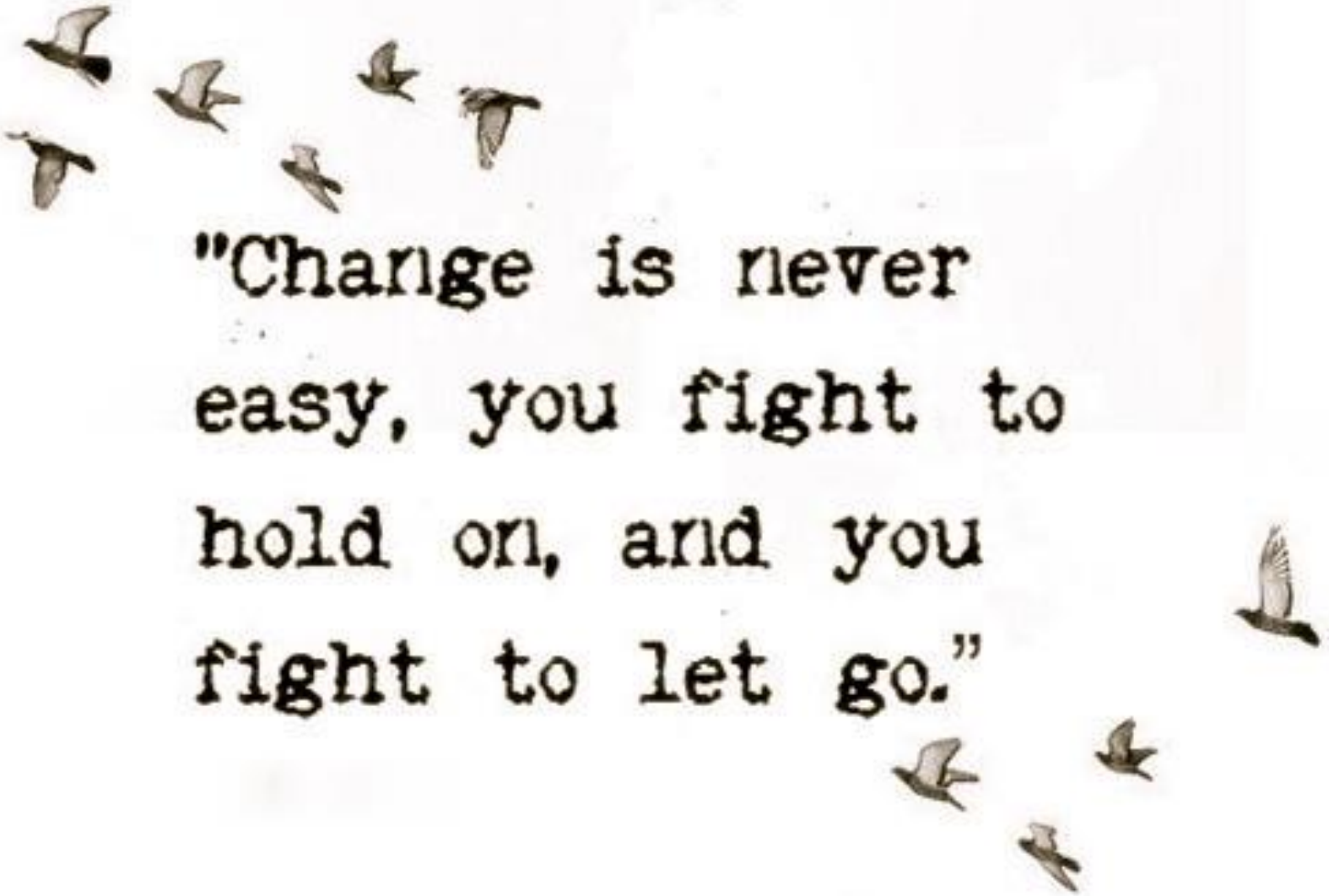
"Change is never
easy, you fight to
hold on, and you
fight to let go."

