Pharmaceutical Biotechnology

(PCS521)

Lecture Four

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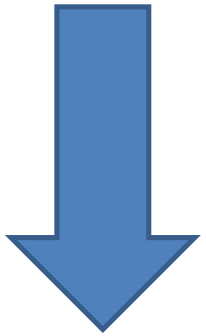
Different Molecular Techniques

- Nucleic acid Extraction
- Conventional PCR
- Gel Electrophoresis

Nucleic Acid Isolation



PCR

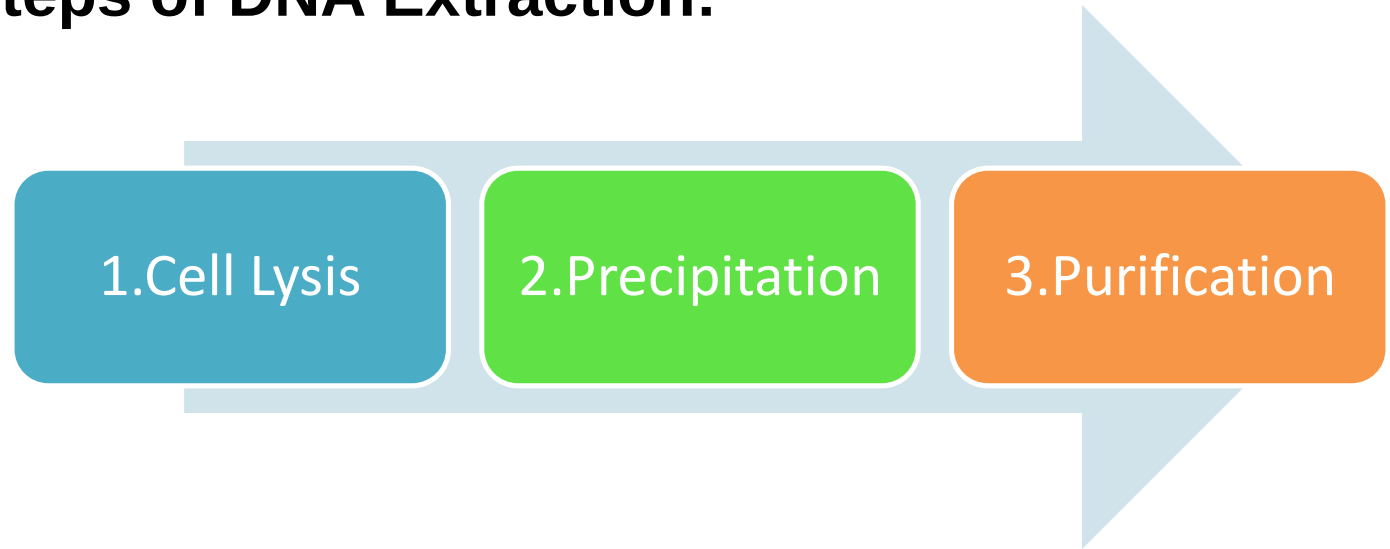
Conventional PCR
Real-Time (Quantitative) PCR
Reverse transcriptase PCR
Nested PCR
Multiplex PCR
Etc…..

1- Nucleic Acid Isolation

Nucleic Acid Isolation

- **DNA** is found within the **cell nucleus** while **RNA** is found either in the **nucleus or cytoplasm**.
- To study DNA, we first have to get it out of the cell.
- **AIM**: The DNA extraction process frees DNA from the cell and then separates it from cellular fluid and proteins, leaving behind pure DNA.

- **Basic Steps of DNA Extraction:**



1. Cell Lysis

- In this step, the cell and the nucleus are "**lysed**" to release the DNA inside.
- Cell lysis results in the release of all organic components within the cell into solution, such as nucleic acids, proteins, carbohydrates, lipids (the most abundant of these being proteins). There are two ways to do this:

a. **Mechanical disruption:** breaks open the cells. This can be done with a tissue homogenizer or with Sonication. Mechanical disruption is particularly important when using plant cells because they have a tough cell wall.

b. Lysis using **Detergents** (such as Sodium Dodecyl Sulphate - SDS) & **Enzymes** (such as Proteinase K) to free the DNA and dissolve cellular proteins.
   - Phospholipids from the cell membrane and the nucleus are disrupted with detergents and surfactants.
   - Breaking down proteins by adding a protease
   - In addition, RNase enzyme may be added to break down RNA.

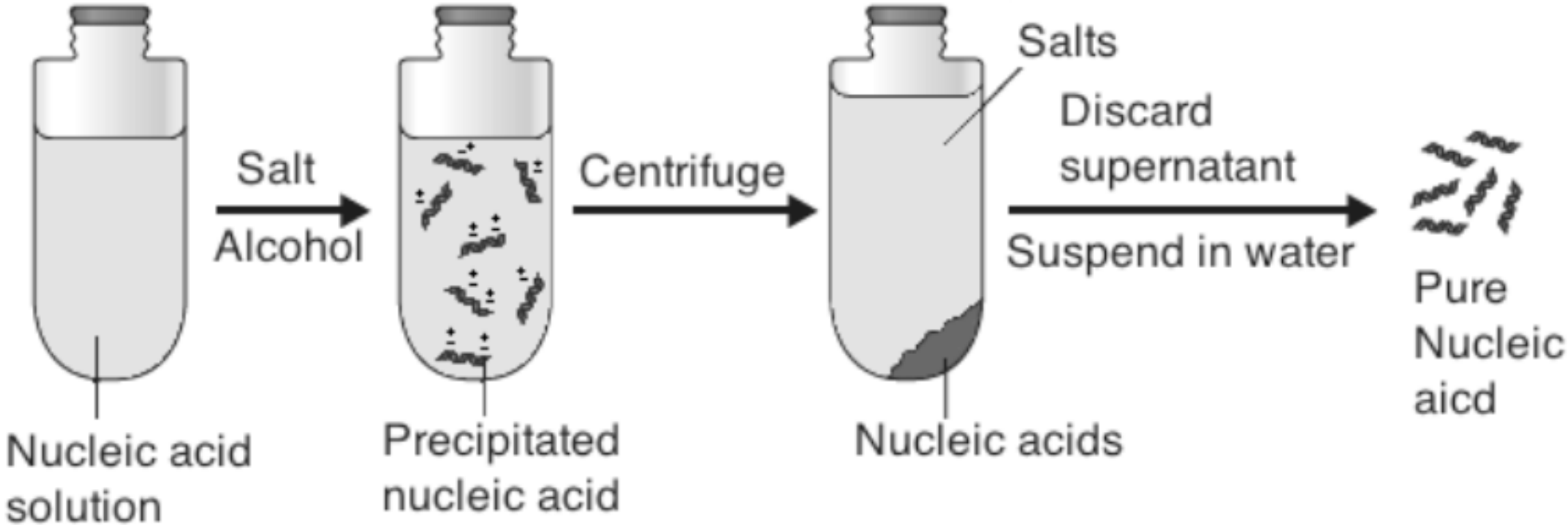
2. Precipitation

Now DNA is mixed with mashed up cell parts and debris. **Precipitation aims to separate the DNA from this cellular debris.**

1) First, **Na+ ions (e.g. sodium acetate)** may be added to neutralize the negative charges on the DNA molecules, which makes them more stable and less water soluble.

2) Next, **alcohol (such as 70% ethanol or isopropanol)** is added, which facilitates Na+ to interact with the PO3- (in DNA). Alcohol also removes the hydration shell of H2O molecules around the phosphate. The total effect is that the DNA molecules can come closer together due to neutralization of charge and removal of water that eventually leads to the precipitation from the solution.

3) Upon **centrifugation**, DNA will aggregate together forming a pellet.

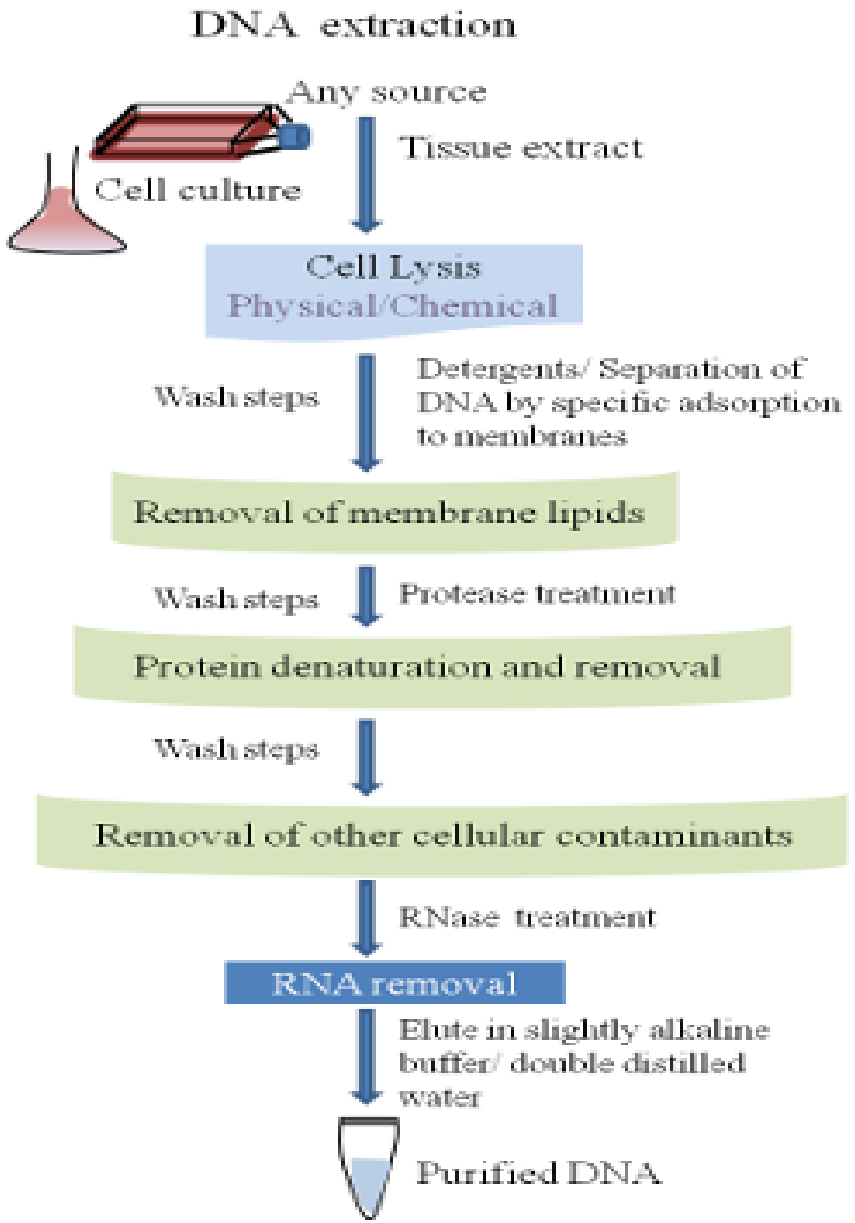

Salt
Alcohol
Centrifuge
Salts
Discard
supernatant
Suspend in water
Pure
Nucleic
aicd
Nucleic acid
solution
Precipitated
nucleic acid
Nucleic acids

2. Purification

- Now that DNA has been separated from the aqueous phase, it can be **rinsed with alcohol** to purify it & remove any remaining unwanted material and cellular debris.

