Chapter V:

Nucleic acid sequence determination: **Sequencing**

DNA sequencing

- Maxam and Gilbert chemical method
- Sanger enzymatic method
- Automated Sequencing
- Pyrosequency
- Next generation of sequencing:
  - High-throughput sequencing

DNA sequencing

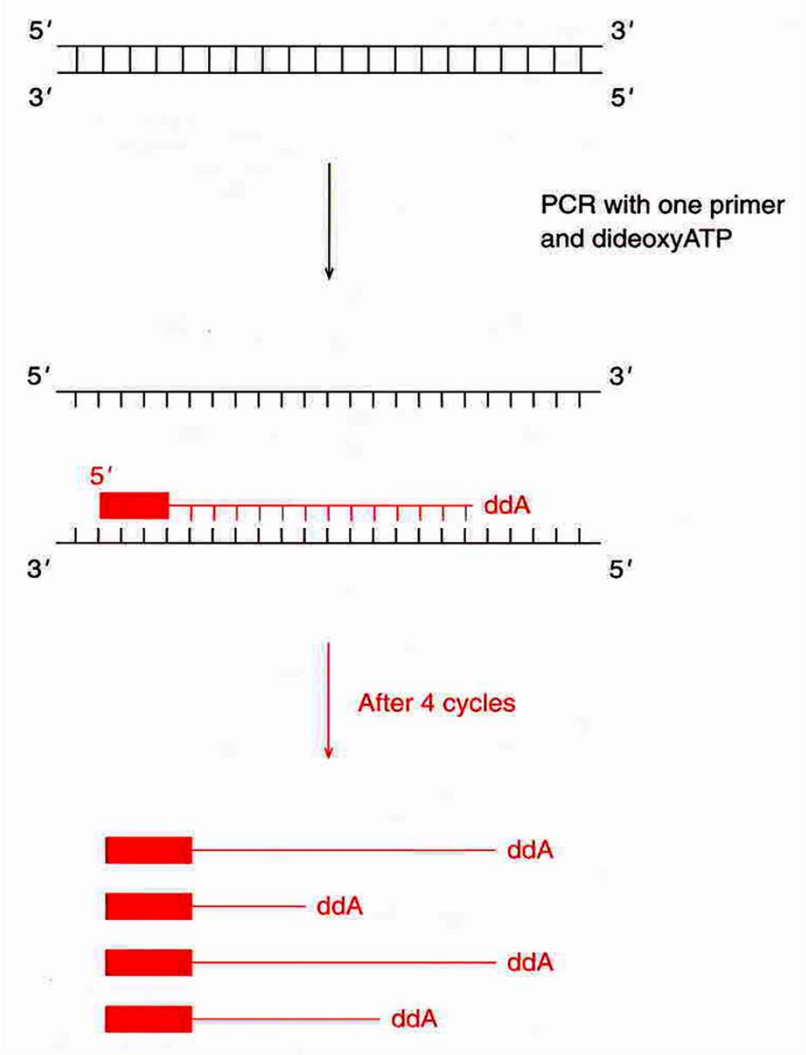
- DNA sequencing involves determining the order of nucleotides in a given DNA fragment.
- Used to identify and diagnose genetic diseases and in microbiology.
- An important tool for species classification.

Sanger sequencing

The most commonly used method (developed in 1977).
For Sanger sequencing you need:

1) DNA Matrix
2) A primer for DNA synthesis
3) DNA polymerase
4) deoxynucleotide triphosphates (dNTPs) and dideoxynucleotide triphosphates (ddNTPs) (32P)

Sequencing cycle:



