Sequencing Technology

Sequencing Technology

- Sanger
- NGS
  - Illumina
- 3rd Generation
  - Nanopore
  - PacBio

What is a read? What is a library?

- Definition of “read”: A single sequence from one fragment in the sequencing library (one cluster, bead, *etc*.)
- If generating paired reads, then 2 reads derived from each fragment in the library
- Definition of “library”: A collection of DNA fragments that have been prepared to be sequenced
- Definition of “coverage”: The number of reads spanning a particular base in the genome

Where does library come from?

› Cell. 1978 Oct;15(2):687-701. doi: 10.1016/0092-8674(78)90036-3.

The isolation of structural genes from libraries of eucaryotic DNA

T Maniatis, R C Hardison, E Lacy, J Lauer, C O'Connell, D Quon, G K Sim, A Efstratiadis

PMID: 719759 DOI: 10.1016/0092-8674(78)90036-3

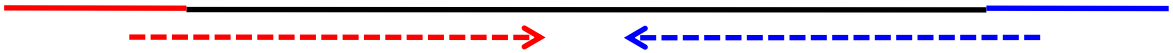
Abstract

We present a procedure for eucaryotic structural gene isolation which involves the construction and screening of cloned libraries of genomic DNA. Large random DNA fragments are joined to phage lambda vectors by using synthetic DNA linkers. The recombinant molecules are packaged into viable phage particles in vitro and amplified to establish a permanent library. We isolated structural genes together with their associated sequences from three libraries constructed from Drosophila, silkmoth and rabbit genomic DNA. In particular, we obtained a large number of phage recombinants bearing the chorion gene sequence from the silkmoth library and several independent clones of beta-globin genes from the rabbit library. Restriction mapping and hybridization studies reveal the presence of closely linked beta-globin genes.

Types of reads

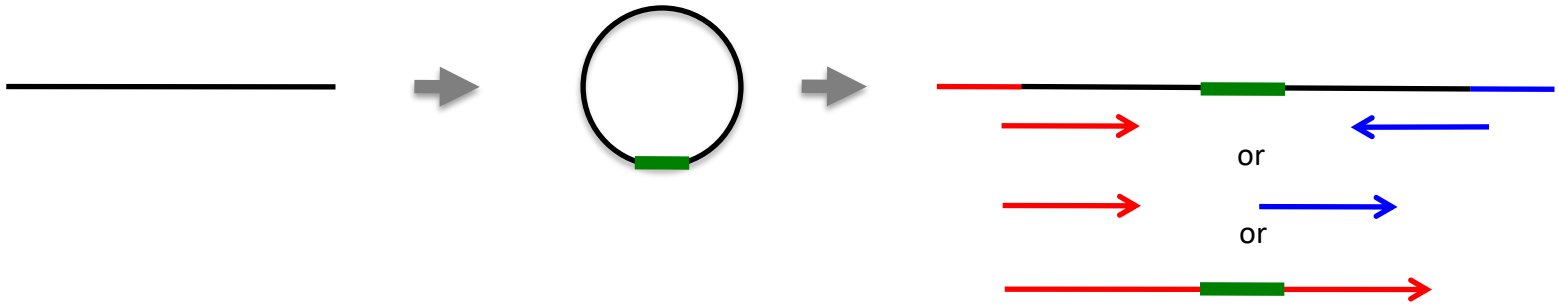
- Fragment reads (come from fragment libraries)
  - Single read in one direction from a fragment

- Paired end reads (come from fragment libraries)
  - Two reads from opposite ends of the same fragment
  - Reads point towards each other



Types of reads

- Mate Pair Reads (come from Jumping Libraries)
  - Long fragment of DNA is circularized
  - Junction is captured (e.g., by biotinylated adapter)
  - Remainder is cleaved (many methods)
  - Ends are sequenced
  - Read orientations depend on the exact method



Types of reads

- Linked reads (several methods)
  - Long (10-100kb) DNA isolated
  - DNA is labeled (barcode, mutation, etc.)
  - Read pairs generated from specific long fragments
  - Sequence normal read pairs
  - Can use reads normally for alignment/assembly
  - Can also group reads by haplotype of origin
  - In some methods, can assemble the long fragment

Course Outline

- Terminology
- History of Sequencing
- Current Sequencing Technologies
- Prepping DNA for Sequencing
- General Study Design Considerations
- Considerations for Specific Sequencing Assays

Sanger Sequencing (1977)

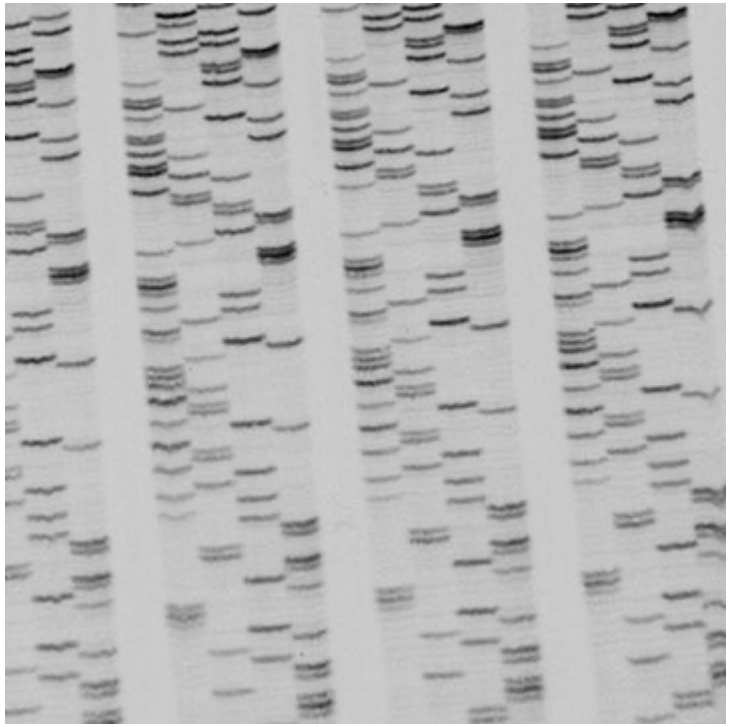
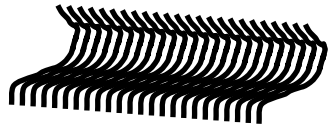
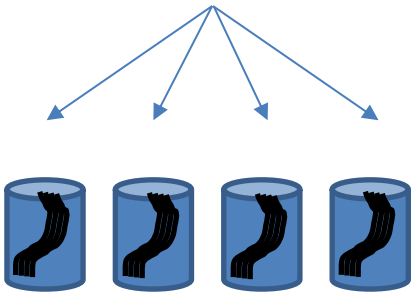


Image credit: https://unlockinglifescode.org/timeline/11

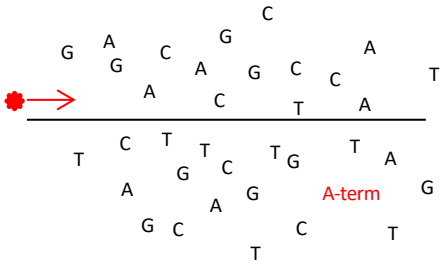
How Sanger Sequencing Works



Single-stranded DNA



Split into four reactions



Add labeled primer and nucleotides, one of which is sometimes terminated

Allow the strands to extend

A	T	A	T	A	T
G	C	G	C	G	C
C	G	C	G	C	G
T	A	T	A	T	A
G	C	G	+	G	C
A	T	A		A	T
C	G	C		C	G
T	A	T		T	A
A	T	A		A	+
G	C	G		G	
A	T	A		A	
T	A	T		T	
C	+	C		C	
G		G		G	

Automation of Sanger (1986)

- Replacement of radioactive label with laser-excitable fluorescent dyes
- Allowed all four nucleotides to be run in a single lane of a gel
- Base sequence could be read off with a camera as the fluorescing strands passed a certain point near the end of the gel
- Signal from each lane could be converted to a nucleotide sequence by a computational process called base calling

