DNA Sequencing and Data Analysis

Prof Noam Shomron

Amit Levon

Lecture 3, April 24, 2025

DNA Sequencing and Data Analysis

Modern DNA Sequencing, 2nd Wave
File Formats, Tools

Thursday 18:30 to 21:00

C.L03

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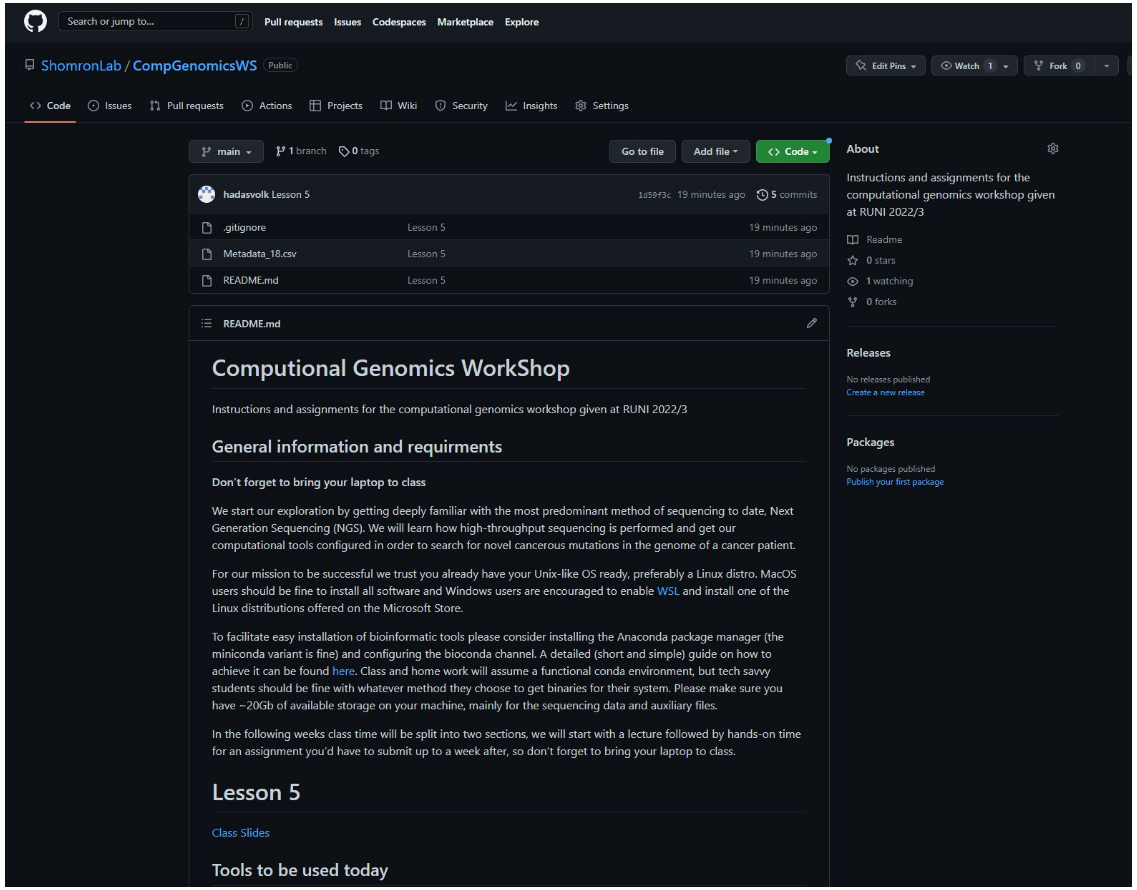
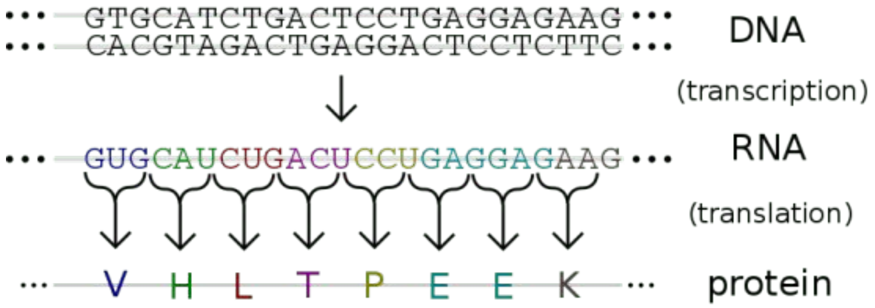
Class & Home Assignment

github.com/ShomronLab/CompGenomicsWS

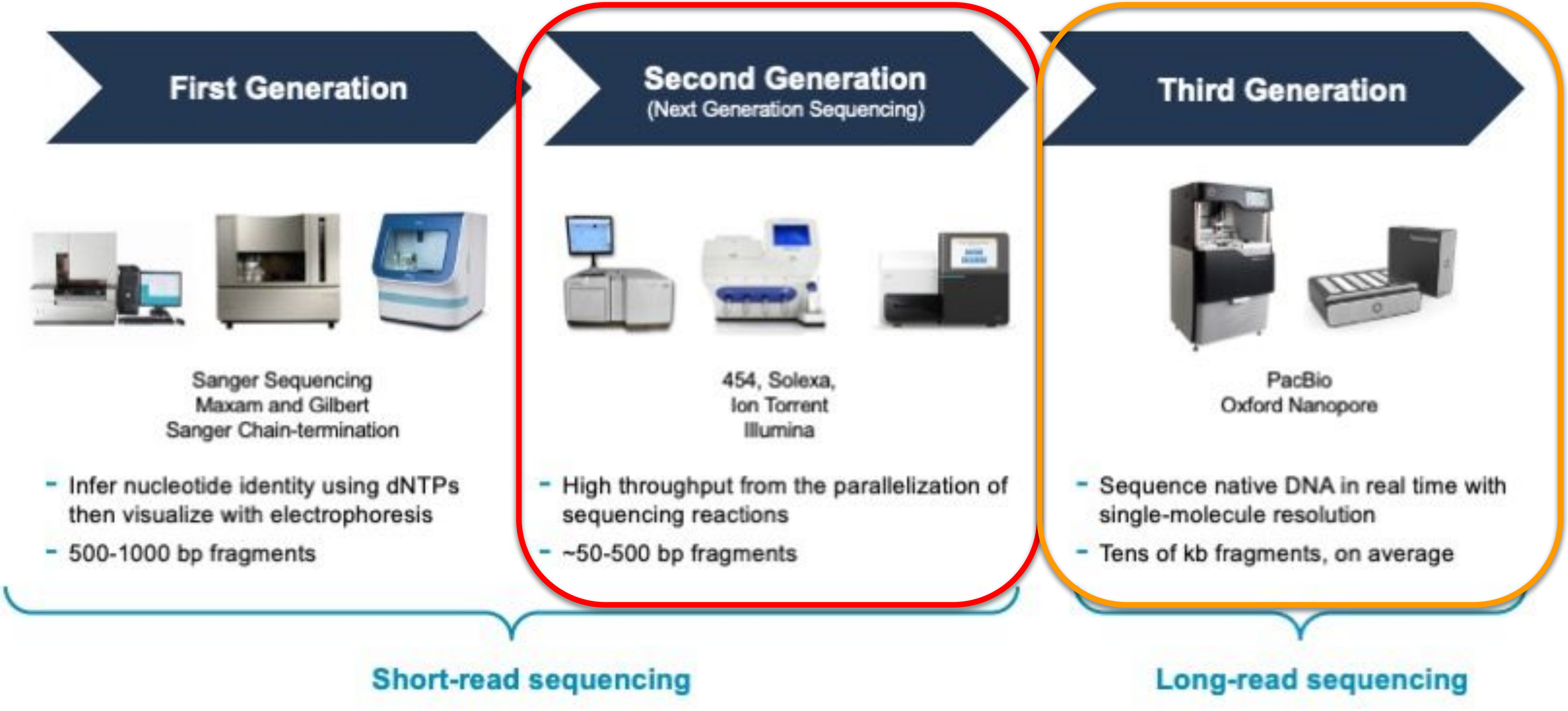
Home assignments are submitted via Moodle up to two weeks!!!



DNA Sequencing (ריצוף דנ"א)



The Evolution of DNA Sequencing Tools



pacbio

The First Sequencing Efforts

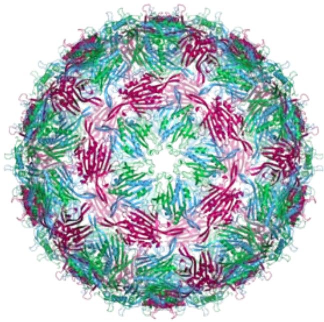
Highly abundant single-stranded RNA (ssRNA) - ribosomal RNA (rRNA), transfer RNA (tRNA) & phages.

1965 - first full sequence of yeast tRNA[1].

1972 - first protein-coding sequence[2].

1976 - first full (RNA) phage genome[3] - 3,569 nucleotides.

Main technology - 2D fractionation of radioactive nucleotides[4].



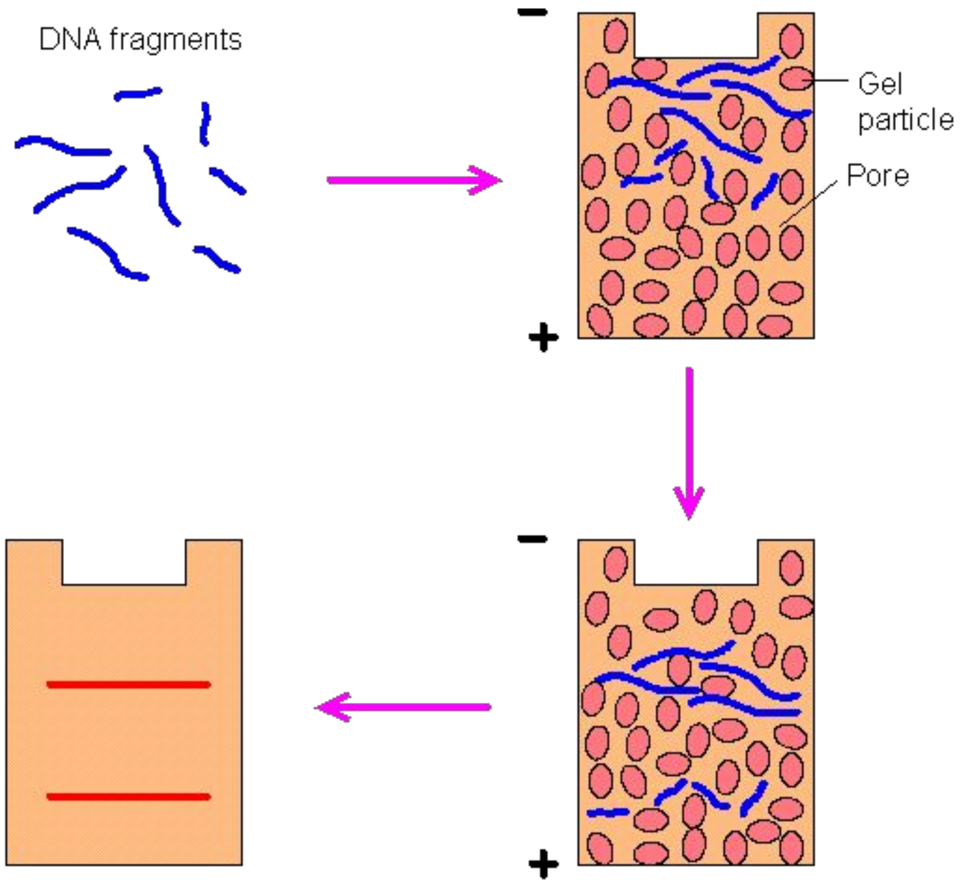
1. Holley, Robert W., et al. "Structure of a ribonucleic acid." *Science* (1965): 1462-1465.
2. Jou, W. Min, et al. "Nucleotide sequence of the gene coding for the bacteriophage MS2 coat protein." Nature 237.5350 (1972): 82.
3. Fiers, Walter, et al. "Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene." Nature 260.5551 (1976): 500.
4. Sanger, F., G. G. Brownlee, and B. G. Barrell. "A two-dimensional fractionation procedure for radioactive nucleotides." Journal of molecular biology 13.2 (1965): 373-IN4.

1970's - 1st Generation Sequencing (Sanger)

Chain termination method[1]

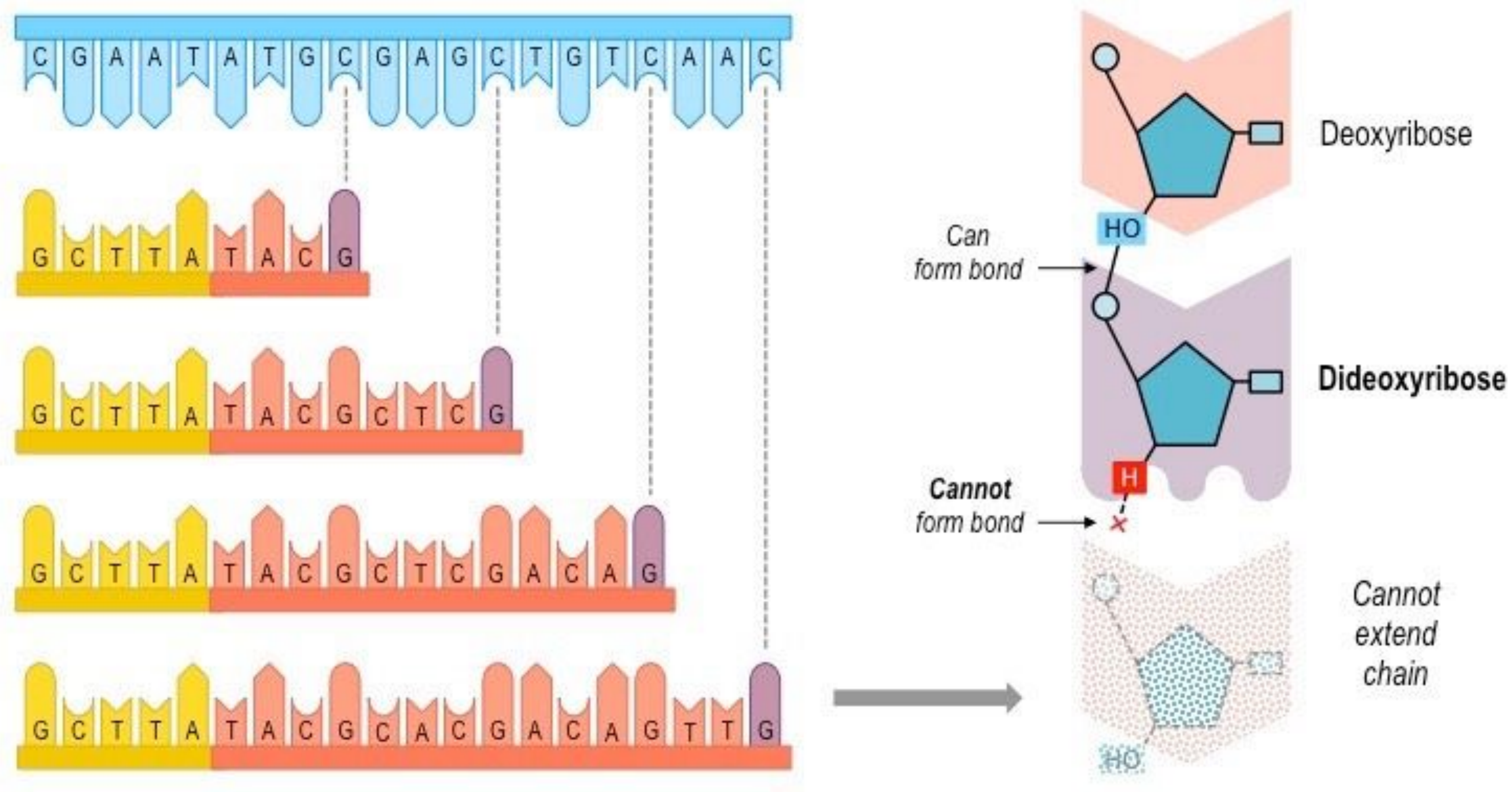
Both used polyacrylamide gels

First DNA phages sequenced



1. Sanger, Frederick, Steven Nicklen, and Alan R. Coulson. "DNA sequencing with chain-terminating inhibitors." Proceedings of the national academy of sciences 74.12 (1977): 5463-5467.

1970’s - 1st Generation Sequencing



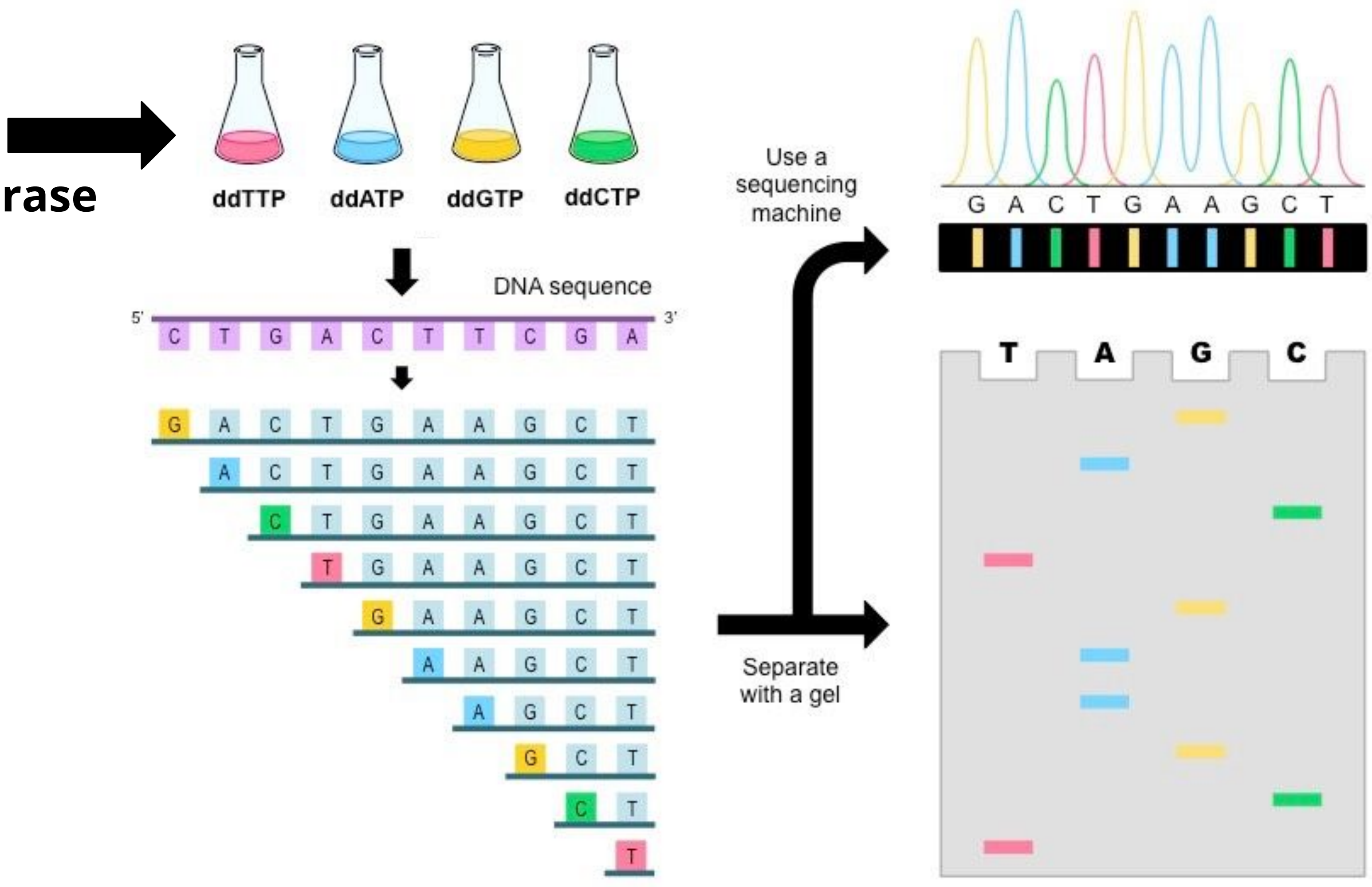
Sequence terminates when the ddNTP is incorporated
Fragment lengths reflect base position in sequence

