Principles of DNA Sequencing

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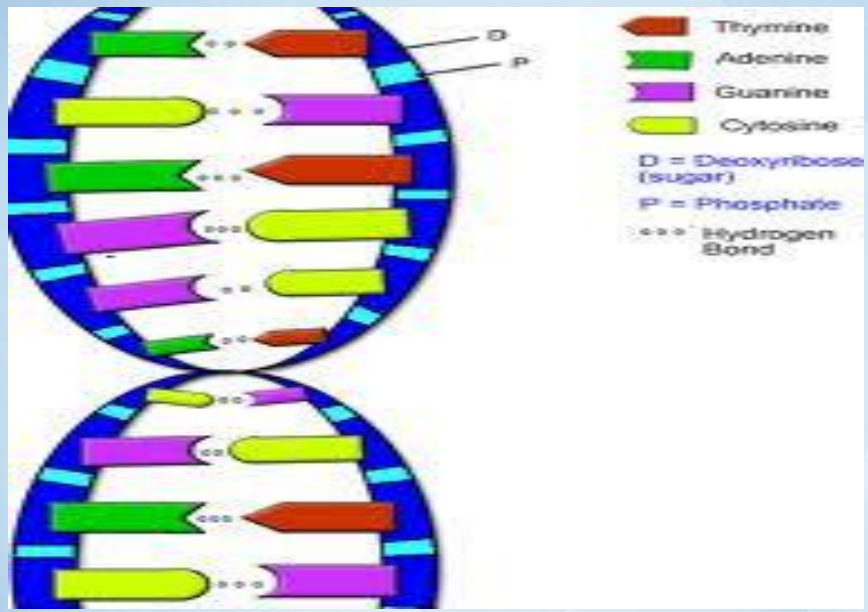
What is DNA Sequencing?

- DNA Sequencing is finding the order of nucleotides in a fragment of DNA



- It is involving various biochemical, biophysical and computational techniques to determine the order of the nucleotide bases- adenine, guanine, thymine & cytosine in a molecule of DNA.

Methods of DNA Sequencing

Sanger Method

DNA sequencing by enzymatic synthesis

Nobel Prize 1958, seq. of insulin

Nobel Prize 1980, DNA seq.

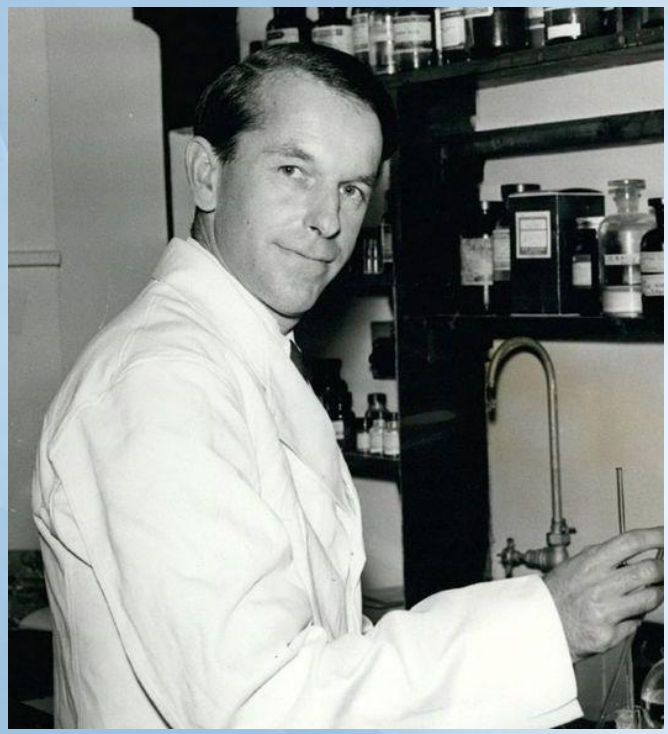
Maxam–Gilbert Method

DNA sequencing by chemical degradation

Nobel Prize 1980, DNA sequence

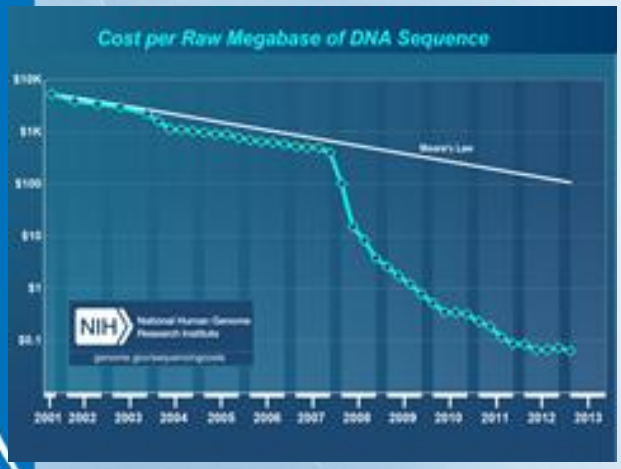
Modern sequencing equipment uses the principles of the Sanger technique

Sanger sequencing



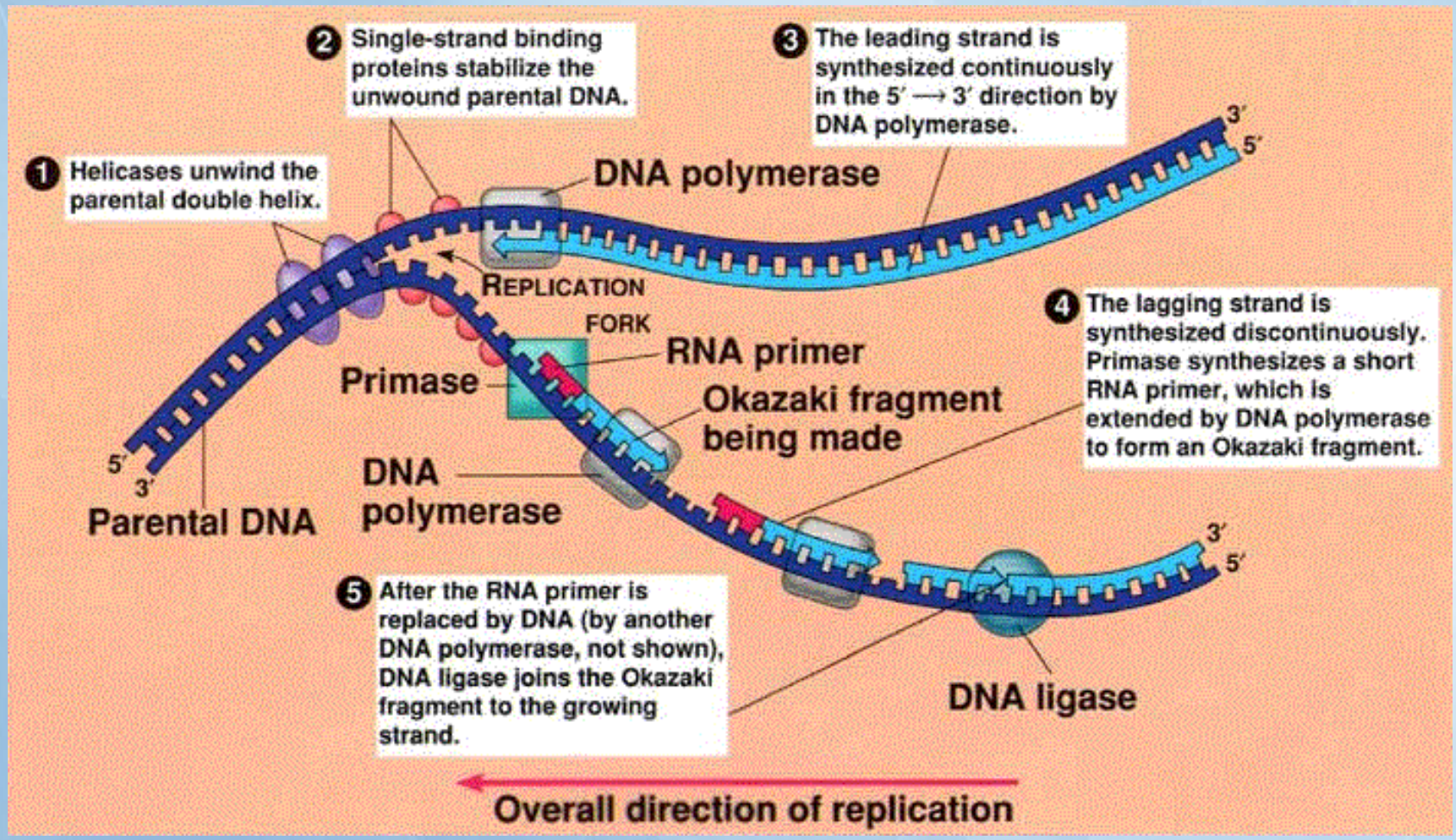
- Frederick Sanger
- British biochemist
- Recipient of the Nobel Prize ***TWICE***
- 1958 - structure of proteins, Insulin
- 1980 - determination of base sequences in nucleic acids

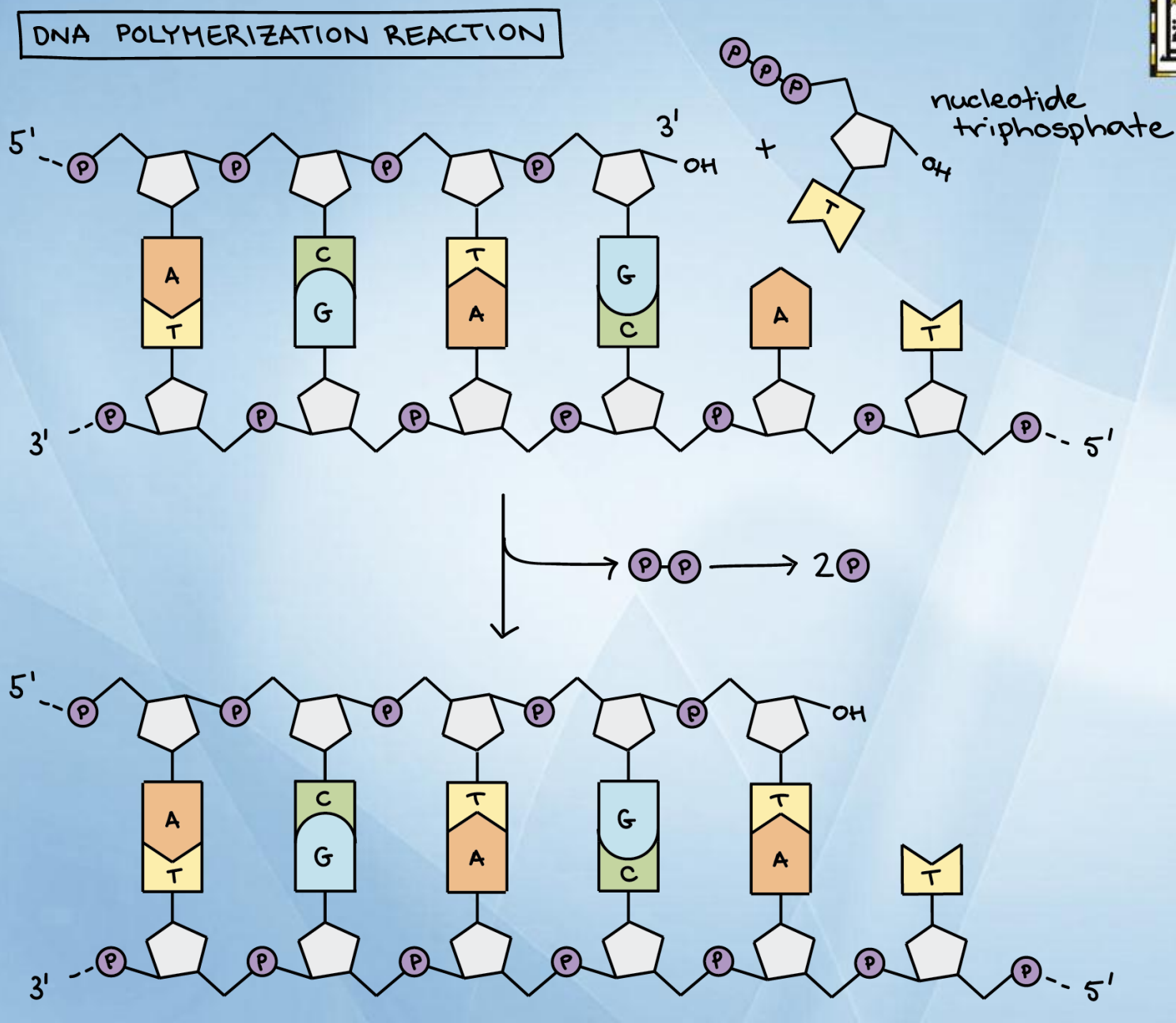
Sequencing Cost

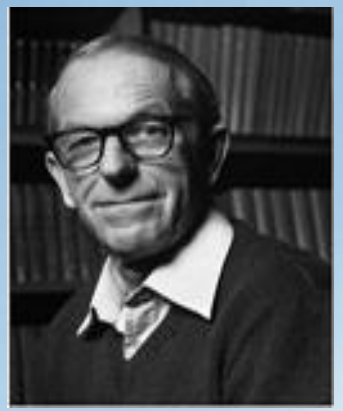


Date	Cost per Mb	Cost per Genome
Sep-01	$5,292.39	$95,263,072
Sep-02	$3,413.80	$61,448,422
Oct-03	$2,230.98	$40,157,554
Oct-04	$1,028.85	$18,519,312
Oct-05	$766.73	$13,801,124
Oct-06	$581.92	$10,474,556
Oct-07	$397.09	$7,147,571
Oct-08	$3.81	$342,502
Oct-09	$0.78	$70,333
Oct-10	$0.32	$29,092
Oct-11	$0.09	$7,743
Oct-12	$0.07	$6,618
Jan-13	$0.06	$5,671