GE&MBR Center
GENETIC ENGINEERING and MOLECULAR BIOLOGY RESEARCH CENTER
مركز بحوث الهندسة الوراثية والبيولوجيا الجزيئية

Molecular Biology Research Unit

جامعة أسيوط

BASICS OF PCR

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The Molecular Assays

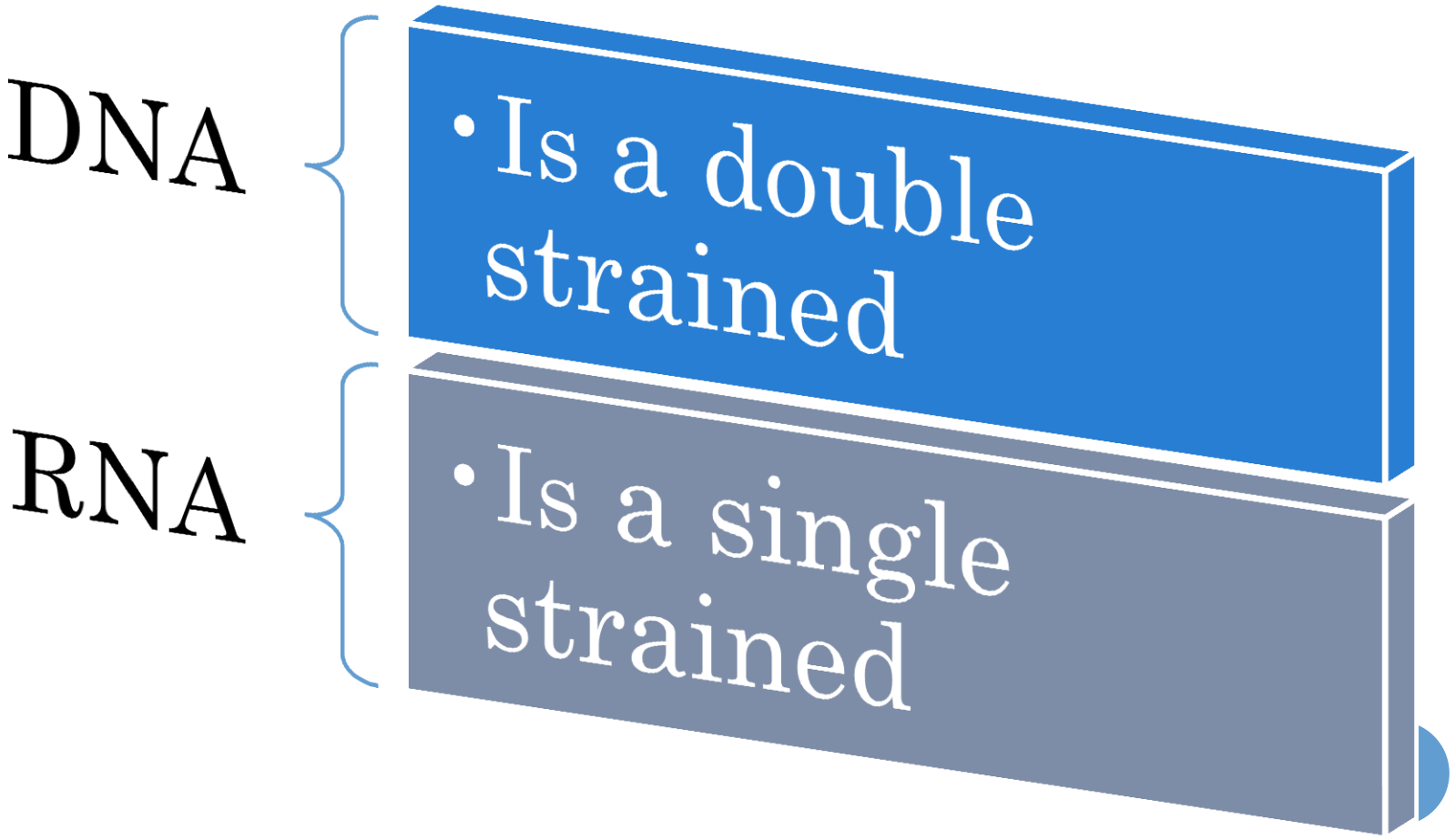
At the Beginning: **This type of the tests used for detection of only fastidious or uncultivated M.O.**

But Now: **It become one of the most important diagnostic tools for detection and characterization of M.Os.**