Chain termination by dideoxynucleotides

https://ib.bioninja.com.au

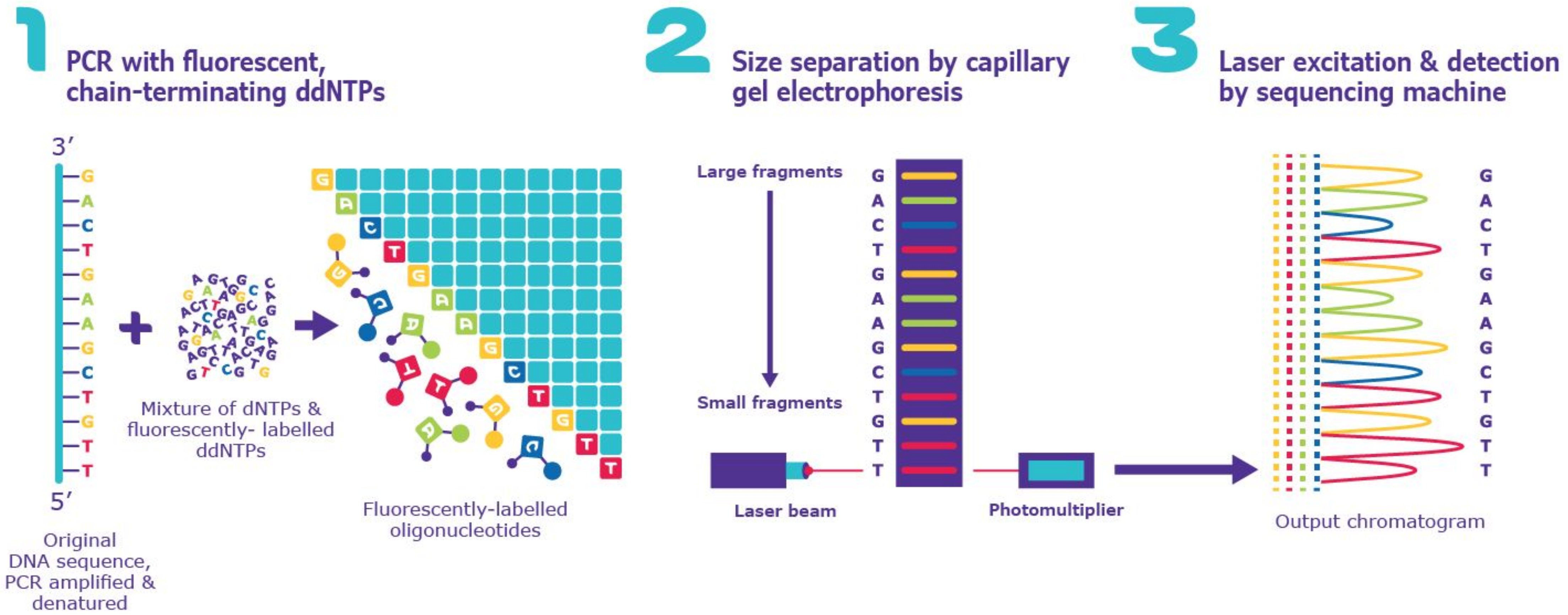
The Chain Termination Method - Sanger Sequencing

Ingredients:
(1) Target DNA
(2) Primer
(3) DNA Polymerase
(4) 4 dNTPs



https://ib.bioninja.com.au

The Chain Termination Method - Sanger Sequencing



The Chain Termination Method - Sanger Sequencing

SBS - sequencing by synthesis

Produces reads 500-1,000 bp long

First commercial machines

Still used today!

Can sequence ~70k bp/hour

Cost: ~$500/1Mbp

Used for various Whole Genome Projects

Shotgun sequencing

The Human Genome Project

Reminder: human genome size is ~3Gb

Started 1990

First genome draft - 2000

Project completion - 2003

Largest ever biological collaborative project

20 sequencing centers around the world

Entirely based on Sanger sequencing

Estimated cost: $2.7 billion ($5 billion today)

For every dollar invested by the U.S. government in the Human Genome Project, ~$141 was returned to the economy - ~$380.7 billion!

2000s - 2nd Generation Sequencing (NGS)

= Massively Parallel Sequencing (MPS) = High-Throughput Sequencing

1. Pyrosequencing[1] - “454 sequencing”
2. Illumina (Solexa) method

Allow massively parallel sequencing.

Produce short reads - usually 50-250 bp.

Deep sequencing - each genomic region is sequenced multiple times.

1. Ronaghi, Mostafa, Mathias Uhlén, and Pål Nyrén. "A sequencing method based on real-time pyrophosphate." Science 281.5375 (1998): 363-365.
2. Canard, Bruno, and Robert S. Sarfati. "DNA polymerase fluorescent substrates with reversible 3′-tags." Gene 148.1 (1994): 1-6.

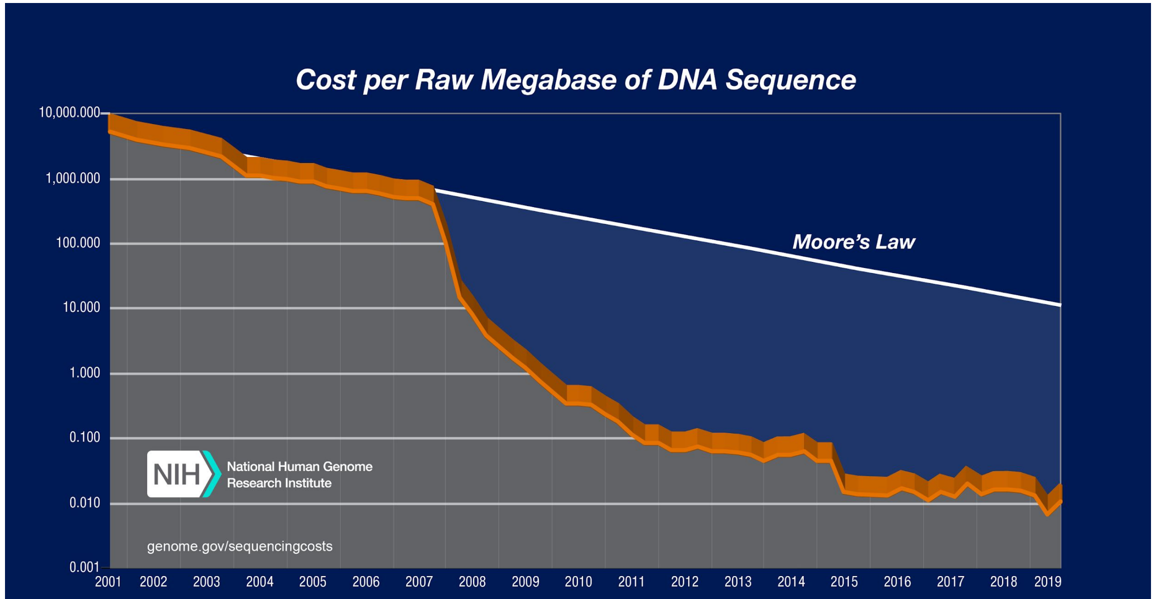
Illumina Technologies - Standard for NGS

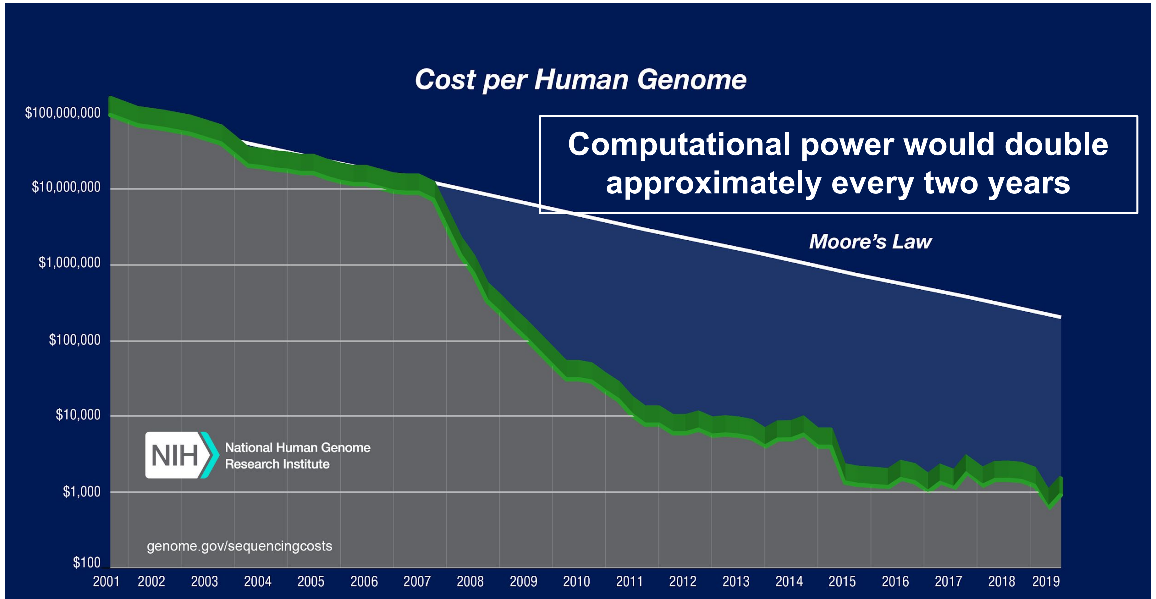
By far the most popular sequencing technology.

Up to 120 Gb/hour.

Significantly reduced sequencing costs (~250$ these days).

Allowed the sequencing of numerous species and samples.





The cost of sequencing the human genome has decreased at a rate that far outpaces Moore's Law.

TheMarker | TechNation

אולטימה ג'נומיקס גייסה 600 מיליון דולר בחשאי – ופיתחה מכונה המאפשרת ריצוף גנטי ב-100 דולר בלבד

את החברה הקים היזם הישראלי גלעד אלמוגי לפני חמש שנים וחצי, אך היא לא נחשפה עד כה, אף שגייסה הון מכמה מהמשקיעים הבולטים בעמק הסיליקון ■ המכונה שפיתחה החברה מאפשרת ריצוף גנום שלם של אדם בחמישית המחיר בשוק

Ultima Genomics
14,006 followers
2d • Edited •

Ultima is gearing up for the full commercial availability of the UG 100™ at the upcoming AGBT - Advances in Genome Biology and Technology (AGBT) on February 5, 2024. We will also be unveiling our ppmSeq™ Technology with industry-leading raw read accuracy for calling SNVs for rare event detection applications.

Hear why our early access customers are excited about the launch of the UG 100™ and the era of the $100 genome and beyond.

https://lnkd.in/gANE_9Cv

Ultima Genomics Announces End of Early Access and Upcoming Launch of UG 100™
businesswire.com • 5 min read

Cost-efficient whole genome-sequencing using novel mostly natural sequencing-by-synthesis chemistry and open fluidics platform

Authors: Gilad Almogy[1], Mark Pratt[1], Florian Oberstrass[1], Linda Lee[1], Dan Mazur[1], Nate Beckett[1], Omer Barad[1], Ilya Soifer[1], Eddie Perelman[1], Yoav Etzioni[1], Martin Sosa[1], April Jung[1], Tyson Clark[1], Eliane Trepagnier[1], Gila Lithwick-Yanai[1], Sarah Pollock[1], Gil Hornung[1], Maya Levy[1], Matthew Coole[2], Tom Howd[2], Megan Shand[2,3], Yossi Farjoun[3], James Emery[3], Giles Hall[3], Samuel Lee[3], Takuto Sato[2,3], Ricky Magner[2,3], Sophie Low[2], Andrew Bernier[2], Bharathi Gandi[2], Jack Stohlman[2], Corey Nolet[2], Siobhan Donovan[2], Brendan Blumenstiel[2], Michelle Cipicchio[2], Sheila Dodge[2], Eric Banks[3], Niall Lennon[2], Stacey Gabriel[2], Doron Lipson[1]

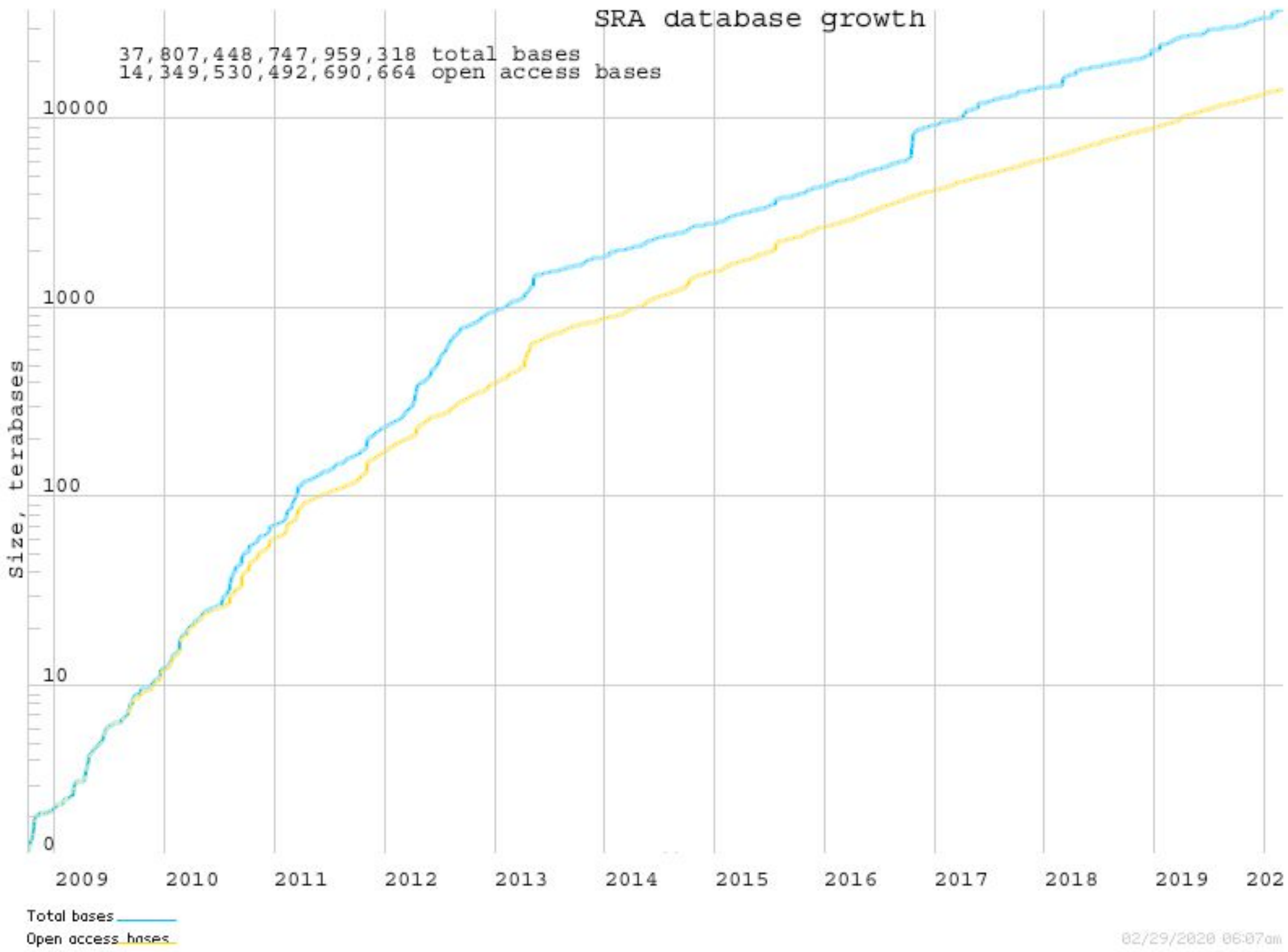
[1] Ultima Genomics, 7979 Gateway Blvd, Newark, CA 94560
[2] Genomics Platform, Broad Institute of MIT and Harvard, Cambridge, MA 02141
[3] Data Sciences Platform, Broad Institute of MIT and Harvard, Cambridge, MA 02141

Introducing the UG 100™

The $100 genome is just the beginning

https://www.businesswire.com/news/home/20240130828872/en/Ultima-Genomics-Announces-End-of-Early-Access-and-Upcoming-Launch-of-UG-100%E2%84%A2

Number of Bases Deposited in Sequence Read Archive (SRA)



Some Sequencing Projects From 2022

nature communications

Explore content ⌄ About the journal ⌄ Publish with us ⌄

nature > nature communications > articles > article

Article | Open Access | Published: 04 March 2022

Whole-genome sequencing of 1,171 elderly admixed individuals from Brazil

nature genetics

Explore content ⌄ About the journal ⌄ Publish with us ⌄

nature > nature genetics > articles > article

Article | Open Access | Published: 22 September 2022

Improved pea reference genome and pan-genome highlight genomic features and evolutionary characteristics

ZR ZOOLOGICAL RESEARCH www.zoores.ac.cn

Zool Res. 2022 Jan 18; 43(1): 78–80. PMCID: PMC8743251
doi: 10.24272/j.issn.2095-8137.2021.266 PMID: 34877831

Whole-genome resequencing infers genomic basis of giant phenotype in Siamese fighting fish (*Betta splendens*)

What can we do with NGS?