Source - NHGRI : http://www.genome.gov/sequencingcosts/

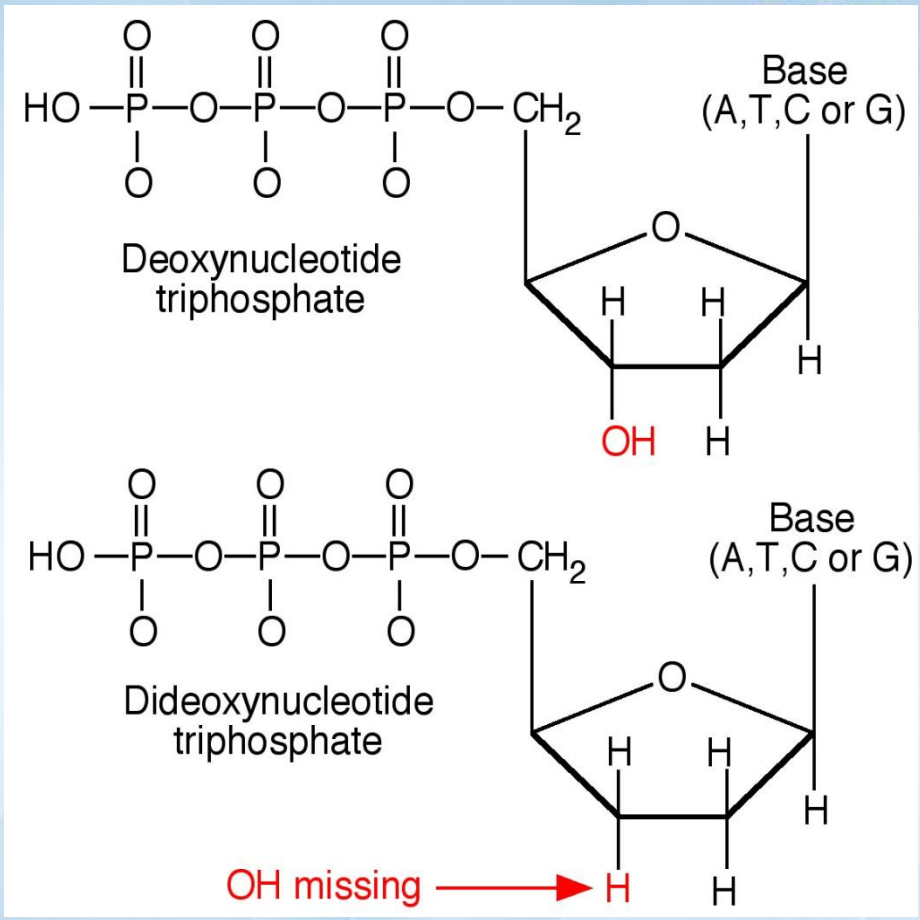

2 Single-strand binding proteins stabilize the unwound parental DNA.
3 The leading strand is synthesized continuously in the 5′ ⟶ 3′ direction by DNA polymerase.
1 Helicases unwind the parental double helix.
DNA polymerase
3′
5′
REPLICATION FORK
RNA primer
Primase
Okazaki fragment being made
4 The lagging strand is synthesized discontinuously. Primase synthesizes a short RNA primer, which is extended by DNA polymerase to form an Okazaki fragment.
5′
3′
Parental DNA
DNA polymerase
3′
5′
5 After the RNA primer is replaced by DNA (by another DNA polymerase, not shown), DNA ligase joins the Okazaki fragment to the growing strand.
DNA ligase
Overall direction of replication


DNA POLYMERIZATION REACTION
nucleotide triphosphate
5'
3'
OH
A
T
C
G
T
A
G
C
A
T
P
2P



The Sanger method

Uses dideoxy nucleotides to terminate DNA synthesis.



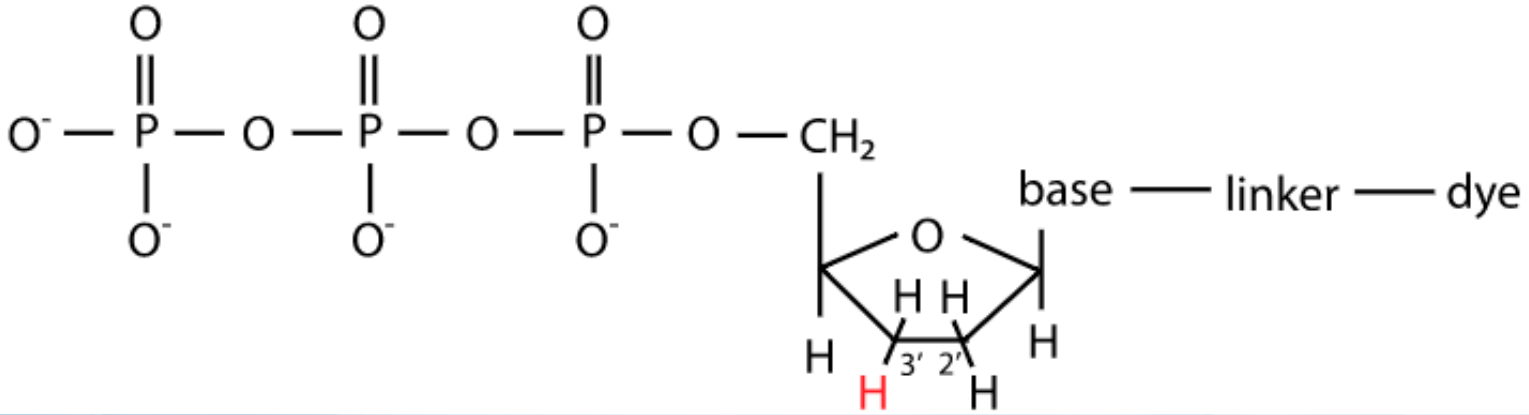
Because they lack the –OH, replication stops

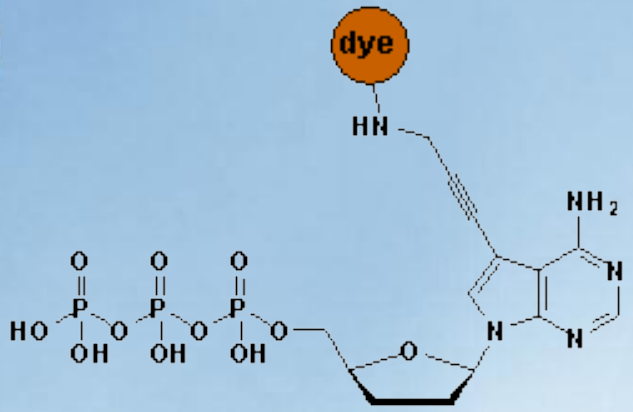
ddNTPs are the terminator molecules…

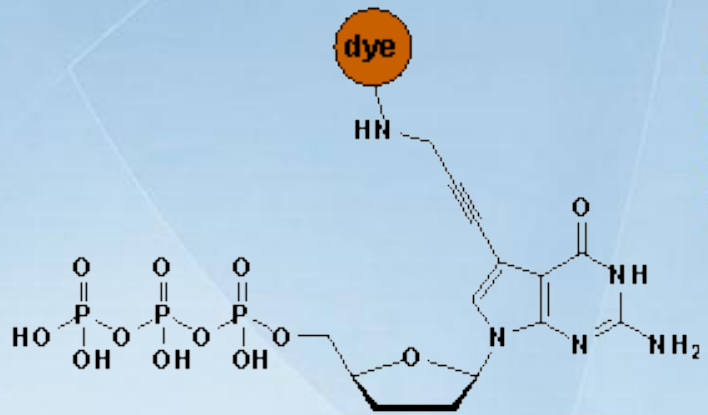
- **dd ATP**
- **dd GTP**
- **dd CTP**
- **dd TTP**

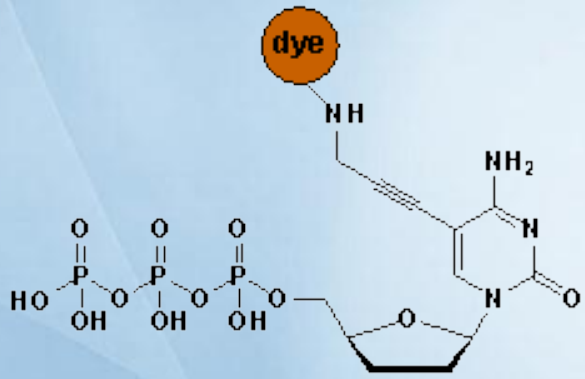
Bigdye terminator

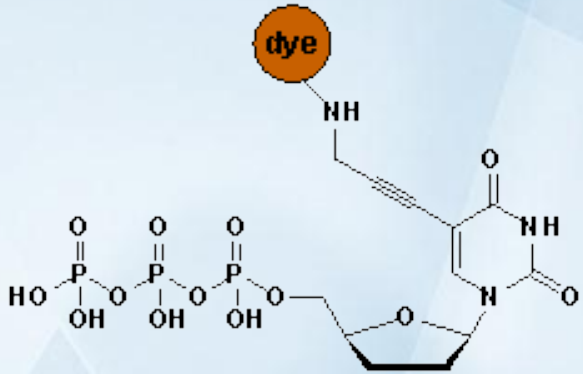
Sanger fluorescent dideoxynucleotide (ddNTP)





