CS123A Bioinformatics Module 5 – Week 14 – Presentation 2

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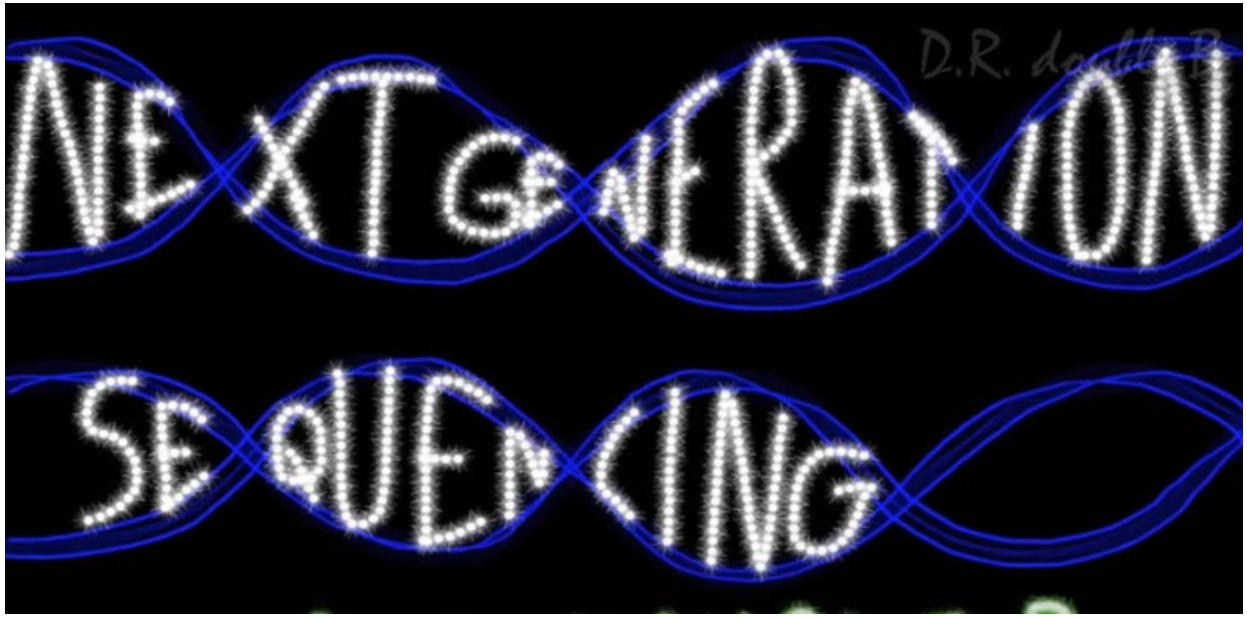
San Jose State Univ



Agenda

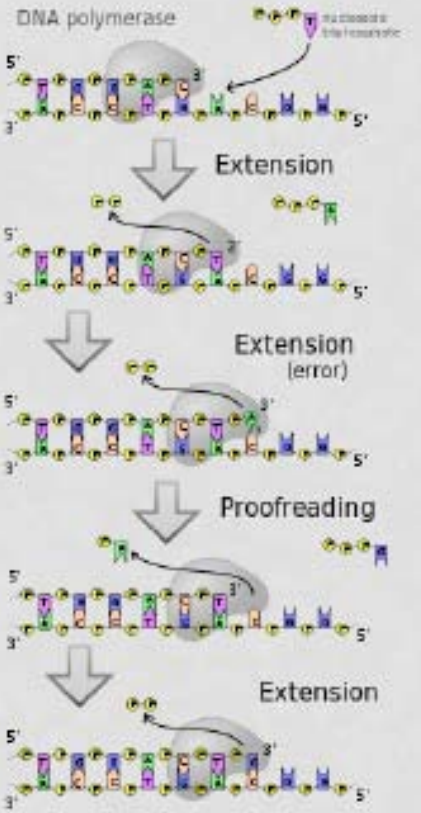
- Introduction to Next Generation Sequencing (NGS) continued
  - Sanger sequencing

Sanger Sequencing

Quick DNA Review

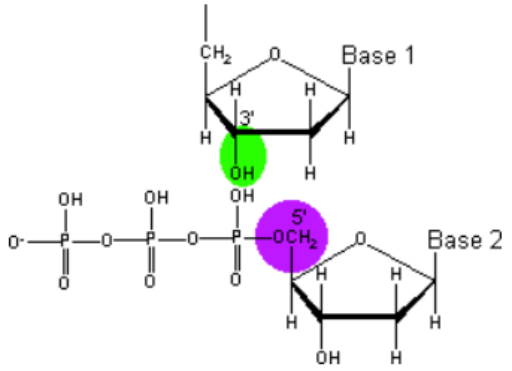
Natural Synthesis of DNA

- 5' to 3'
- By extension of an existing DNA or RNA strand (primer)
- Using a complementary (3' to 5') strand as template
- By addition of dNTP's (releasing pyrophosphate, PP_i)
- IF polymerase has 3'→5' exonuclease activity, it can "proofread" (correct errors)

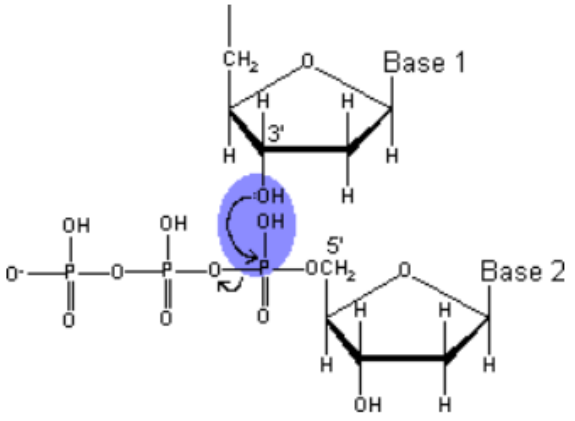


DNA Synthesis Process

- The 5’ group of a nucleotide triphosphate is held close to the 3’ hydroxyl group of a nucleotide chain.

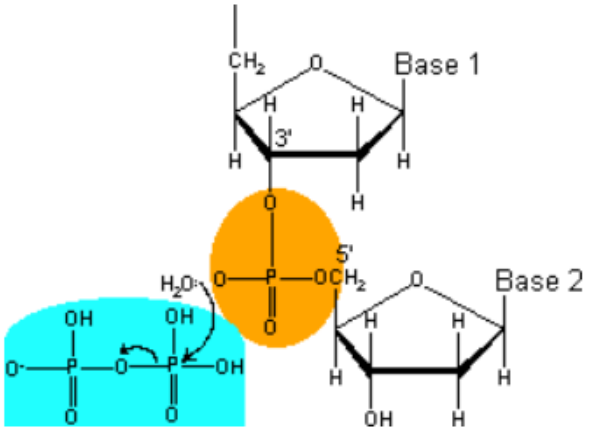


- The 3’hydroxyl group forms a bond to the phosphorous atom of the free nucleotide closest to the 5’ oxygen atom. Meanwhile the bond between the first phosphorus atom and the oxygen atom linking it to the next phosphate group breaks.