- At this point the purified DNA in pellet is then **re-dissolved in water** for easy handling and storage.

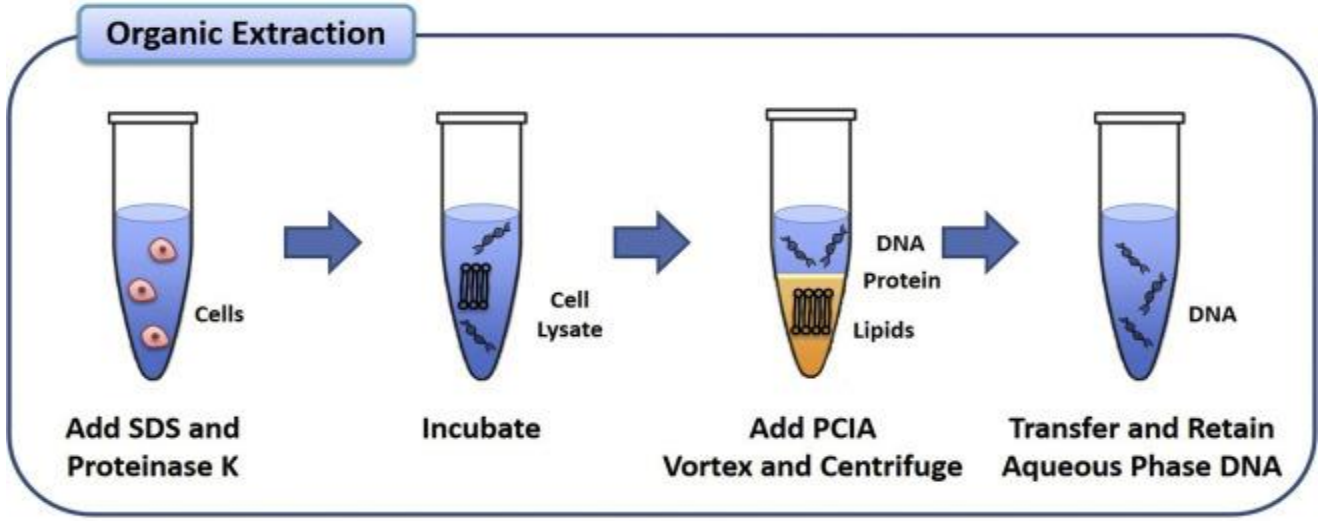
N.B: Mini-column purification is another method that relies on the fact that the nucleic acids may bind (adsorption) to the solid phase (e.g. silica)



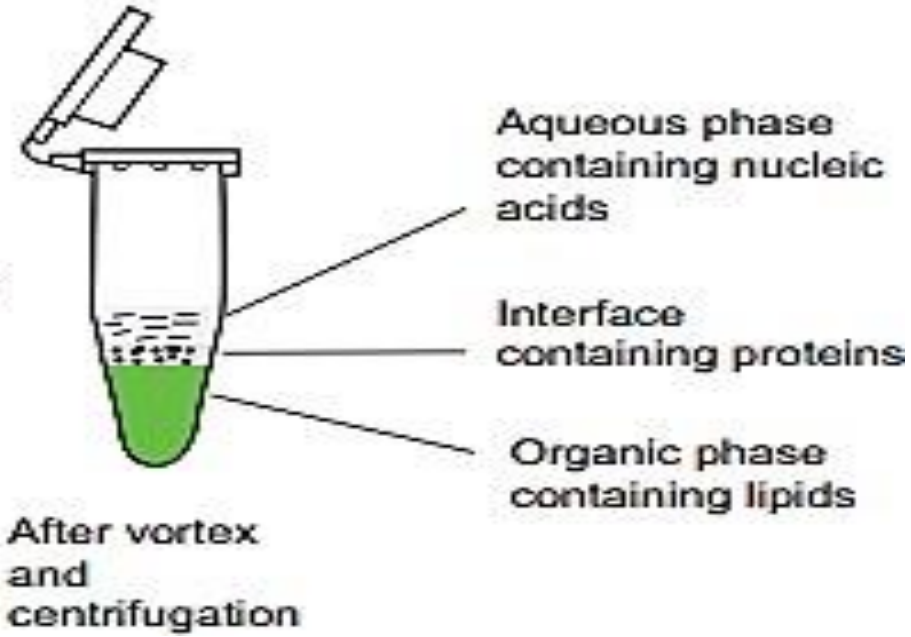
Organic Phenol–Chloroform extraction method

1. **Cell Lysis:** sodium dodecylsulfate (SDS) + proteinase K (for the enzymatic digestion of proteins and non-nucleic acid cellular components)

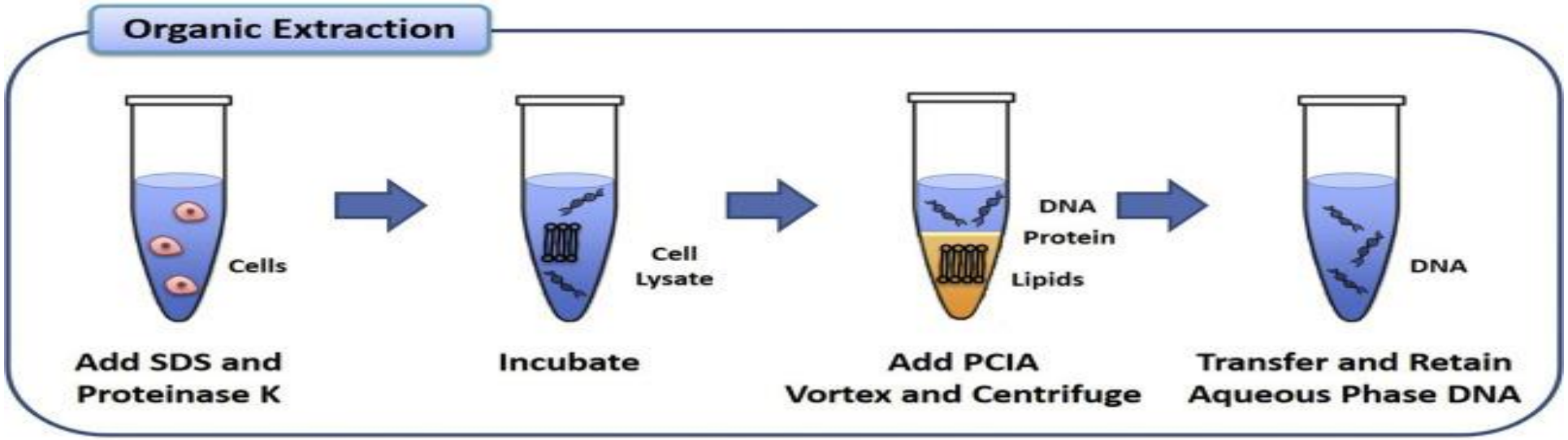
2. A mixture of **phenol: chloroform: isoamyl alcohol** (25:24:1) is then added to promote the partitioning of lipids and cellular debris into the organic phase, leaving isolated DNA in the aqueous phase. Chloroform mixed with phenol is more efficient at denaturing proteins than either reagent is alone.



3. When the mixture is vortexed and centrifuged, the denser organic layer containing lipids settles at the bottom of the tube while aqueous phase containing nucleic acids remains at the top. Proteins may be found at the interface of the aqueous and organic phase.



4. The aqueous phase containing the purified DNA can be transferred to a clean tube for analysis.



2- Polymerase Chain Reaction (PCR)

PCR: Where does the name come from?

Polymerase: DNA polymerase enzyme

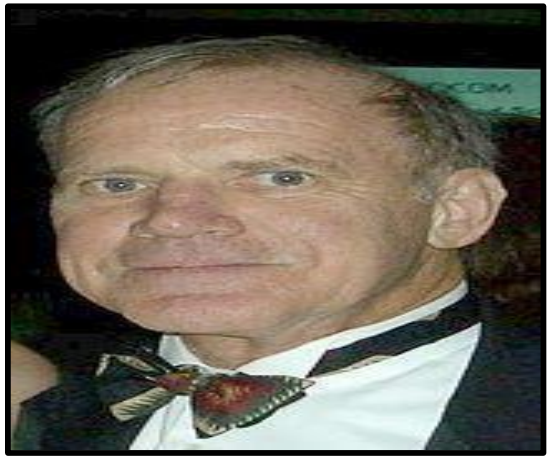
DNA polymerase duplicates DNA

Chain Reaction: The product of a reaction is used to amplify the next reaction

Results in rapid increase in the amount of product

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (**PCR**) is a molecular biology technique used in the lab to amplify (make millions of copies) of a particular section of DNA. It is a revolutionary method developed by Kary Mullis in the 1980s.



Kary Mullis

1. **The developer of PCR Technique – 1987**
2. **Published paper - 1990.**
3. **Nobel Prize- 1993.**

The Unusual Origin of the Polymerase Chain Reaction

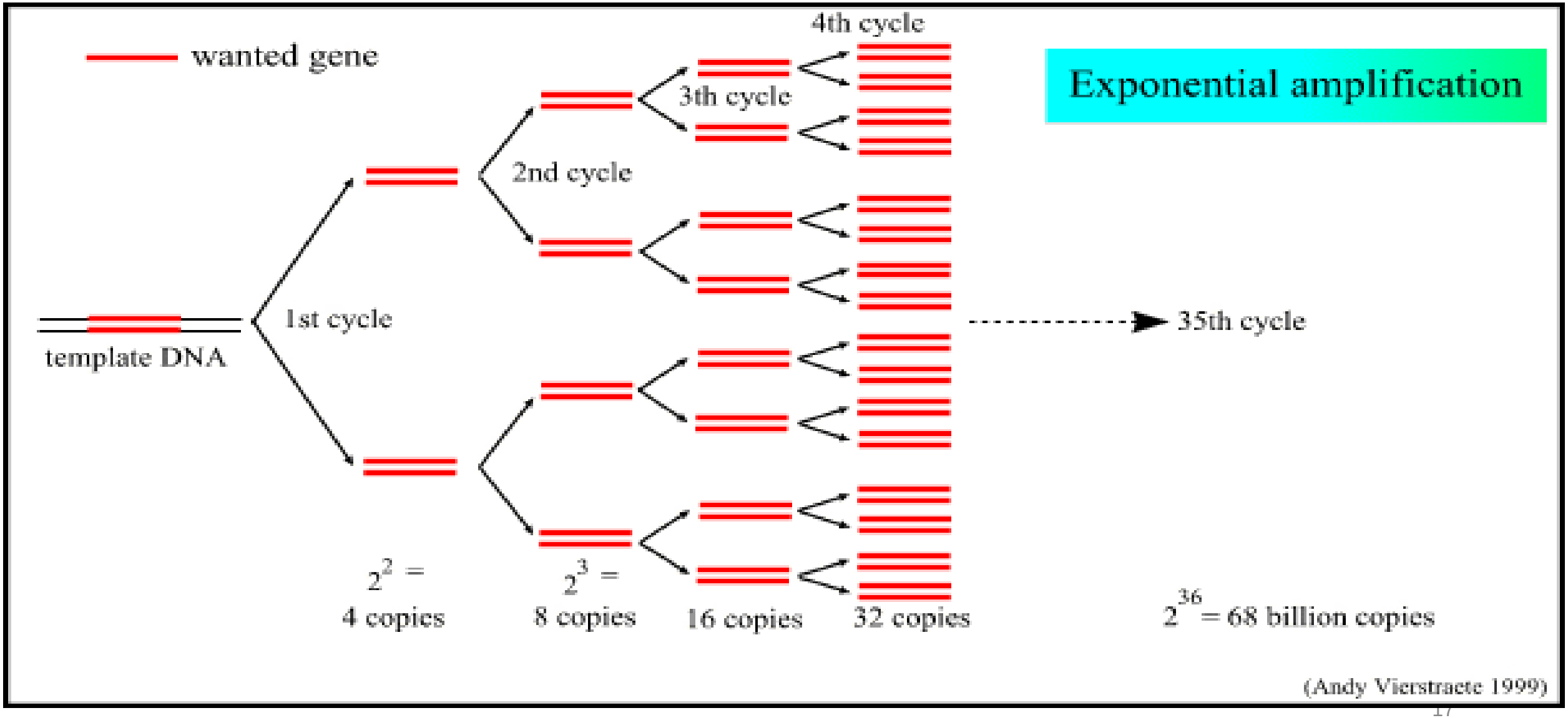
Polymerase Chain Reaction Technique

- It is fast technique for amplifying a specific segment of DNA into many fragments with identical size and sequence by enzymatic method and special cycling conditions.
- This requires the use of a special device called a PCR thermal cycler.

