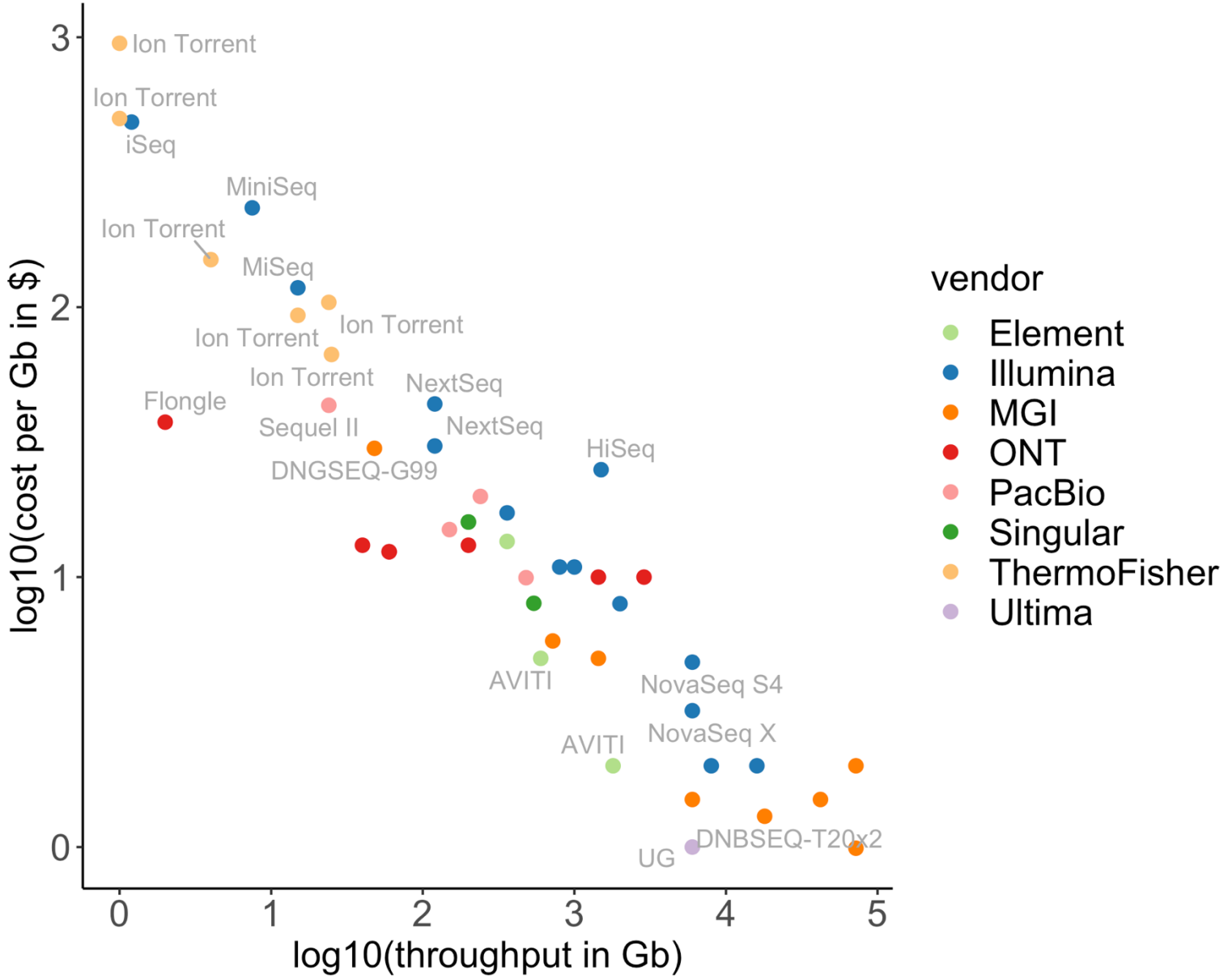
NGS – variant analysis

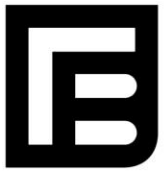
Sequencing and alignment



drawn from: https://docs.google.com/spreadsheets/d/1GMMfhyLK0-q8XkIo3YxlWaZA5vVMuhU1kg41g4xLkXc/ Albert Vilella

Sequencing-by-synthesis

- 2nd generation sequencing
- Massive throughput: up to $500x10^9$ bases/run
- Illumina still most used platform today



Element Biosciences

illumina®

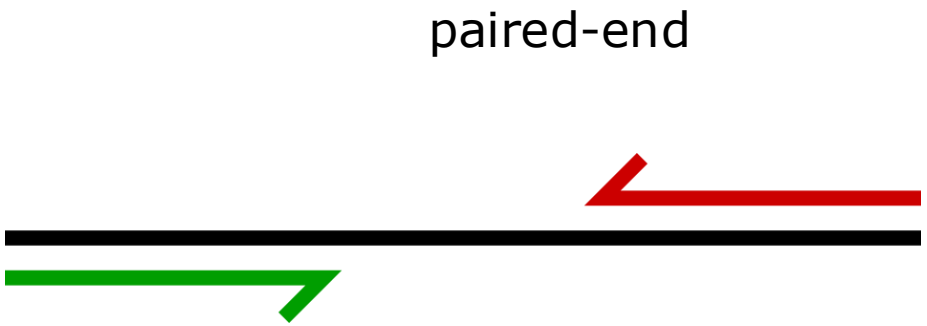
SINGULAR GENOMICS

Sequencing-by-synthesis

- 2nd generation sequencing
- Massive throughput: up to $500x10^9$ bases/run
- Most used platform today

