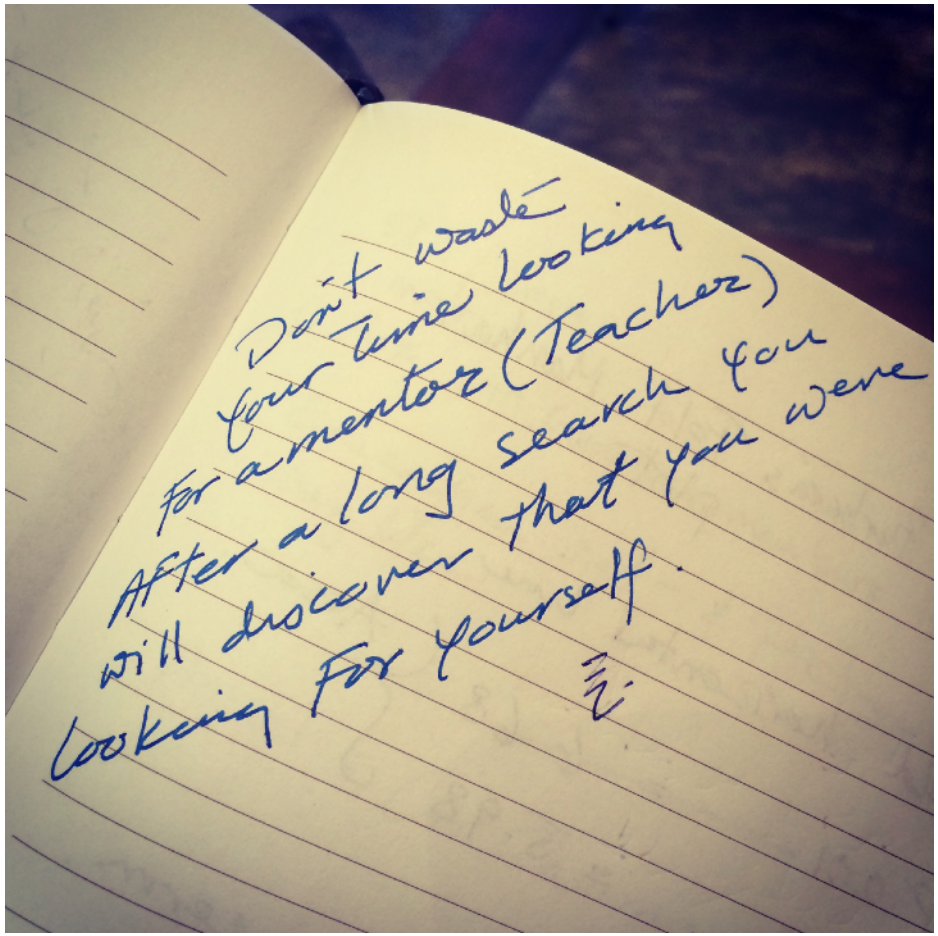
Molecular techniques

III. PCR and DNA sequencing

Course 281

Lessons for life



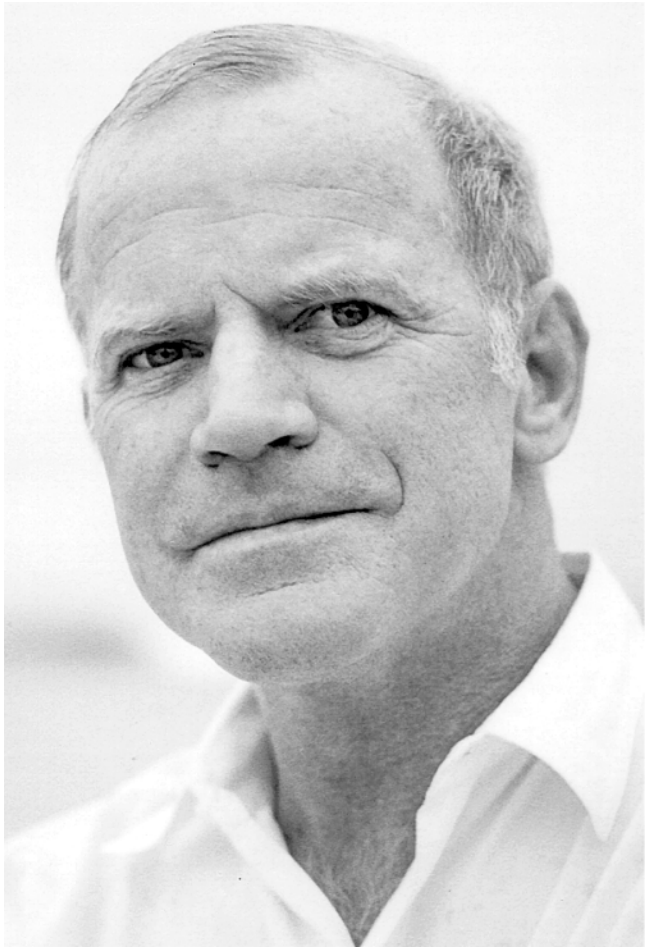
AIMS

- Understand the process of PCR and the components needed for the reaction.
- Understand why PCR is important in molecular biology.
- Understand the importance of DNA sequencing.
- Understand Sanger sequencing method as an example of DNA sequencing.

Polymerase chain reaction

• Polymerase Chain Reaction (**PCR**) allows the amplification (copying) of small amounts of DNA millions of copies.

• The method was developed by Kary Mullis (1983) and he was awarded the Nobel Prize for his invention.

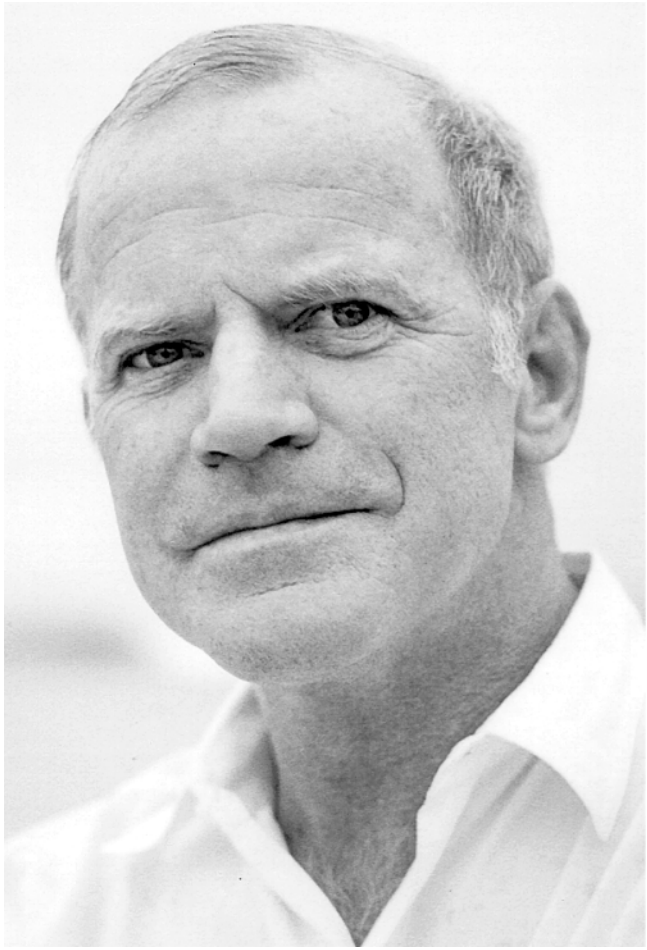


Polymerase chain reaction

- The process of PCR is similar to the process of DNA replication except it is done in tubes rather than living cells.

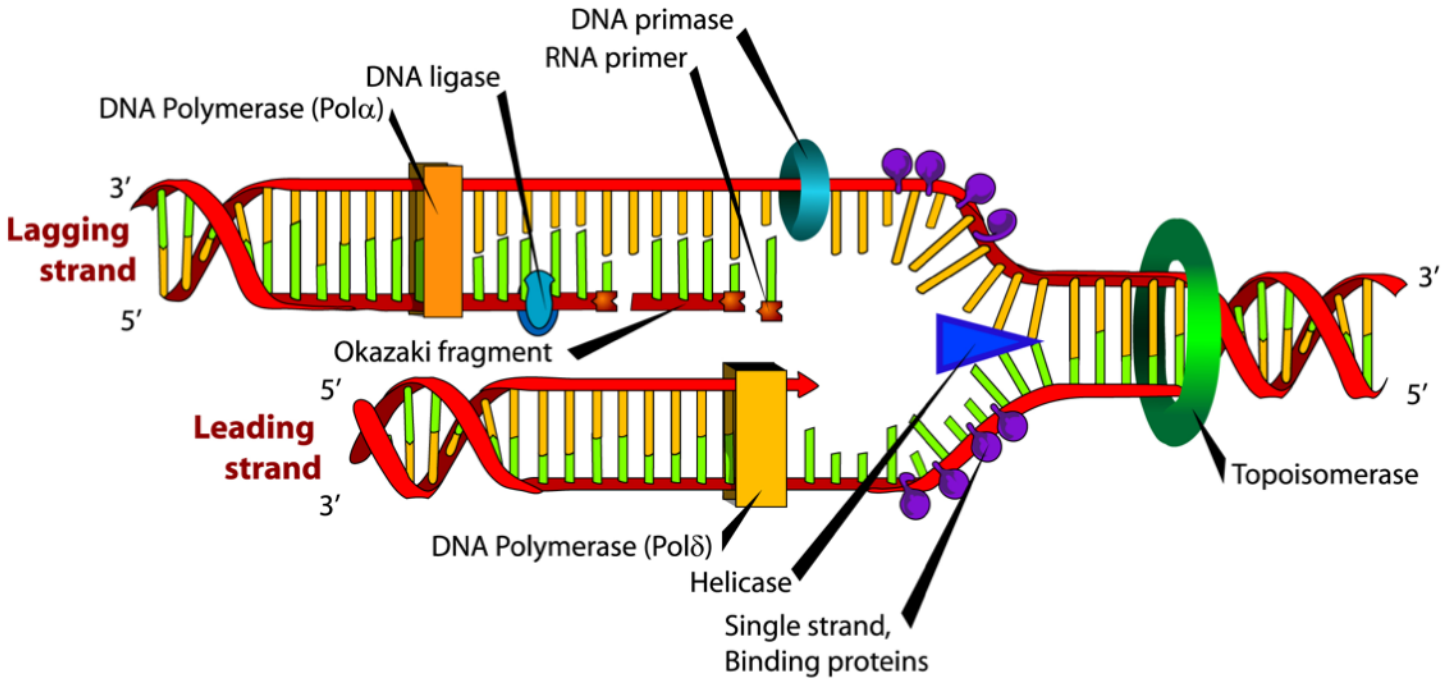
- It is considered in many cases the first step before any genetic analysis.

- Many methods and applications involve PCR.



DNA replication and PCR

- DNA replication in the cells involves making an identical copy of the genome (DNA).
- PCR uses the same procedure but to generate millions of copies of a small section of the genome in a tube!



Why PCR?

PCR is used:

1. To amplify small quantities of DNA.
2. For DNA quantification.
3. For genetic profile analyses:
    - RFLP
    - Microsatellite
    - Mitochondrial DNA genotyping and sequencing.
4. For sequencing small section of the genome or the genome.