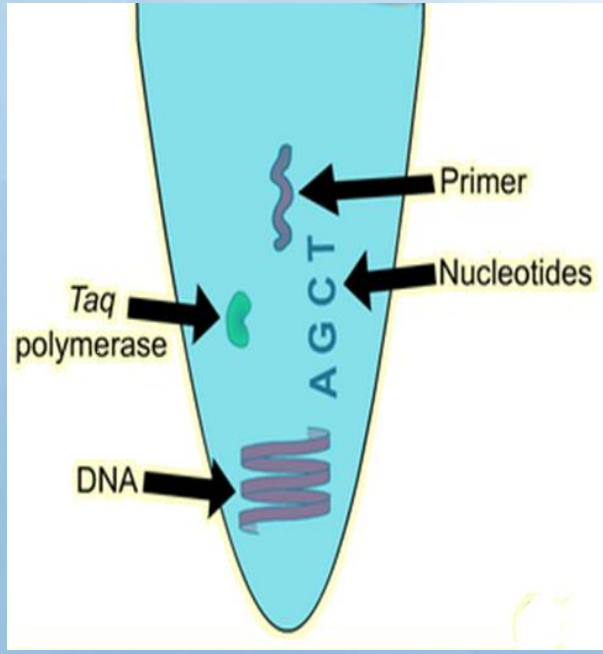


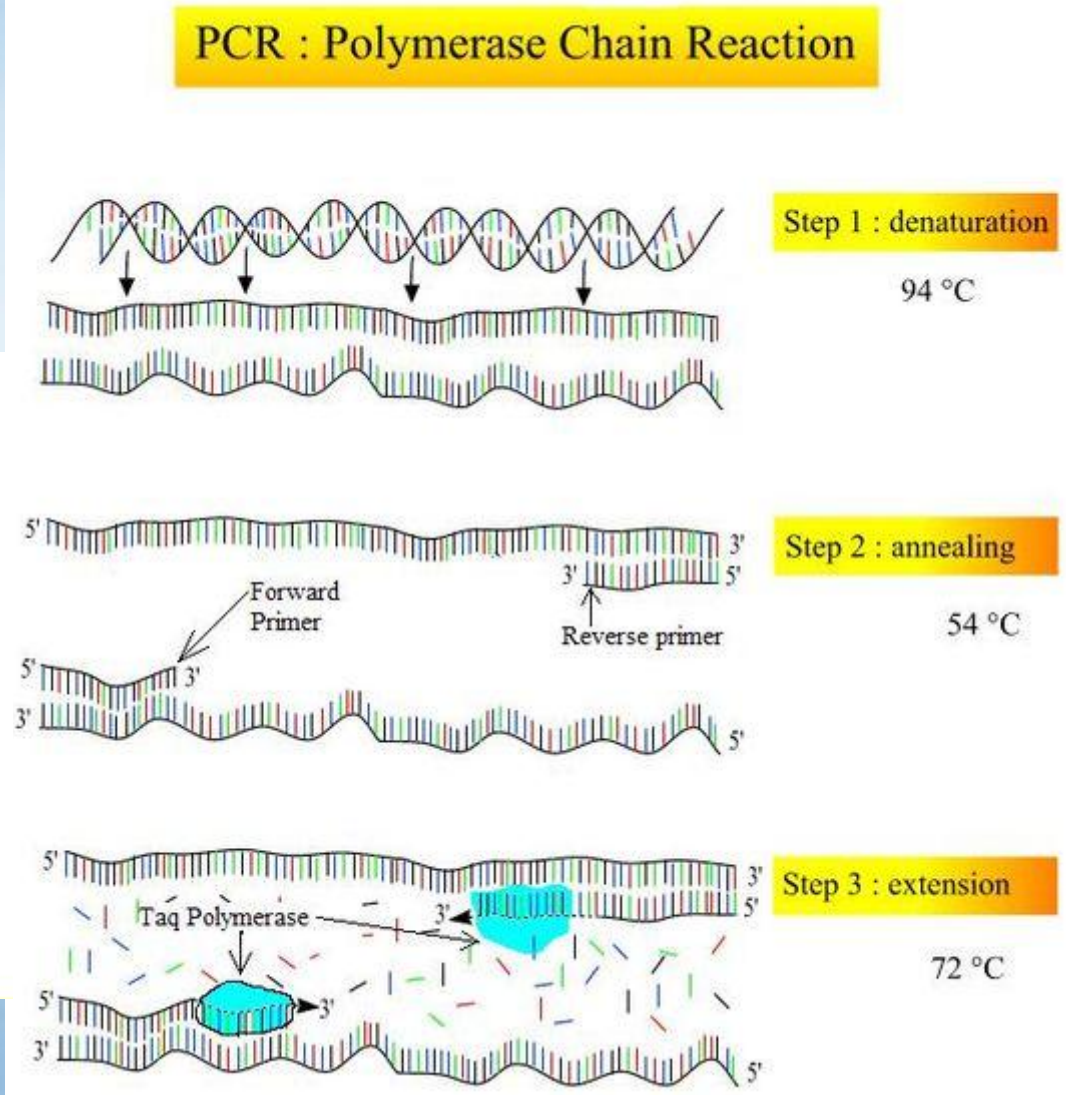




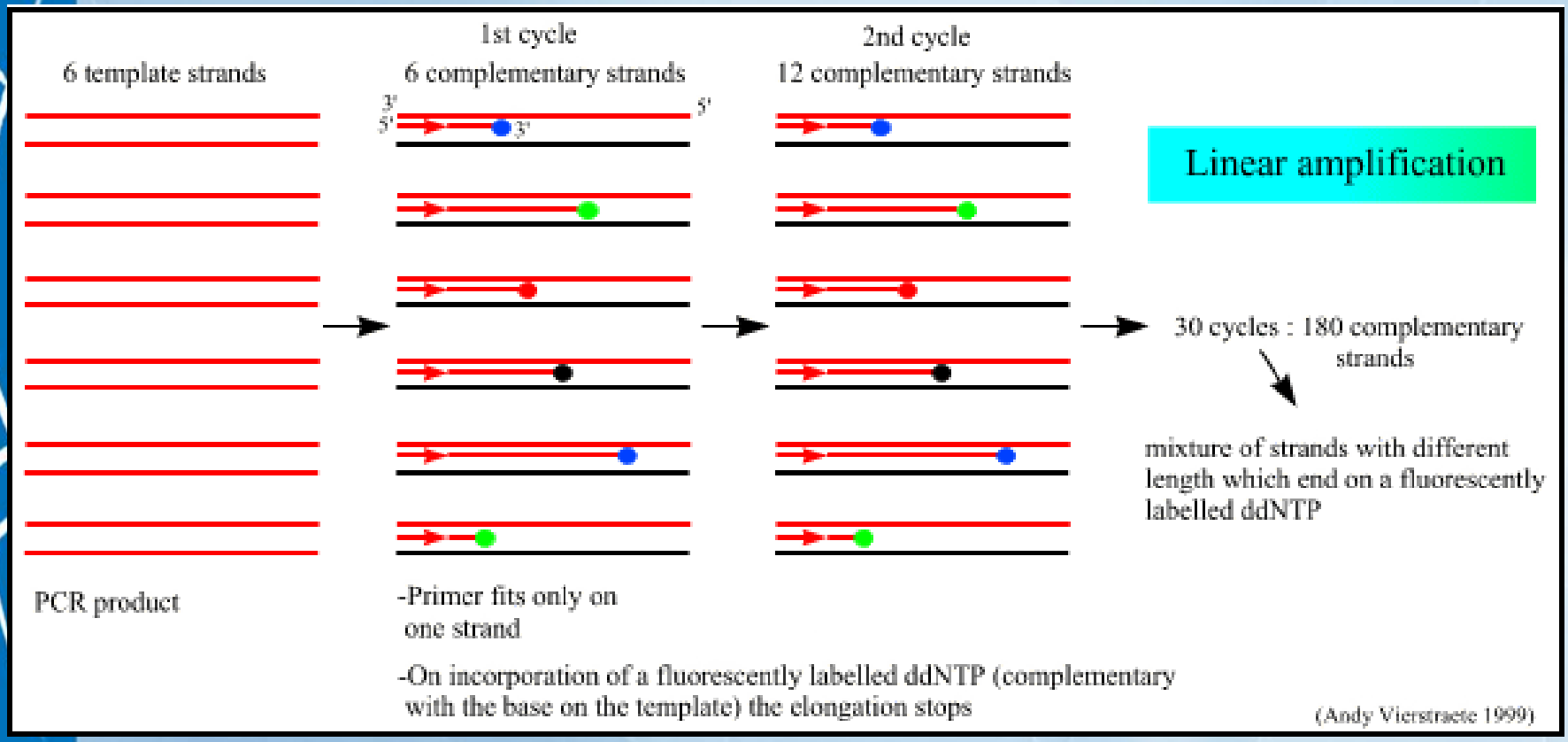
Terminator	Acceptor Dye	Emission Peak (nm)	Electropherogram Color
ddATP	dichloroR6G	565	green
ddCTP	dichloroROX	630	blue
ddGTP	dichloroR110	535	black
ddTTP	dichloroTAMRA	600	red

What will happen if ddATP, ddGTP, ddCTP, ddTTP are added ?



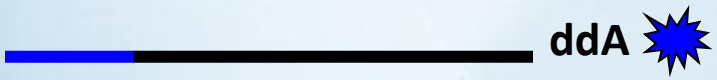


What will happen if ddATP, ddGTP, ddCTP, ddTTP are added ?