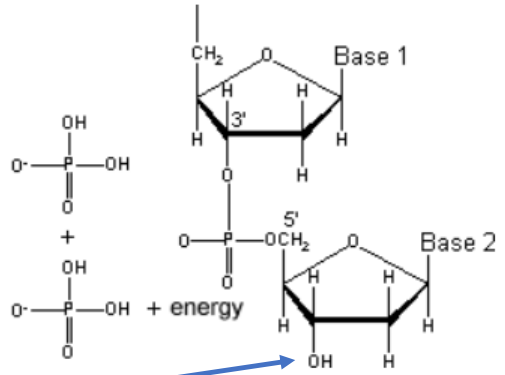


DNA Synthesis Process *(cont.)*

- A new phosphodiester bond now joins the two nucleotides. A pyrophosphate group has been liberated.



- The pyrophosphate group is hydrolyzed (split by the addition of water), releasing a lot of energy and driving the reaction forward to completion.



Remember this hydroxyl group on the 3' C. If OH is missing it is called A ddN (di-deoxy-nucleotide).

Chain-termination (Sanger) methods

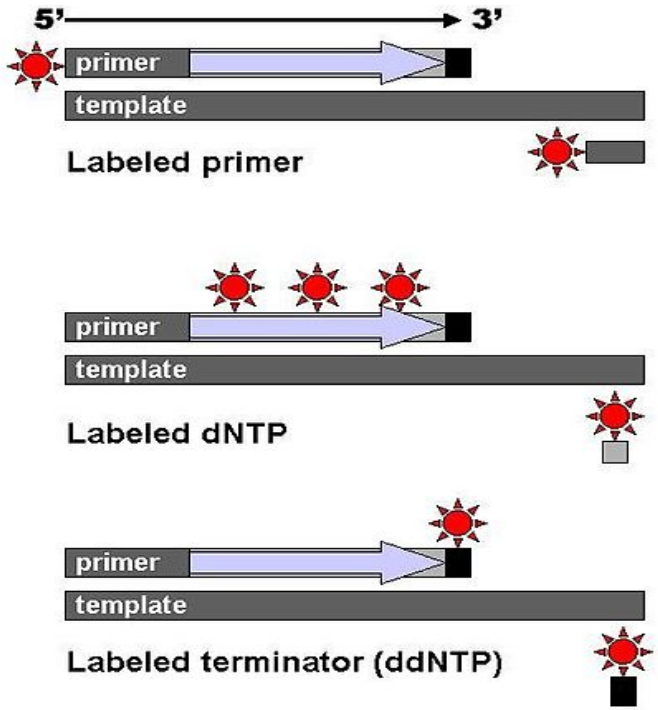
- Because the chain-terminator method (or Sanger method) is more efficient and uses fewer toxic chemicals and lower amounts of radioactivity than Maxam/Gilbert, it rapidly became the method of choice.

- Sanger F, Nicklen S, Coulson AR (December 1977). "DNA sequencing with chain-terminating inhibitors". *Proc. Natl. Acad. Sci. U.S.A.* **74** (12): 5463–7 (assigned reading)

Chain-termination (Sanger) methods

- The classical chain-termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal deoxynucleotidephosphates (dNTPs), and modified nucleotides (dideoxyNTPs) that terminate DNA strand elongation.
- These ddNTPs were also radioactively labelled for detection in automated sequencing machines. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase.
- To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP) which are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length.
- The newly synthesized and labelled DNA fragments are heat denatured, and separated by size (with a resolution of just one nucleotide) by gel electrophoresis on a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography, and the DNA sequence can be directly read off the X-ray film.

Chain Termination Method

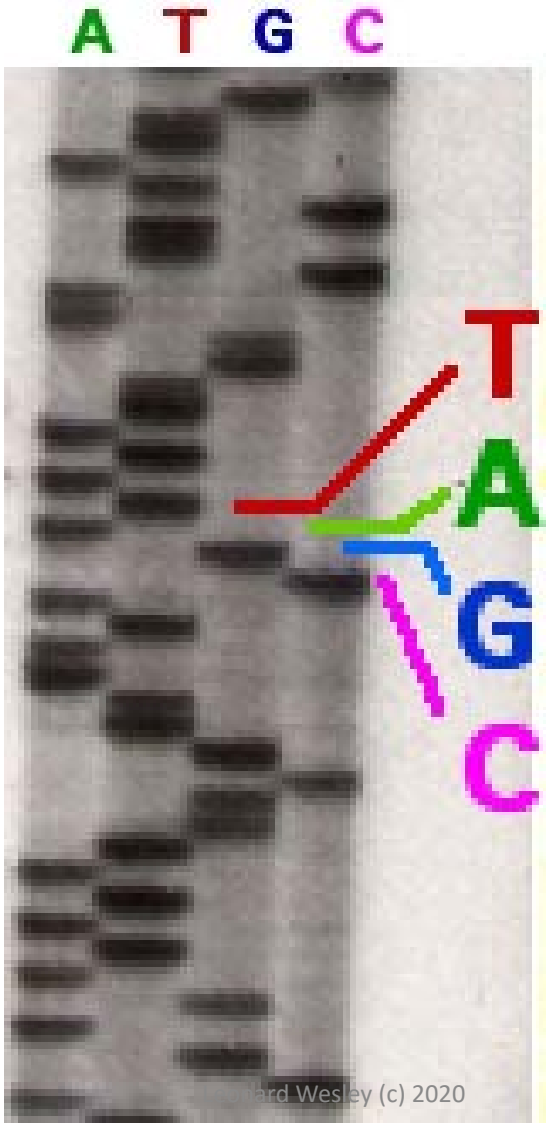


Chain-termination (Sanger) methods

- In the image on the next slide, X-ray film was exposed to the gel, and the dark bands correspond to DNA fragments of different lengths.

- A dark band in a lane indicates a DNA fragment that is the result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP).

- The relative positions of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence.


A T G C
T
A
G
C

Chain-termination (Sanger) methods

- Chain-termination methods greatly simplified DNA sequencing.

- For example, chain-termination-based kits are commercially available that contain the reagents needed for sequencing, pre-aliquoted and ready to use.