-Repetitive cycling at specific temperatures allows the amplification of a unique gene to **millions of copies**

- The **newly generated DNA strands serve as template DNA for the next cycle,** leading to an exponential increase in the number of amplicons.

Polymerase Chain Reaction (PCR)



Components Required for the (PCR) Reaction

a. DNA template (sample) to be amplified.

b. DNA polymerase:

- ***Taq* DNA polymerase :** a special enzyme with **high optimal temperature**
- Uses single-stranded DNA as a template.
- Requires **primers** to initiate DNA synthesis.

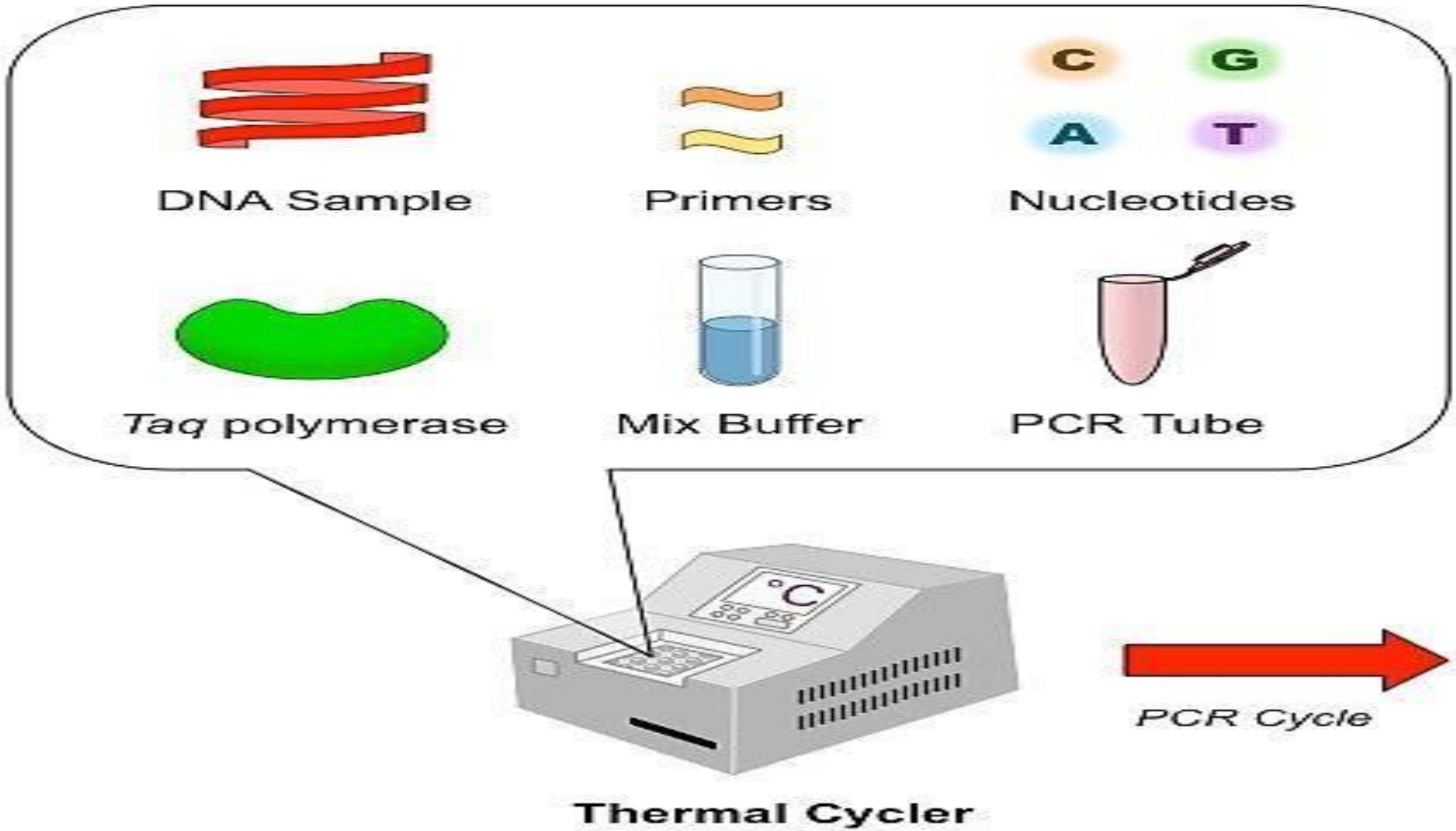
c. Nucleotides: Called Deoxynucleoside triphosphates (dNTPs)- dATP, dTTP, dGTP, dCTP

d- Pair of primers:

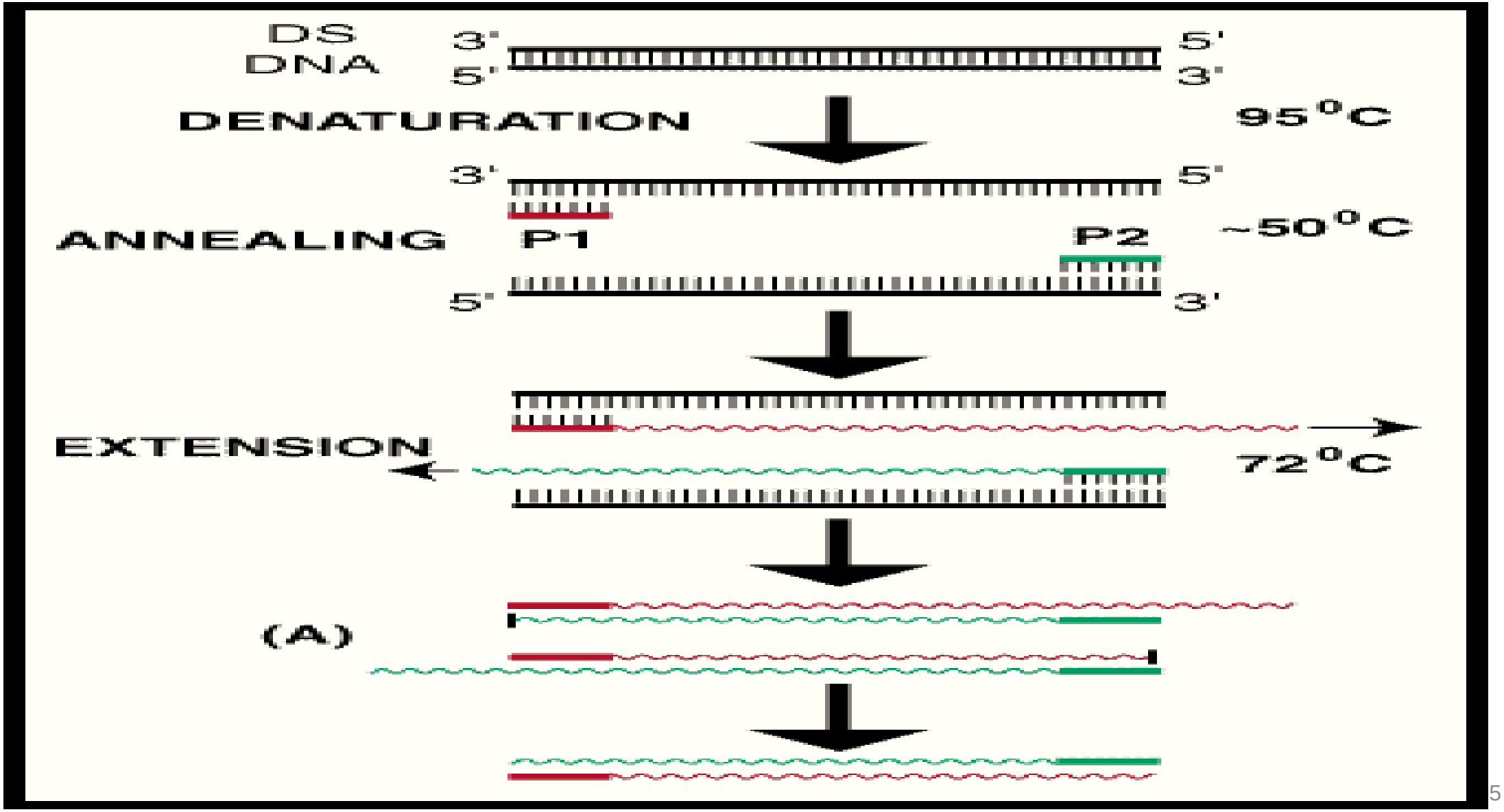
- Generally, **20-30 nucleotides** long.
- Synthetically produced.
- Designed/Chosen to flank the targeted DNA region.
- One primer binds to the 5' end of one of the DNA strands.
- The other primer binds to the 3' end of the anti-parallel DNA strand.
- Delineate the region of DNA you want to be amplified.