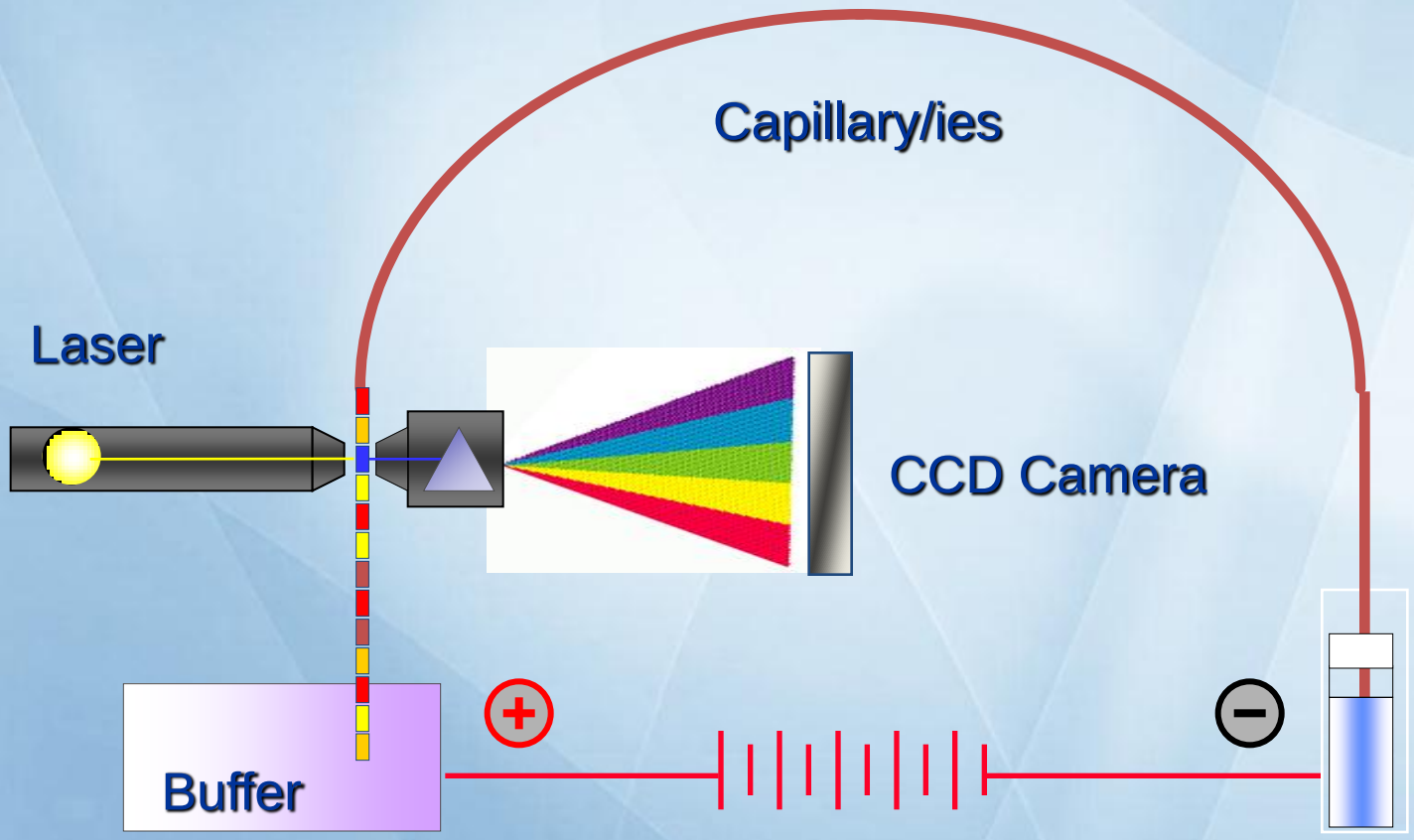


Fluorescent Dyes

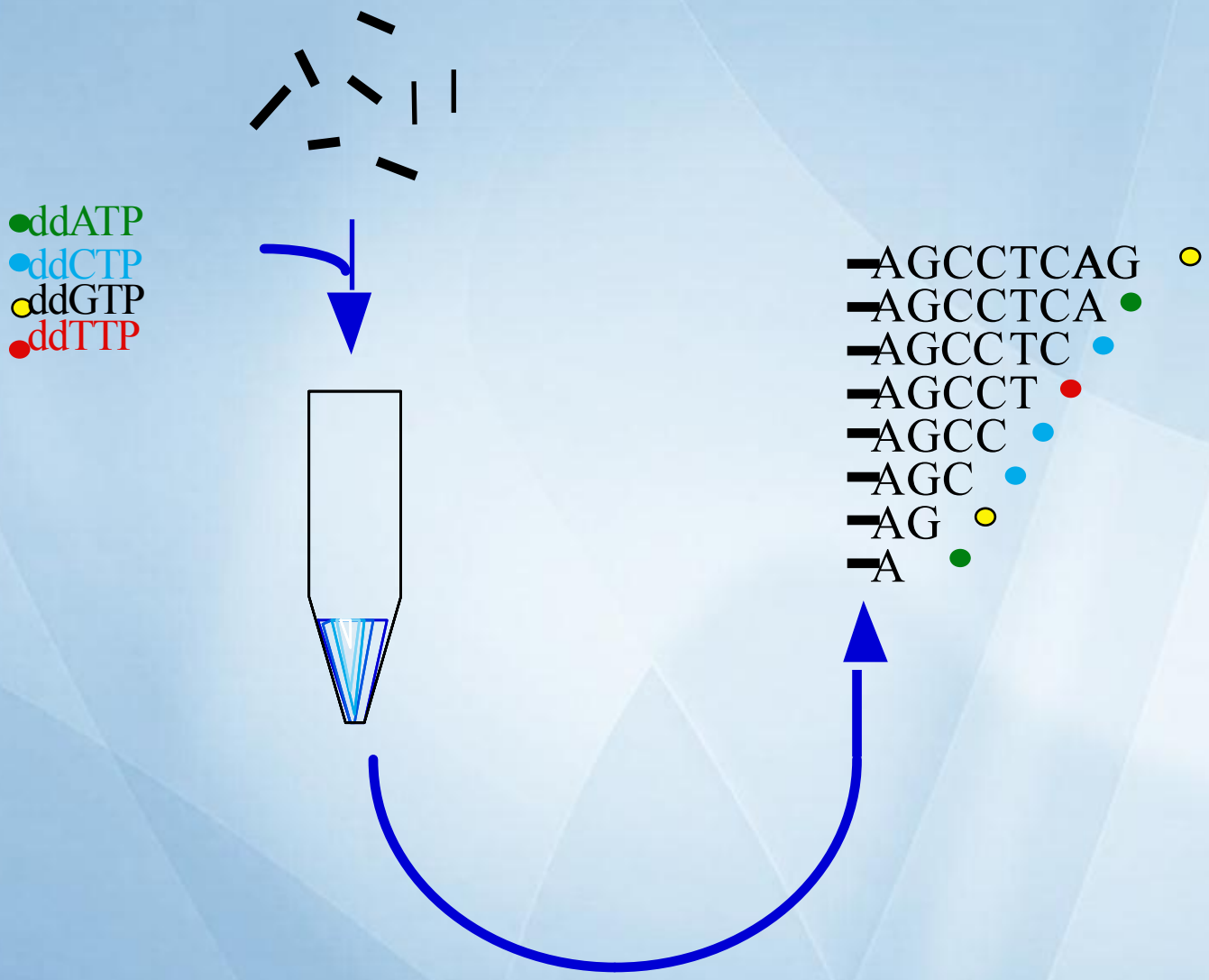
- In dye terminator sequencing, the fluorescent dye molecules are covalently attached to the dideoxynucleotides, labeling the sequencing ladder at the 3′ ends of the chains.



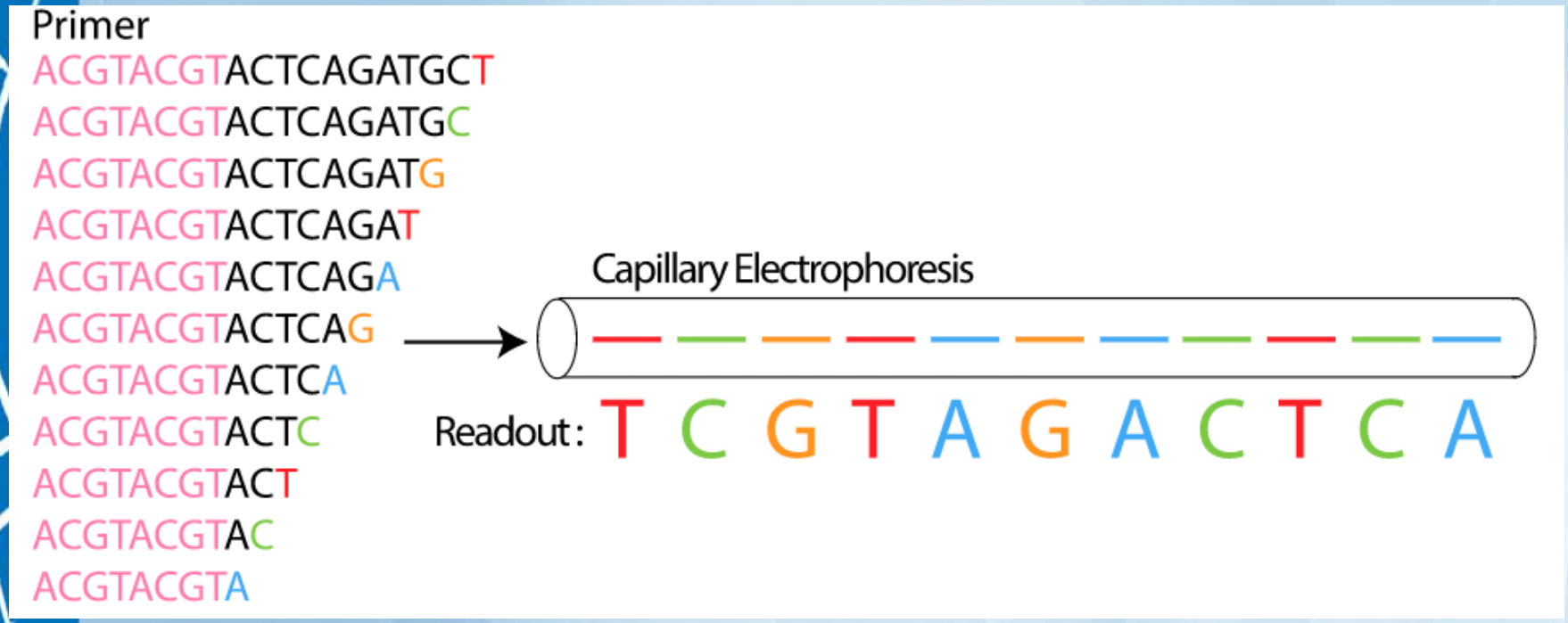
High-throughput seqeuncing: Capillary electrophoresis



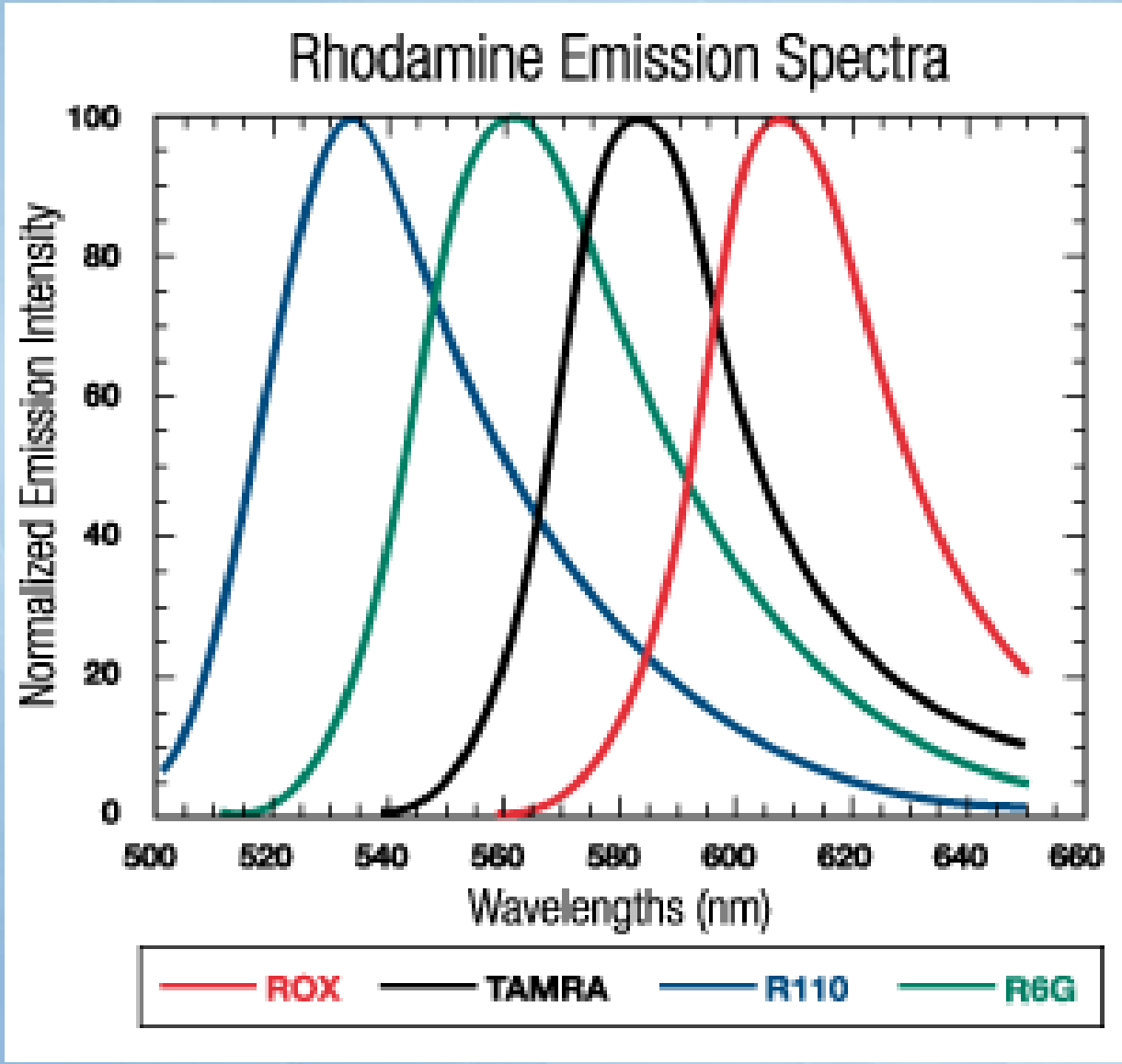
Sequencing Reaction



- Proportion of the dNTPs and ddNTPs 100 : 1

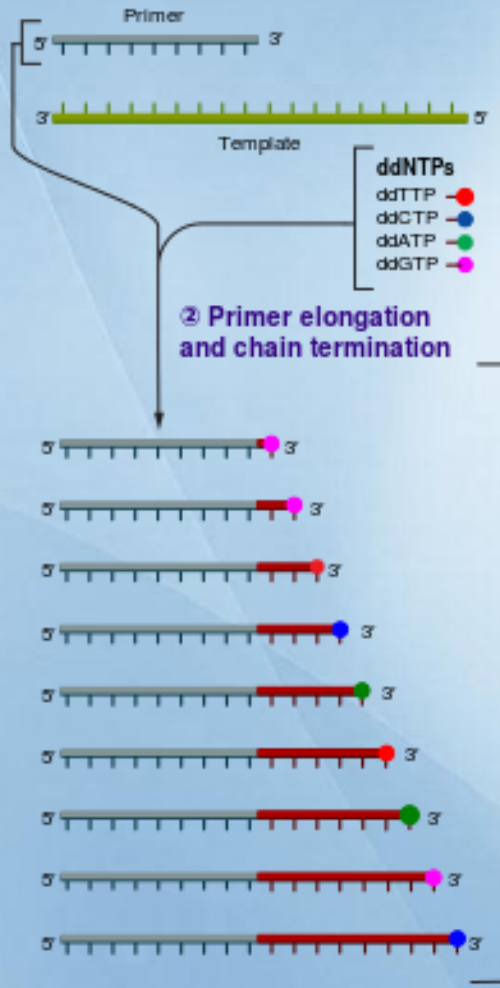

Primer
ACGTACGTACTCAGATGCT
ACGTACGTACTCAGATGC
ACGTACGTACTCAGATG
ACGTACGTACTCAGAT
ACGTACGTACTCAGA
ACGTACGTACTCAG
ACGTACGTACTCA
ACGTACGTACTC
ACGTACGTACT
ACGTACGTAC
ACGTACGTA
Capillary Electrophoresis
Readout: T C G T A G A C T C A