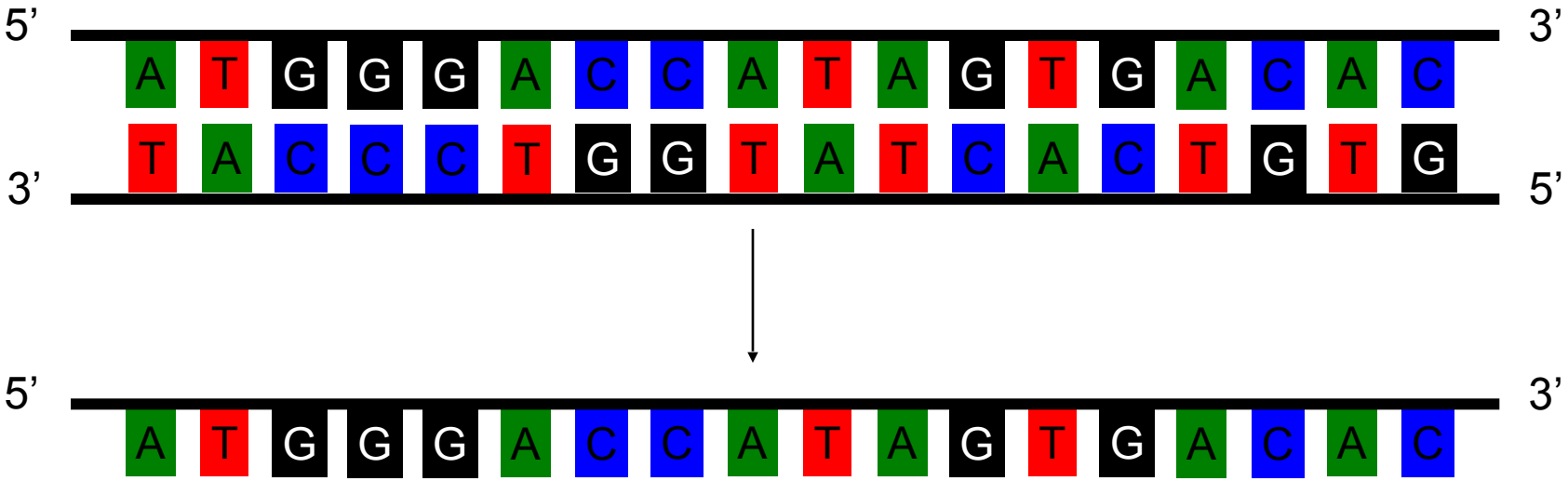
Components

What do we need to replicate (copy) DNA?

1. DNA template.
2. Building block of DNA (dNTPs).
3. DNA copier (an enzyme).
4. 3’OH (primer).

Components: (1) DNA template

• The DNA sample you collect from a crime scene or the one under investigation is the DNA template.

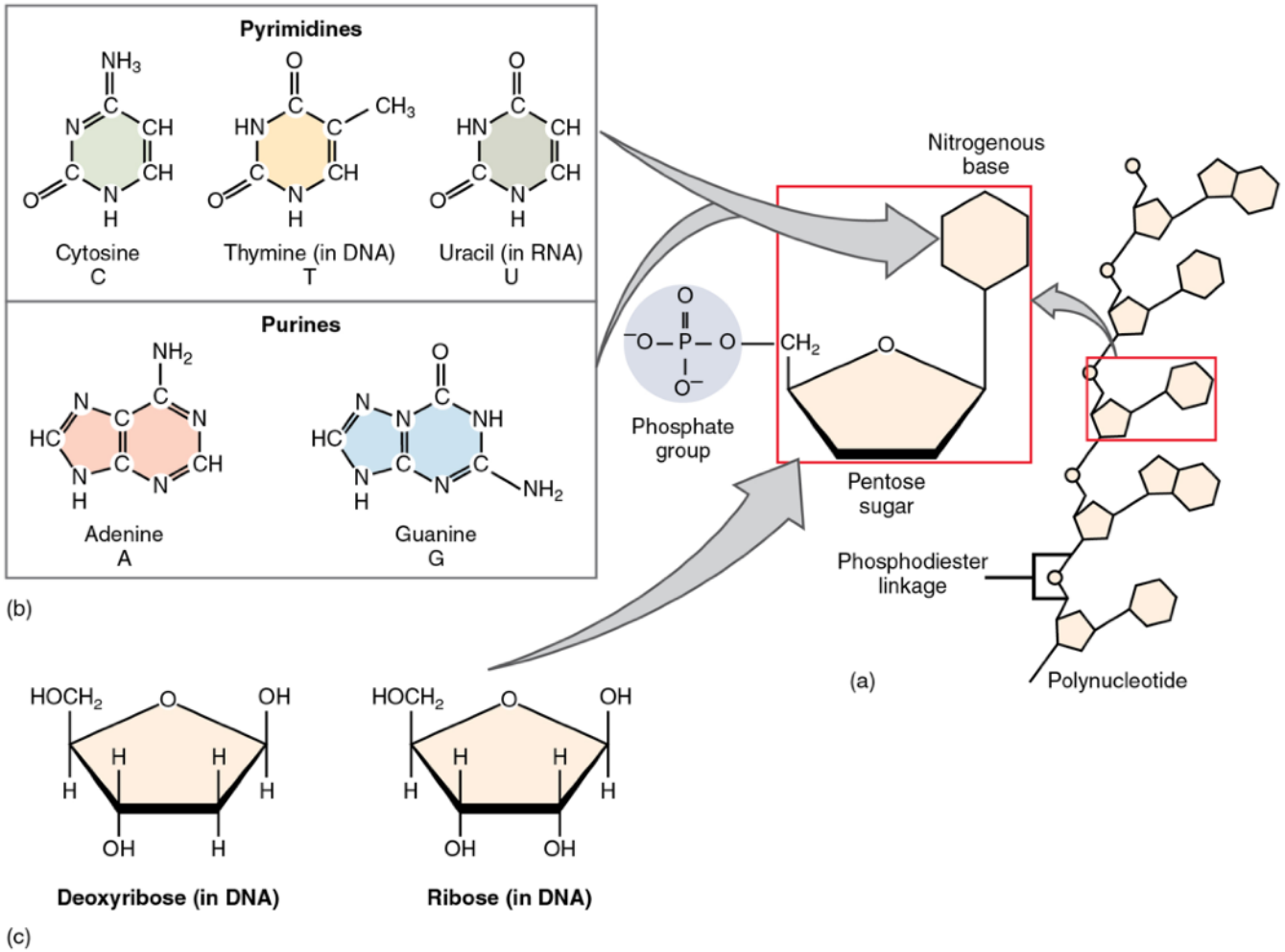


Each strand serves as a **template** for copying.

Remember complementary base-pairing!

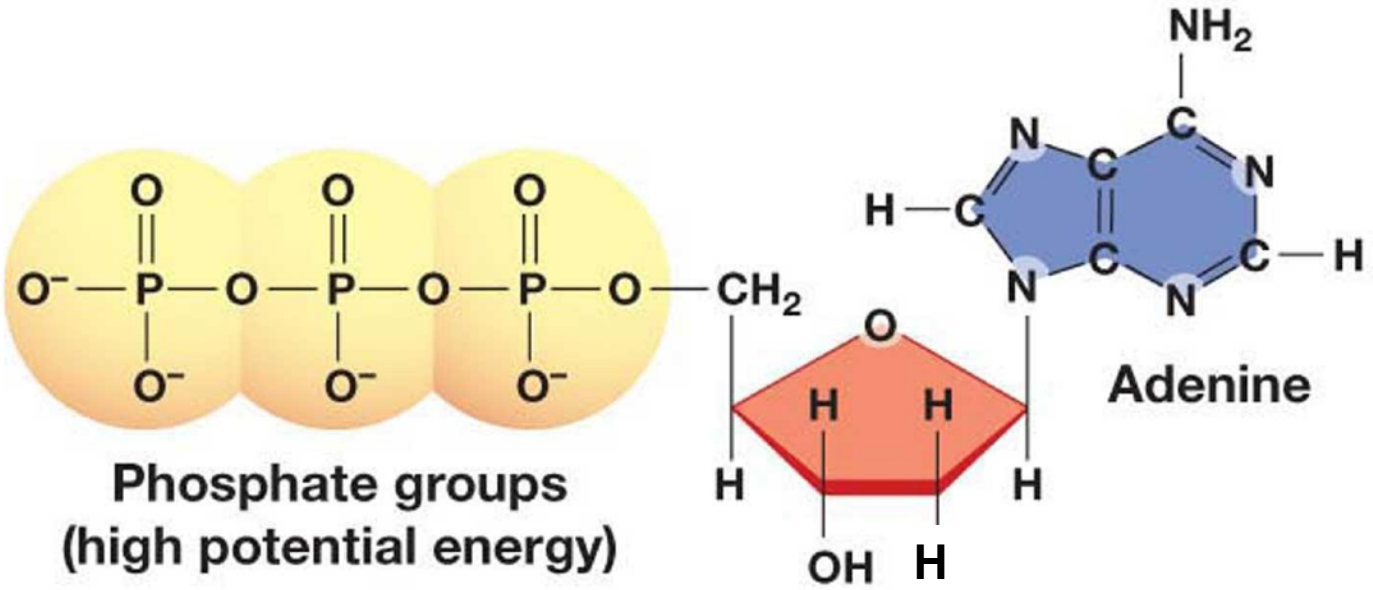
Components: (2) dNTP (building blocks)

Do you remember? DNA is made of nucleotides!



Components: (2) dNTP (building blocks)

Deoxyribonucleoside triphosphate (dNTP)



Four dNTPs serve as the building blocks of DNA (dATP, dTTP, dGTP, dCTP)

