGAGGTCAGGAGT
+
FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF
```




- **Label**
  unique identifier for the single read (instrument ID, run number, chip ID, tile, tile XY …)
- **Q scores**
  phred like quality score for each base sequenced encoded as ASCII character

# Sequences - Base quality in FASTQ files

*PHRED quality*

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

$$P = 10^{\frac{-Q}{10}}$$

| 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 10,000 | 99.99% |

*Quality encoding as ASCII characters*

```
@SRR038845.3 HWI-EAS038:6:1:0:1938 length=36
CAACGAGTTCACACCTTGGCCGACAGGCCCGGGTAA
+SRR038845.3 HWI-EAS038:6:1:0:1938 length=36
BA@7>B=>:>>7@7@>>9=BAA?;>52;>:9=8.=A
```

Quality converted to single ASCII character

PHRED+33 ⇒ ASCII code ⇒ Char

| Char | ASCII code | Phred quality |
|---|---|---|
| B | 66 | 33 |
| A | 65 | 32 |
| @ | 64 | 31 |
| 7 | 55 | 22 |

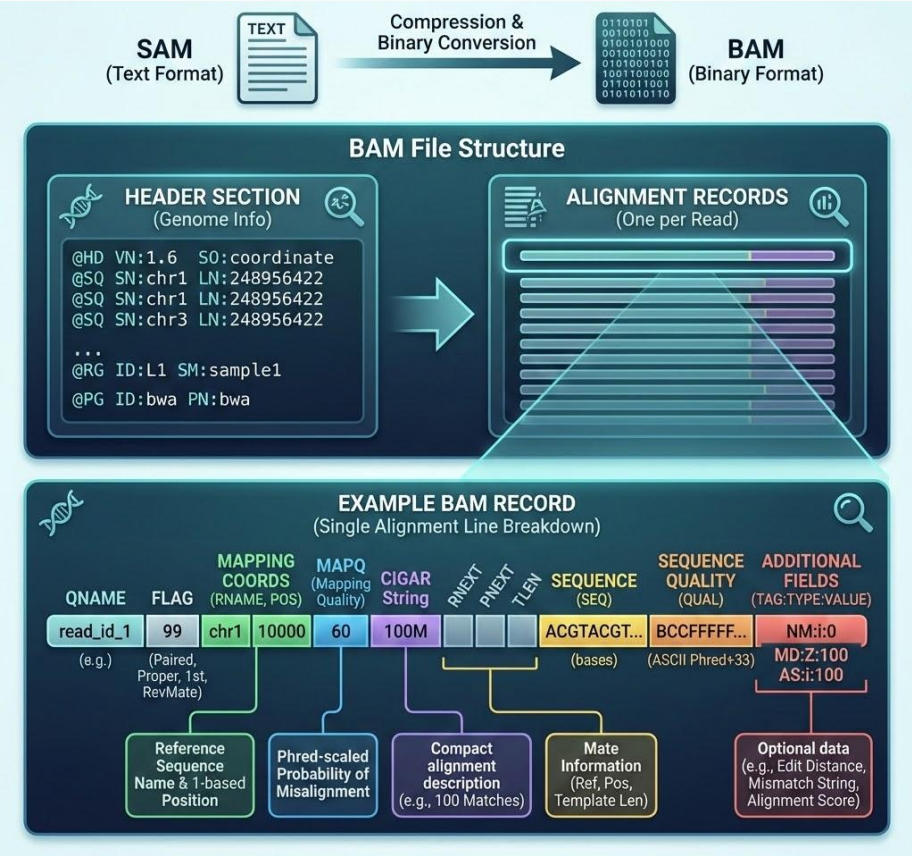
**ASCII Table**

| Dec | Hx | Oct | Html | Chr | Dec | Hx | Oct | Html | Chr |
|---|---|---|---|---|---|---|---|---|---|
| 64 | 40 | 100 | @ | @ | 96 | 60 | 140 | ` | ` |
| 65 | 41 | 101 | A | A | 97 | 61 | 141 | a | a |
| 66 | 42 | 102 | B | B | 98 | 62 | 142 | b | b |
| 67 | 43 | 103 | C | C | 99 | 63 | 143 | c | c |
| 68 | 44 | 104 | D | D | 100 | 64 | 144 | d | d |
| 69 | 45 | 105 | E | E | 101 | 65 | 145 | e | e |
| 70 | 46 | 106 | F | F | 102 | 66 | 146 | f | f |
| 71 | 47 | 107 | G | G | 103 | 67 | 147 | g | g |
| 72 | 48 | 110 | H | H | 104 | 68 | 150 | h | h |
| 73 | 49 | 111 | I | I | 105 | 69 | 151 | i | i |
| 74 | 4A | 112 | J | J | 106 | 6A | 152 | j | j |
| 75 | 4B | 113 | K | K | 107 | 6B | 153 | k | k |
| 76 | 4C | 114 | L | L | 108 | 6C | 154 | l | l |
| 77 | 4D | 115 | M | M | 109 | 6D | 155 | m | m |
| 78 | 4E | 116 | N | N | 110 | 6E | 156 | n | n |