Fluorescent slab gel image

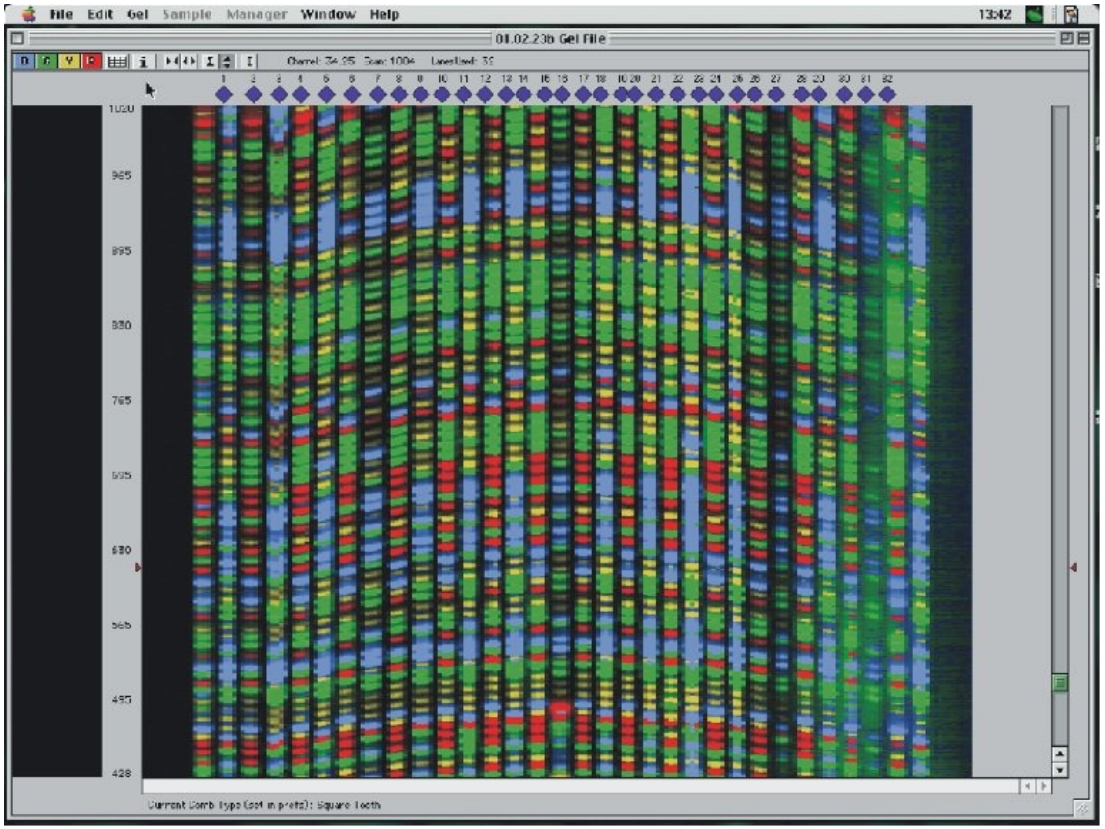


Image credit: http://www.mun.ca/biology/scarr/How_it_works.htm

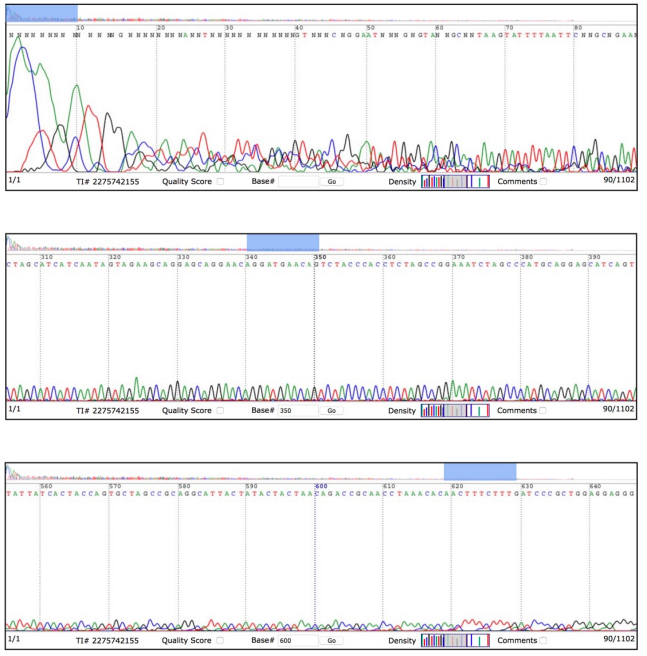
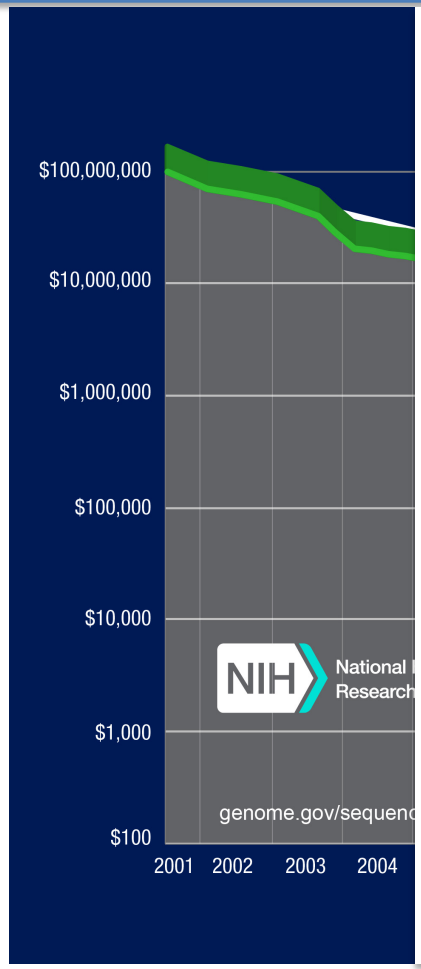


Image credit: NCBI Trace Archive

Capillary Gel Sequencing (1998)

- Replacement of 2D slab gels with an array of enclosed capillaries
- Cleaner signal processing
- Fully automated loading
- Faster run times

Cost Curve for Sanger Gel Sequencing



Other Early Technologies

- Maxam-Gilbert sequencing
- LI-COR
- Molecular Dynamics MegaBACE
- Pyrosequencing
- Mass spectrometry

Statistics of Apex Capillary Sequencing

- 96 reads per run
- 700-1000 bases per read
- Very high base accuracy over most of the read length (<1/100,000)
- ~$1 per read
- ~1 run per hour
- ~2 million bases per machine per day
- Large sequencing centers could do a single mammalian genome to assembly depth in about 2-3 months

Limits on Sanger Gel Performance

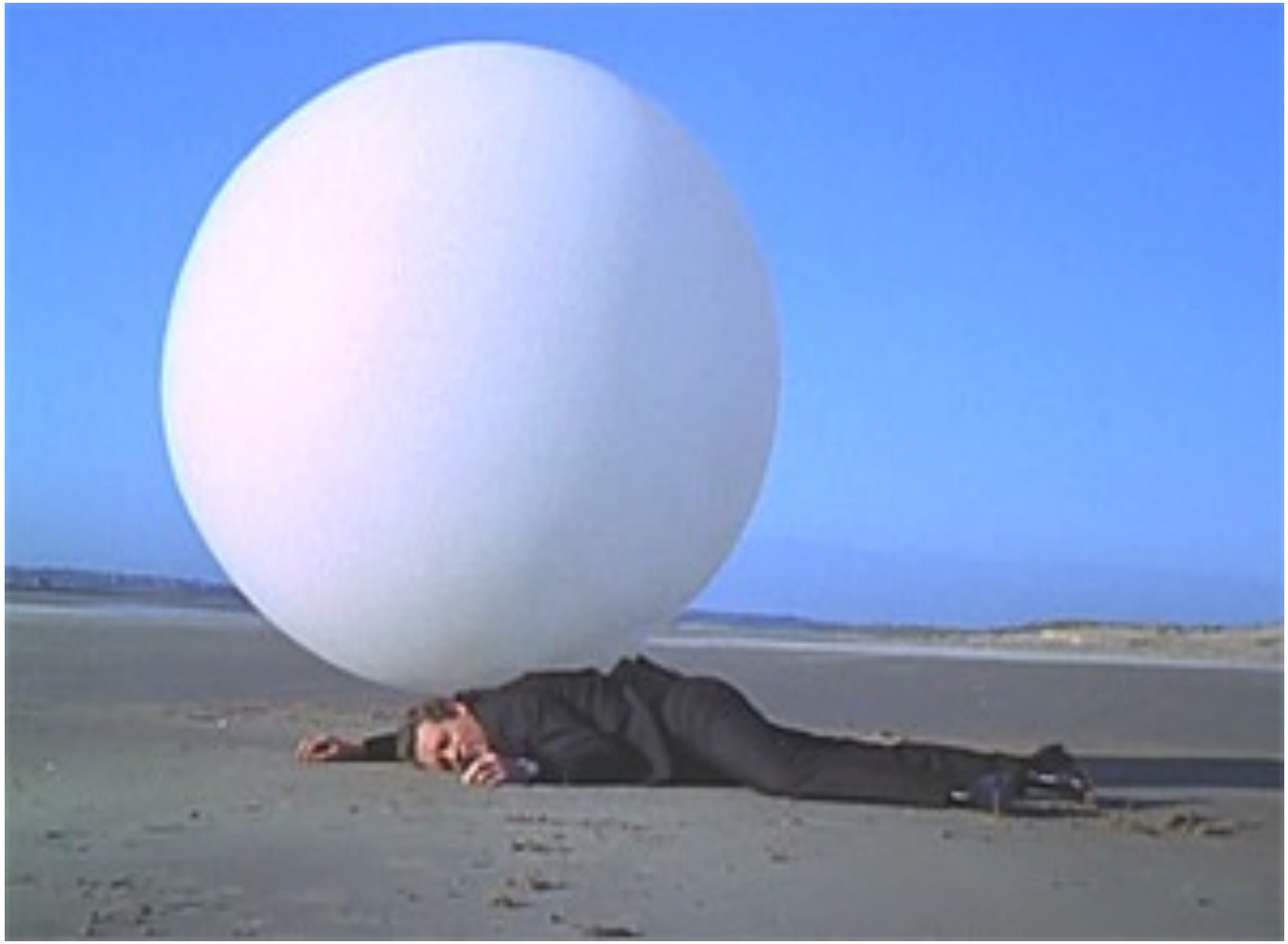
- Tradeoff between loss of signal due to diffusion and loss of resolution at high voltage or short gel length
- Longer gels/capillaries or lower voltages provide better separation of short to medium fragments
- Longer gel run times mean more diffusion of fragments in the gel, which blurs adjacent signal and spreads peaks
- The maximal high quality read length is around 1000 bp

454 Sequencing

- First “Next Generation” or massively parallel technique
- Based on pyrosequencing
- Emulsion PCR DNA prep on beads
- Beads loaded into a picotiter plate for sequencing