- Limitations include non-specific binding of the primer to the DNA, affecting accurate read-out of the DNA sequence; difficulty with runs of the same nucleotide; and DNA secondary structures affecting the fidelity of the sequence.

Sanger Sequencing

- For example, the chain-termination-based "Sequenase" kit from USB Biochemicals contains most of the reagents needed for sequencing, pre-aliquoted and ready to use.

- Limitations include non-specific binding of the primer to the DNA, affecting accurate read-out of the DNA sequence, and DNA secondary structures affecting the fidelity of the sequence.

Sanger Sequencing

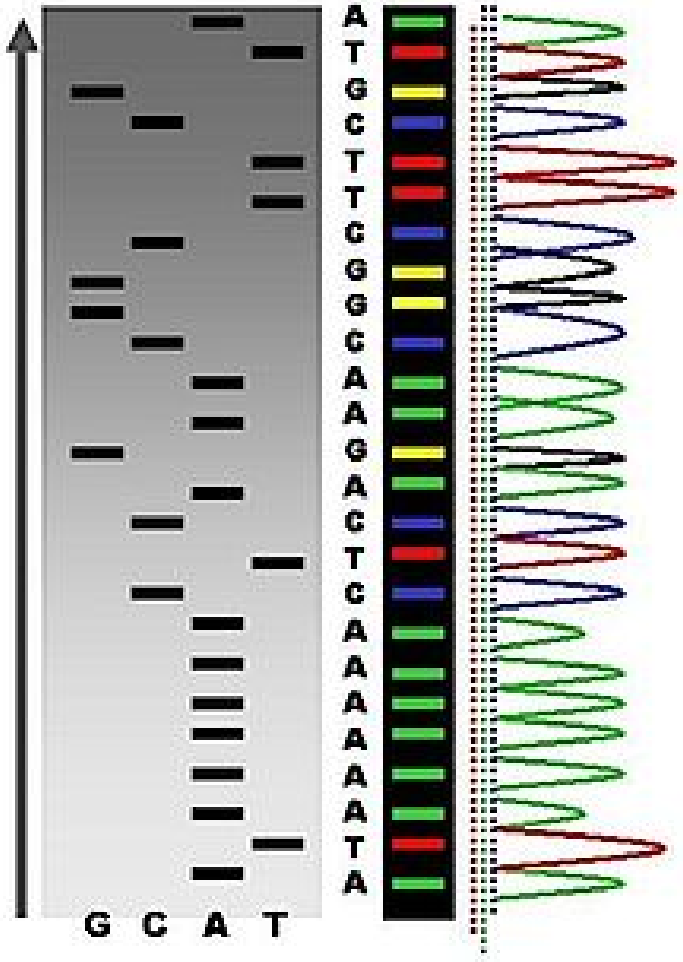