| 79 | 4F | 117 | O | O | 111 | 6F | 157 | o | o |
| 80 | 50 | 120 | P | P | 112 | 70 | 160 | p | p |
| 81 | 51 | 121 | Q | Q | 113 | 71 | 161 | q | q |
| 82 | 52 | 122 | R | R | 114 | 72 | 162 | r | r |

# Aligned sequences - The BAM file

- BAM file stores aligned reads

- For each alignment records we can find
  - *coordinates*
  - *a flag with information about the read mapping*
  - *mapping quality*
  - *CIGAR string*
  - *additional information stored as named fields*



# Short-reads QC

# Reads cleaning

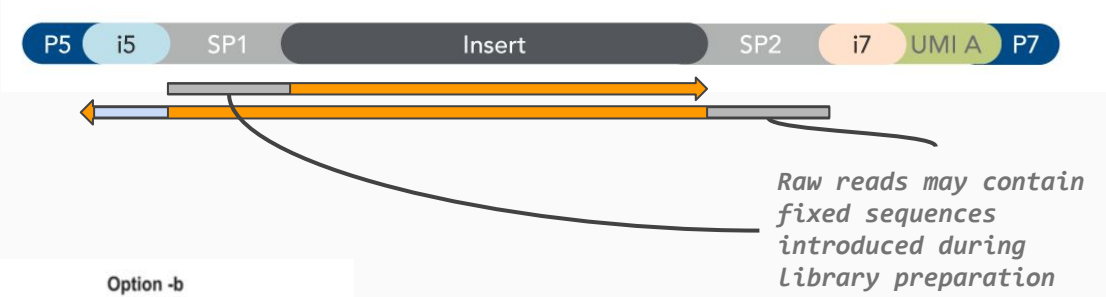
Remove unwanted sequences and poor quality bases

- adapter trimming
- quality trimming
- fixed length trimming

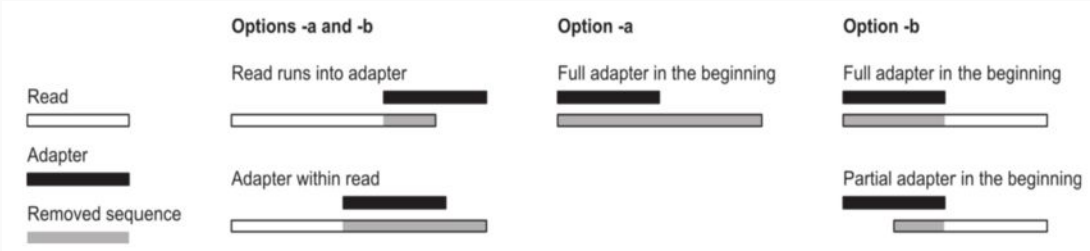
*Useful tools*

*fastp, cutadapt*

# Adapter trimming - remove unwanted fixed sequences

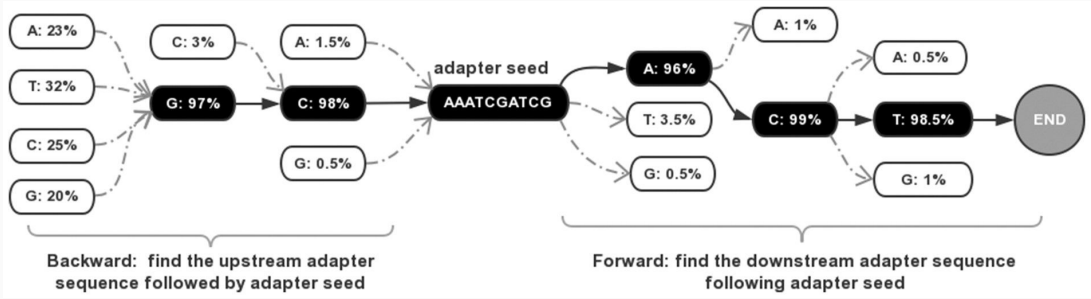



*Example from [cutadapt tool]*

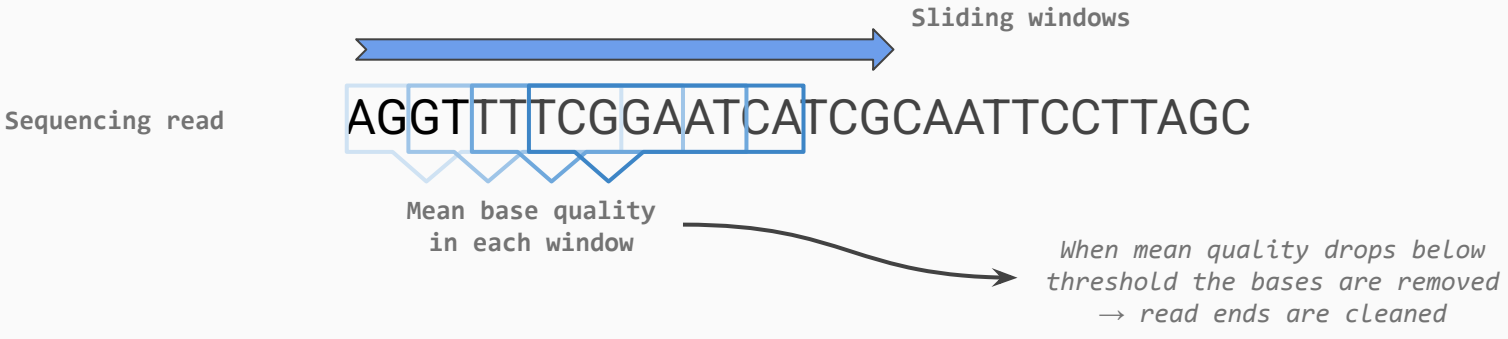