Sequencing-by-synthesis

- 50 – 300 bp
- Paired-end (or single-end)

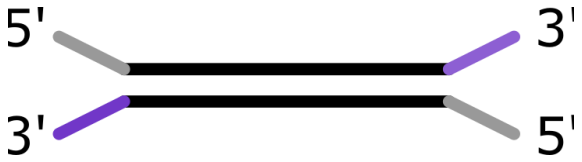


Illumina libray prep

shear DNA

↓

Ligate adapters



↓

Barcode + p5/p7 sites

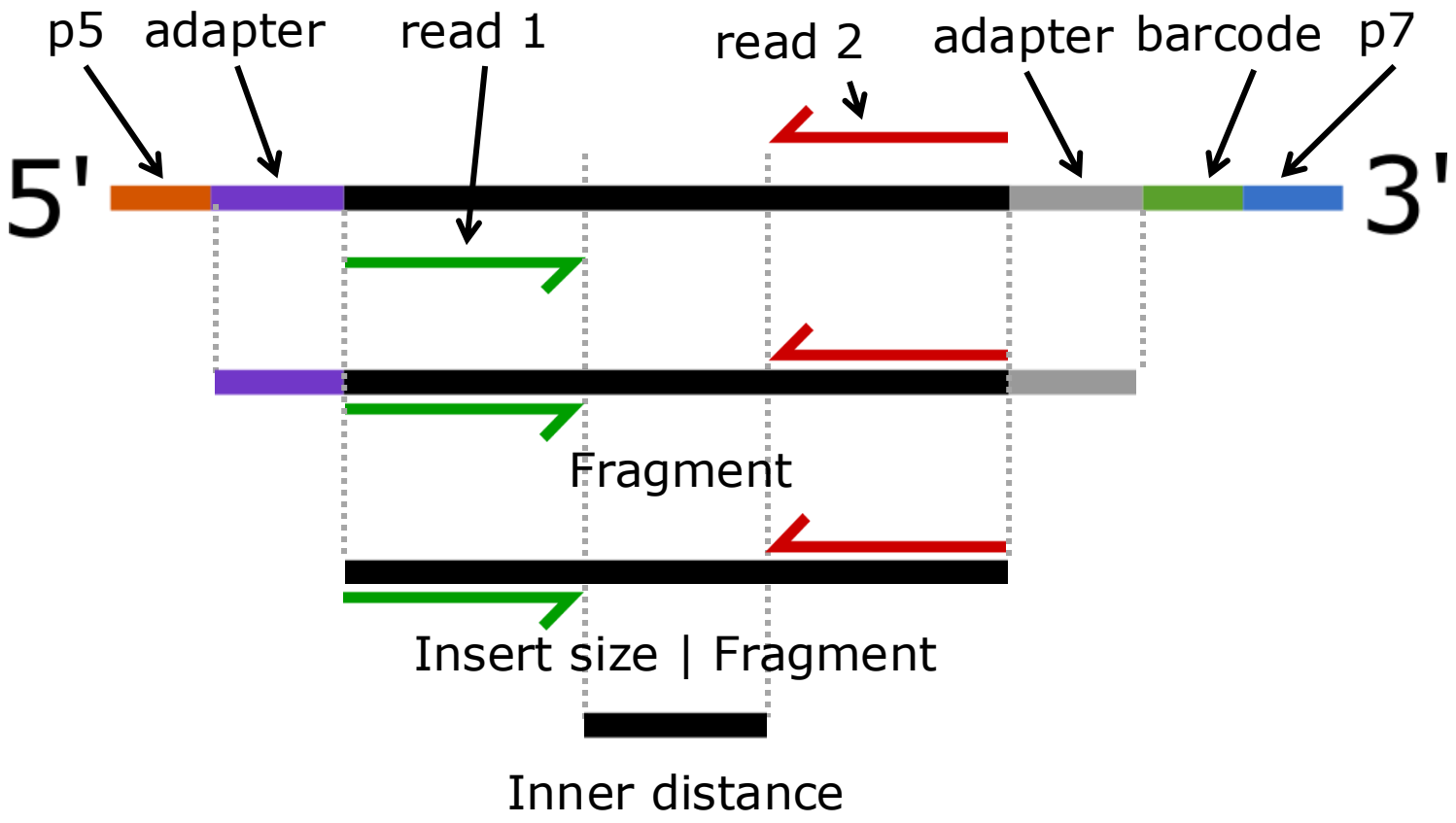
↓

PCR: 8-16 cycles

↓

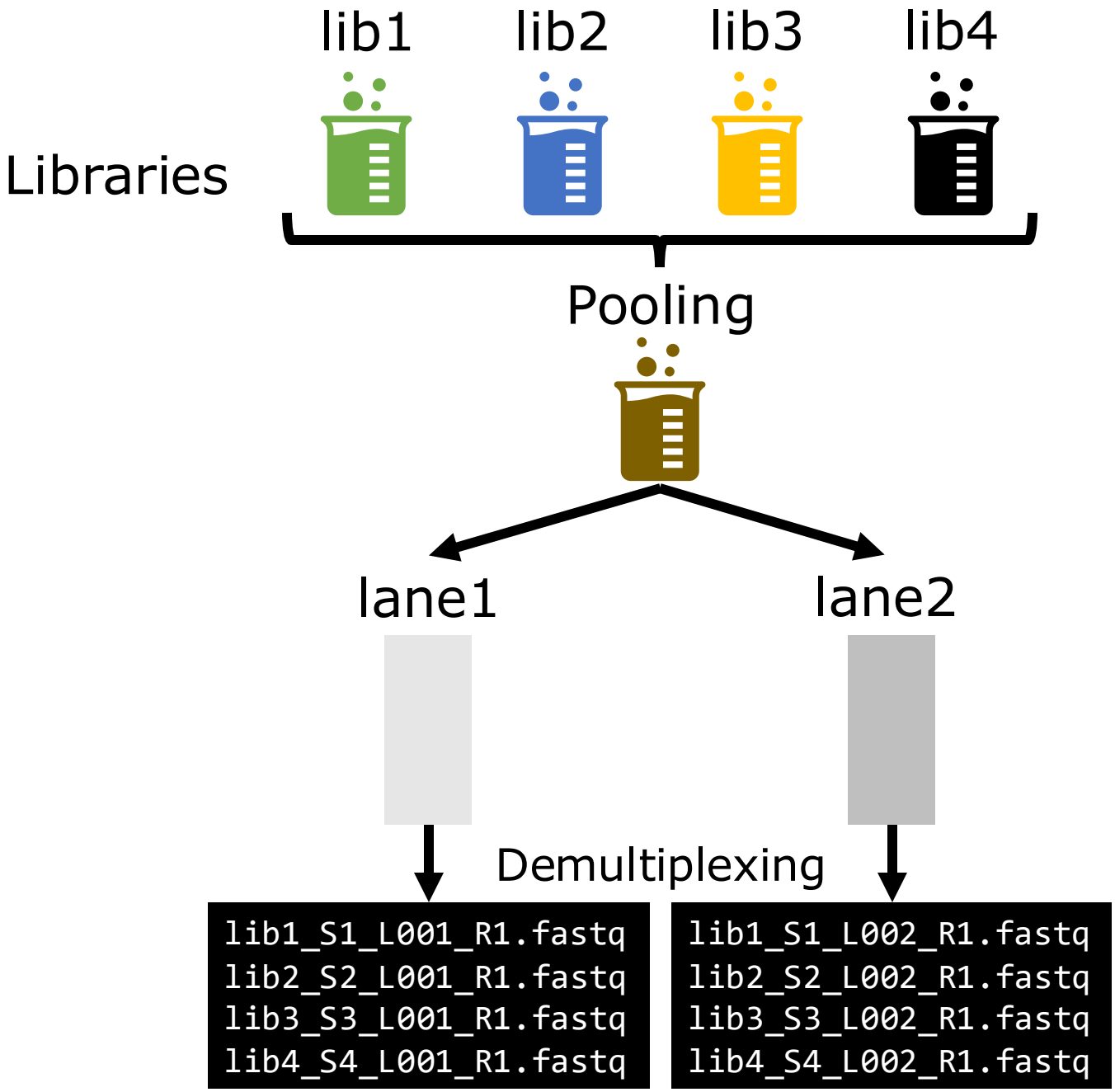
Sequencing

Some definitions



Some more definitions..

- **Library:** fragments from one (c)DNA sample that share a barcode
- **Sequencing run:** complete cycle of generating reads on a machine
- **Flow cell:** physical platform where sequencing reactions take place. Used once in a sequencing run.
- **Lane:** compartment within the flow cell. An Illumina flow cell often has multiple lanes (2 or 4)