Sanger sequence video

https://www.youtube.com/watch?v=e2G5zx-OJIw&feature=em-share_video_user

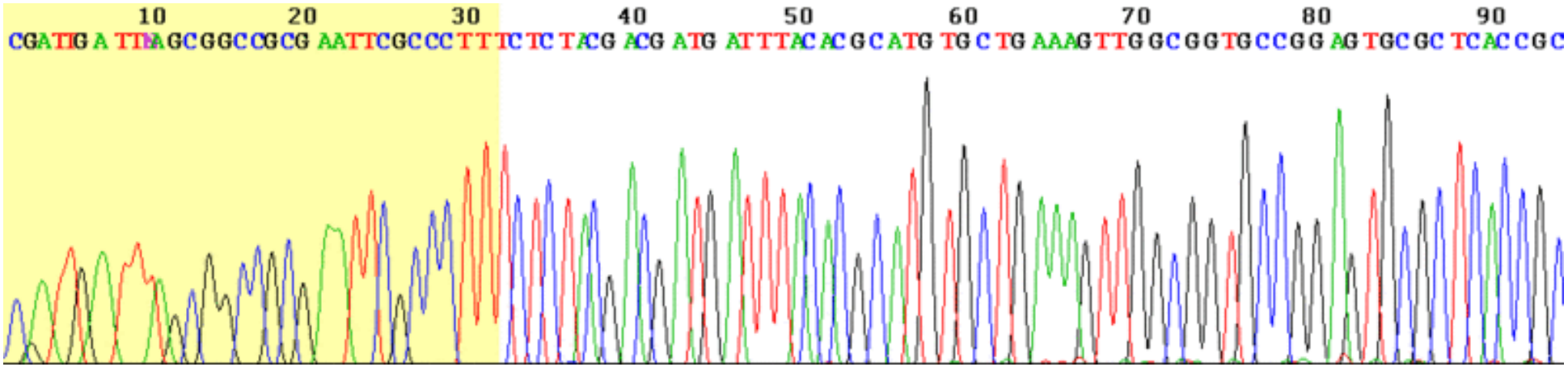
Automated Sequencing – the Beginning

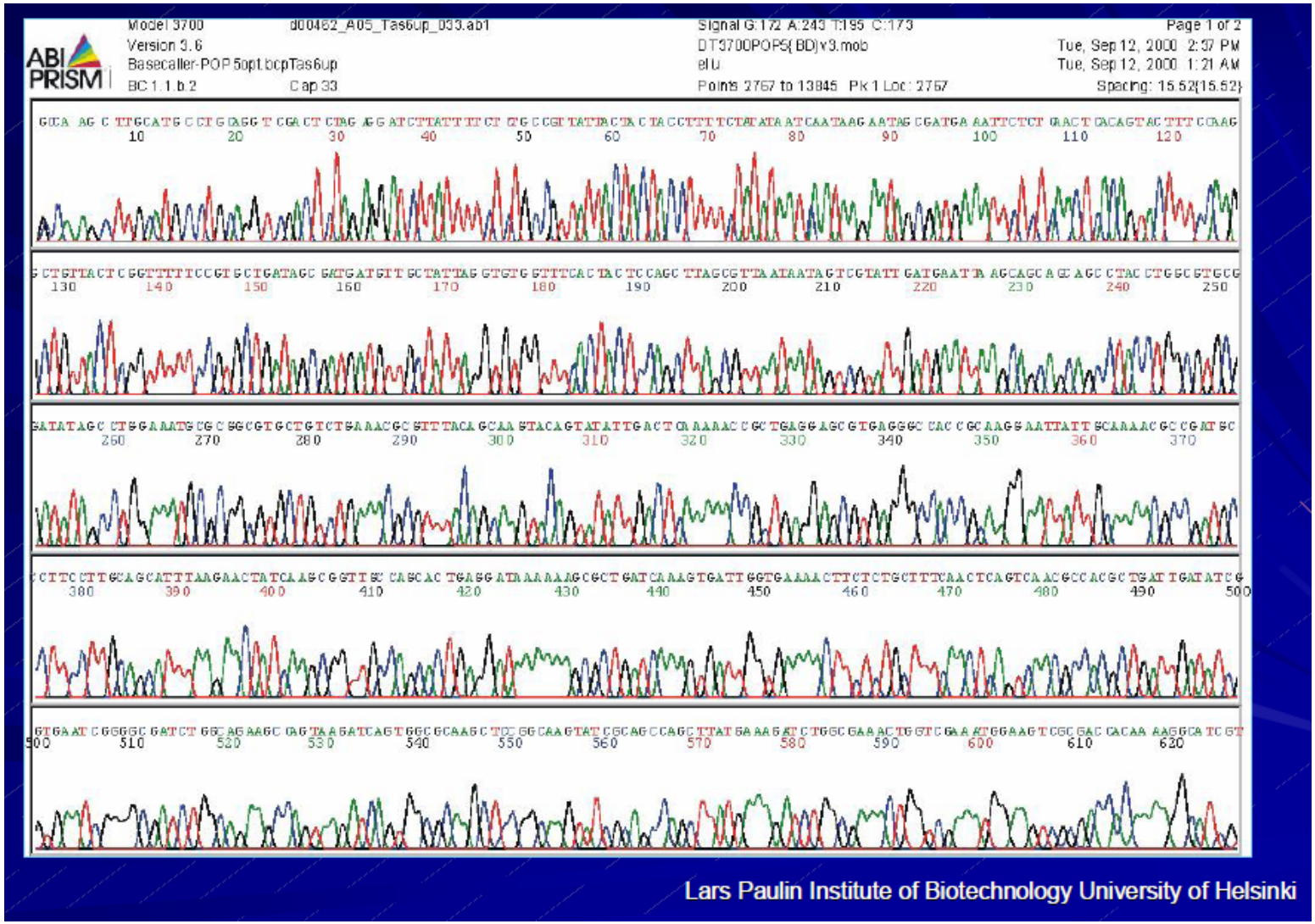
Dye-terminator sequencing

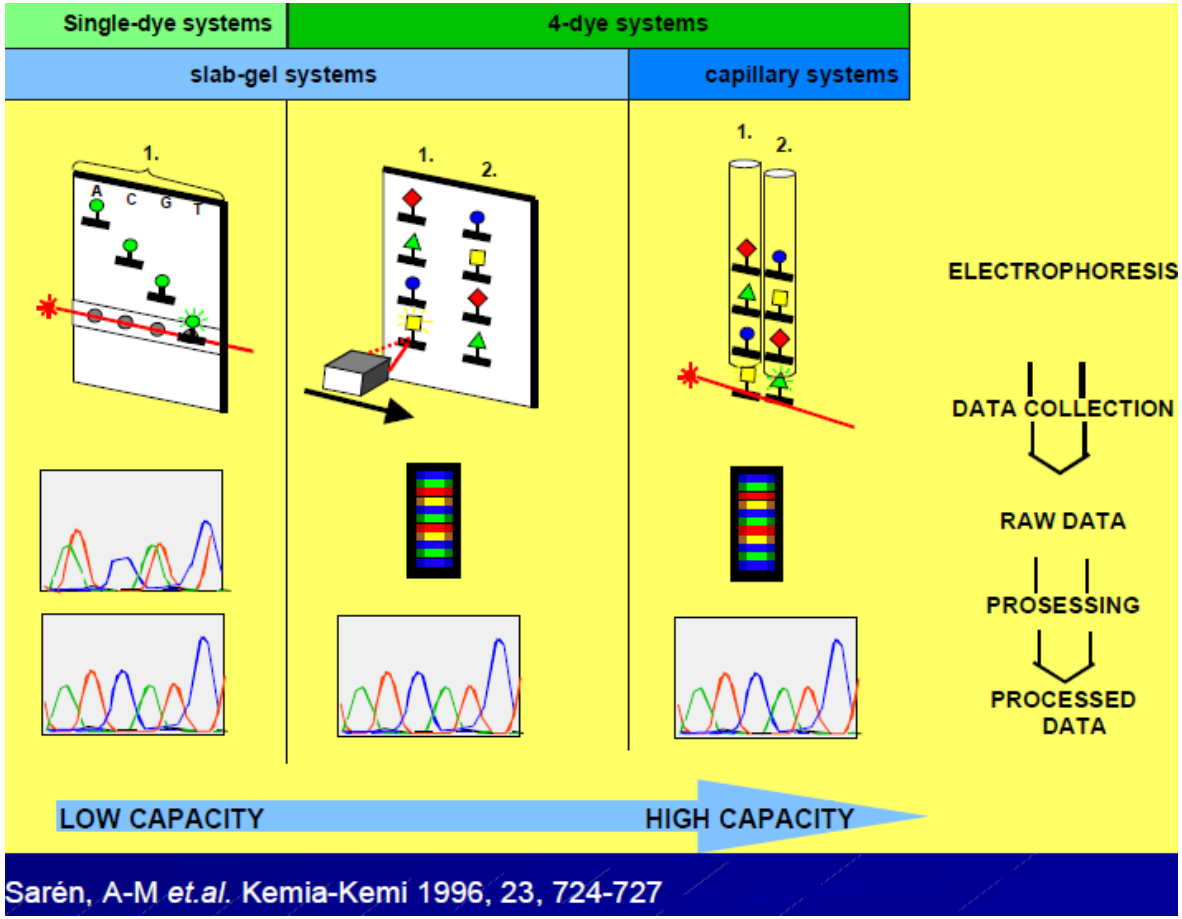
- Led to automation of DNA sequencing
- Initially used a primer labeled at the 5’ end with a fluorescent dye.
- Dye-primer sequencing facilitates reading in an optical system for faster and more economical analysis and automation.
- Later development by L Hood and coworkers of fluorescently labeled ddNTPs and primers set the stage for automated, high-throughput DNA sequencing.


