

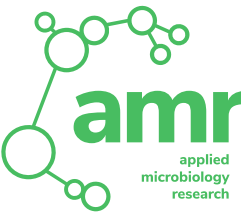


# Bio 296: Microbial Bioinformatics

## Introduction to Sequencing Technologies and DNA extraction

Tim Roloff

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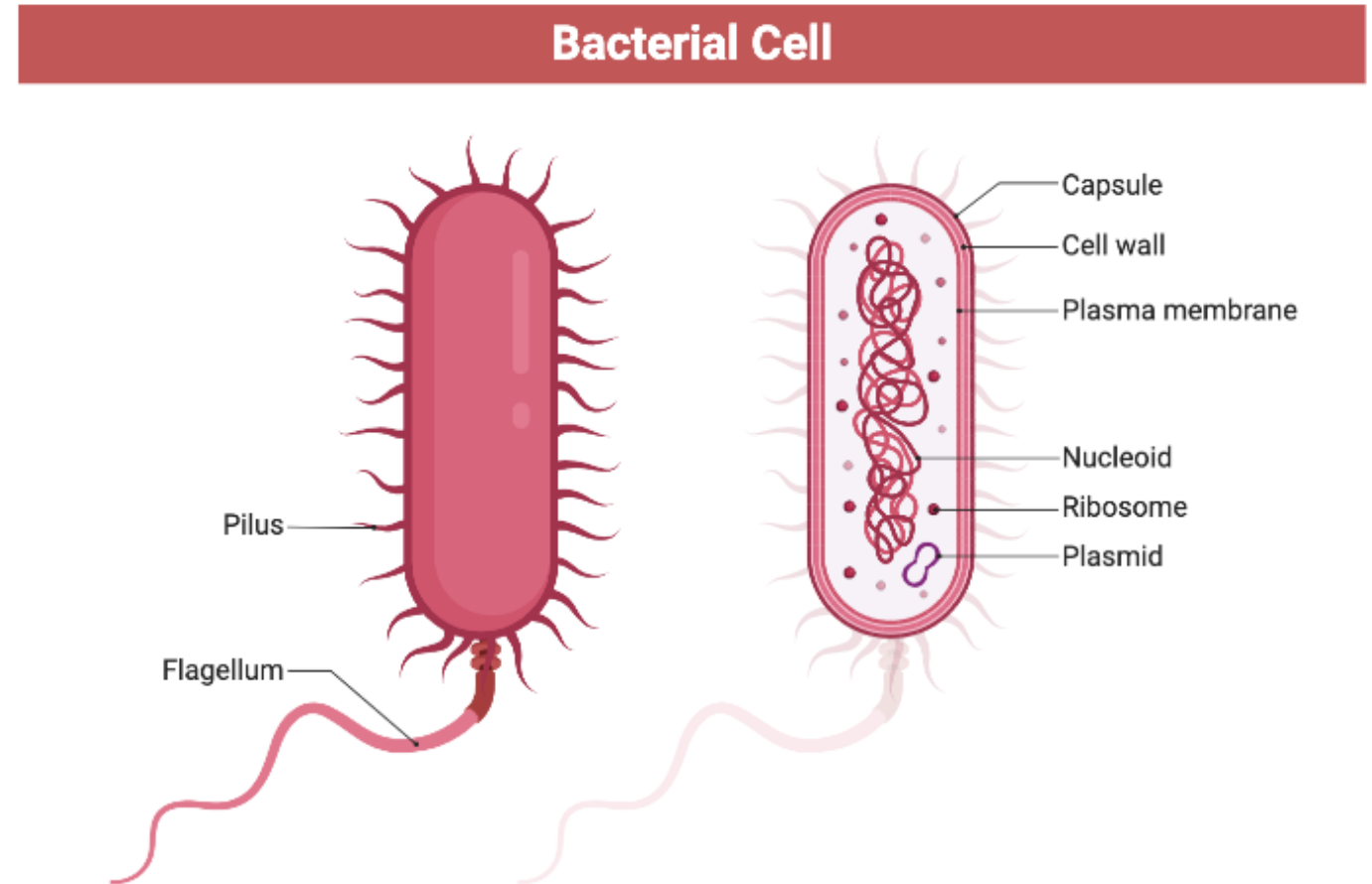


- DNA extraction
- Illumina sequencing
- Nanopore sequencing (ONT)

# DNA extraction

Purify nucleic acids from bacterial cell

- Genomic DNA
- Plasmids
- RNA

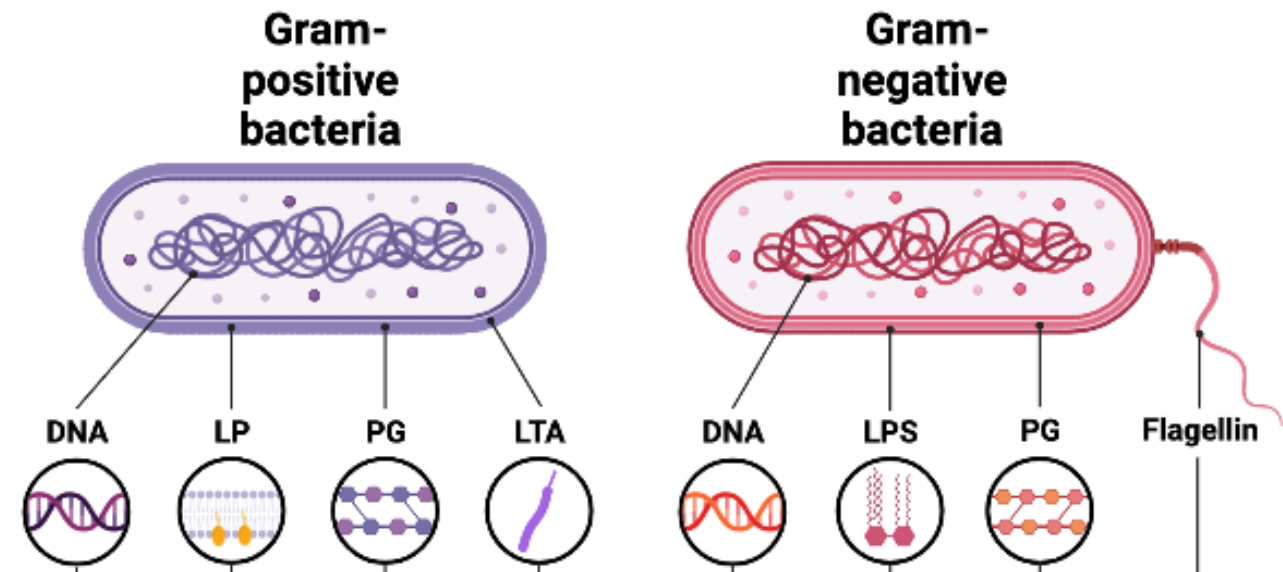


BioRender (2021). Structural Overview of a Bacterial Cell. <https://app.biorender.com/biorender-templates/figures/all/t-5ffdfb47420acf00a02c5e53-structural-overview-of-a-bacterial-cell>

# DNA extraction

- Gram positive and gram negative bacteria have different cell wall compositions
- Gram positives need harsher extraction methods
- Mechanical disruption of cell wall
  - Bead beating
- Enzymatic disruption of cell wall
  - Lysozyme (Muramidase)
  - Proteinase K
  - Lysostaphin (zink endopeptidase) for *Staphylococcus* sp.