e- Water. **f- Buffer/ Mg+2**

PCR Components



Steps of PCR

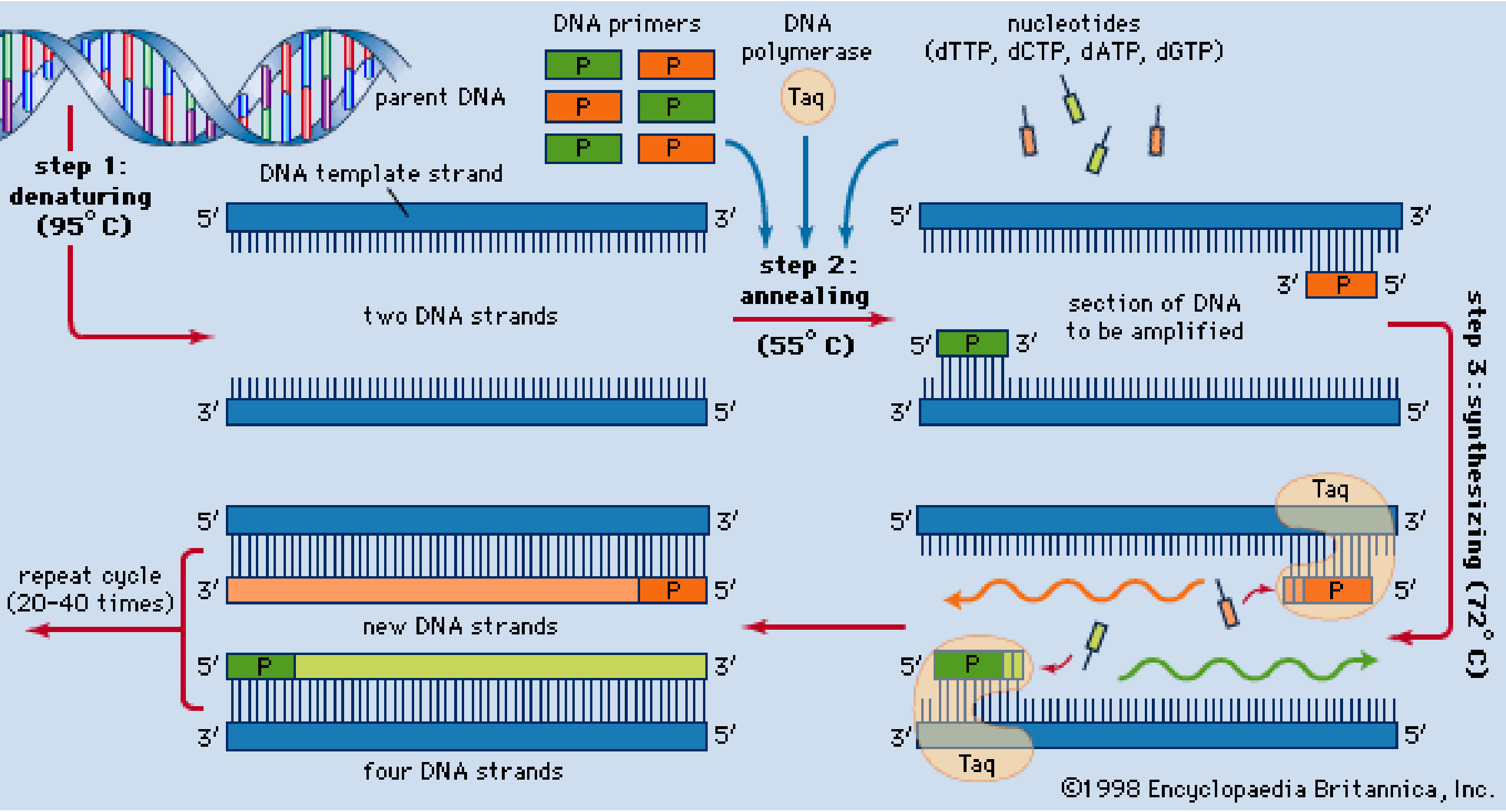

DS DNA
3'
5'
5'
3'
DENATURATION
95°C
3'
5'
ANNEALING
P1
P2
~50°C
5'
3'
EXTENSION
72°C
(A)

Steps of PCR

1. **Denaturation Step:**
   DNA sample is heated to 95^{o}C to separate the two DNA template strands.
2. **Annealing:**
   Cooling to 55^{o}C to allow short single-stranded primers to anneal to complementary sequences on template DNA.
3. **Extension (Synthesis of a new dsDNA):**
   Heating to 72^{o}C; DNA polymerase (Taq) synthesizes new DNA strands creating 2 new double-stranded DNA.

Then: Repeating the cycle:

Each time the cycle repeats the amount of DNA doubles;
After 20 cycles, i.e. 2^{20} =1 million dsDNA copies are formed, and after 30 cycles, i.e. 2^{30} = 1 billion dsDNA copies are formed


DNA primers
DNA polymerase
nucleotides
(dTTP, dCTP, dATP, dGTP)
parent DNA
Taq
step 1:
denaturing
(95° C)
DNA template strand
5′
3′
step 2:
annealing
(55° C)
two DNA strands
section of DNA
to be amplified
P
step 3: synthesizing (72° C)
repeat cycle
(20-40 times)
new DNA strands
four DNA strands
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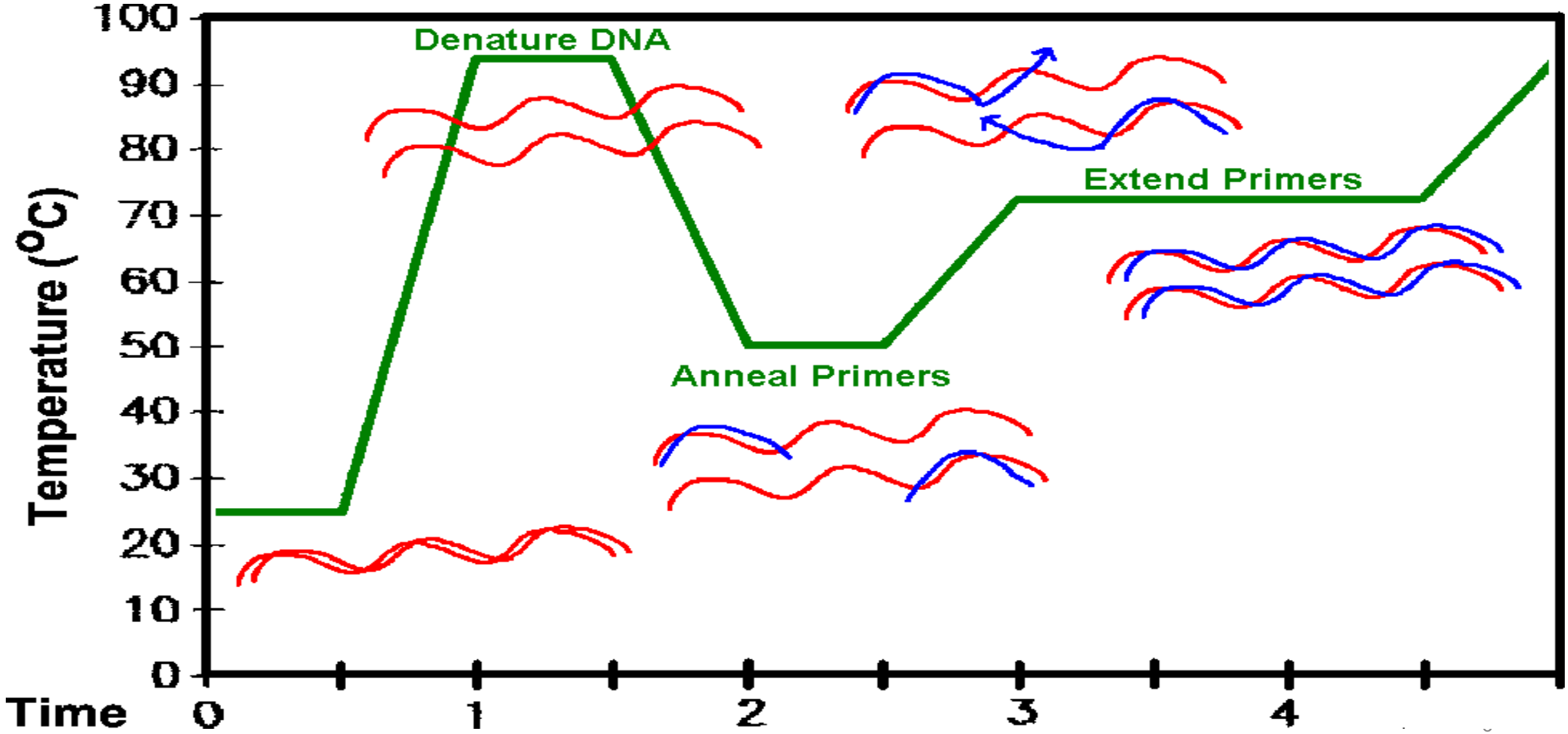
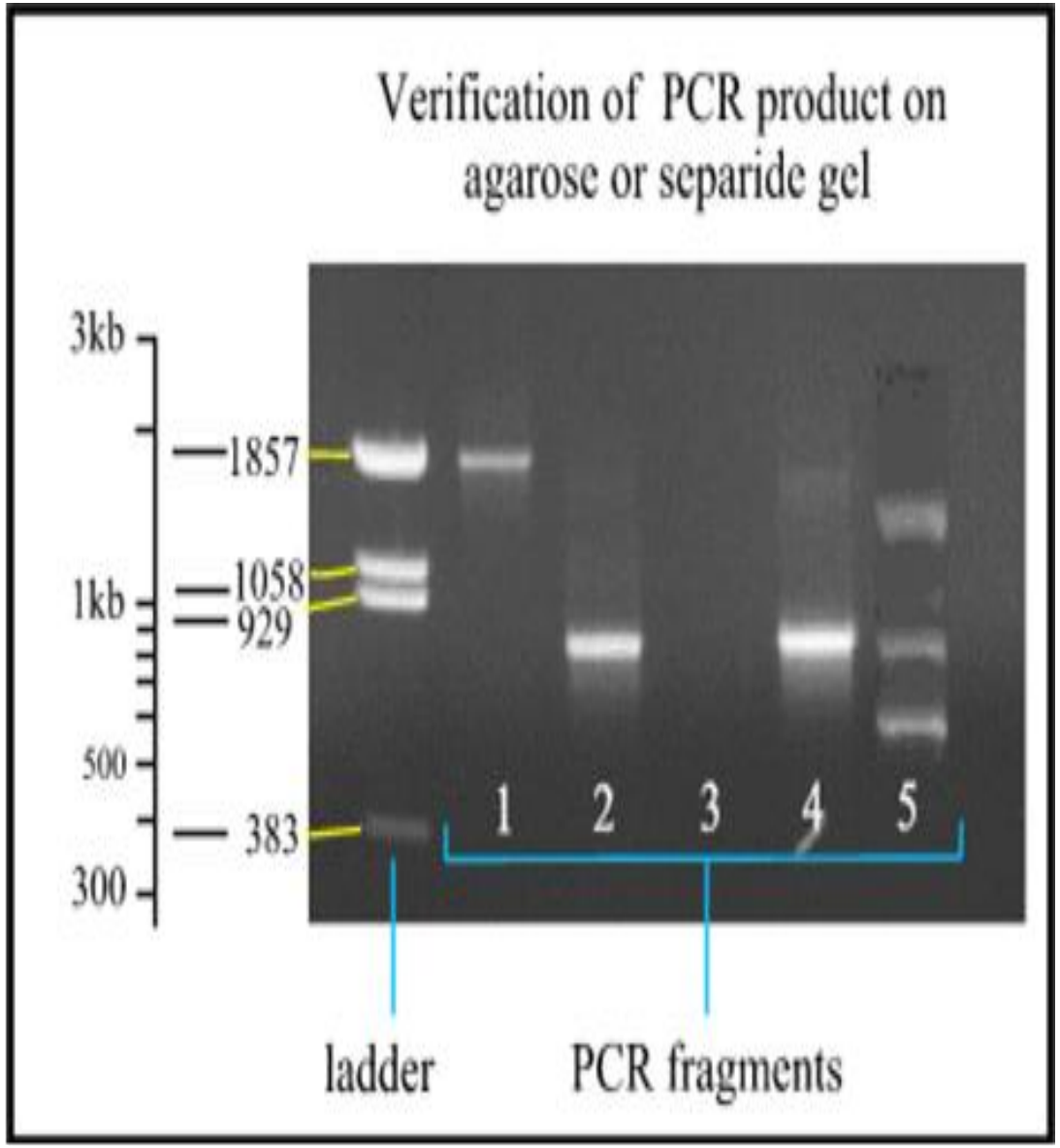
PCR Cycler
(Temperature vs Time (Hr))