Acquire new genomes

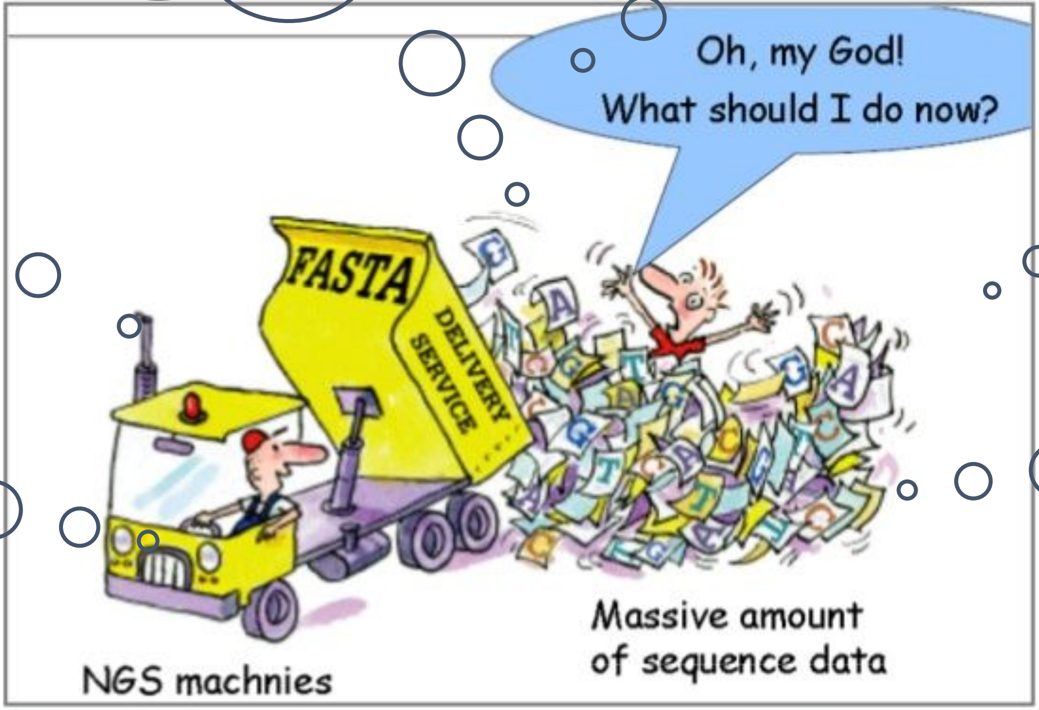
Detect genomic variation

Profile gene expression

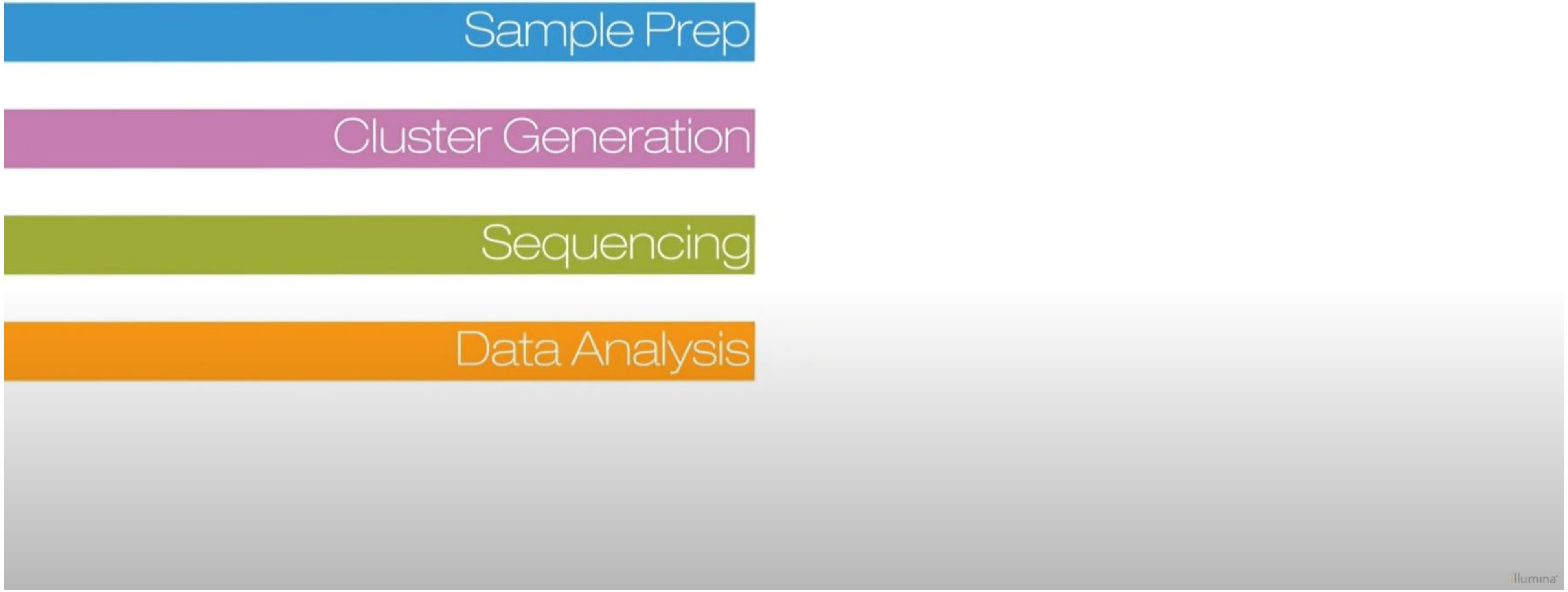
Genotype

Characterize environments

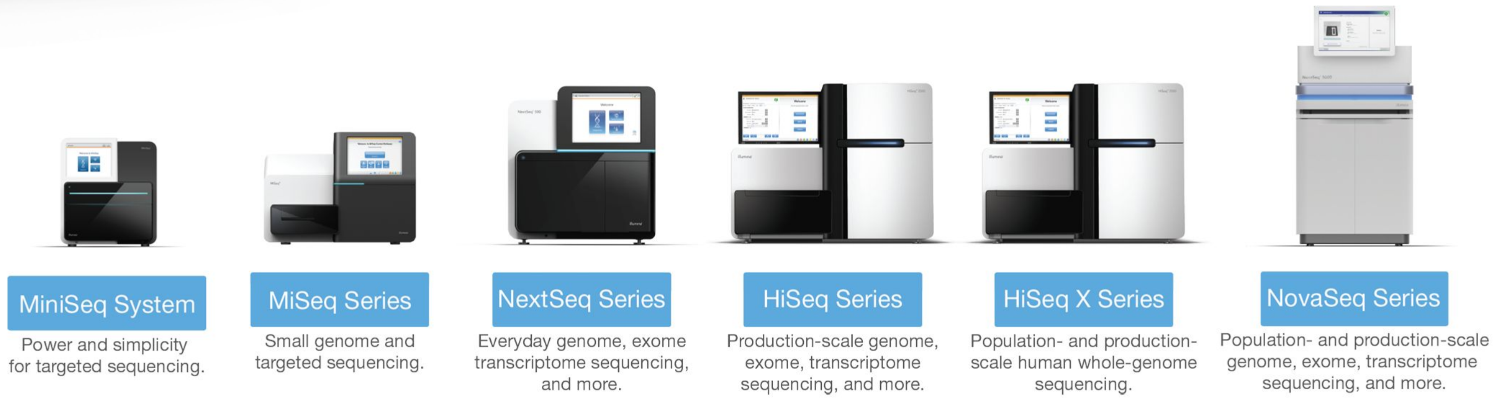
More exotic stuff...



The Illumina Sequencing Method



The Illumina Sequencing Machine



DNA/RNA Extraction

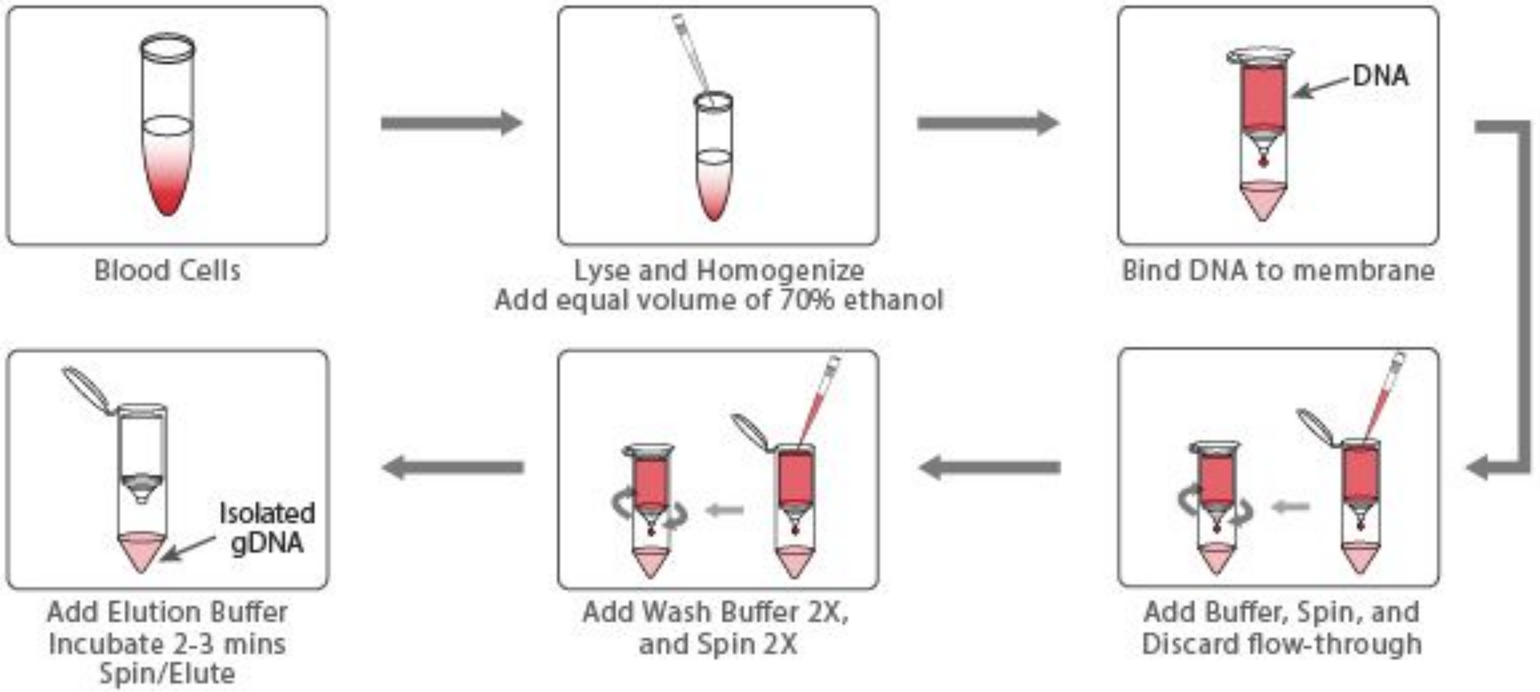
Protocol depends on organism and tissue

Required DNA amount depends on application

DNA should be as intact as possible

- Fragment sizes
- Single strand breaks

May be challenging!



Library Prep – Step A

Many protocols exist

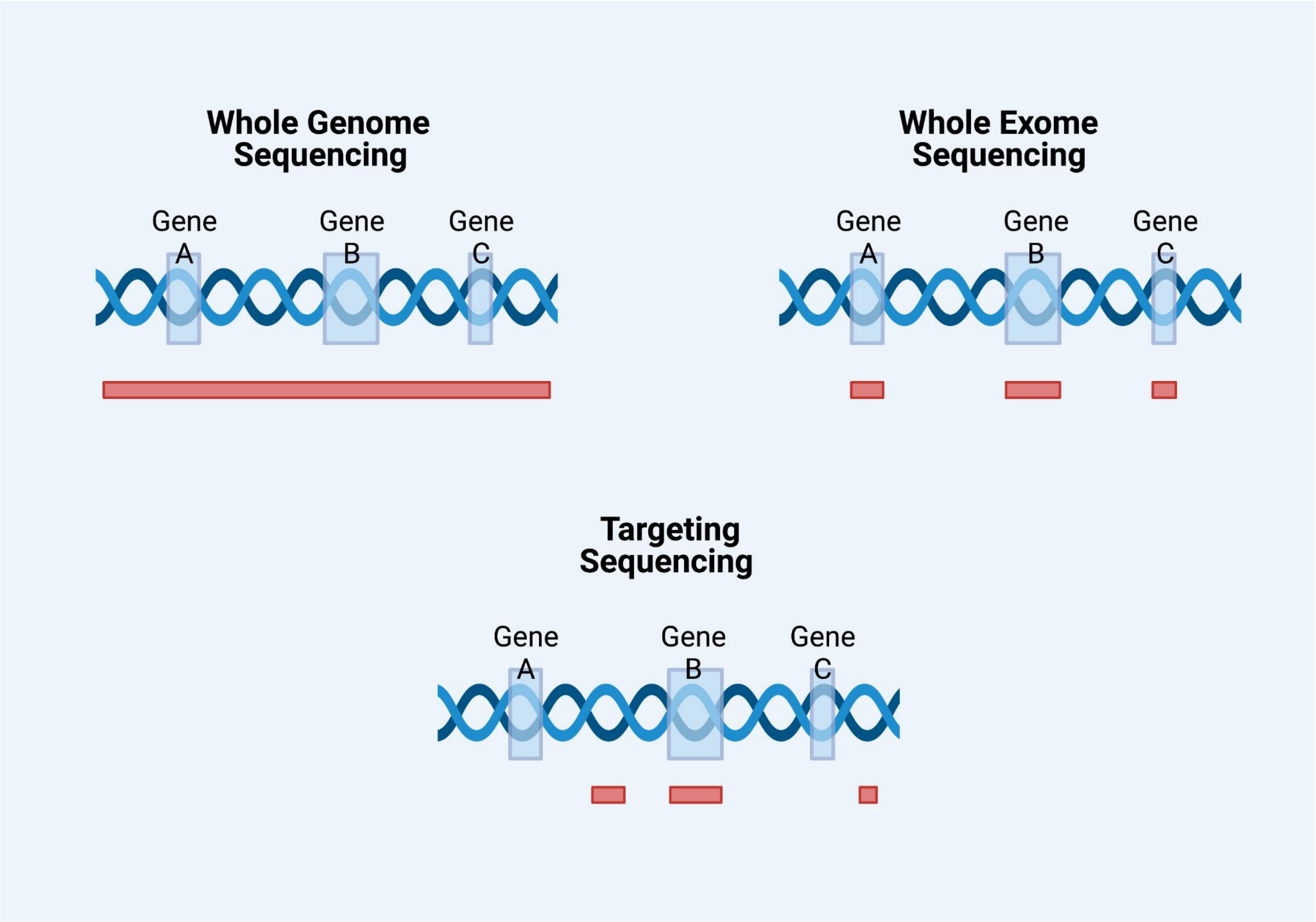
Main goal - fragment and add adapters

Single-end or **paired-end**?

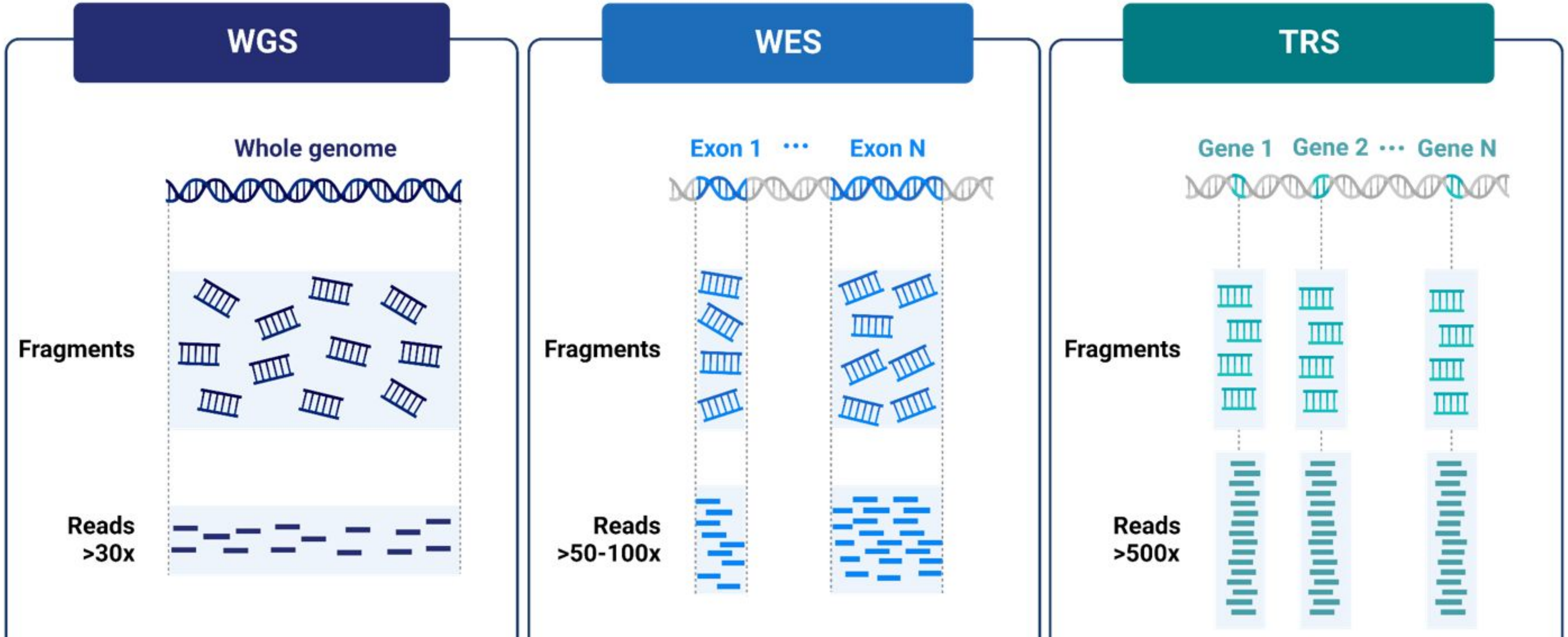
Whole genome sequencing (**WGS**) or **targeted** sequencing?

Insert size is determined

Library Prep – Sequencing Types



Library Prep – Step A

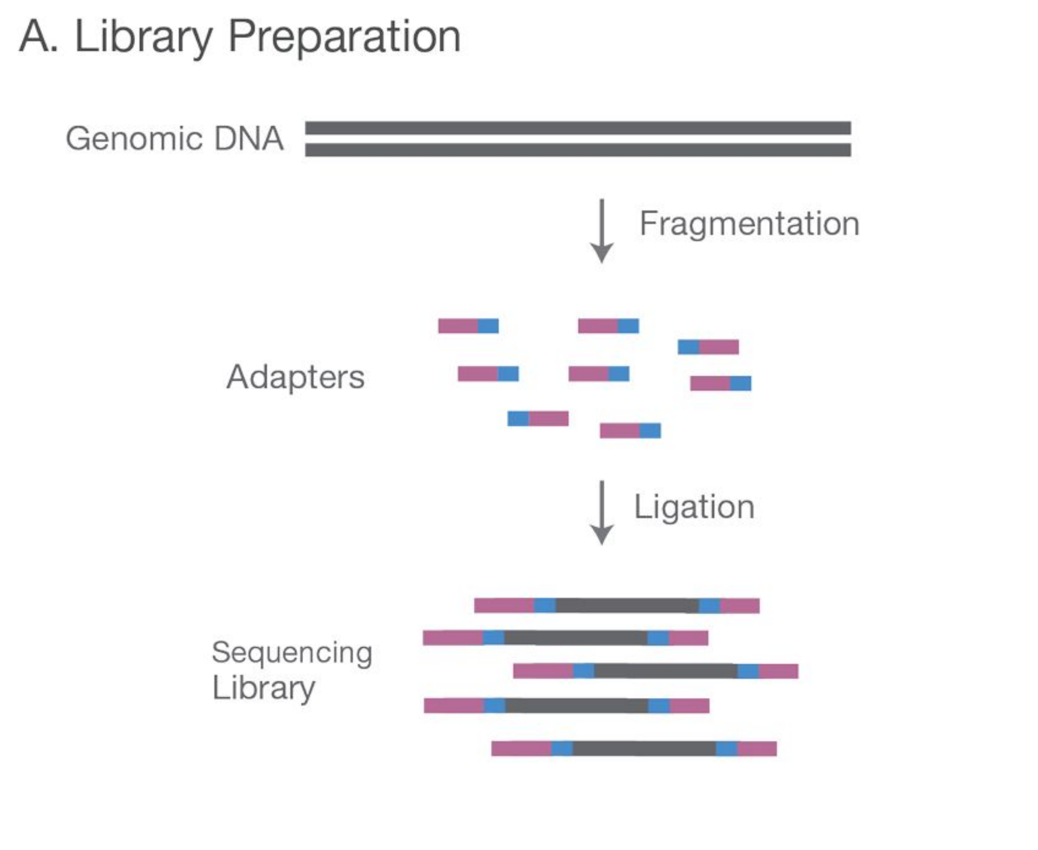


Coverage: All genes and non-coding DNA
Acuracy: Low
Variants: SNVs, InDels, SVs
Cost: Most expensive
Depth: >30X

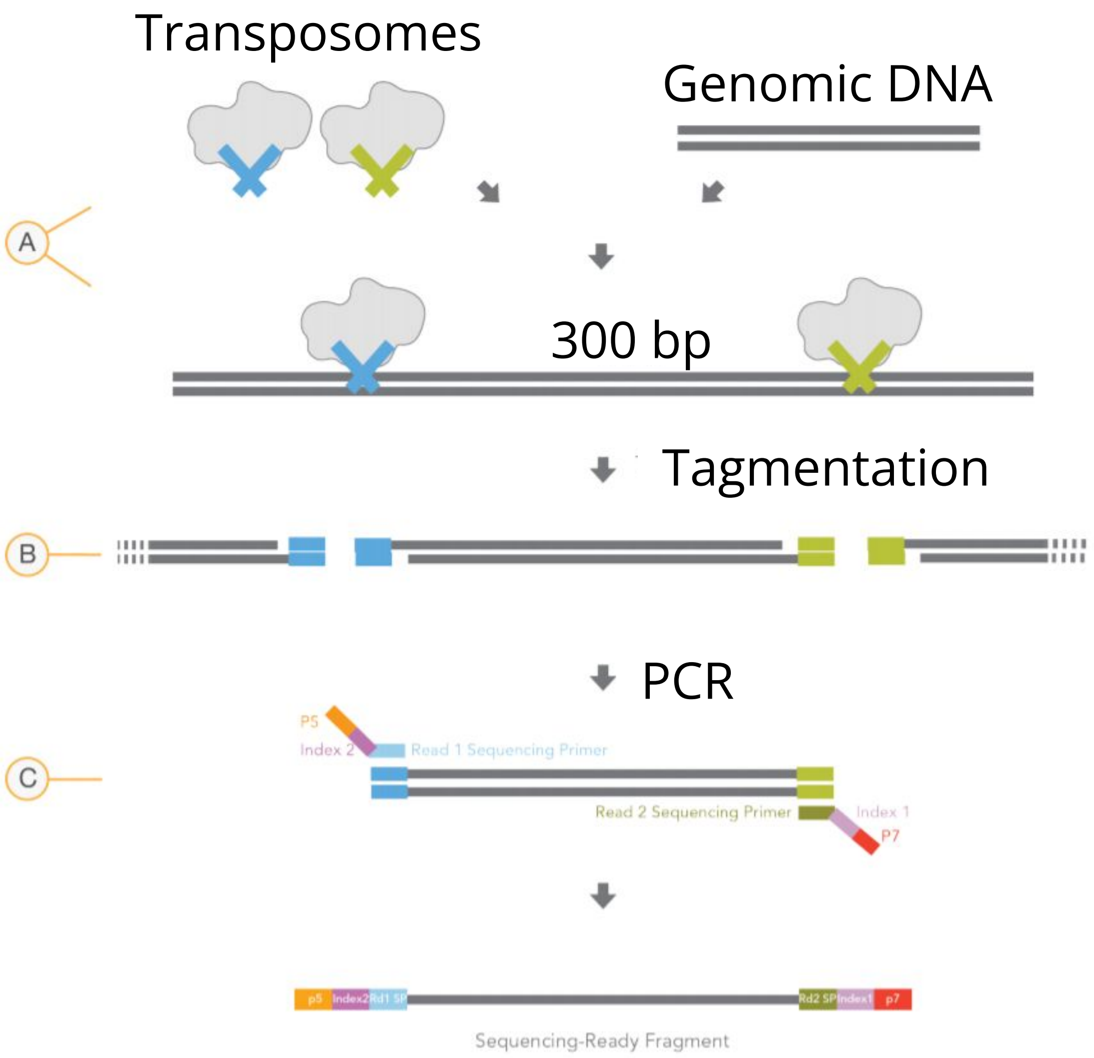
Coverage: Entire exome
Acuracy: Good
Variants: coding SNVs, InDels, CNV
Cost: Cost-effective
Depth: >50-100X