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

Example Dye Sequencing Result




Model 3700
Version 3.6
Basecaller-POP5opt.bcpTas6up
BC 1.1.b.2
d00462_A05_Tas6up_033.ab1
Cap 33
Signal G:172 A:243 T:195 C:173
DT3700POP5(BD)v3.mob
elu
Points 2767 to 13845 Pk 1 Loc: 2767
Page 1 of 2
Tue, Sep 12, 2000 2:37 PM
Tue, Sep 12, 2000 1:21 AM
Spacing: 15.52[15.62]
Lars Paulin Institute of Biotechnology University of Helsinki


Single-dye systems
4-dye systems
slab-gel systems
capillary systems
1.
A C G T
1.
2.
1.
2.
ELECTROPHORESIS
DATA COLLECTION
RAW DATA
PROSESSING
PROCESSED DATA
LOW CAPACITY
HIGH CAPACITY
Sarén, A-M et.al. Kemia-Kemi 1996, 23, 724-727

Automated Sequencing

- The first step toward this goal was achieved in 1985, when Leroy Hood at CalTech attached fluorescent dyes to the primer used in the sequencing reactions; each different color dye (blue, green, yellow, and red) was matched with a different terminator base.

- He and Michael Hunkapiller from Applied Biosystems, Inc. (ABI) built an instrument, dubbed the ABI Model 370, to read the sequence of the dyelabeled fragments. It was equipped with an argon ion laser for exciting the dyes, a flat gel laid between two glass plates (referred to as a "slab" gel) capable of sixteen-lane electrophoresis, and a Hewlett-Packard Vectra computer boasting 640 MB of memory for data analysis.

Automated Sequenicng

- Using fluorescent dyes, all four sequencing reactions could now be loaded into a single gel lane. As the fragments electrophoresed, the beam of the laser focused at the bottom of the gel made the dye-labeled fragments glow as they passed.

- The color of each dye-labeled fragment was then interpreted by the computer as a specific base (A if green, C if blue, G if yellow, and T if red). Over 350 bases could be read per lane. With this new automated approach, a technician could read more sequence in a day than could be read manually in a n entire week!

Limitations and challenges

- Its limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis. This problem has been addressed with the use of modified DNA polymerase enzyme systems and dyes that minimize incorporation variability, as well as methods for eliminating "dye blobs".

- Common challenges of DNA sequencing include poor quality in the first 15–40 bases of the sequence and deteriorating quality of sequencing traces after 700–900 bases. Base calling software typically gives an estimate of quality to aid in quality trimming.

Automated Sequencing Timeline

- Dye-terminator sequencing utilizes labelling of the chain terminator ddNTPs, which permits sequencing in a **single reaction**, rather than four reactions as in the labelled-primer method.
- In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes, each of which with different wavelengths of fluorescence and emission.
- 1986 -First commercialized by ABI - First Automated DNA Sequencer ABI 370 1988– Pharmacia ALF
- 1990 – ABI 373
- 1995– ABI 377, Up to 96 lanes, a four- to fivefold increase in throughput compared with that of the 373 system.
- 1996 – First Capillary DNA Sequencer ABI 310
- 1998– First 96 Capillaryinstruments: MegaBace, ABI 3700
- 2002– ABI 3730, 48 or 96 Capillary

ABI 373

On eBay for $299!!

ABI 373 XL

Increased resolution and read length with longer slab gel

Evolution

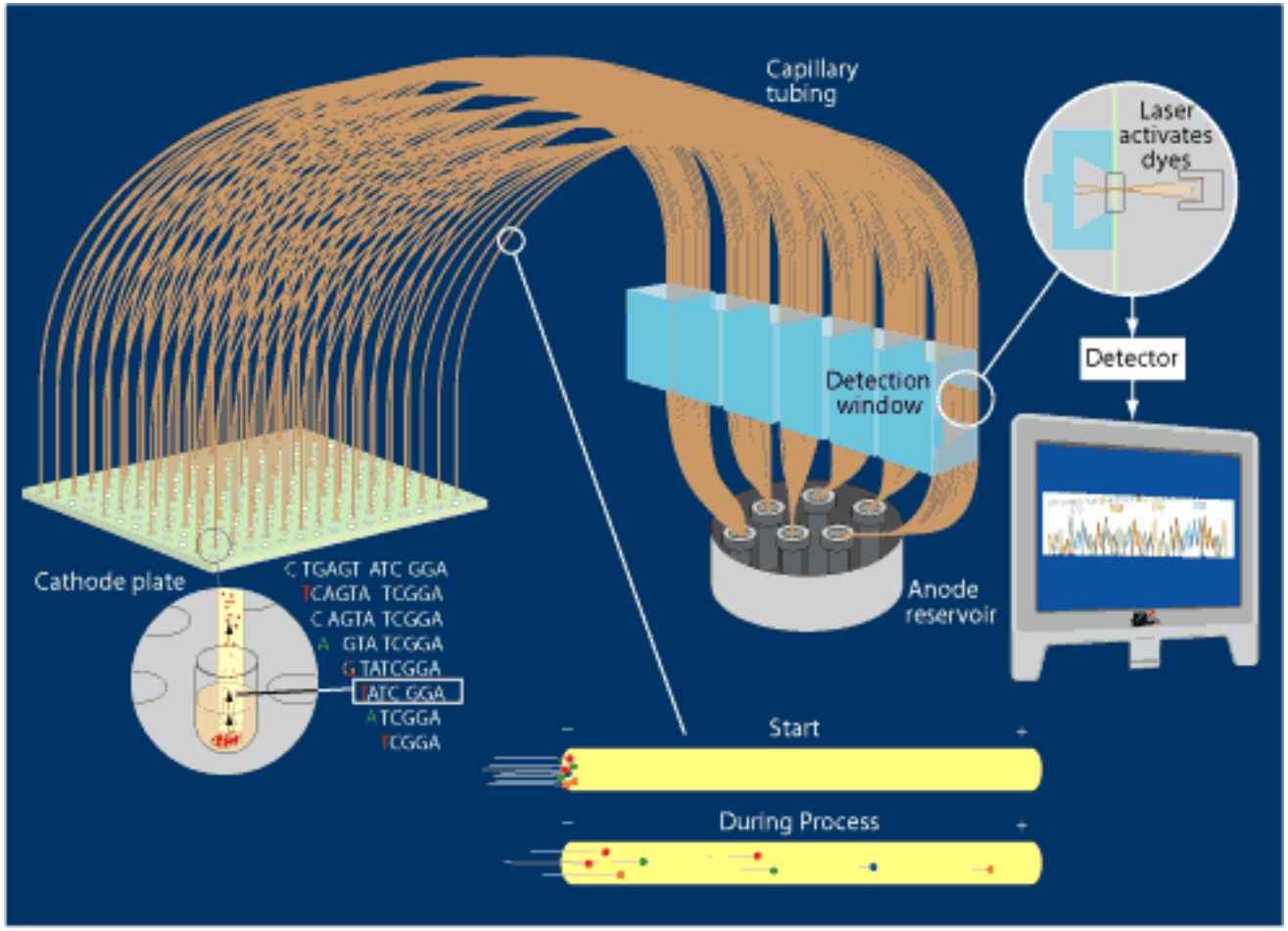
- Shortly after ABI placed its automated DNA sequencer on the market, the Dupont company introduced its own model, the Genesis 2000. Dupont had also developed a new method of labeling sequencing fragments: attaching the fluorescent dyes to the terminator bases. With this innovation, four separate sequencing reactions were no longer required; the entire sequencing reaction could be accomplished in a single tube. However, Dupont failed to effectively compete in the market and sold the rights to the dye terminator chemistry to ABI.
- ABI continued to refine its automated sequencer. More powerful computers, increased gel capacity (to 96 lanes), improvement of the optical systems, enhancement of the chemistry, and the introduction of more sensitive fluorescent dyes increased the reading capacity of the instrument to over 550 bases per lane.
- The ABI PRISM Model 377 Automated Sequencer, introduced in 1995, incorporated these changes and could read, at maximum capacity, over 19,000 bases in a day. Even at this rate, however, the sequencing of entire genomes, as that of humans (3 billion bases in length), was still not practical.