Post-PCR processing

Detection of the target is accomplished by different methods:

- **gel electrophoresis**,
- by use of **labeled probes** (in real time PCR)
- **by end-product** analysis like melt curve analysis of the amplified product

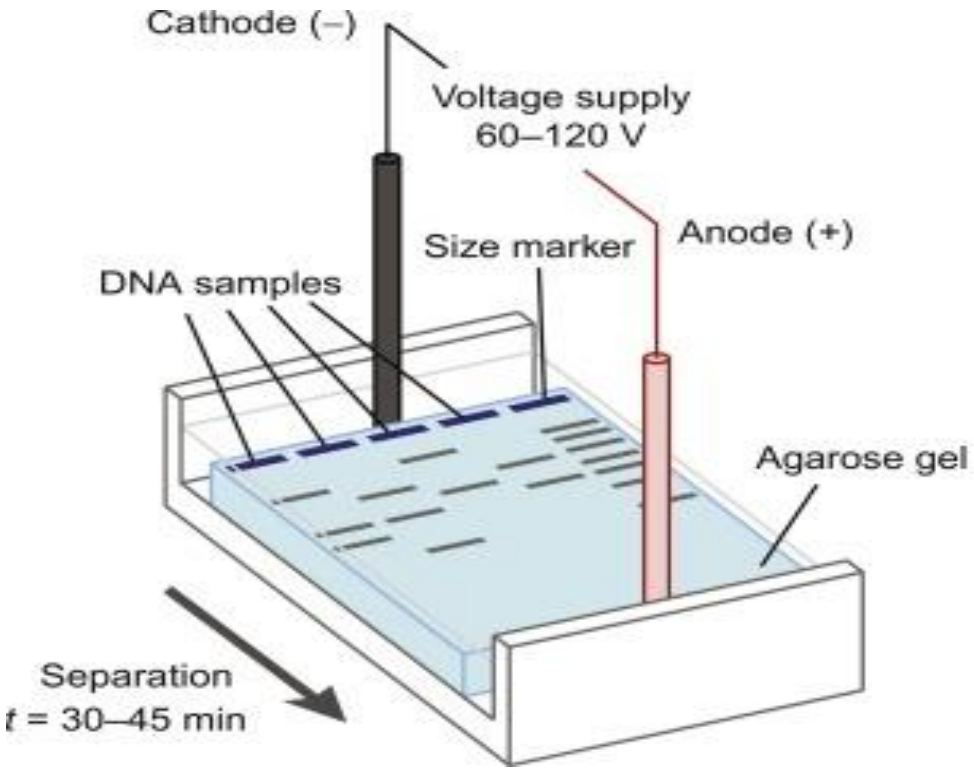


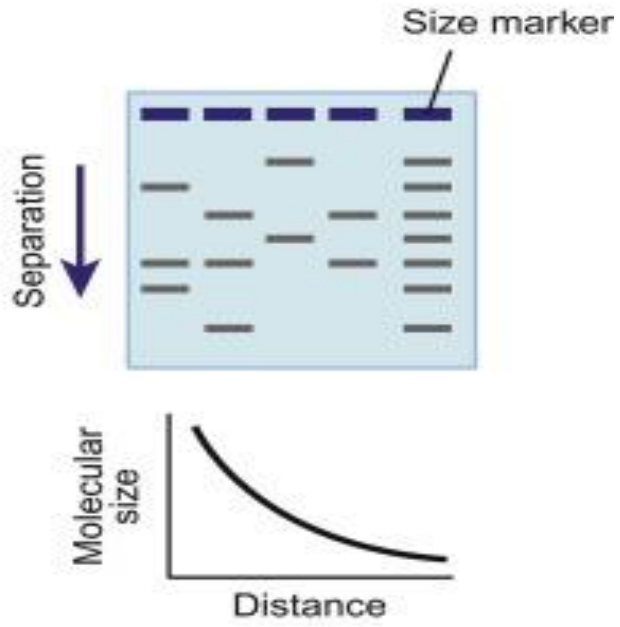
3. Gel Electrophoresis

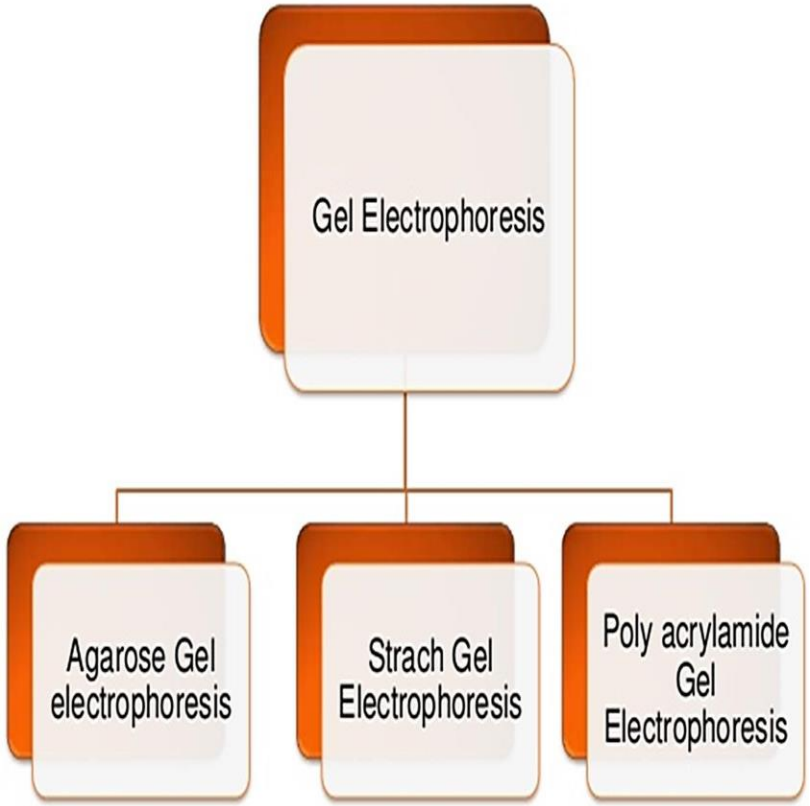
ELECTROPHORESIS

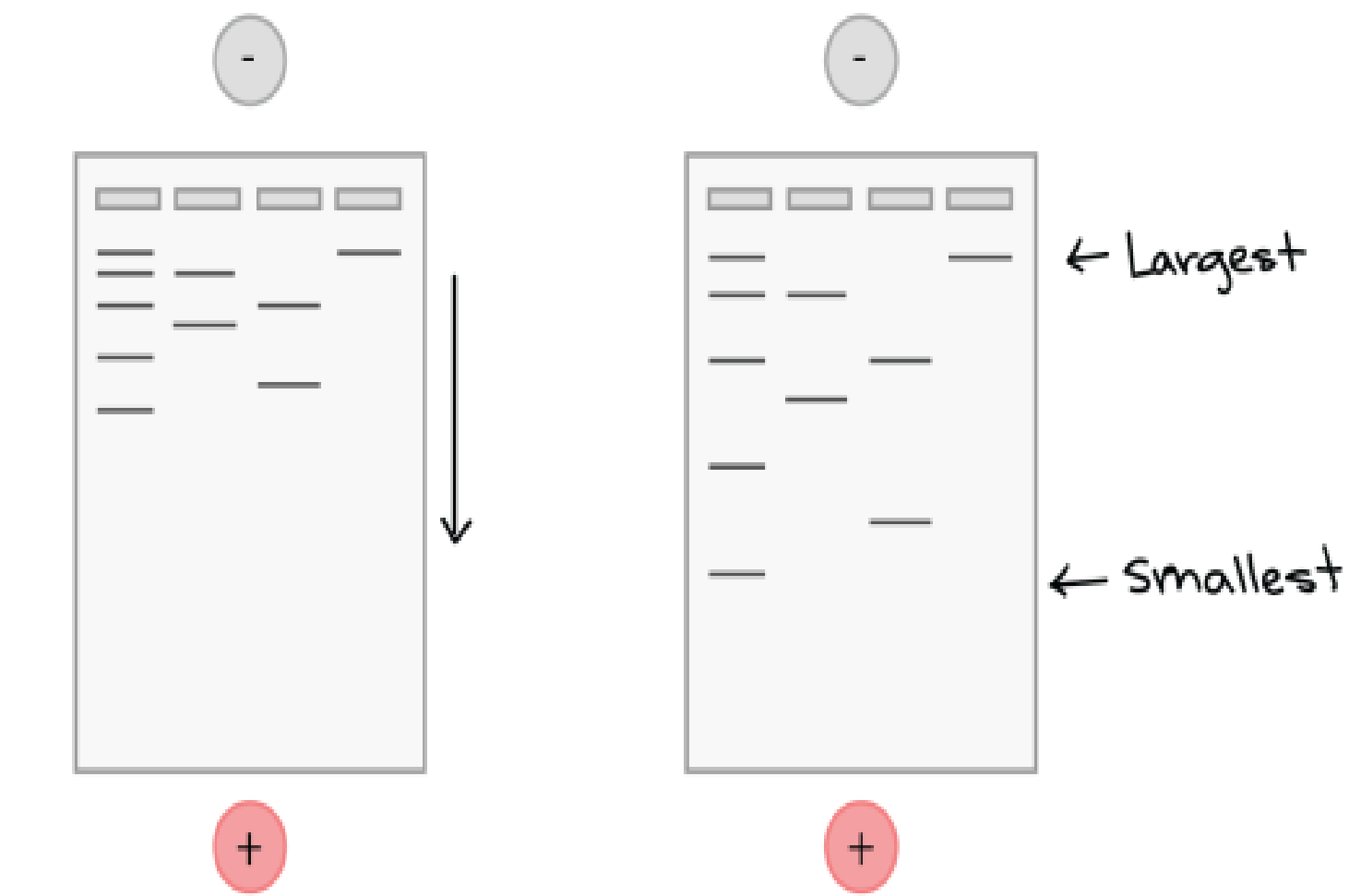
- **Electrophoresis** is a technique used in laboratories in order to separate macromolecules (protein, DNA and RNA) based on size.

- The technique applies an electric charge; so the negatively charged proteins and DNA move towards the positively charged electrode.








-
-
← Largest
← Smallest
+
+
DNA fragments migrate through gel.
The fragments are separated by size.