Coverage: 10-500 genes
Acuracy: High
Variants: SNVs, InDels
Cost: Most cost-effective
Depth: >500X

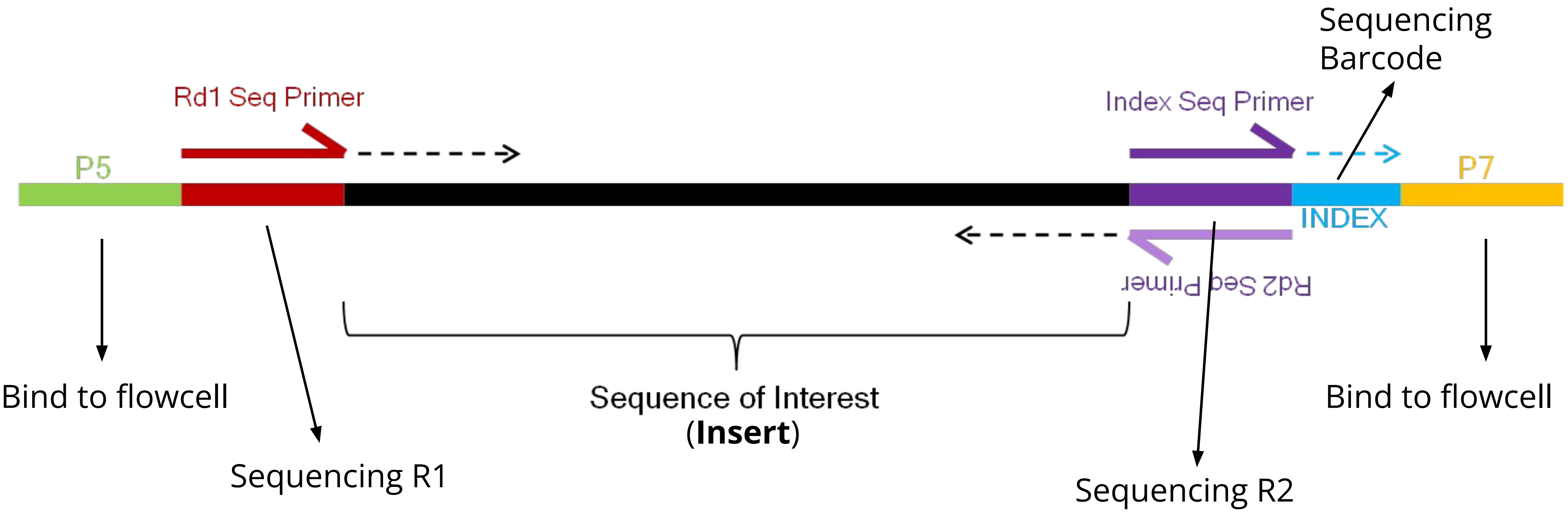
Basic WGS Library Prep Example



NGS library is prepared by fragmenting a gDNA sample and ligating specialized adapters to both fragment ends.

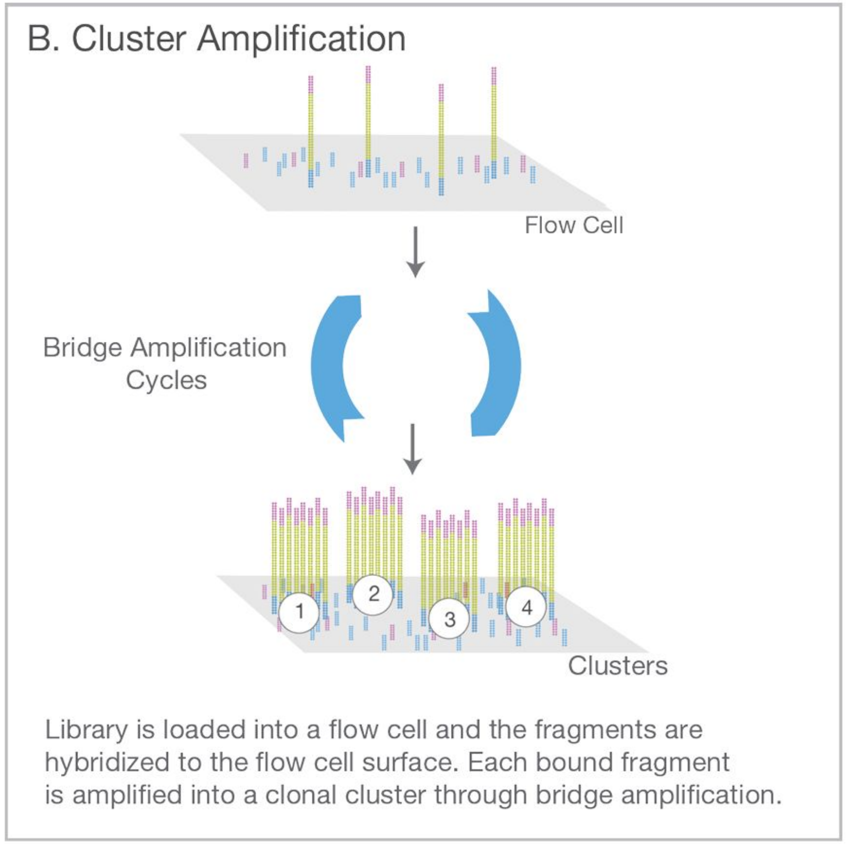


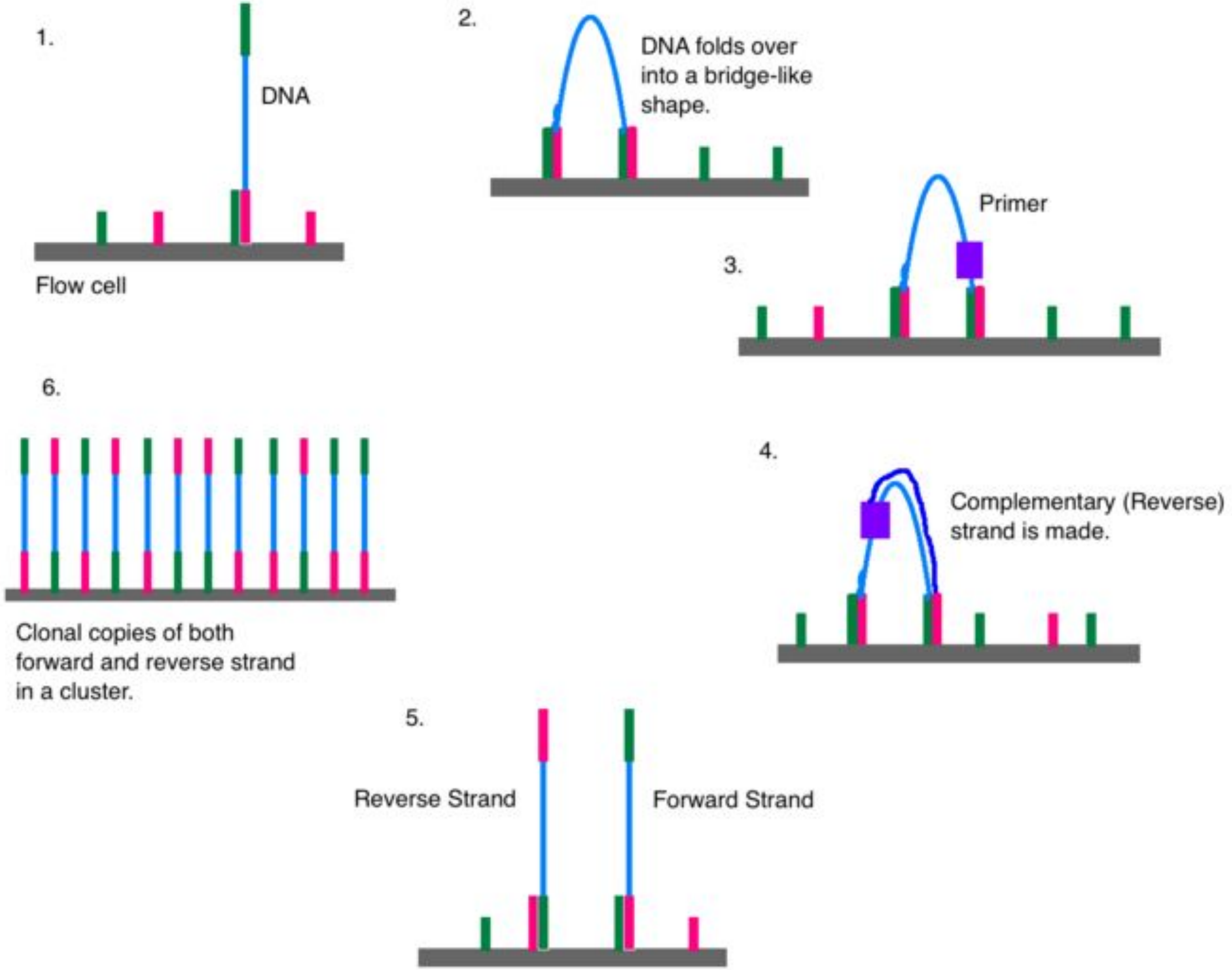
Library Prep What Do We Get



Main Steps in Sequencing

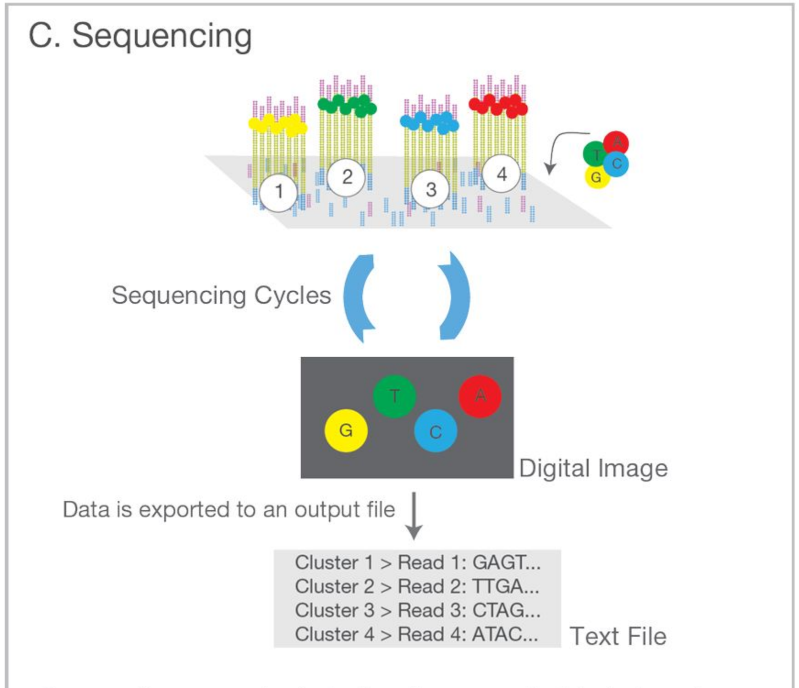
STEP B - Cluster amplification
No sequencing



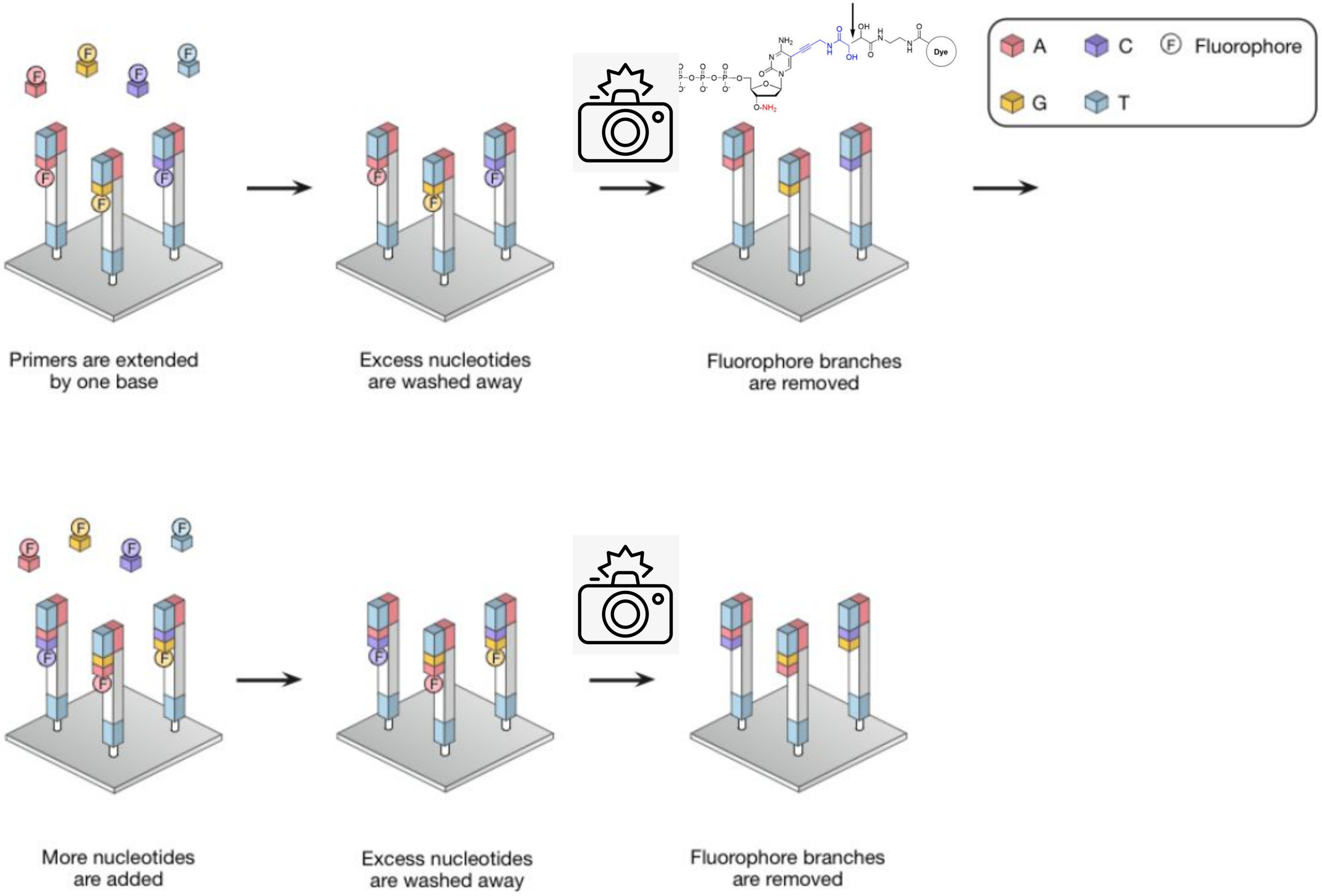


Main Steps in Sequencing

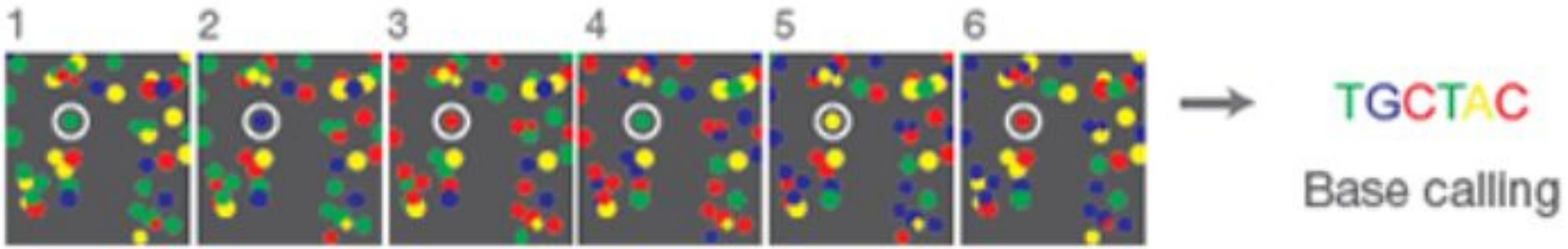
STEP C - Sequencing



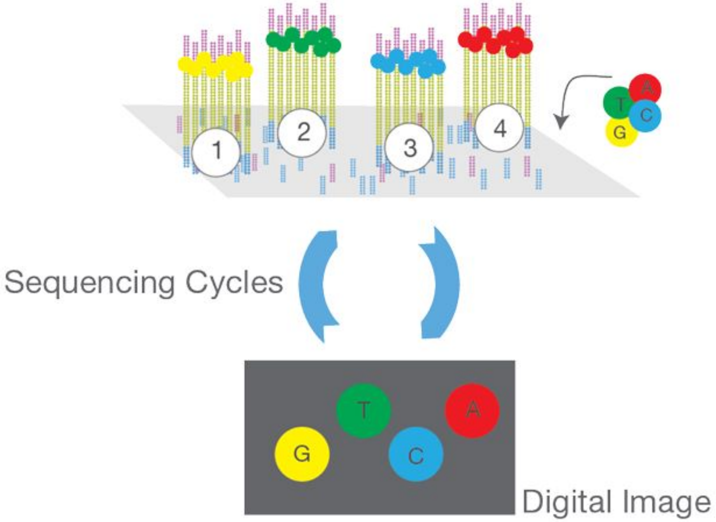
Sequencing reagents, including fluorescently labeled nucleotides, are added and the first base is incorporated. The flow cell is imaged and the emission from each cluster is recorded. The emission wavelength and intensity are used to identify the base. This cycle is repeated "n" times to create a read length of "n" bases.