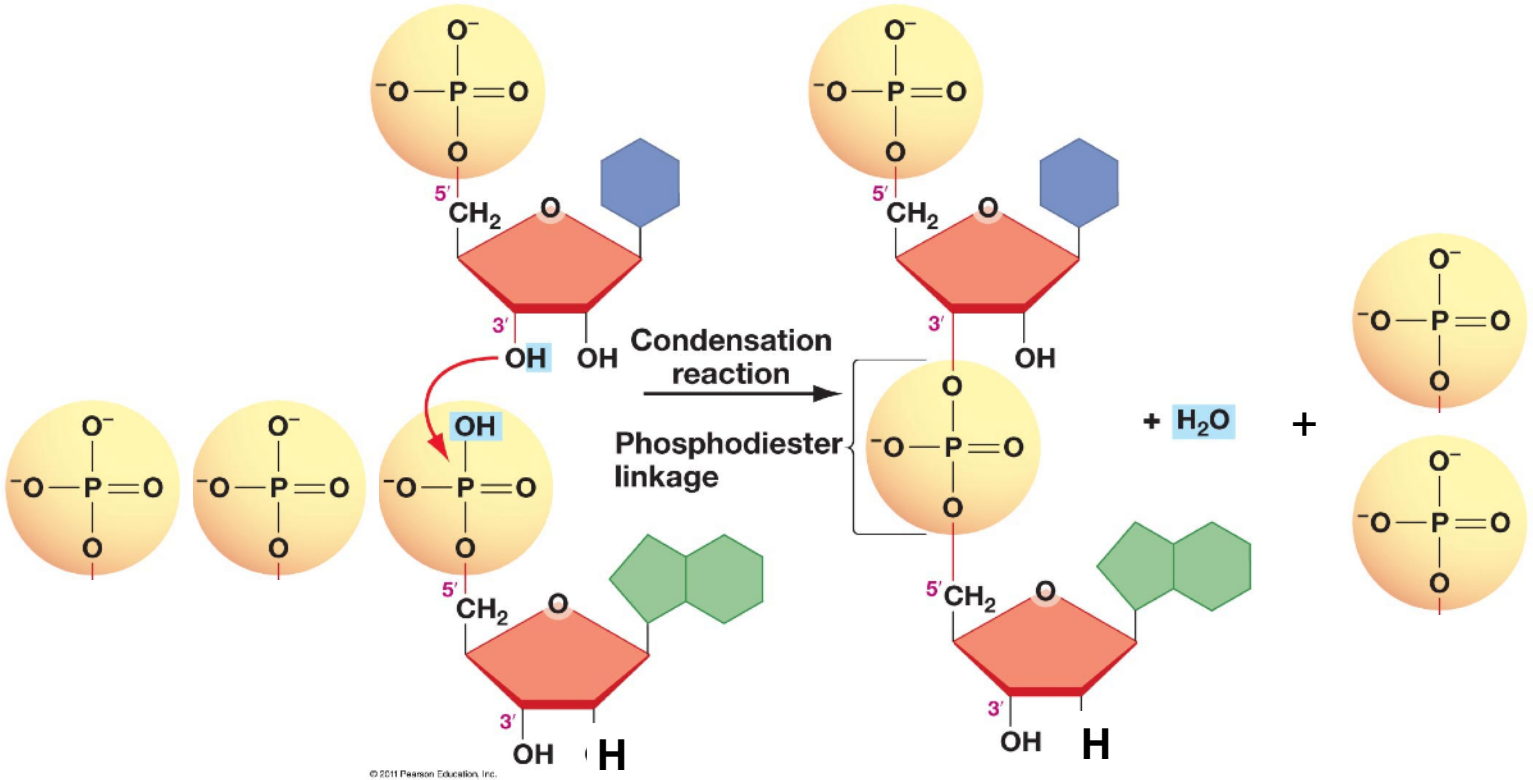
Remember **Nucleotides!**

Components: (2) dNTP (building blocks)

Deoxyribonucleoside triphosphate (dNTP)

Why triphosphate?

For the energy required to for the phosphodiester bond



Components: (3) DNA copier (polymerase)

- DNA polymerase is the DNA copier in the cell.

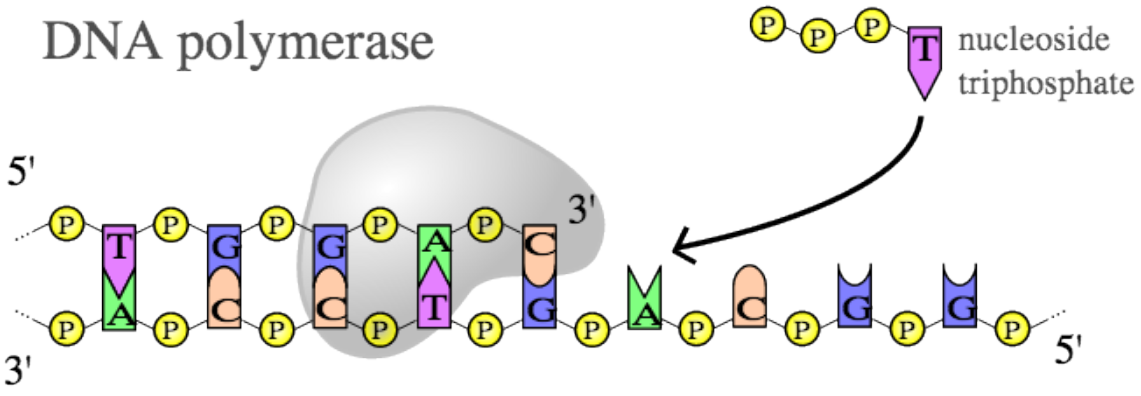
- Uses the dNTPs (DNA building blocks) to make a complementary strand to the template.

- Uses the available 3’-OH of a previous nucleotide and 5’phsphate from dNTP to form a phosphodiester bond.

Components: (3) DNA copier (polymerase)

• Each time DNA Pol finds the correct complementary dNTP and catalyzes the reaction linking the new nucleotide.

Remember DNA Pol needs 3'-OH



Components: (4) primer (3’ OH)

Primers are short piece of polynucleotide

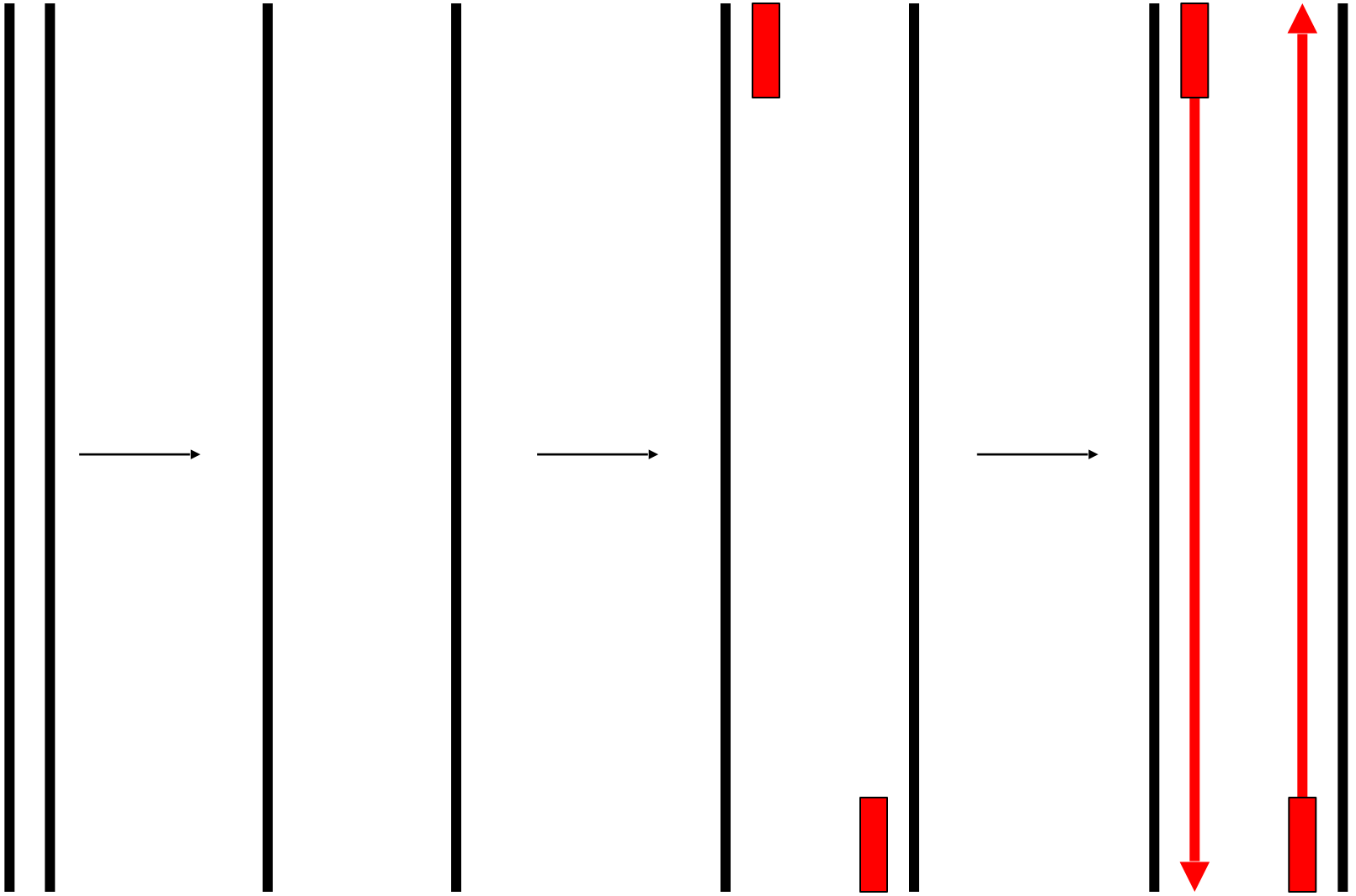
In order for the DNA copying machine to work and add nucleotides,

a 3’-OH needs to be available to form a phosphodiester bond!

PCR Process

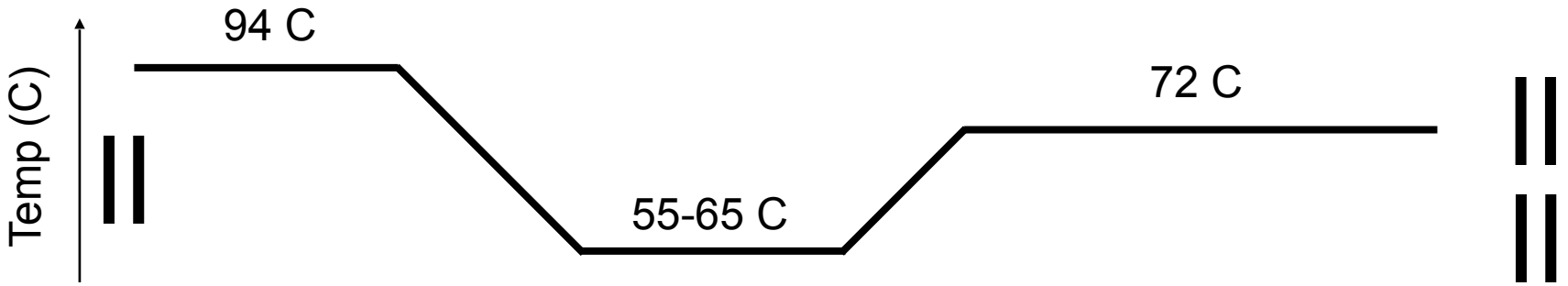
- Three steps are involved in PCR:
    1. **DNA template denaturation:** separation of the two strands of DNA.
    2. **Primers annealing:** small oligonucleotide attaches to each separated strand providing the 3’OH for DNA polymerase.
    3. **DNA polymerization (extension):** DNA polymerase extends the primers on both strands and adds nucleotides.

PCR Process



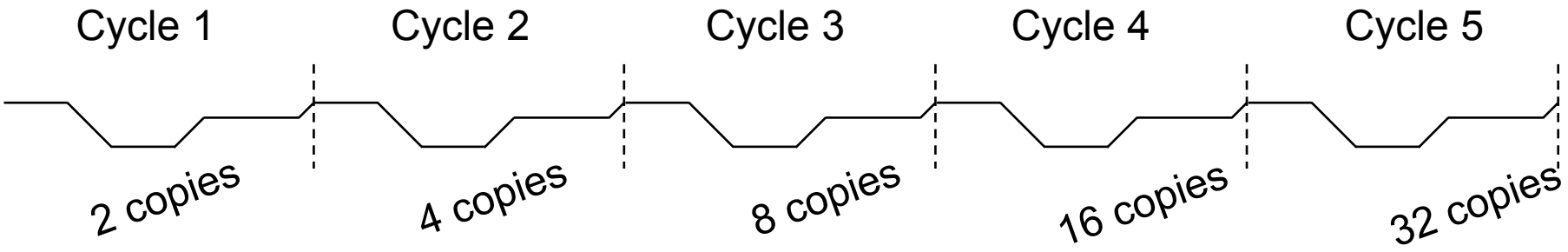
1. DNA denaturation
2. Primers annealing
3. DNA extension

PCR cycles



1. DNA denaturation 2. Primers annealing 3. DNA extension

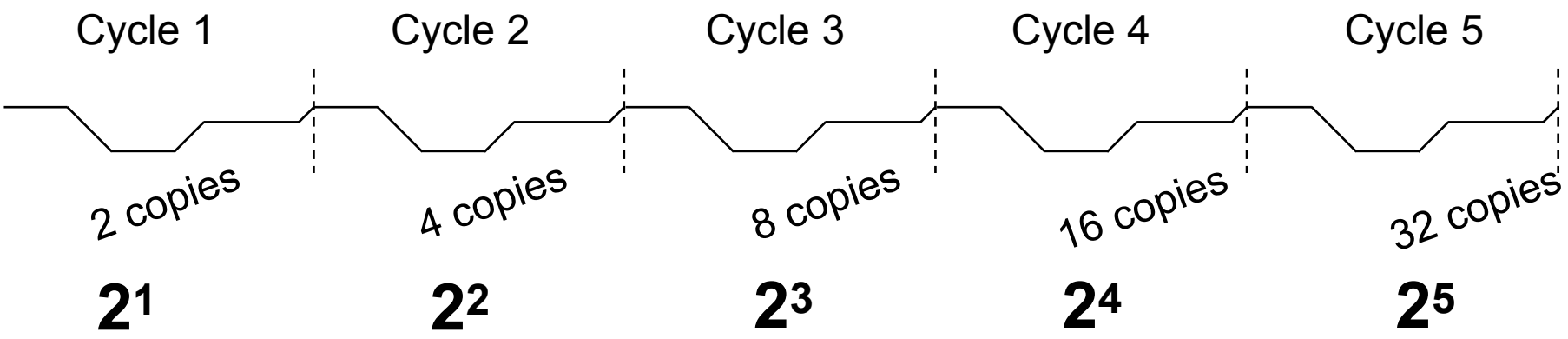
What happens if we repeat this cycle many times?