Fluorescent end labeling of DNA

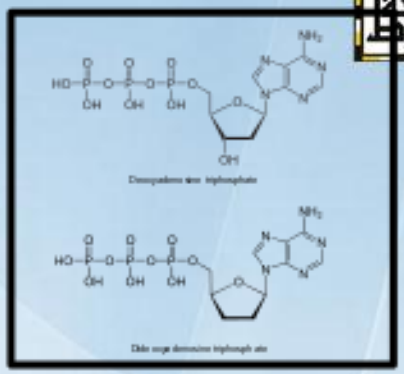


① Reaction mixture

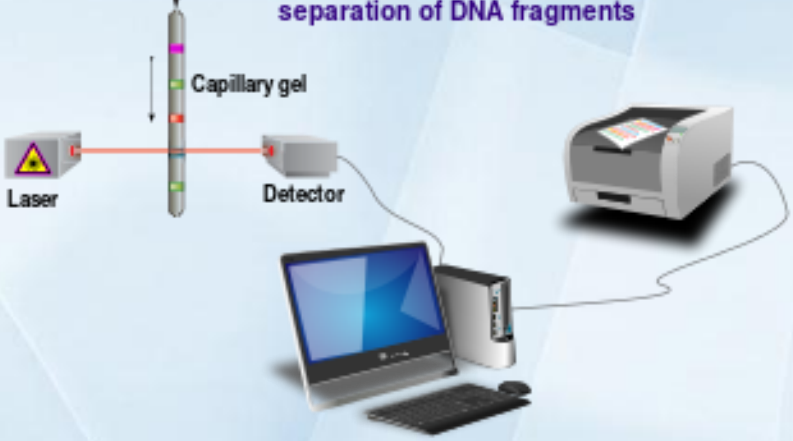
- Primer and DNA template
- DNA polymerase
- ddNTPs with flourochromes
- dNTPs (dATP, dCTP, dGTP, and dTTP)

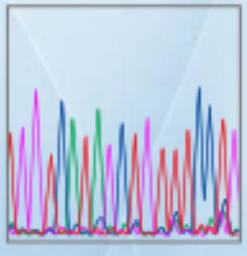


② Primer elongation and chain termination



③ Capillary gel electrophoresis separation of DNA fragments





Chromatograph

④ Laser detection of flourochromes and computational sequence analysis

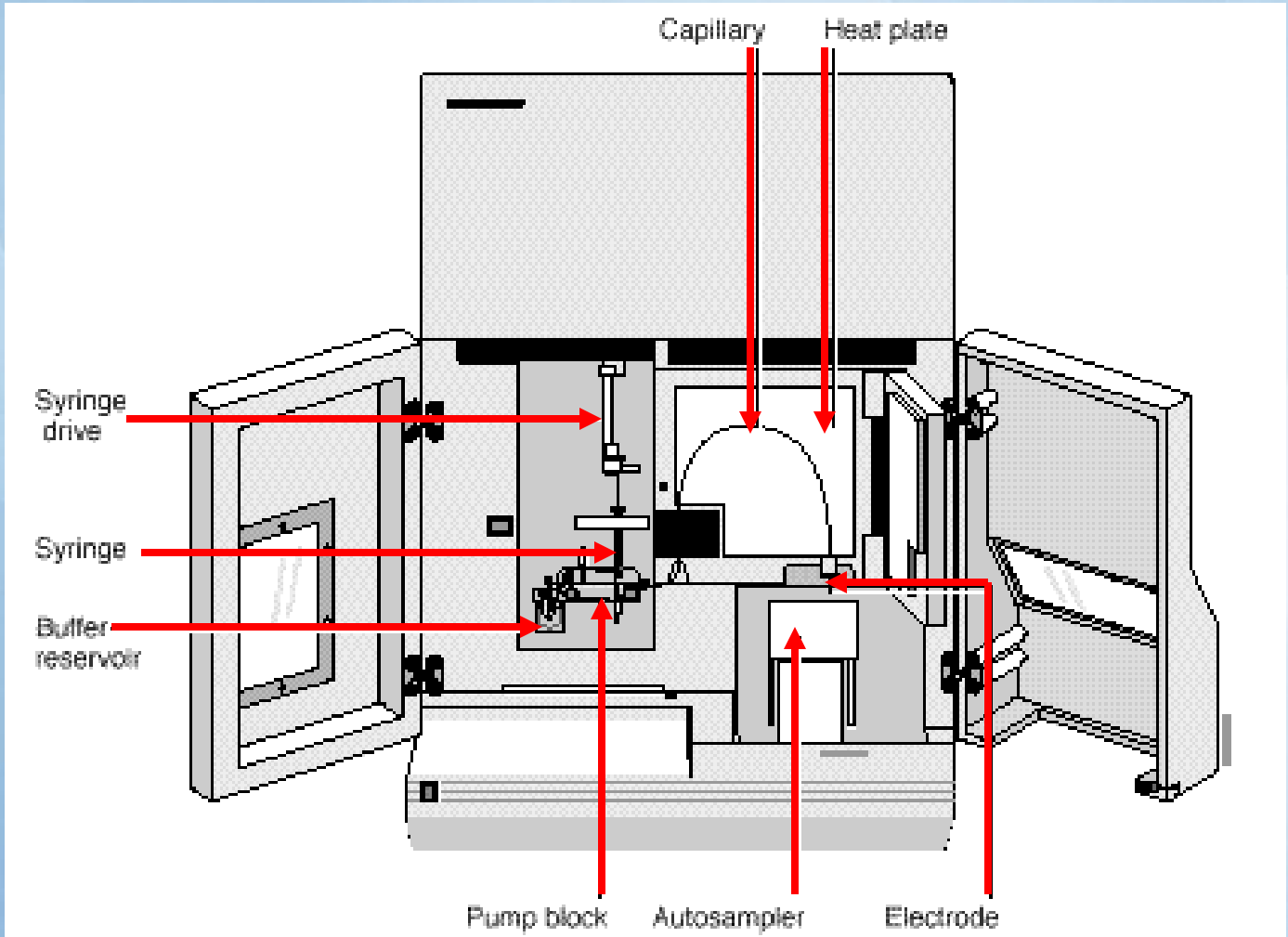
When voltage applied, strands separate by size in capillary, smallest go through first