LP - Lipopeptides  
LPS – Lipopolysaccharid  
PG – Peptidoglycan  
LTA – Lipoteichoic acid



– BioRender (2021). Recognition of Pathogen-Associated Molecular Patterns (Bacteria). <https://app.biorender.com/biorender-templates/figures/all/t-609beda351a1e400aad4193c-recognition-of-pathogen-associated-molecular-patterns-bacter>

# DNA extraction

- Manual kits or extraction robots
- Robots used at IMM
  - QIAGEN QIA Symphony
  - Promega Maxwell
  - QIAGEN QIAcube

Different robots support different protocols

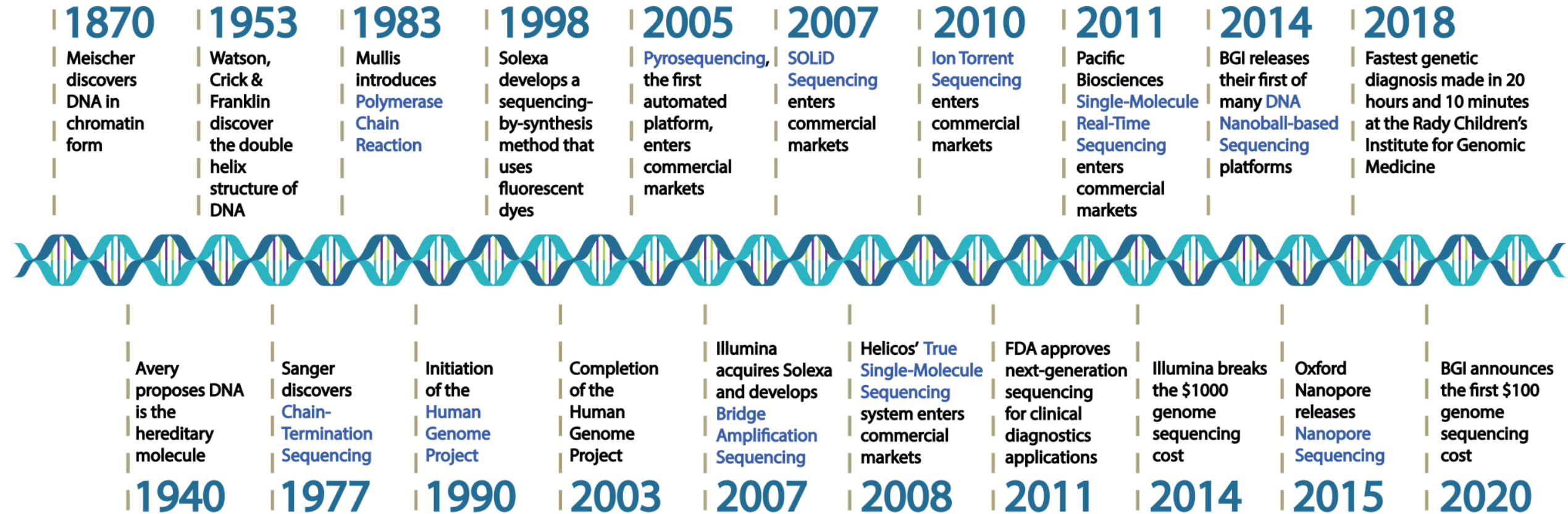
- Input type
- Throughput
- Manual intervention



## Further considerations:

- Amount and concentration of DNA needed
  - Input e.g. for library prep
- Fragment length needed e.g. for long-read sequencing
  - Bead beating will reduce fragment size
- DNA or total nucleic acid (TNA)
- Purity required for subsequent steps
- Number of samples to be extracted

# A history of DNA sequencing

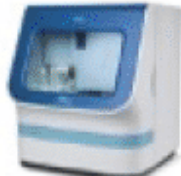
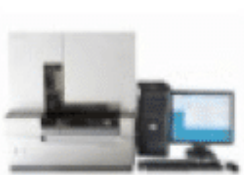


# Sequencing technologies over time

## First generation

## Second generation (next generation sequencing)

## Third generation



Sanger sequencing  
Maxam and Gilbert  
Sanger chain termination

Infer nucleotide identity using dNTPs,  
then visualize with electrophoresis

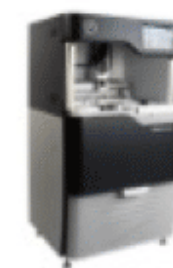
500–1,000 bp fragments



454, Solexa,  
Ion Torrent,  
Illumina

High throughput from the  
parallelization of sequencing reactions

~50–500 bp fragments



PacBio  
Oxford Nanopore

Sequence native DNA in real time  
with single-molecule resolution

Tens of kb fragments, on average

Short-read sequencing

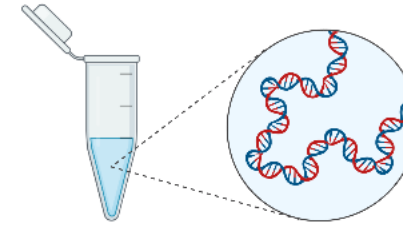
Long-read sequencing



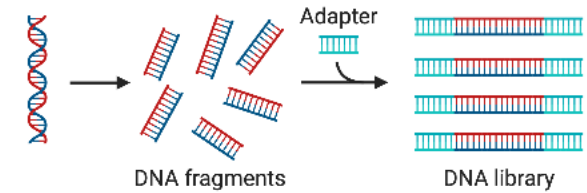
# Illumina sequencing workflow

- Starting from pure nucleic acids
- Library preparation
- Sequencing
- Data analysis

**Step 1:**  
DNA extraction

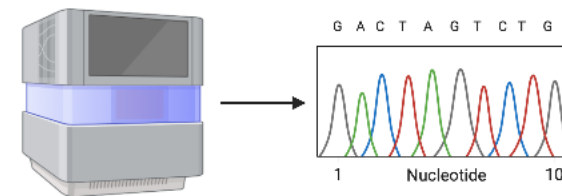


**Step 2:**  
Library preparation



## Next Generation Sequencing Workflow

**Step 3:**  
Sequencing



**Step 4:**  
Analysis



## Library preparation

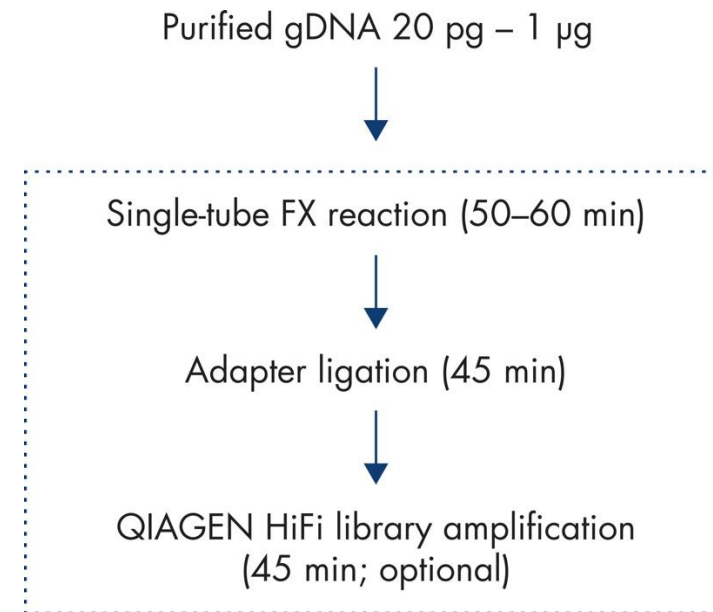
- List of current library prep methods
- Choose method based on question
- Many methods available as kits
  - Important for diagnostic lab (standardisation, reproducibility)