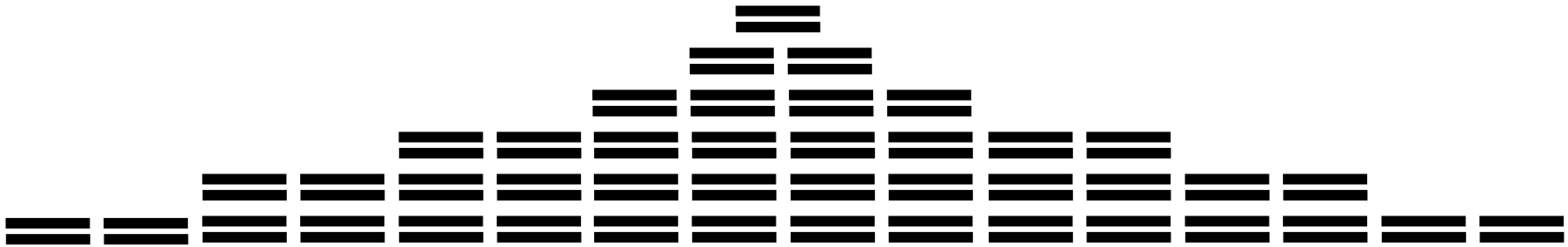
PCR cycles

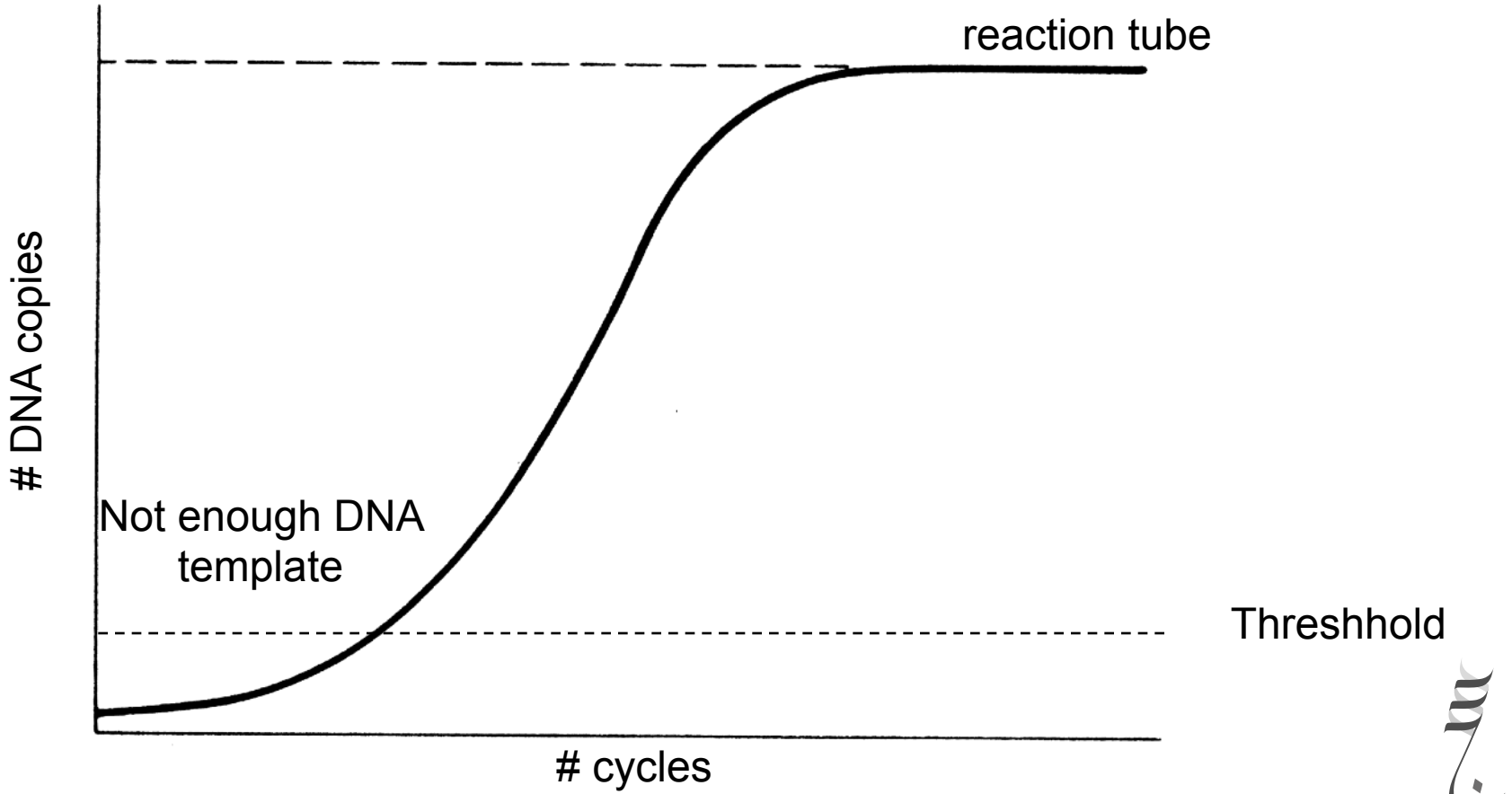
Exponential growth in the number of copies generated.

The number of copies you get at the end of your PCR will be $2^{\#cycles}$ ($2^{36\ cycles}$ = 68 billion copies)



PCR – how many copies?





Problems!

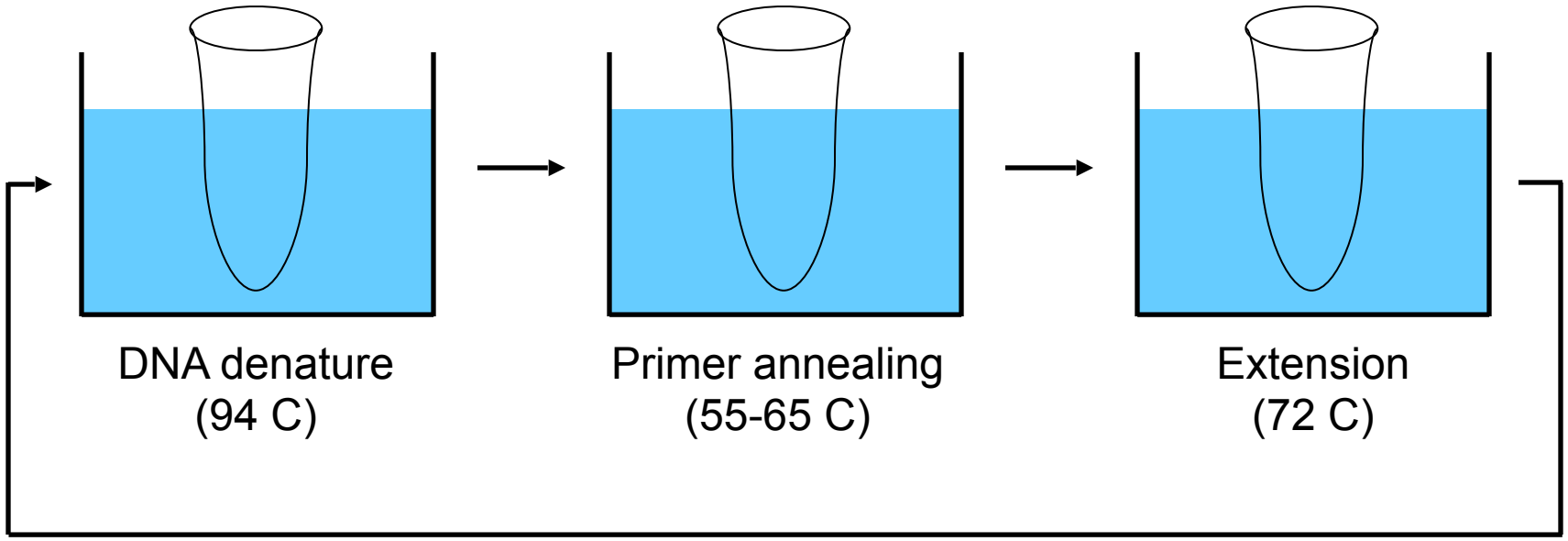
There were some difficulties with this system:

1. Three water-baths with three different temperature.

2. DNA polymerase denatures at 94 C.

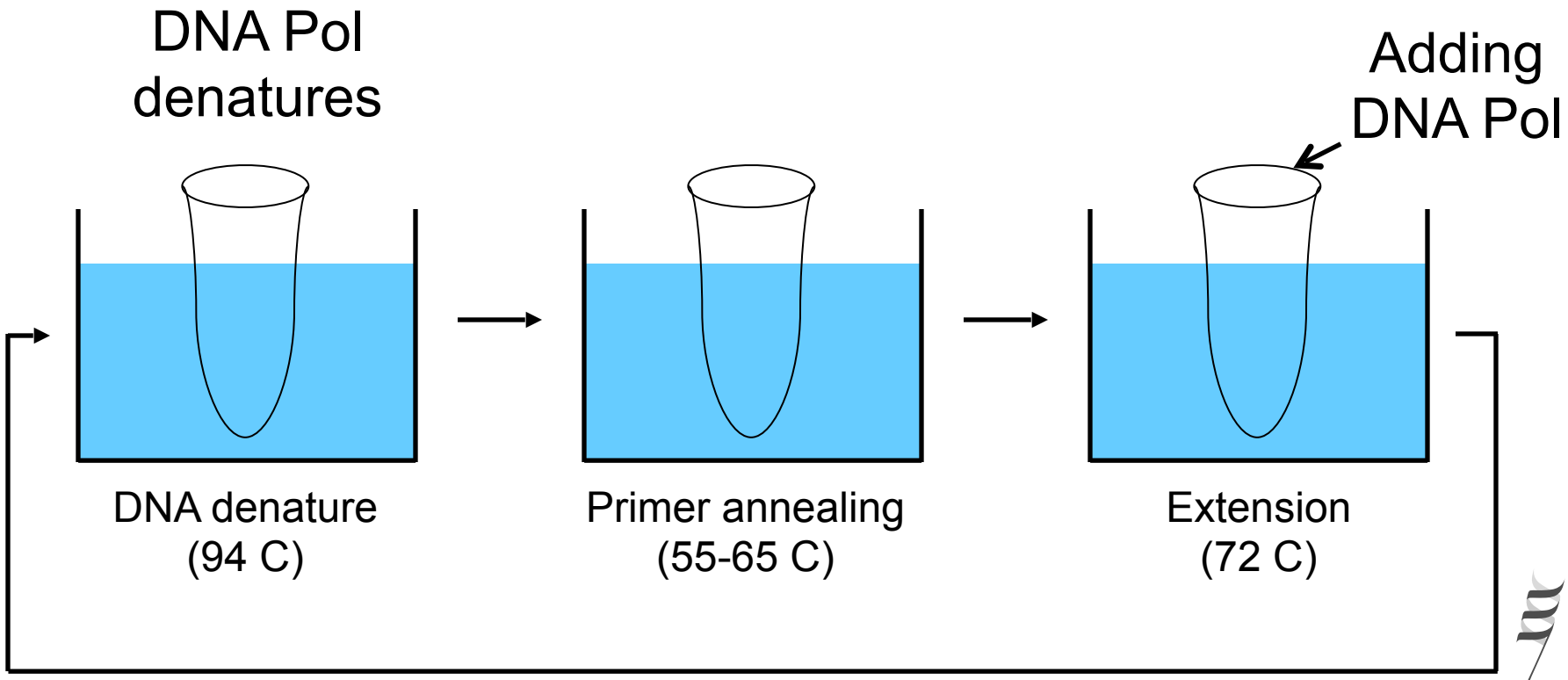
Problems!

- The sample has to be transferred into multiple water baths to accommodate the needed temperature.



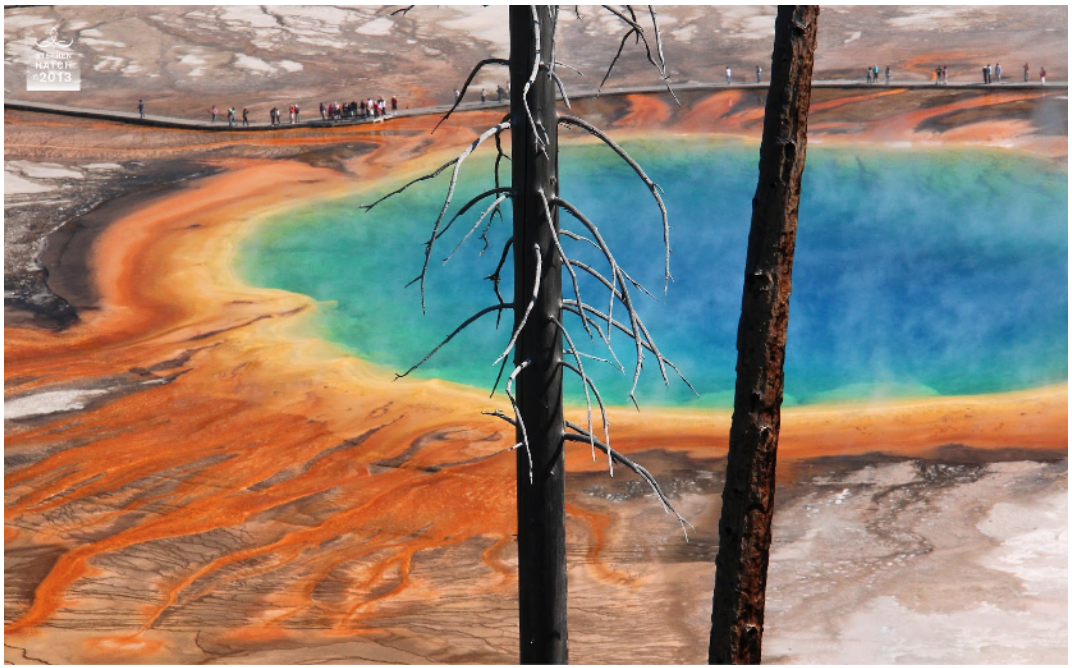
Problems!

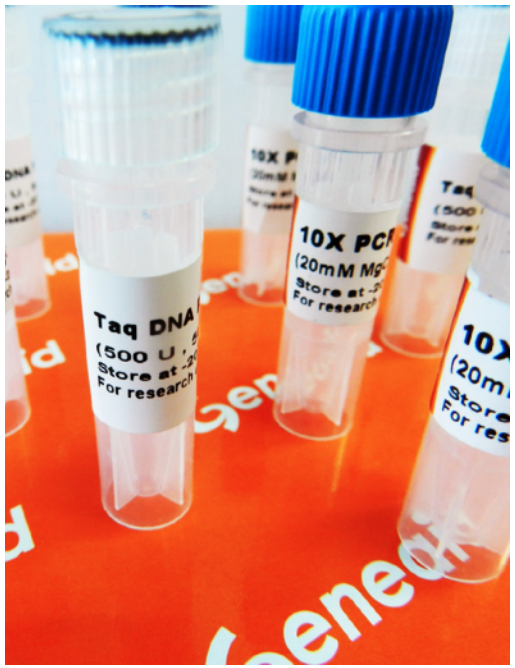
- DNA polymerase needs to be added in every cycle because DNA polymerase denatures at high temperature.



Improvement 1

- Using *Thermus aquaticus* (Taq) polymerase.
- Taq polymerase is heat stable and the cycles can take place without the polymerase being destroyed during the denaturation phase





Improvement 2

Replacing old machine (water baths) with a thermocycler

To consider

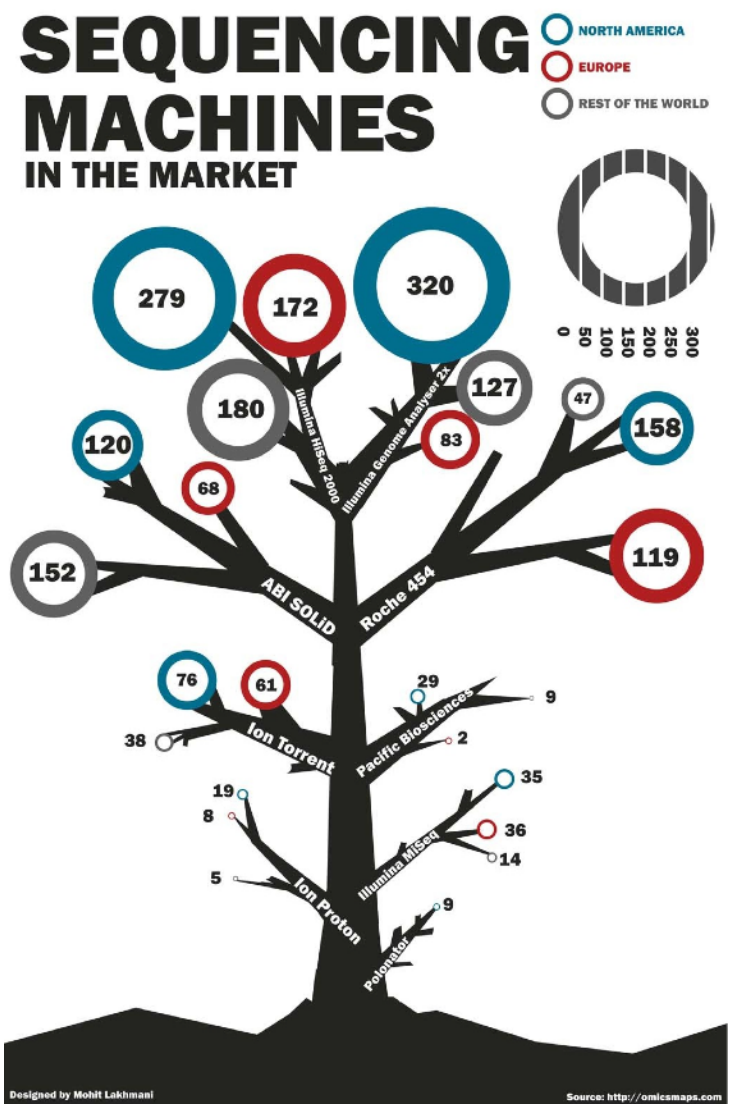
- Length and GC content of your primer.
- Compatibility of your forward and reverse primers.
- Primer's sequences do not complement each other (primer dimer).
- Annealing temperature of both primers should be the same.
- Length of the target DNA piece (the longer the target the longer the extension time).
- DNA polymerase, primers and other chemicals' concentration should be precisely calculated.

What is DNA sequencing?

It is reading the letters of the book.
It is reading the exact nucleotide sequence of the genome.

Sequencing methods available now!

1. Maxam and Gilbert chemical degradation method (extinct).
2. Sanger sequencing (dideoxy or chain termination method).
3. Illumina sequencing.
4. SOLiD sequencing.
5. Pyrosequencing.
6. Ion Torrent method.
7. Single molecule sequencing.

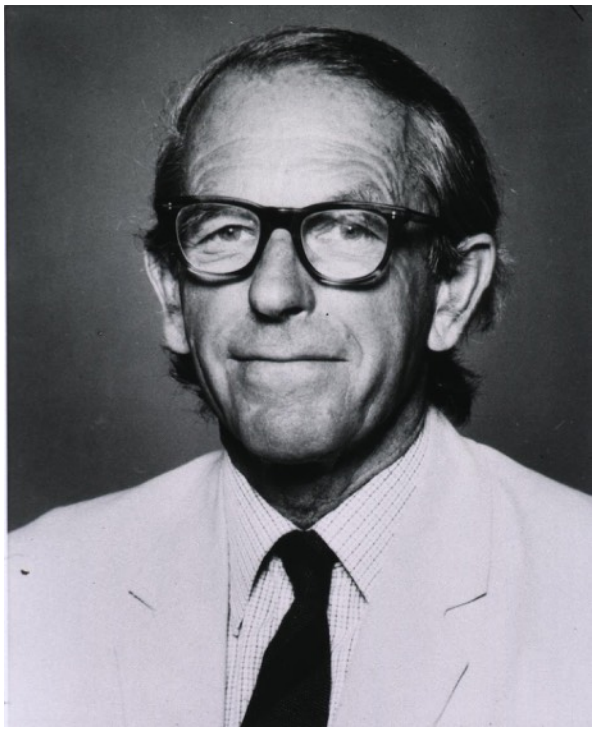


Why DNA sequencing?

- DNA sequencing can be considered the ultimate characterization of gene(s) or fragment(s) of DNA.