Output of the Sequencing Machine

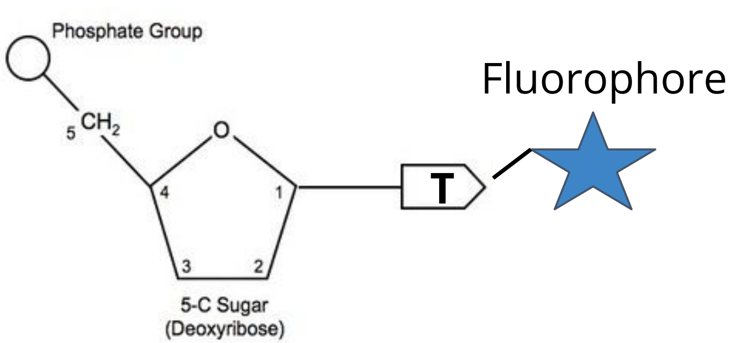


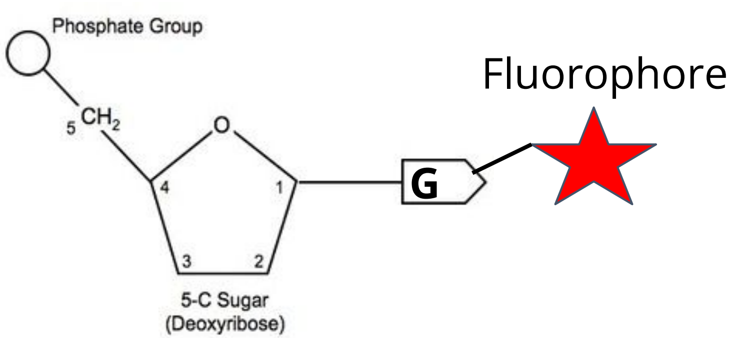
- Each flow cell cluster results in a read or read pair

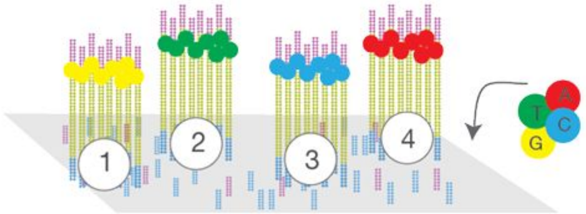


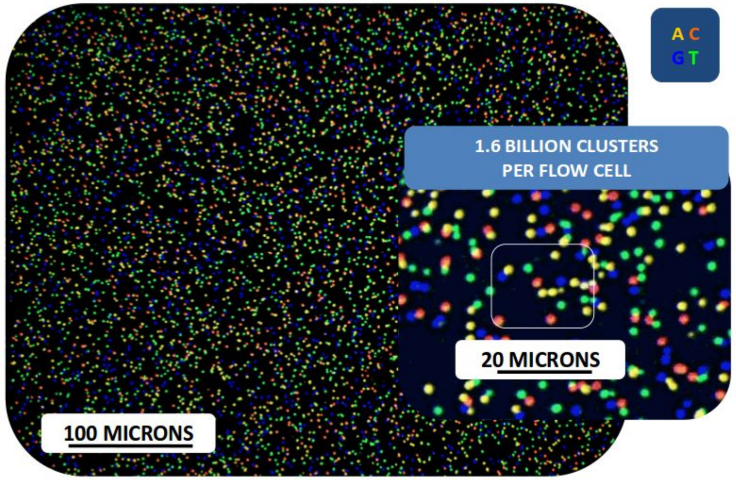
Low Quality Base Calling

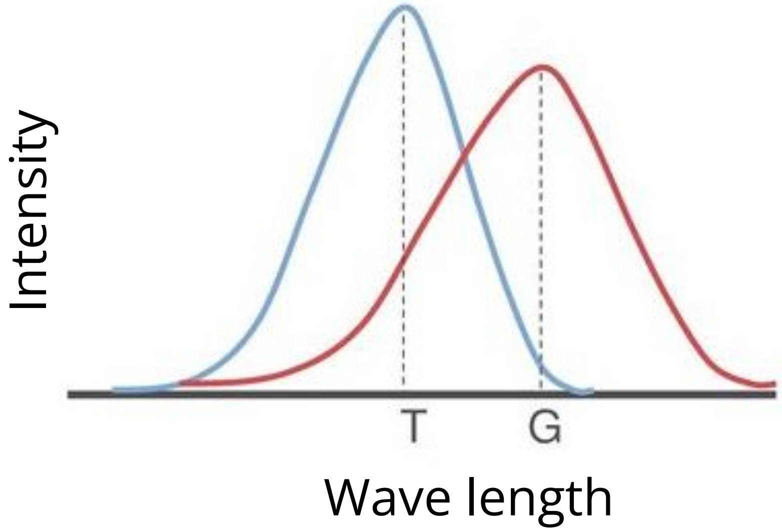
- Illumina machines make mistakes!
- Rate of errors: ~1/1000
- Reasons:
    - Color cross-talk
    - Clusters cross-talk
    - Phasing

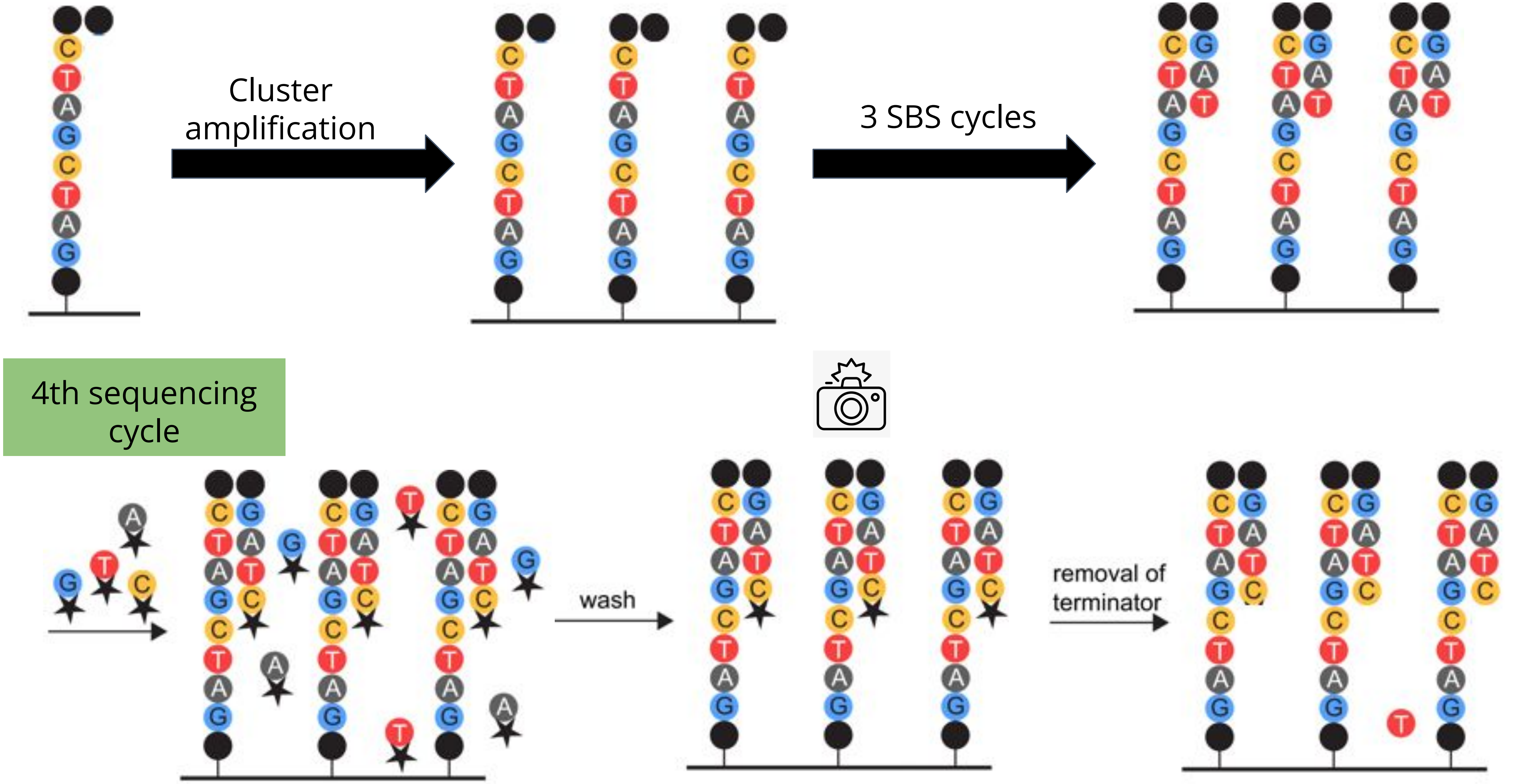


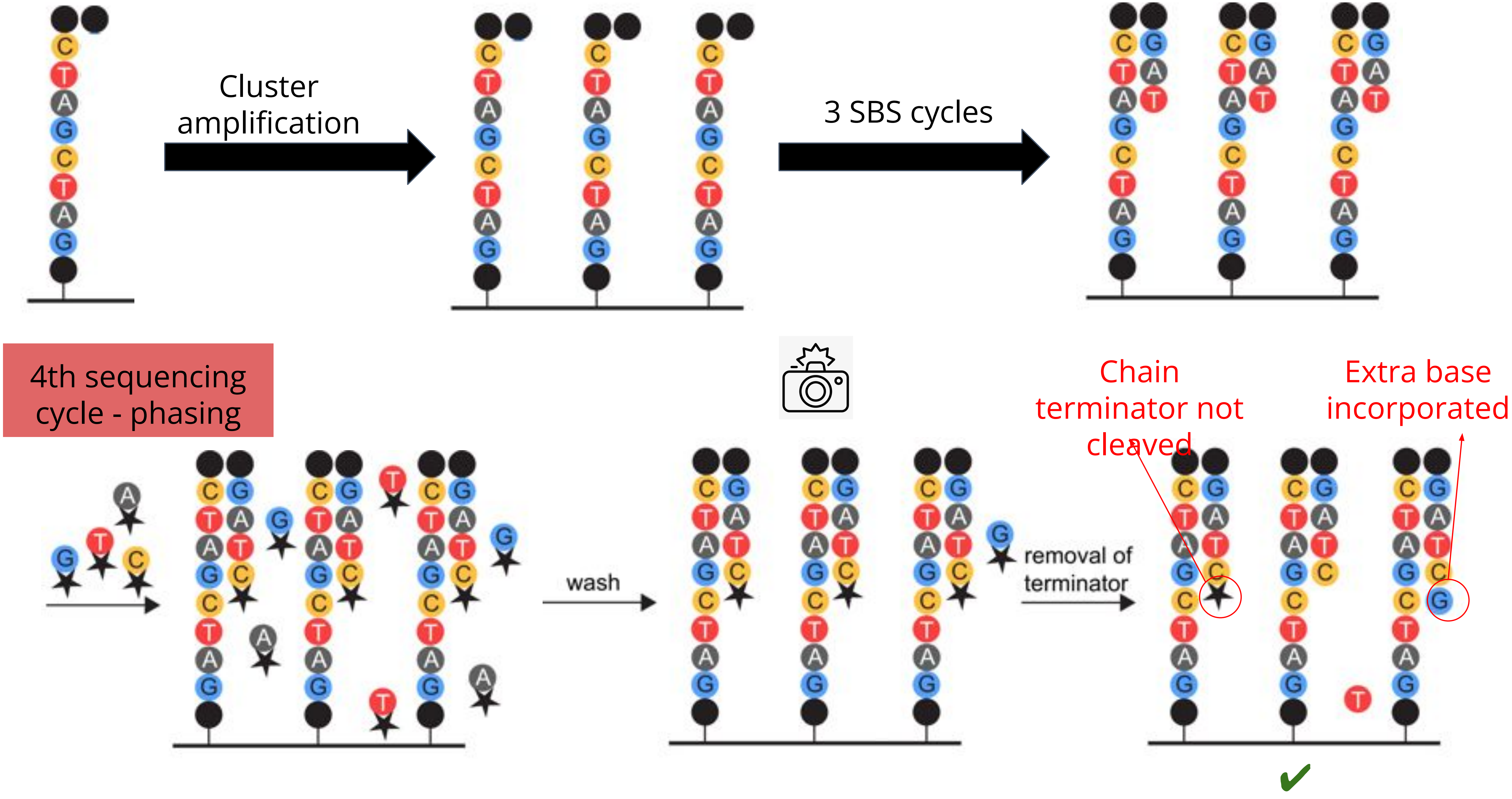






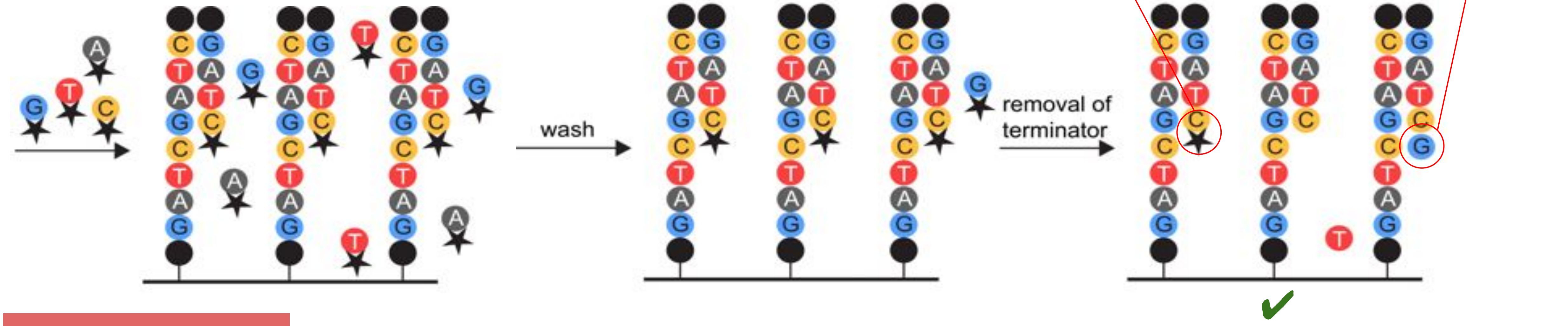



Cluster amplification
3 SBS cycles
4th sequencing cycle
wash
removal of terminator

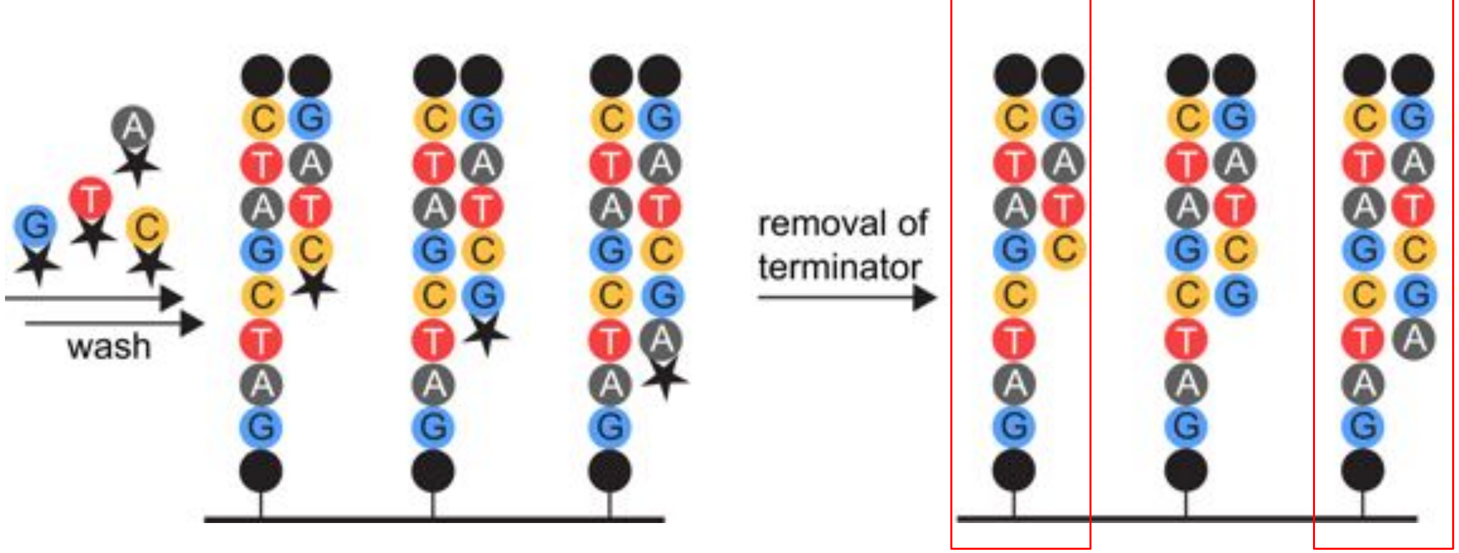


Cluster amplification
3 SBS cycles
4th sequencing cycle - phasing
wash
removal of terminator
Chain terminator not cleaved
Extra base incorporated

4th sequencing cycle - phasing



5th sequencing cycle - phasing



Low or Biased Yield

- Too few reads

→Usually caused by non-optimal cluster density

- Some genomic fragments sequenced more often than others

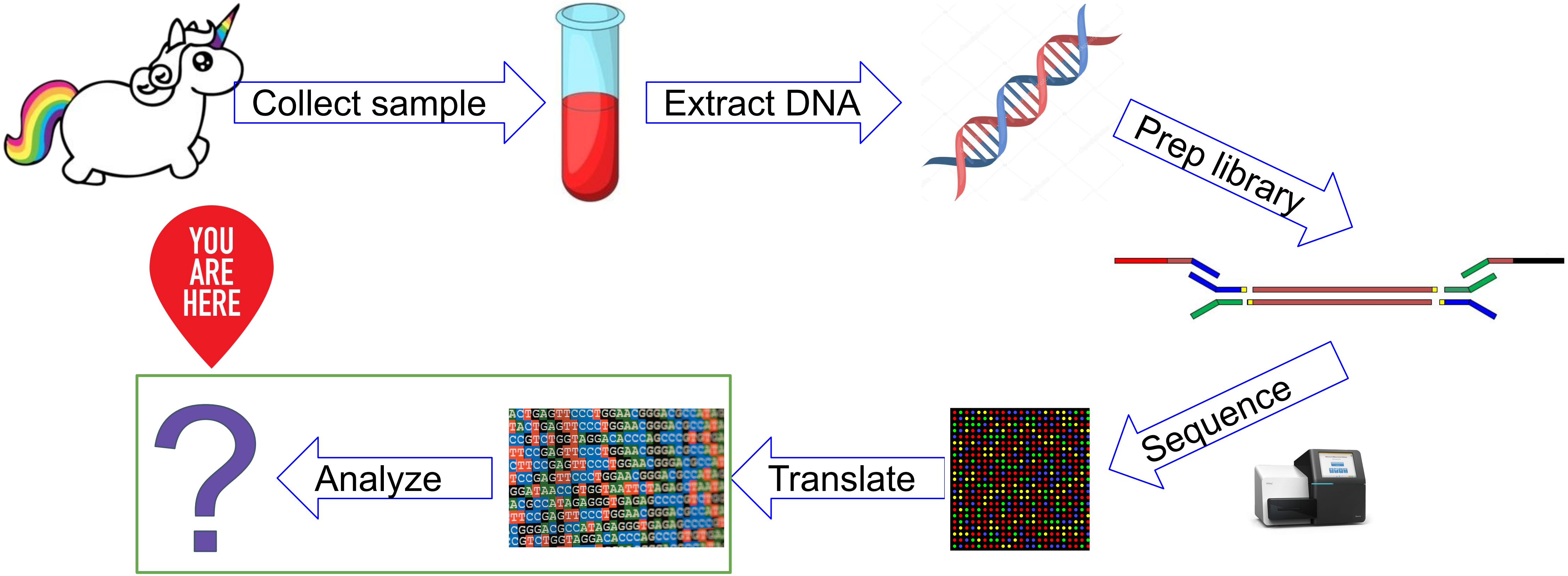
→Usually caused by library prep issues or PCR duplication

Illumina Sequencing Instruments

	iSeq	MiniSeq	MiSeq	NextSeq	HiSeq 4000	HiSeq X	NovaSeq
Run Time	9–17.5 hrs	4–24 hours	4–55 hours	12–30 hours	< 1–3.5 days	< 3 days	13-44 hours
Maximum Output	1.2 Gb	7.5 Gb	15 Gb	120 Gb	1500 Gb	1800 Gb	6000 Gb
Maximum Reads Per Run	4 million	25 million	25 million	400 million	5 billion	6 billion	20 billion
Maximum Read Length	2 × 150 bp	2 × 150 bp	2 × 300 bp	2 × 150 bp	2 × 150 bp	2 × 150 bp	2 x 250

***Reminder**: human genome size is ~3Gb*

NGS - From Organism to Sequence



Main Steps in Sequencing

D. Alignment and Data Anaylsis

Reads

```
  ATGGCATTGCAATTTGACAT
   TGGCATTGCAATTTG
AGATGGTATTG
 GATGGCATTGCAA
     GCATTGCAATTTGAC
  ATGGCATTGCAATT
AGATGGCATTGCAATTTG
```

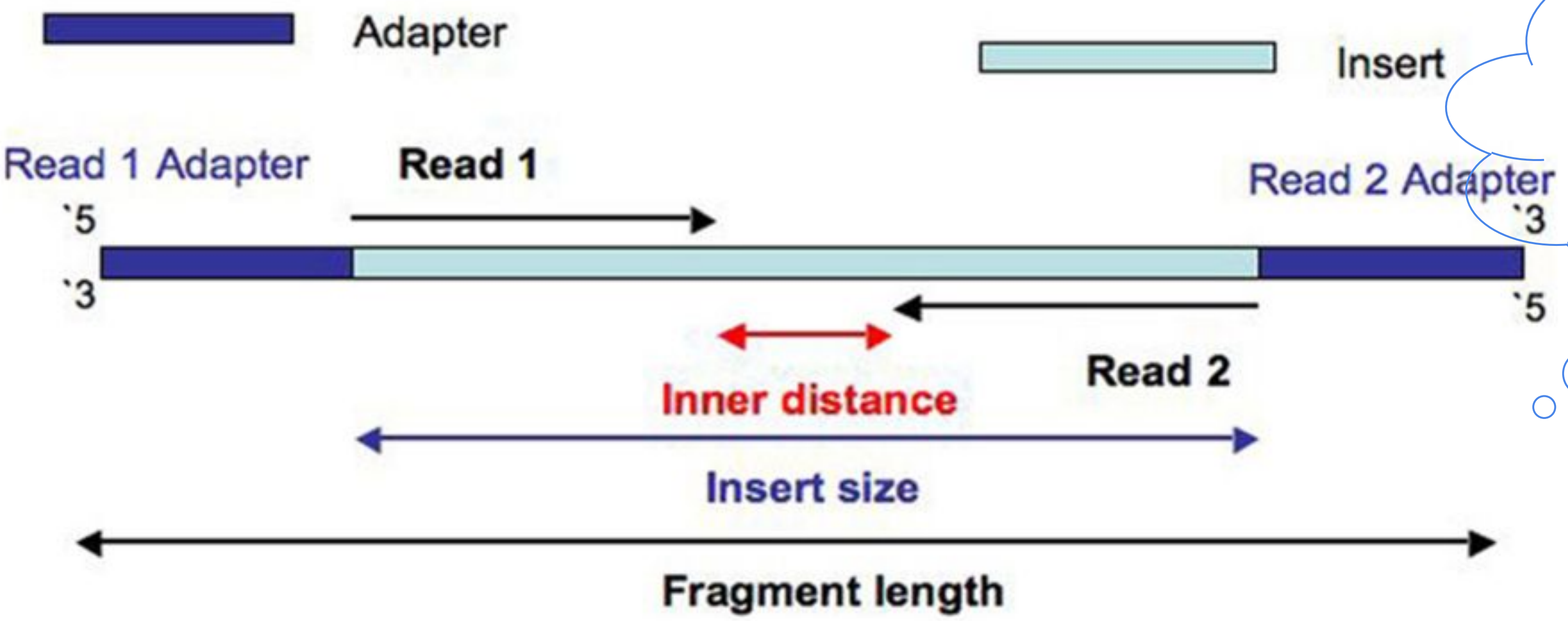
Reference Genome

```
AGATGGTATTGCAATTTGACAT
```

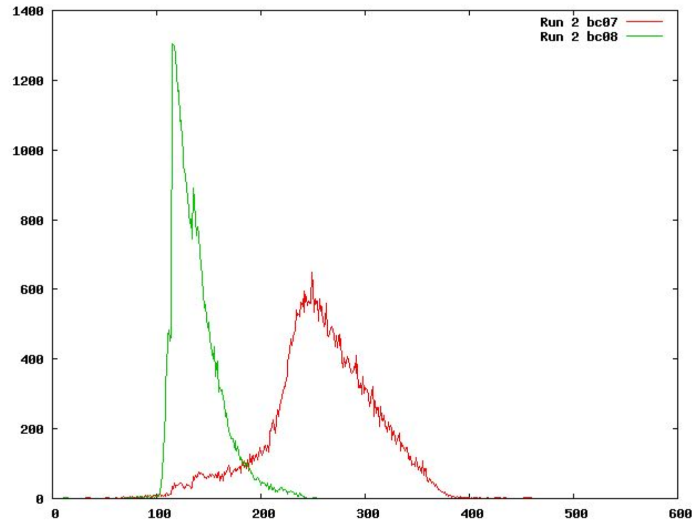
"Bioinformatics"

Reads are aligned to a reference sequence with bioinformatics software. After alignment, differences between the reference genome and the newly sequenced reads can be identified.