# Library prep method used at IMM

## QIAseq FX workflow:

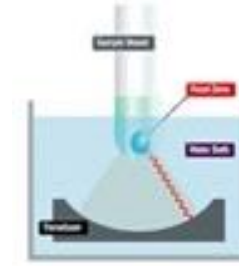
- Fragment DNA (enzymatic)
- End repair
- Adapter ligation / barcoding
- Amplification
- Quality control
  - Qubit / Fragment analyzer
- Pooling



# Library prep

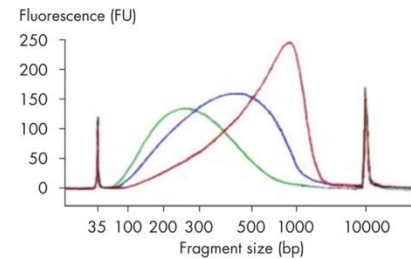
Fragmentation – ideally uniform and sequence independant

Mechanical shearing (Covaris)

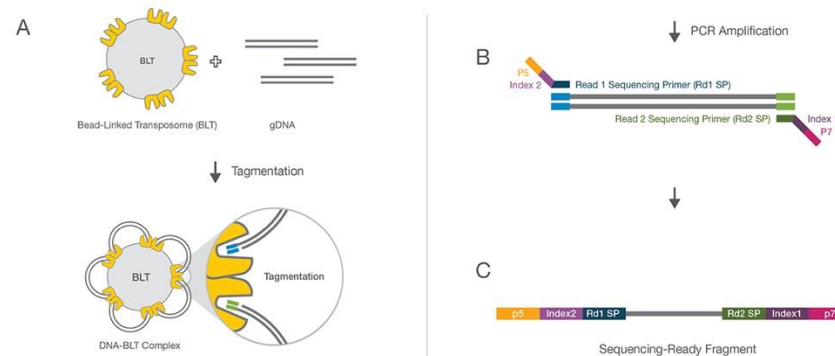


Enzymatic

- Time dependant



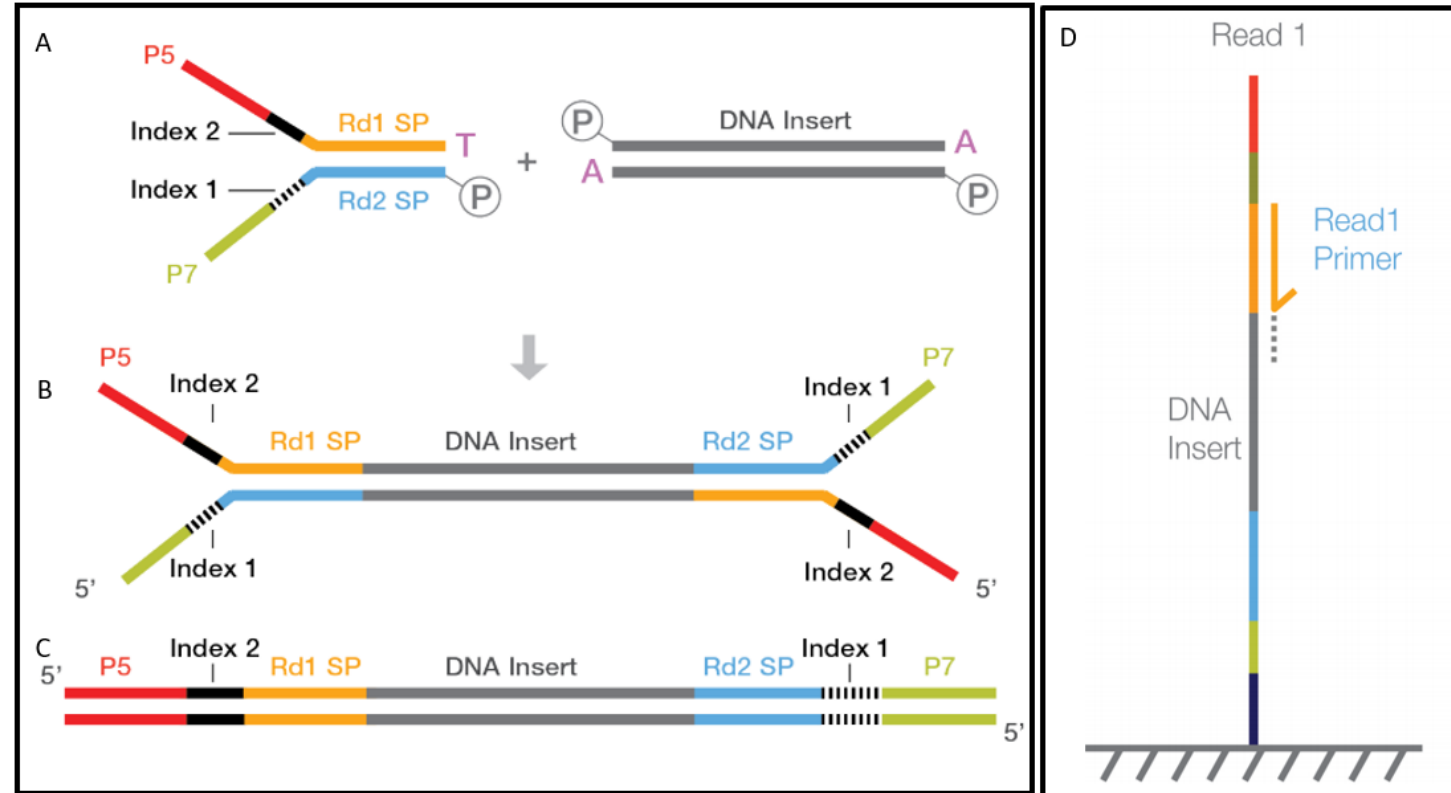
Tagmentation



# Library prep

Final library contains:

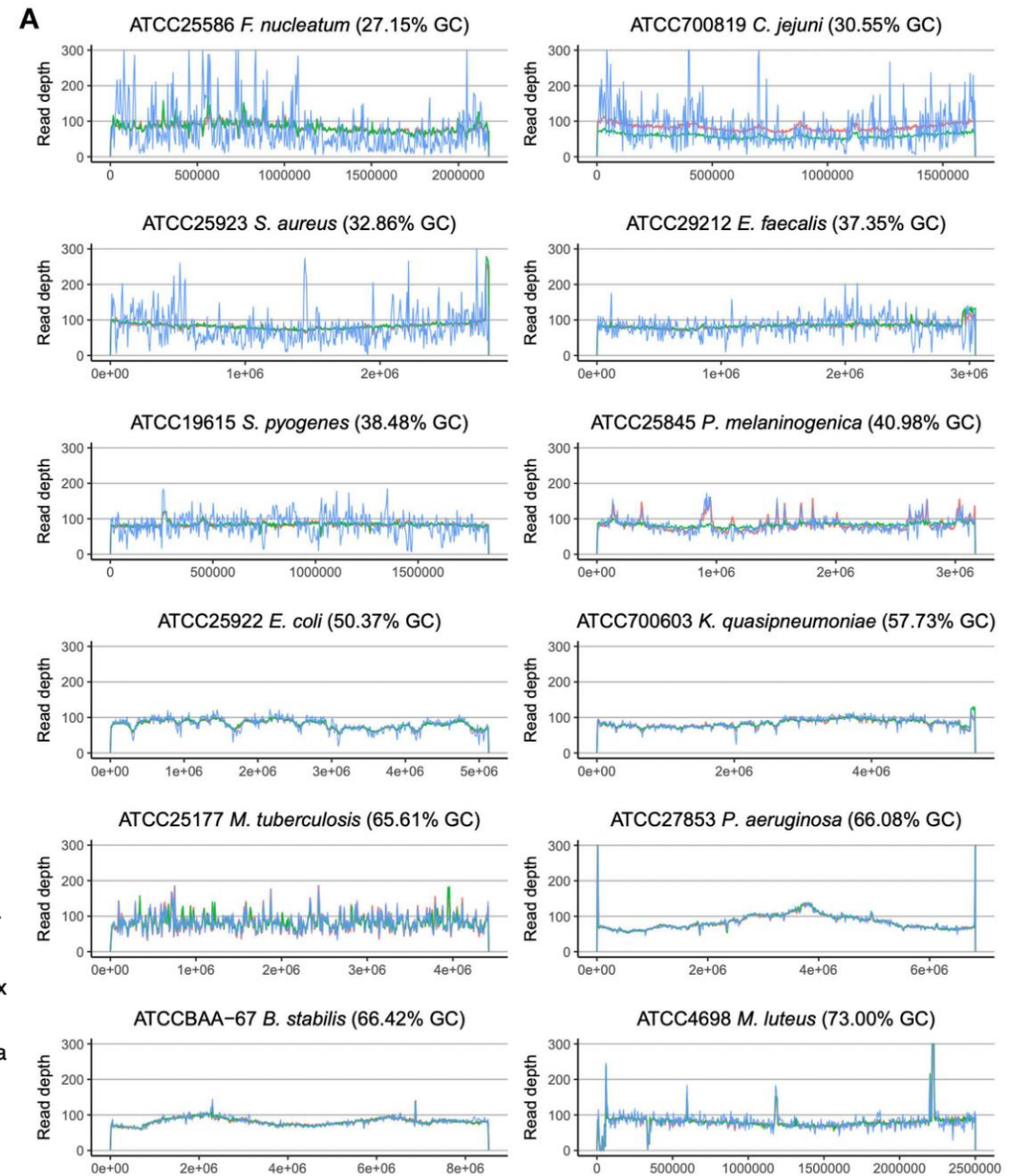
- DNA insert
- P5 and P7 adapter
- Indices (Index 1 / 2)
- Sequencing primer binding sites (Rd1/Rd2 SP)



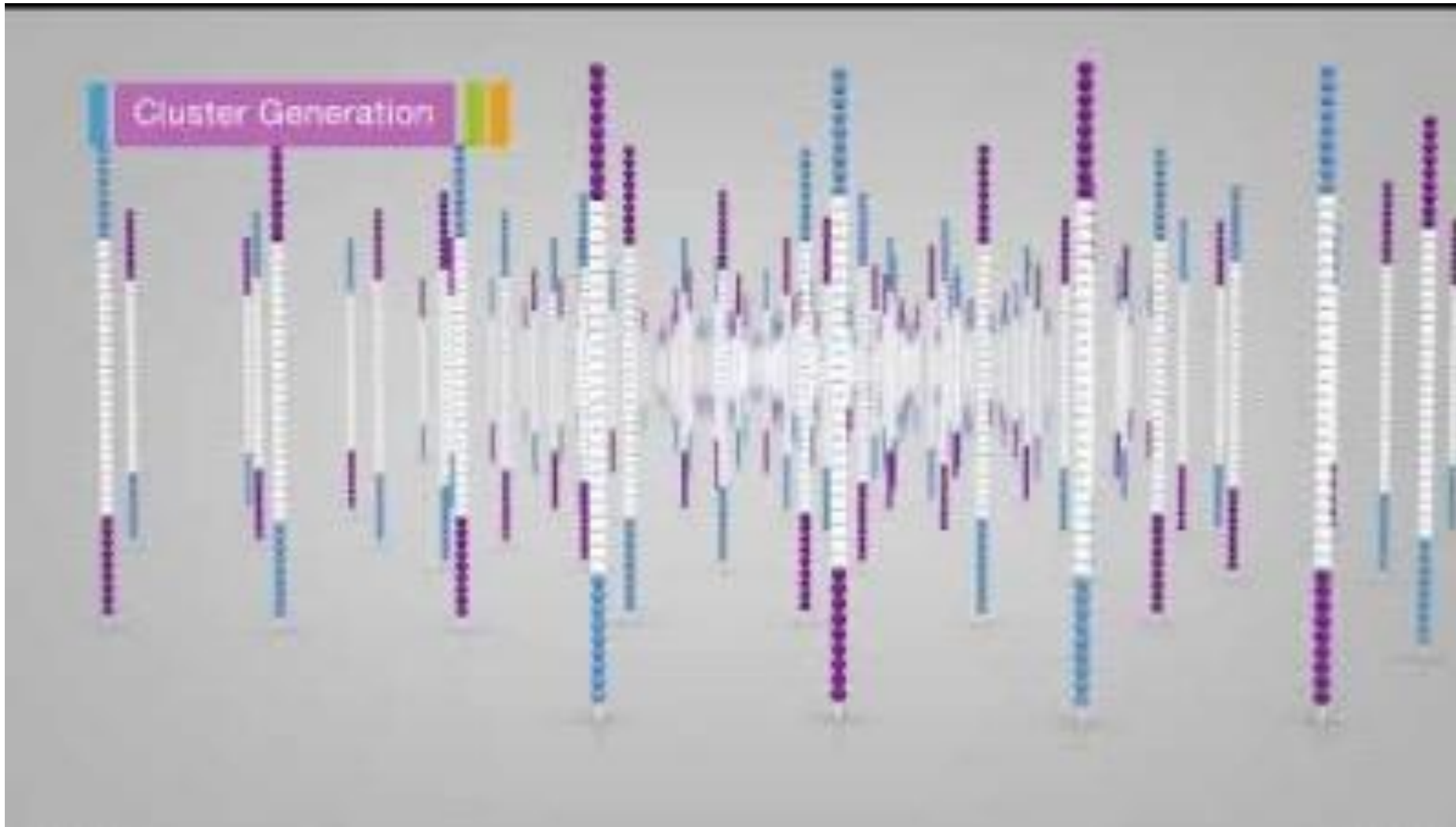


# Library prep - find the best kit

- Panel of 12 ATCC strains with complete genomes published:
  - variable genome sizes
  - variable %G+C
  - plasmid content
  - ARG content
- Tested:
  - 3 kits: Illumina DNA Prep (flex), QIAseq Fx (Qia) and Illumina Nextera XT (XT)
  - evenness of coverage
  - insert size distribution
  - base composition of reads
  - Assemblies with subsampling
  - QC, kmer content, core genes
- 50x acceptable for flex and Qia; 100x for XT