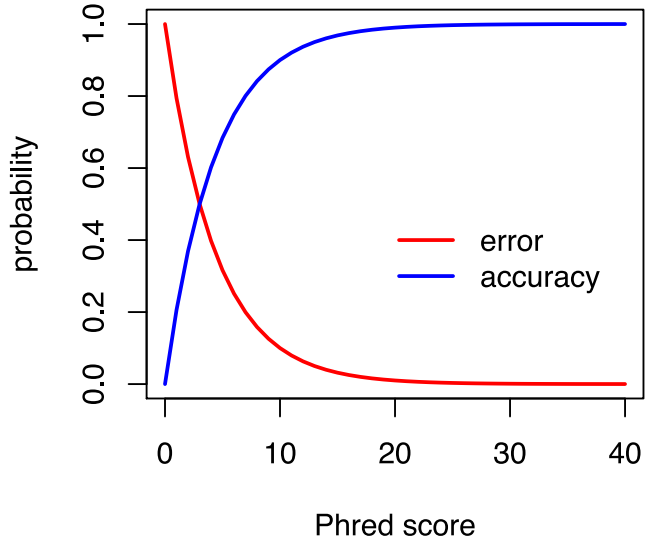

Libraries
lib1
lib2
lib3
lib4
Pooling
lane1
lane2
Demultiplexing
lib1_S1_L001_R1.fastq
lib2_S2_L001_R1.fastq
lib3_S3_L001_R1.fastq
lib4_S4_L001_R1.fastq
lib1_S1_L002_R1.fastq
lib2_S2_L002_R1.fastq
lib3_S3_L002_R1.fastq
lib4_S4_L002_R1.fastq

fastq

fasta + basequality (fasta + q = fastq)

$$BASEQ = -10log_{10}\Pr\{base\ is\ wrong\}$$

$$-10log_{10}(0.01) = 20$$
$$-10log_{10}(0.1) = 10$$
$$-10log_{10}(0.5) = 3$$



Question

fastq files

reads.fastq

```
@D00283R:66:CC611ANXX:4:2311:2596:2330 1:N:0:TCCGGAG
ACTCTACGCTCAATAAAGATTTCTGATACGGCTCCTGAAATGCAGAATGAGT
+
B/<<<B<FFFFFFFFFBBFFFBFFFFBFFFF/FFFFFFFF/BFFFFFBFFF
```