- **DNA Sequencing is used for:**
  - Mapping genomes
  - Determining gene structure and thus function
  - Detecting polymorphism (single nucleotide polymorphism SNP)
  - Analyzing genetic variation
  - Predicting the possible product(s) of DNA fragments
  - Many purposes depending on the questions one is asking

Sanger sequencing (the great method)



1. Fredrick Sanger has developed a sequencing method and received a Noble prize for it.

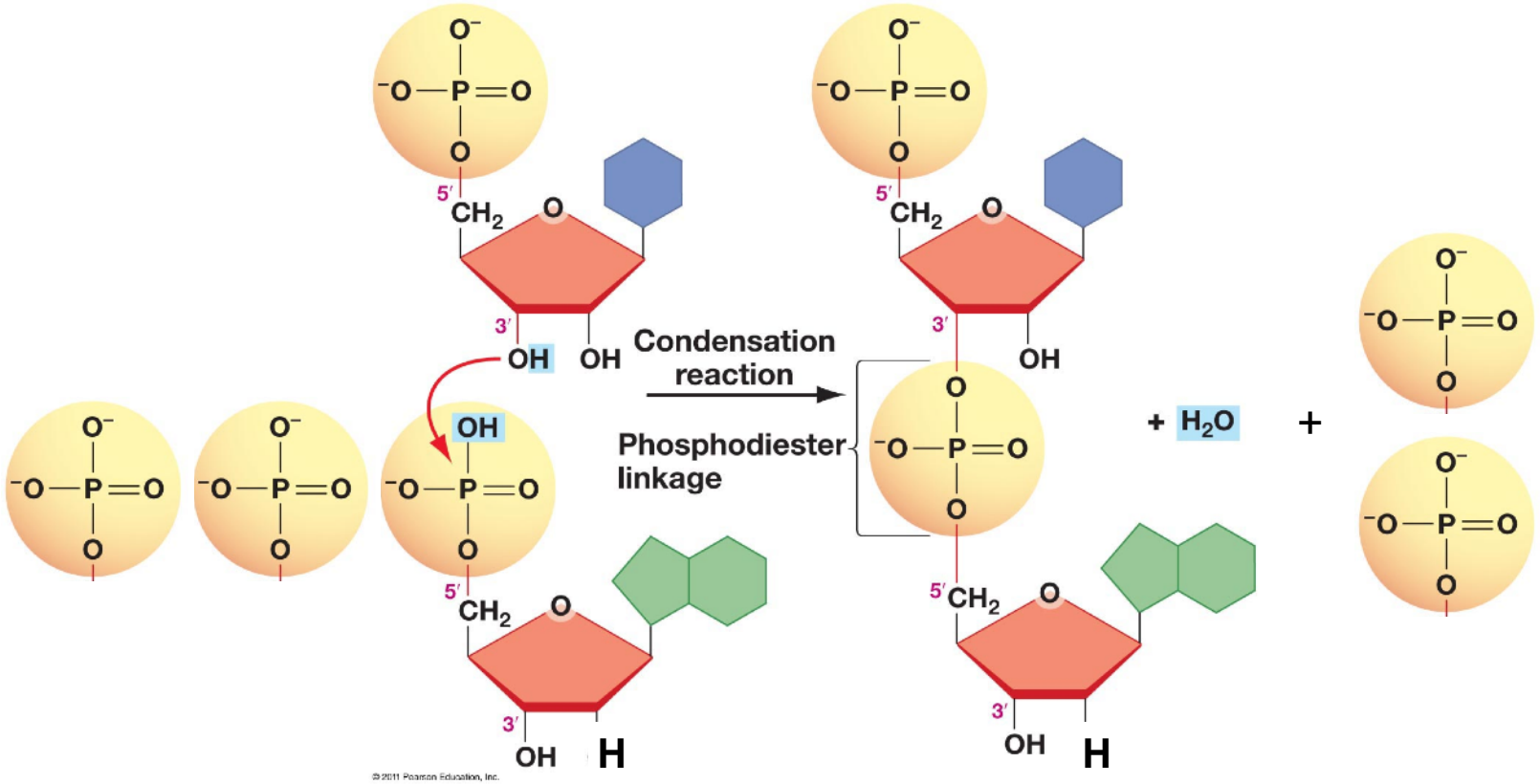
2. Sanger sequencing method is also called **Chain Termination Method** and **Dideoxy sequencing method.**

Sanger sequencing (the great method)

- **Employs:**
  - specific primers
  - dNTPs
  - ddNTPs
  - DNA polymerase
  - DNA template

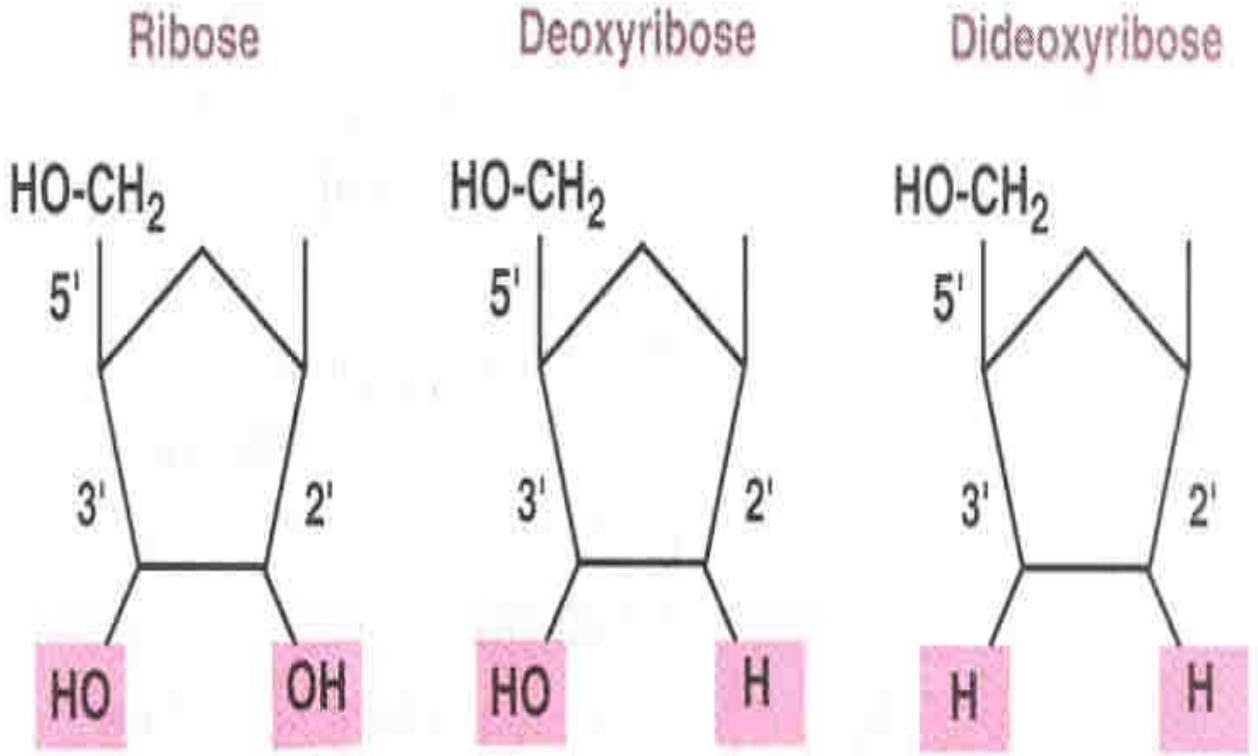
DNA synthesis

DNA synthesis requires the availability of a 3’-OH and energy



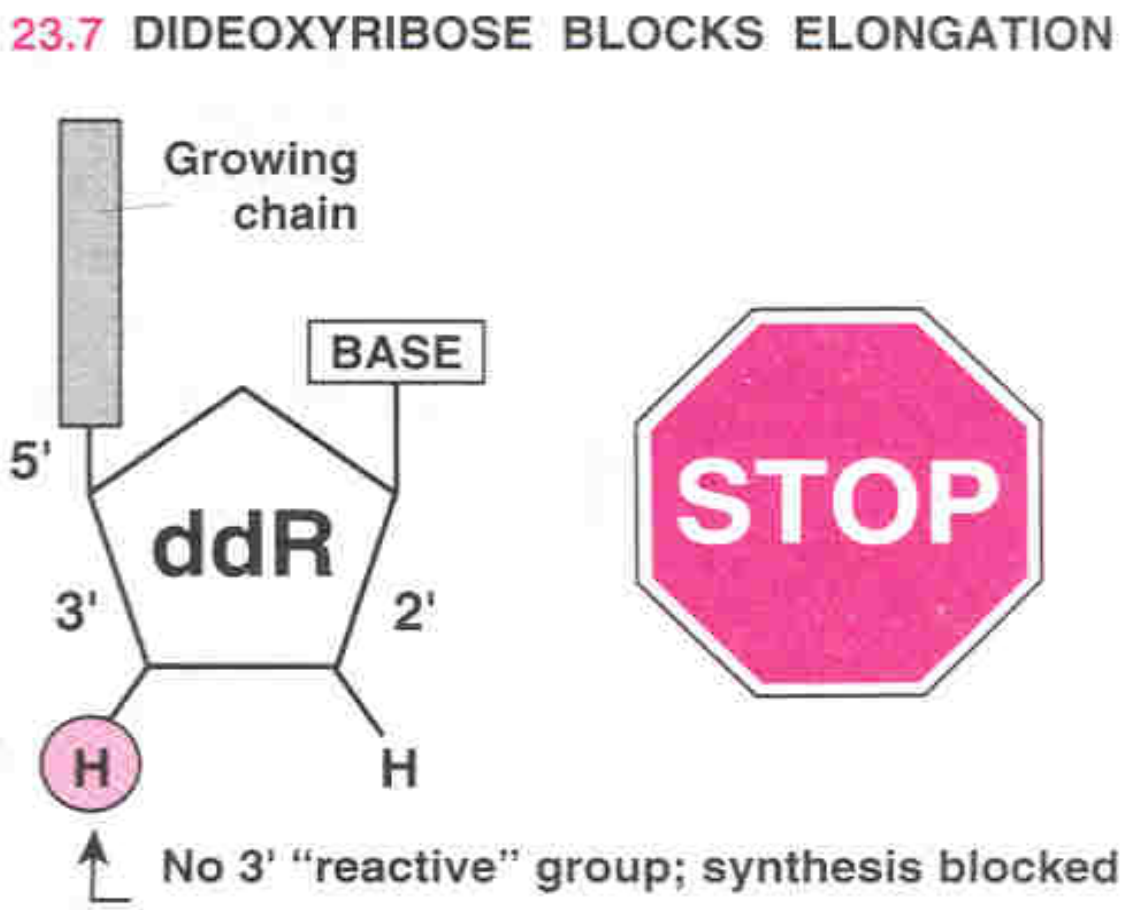
DNA synthesis

Difference in OH location in sugar and consequences



DNA synthesis

The absence of OH group on the 3’ carbon of the sugar blocks further addition of nucleotides