454 Sequencer

Rover



Emulsion PCR

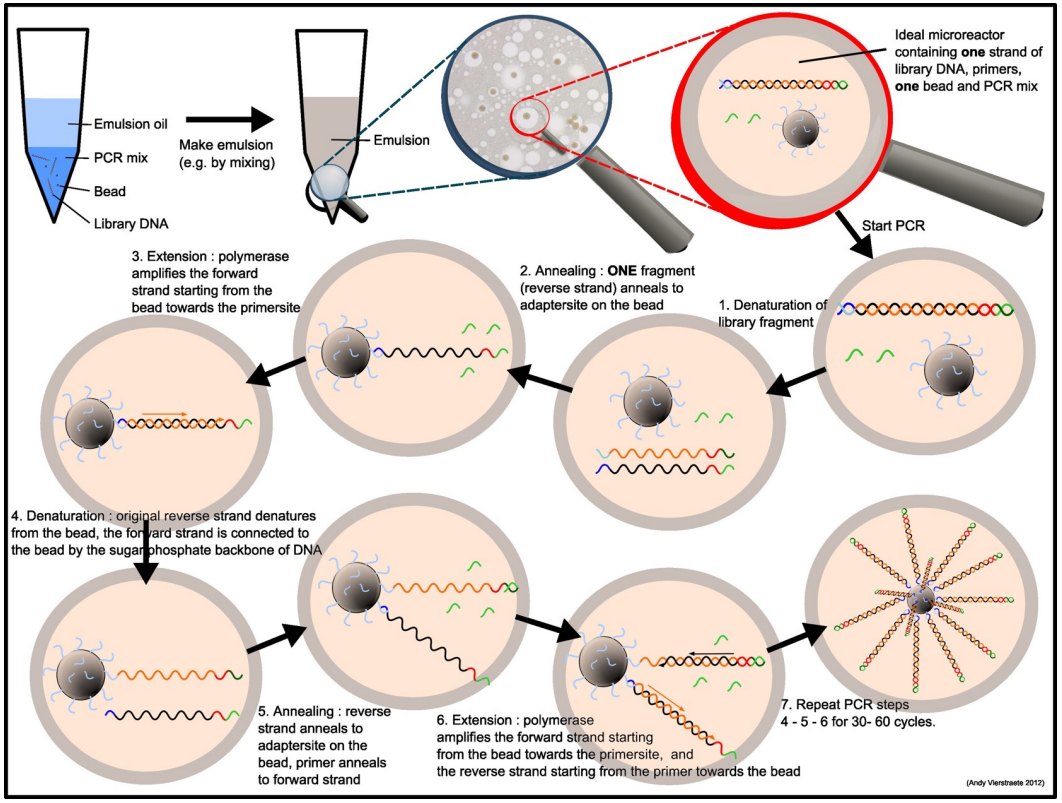
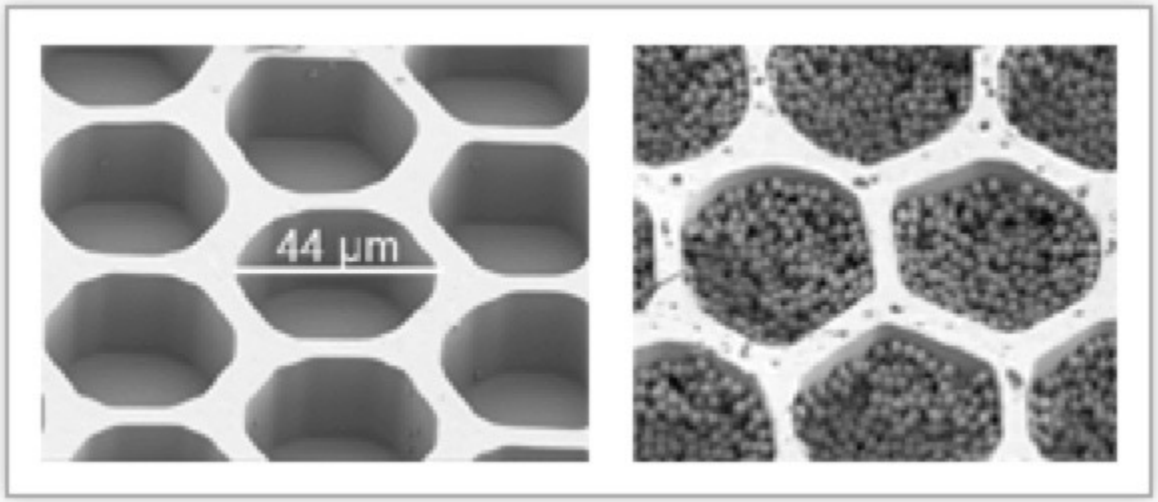


Image credit:
https://users.ugent.be/~avierstr/nextgen/nextgen.html

454 Picotiter Plate



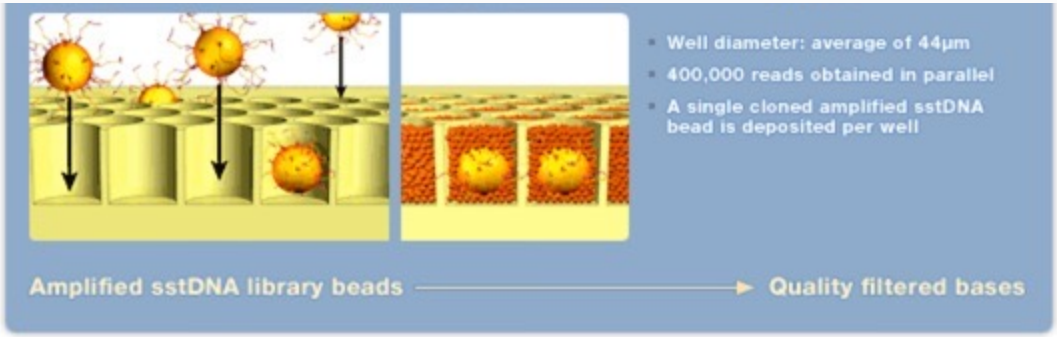
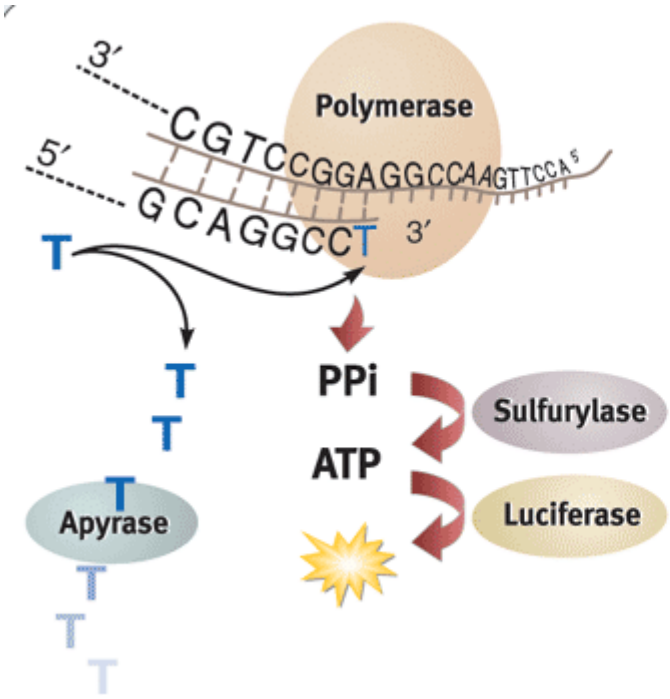
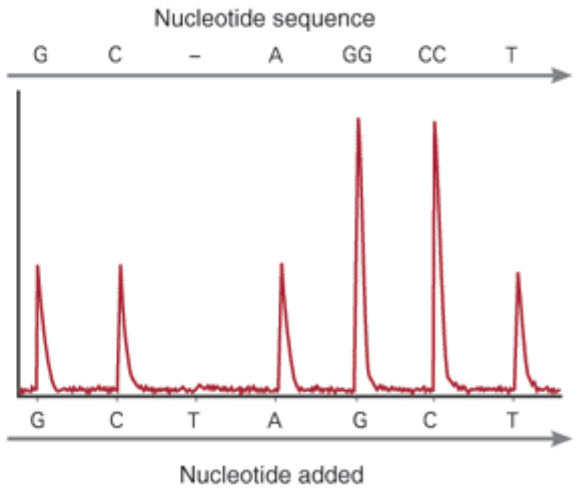


Image credit: http://www.mbio.ncsu.edu/MB451/lectureModules/molecularEcology/molecularSurveys/454/454.html