Automated Sequencing – From Genes to Genomes

Capillary Sequencers

- Working with the Model 377 Automated Sequencer, a laboratory technician had to pour the slab gels and mount them on the instrument. This process alone was time-consuming and cumbersome. In addition, the technician had to add each sequencing reaction into each individual lane of the gel prior to the run. The MegaBase, developed by Molecular Dynamics, and the ABI Model 3700 Automated Sequencer, developed by ABI, addressed these limitations by using multiple capillaries, thin, hollow glass tubes filled with a gel polymer.

- The ABI PRISM Model 3700 Automated Sequencer, developed with the Hitachi Corporation and having a price tag of $300,000, uses ninety-six capillaries, each not much wider than a strand of human hair. The capillaries are automatically cleaned and filled with fresh gel polymer between each electrophoresis run. The instrument is also equipped with a robot arm that automatically loads the sequencing reactions into the capillaries, greatly decreasing the amount of human labor required for its operation. The Model 3700 Automated Sequencer can read over 400,000 bases in a day, a greater than twenty-fold increase over the maximum capacity of the Model 377. Beginning in September 1999 and using 300 of these instruments, the Celera Corporation had sequenced the entire human genome five times over within four months.

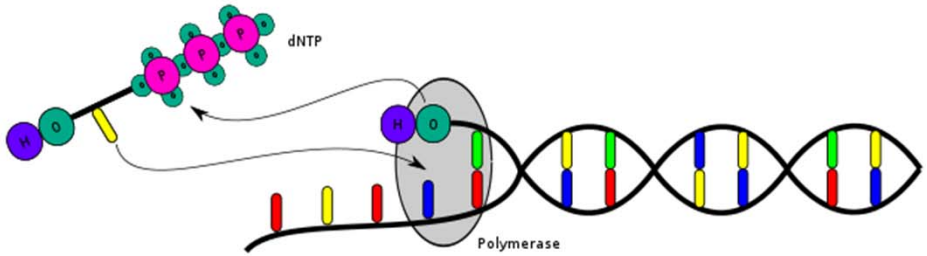

Capillary tubing
Laser activates dyes
Detector
Detection window
Cathode plate
C TGAGT ATC GGA
TCAGTA TCGGA
C AGTA TCGGA
A GTA TCGGA
G TATCGGA
TATC GGA
ATCGGA
TCGGA
Anode reservoir
Start
During Process

A Little History

- Between late 2007 and the present, DNA sequencing has experienced a “technological singularity”
  - In Sep-2007, the $1,000 human genome sequence was expected to arrive around Sep-2023
  - Instead, it is arriving NOW! Cost now $300 - $1K.
- Numerous business plans, research approaches and sequencing alternatives that made sense in 2007 have rapidly been relegated to the trash bin of history
- The technology is moving so rapidly that many of the “cutting edge” technologies mentioned in the course text (2015/2016) have already been discarded or replaced…

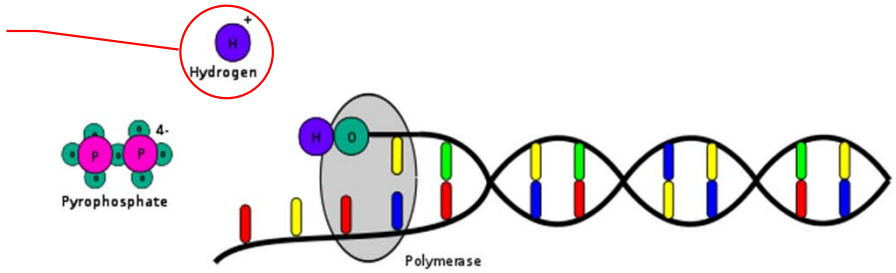
Additional NGS Technologies

Ion Torrent



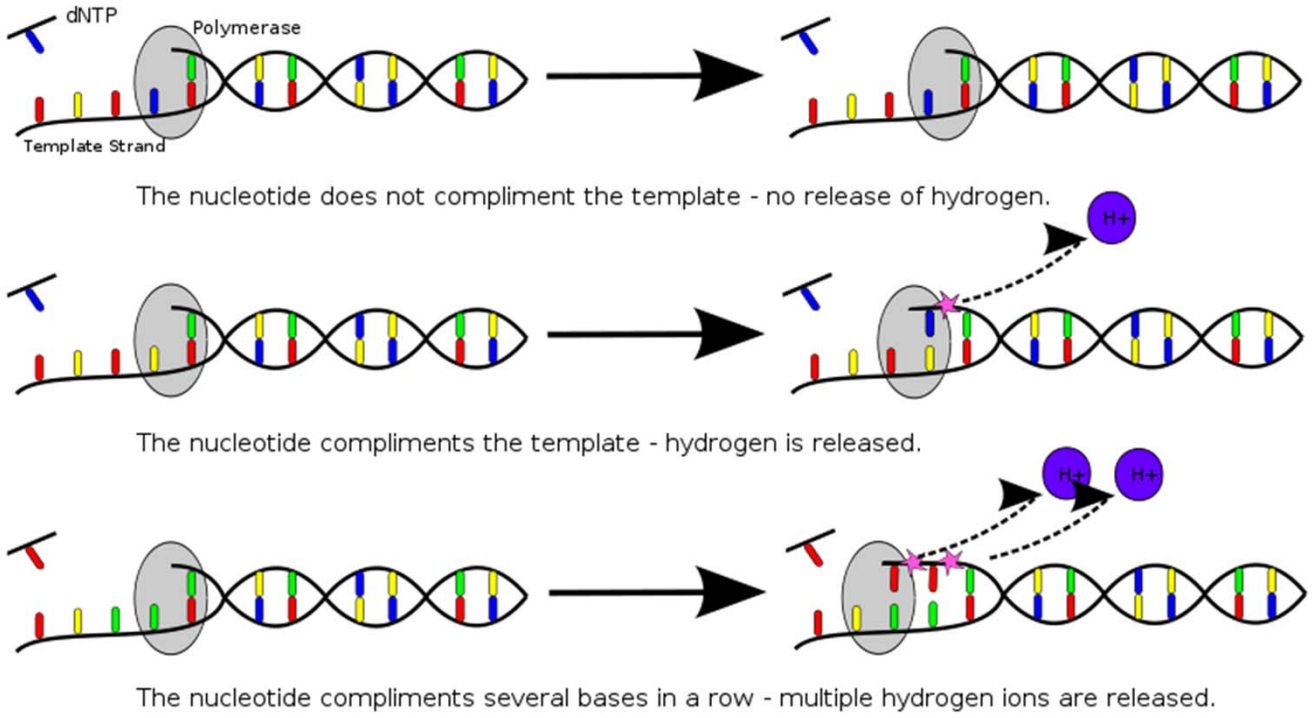
Polymerase integrates a nucleotide.