# Illumina: Sequencing by Synthesis



# Illumina: the sequencer portfolio



iSeq 100



MiniSeq



MiSeq Series +



Miseq i100



NextSeq 1000 & 2000



NovaSeq 6000 Series +



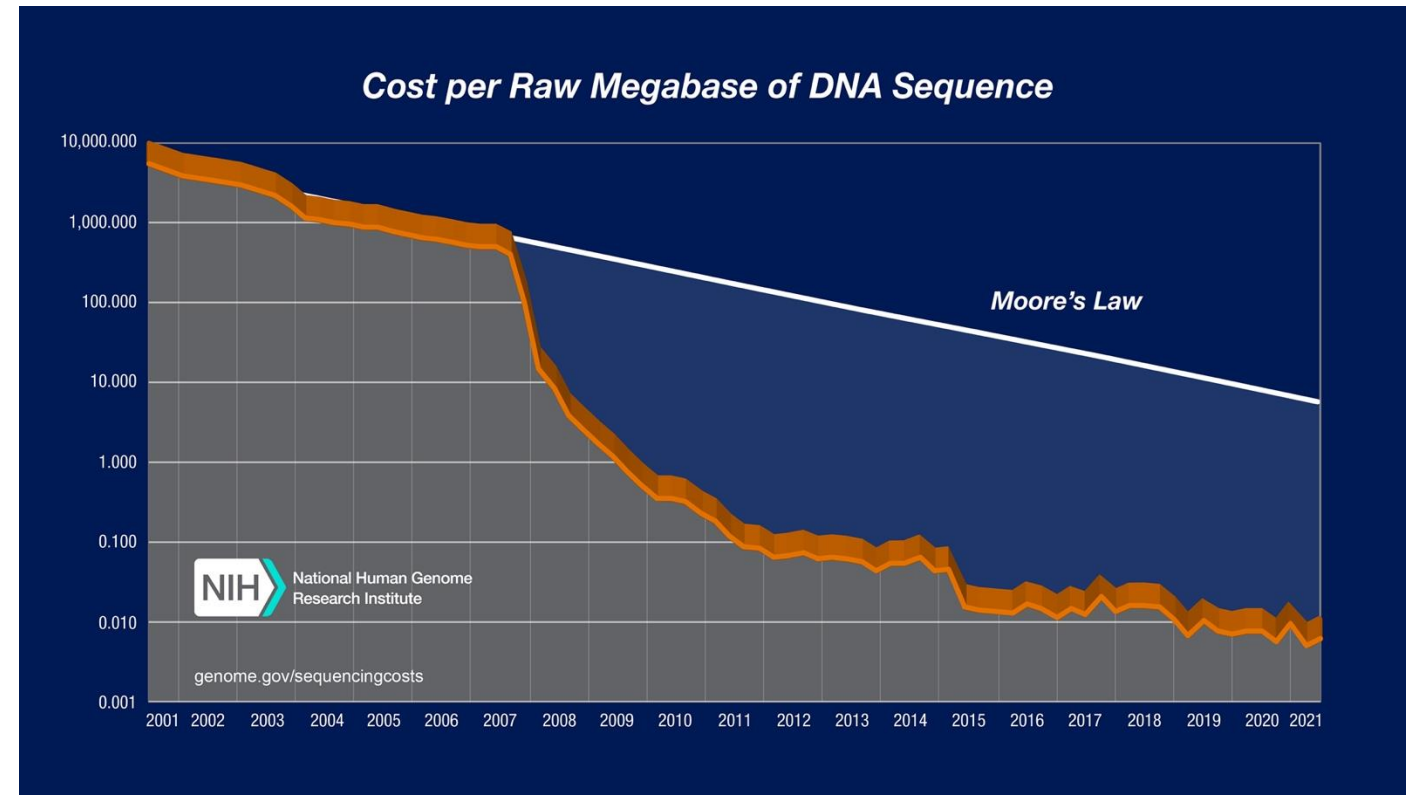
NovaSeq X Series

1.2 Gb	7.5 Gb	15 Gb	120 Gb	360 Gb	6 Tb	16 Tb
2 x 150 bp	2 x 150 bp	2 x 300 bp	2 x 300 bp (or more?)	2 x 150 bp (2 x 300 bp)	2 x 250 bp	2 x 150 bp
19 h	24 h	55 h	15.5 h	48 h	44 h	48 h
20k CHF	50k CHF	125k CHF	105k CHF	200k CHF		985k CHF
	4 h 1 x 100bp	Long fragments	Fast & flexible	Long fragments		
Test libraries	Few bacterial genomes	16S amplicon	Transcriptomes Eukaryotic genomes, 16S	Transcriptomes Eukaryotic genomes	Transcriptomes Eukaryotic genomes	Human genomes



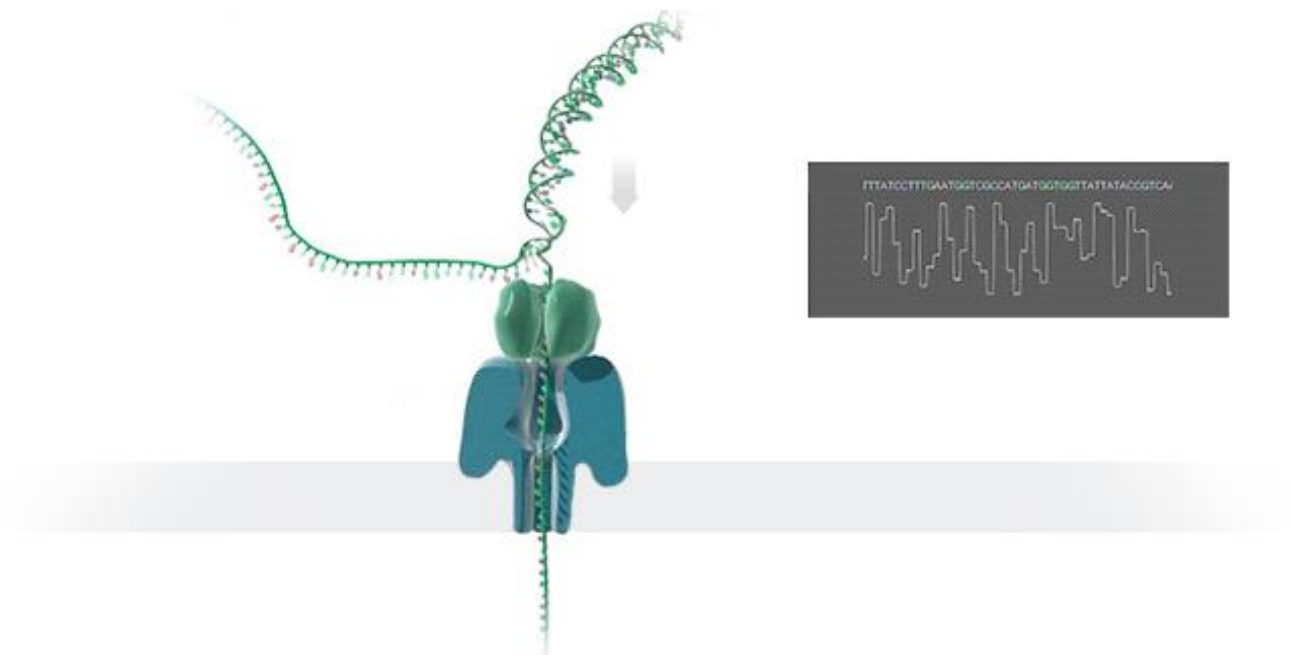
# Illumina sequencing cost

- Sequencing cost is constantly dropping
- Recently more competitors (again) (MGI, Nanopore, Roche!)
- 100\$ human genome announced in 2022