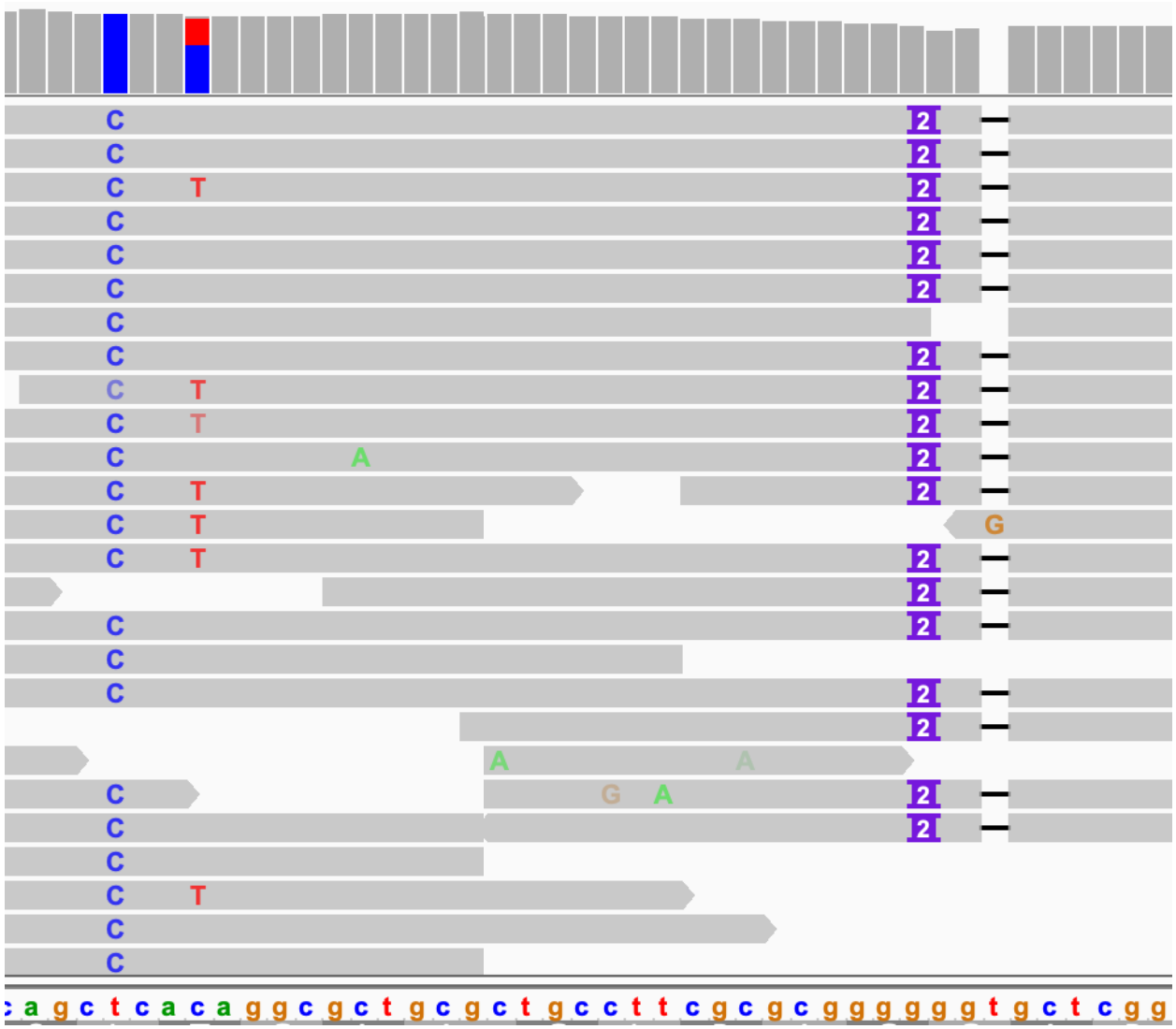
title, starts with @

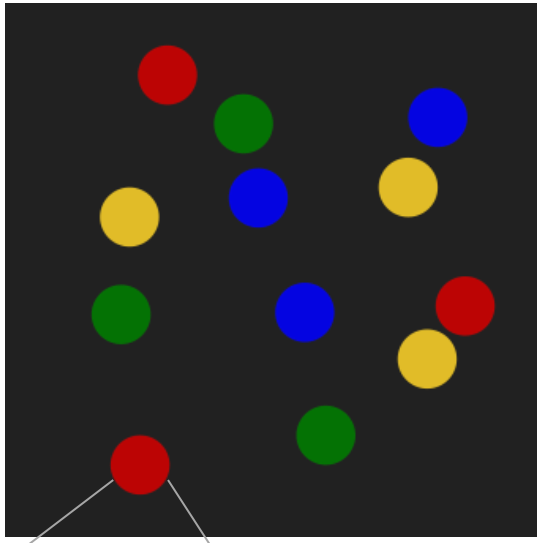
nucleotide sequence

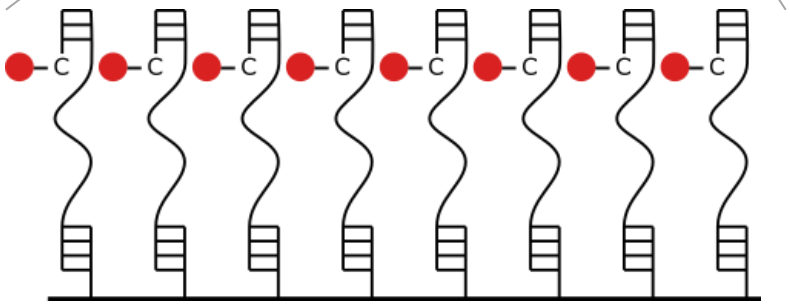
optional description

base quality

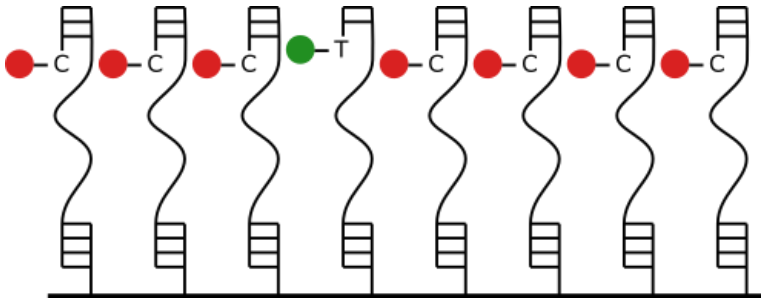
```
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHI
|                              |    |        |
0.2.....................26...31........41
```







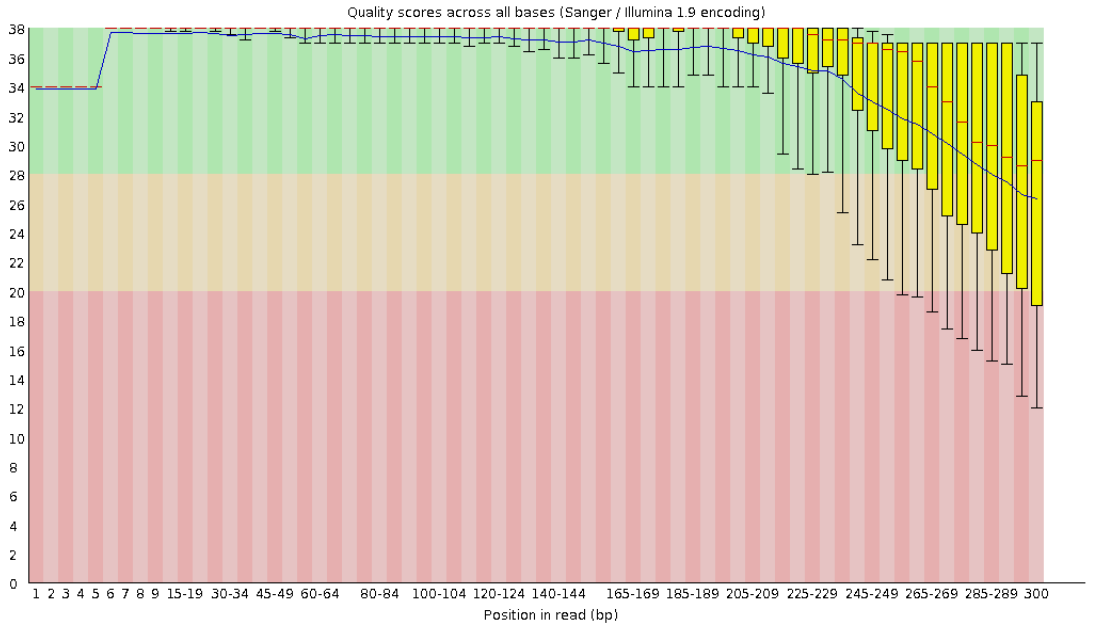
in phase



out of phase

Illumina - limitations

- Bridge amplification
- Lengths are limited by out-of-phase of signal



Long reads (3rd generation)