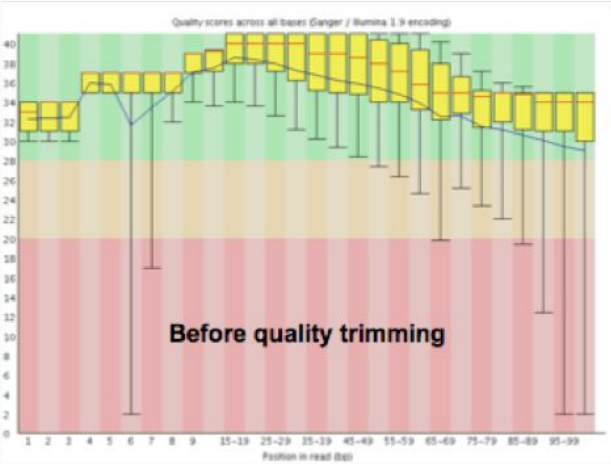

Trimming tools scan the read ends removing fixed sequences

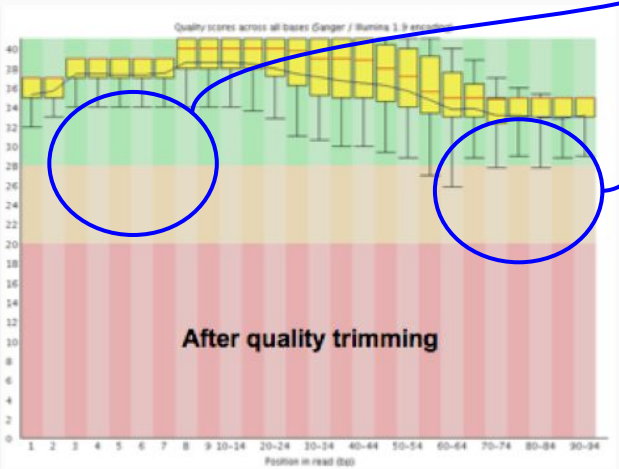
*Based on adapter known sequences the algorithm search for partial matches and then extend the matching region to completely remove adapter sequences*



# Quality trimming - remove low quality bases from read ends

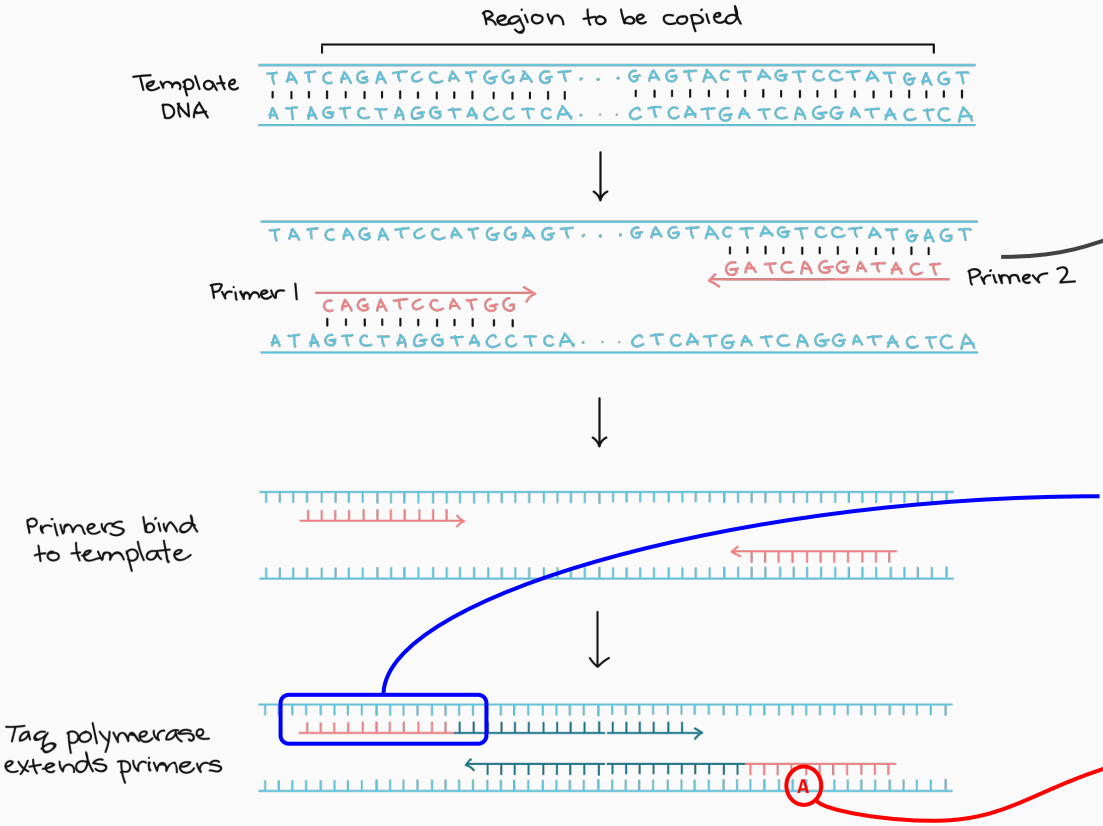



https://h3abionet.github.io/H3ABionet-SOPs/RNA-Seq-2-3.html

# Fixed length trimming - remove a fixed amount of bases from read ends




A library is generated started from targeted amplification

*Primers are used to amplify region(s) of interest*

*The primer sequence and NOT the real DNA sequence is included in the amplified fragment*
*→ primers should be trimmed!*

***A variant present in this region will not be detected (masked by the primer)***

https://www.khanacademy.org/science/ap-biology/gene-expression-and-regulation/biotechnology/a/polymerase-chain-reaction-pcr