95° C: Denatured DNA

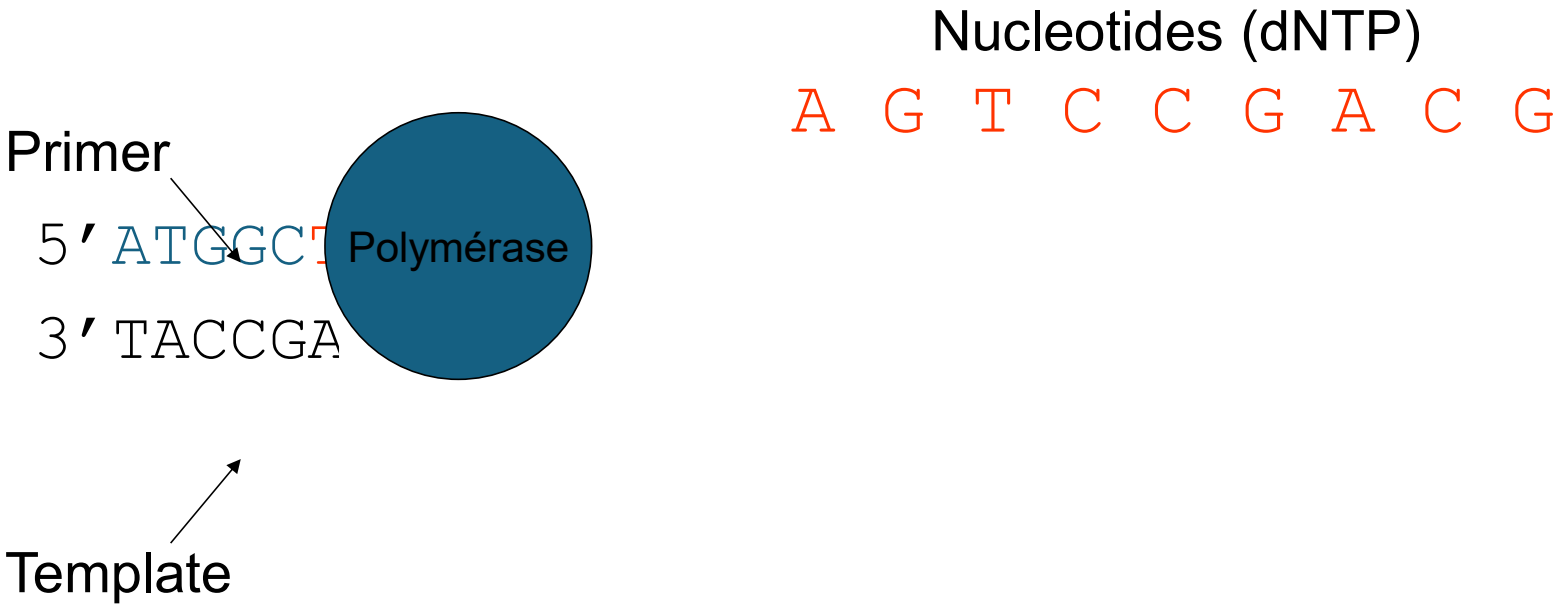
45° C: primer hybridization

60-72° C: primer elongation by thermostable DNA polymerase

Repeat 25 times (25 cycles)