- Crux: maximizing signal from a single-molecule base read-out
- Single molecule, so no out-of-phase signal
- Two frequently used platforms:
  - PacBio SMRT sequencing
  - Oxford Nanopore Technology

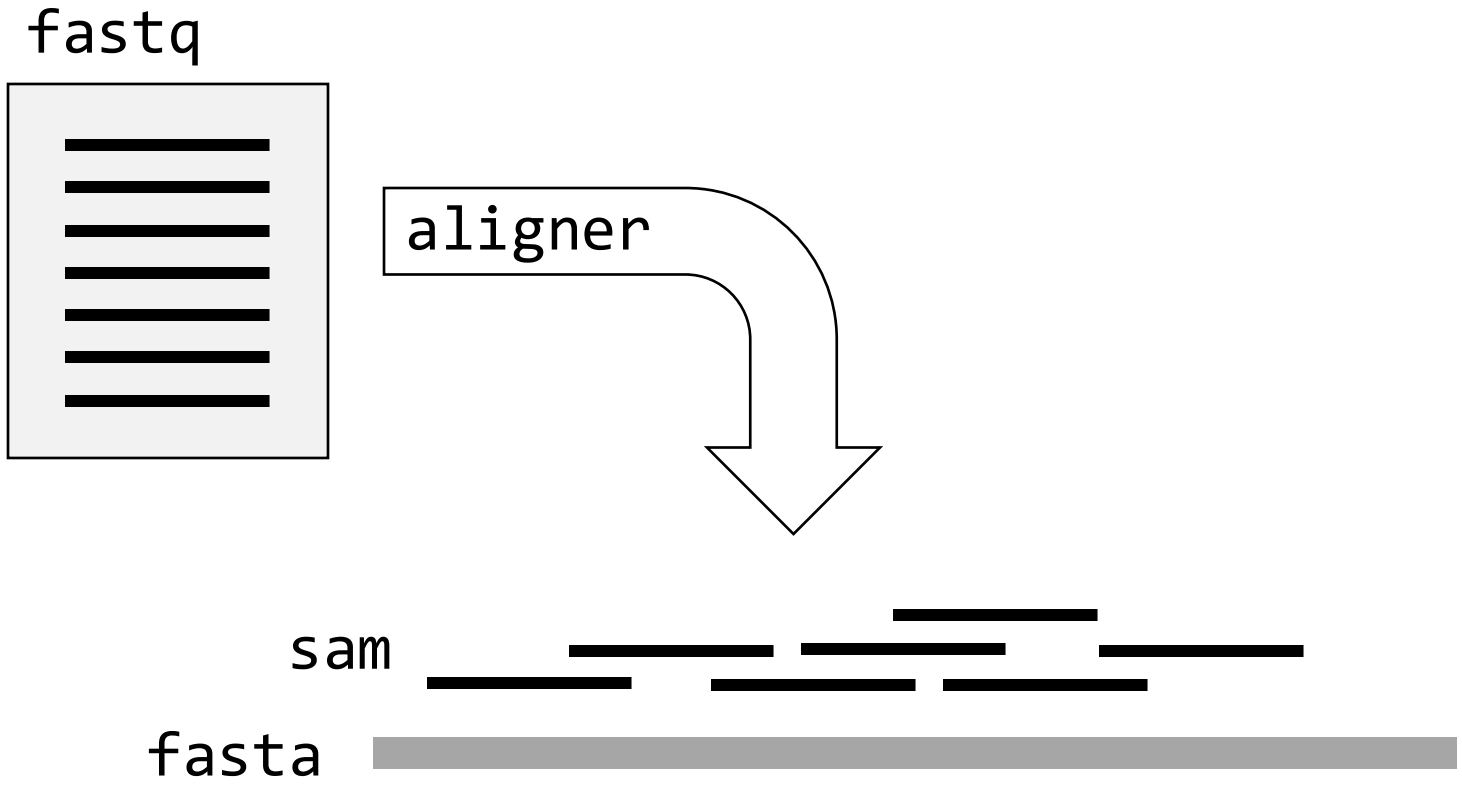
Long reads

- More error -> difficulties for variant analysis
- But:
  - PacBio HiFi: high baseQ + no bias
  - Long reads can have higher mapping qualities
  - Long reads improve haplotyping

What to sequence?