# Short-reads QC

- raw reads length and quality
- GC content
- adapter content
- mapping statistics

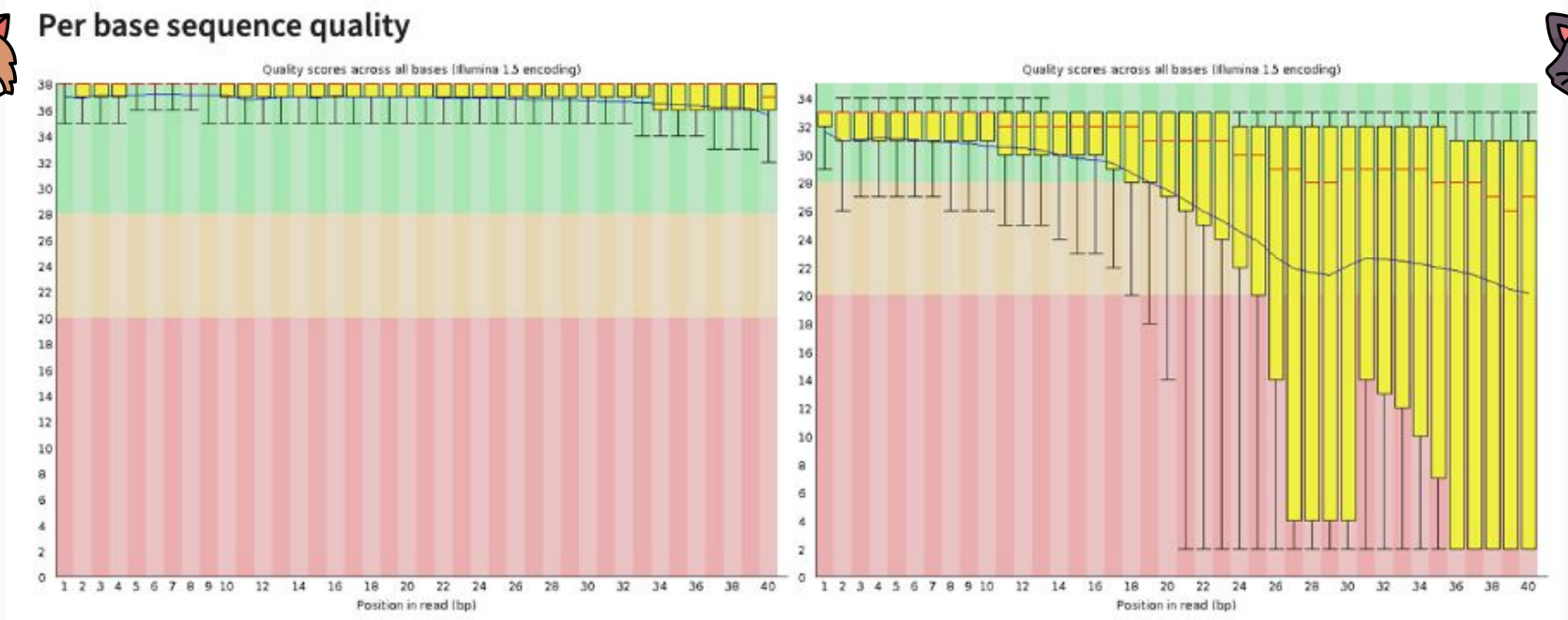
*Useful tools*

*fastp, fastQC, samtools*

# Reads QC - Per base sequence quality across the reads

fastQC / FASTP

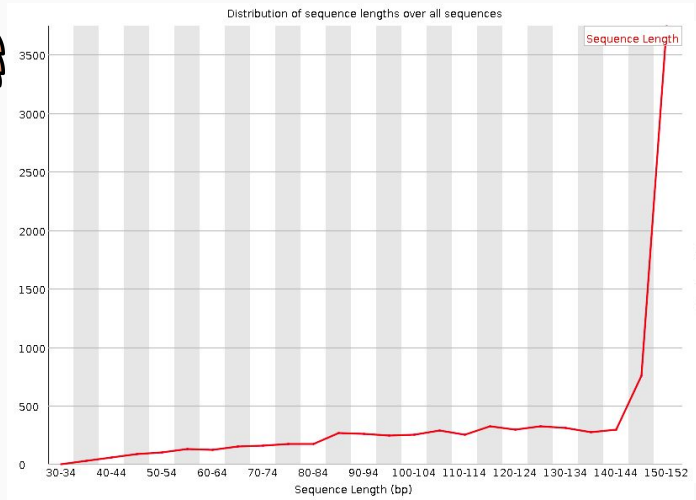
- Ideally, base quality should be >= 30 across all the read
- A little decrease in quality is expected toward the end of the read
- Base quality is considered during variant calling and can affect variant caller performances

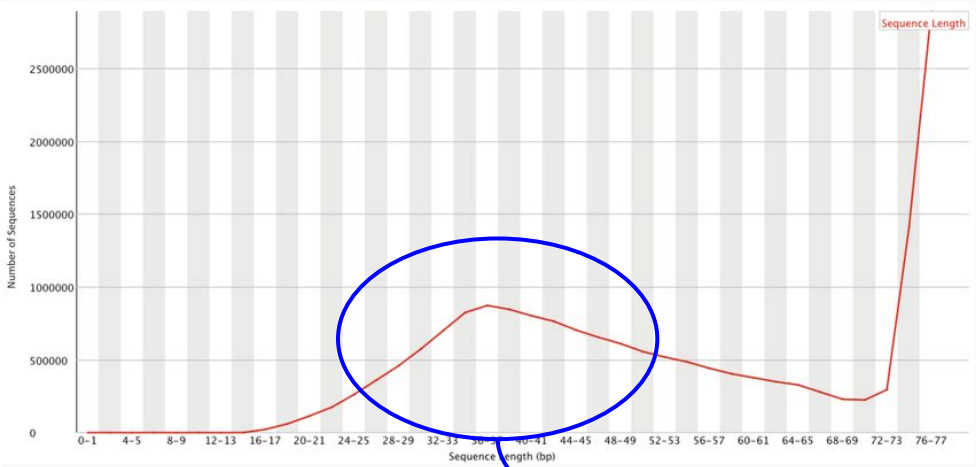


# Read QC - sequence length distribution

fastQC / FASTP

- Most read should have the expected read length
- A small tail on the left is acceptable, usually indicate trimming was performed



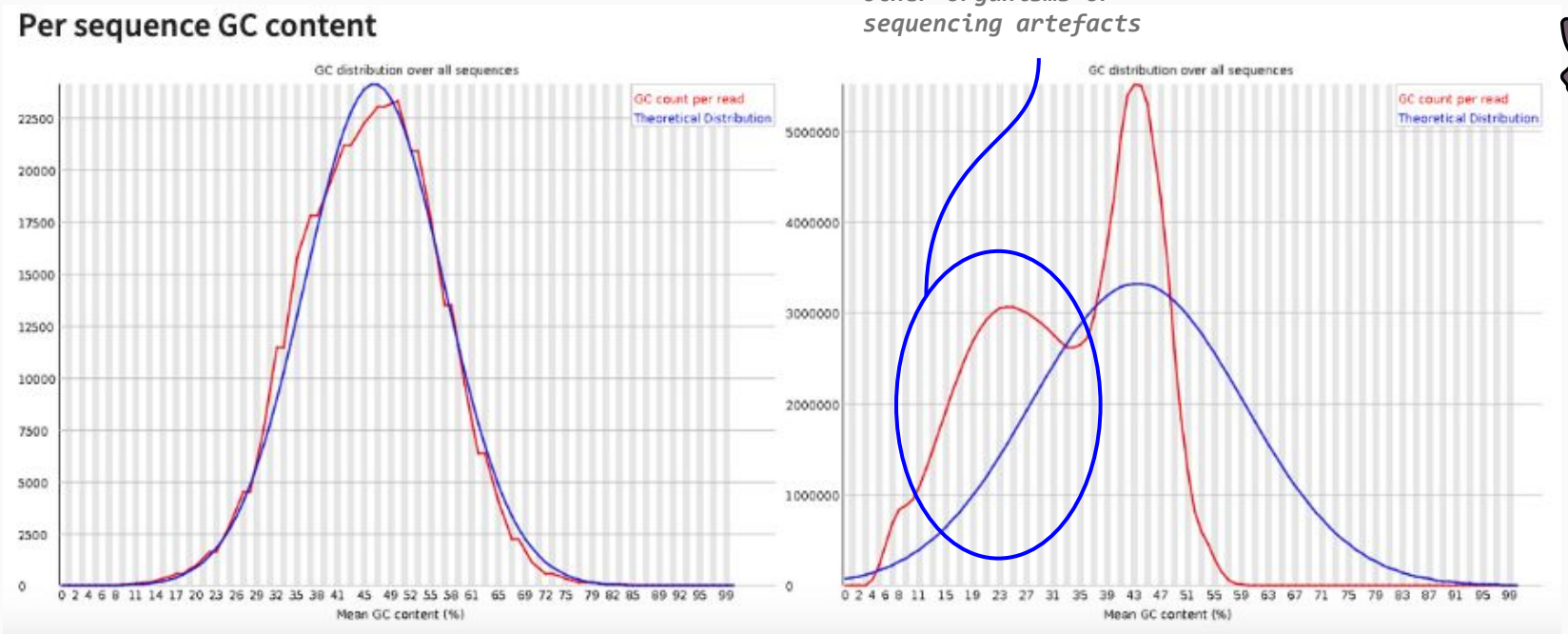