Pyrosequencing





454 Output: the Flowgram

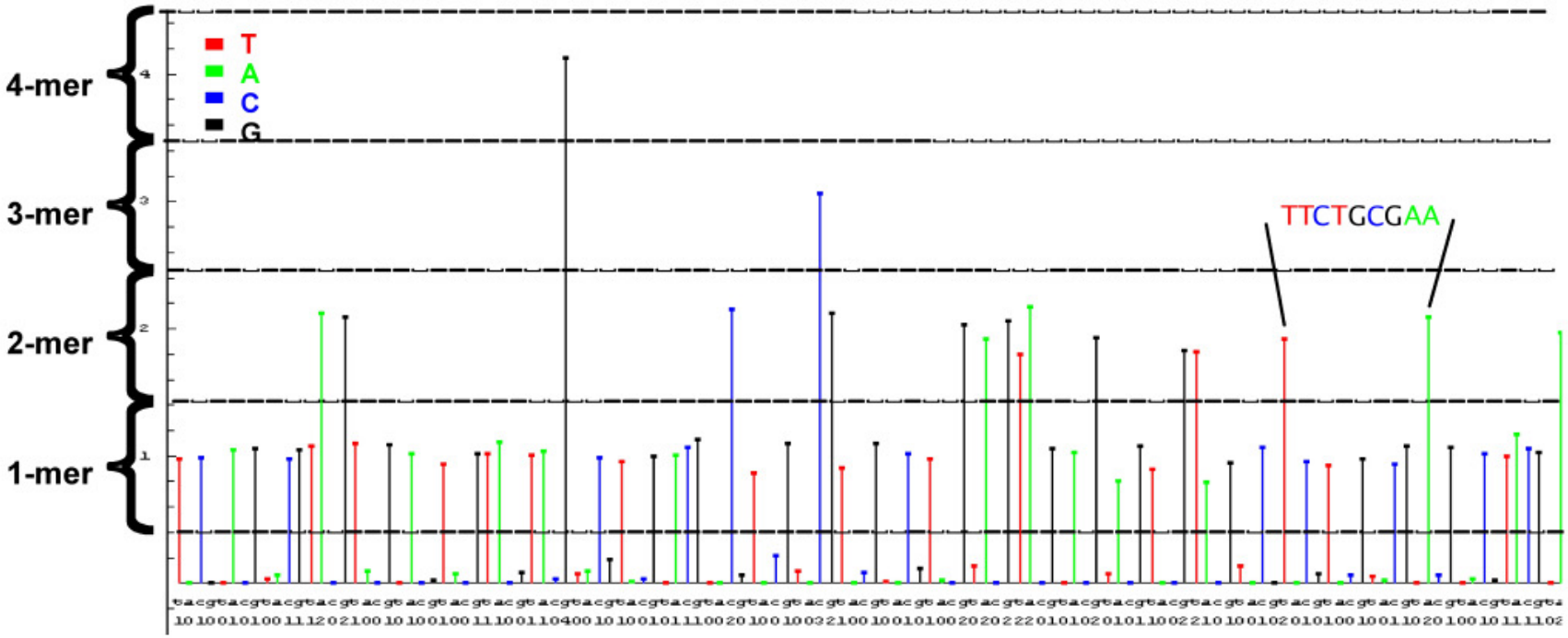
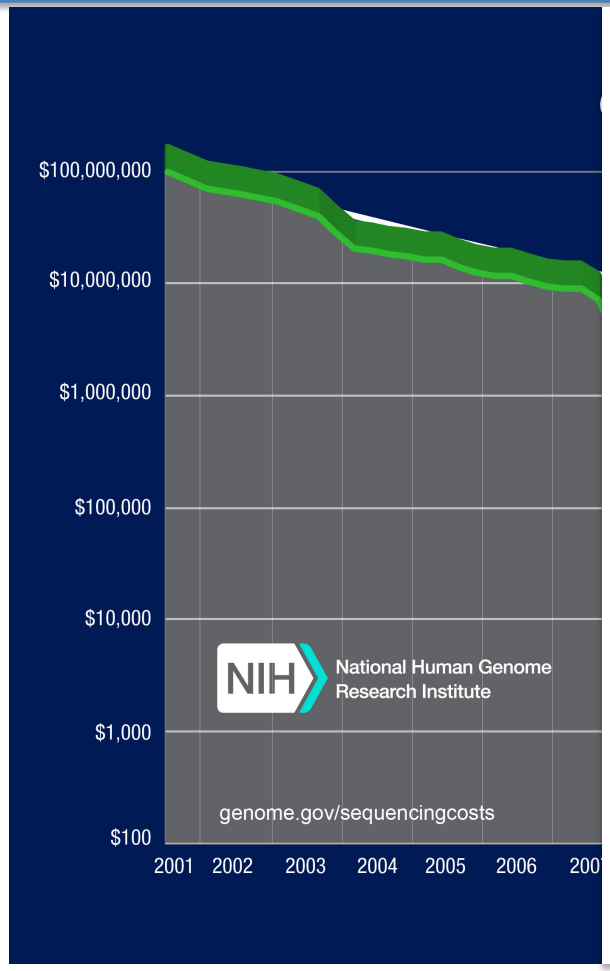


Image credit: https://contig.wordpress.com/2010/10/28/newbler-input-i-the-sff-file/

Cost Curve for 454 Sequencing



Statistics of Apex 454 Sequencing

- 1 million reads per run
- 400-500 bases per read (750?)
- High error rate (~1.5%), very motif dependent (homopolymers)
- Cost several thousand dollars per run
- ~10 hours per run
- ~1 billion bases per machine per day

Limits on 454 Performance

- Failure to accurately read homopolymers or sequences near homopolymers; physical limit on ability to read the full incorporation
- Loss of signal over time
- Signal/noise degradation due to asynchrony of extending strands
- Length of fragment that could be amplified on bead in emPCR
- No ability to sequence the second strand of DNA or do non-contiguous reads

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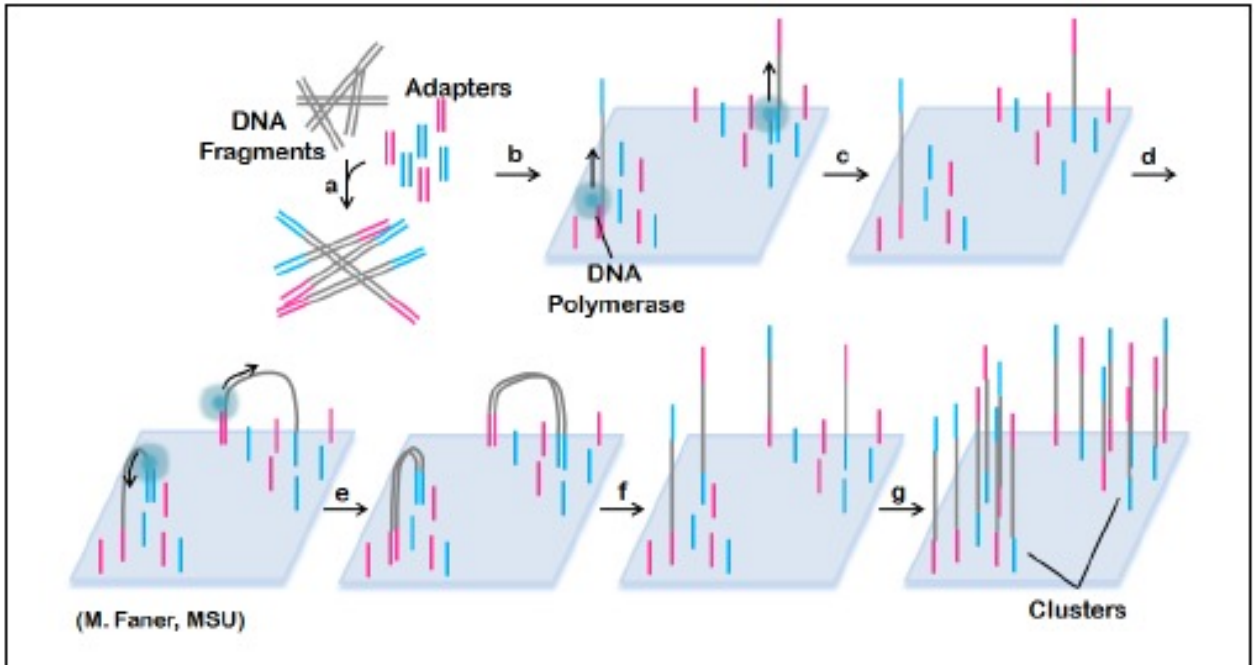
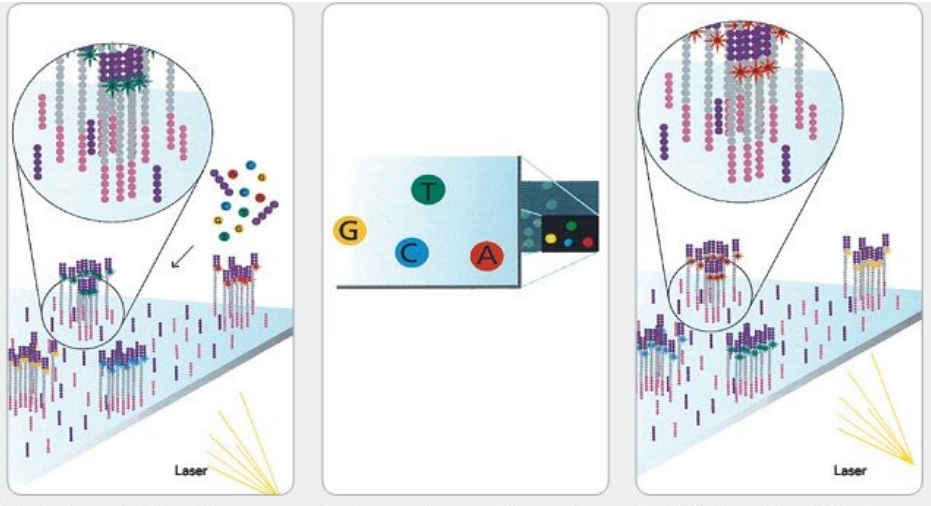
Illumina (Solexa) Sequencing

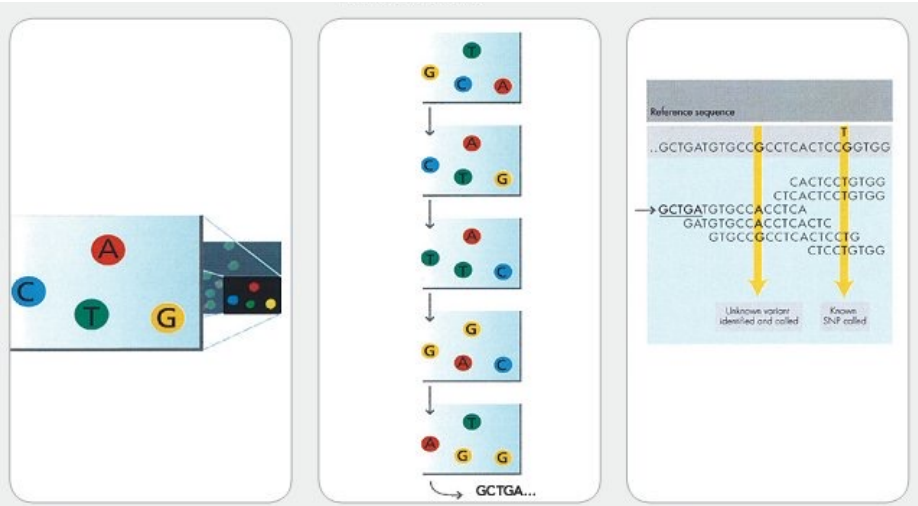
- Sequencing of DNA strands amplified *in situ* on a glass slide
- Use reversible terminators to sequence one base at a time