Ion Torrent

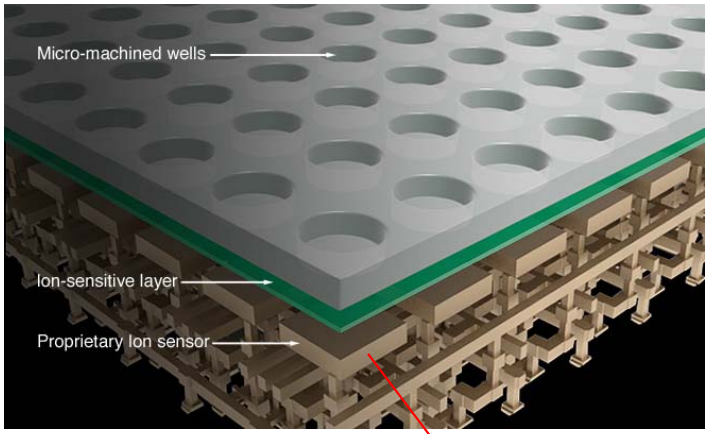


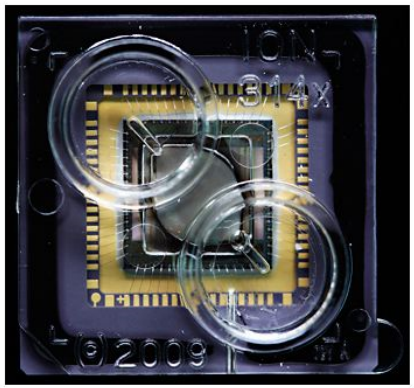
Hydrogen and pyrophosphate are released.

Synthesis With 1 dNTP



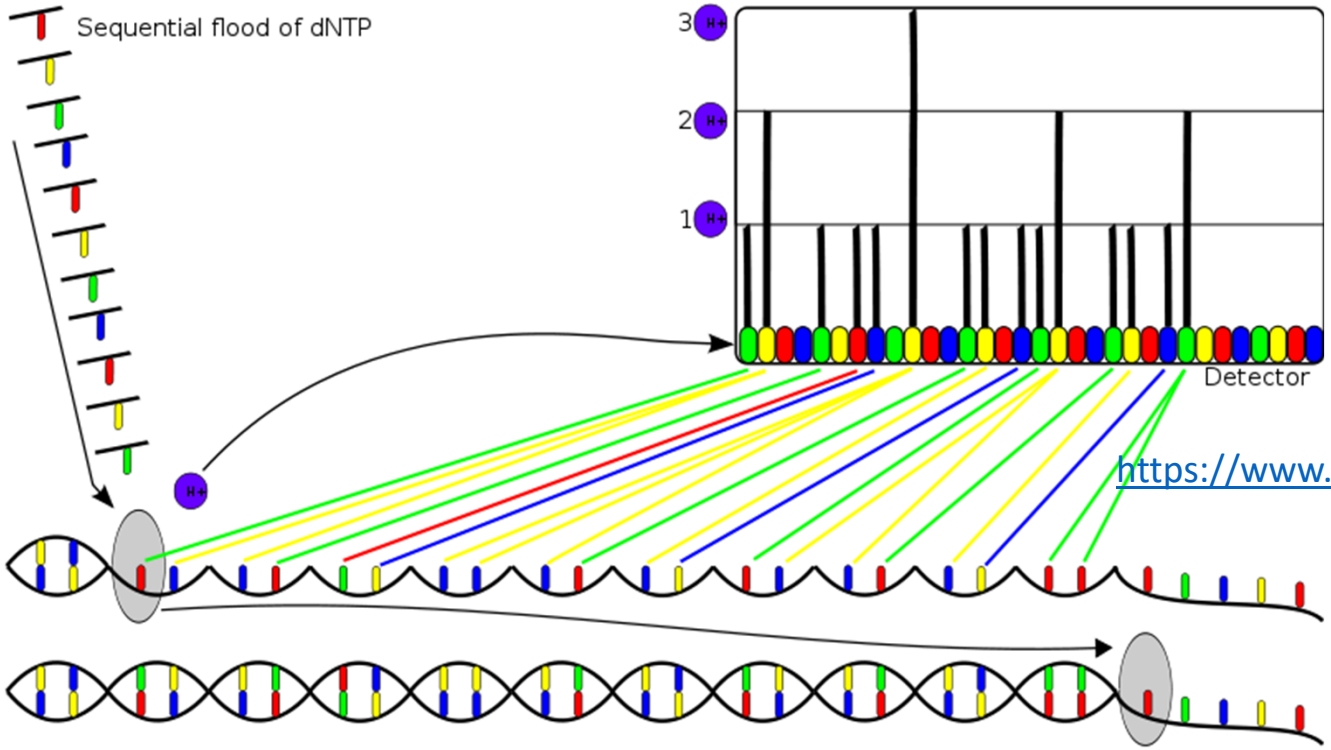
Measuring pH Changes





Use Ion-Sensitive Field Effect Transistor (ISFET) technology to measure pH change electronically

Ion Torrent Sequence Calling

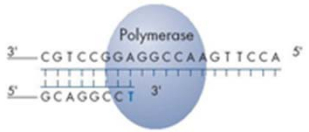


Ion Torrent Video

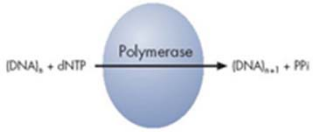
https://www.youtube.com/watch?v=WYBzbxIfuKs

Pyrosequencing

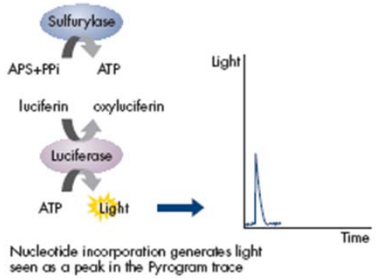
Step 1
A sequencing primer is hybridized to a single-stranded PCR amplicon that serves as a template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase as well as the substrates, adenosine 5' phosphosulfate (APS), and luciferin.