*Sequencing problems, low quality reads that got trimmed, adapter dimers*

# Reads QC - reads GC content

- Distribution should follow the expected for the sequenced organism
- Peak around 45% for human genome samples

**fastQC / FASTP**

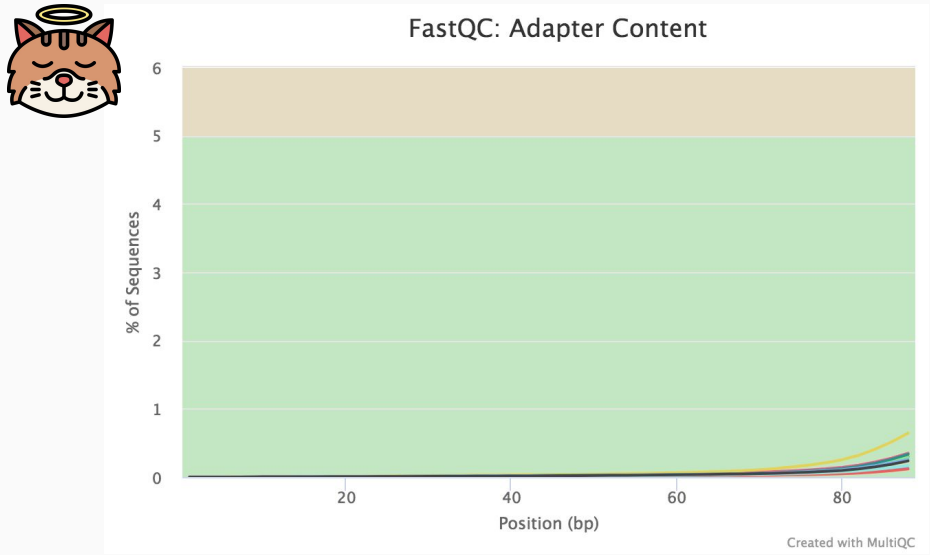
*Contamination from DNA of other organisms or sequencing artefacts*

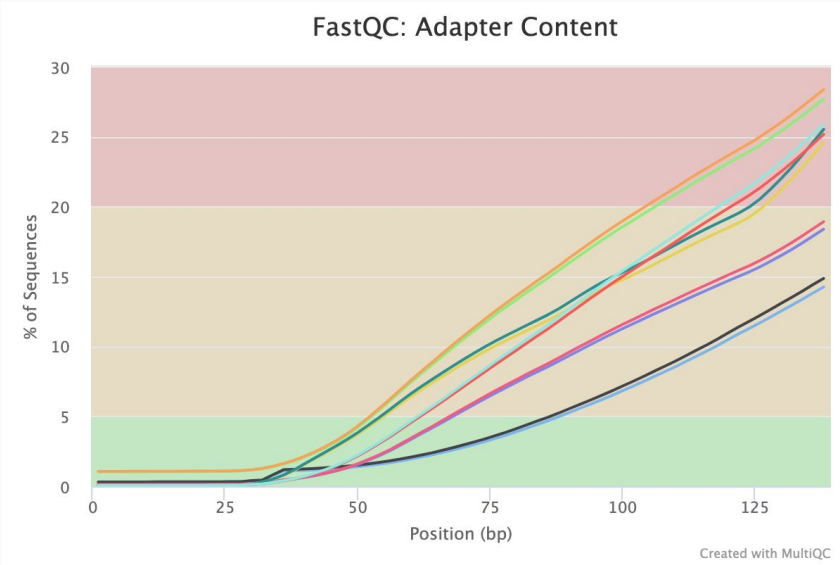


# Read QC - residual adapter content

fastQC / FASTP

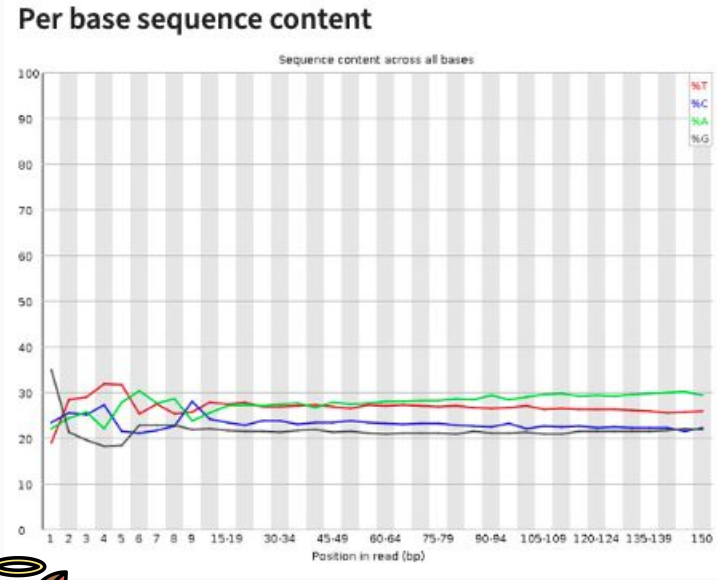
- Low presence of adapter sequences at the read ends
- Residual adapters can be cleaned by trimming



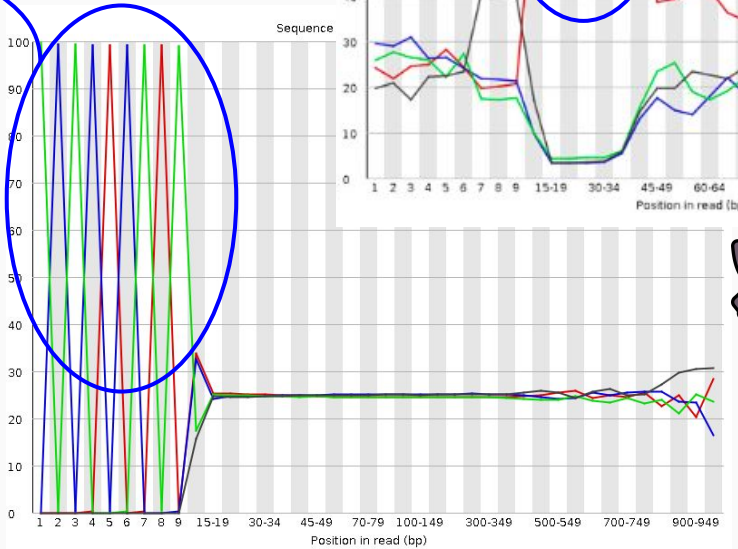



# Reads QC - Per base sequence content across the reads

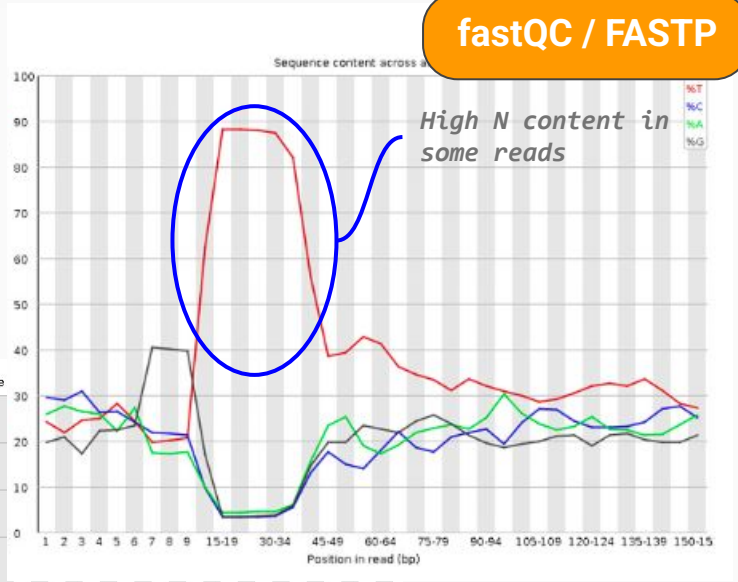
In most cases random sequences are expected
→ balanced base composition across the reads




*Residual fixed elements or sequencing artefacts*

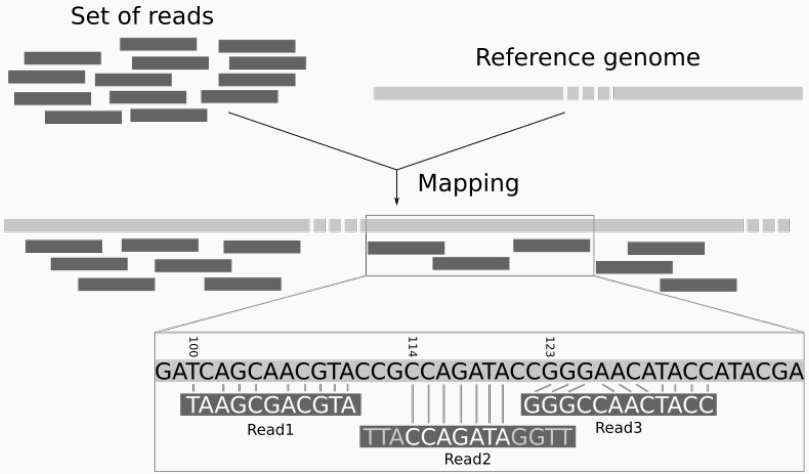