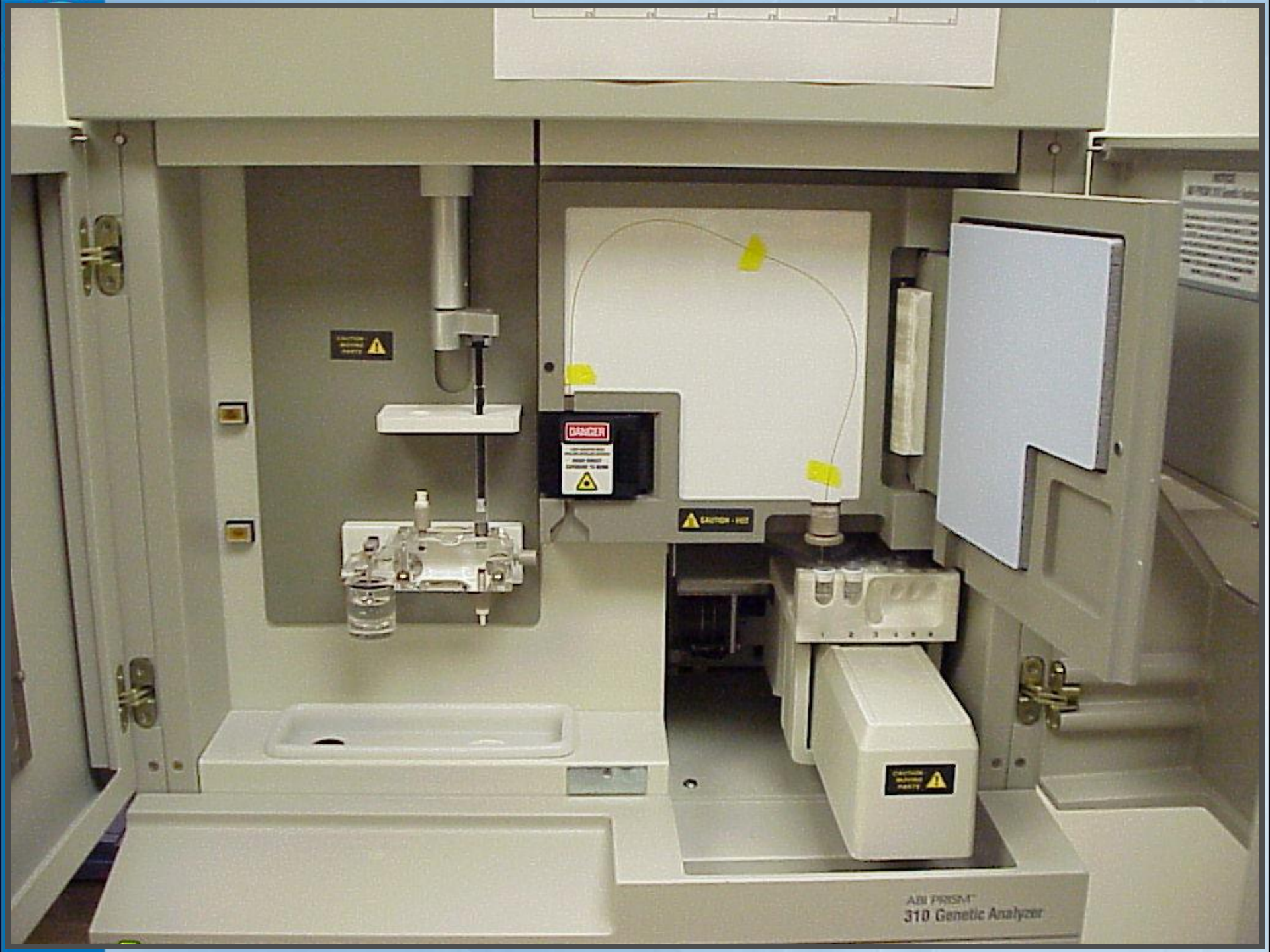
ABI prism 310

Capillary electrophoresis

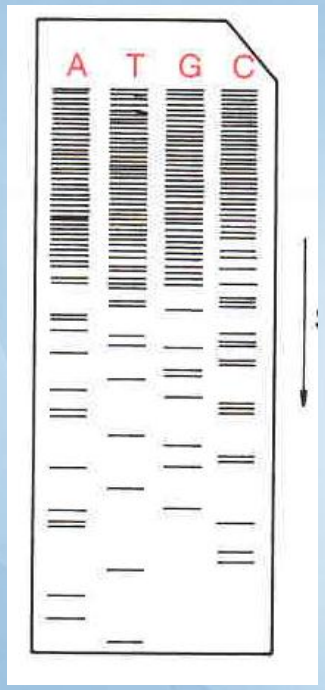
ABI prism 310

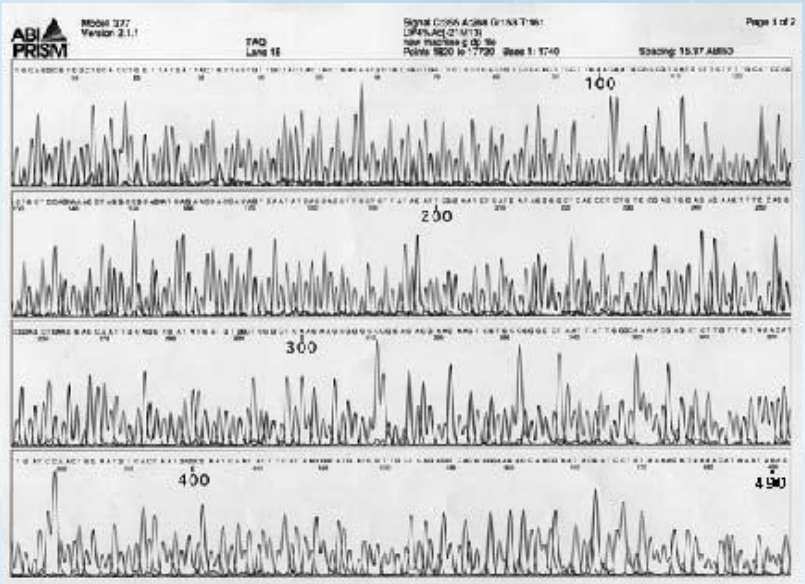
Capillary electrophoresis




DANGER
CAUTION - HOT
ABI PRISM
310 Genetic Analyzer

Automated DNA sequencing

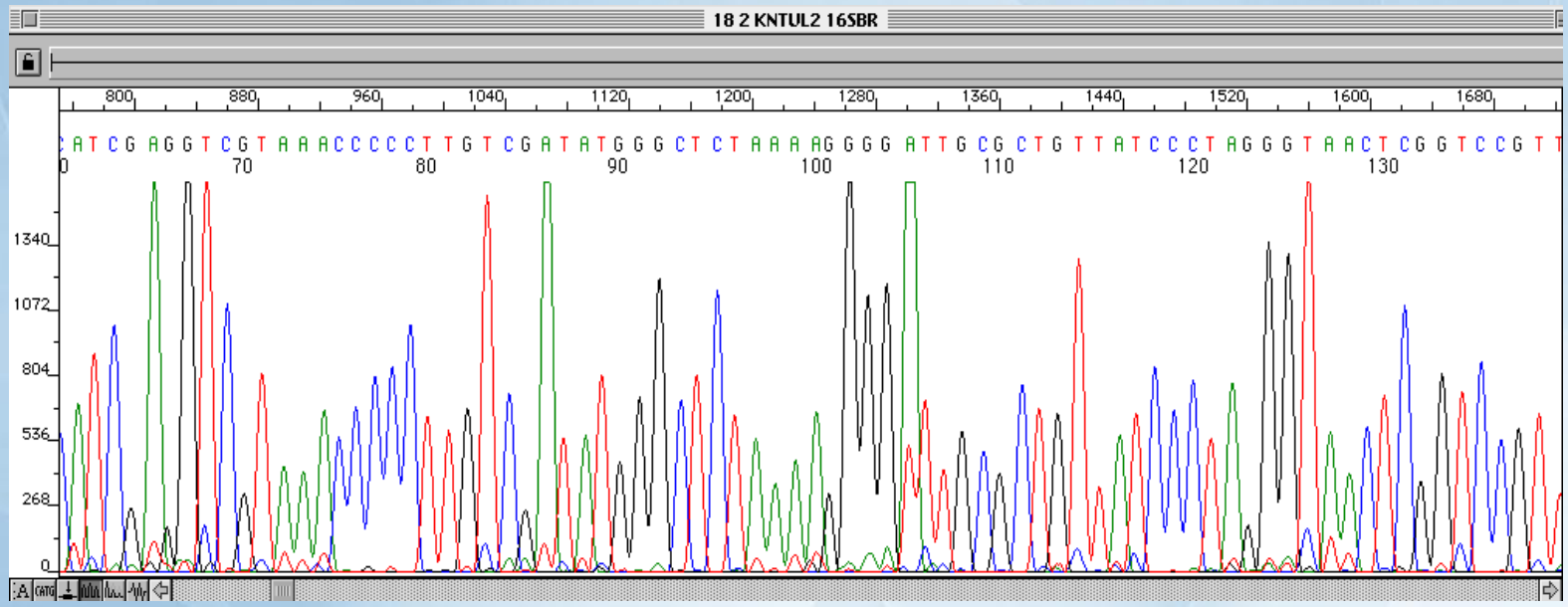




A computer read-out of the gel generates a “false color” image
where each color corresponds to a base. Then the intensities are
translated into peaks that represent the sequence.

Data output

Data in electropherogram format shows peaks .abi file
Free software sequence scanner v1.0 (Life Tech).

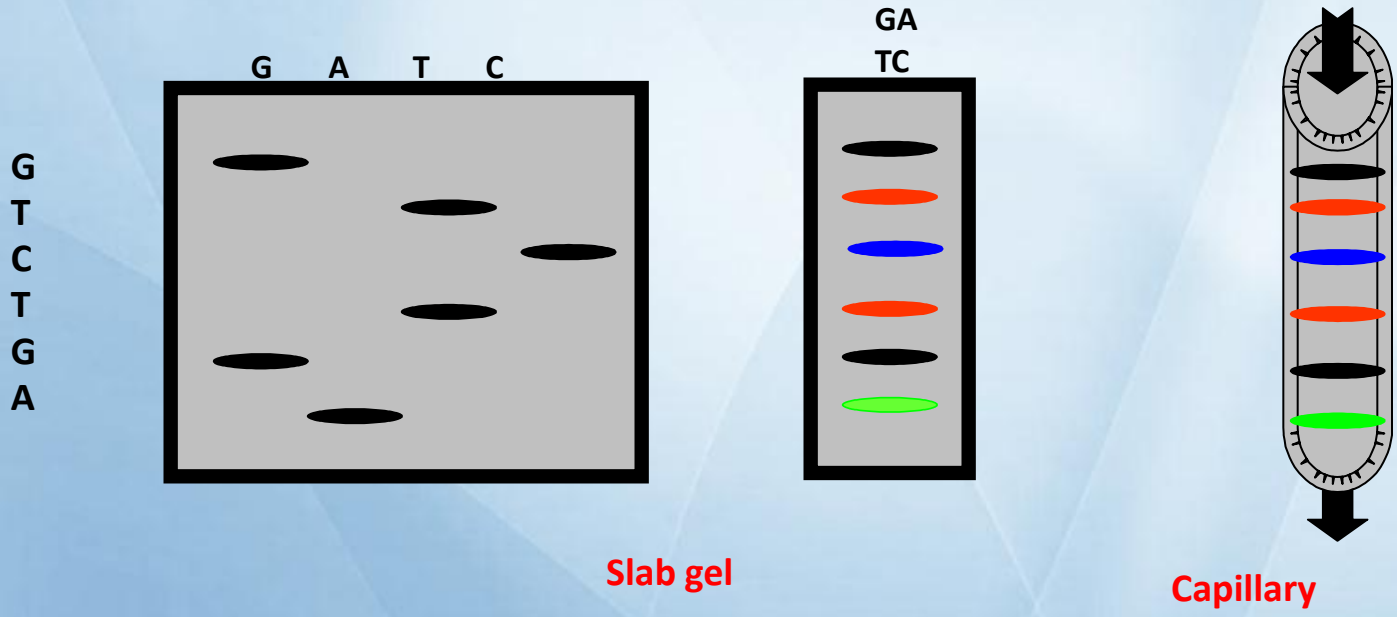


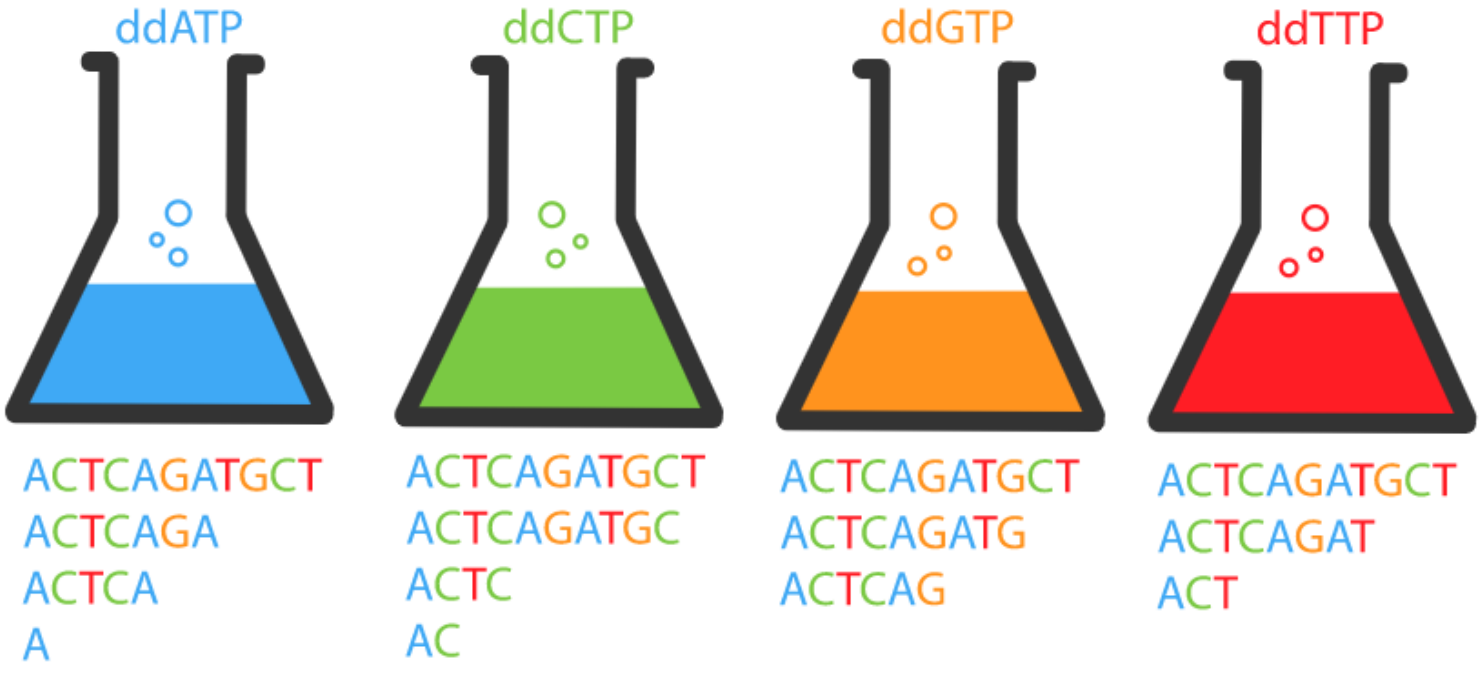
Data in sequence file format shows text .seq file