Bridge Amplification



Reversible Terminator Sequencing





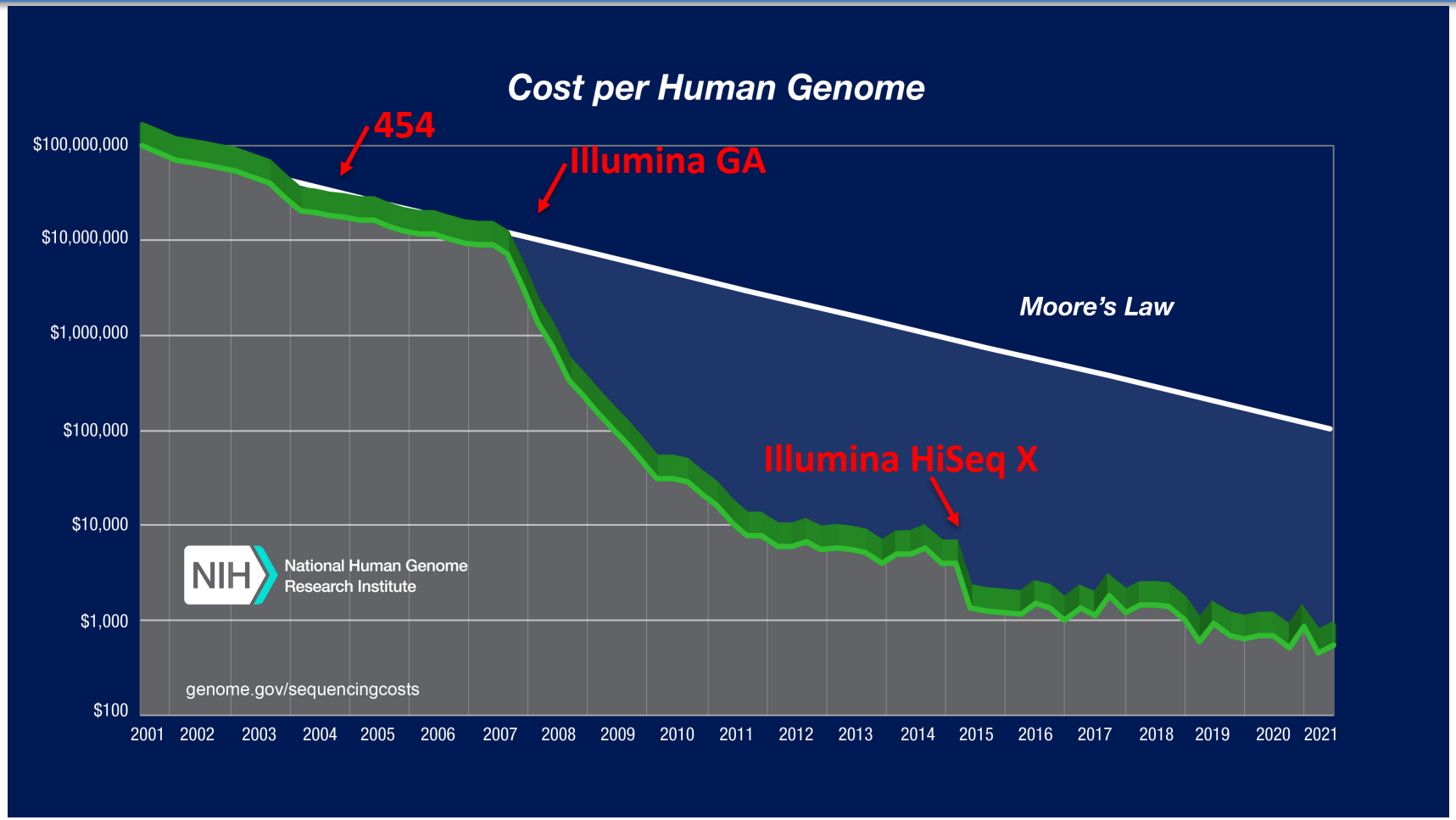
Statistics of Apex (so far) Illumina

- 10 billion (25B) read pairs per run
- 300 bases per read pair
- Relatively low error (<1%), some context dependency
- Cost ~$30,000 per run (for NovaSeq on largest flow cell size)
- ~2-3 days per run
- ~1 trillion (4T) bases per machine per day

Limits on Illumina Performance

- Loss of signal over time
- Signal/noise degradation due to asynchrony of extending strands
- Viability of sequencing reagents over the course of a run
- Length of fragment that could be amplified into clusters on the slide

Cost Curve for Illumina Sequencing

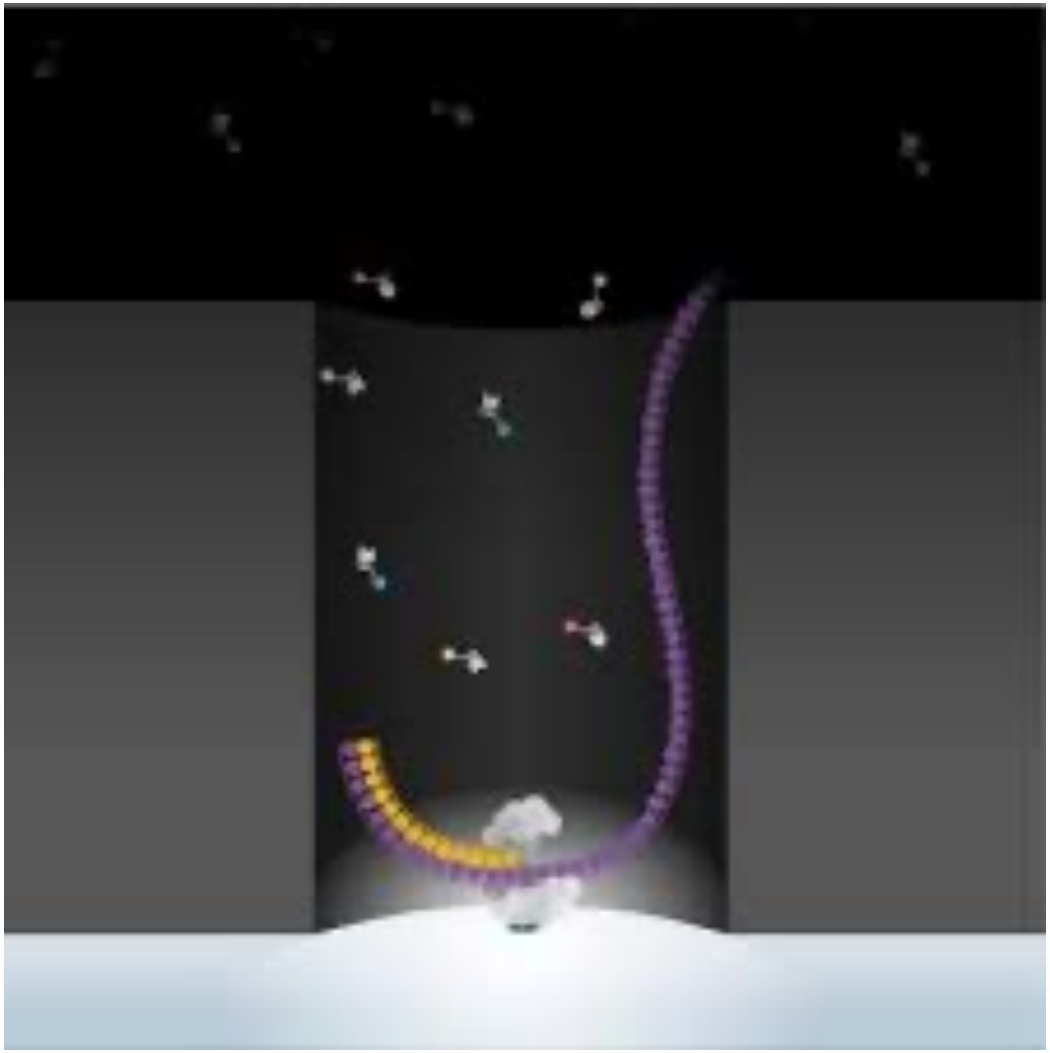


Sequencing Technology

Single Molecule (Third Generation) Methods

- Pacific Biosciences SMRT (Single Molecule Real Time) sequencing
  - Uses a polymerase anchored in a zero-mode waveguide
  - Images all wells at the same time in real time with digital video
  - Interprets bases by the light signal visible at incorporation
  - Very large instrument
- Oxford Nanopore Technologies
  - Uses protein nanopores in synthetic membrane to thread DNA through
  - Current sensors measure change in fluid current flow through pore to differentiate groups of multiple bases as they occupy the pore
  - Very small instrument (attaches to compute like a USB drive)