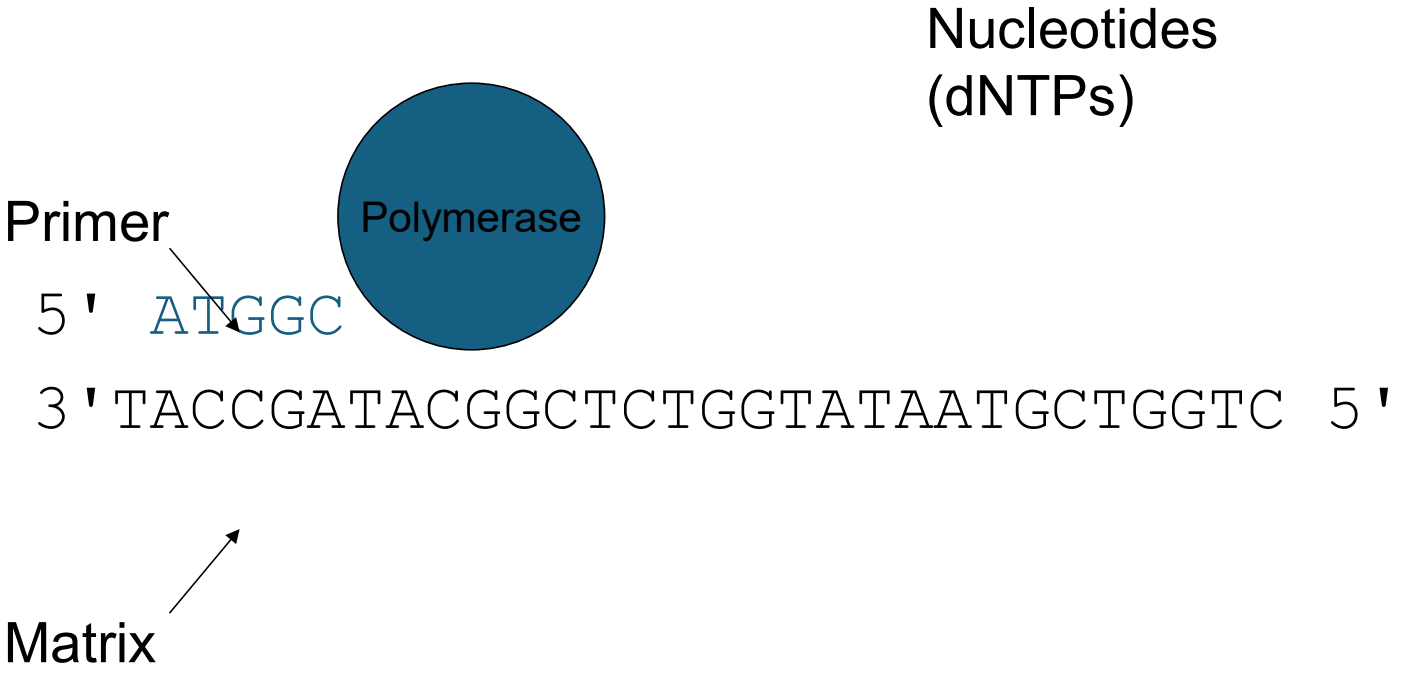
Sanger sequencing

- Polymerisation of DNA



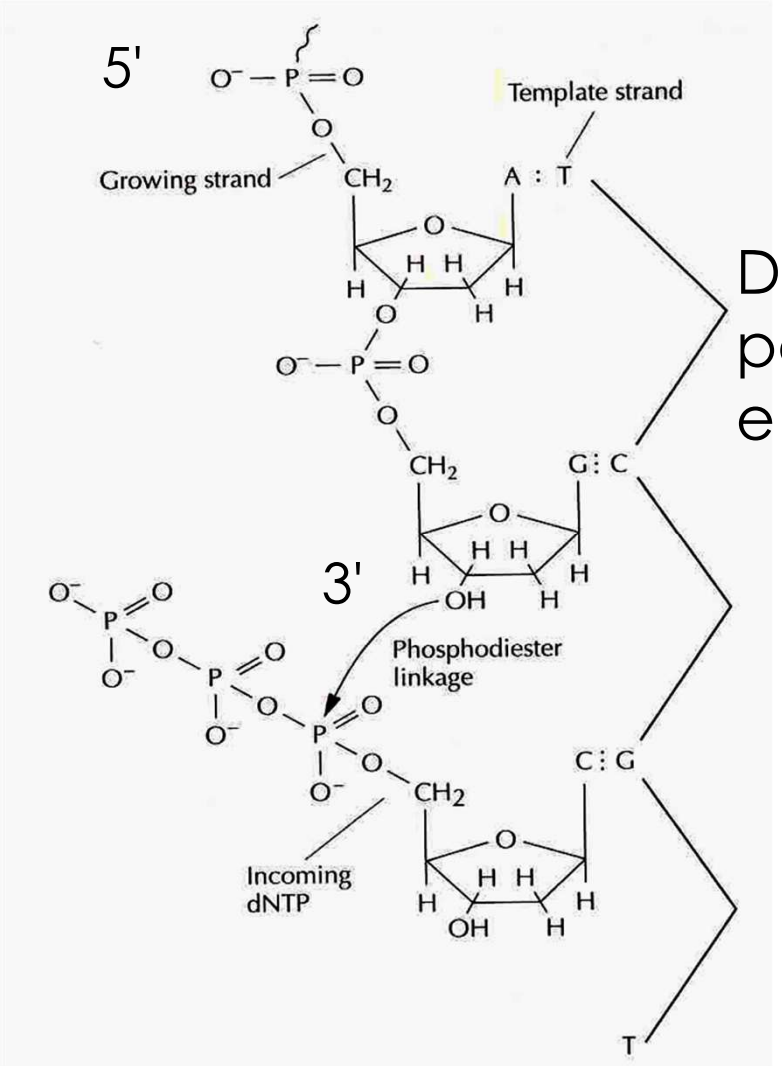
Sanger sequencing

- DNA polymerization



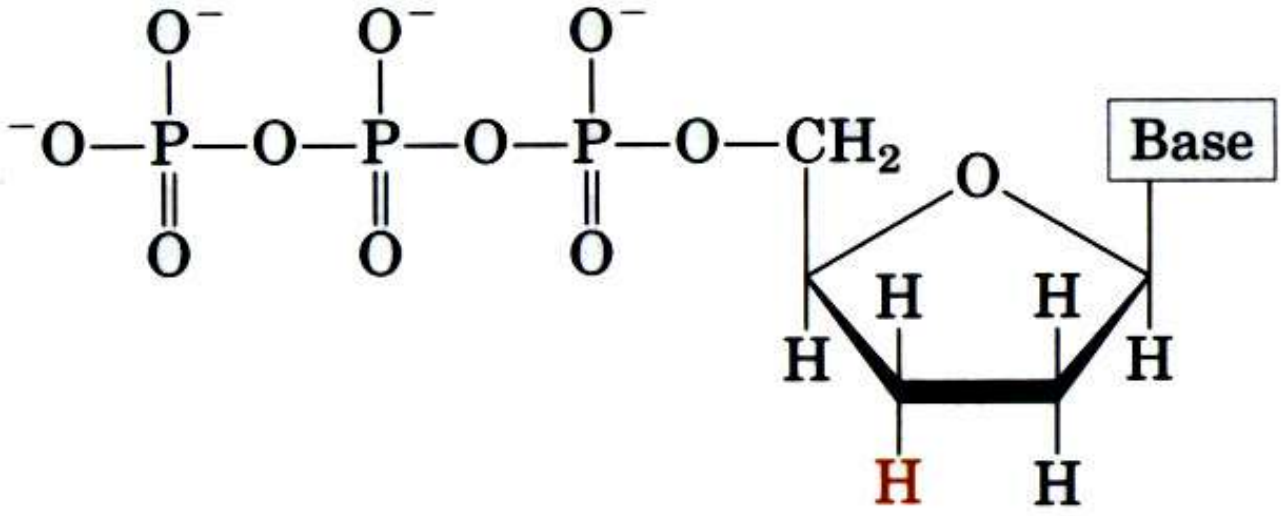
Sanger sequencing

Primer elongation with DNA polymerase in the presence of the four dNTPs, with a limited amount of radioactive dideoxy NTPs (ddNTPs)