<https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data>

# Nanopore sequencing



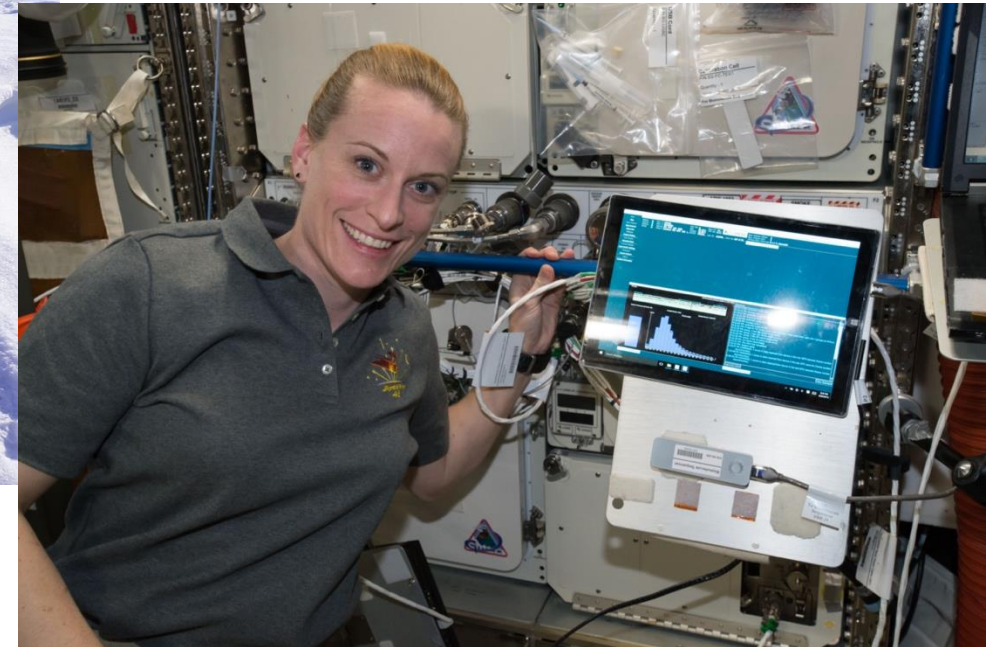
# Nanopore sequencing – sequencing everywhere



RAIN FORREST BRAZIL



CANADIAN HIGH ARCTIC



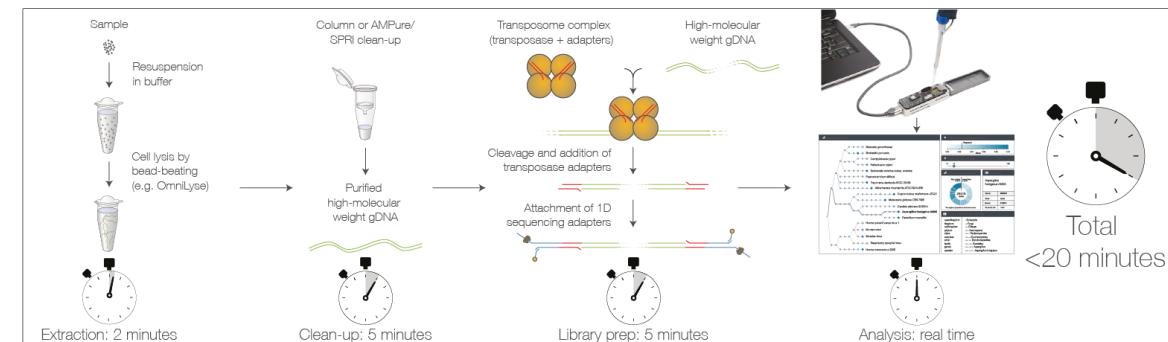
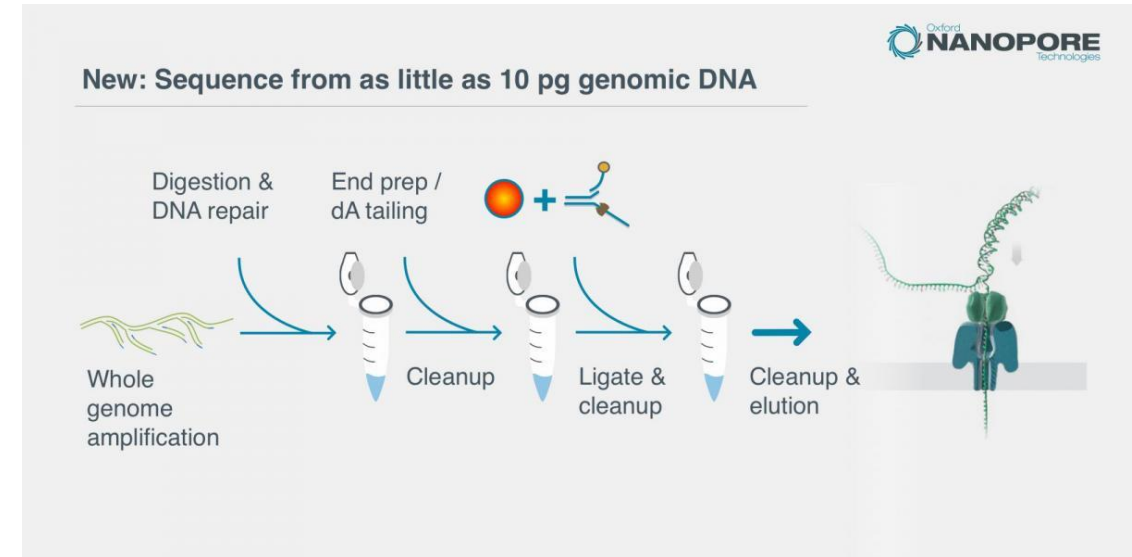
INTERNATIONAL SPACE STATION (ISS)

# Nanopore DNA extraction

- Long read sequencing requires good (long) input DNA
  - Check DNA size distribution on a gel
- Sequencing length mainly limited by library size
- Many extraction methods developed for PCR or Illumina sequencing