Movie

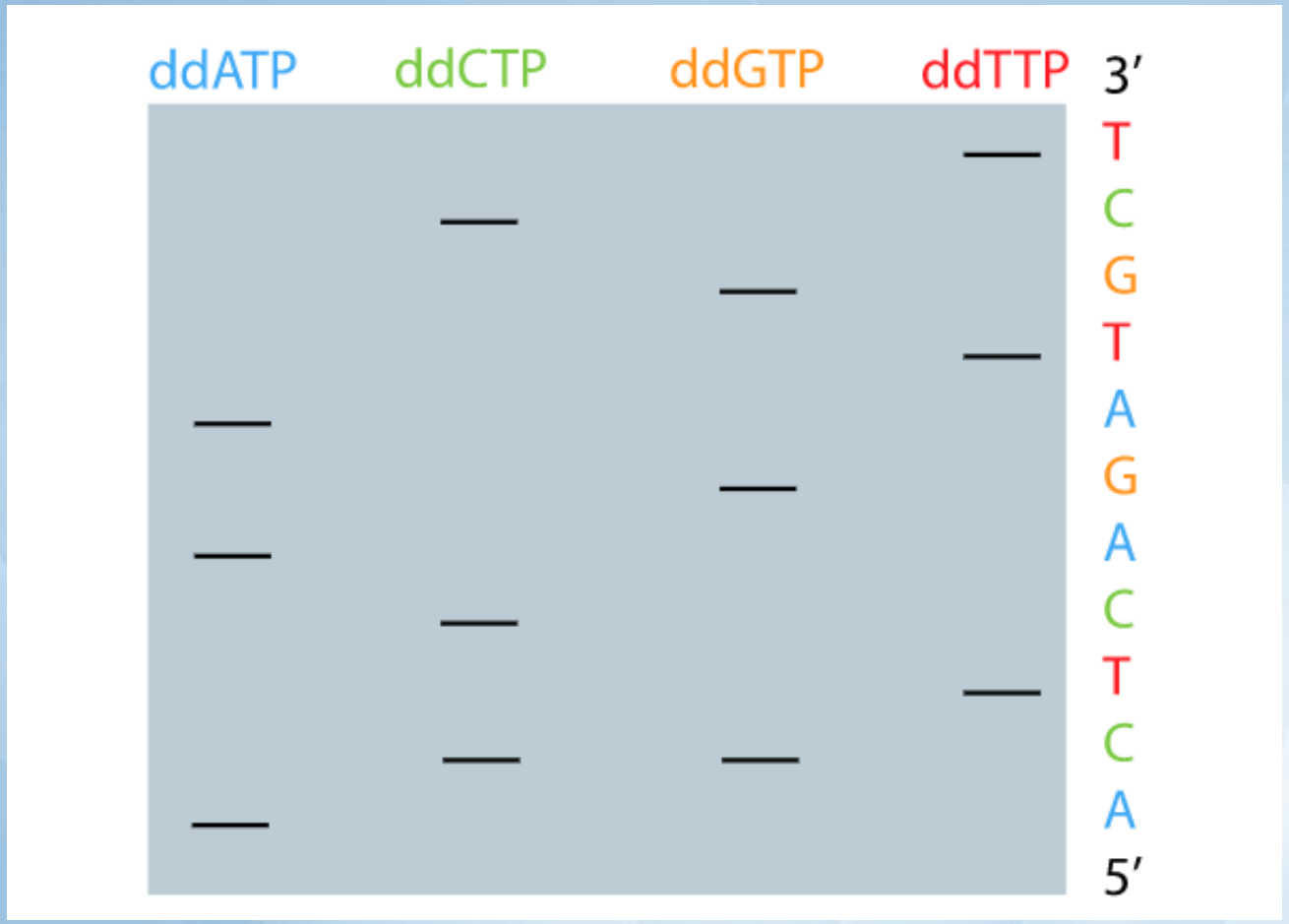
Dye Terminator Sequencing

The DNA ladder is resolved in one gel lane or in a capillary.



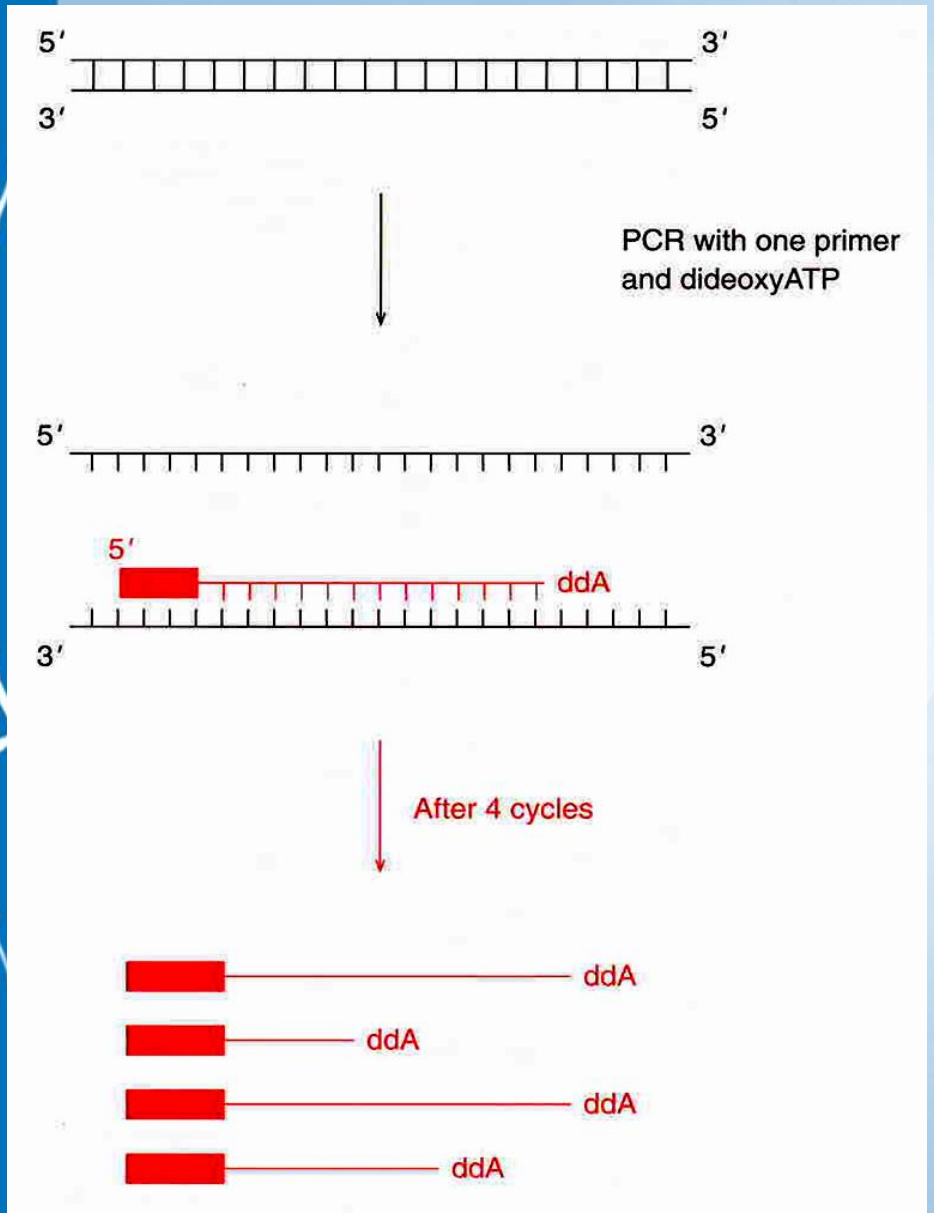


ACTCAGATGCT



ACTCAGATGCT

cycle sequencing: denaturation occurs during temperature cycles



94°C:DNA denatures

45°C: primer anneals

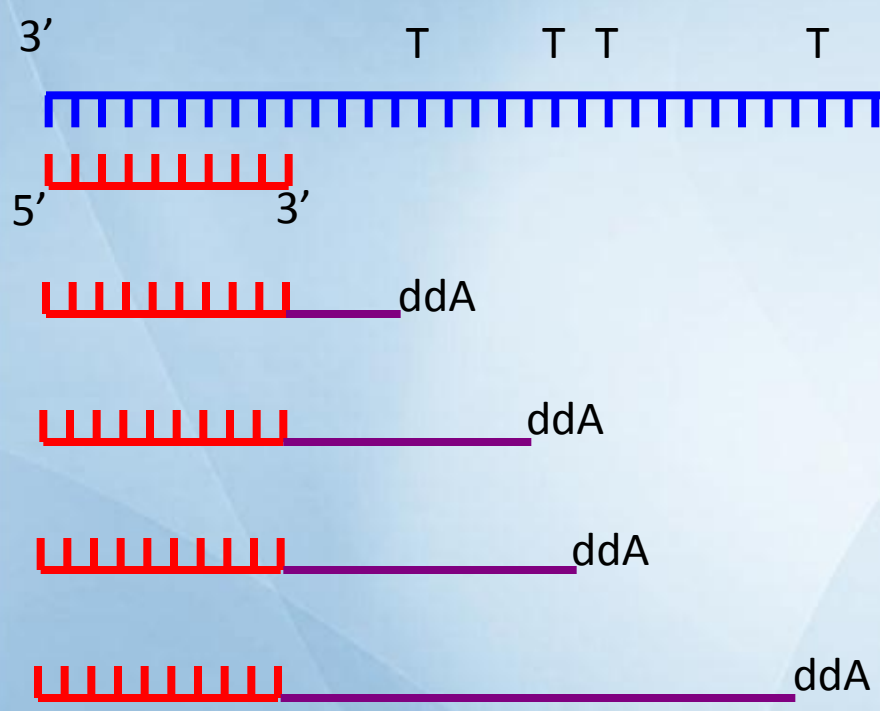
60-72°C: thermostable DNA pol extends primer