DNA polymerase direction

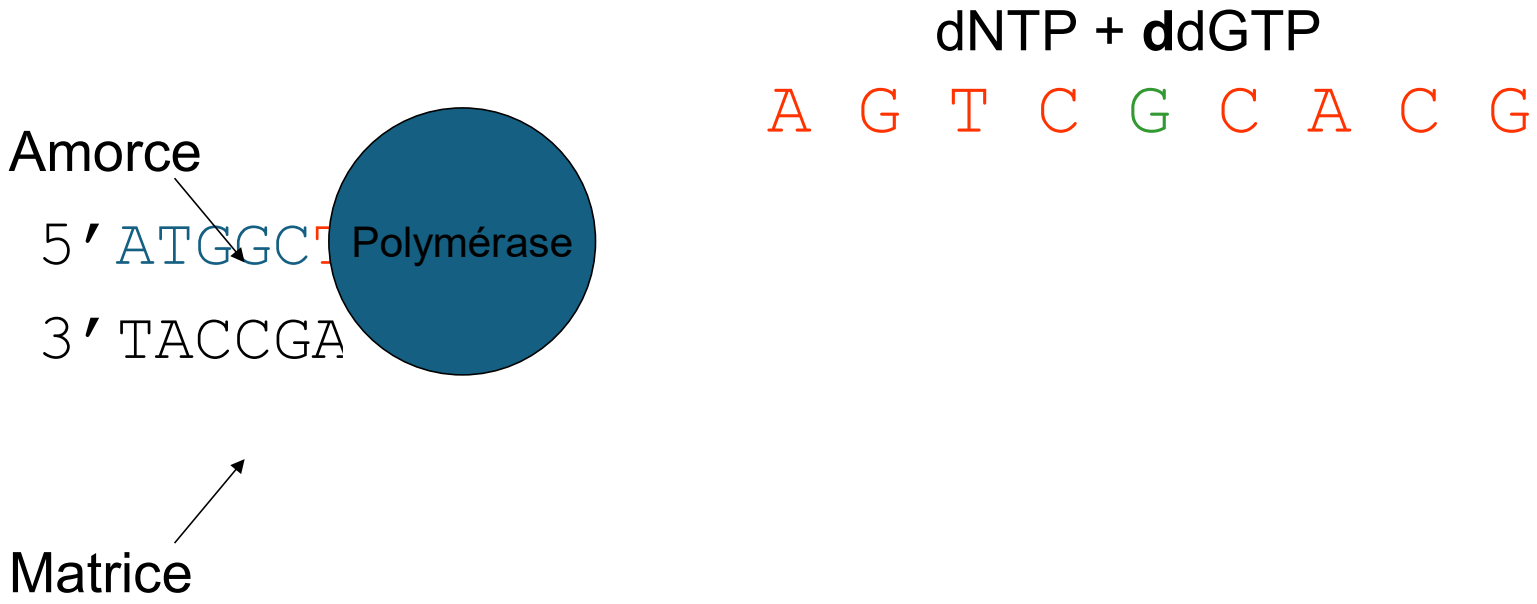
Dideoxy Nucleotide



ddNTP Analog

Sanger sequencing

- Sequencing reaction (ddGTP)



Sanger sequencing

- Sequencing reaction (ddGTP)

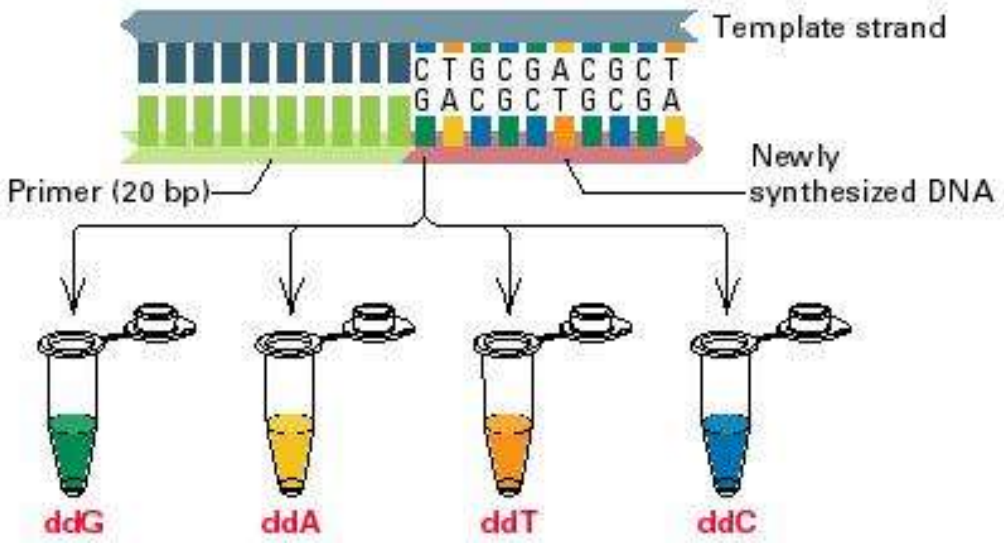
Random incorporation of a ddNTP stops synthesis

Sanger sequencing

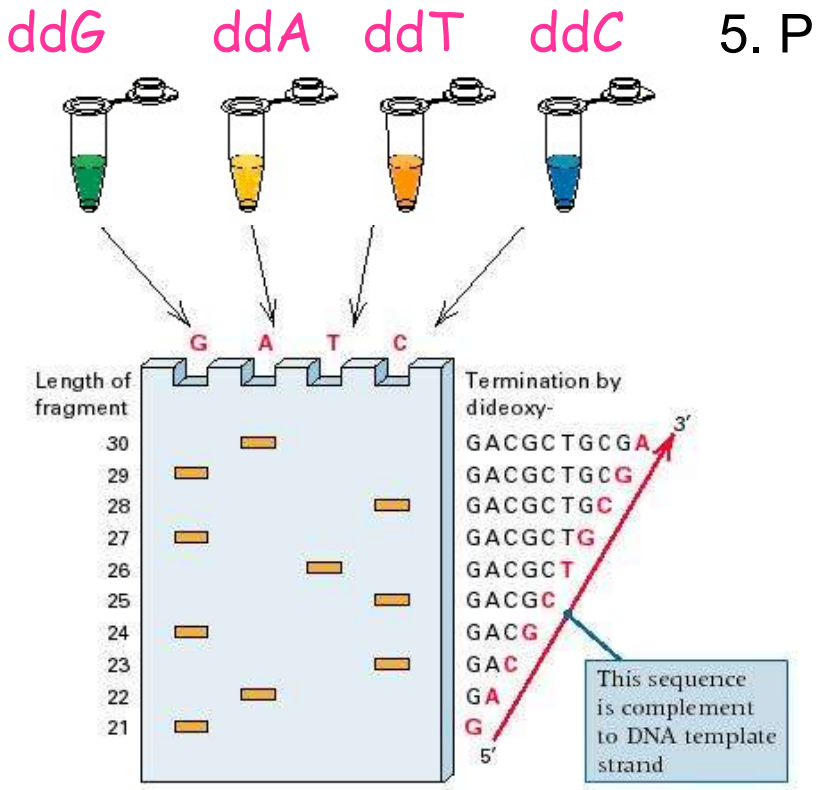
- Product separation

Sanger's Method

1. Prepare a large quantity of DNA. Denature and add the primer, the 4 dNTPs, and DNA polymerase.
2. Make 4 aliquots and add one of the radioactive ddNTPs (S35 or P32) to each tube
. 3. Elongation and termination by incorporation of specific dideoxynucleotides.