# Nanopore library prep

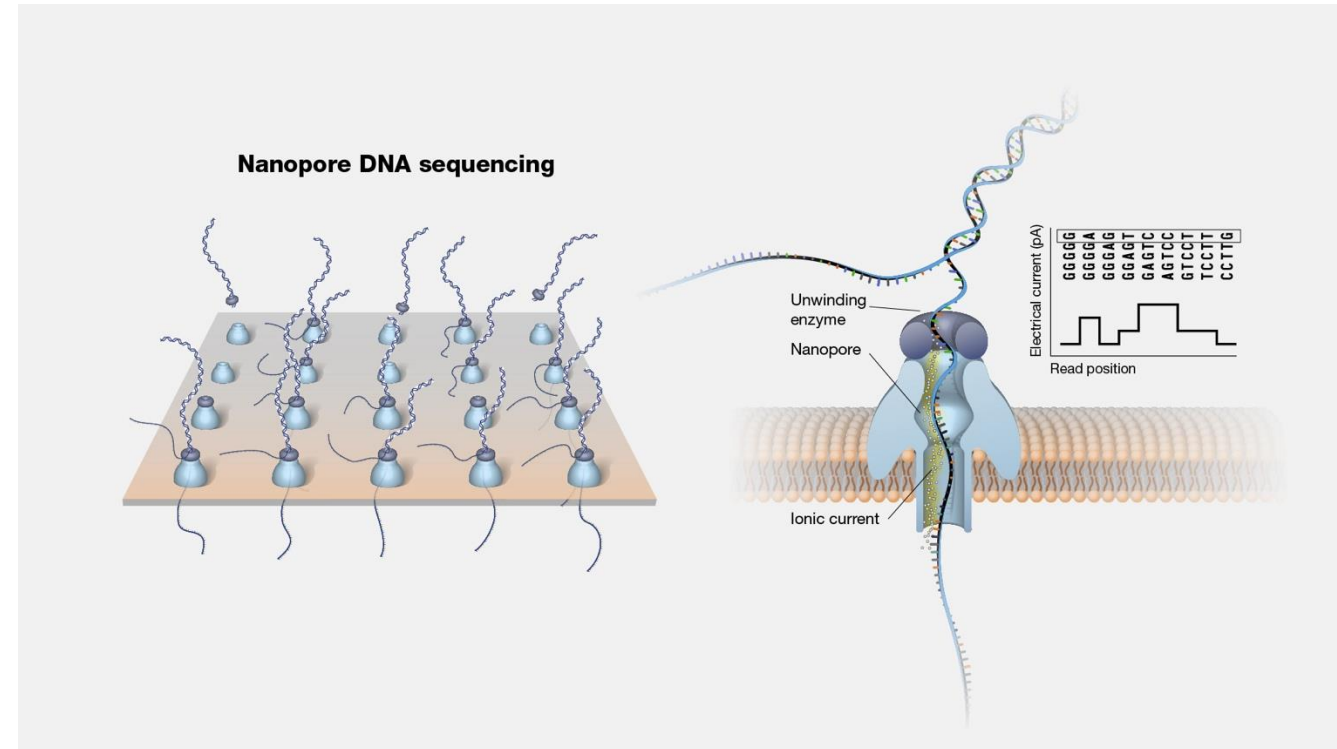
- Add adapter and motor protein to DNA fragments
- Currently 2 strategies
  - Ligation
    - Longer libraries
    - 4-5 h protocol
    - Good for de-novo sequencing
  - Tagmentation
    - Used in this course
    - Shorter libraries
    - Fast – 20 minutes
    - Good to sequence plasmids



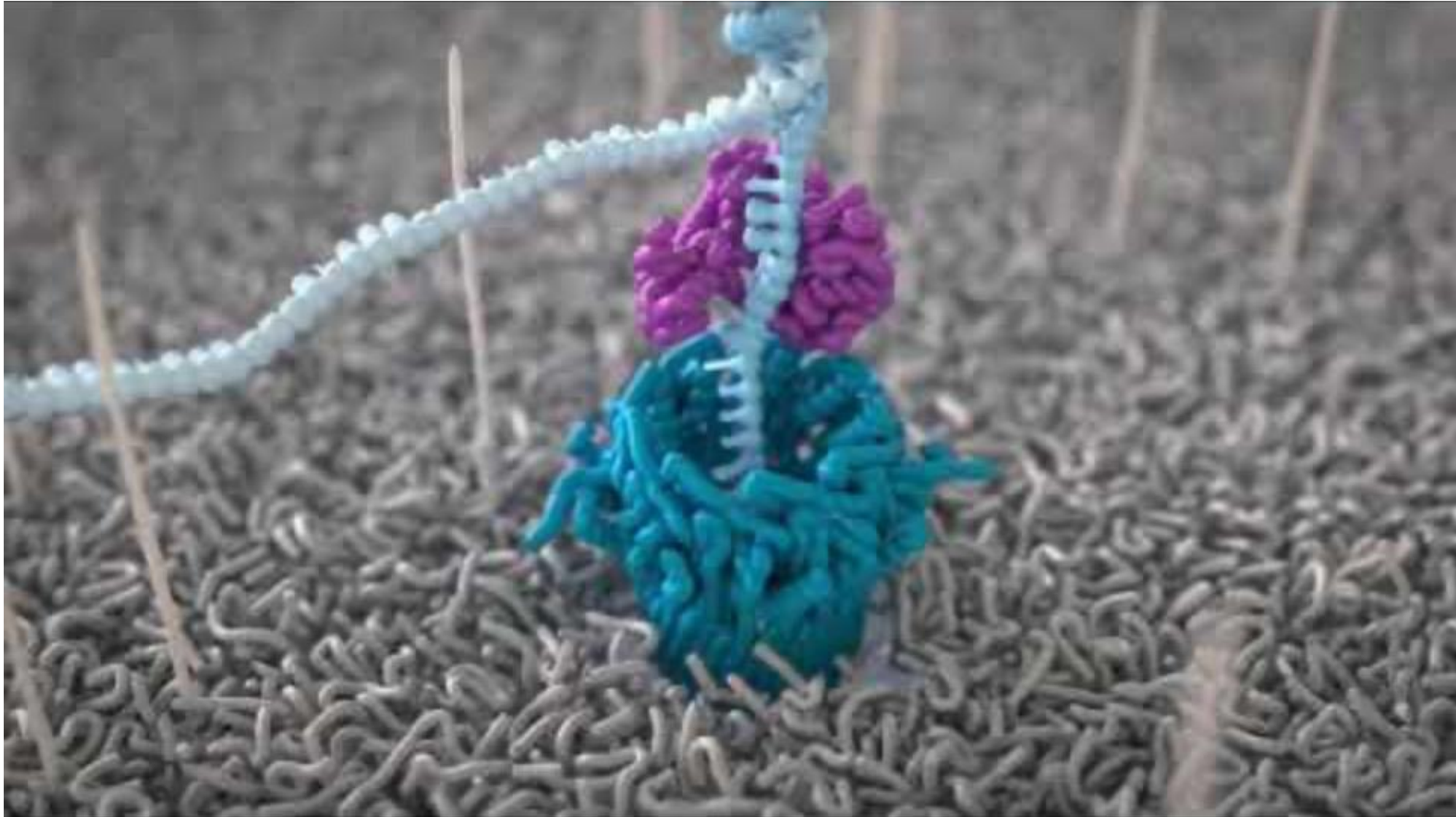


# Nanopore sequencing

- Use pores to “read” the DNA
- Pores embedded in membrane
- Apply tension to membrane to create a constant flow of ions through the pore which can be measured by electrodes
- Motor protein moves DNA through the pores
- While DNA passes through the pore, the flow of ions is changed depending on the base that is currently in most narrow site of the pore
- Even modifications of bases (e.g. methylation) can be measured



# Nanopore sequencing



## Nanopore sequencing - limitations

# Data quality!

Originally 5% error rate (1 in 20 bases wrongly called)