Terminology



Are Read1 and Read2 reverse-complement of each other?

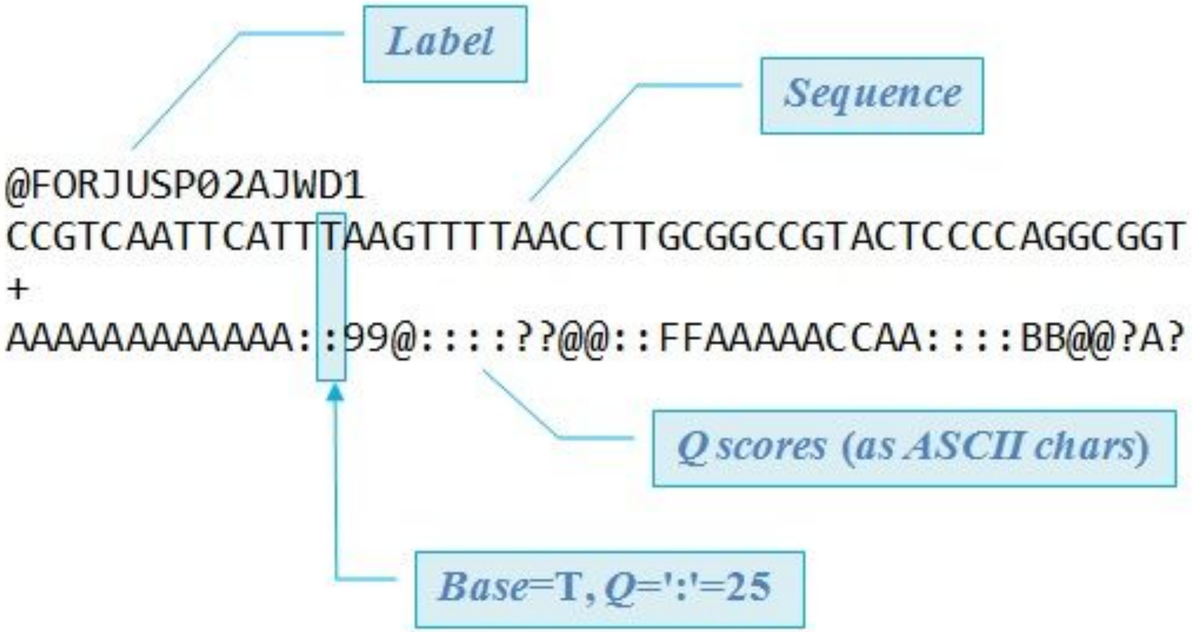


Read length - fixed - typically 50 < L < 250
Insert size - distributed - limitation - up to 700 bp

Sequencing Yield DNA Sequences in FASTQ Format

DNA

```
@seq1
ACCTTCGAACGGCGGGGGGTTACAA
+
!''*((((***+))%%%++).1***
@seq2
TGGAACCGAACGGCCCCGGTTACAT
+
!''*!!!!***+))+++++).1***
And so on...
```



The FASTA Format

FASTA format is the most basic format for reporting a sequence and is accepted by almost all sequence analysis program

Sequence ID `>1 dna:chromosome chromosome:GRCh38:1:1:248956422:1 REF`

Sequence

```
CGGTGGCTCACGCCTGTAATCCCAACACTTTGGGAGGCCAAAGCAGGTGGATTACCTGAG
ATCAGGAGTTCGAGACCAGCCTGGCCAACATGGTGAAACCCTGTCTCTACTAAAAATACA
```

en.wikipedia.org/wiki/FASTA_format

The FASTQ Format

A “standard” format for storing and defining sequences from next-generation sequencing technologies.

Sequence ID	`@SEQ_ID`
Sequence	`GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT`
<separator>	`+`
Quality scores	`!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65`

en.wikipedia.org/wiki/FASTQ_format

The FASTQ Format's Sequence Identifier

```
@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

FASTQ Quality Scores: Estimate of Confidence in Each Base

Sequence ID	@SEQ_ID
Sequence	GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAACTCACAGTTT
<separator>	+
Quality scores	!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>>CCCCCCC65

↓

Qualities are based on the Phred* scale and are *encoded*

$$Q = -10 * \log_{10}(P_{err})$$

Q = Phred quality score

P_{err} = The probability that the base call is incorrect

*The Ph in Phred comes from Phil Green, [the inventor of the encoding]

Phred Quality Score Calculation

$$Q = -10*\log_{10}(P_{err})$$

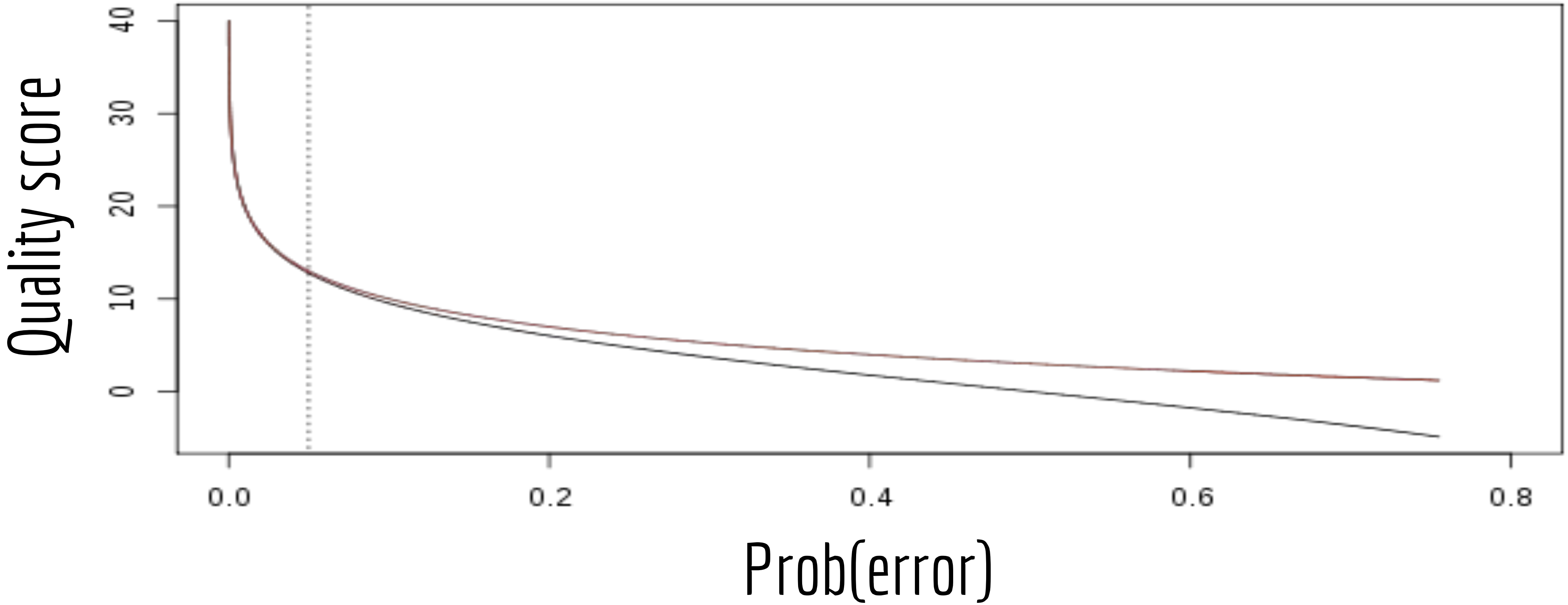
Error probability (P_{err})	$\log_{10}(P_{err})$	Phred quality score
1	0	0
0.1	-1	10
0.01	-2	20
0.001	-3	30
0.0001	-4	40

Phred Quality Score Calculation

Phred 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%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

A Higher Quality Score is Better

>=20 is considered "good"



Q-Score Encoding - ASCII Table

Dec	Hex	Char	Dec	Hex	Char	Dec	Hex	Char	Dec	Hex	Char
0	00	Null	32	20	Space	64	40	@	96	60	`
1	01	Start of heading	33	21	!	65	41	A	97	61	a
2	02	Start of text	34	22	"	66	42	B	98	62	b
3	03	End of text	35	23	#	67	43	C	99	63	c
4	04	End of transmit	36	24	$	68	44	D	100	64	d
5	05	Enquiry	37	25	%	69	45	E	101	65	e
6	06	Acknowledge	38	26	&	70	46	F	102	66	f
7	07	Audible bell	39	27	'	71	47	G	103	67	g
8	08	Backspace	40	28	(	72	48	H	104	68	h
9	09	Horizontal tab	41	29	)	73	49	I	105	69	i
10	0A	Line feed	42	2A	*	74	4A	J	106	6A	j
11	0B	Vertical tab	43	2B	+	75	4B	K	107	6B	k
12	0C	Form feed	44	2C	,	76	4C	L	108	6C	l
13	0D	Carriage return	45	2D	-	77	4D	M	109	6D	m
14	0E	Shift out	46	2E	.	78	4E	N	110	6E	n
15	0F	Shift in	47	2F	/	79	4F	O	111	6F	o
16	10	Data link escape	48	30	0	80	50	P	112	70	p
17	11	Device control 1	49	31	1	81	51	Q	113	71	q
18	12	Device control 2	50	32	2	82	52	R	114	72	r
19	13	Device control 3	51	33	3	83	53	S	115	73	s
20	14	Device control 4	52	34	4	84	54	T	116	74	t
21	15	Neg. acknowledge	53	35	5	85	55	U	117	75	u
22	16	Synchronous idle	54	36	6	86	56	V	118	76	v
23	17	End trans. block	55	37	7	87	57	W	119	77	w
24	18	Cancel	56	38	8	88	58	X	120	78	x
25	19	End of medium	57	39	9	89	59	Y	121	79	y
26	1A	Substitution	58	3A	:	90	5A	Z	122	7A	z
27	1B	Escape	59	3B	;	91	5B	[	123	7B	{
28	1C	File separator	60	3C	<	92	5C	\	124	7C	|
29	1D	Group separator	61	3D	=	93	5D	]	125	7D	}
30	1E	Record separator	62	3E	>	94	5E	^	126	7E	~
31	1F	Unit separator	63	3F	?	95	5F	_	127	7F	□

Formula for getting PHRED quality from encoded quality:

Q = ascii(char) - 33

Example:

!+EJ

ASCII	33	43	69	74
-33	0	10	36	41

Is My Data Any Good?

FastQC

- A common first step when getting your fastq data
- Generates a simple HTML report
- Can help detect issues with the data

Running:

```
fastqc <file1.fastq> <file2.fastq> … <file n.fastq>
```

For more options:

```
fastqc -h | less
```

The FastQC Report

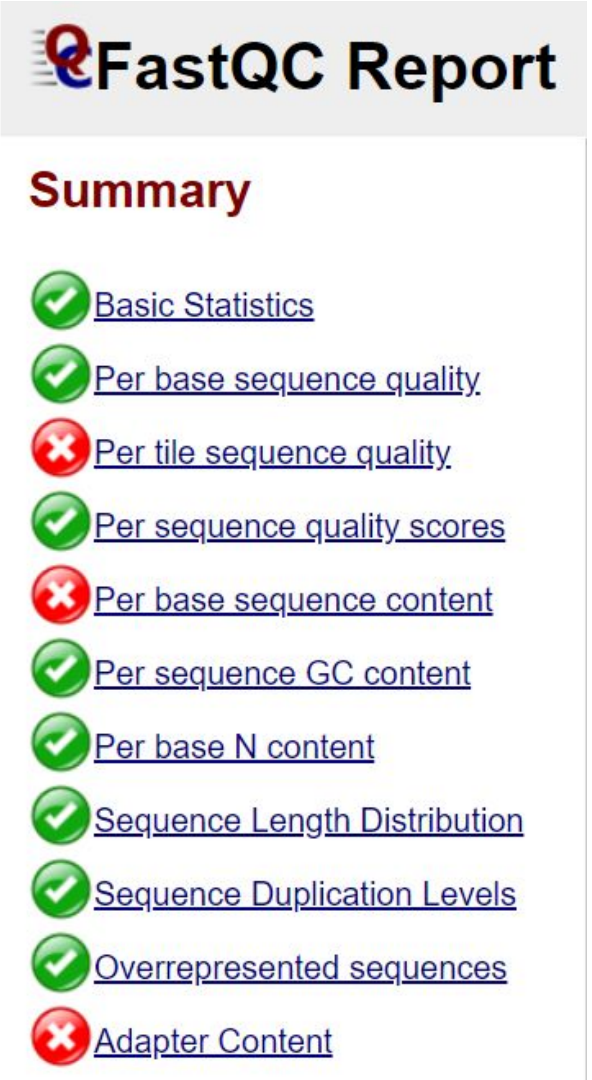
View using your favorite web browser

Contains multiple analysis modules

Issues “Warning” and “Failure” messages per module

Remember to look both at R1 and R2

www.bioinformatics.babraham.ac.uk/projects/fastqc

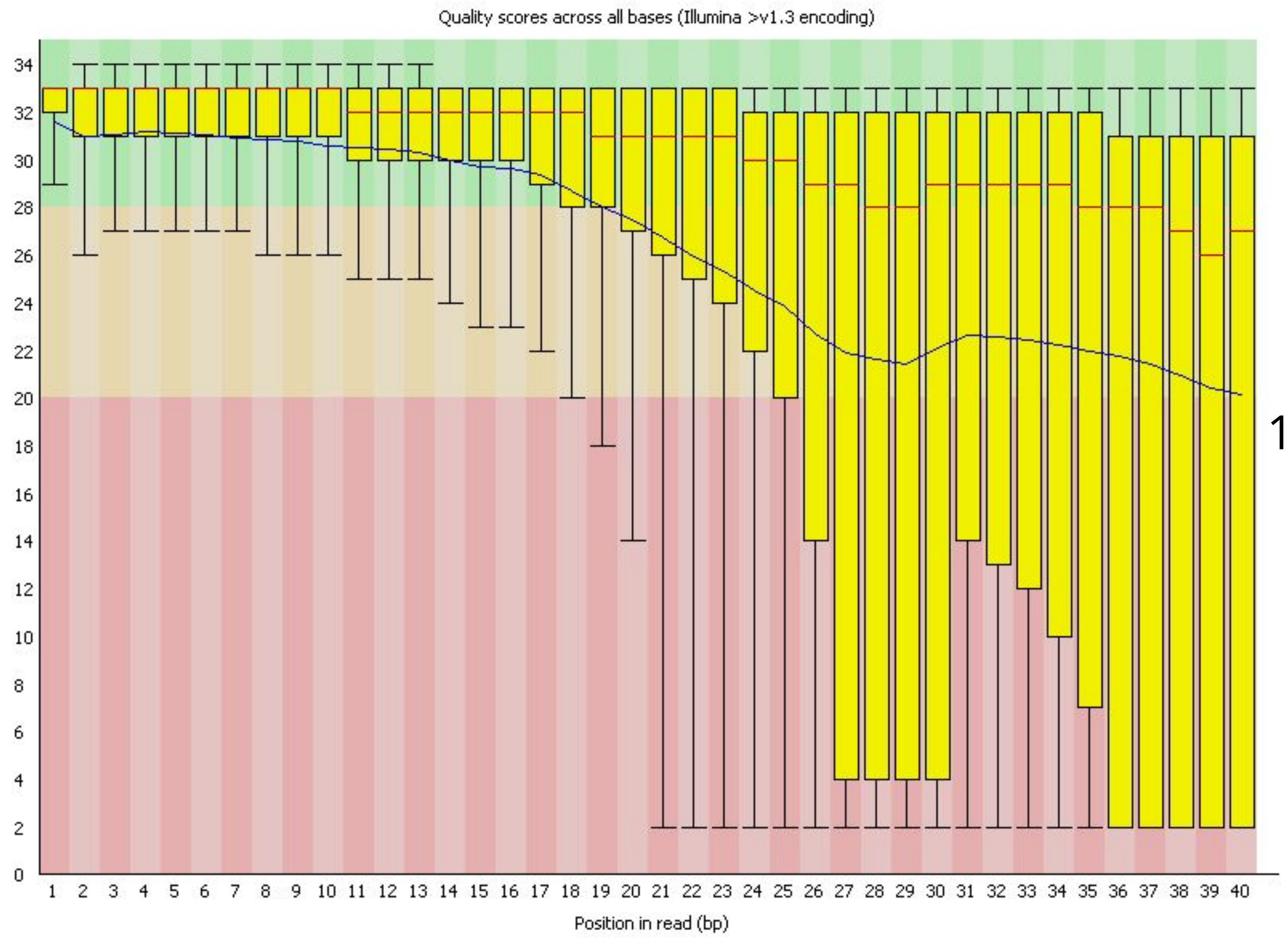


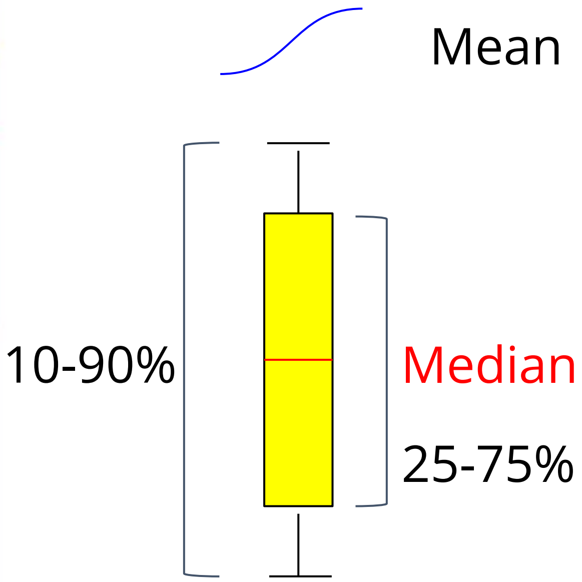
Basic Statistics

Basic Statistics

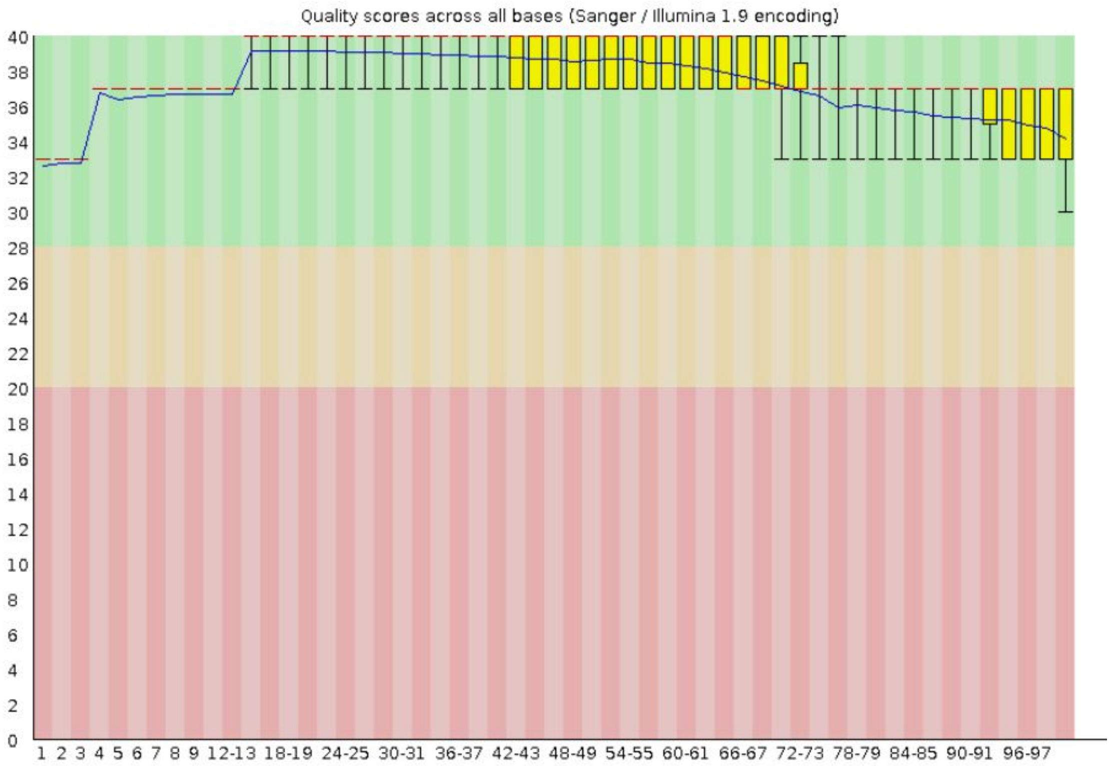
Measure	Value
Filename	ERR2834525_1.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	3161562
Sequences flagged as poor quality	0
Sequence length	101
%GC	41

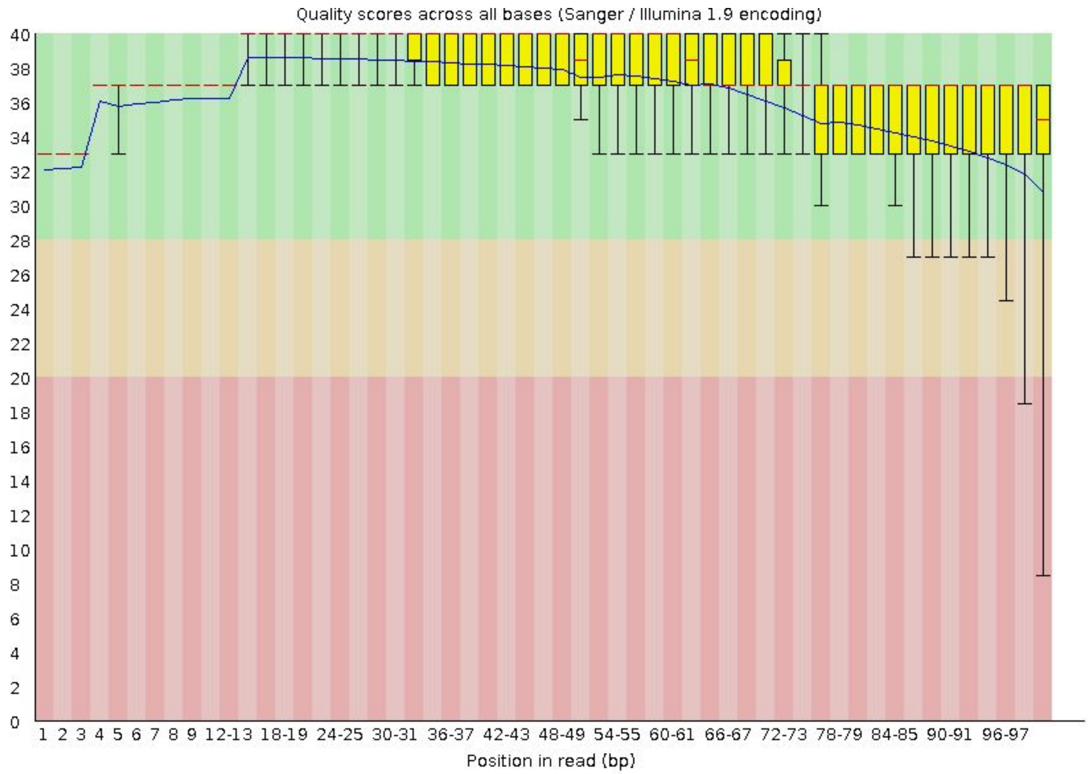
Per Base Sequence Quality



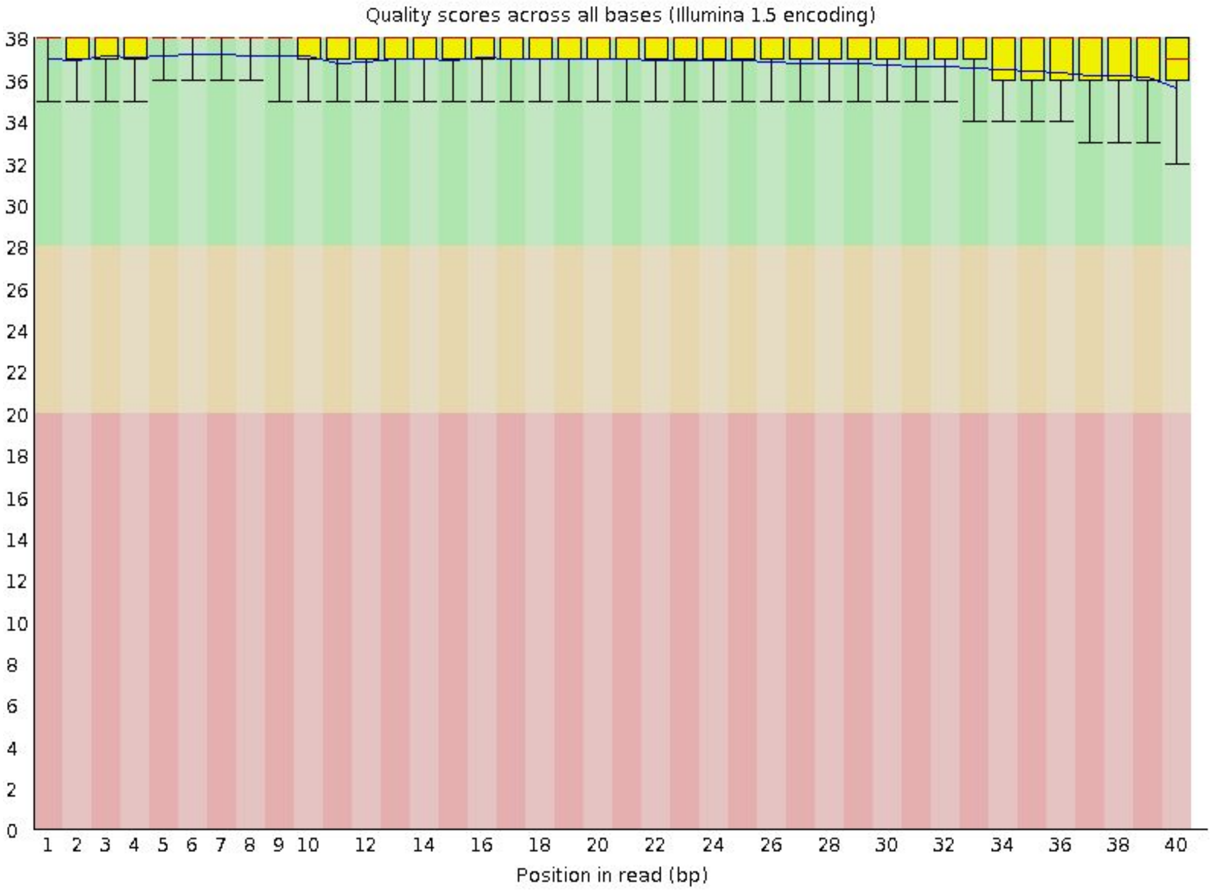


Per Base Sequence Quality - R1 vs. R2



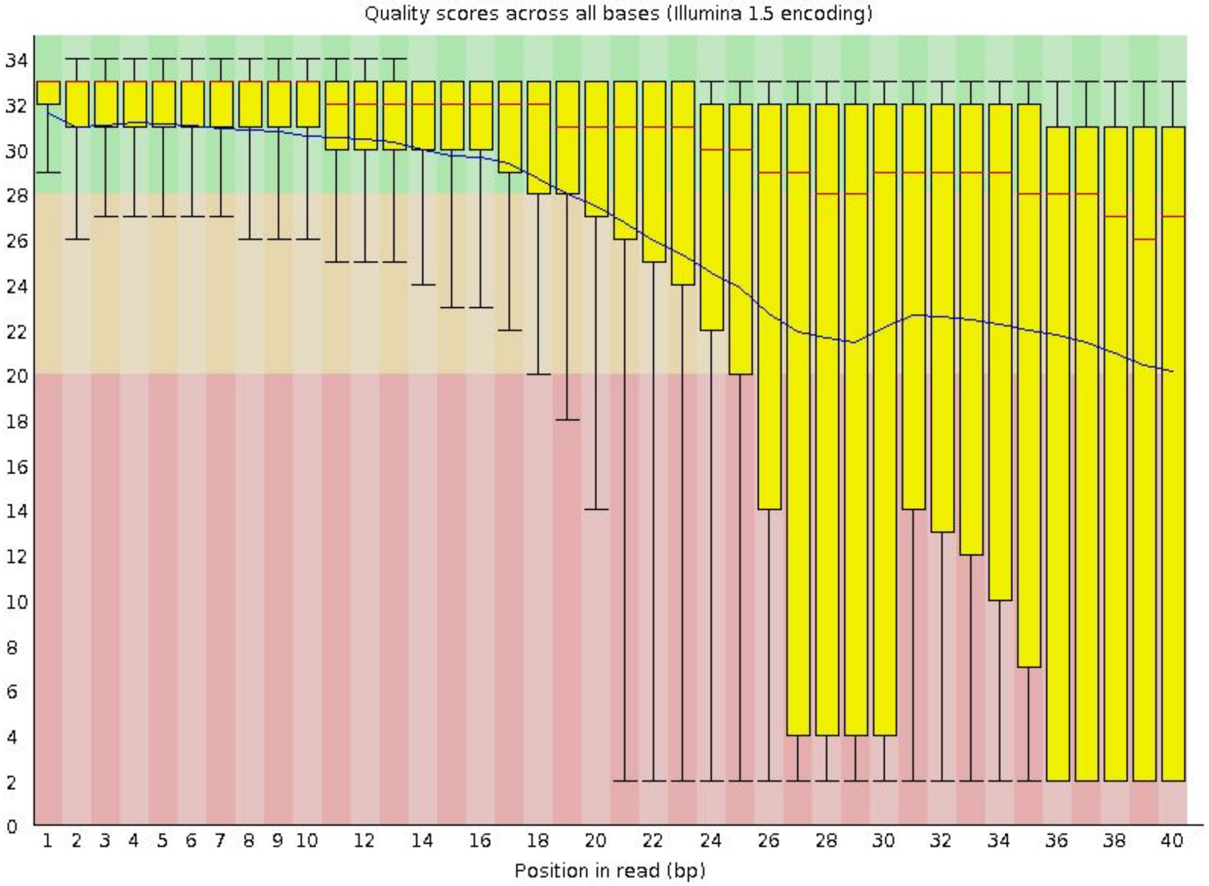


Good



Quality scores across all bases (Illumina 1.5 encoding)
Position in read (bp)

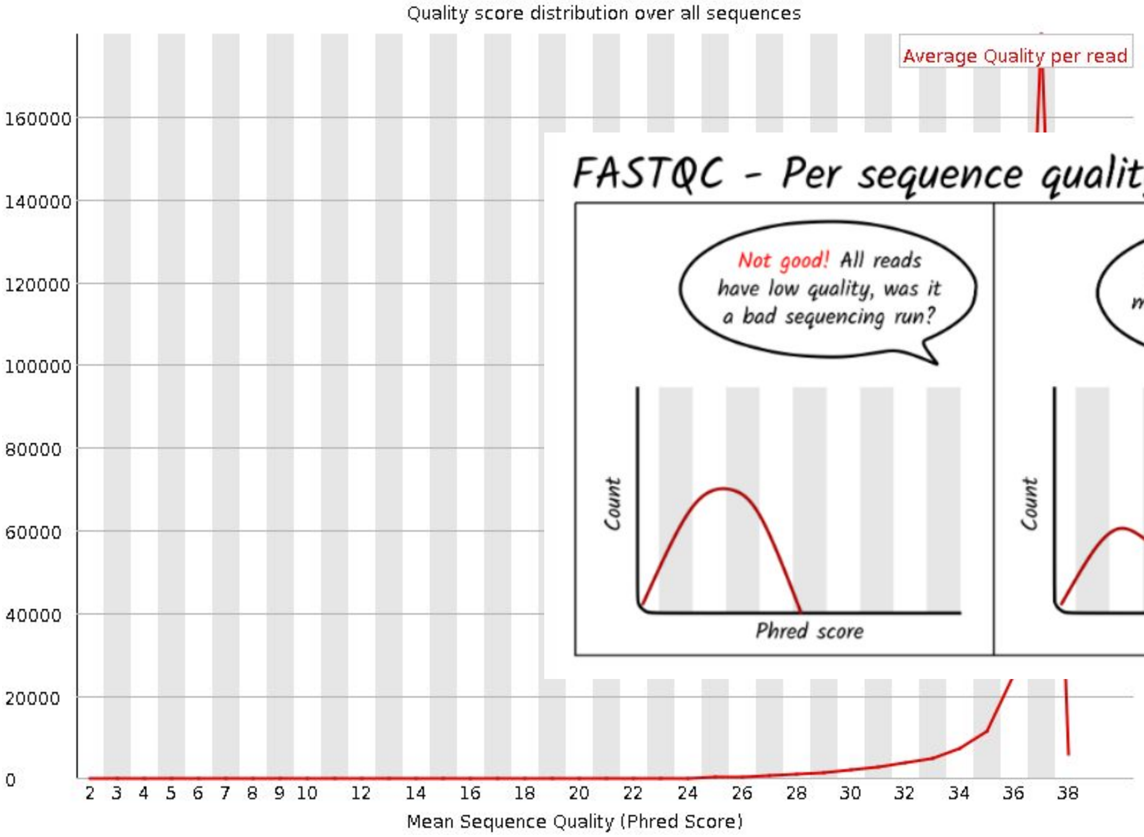
Bad

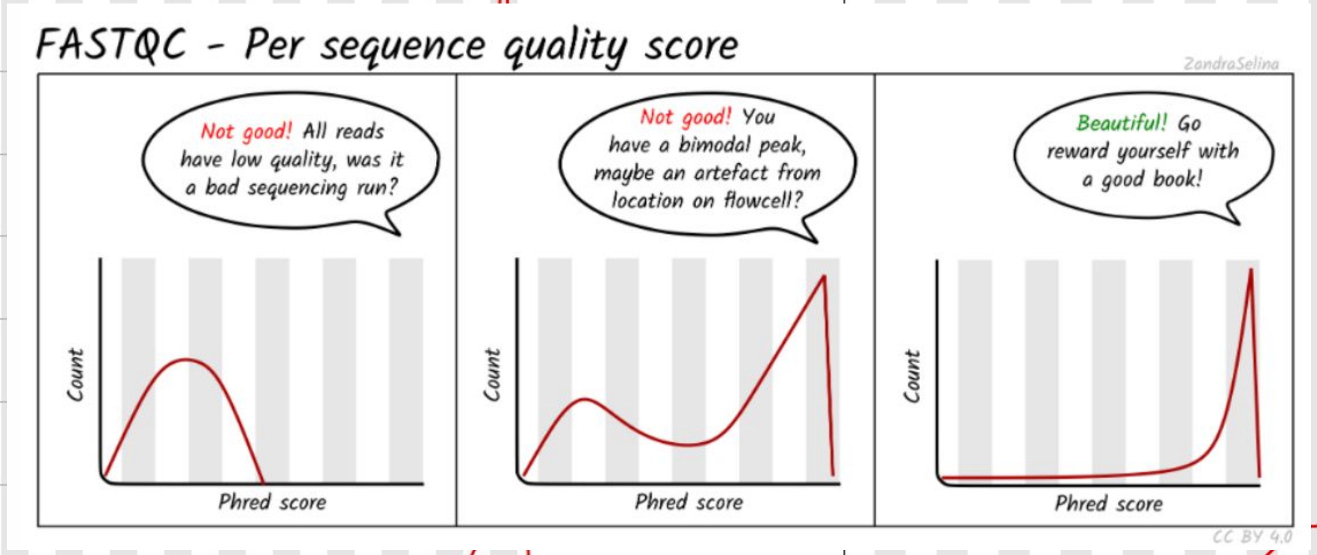


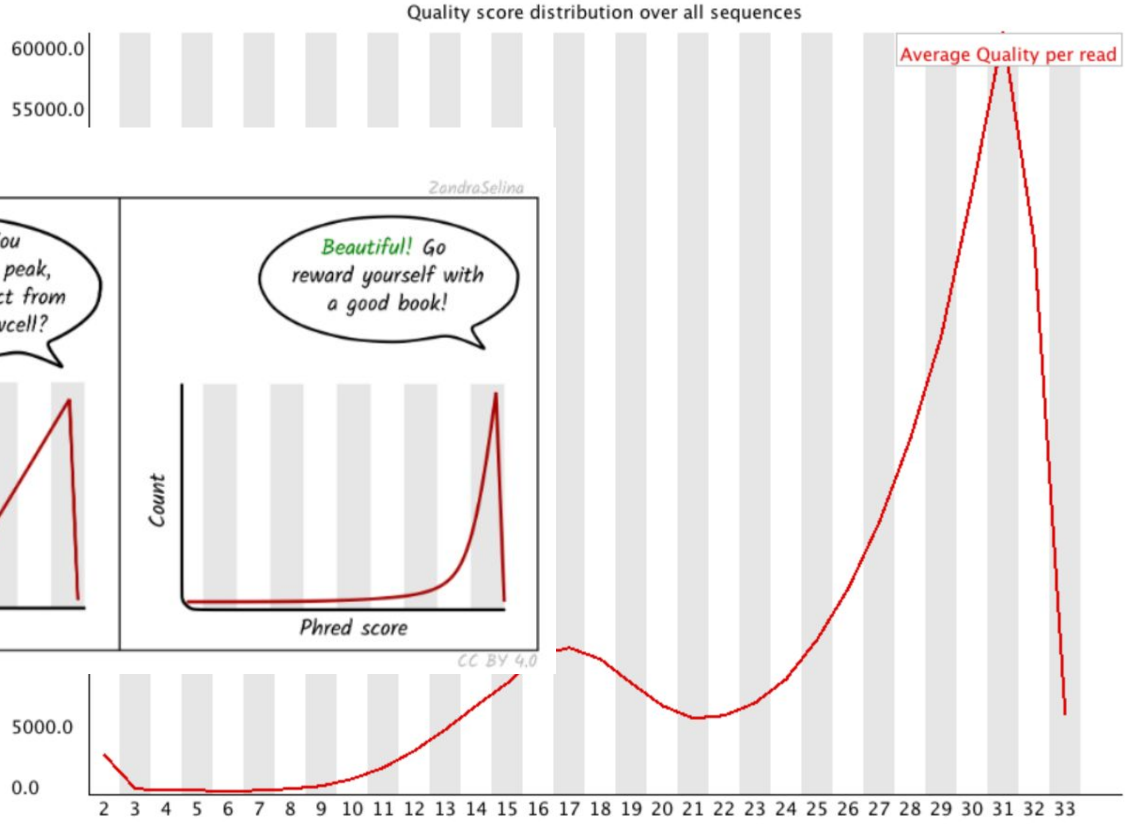
Quality scores across all bases (Illumina 1.5 encoding)
Position in read (bp)

Mean Read Quality

Good **Bad**

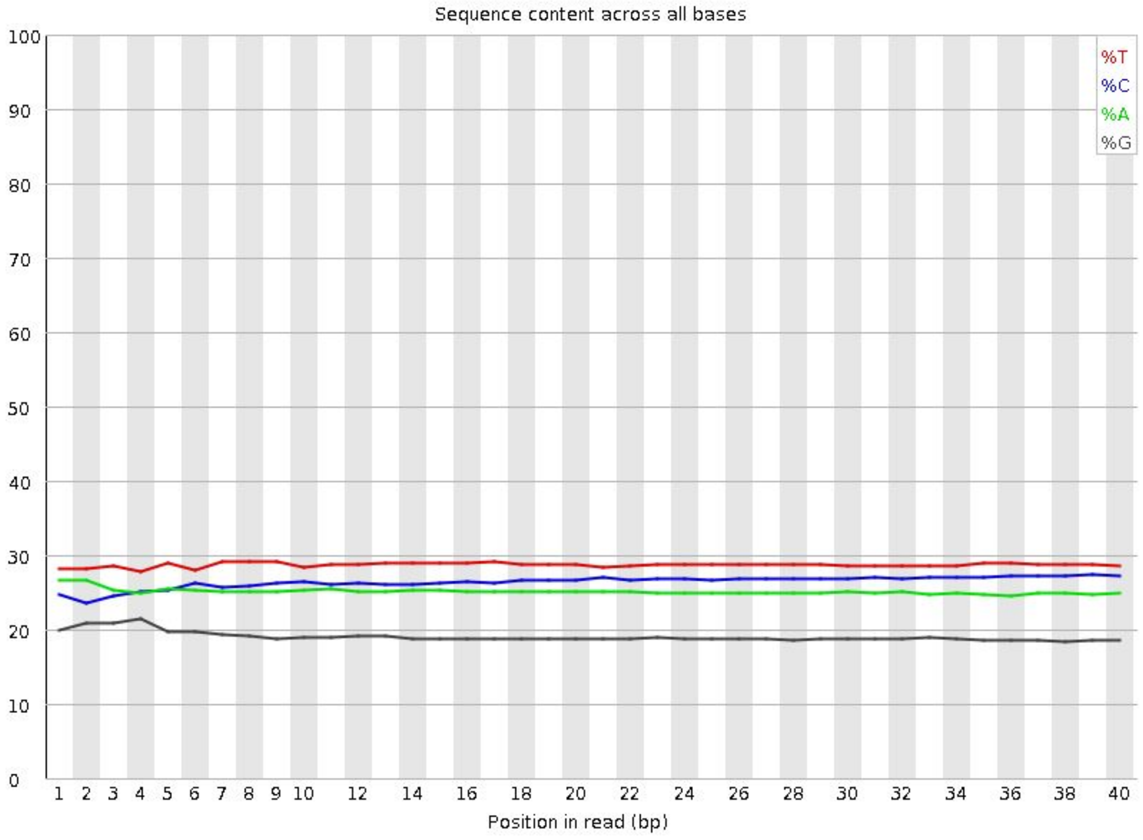




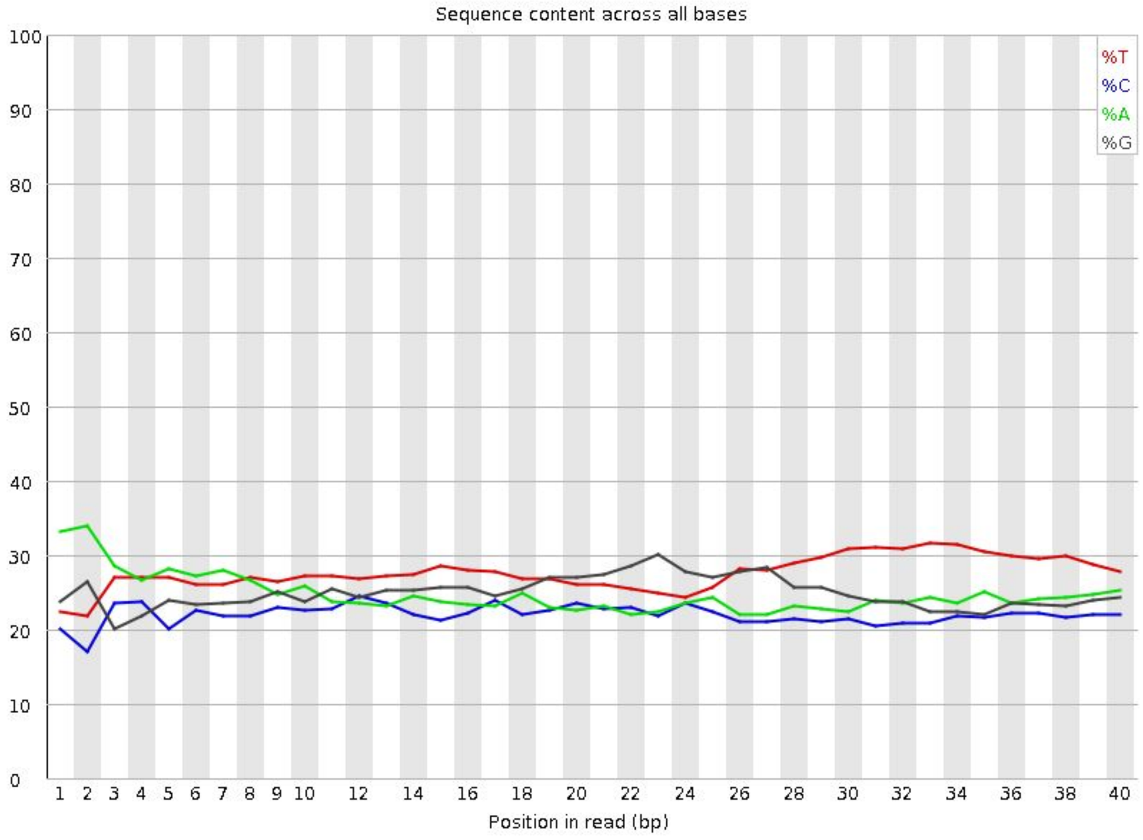


Per Base Sequence Content

Good

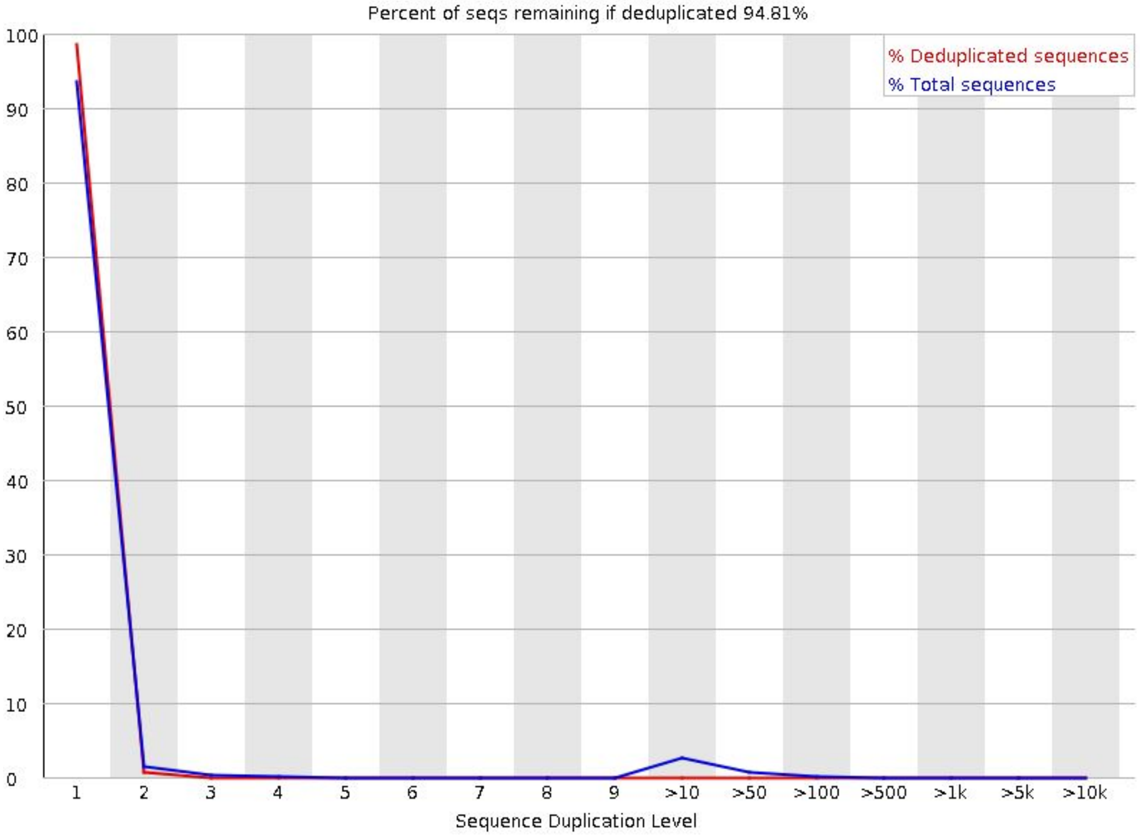


Bad

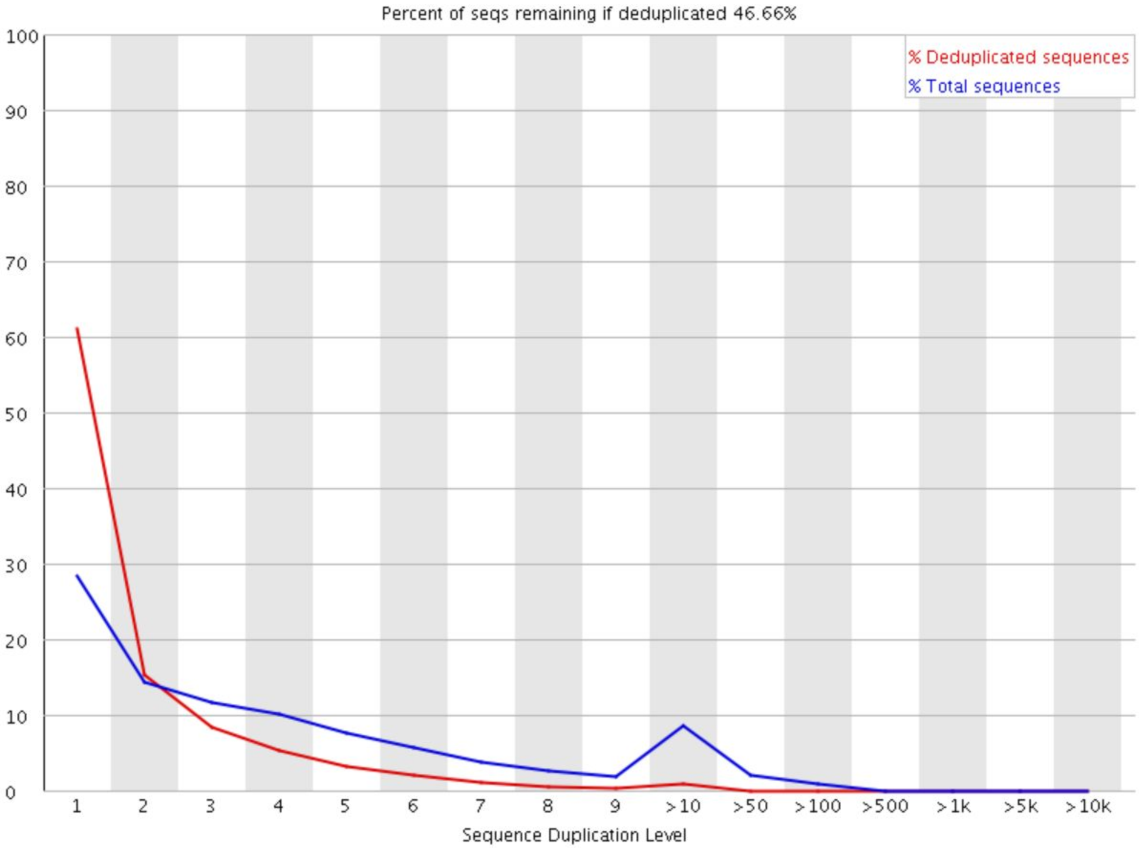


Duplicate Reads

Good

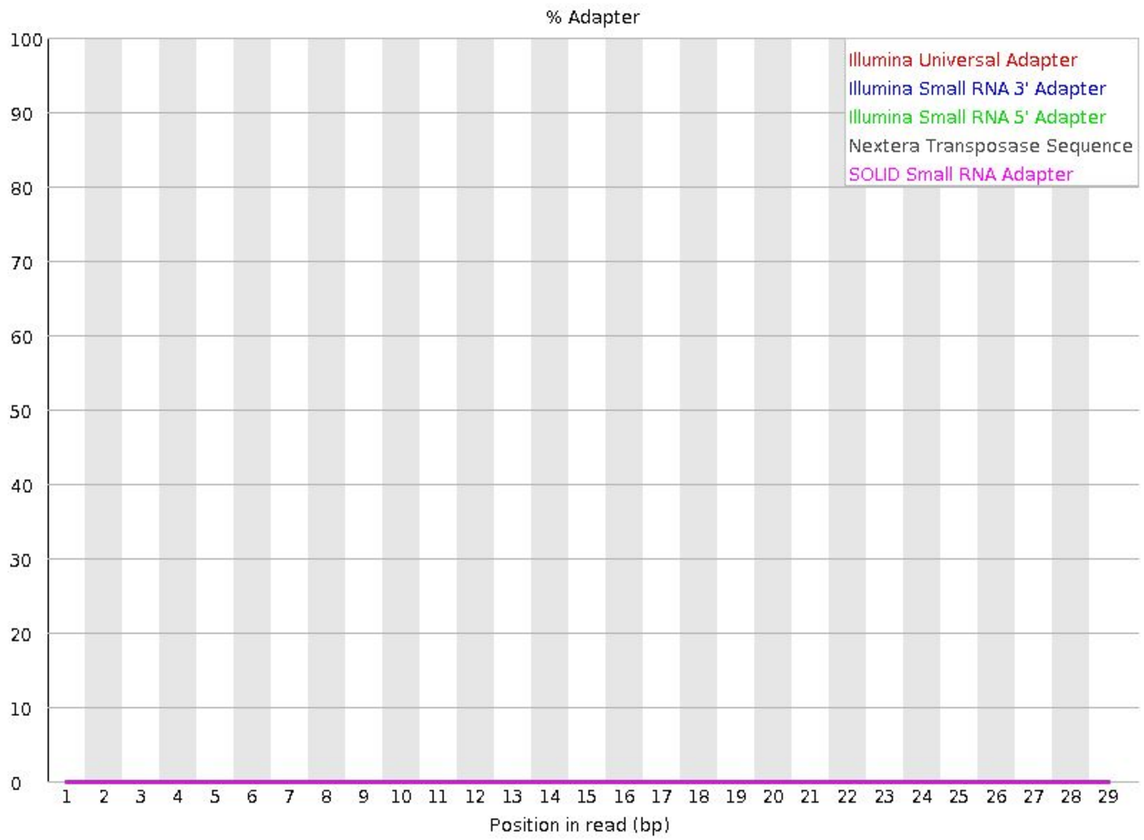


Bad

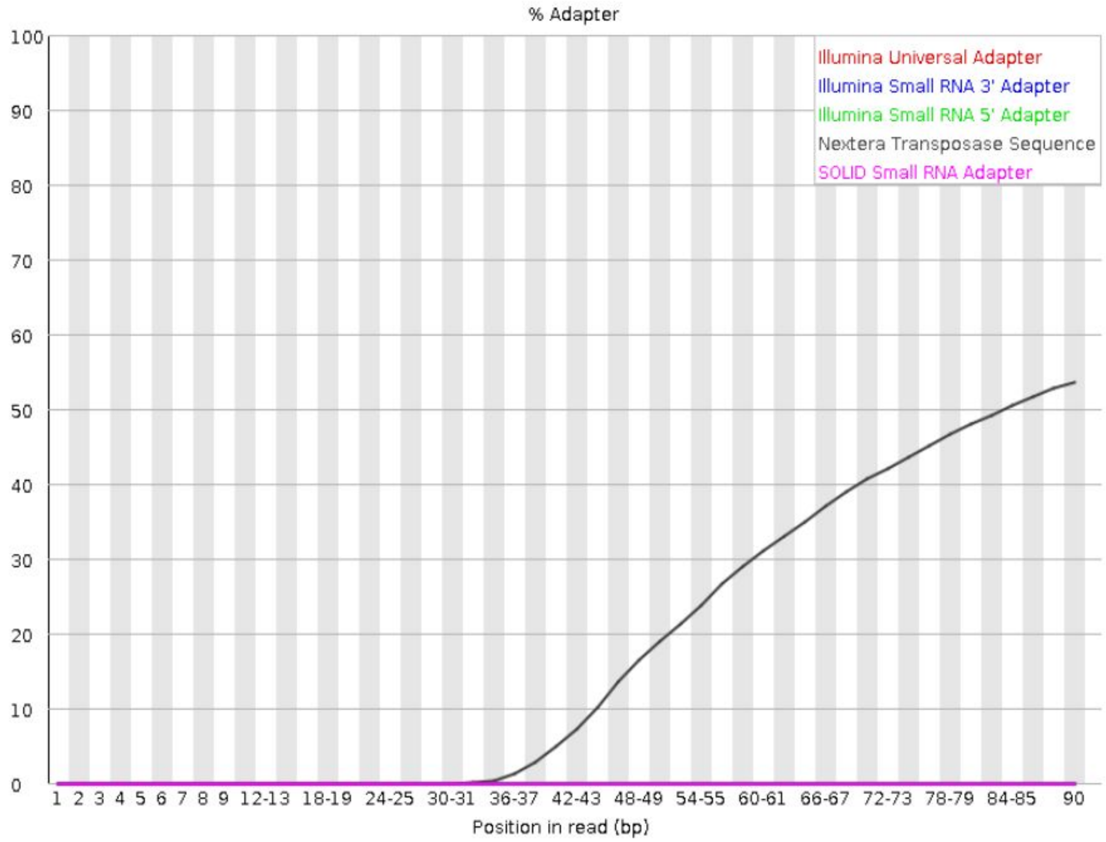


Adapter Sequence

Good



Bad



Warnings and Alerts

Use FastQC report to decide on next steps

Warnings might not affect downstream analysis

Mainly useful when comparing results

Our Toy Data

Cervical Adenocarcinoma Whole Exome Sequencing

Matched normal-tumor samples of a 55 year old female patient

https://www.ncbi.nlm.nih.gov/sra/?term=ERS5326207

ERX4703404: NextSeq 550 paired end sequencing; Cervical Adenocarcinoma Whole Exome sequencing