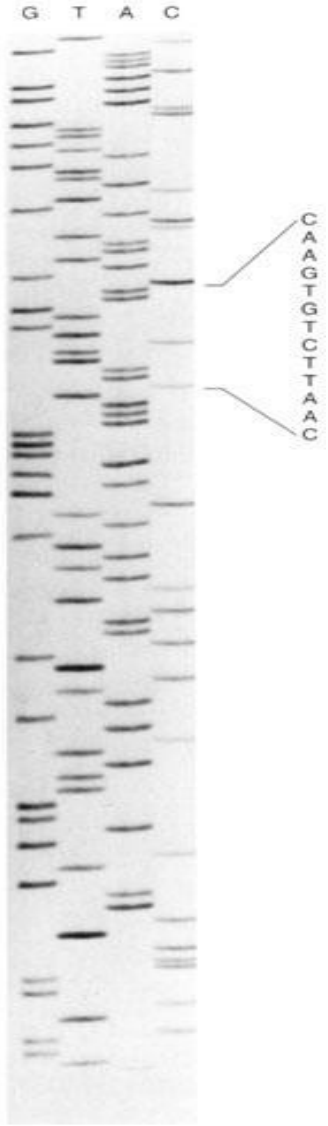
Sanger's Method



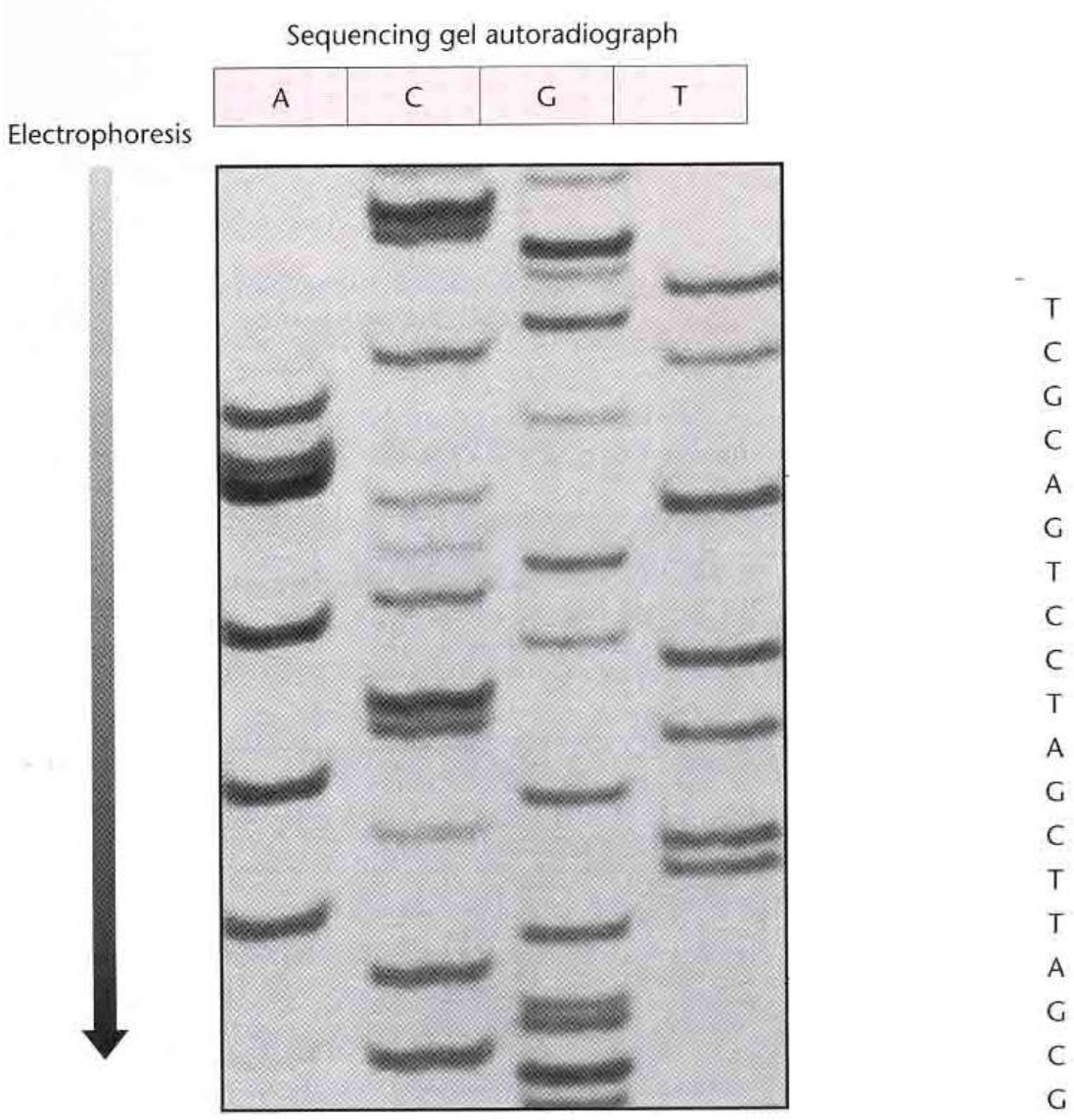
5. Preparation of the polyacrylamide gel and loading onto the gel.
6. Electrophoretic migration.