Agarose gel equipment/chemicals

An electrophoresis chamber and power supply

Gel casting trays

Sample combs

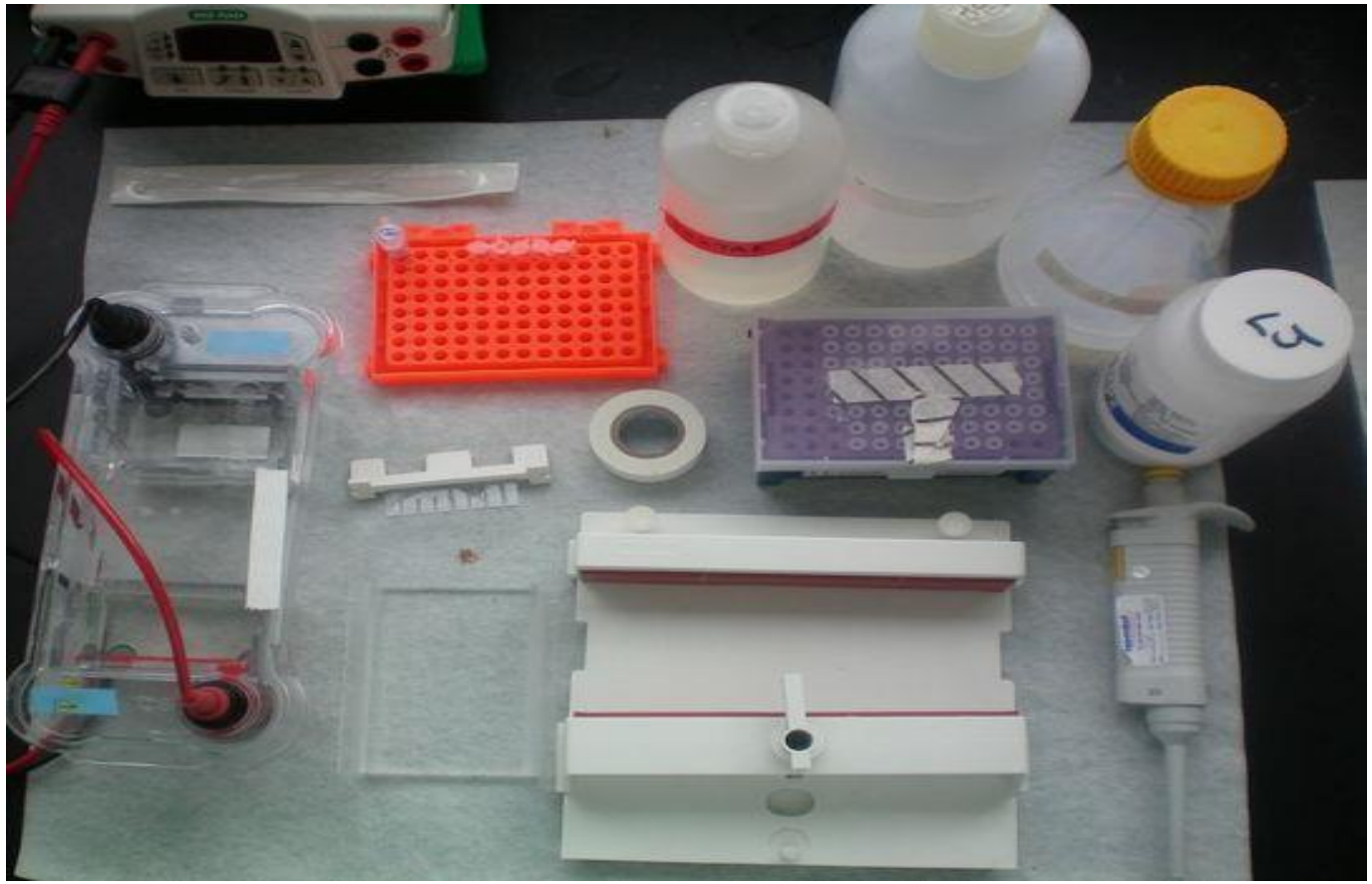
Agarose

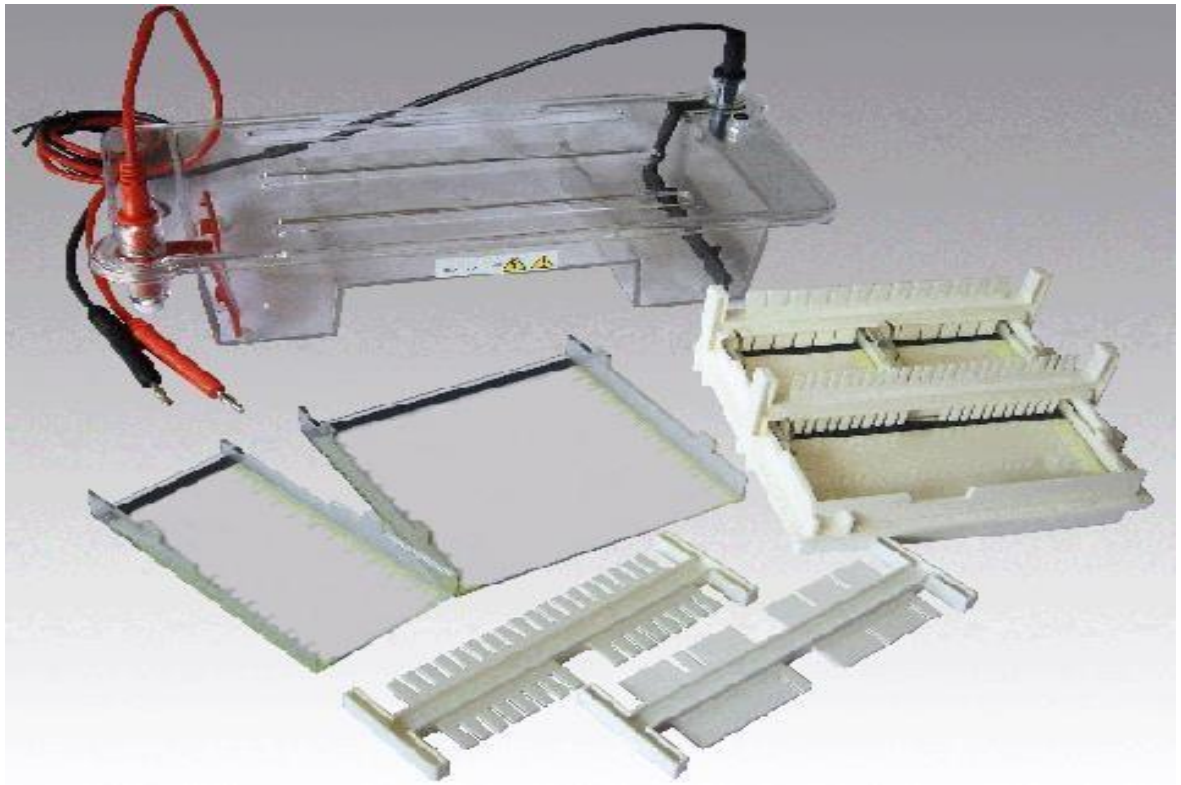
Electrophoresis buffer

Loading buffer

Staining

UV Transilluminator

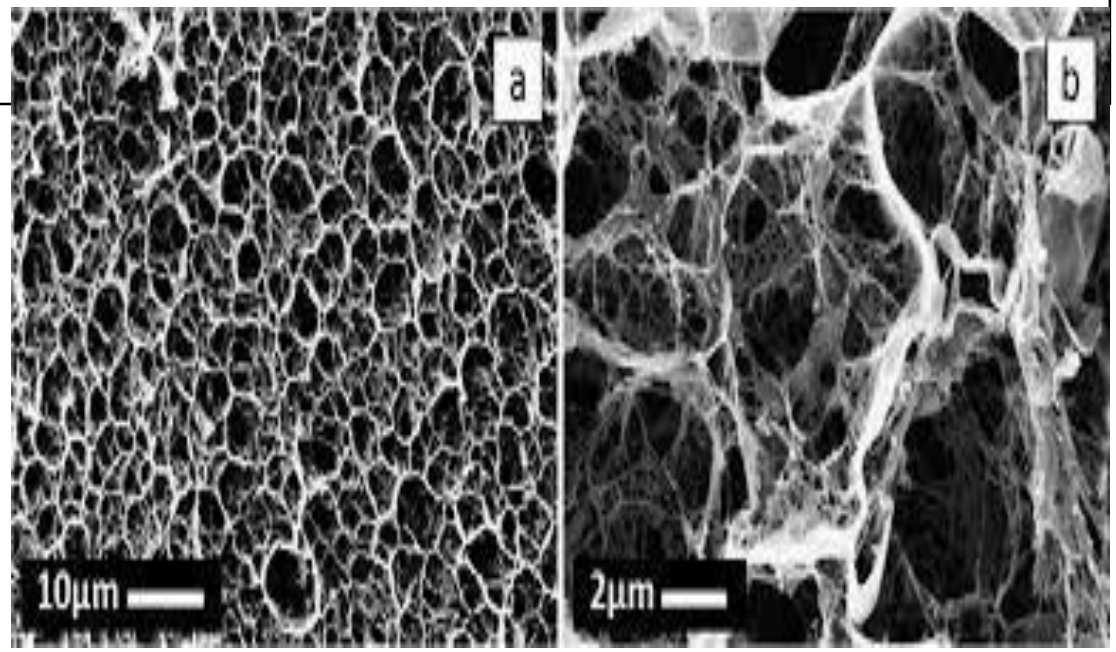




1- Agarose

Advantages	Disadvantages
Nontoxic gel medium	High cost of agarose
Gels are quick and easy to cast	Fuzzy bands
Good for separating large DNA molecules	Poor separation of low molecular weight samples
Can recover samples by melting the gel, digesting with enzyme agarose or treating with chaotropic salts	

Concentration of agarose (%)	DNA size range (bp)
0.2	5000-40000
0.4	5000-30000
0.6	3000-10000
0.8	1000-7000
1	500-5000
1.5	300-3000
2	200-1500
3	100-1000



2- Electrophoresis Buffer Solutions

- Effective separation of nucleic acids by agarose gel electrophoresis depends upon the effective maintenance of pH within the matrix.

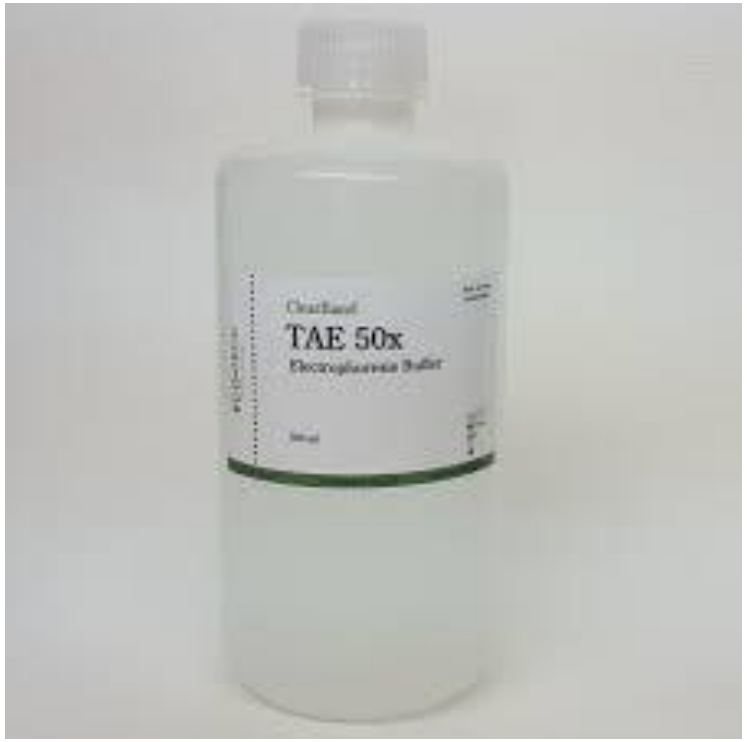
- Therefore, **buffers** are an integral part of any electrophoresis technique.