- Whole genome/metagenome
- Reduced representation:
  - Bait capture/whole exome sequencing
  - Restriction Enzyme based (e.g. RAD seq)
  - Amplicon sequencing
  - RNA-seq

Read alignment (phred)



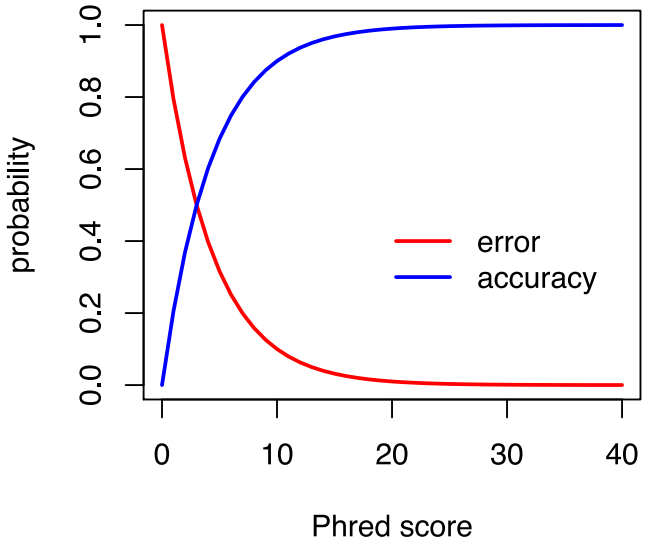
Software

- Basic alignment:
  - `bowtie2`
  - `bwa-mem`
  - `dragen`
- Long reads:
  - `minimap2`

Mapping quality

$$MAPQ = -10log_{10}\Pr\{mapping\ position\ is\ wrong\}$$

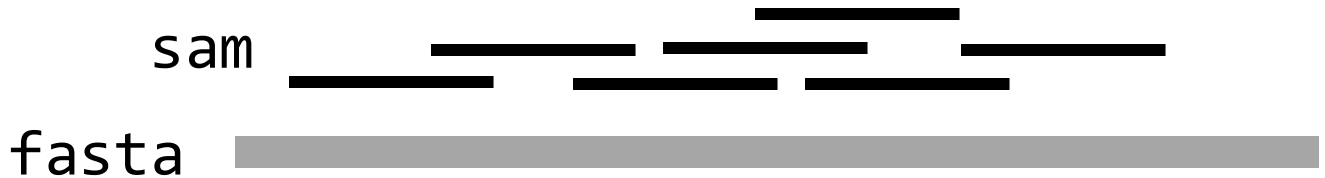
$$-10log_{10}(0.01) = 20$$
$$-10log_{10}(0.5) = 3$$



Question

sam

sequence alignment format



sam header

```
@HD     VN:1.0  SO:coordinate
@SQ     SN:U00096.3     LN:4641652
@PG     ID:bowtie2      PN:bowtie2      VN:2.4.1        CL: bowtie2-
align-s --wrapper basic-0 -x ref.fasta -1 reads_1.fastq -2
reads_2.fastq"
```

SAM column	**example**
read name	SRR519926.5
flag	89
reference	chr20
start position	61
mapping quality	42
CIGAR string	150M
reference name mate is mapped	=
start position mate	476
fragment length	515
sequence	CATCACCATTCCCAC
base quality	@>4:4C@89+&9CC@
optional	AS:i:-2
optional	RG:Z:rg1