Sanger's method

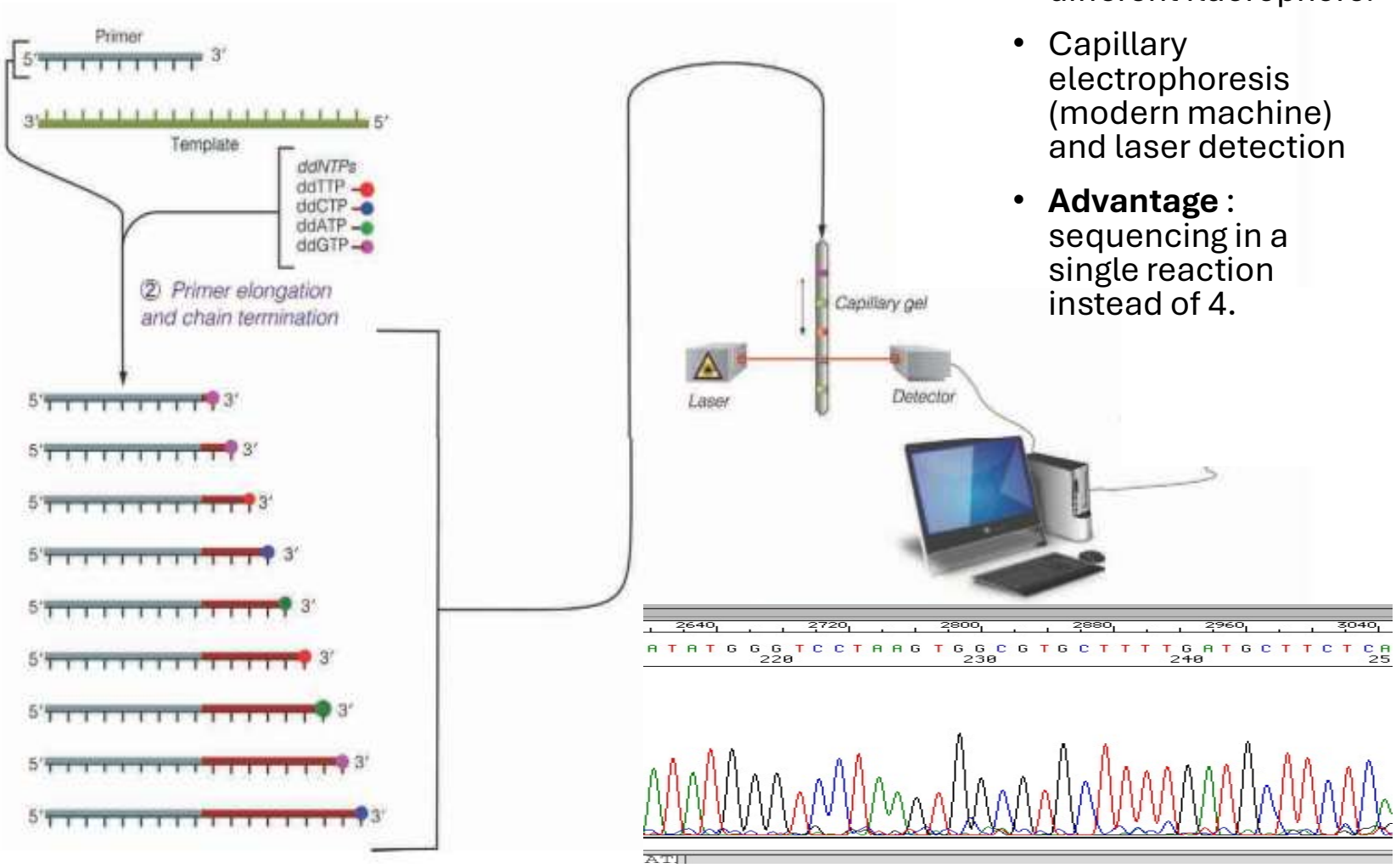
7. Transfer of the gel onto filter paper
   (Whatman 3MM).
   Development of the autoradiographic film.
   Computer capture.



Typical read length: 400 nucleotides.
Total experiment duration:
approximately 3 days.


Sequencing gel autoradiograph
A
C
G
T
Electrophoresis
T
C
G
C
A
G
T
C
C
T
A
G
C
T
T
A
G
C
G
G

Automation of the Sanger method



- Each ddNTP is labeled with a different fluorophore.
- Capillary electrophoresis (modern machine) and laser detection
- **Advantage** : sequencing in a single reaction instead of 4.