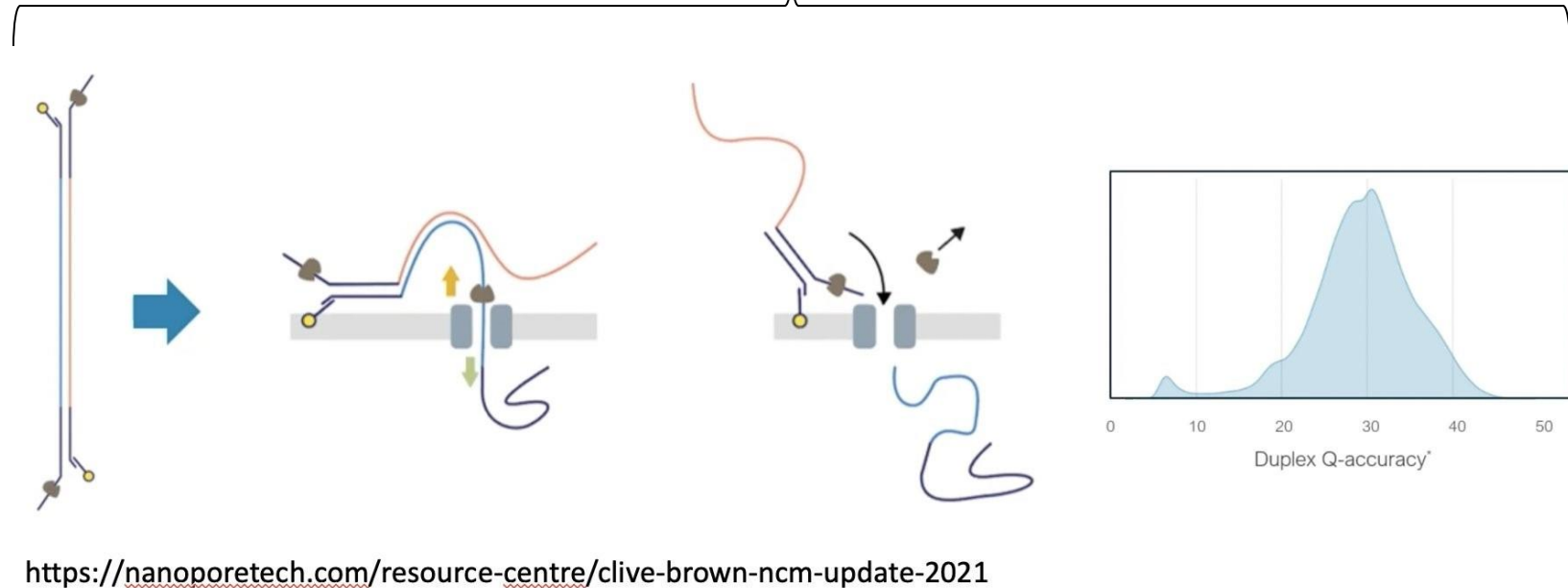
# Current flowcell version R10.4.1

based on base caller (fast / super accuracy / duplex)

# Less automation

## Less standardization

# Constant development





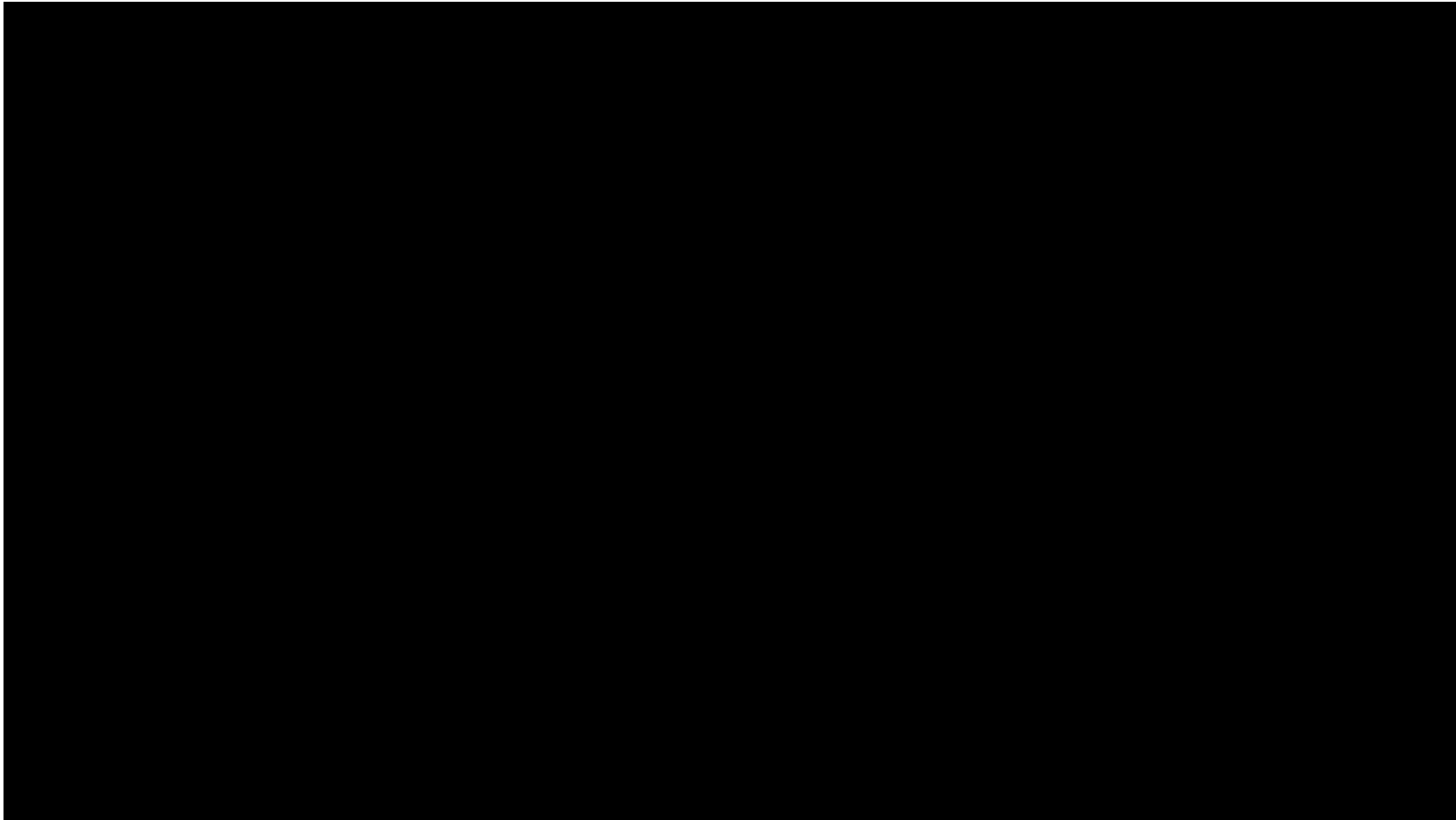
# Nanopore sequencing – adaptive sequencing

Select what you want to sequence

- Positive selection
- Negative selection

Max around 15-fold  
enrichment

Only works on fragments  
> 400bp



<https://doi.org/10.1186/s13059-021-02582-x>