E.g.

Tris/Acetate/EDTA (TAE)

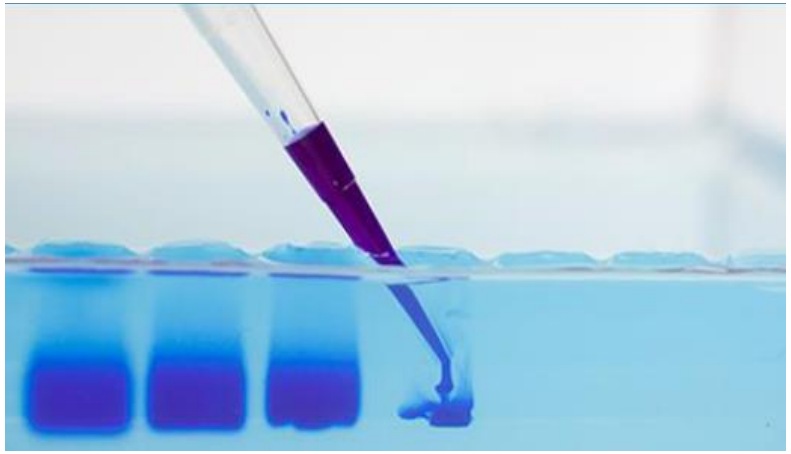
Tris/Borate/EDTA (TBE)

at a concentration of approximately 50 mM (**pH 7.5-7.8**).



3- Loading buffer

- This is the buffer to be added to the DNA fragment that will be electrophoresed.
- This buffer contains **glycerol** or **sucrose** to increase the density of the DNA solutions; otherwise, the samples would dissolve in running buffer tank and not sink into the gel pocket.
- Loading buffer also contains **dyes** that facilitate observation of the sample during gel loading and electrophoresis, such as **bromophenol blue** or **xylene cyanol**. These molecules are small, hence migrate quickly through the gel during electrophoresis, indicating the progress of electrophoresis.



4- Voltage/current applied

- The **higher** the voltage/current, the **faster** the DNA migrates.

- It is usually recommended to set the voltage at **5-15 Volts per cm of Gel**.

- **If the voltage is too high**:
  - **band streaking** may result.
  - **increase in buffer temperature in very short time** leads to the **melting of the gel**, **DNA bands smiling**, **decrease of DNA bands resolution** and **fuse blowout**.

- **If the voltage is too low**, **the mobility of small (≤1kb) DNA is reduced** and **band broadening** will occur due to dispersion and diffusion.

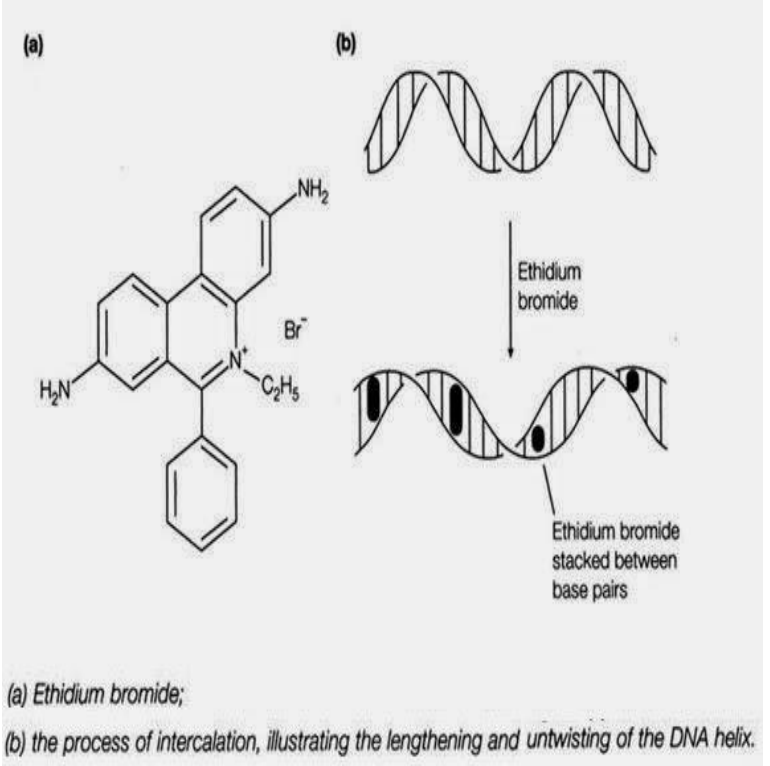
Visualizing the DNA

- The separated DNA species in the gel is visualized and localized by using **Ethidium bromide stain** (interchalating fluorescent dye) in very low concentrations under UV light.

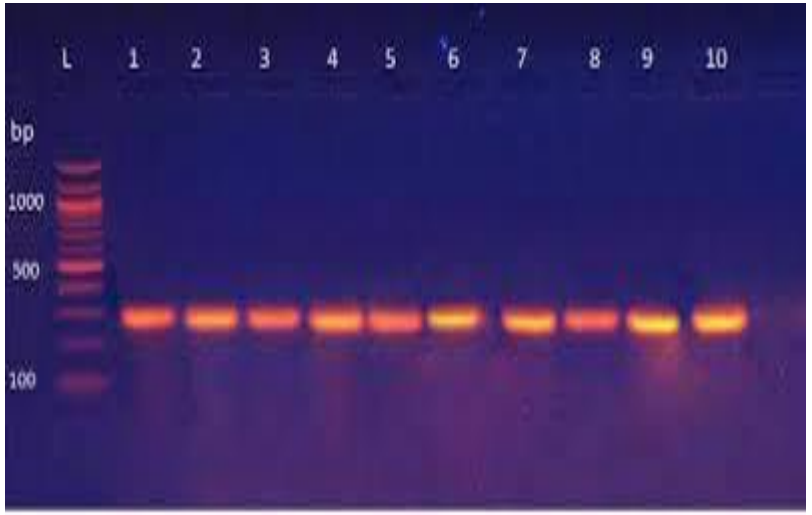
- The dye can be included in both the running buffer tank and the gel, the gel alone, or the gel can be stained after DNA separation.

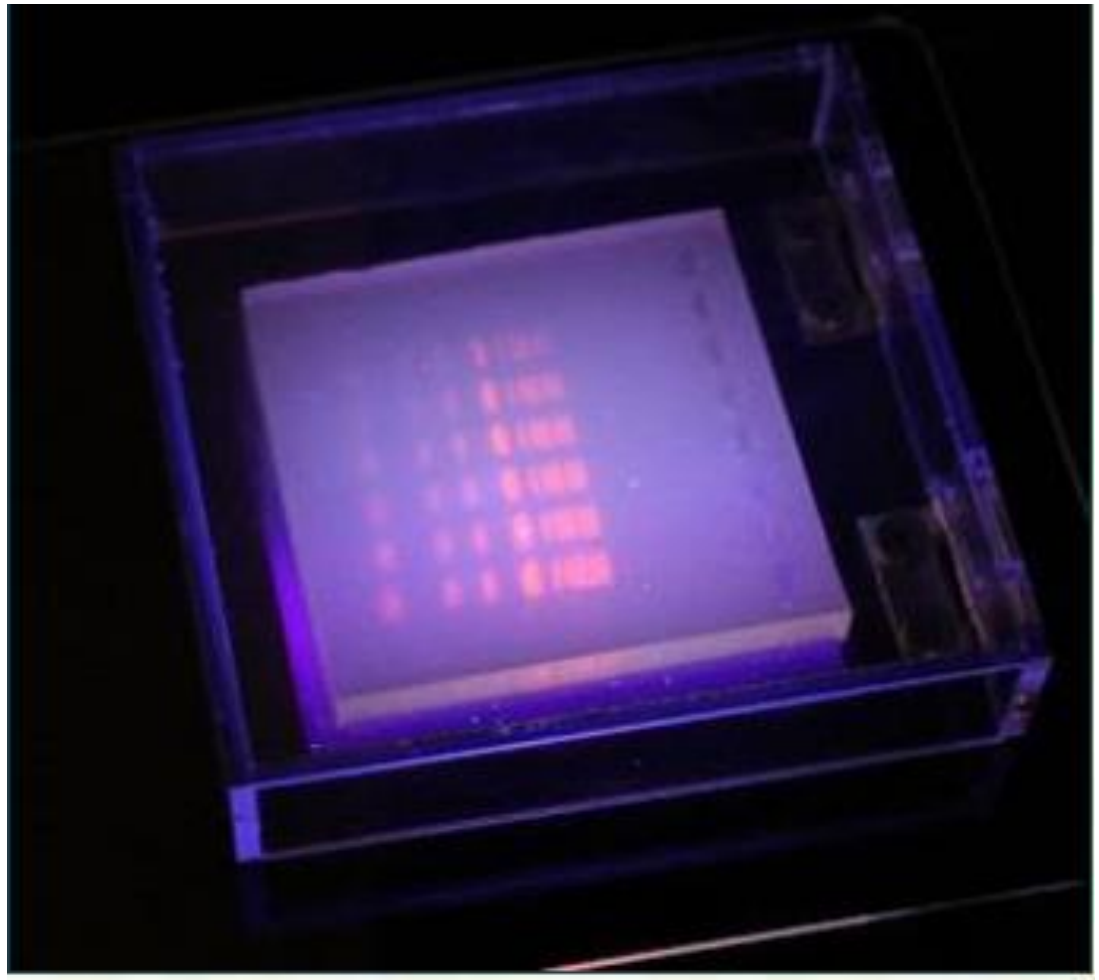
- Instant photos are taken from the gels under UV light in a dark room.