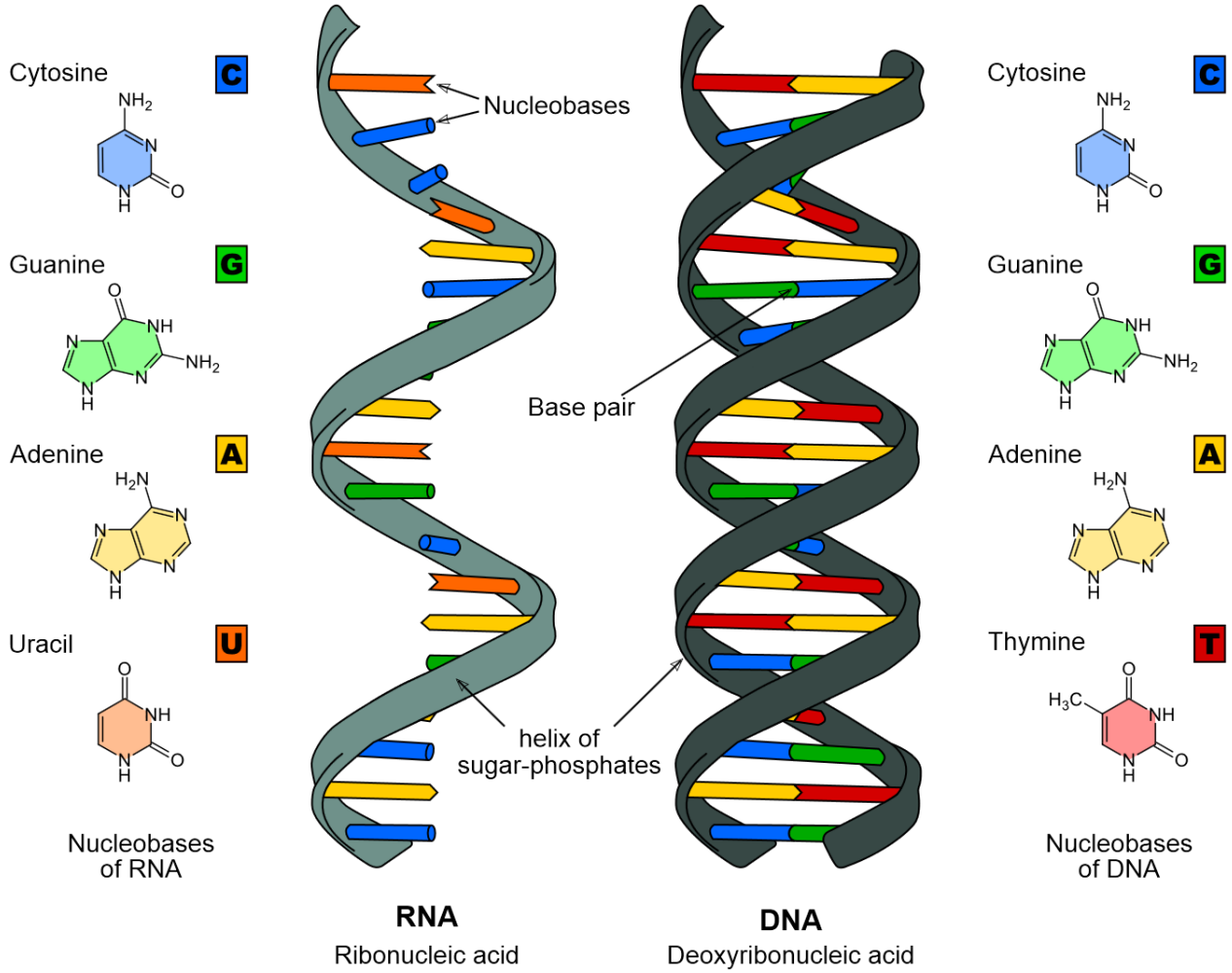
Polymerase Chain Reaction (PCR)

- **It is a molecular technology aim to amplify a single or few copies of the DNA to thousands or millions of copies.**
- **Developed in 1983 by Kary Mullis, In 1993, Mullis was awarded the Nobel prize in Chemistry.**
- **PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.**
- **These include diagnosis of infectious diseases, DNA sequencing and DNA-based phylogeny.**