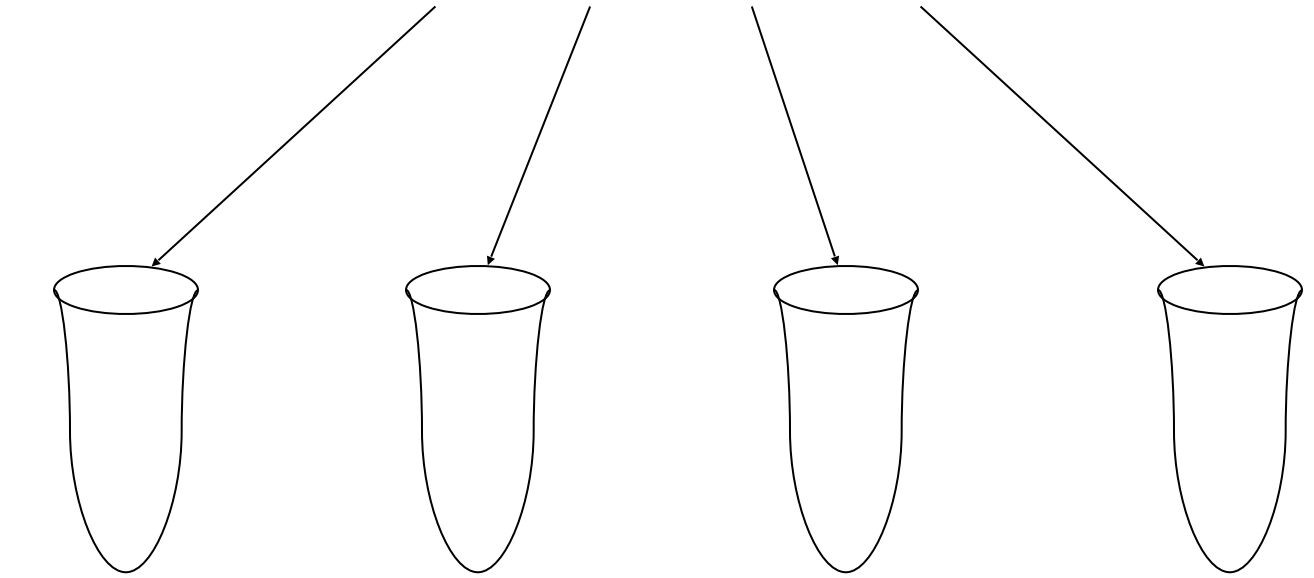
Sanger sequencing procedure

DNA Template

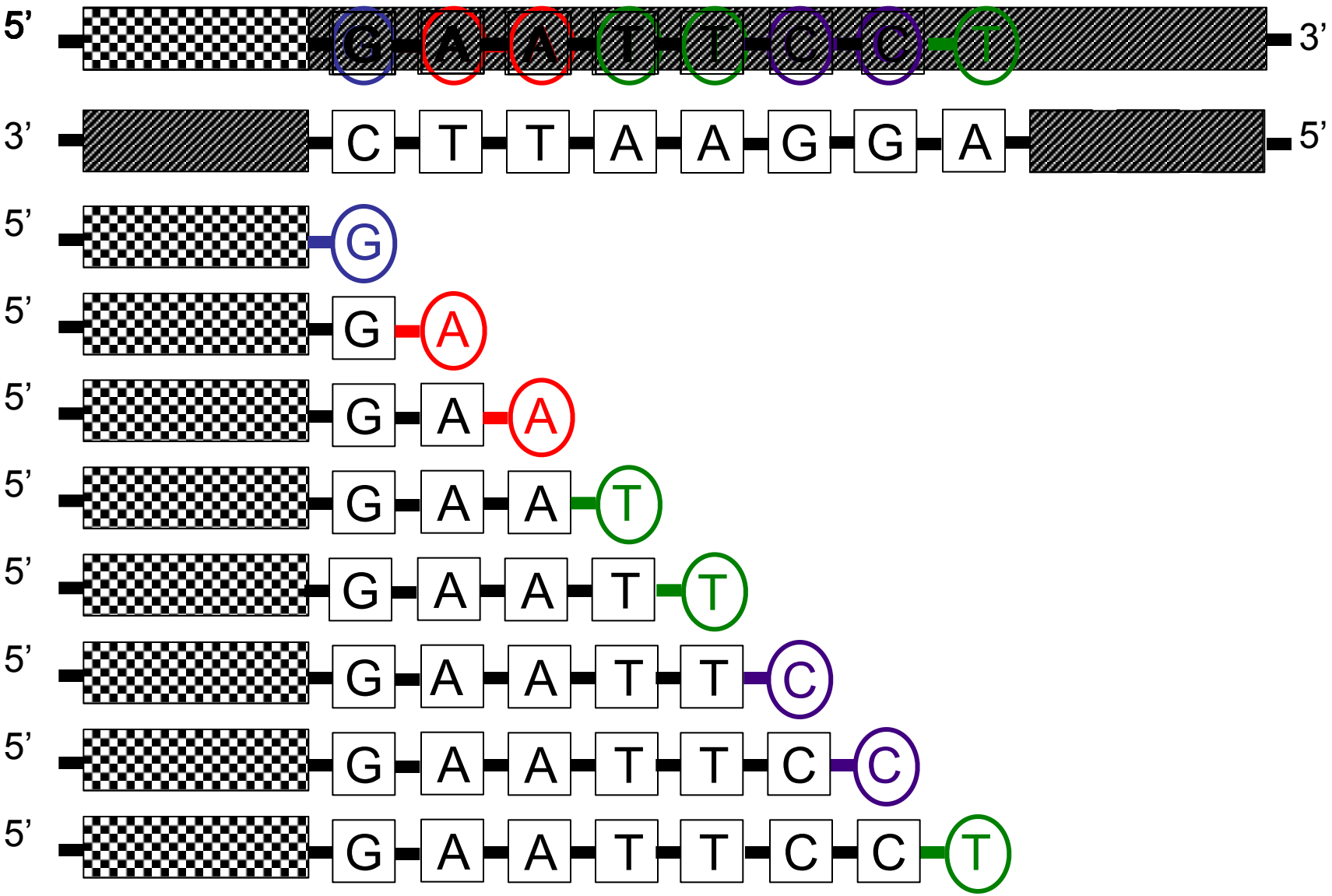
Polymerase

Excess dNTPs

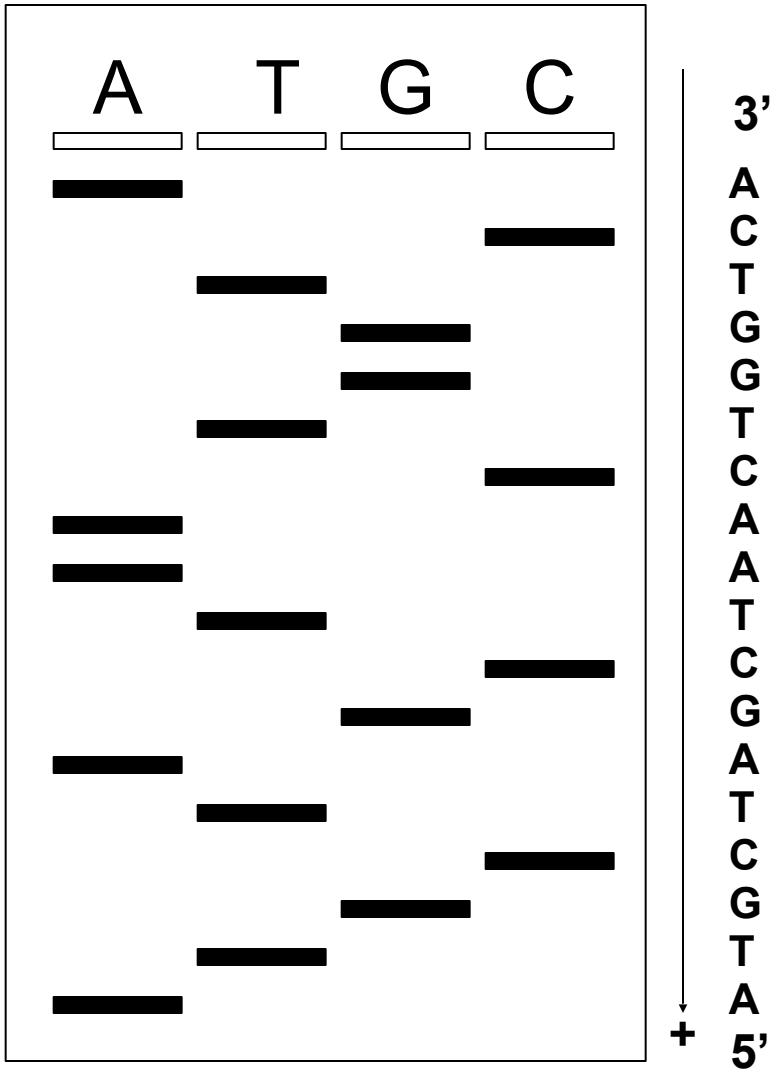
Primer



Sanger sequencing procedure

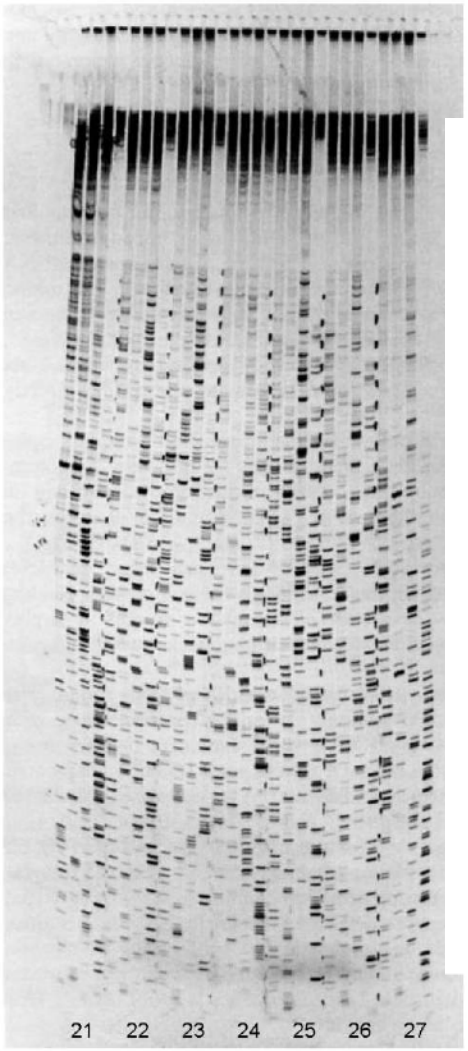


Sanger sequencing procedure



- Analysis using high resolution polyacrylamide gel electrophoresis.
- Fragments are detected using radioactive markers and autoradiography.