PacBio SMRT

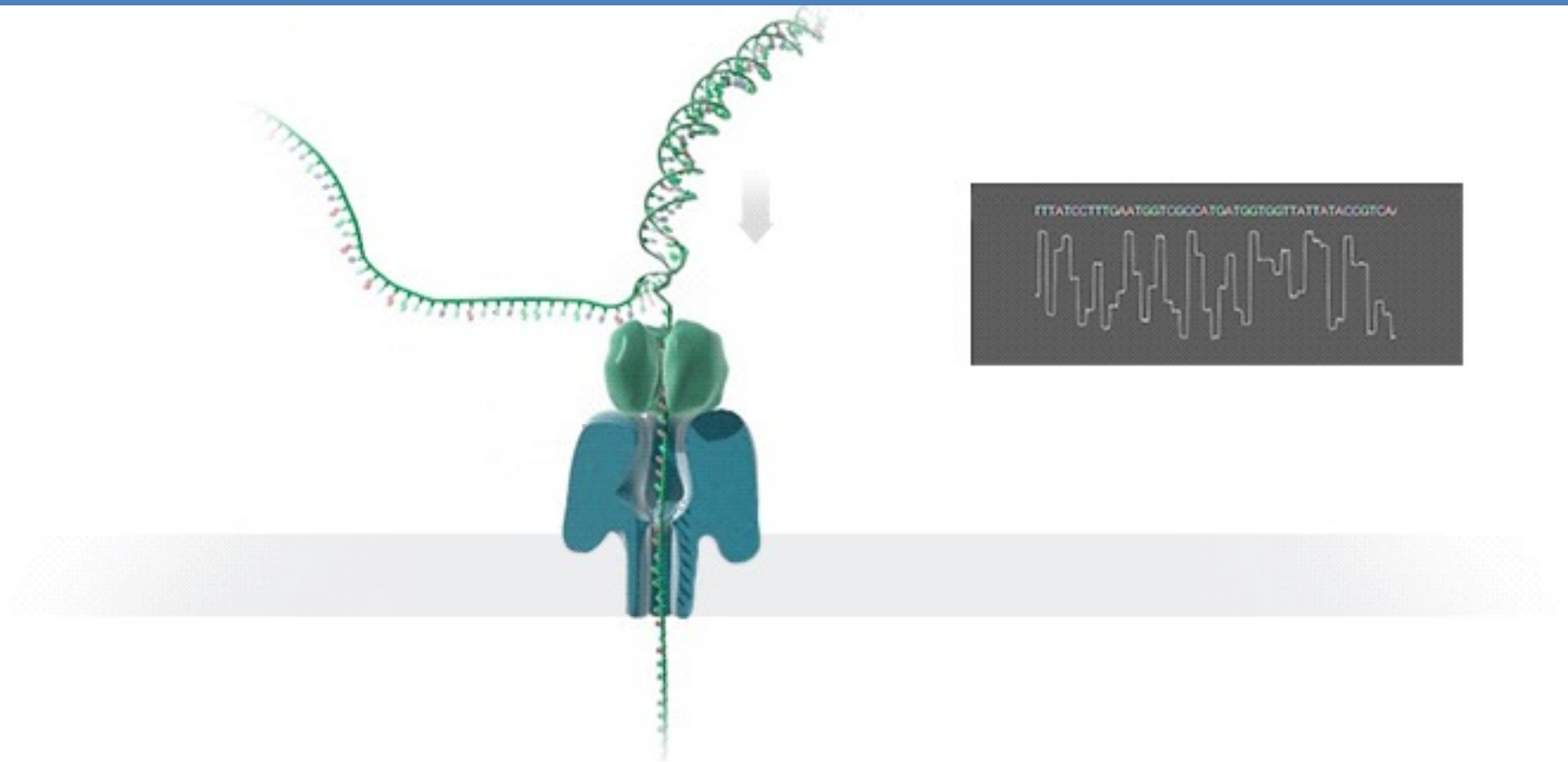


https://www.youtube.com/watch?v=WMZmG00uhwU&feature=youtu.be

PacBio “Hi-Fi” Reads

- Sized libraries, 10-20kb long
- Generate circular consensus on these
- Can read each linear piece 7-10 times
- Generates very high accuracy long reads
- Can assemble easily and even distinguish highly similar repeats

Oxford Nanopore



Current State of Oxford Nanopore

- Very long reads (100,000+, >1 Mbp)
- High error rates (10%+), non random
- Can read both strands to improve accuracy (<1% error, but not all reads)
- Low throughput
- Run times variable (few hours to 3+ days)
- Higher costs per base than Illumina (2-fold)
- Reads end when pores die

Limitations of Single Molecule Techniques

- Single molecule means no redundancy, so error rates will be high unless the same molecule can be read more than once
- Methods of detection are hard to massively parallelize
- Currently, these techniques actually require large amounts of DNA
- Getting very long reads requires very high quality input DNA
- Data processing and base calling is more expensive

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- Terminology
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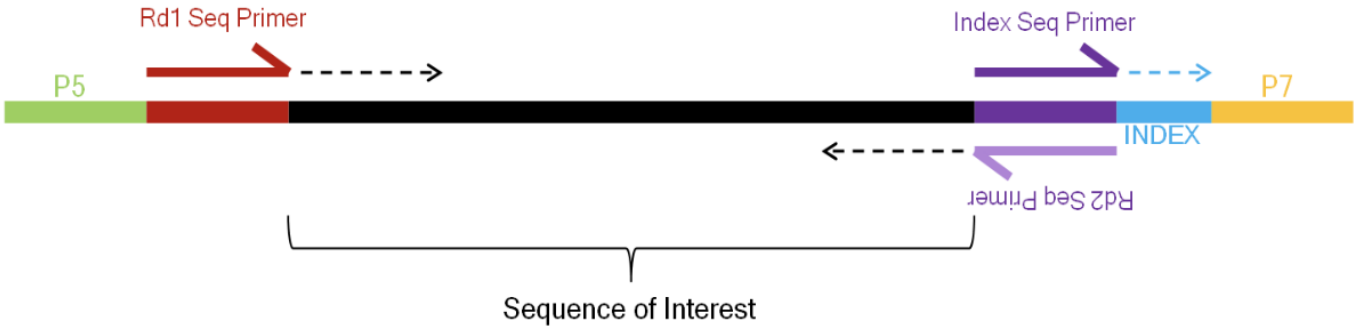
Prepping DNA for Sequencing

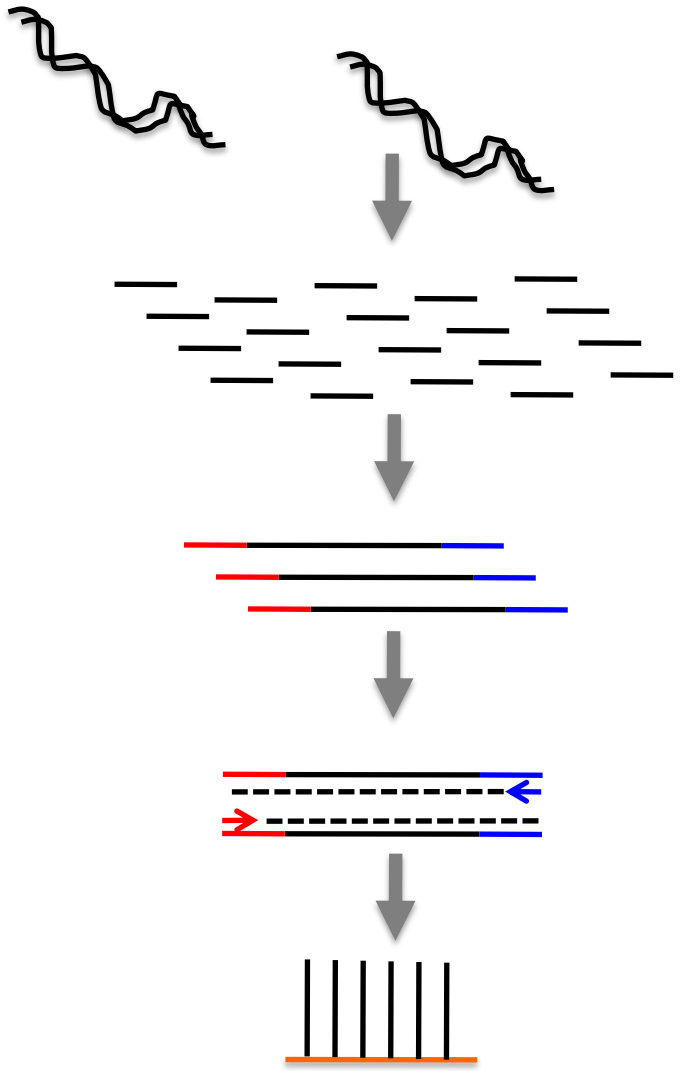
- Steps of library construction and sequencing
- Making Fragment libraries (to generate fragment or paired end reads)
- Making Jumping libraries (to generate mate pair reads)
- Pooling with or without barcoding
- Possible artifacts of library construction
  - PCR-based artifacts
  - Sequencing of primers, adapters, and tags

Steps of Library Construction

- Add adapters containing:
  - Barcodes (for multiplexing)
  - Sequencing primers
  - Amplification primers
  - Sequence for substrate attachment
- Amplify fragments by universal PCR
- Optionally pool barcoded libraries

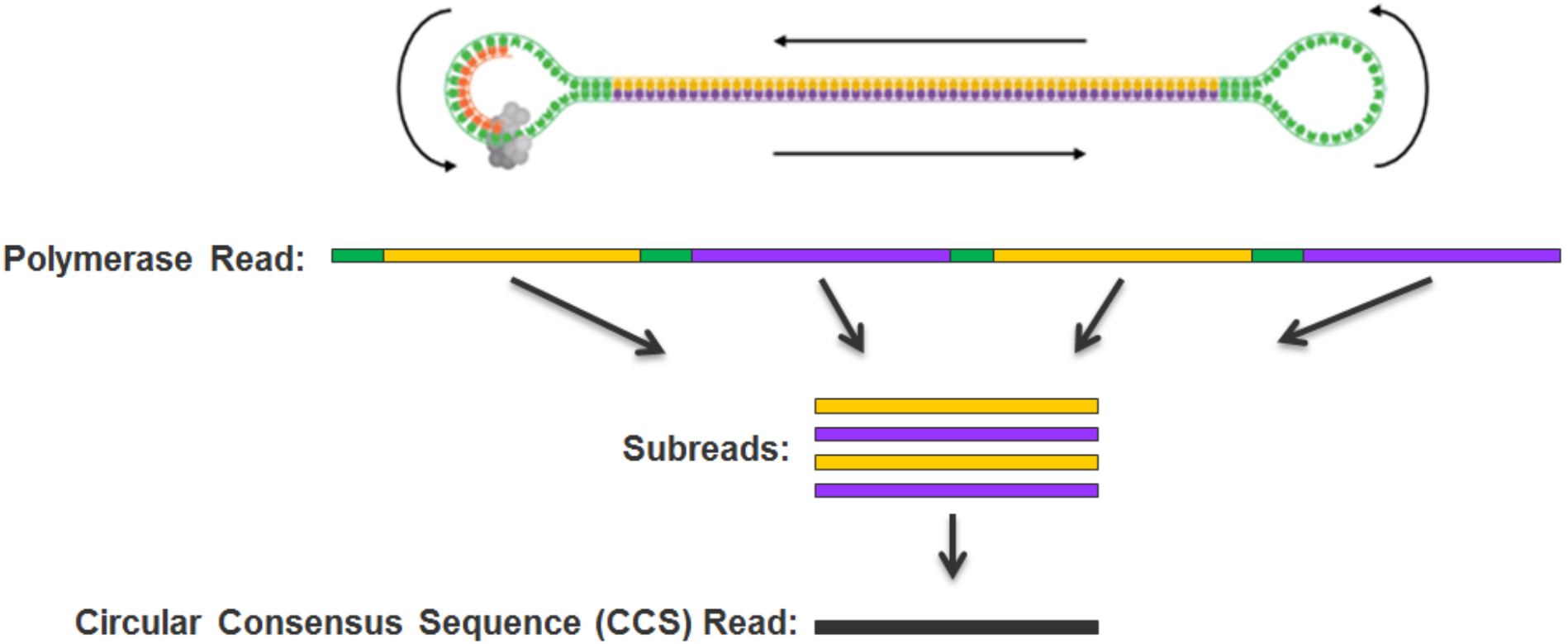


Steps of Fragment Library Construction



- Extract DNA
- Fragment and possibly size select (300-600 bp)
- Add adapters
- Amplify
- Select single molecules
- Amplify in clusters/beads