Question

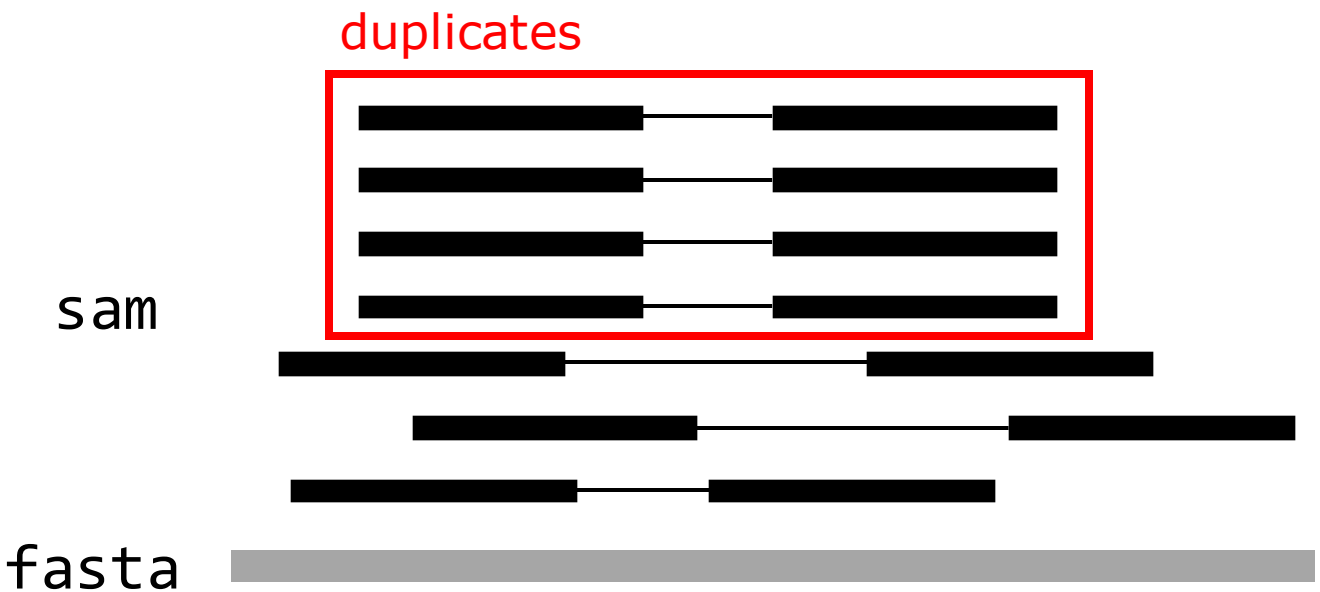
Read groups

- Have multiple groups of reads in a bam file
- Add metadata to alignments:
  - Samples
  - Libraries
  - Lanes
  - ..

Read groups

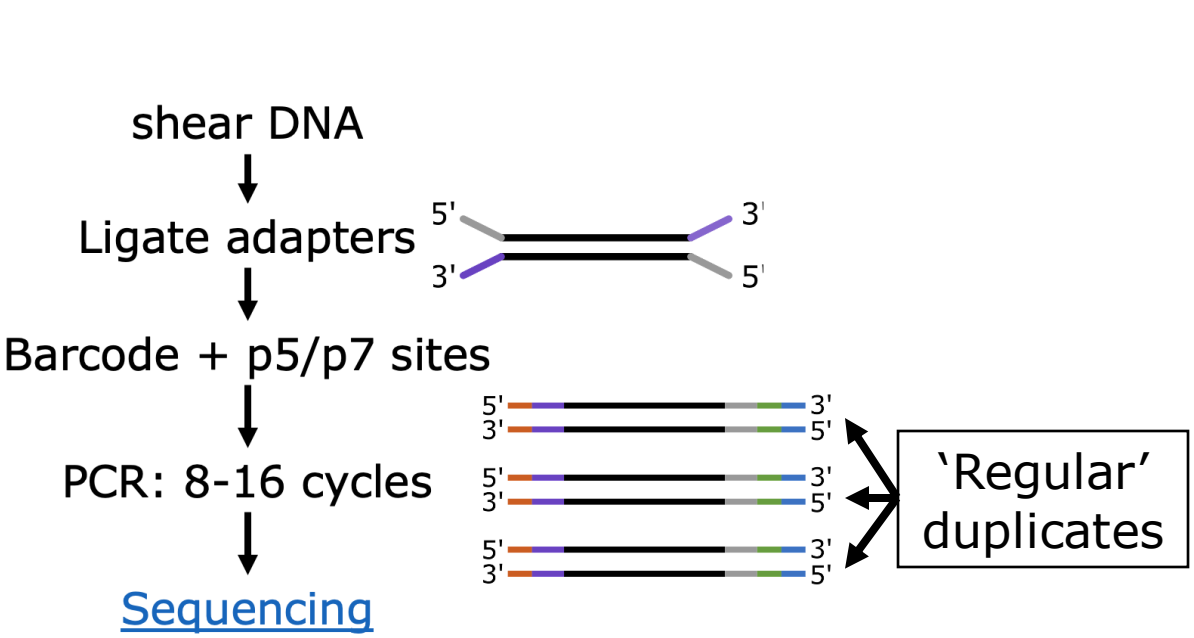
```
@RG ID:rg1 LB:lib1 SM:sampleA
@RG ID:rg2 LB:lib2 SM:sampleA
read1     456345    chr20     RG:Z:rg1
read2     456348    chr20     RG:Z:rg2
read3     456357    chr20     RG:Z:rg2
read4     456359    chr20     RG:Z:rg1
```

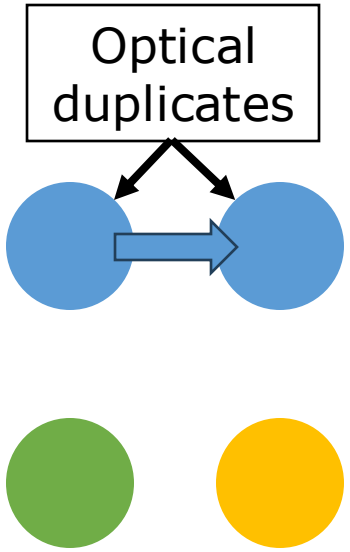
Marking duplicates



Marking duplicates

- 'Regular' duplicate: originates from PCR during library prep
- Optical duplicate: originates from bridge amplification





Marking duplicates

- Variant calling: each read is an independent observation of the genome
- Duplicates (can) have the same molecular origin -> not independent
- In a high-quality library, removing duplicates probably doesn't have a big effect on variant analysis

Ebbert MTW et al. (2016) Evaluating the necessity of PCR duplicate removal from next-generation sequencing data and a comparison of approaches. BMC Bioinformatics.

Unique Molecular Identifiers

- UMI added before PCR reaction
- Detect PCR duplicates and PCR errors