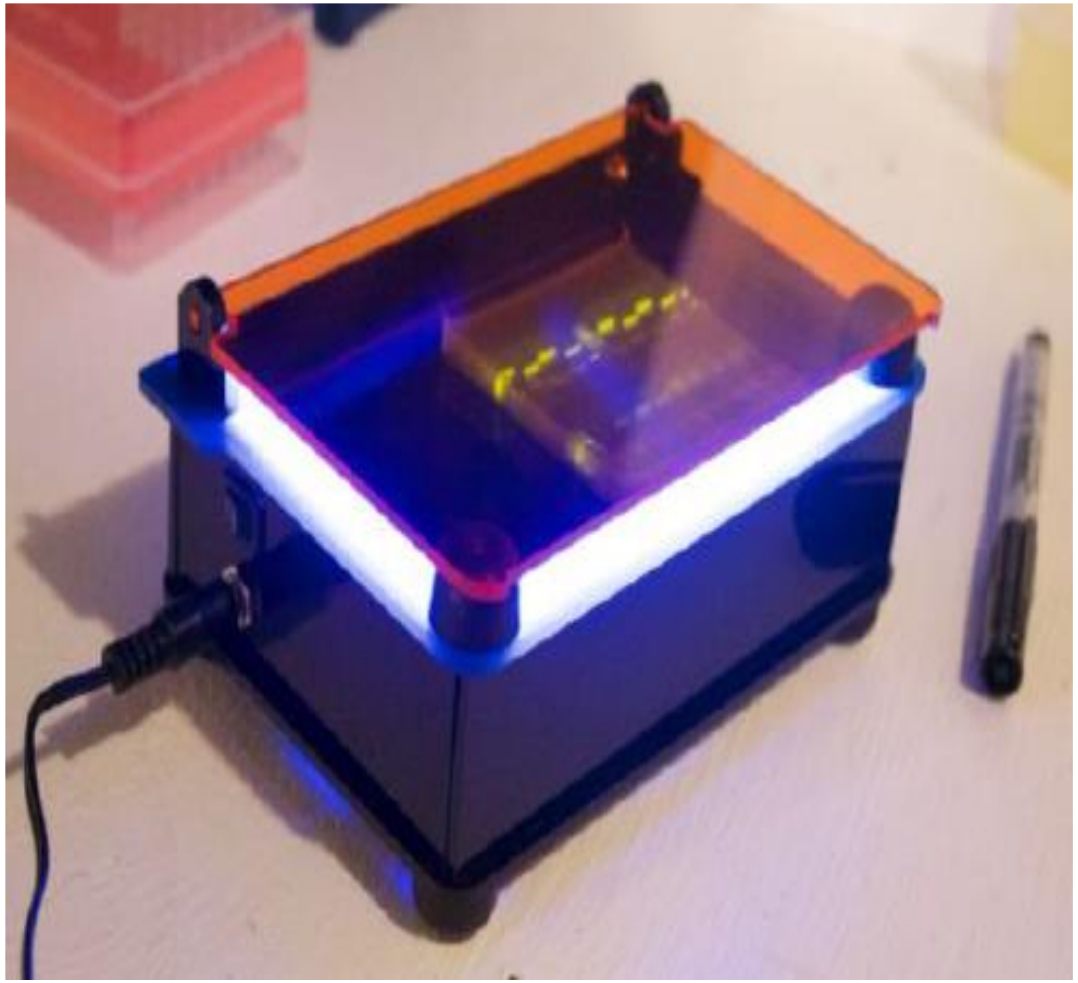
- It is important to note that ethidium bromide is a **potent mutagen** and should be handled with great care.



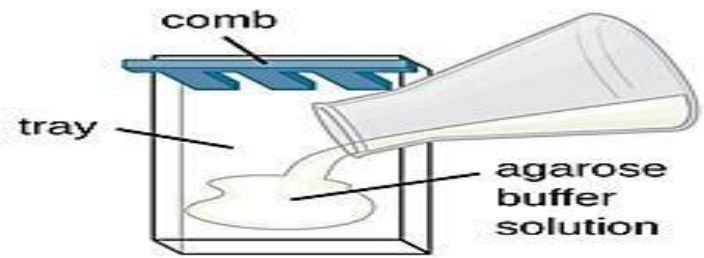
(a) Ethidium bromide;
(b) the process of intercalation, illustrating the lengthening and untwisting of the DNA helix.



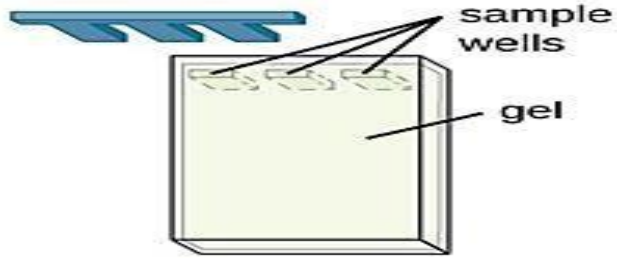




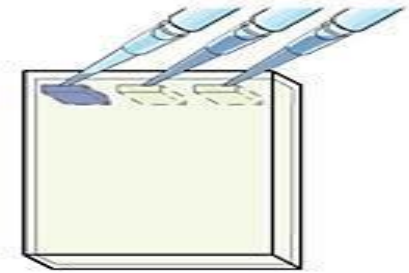
1. An agarose and buffer solution is poured into a plastic tray. A comb is placed into the tray on one end.



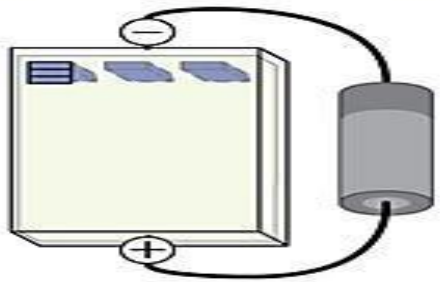
2. The agarose polymerizes into a gel as it cools. The comb is removed from the gel to form wells for samples.



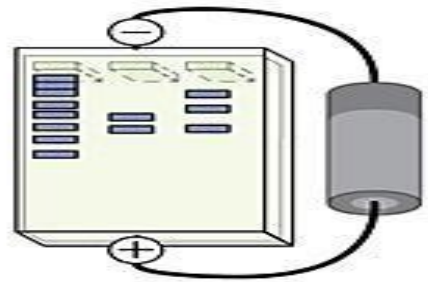
3. DNA samples colored with a tracking dye are pipetted into the wells.



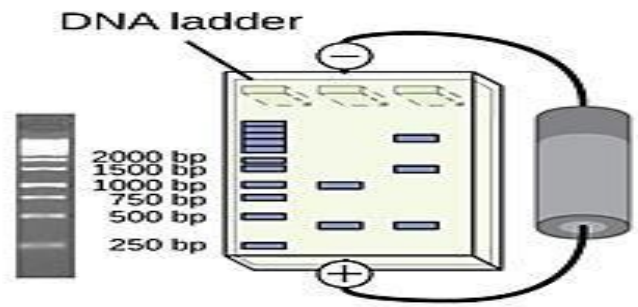
4. The tray is placed into a chamber that generates electric current through the gel. The negative electrode is placed on the side nearest the samples. The positive electrode is placed on the other side.



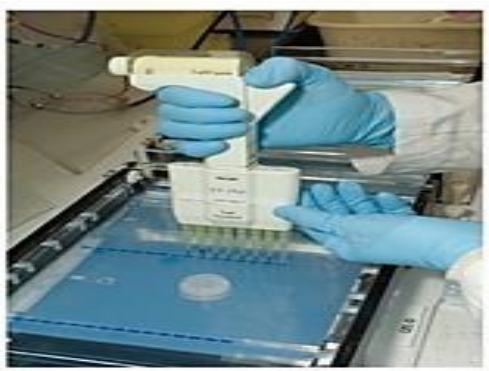
5. DNA has a negative charge and will be drawn to the positive electrode. Smaller DNA molecules will be able to travel faster through the gel.



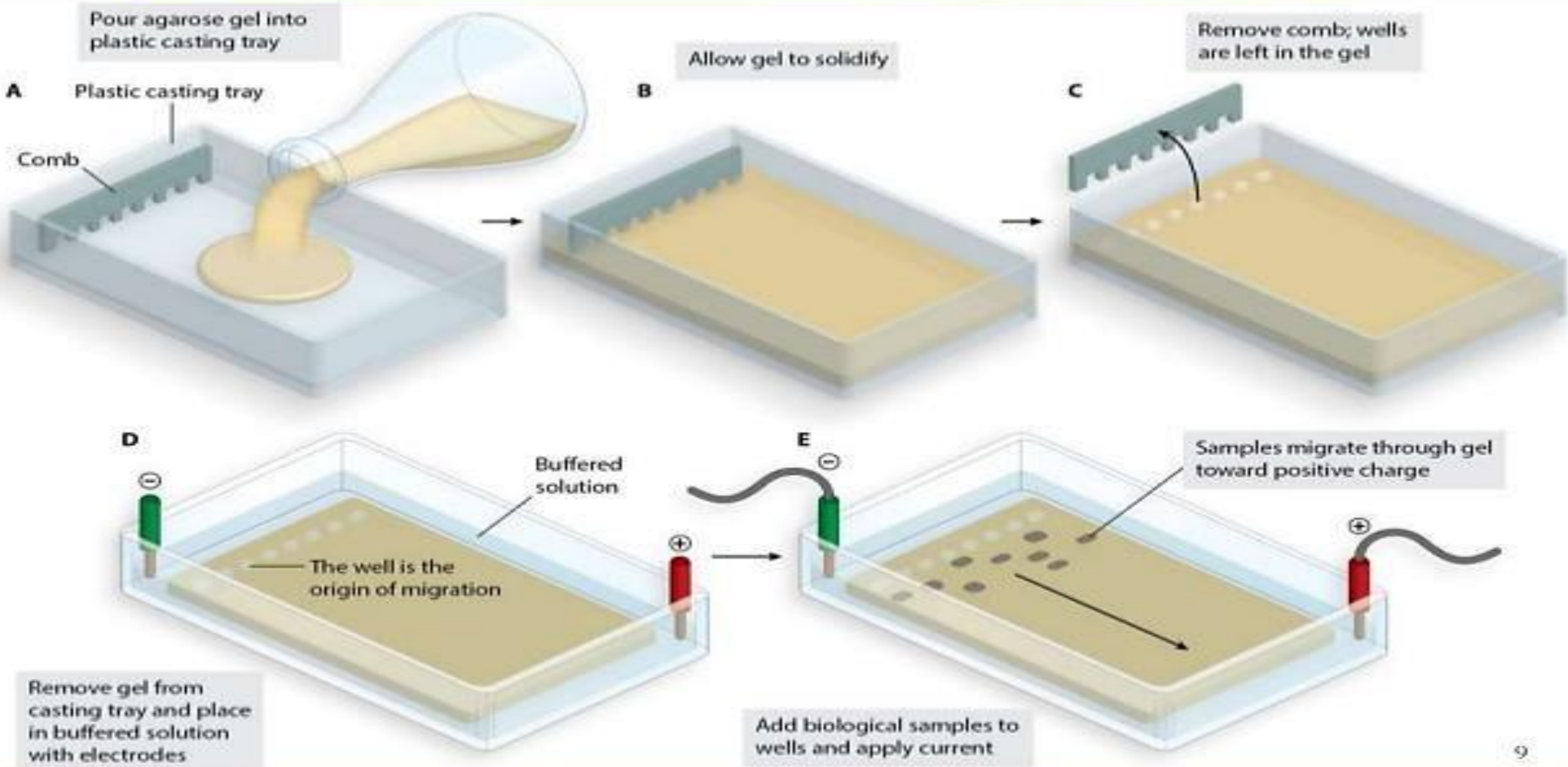
6. One well, called a DNA ladder, will contain DNA fragments of known sizes. This ladder is used to determine the sizes of other samples.



(a)



Preparation & running of gel



Applications

1- Analysis of PCR products,
e.g. in molecular genetic diagnosis, genetic fingerprinting, etc…

2- Separation of DNA fragments for extraction and purification.

3- Estimation of the size of DNA molecules using a DNA marker/ladder which contains DNA fragments of various known sizes.

4- Separation of restriction enzyme digested genomic DNA.

5- Allows for a rough estimation of **DNA quantity and quality.**


Thank
you