Steps of PacBio Library Construction



- Extract DNA, fragment and size select (25-50+ kb)
- Add hairpin adapters to both ends

Pooling with barcoding

- Unique DNA tags identify samples
- Allows multiple distinct samples on one run/lane
- Advantages:
  - Reduced cost of sequencing for small samples
  - Analysis is identical to unpooled data
- Disadvantages:
  - Some small throughput loss due to barcode fails
  - Data mis-assignment from bad barcode reads
  - Increased per sample cost for library construction

Pooling without barcoding

- Mix input DNA without identification
- No way to definitively separate data from different samples afterwards
- Advantages:
  - Single library prep for a number of samples
  - No yield lost to barcodes
- Disadvantages:
  - Loss of all individual associations
    - Loss of ability to use replicates!
  - No check on accuracy of pooling

PCR-based artifacts

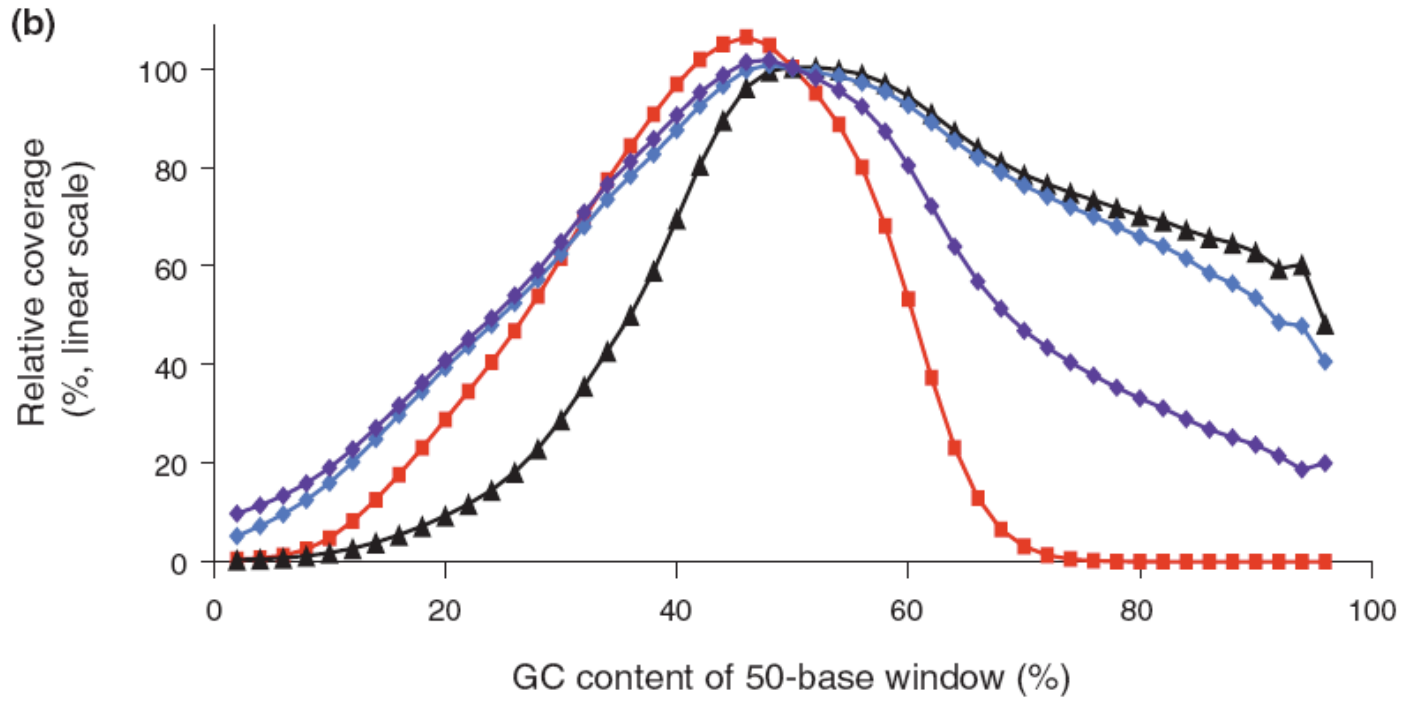
- Most libraries are PCR amplified during construction
- After library construction, single molecules are isolated and then amplified again for sequencing
- Errors from library construction PCR will not be detectable as sequencing errors
- Regions with secondary structure or extreme GC content:
  - Will amplify poorly and be underrepresented
  - May form small or weak clusters with poor sequence quality
- PCR may form chimeric sequences (especially in targeted designs)
- PCR amplification may result in duplicated sequences

PCR Errors: How Much PCR?

- You may be doing more PCR than you think
- Initial amplification of sample
- Targeting PCR
- Library amplification
- 100 rounds of PCR is equivalent to a 2 order of magnitude drop in polymerase accuracy

PCR-based artifacts: PCR bias

- Most PCR protocols work best for ~50% GC
- Extreme GC sequences are underrepresented



Red = standard PCR protocol
Other colors = modified PCR protocols

From Aird et al., Genome Biology *(2011)*

PCR-Free Libraries

- No PCR amplification in library construction
  - Not the same as no PCR, depending on other steps
- More uniform coverage by GC
- Fewer regions of 0 coverage
- Still some bias as cluster formation is PCR-like
- Requires more DNA (1-2 µg *vs*. 100-200 ng)

Considerations before starting a sequencing experiment

- What is the question you want to answer?
- How do you decide how much data to generate to answer your question?
  - Sensitivity (e.g. number of False Negatives)
  - Specificity (e.g. number of False Positives)
  - Cost
- Which factors influence the amount of data you generate?

What is the question you want to answer?

- What scientific result do you want?
- Is there an hypothesis you want to test?
  - Early sequencing was “hypothesis free” (i.e. the genome was the goal)
  - Now, it is affordable to sequence for a specific aim (i.e. What sequence do you need for that aim?)
- Understanding this shapes many decisions in designing the experiment

Why choose one type of read?

- Fragments
  - Fastest runs (one read per fragment), least cost
  - Some technologies only make one read
- Paired reads
  - More data per fragment
  - Help with assembly and alignment
  - Same library steps as fragments, but yields more data

Why choose one type of read?

- Long reads (PacBio or Oxford Nanopore)
  - Advantages over short reads:
    - Can phase variants over long distances
    - Much better structural variant calling
    - Much easier to get contiguous assemblies
    - Can resolve full length transcripts for RNA-Seq
    - Can get methylation information from reads
  - Drawbacks
    - More expensive for the same coverage
    - High error rate (except PacBio HiFi, but much more expensive)
    - Requires high molecular weight DNA, and a lot of it
    - Fewer reads (but can concatenate)

Number of reads

- How much data do you need to generate to answer your question?
- This depends on the level of completeness & accuracy you want
- You have to decide before beginning the experiment what level of completeness & accuracy you want, and this determines how much data to generate
- Analogy: Trying a protocol in the lab that requires 1ug of DNA with 0.1ug may end up working, but it may not

Complexity of library

- Definition of “complexity”: the number of distinct fragments in the library
- After amplification, you may have many copies of the same initial fragment (which does not increase complexity)
- For most experiments, sequencing the same fragment multiple times is not useful and may be detrimental to your analysis