1 ILLUMINA (NextSeq 550) run: 28.9M spots, 5.8G bases, 2.3Gb downloads

Design: Cervical Adenocarcinoma Whole Exome sequencing

Submitted by: ADVANCED CENTRE FOR TREATMENT RESEARCH AND EDUCATION IN CANCER (ADVANCED CENTRE FOR TREATMENT RESEARCH AND EDUCATI)

Study: Cervical Adenocarcinoma Whole Exome sequencing
PRJEB41309 • ERP125057 • All experiments • All runs
hide Abstract
Raw data of exome sequencing of 17 paired samples and 1 orphan tumor sample, total 18 samples

Sample: Sample 8
SAMEA7569583 • ERS5326207 • All experiments • All runs
Organism: Homo sapiens

Library:
Name: Sample 8_p
Instrument: NextSeq 550
Strategy: WXS
Source: GENOMIC
Selection: RANDOM
Layout: PAIRED
Construction protocol: 17 paired and 1 orphan tumor were collected from ACTREC-TMC. DNA was extracted from tumor tissue and the matched normal tissue or blood using DNeasy tissue extraction kit (Qiagen) and QIAamp DNA blood mini kit (Qiagen) following manufacturer's instruction. Genomic DNA was sheared using covaris to generate 150-500 bp fragment size. The fragment ends were repaired followed by adenylation at 3'end and sample was purified using AMPure XP beads. The fragments were ligated to adaptor and amplified by PCR. The generated library is then hybridized with SureselectTarget Enrichment system kit and hybrids are separated using streptavidin coated magnetic beads. Then the sample was PCR amplified using indexing primers and purified. The prepared libraries were loaded on Illumina flowcell to generate clusters

Experiment attributes:
Experimental Factor: genotype: germline genotype
Experimental Factor: sampling site: normal tissue adjacent to neoplasm

Runs: 1 run, 28.9M spots, 5.8G bases, 2.3Gb

Run	# of Spots	# of Bases	Size	Published
ERR4833597	28,927,775	5.8G	2.3Gb	2022-02-13

Basic Command Line & Conda

Live Session amitlevon@nshomron2.tau.ac.il