Sanger sequencing - Gel



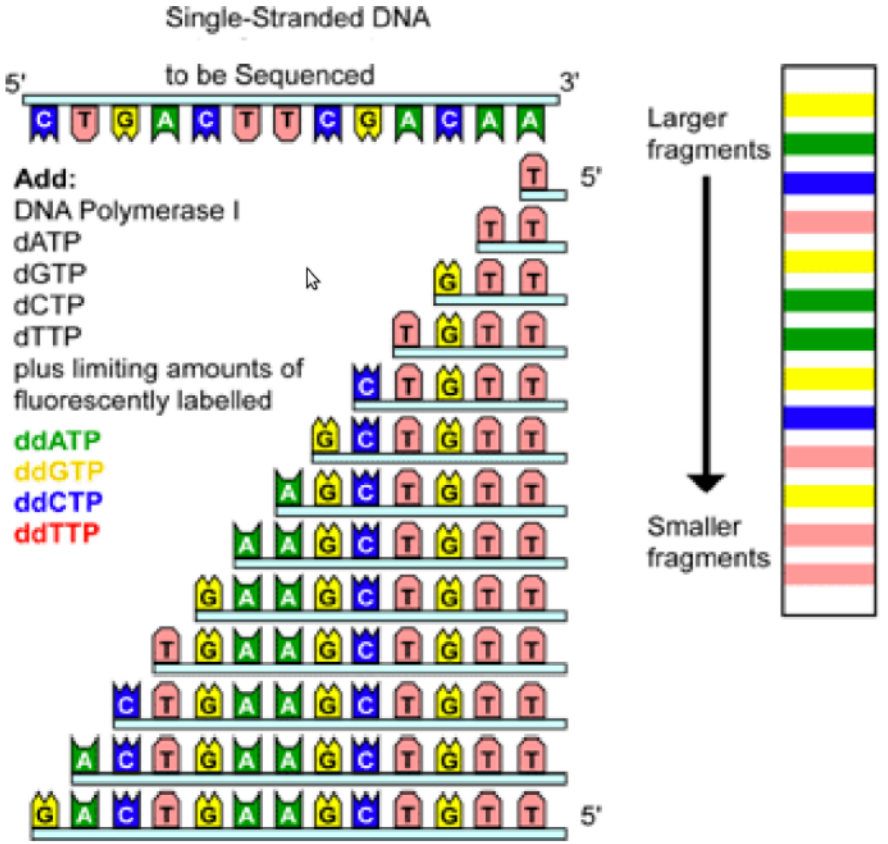
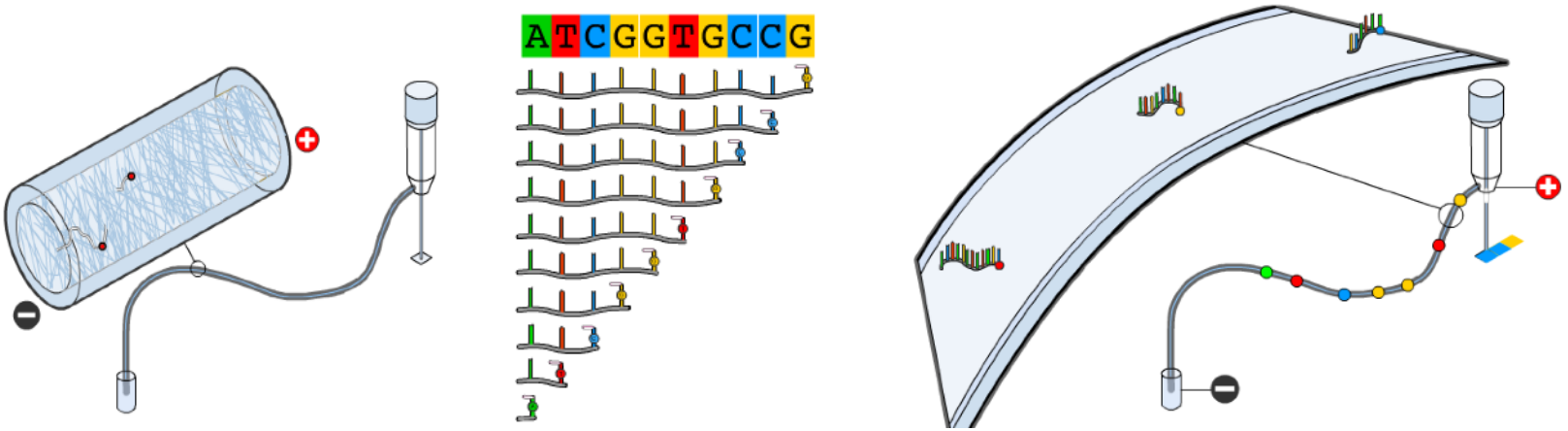


FIGURE 2.8. An autoradiogram (X-ray film) of a DNA sequencing gel. Each sequence requires Four lanes, one for each base.

Sanger sequencing - Automated

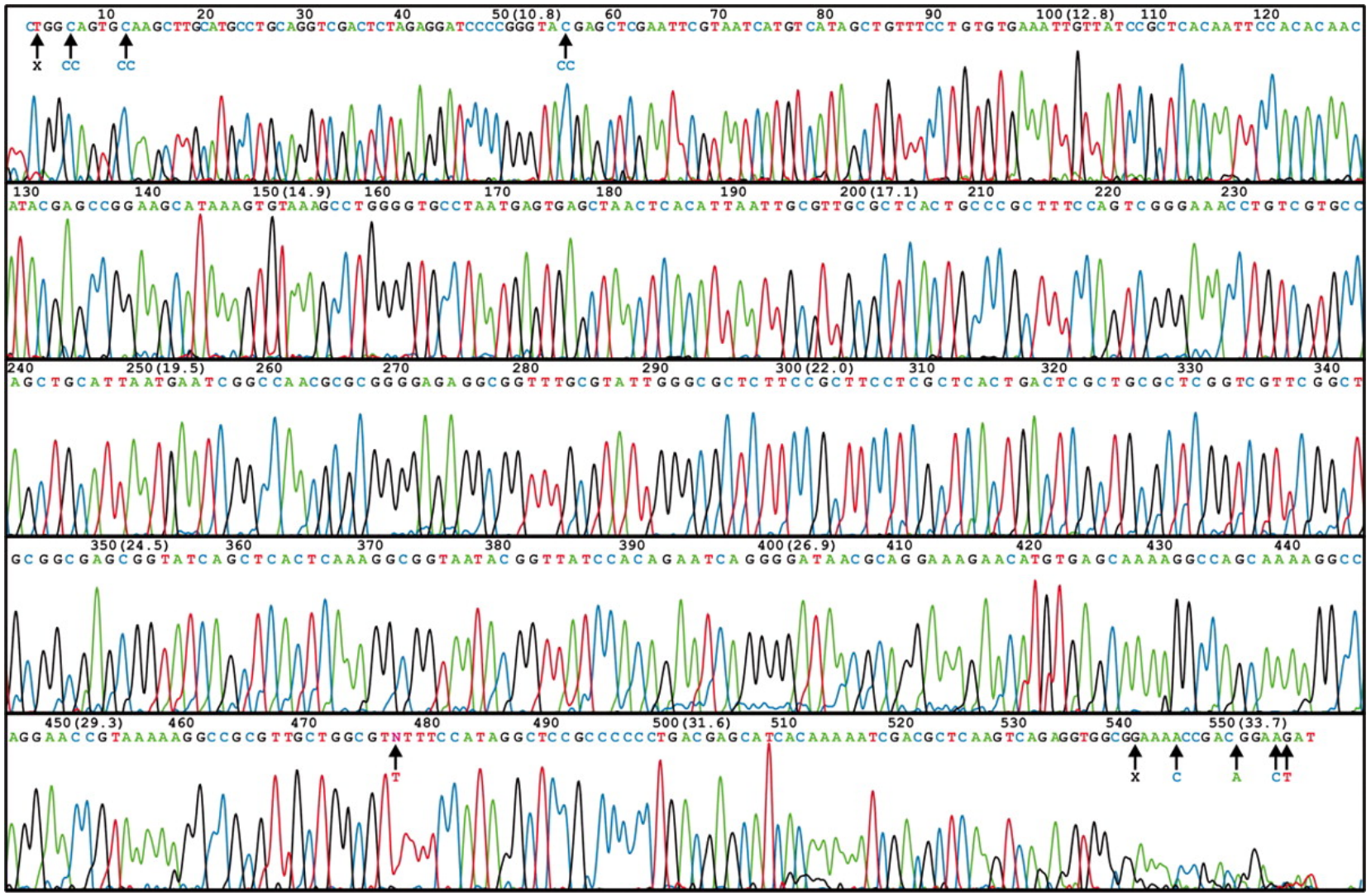
• Each dideoxy nucleotide is attached to a florescent marker.

• At the end of each cycle, a laser beam can detect the florescent marker and thus record the position of the nucleotide.



Sanger sequencing - Automated

Chromatogram - Automated



To know

PCR

DNA polymerase

Sanger sequencing

Capillary electrophoresis

ddNTPs

DNA copies

annealing

Polymerase chain reaction

thermocycler

denaturation

Automated sanger sequencing

primer

3’-OH

Polyacrylamide gel

2 #cycle

chromatogram

DNA template

dNTPs

extension

Chain termination sequencing

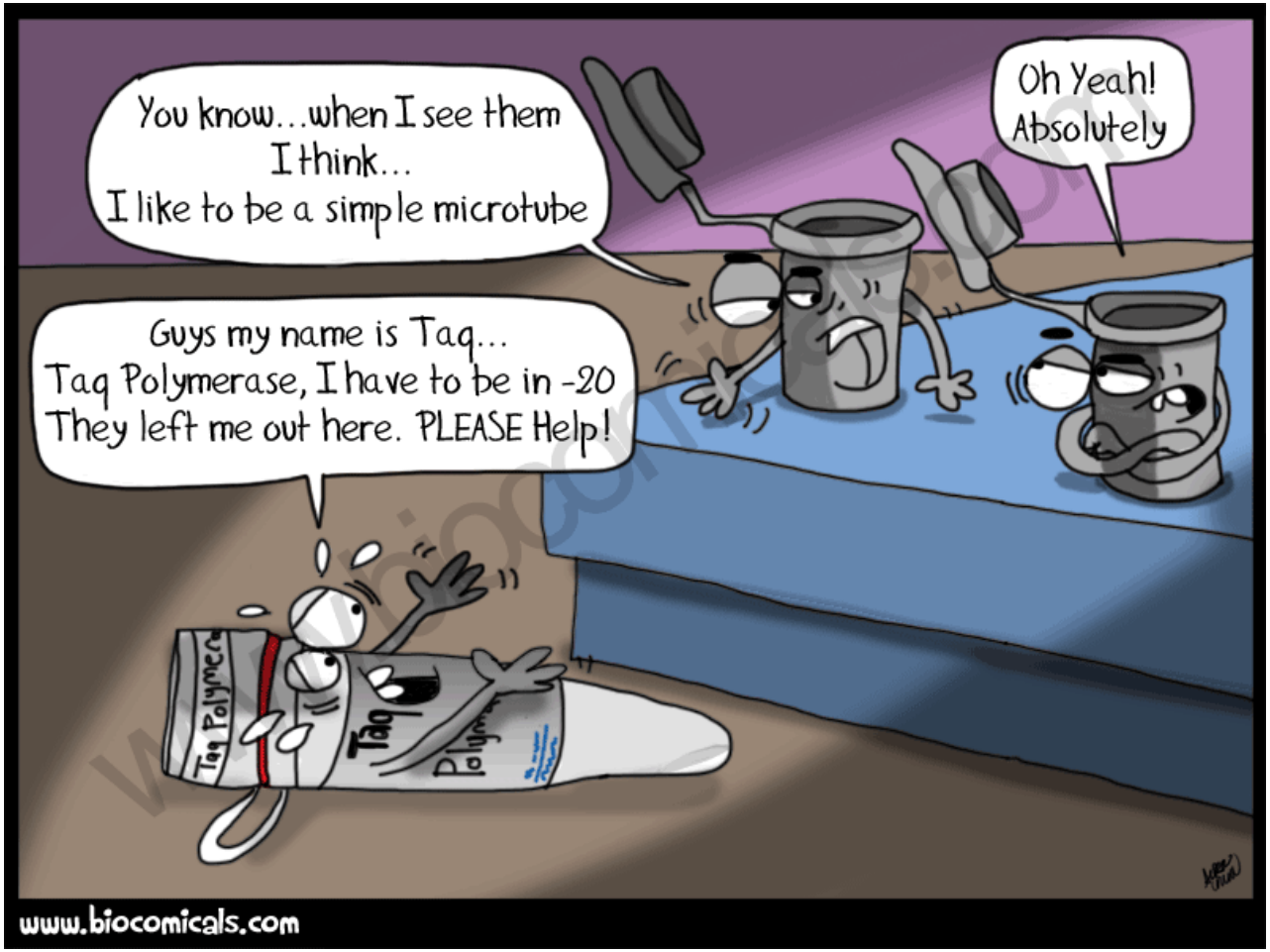
Dideoxy sequencing

Taq polymerase

Expectations

- You know PCR's components and process.
- You know the phases of PCR and what happens in each phase.
- You know how important it is for molecular applications.
- You know DNA sequencing using Sanger method.

For a smile


You know...when I see them
I think...
I like to be a simple microtube
Oh Yeah!
Absolutely
Guys my name is Taq...
Taq Polymerase, I have to be in -20
They left me out here. PLEASE Help!
Taq Polymera
Taq
www.biocomicals.com