**fastQC / FASTP**




*High N content in some reads*

# Aligned reads QC - mapping statistics




**samtools flagstat**

## All libraries

- Mapping quality distribution
  *(>= 30 for good mapping)*
- Fraction of mapped reads
  *(>= 90% in good samples)*

## Paired-end libraries

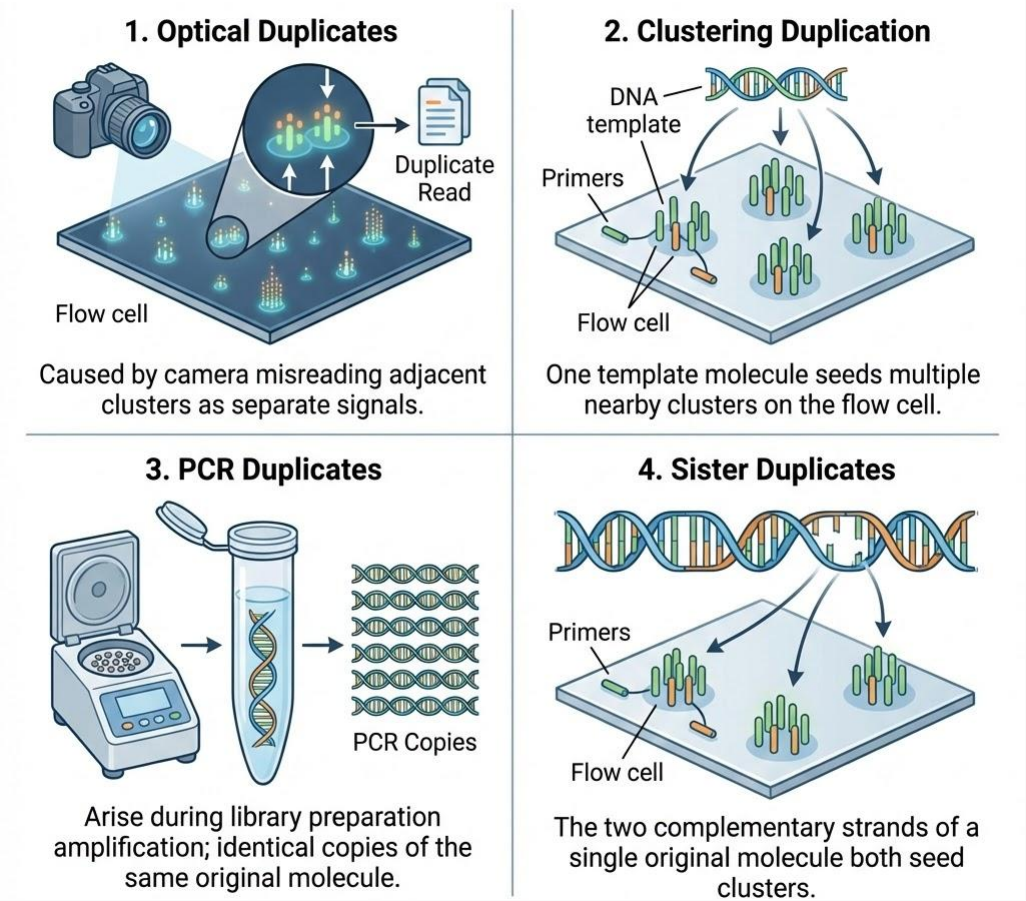
- Insert size distribution
  *(distance between F/R read)*
- Fraction of reads with a proper pair
  *(>= 90% usually)*

# Reads processing and sample QC

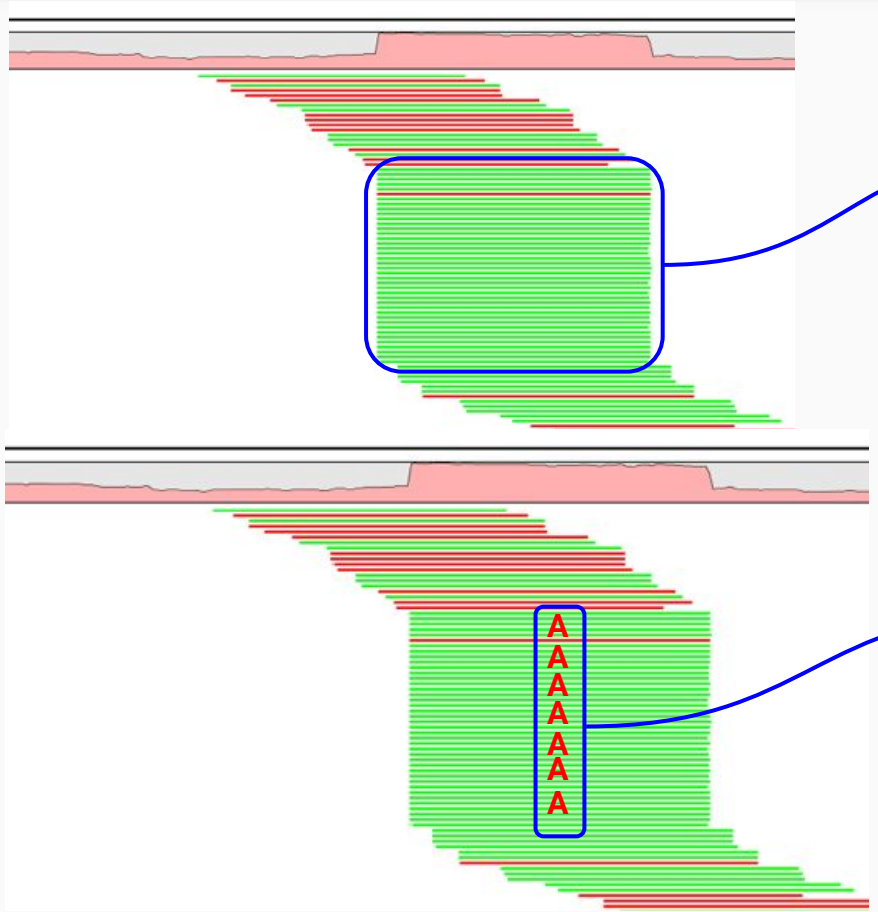
Further clean of sequencing artifacts and check for sample contamination

- duplicated reads
- UMI decomposition
- sex check
- contamination estimates

# Sequence duplicates in short reads data



# PCR or optical duplicates



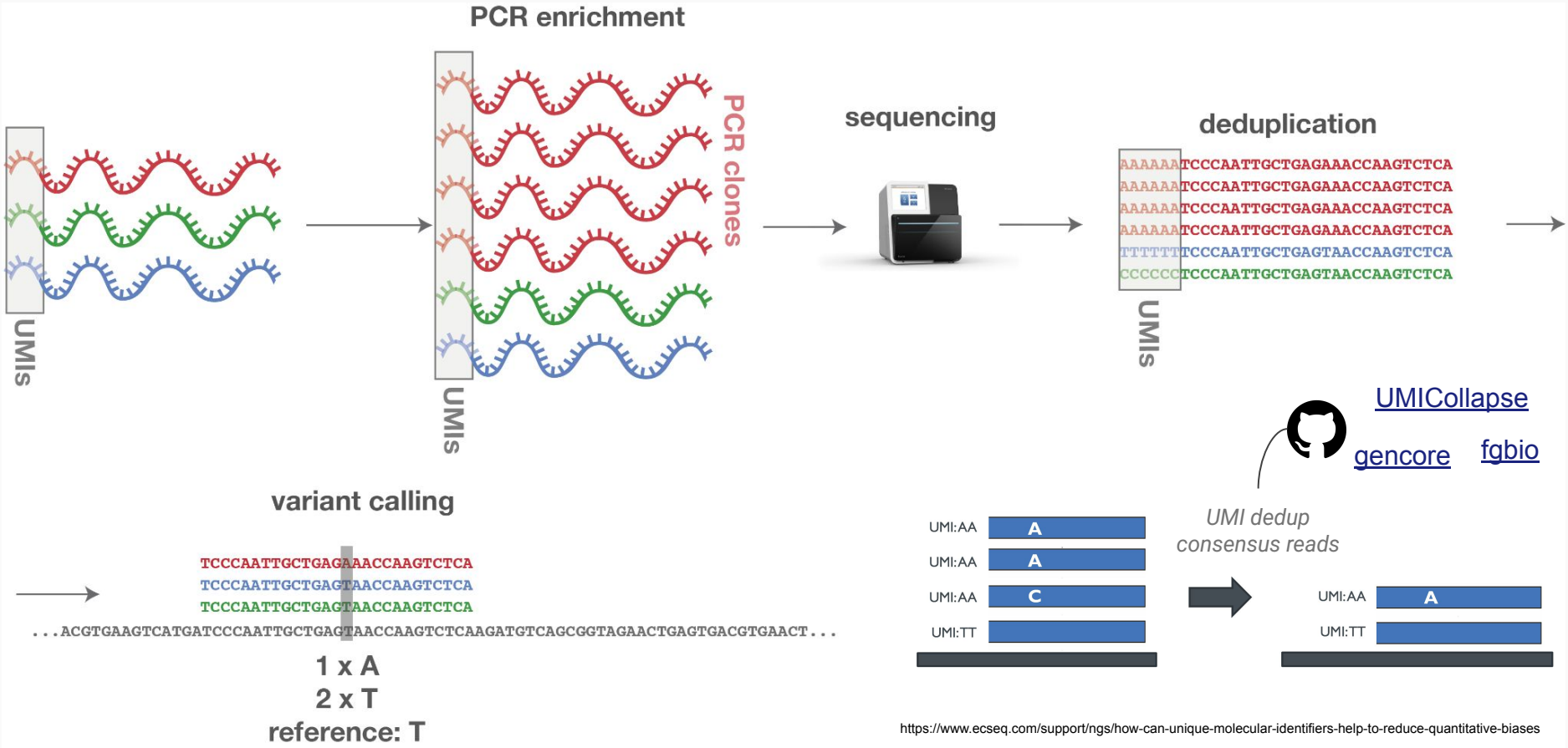

**samtools flagstat**

**Duplicated reads**
A pile or reads that actually represent multiple identical copies originated from the same DNA molecule!