Pyrosequencing

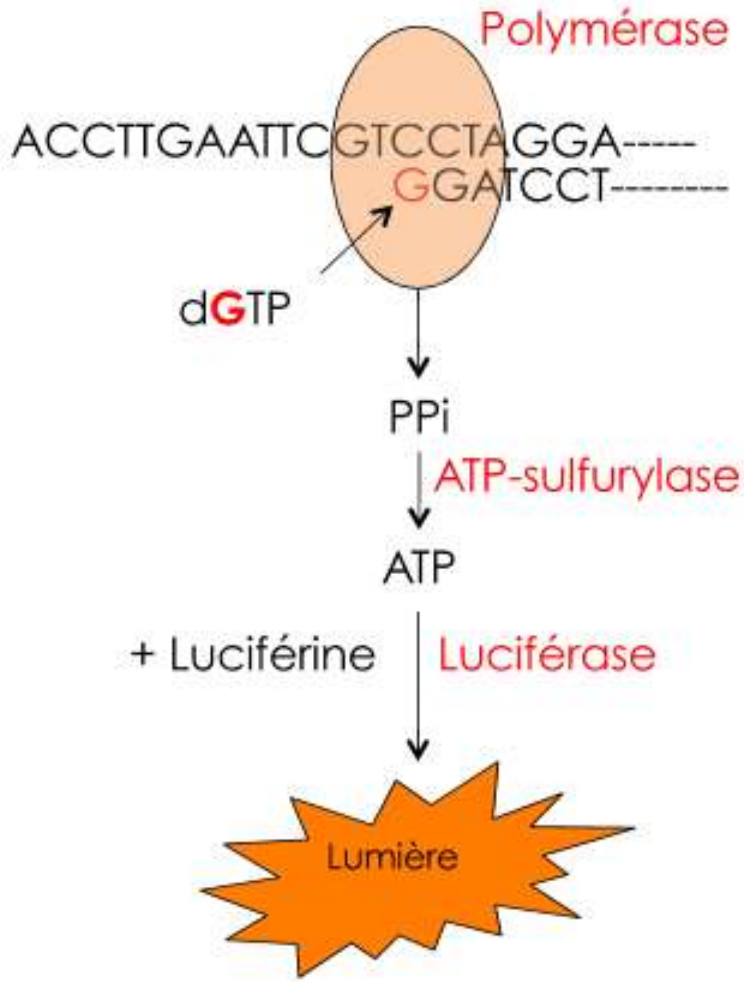
Pyrosequencing

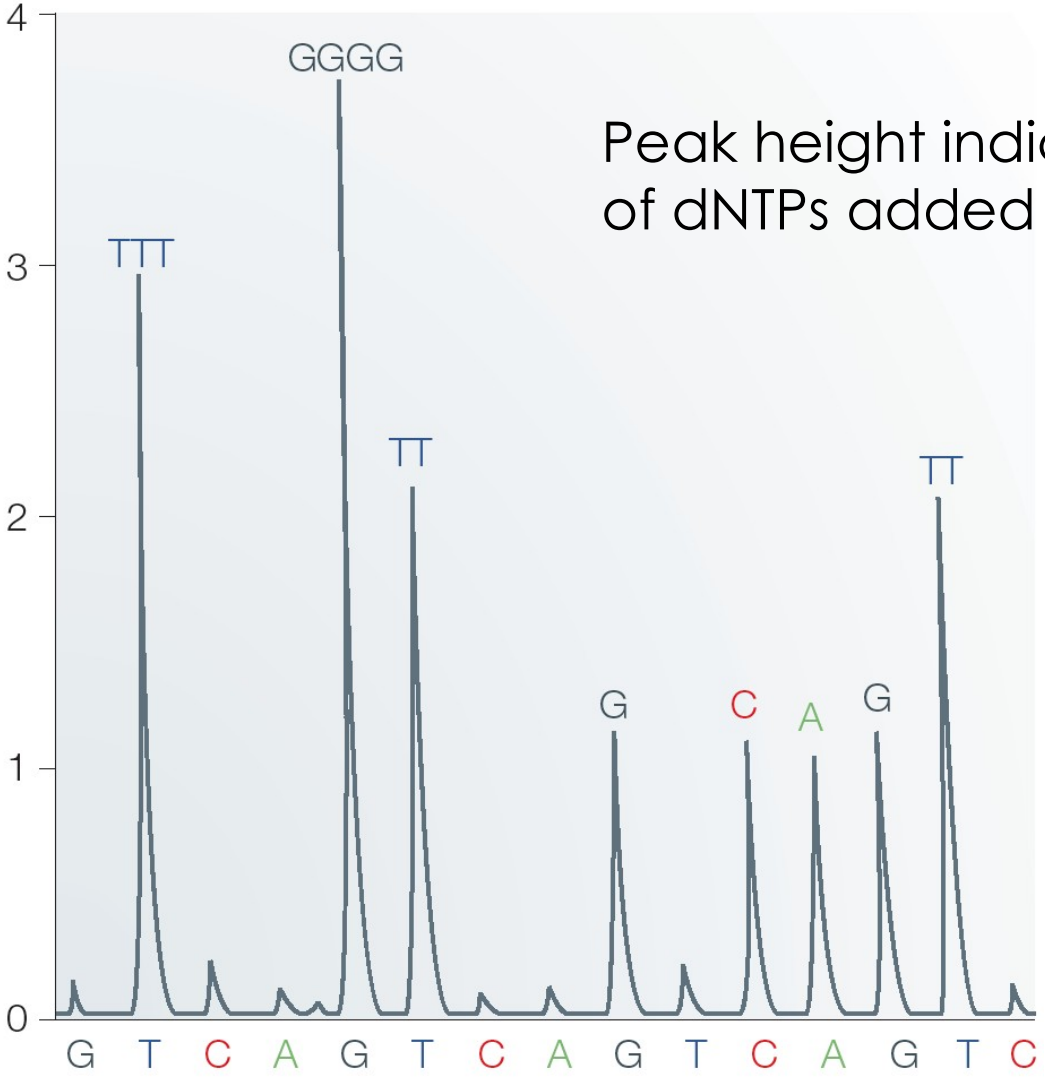
- Based on a principle of sequencing by synthesis, as opposed to sequencing by "termination" (Sanger method)

- Sequencing of a single-strand DNA by synthesis of the complementary strand, base by base, detecting at each step the activity of the polymerase by another chemiluminescent enzyme: luciferase.