Repeat 25-35 times

Advantages: don't need a lot of template DNA

Disadvantages: DNA pol may incorporate ddNTPs poorly

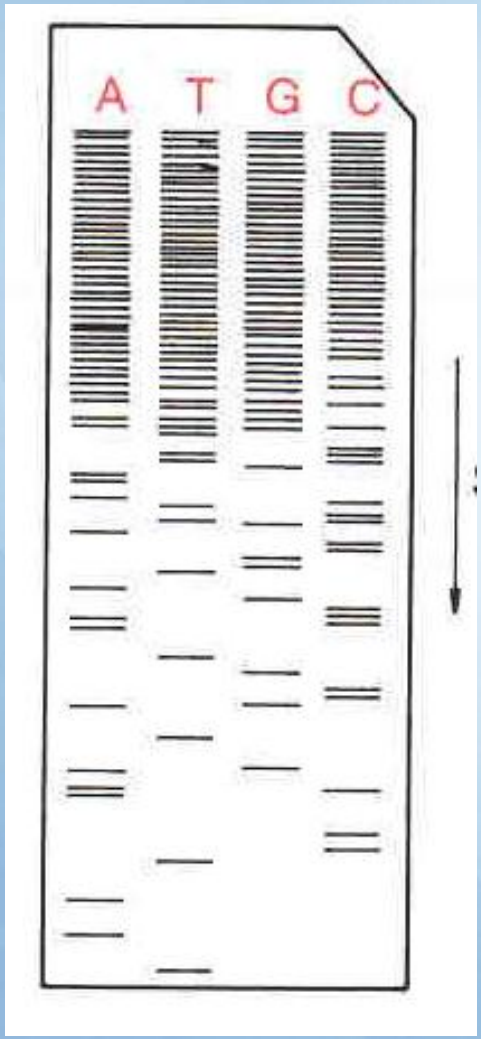
Sanger dideoxy sequencing: basic method

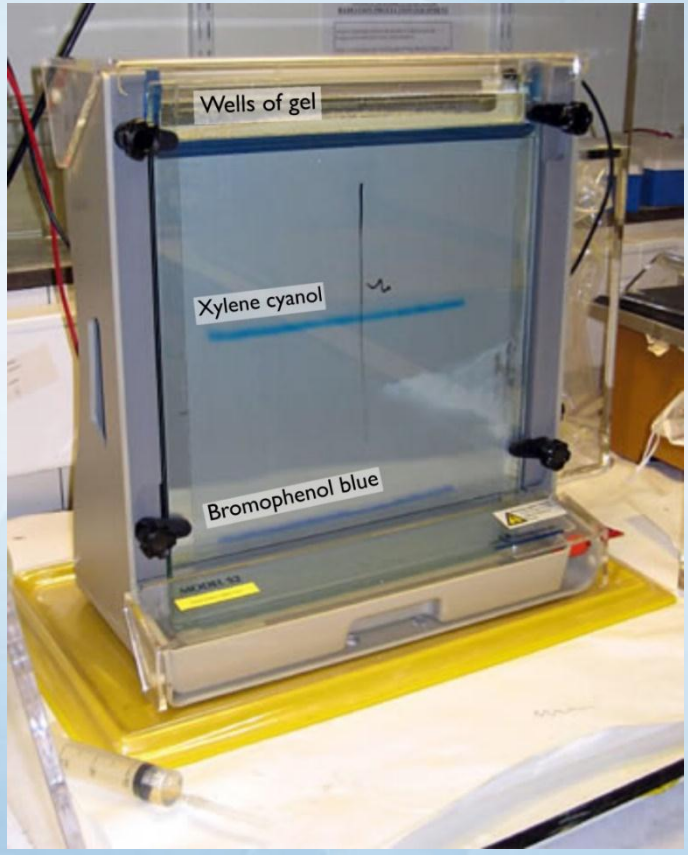


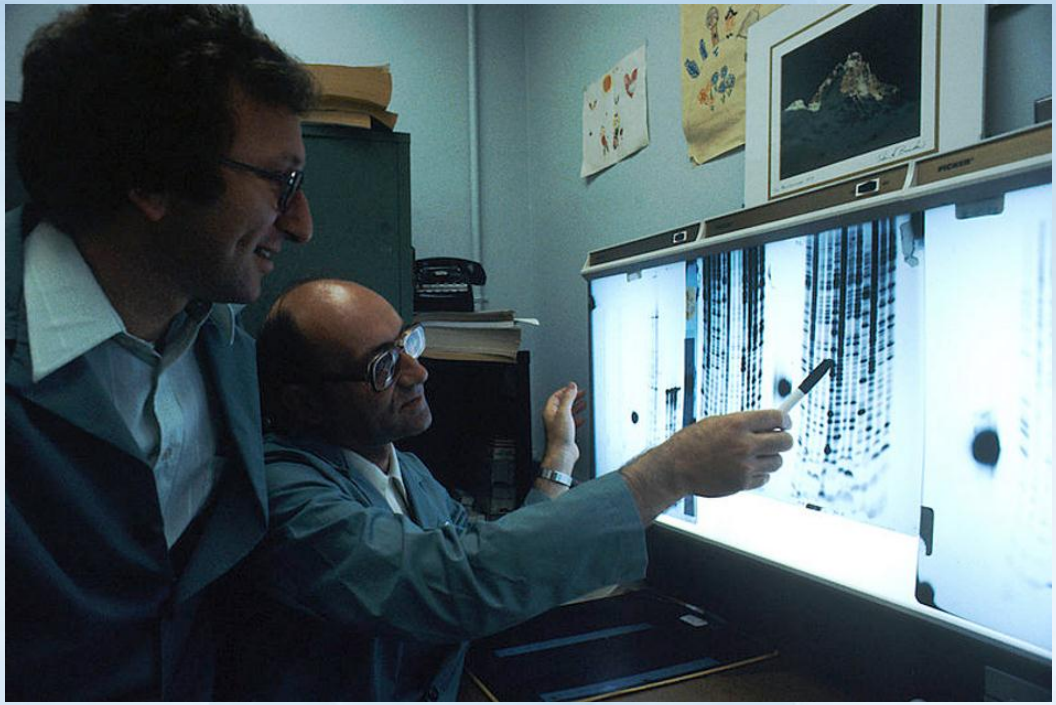
ddATP in the reaction: anywhere there’s a T in the template strand, occasionally a ddA will be added to the growing strand

DNA sequencing gels: old school

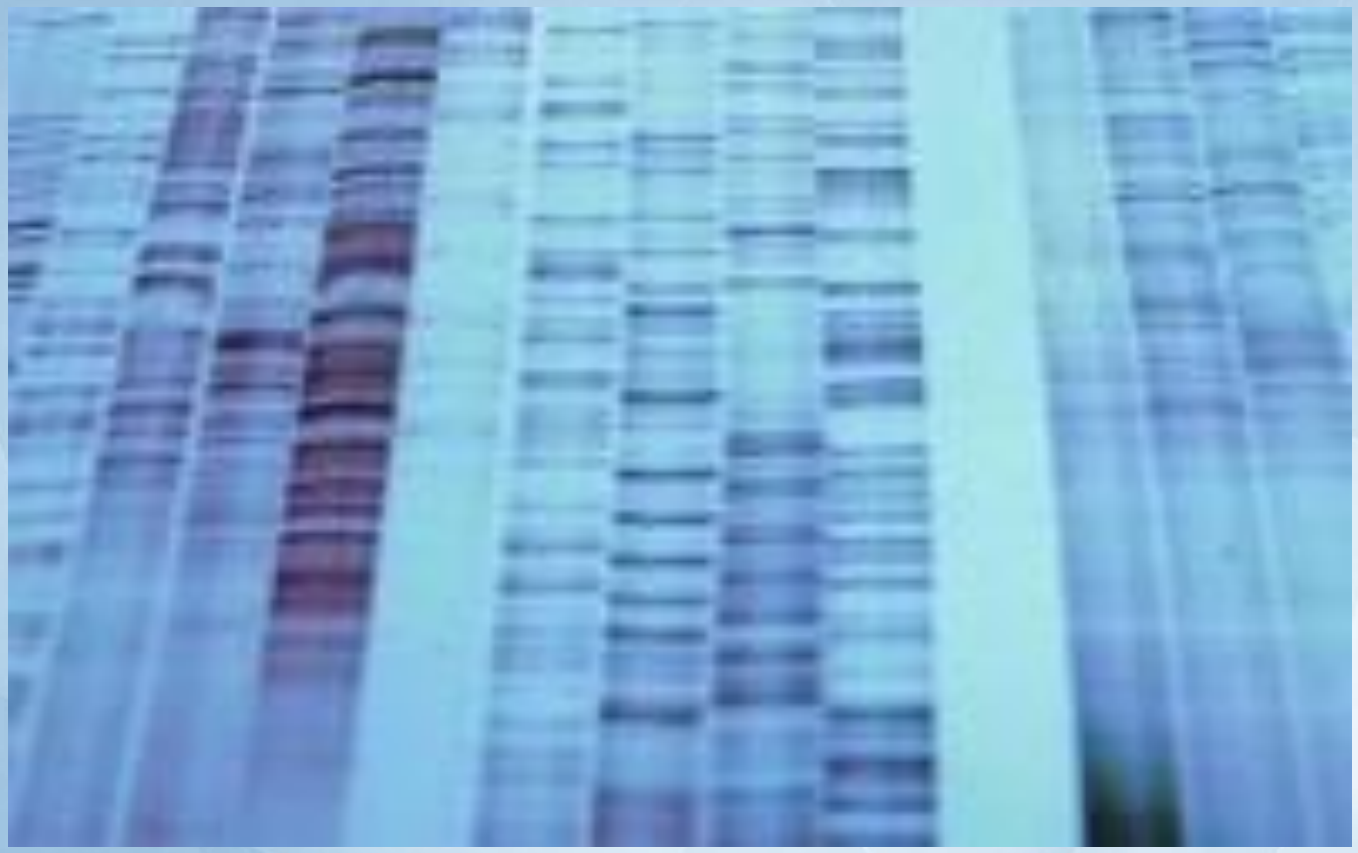
Analyze sequencing products by gel electrophoresis, autoradiography





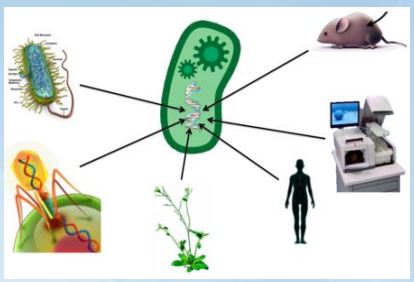


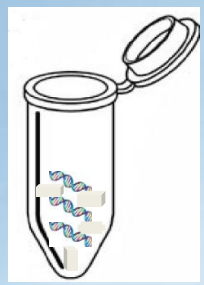
A sequencing gel



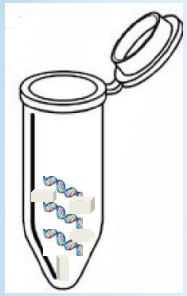
Sequencing workflow

1. DNA isolation

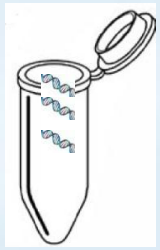




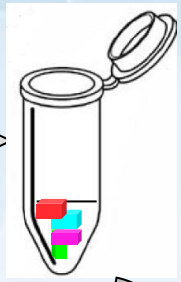
2. PCR amplification of the target gene



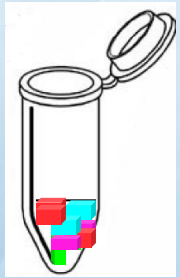
3. Purify PCR product



4. Set up and Perform sequencing reaction

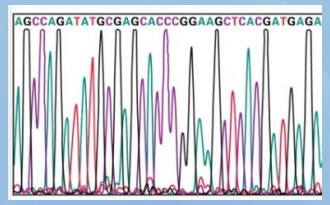


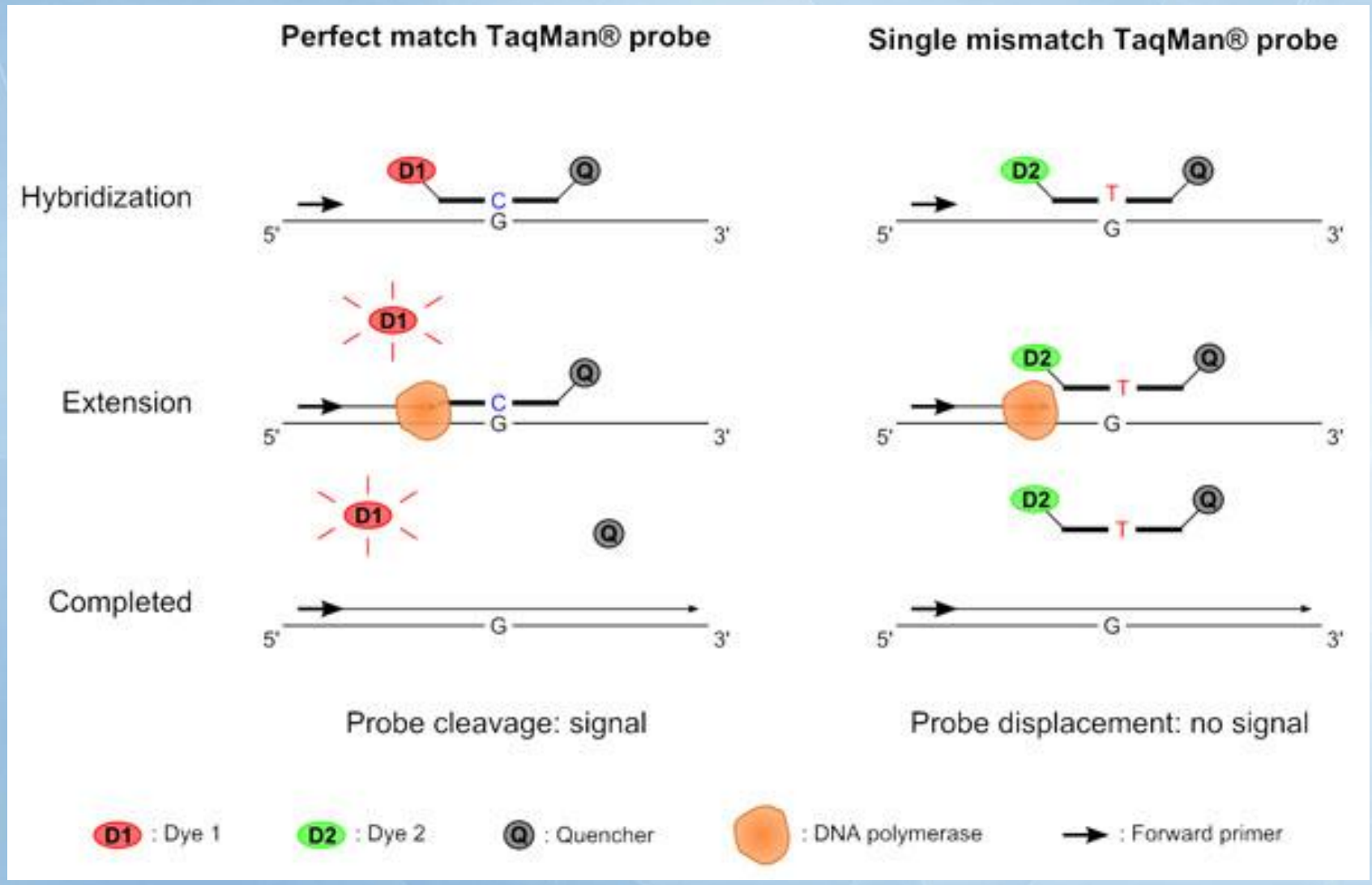
5. Purification PCR product



6. Resolve sequence fragments

7. Read order of terminators (DNA sequence)




Perfect match TaqMan® probe
Single mismatch TaqMan® probe
Hybridization
Extension
Completed
D1
D2
Q
C
T
G
5'
3'
Probe cleavage: signal
Probe displacement: no signal
D1 : Dye 1
D2 : Dye 2
Q : Quencher
: DNA polymerase
: Forward primer