*They can be identified as group of reads that have identical start/end points. They are marked and ignored during variant calling. Available tools: picard, samblaster*

Duplicated reads may affect genotyping when the duplicated molecule contains an error / SNVs

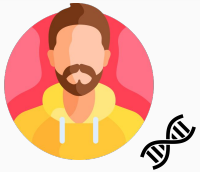
# UMI based read deduplication to increase accuracy

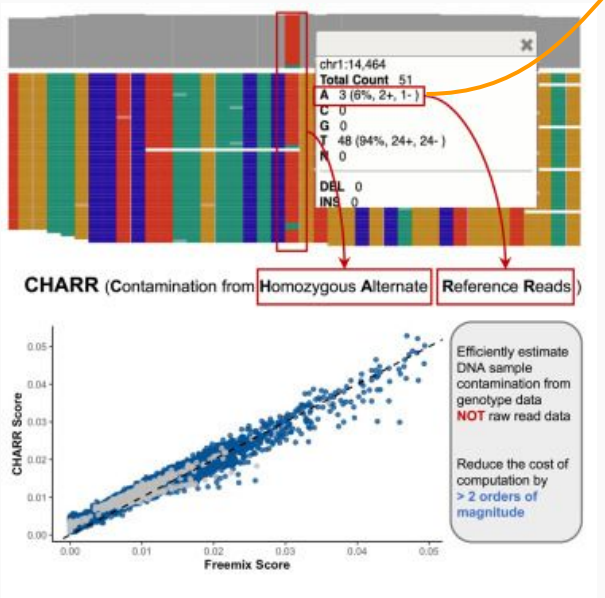



https://www.ecseq.com/support/ngs/how-can-unique-molecular-identifiers-help-to-reduce-quantitative-biases

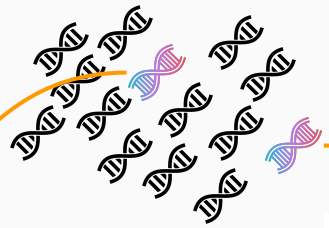
# Additional sample-level QC - detect contamination

Target sample



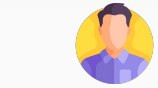



Increased fraction of “unexpected” alleles in homozygous genotypes

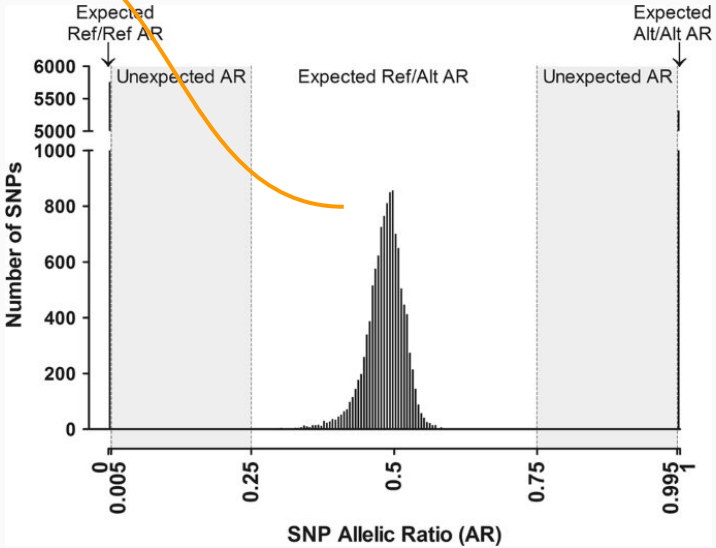


Sequenced material

Contaminating sample

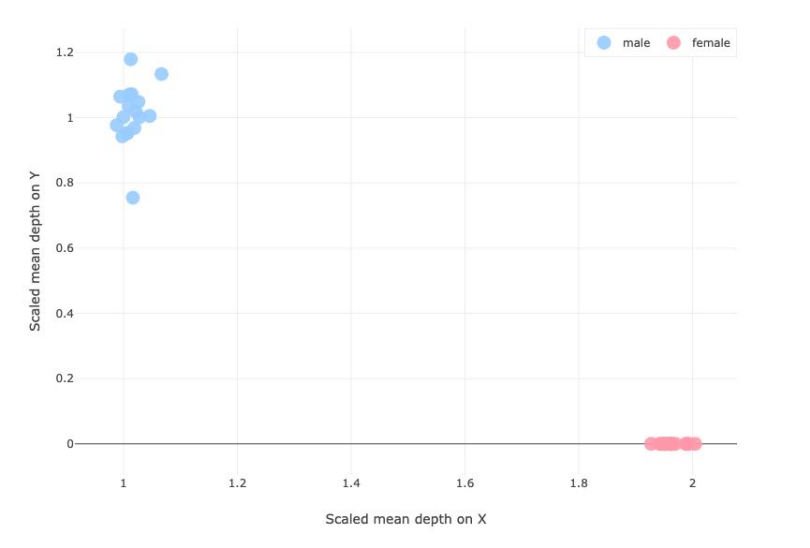


**bcftools stat**



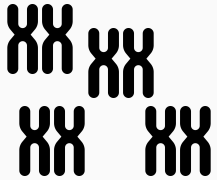

Increased N of heterozygous genotypes
Increased het GT with outlier AR

# Additional sample-level QC - sex inference




**SCALED MEAN DEPTH**

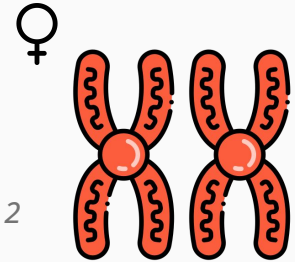
$$\frac{\textit{mean depth chrX}}{\textit{mean depth autosomes}} x\ 2$$



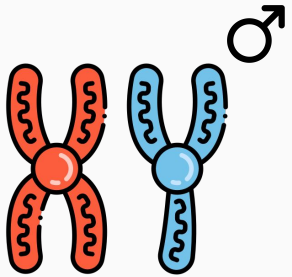
autosomes

mean depth
*expected depth for a diploid chromosome*

***Female***
*Diploid X*
*scaled depth = 2*



***Male***
*Haploid X*
*scaled depth = 1*
*Haploid Y*
*scaled depth = 1*



**samtools stat**