Pyrosequencing

- Nucleotides (dNTPs) added one after the other (≠Sanger sequencing)
- If it is the correct nucleotide: incorporation and release of a pyrophosphate (PPi)
- PPi to ATP by the action of ATP-sulfurylase
- ATP provides the energy needed for the conversion of luciferin by luciferase. This reaction generates visible light, the intensity of which is proportional to the amount of ATP.





Peak height indicates the number of dNTPs added

sequence: TTT GGGG TT G C A G TT

Next-generation sequencing: High-throughput sequencing

High-throughput sequencing technologies

Whole genome sequencing

High-throughput sequencing: Principle

Whole genome sequencing

Biological material: DNA or RNA

Common steps across different technologies:

-Enzymatic DNA fragmentation

-Preparing a DNA library
by linking adapters

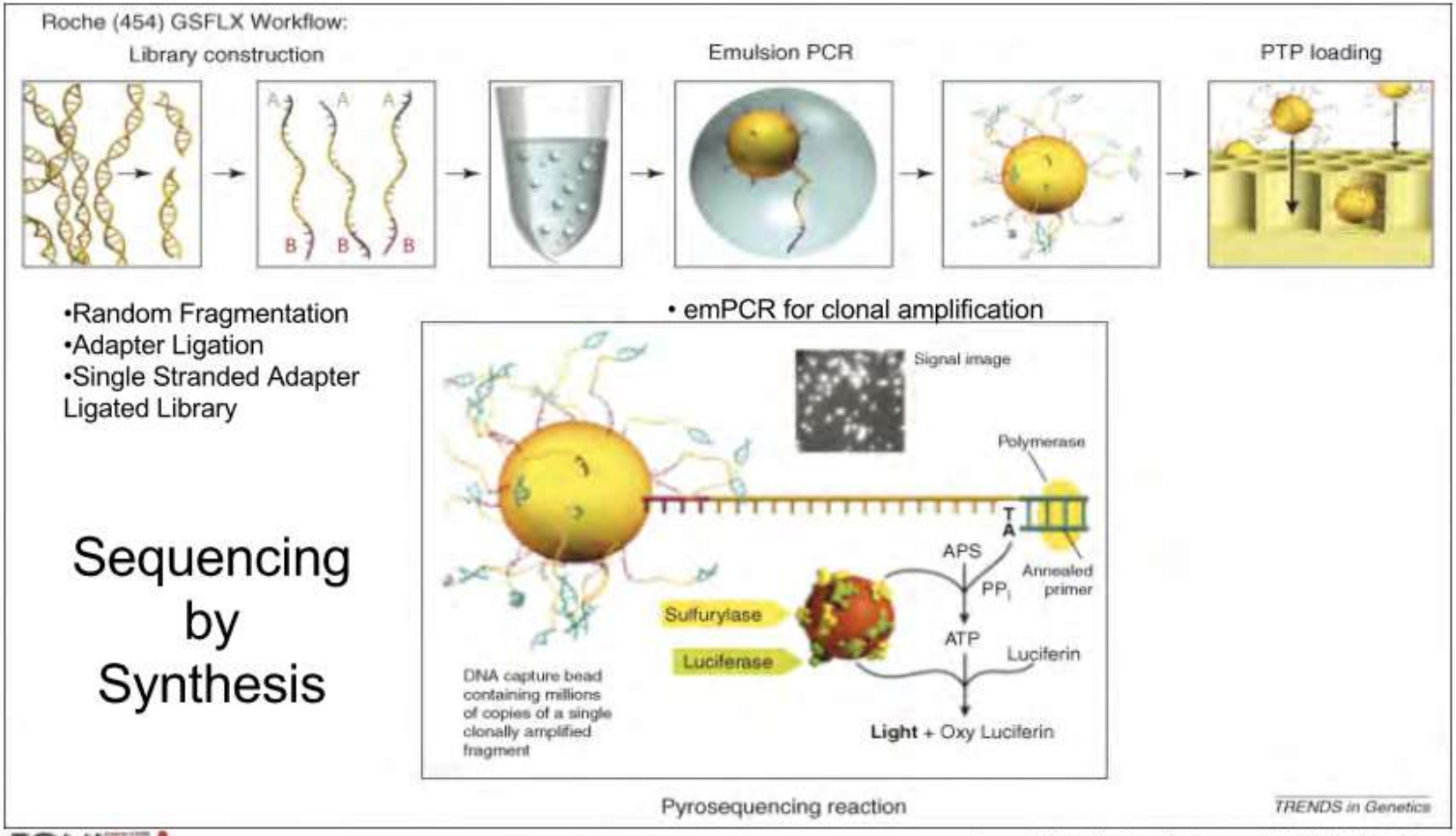
-Amplification

-Sequencing generating signals (luminescent)
(or fluorescent)

-Detection of emitted signals and conversion
in sequence

High-throughput sequencing: Principle

Exp: Roche 4.5.4 GS20 technology



From E. Mardis, Trends Genet., 24:133-141, 2008

High-throughput sequencing: Principle

Exp: Roche 4.5.4 GS20 technology