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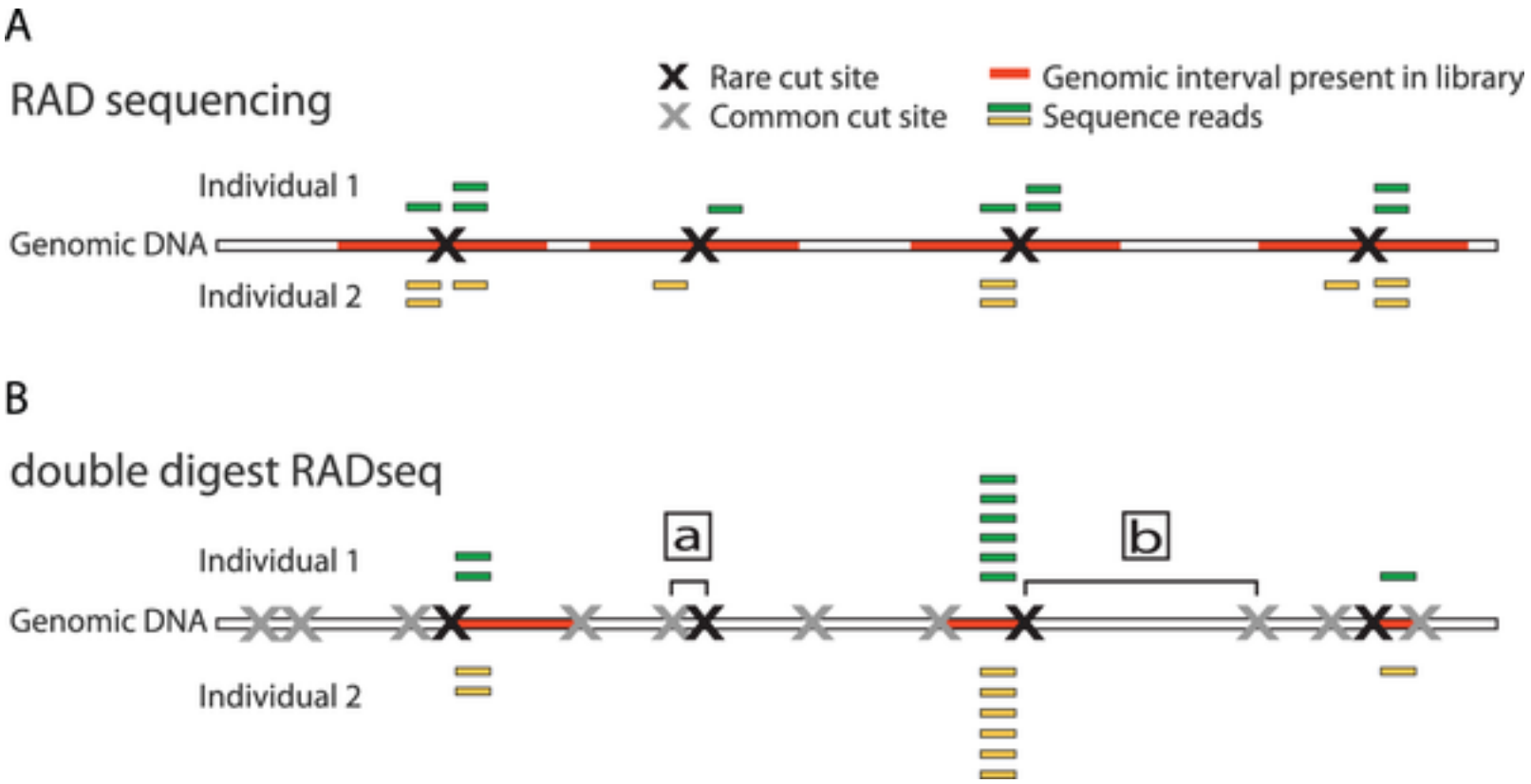
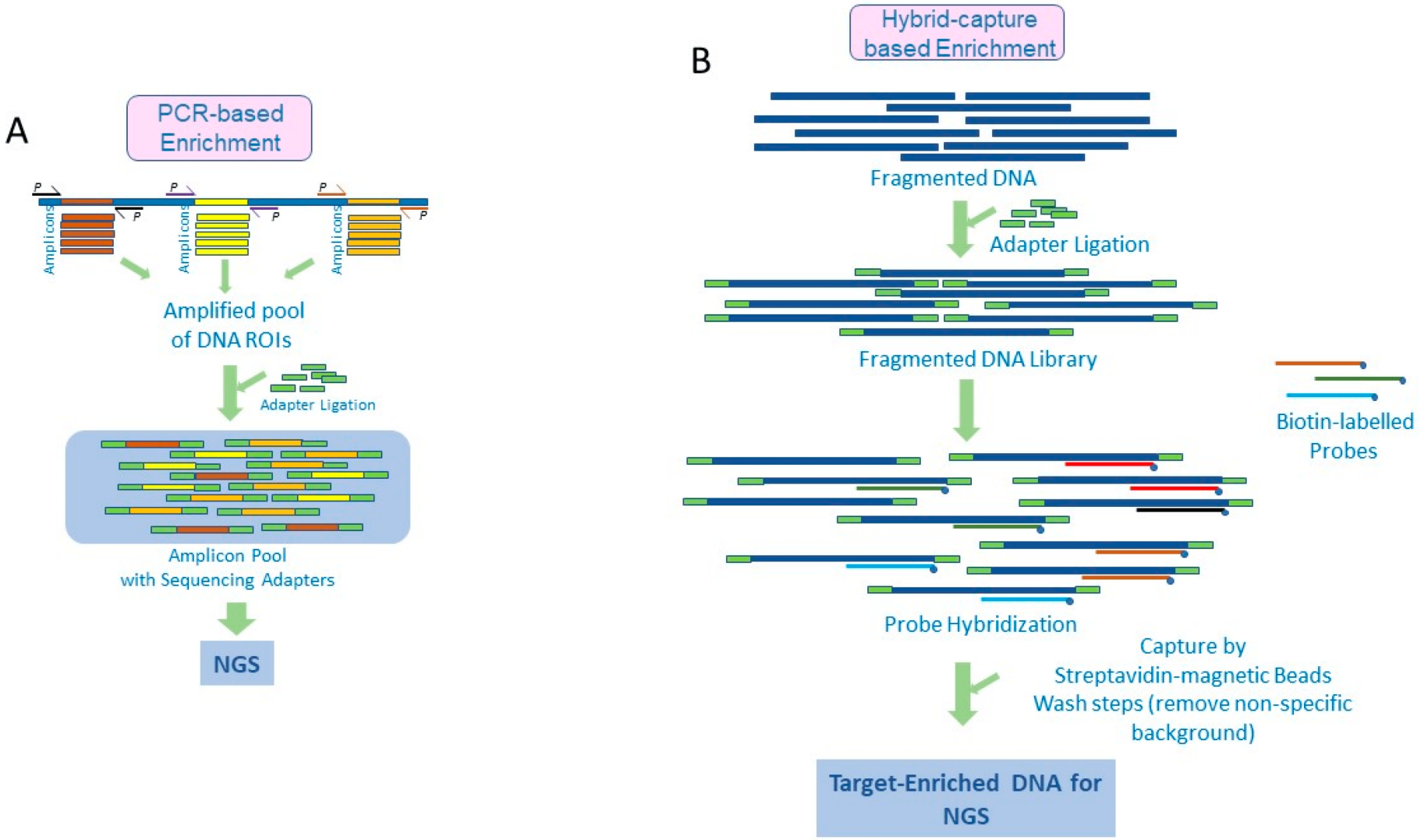
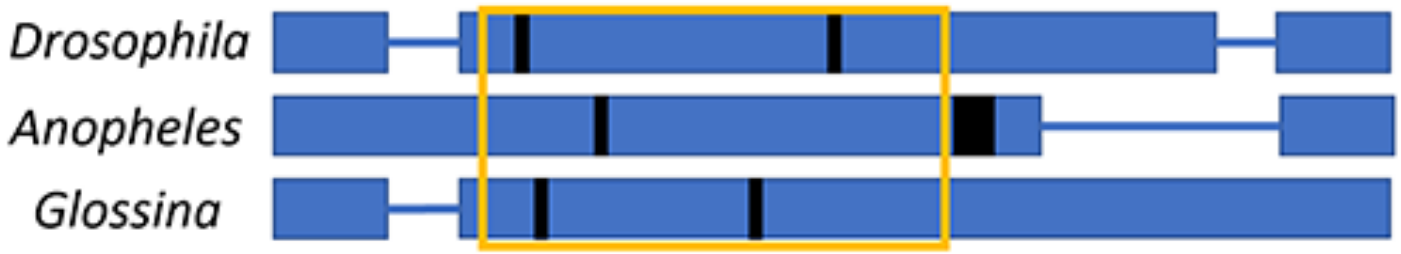
Types of sequencing experiments

- Genome assembly
  - Reference genome
  - RNA-seq
- Phylogenetics
  - WGS/ WGRS
  - Reduced representation
    - Rad-seq
    - Ultraconserved Elements
    - Anchored Hybrid Enrichment
    - Amplicon sequencing

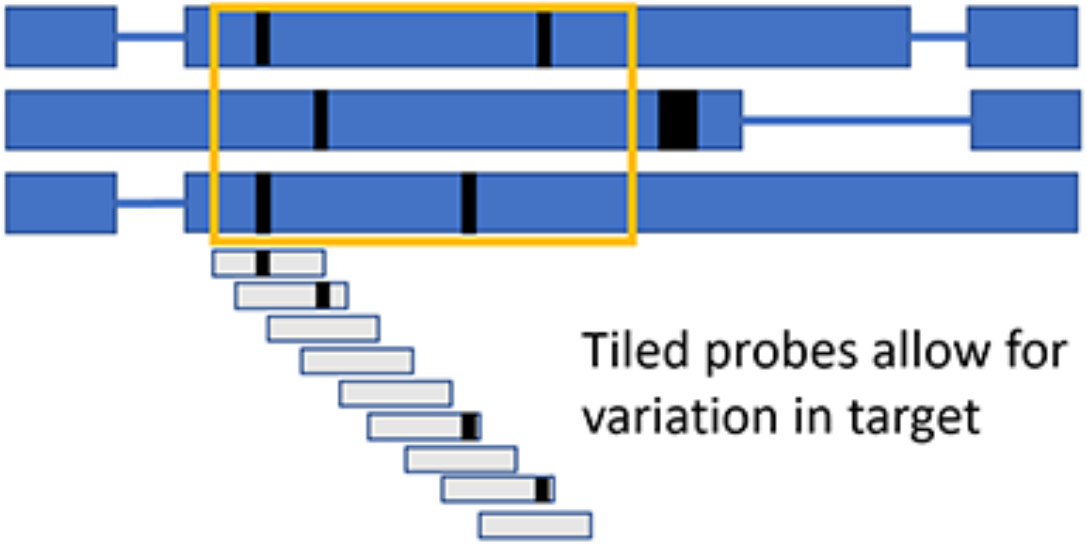
RAD-Seq


A
RAD sequencing
Rare cut site
Common cut site
Genomic interval present in library
Sequence reads
Individual 1
Genomic DNA
Individual 2
B
double digest RADseq
a
b
Individual 1
Genomic DNA
Individual 2


A
PCR-based Enrichment
P
Amplicons
Amplified pool of DNA ROIs
Adapter Ligation
Amplicon Pool with Sequencing Adapters
NGS
B
Hybrid-capture based Enrichment
Fragmented DNA
Adapter Ligation
Fragmented DNA Library
Biotin-labelled Probes
Probe Hybridization
Capture by Streptavidin-magnetic Beads
Wash steps (remove non-specific background)
Target-Enriched DNA for NGS



Anchor Hybrid Enrichment Target
Protein Coding
Conserved, but not “ultra conserved”



Tiled probes allow for variation in target

Ultraconserve Elements