Step 2
The first deoxribonucleotide triphosphate (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxyribo-nucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.

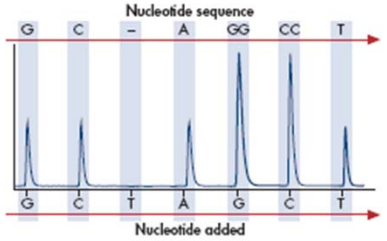


Step 3
ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) chip and seen as a peak in the raw data output (Pyrogram). The height of each peak (light signal) is proportional to the number of nucleotides incorporated.



Step 4
Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added.

Step 5
Addition of dNTPs is performed sequentially. It should be noted that deoxyadenosine alfa-thio triphosphate (dATP·S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.



[Pyrosequencing video](https://www.youtube.com/watch?v=bNKEhOGvcaI)

https://www.youtube.com/watch?v=bNKEhOGvcaI

Illumina Sequencing

https://www.youtube.com/watch?v=fCd6B5HRaZ8

Illumina Sequencing Technology

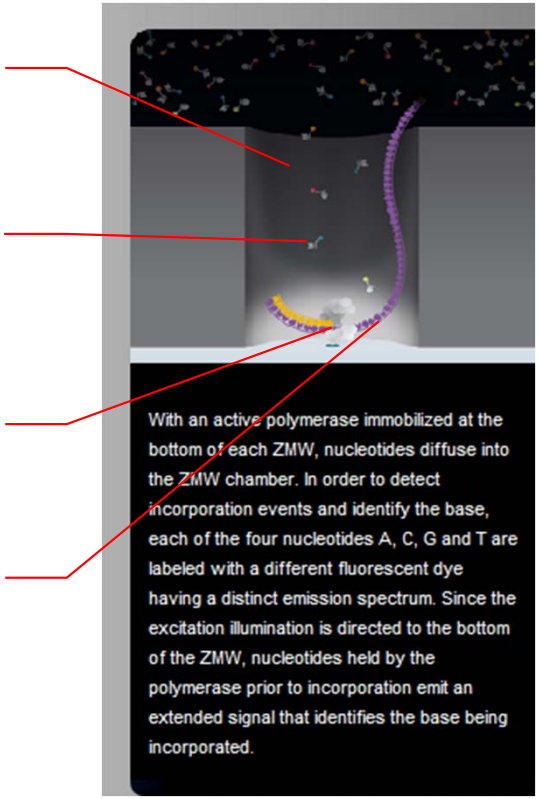
PacBio Sequencing

Zero Mode Waveguide: restricts fluorescence detection to a very narrow layer at bottom of nanowell

Phospho-labelled dNTP's: each dNTP is labelled with a uniquely colored fluorophore at the γ-phosphate; incorporation releases the label, which then diffuses out of the detection zone

Anchored DNA Polymerase: holds template and nucleotide being incorporated in detection zone for several milliseconds

Long, Circular Template: allows template to be read many times ("rolling circle" mechanism)



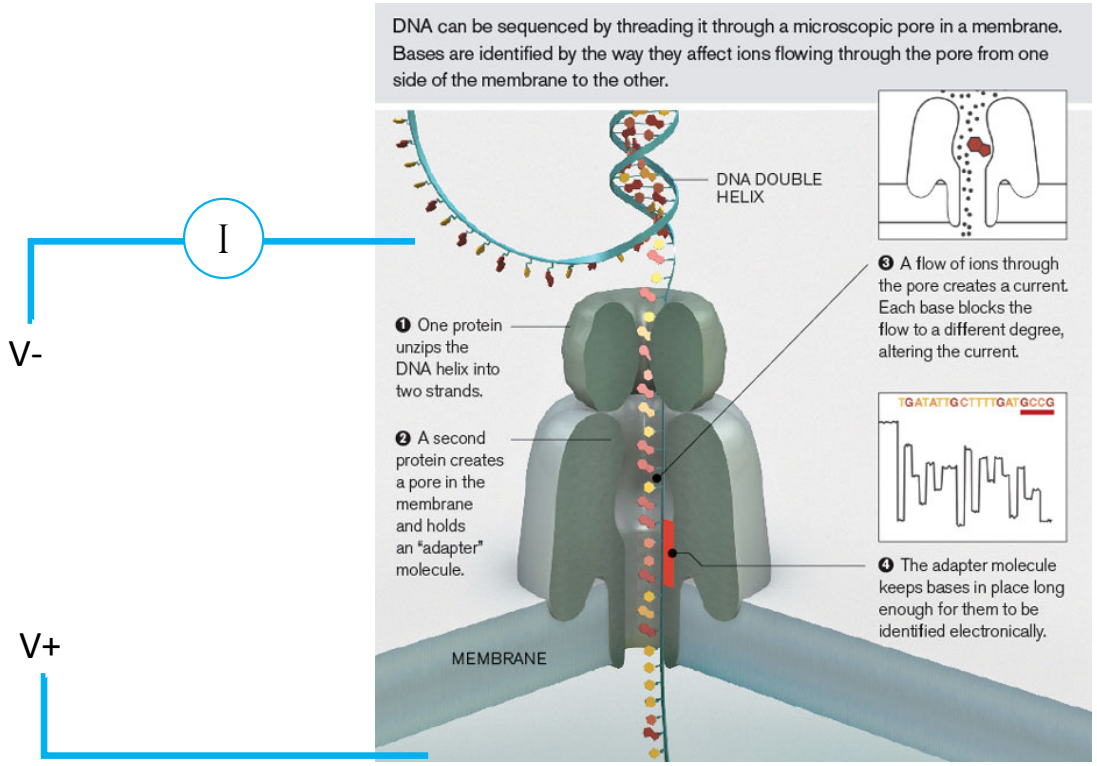
PacBio Video

http://www.youtube.com/watch?v=v8p4ph2MAvI

Oxford Nanopore Sequencing

https://www.youtube.com/watch?v=E9-Rm5AoZGw

Nanopore Sequencing Video



Next Step: Assembly Of NGS Reads