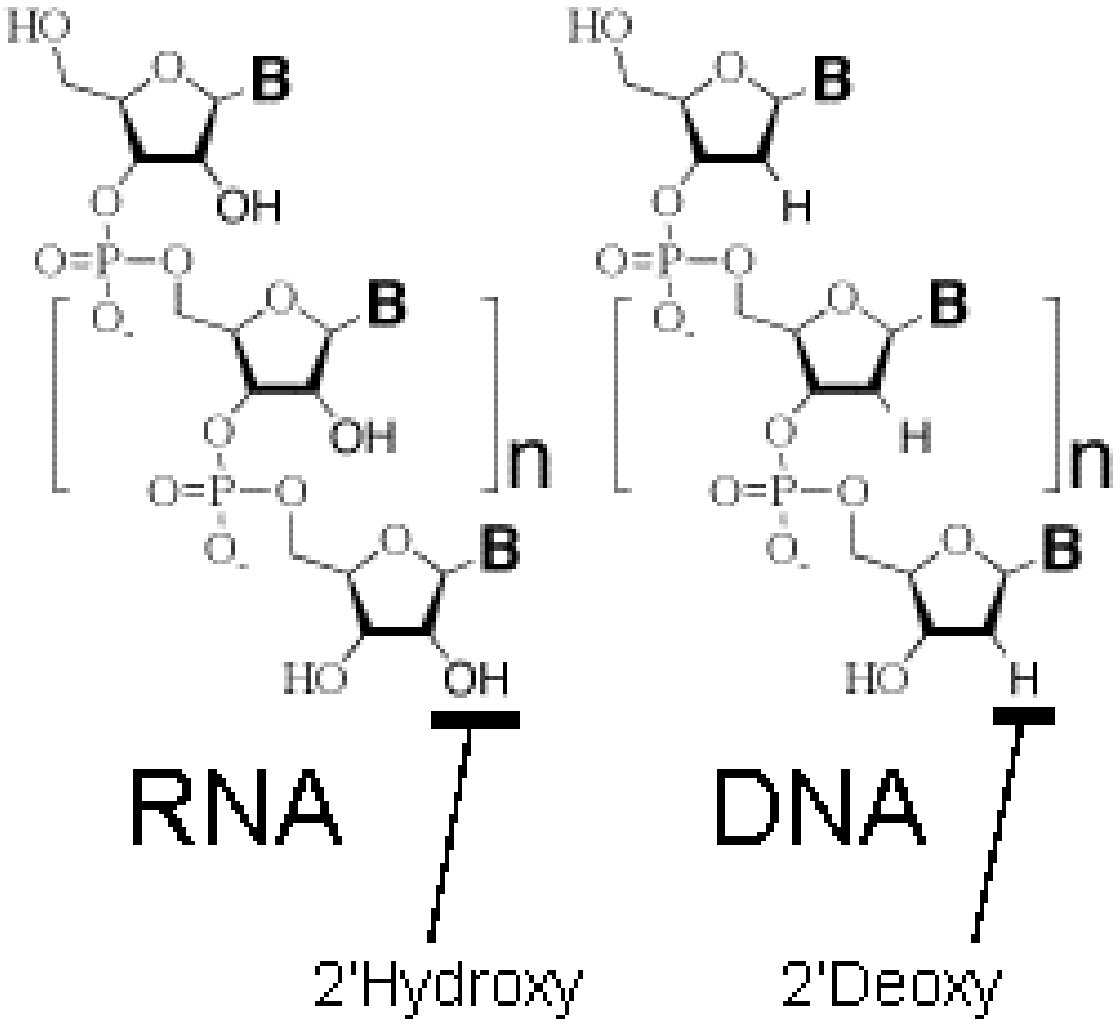
The differences between DNA & RNA



Nucleic Acids (DNA & RNA)



DNA and RNA Breaks (Nucleotides)



3x3

- **DNA/RNA Extraction.**
- **Amplification →**
  - **Denaturation**
  - **Annulling**
  - **Extension**
- **Electrophoresis**

DNA Extraction

What is DNA Extraction?

It is the removal of deoxyribonucleic acid (DNA) from the cells in which it normally resides.

What is it used for?

It is often an early step in many diagnostic processes (PCR, LAMP, ….. etc).

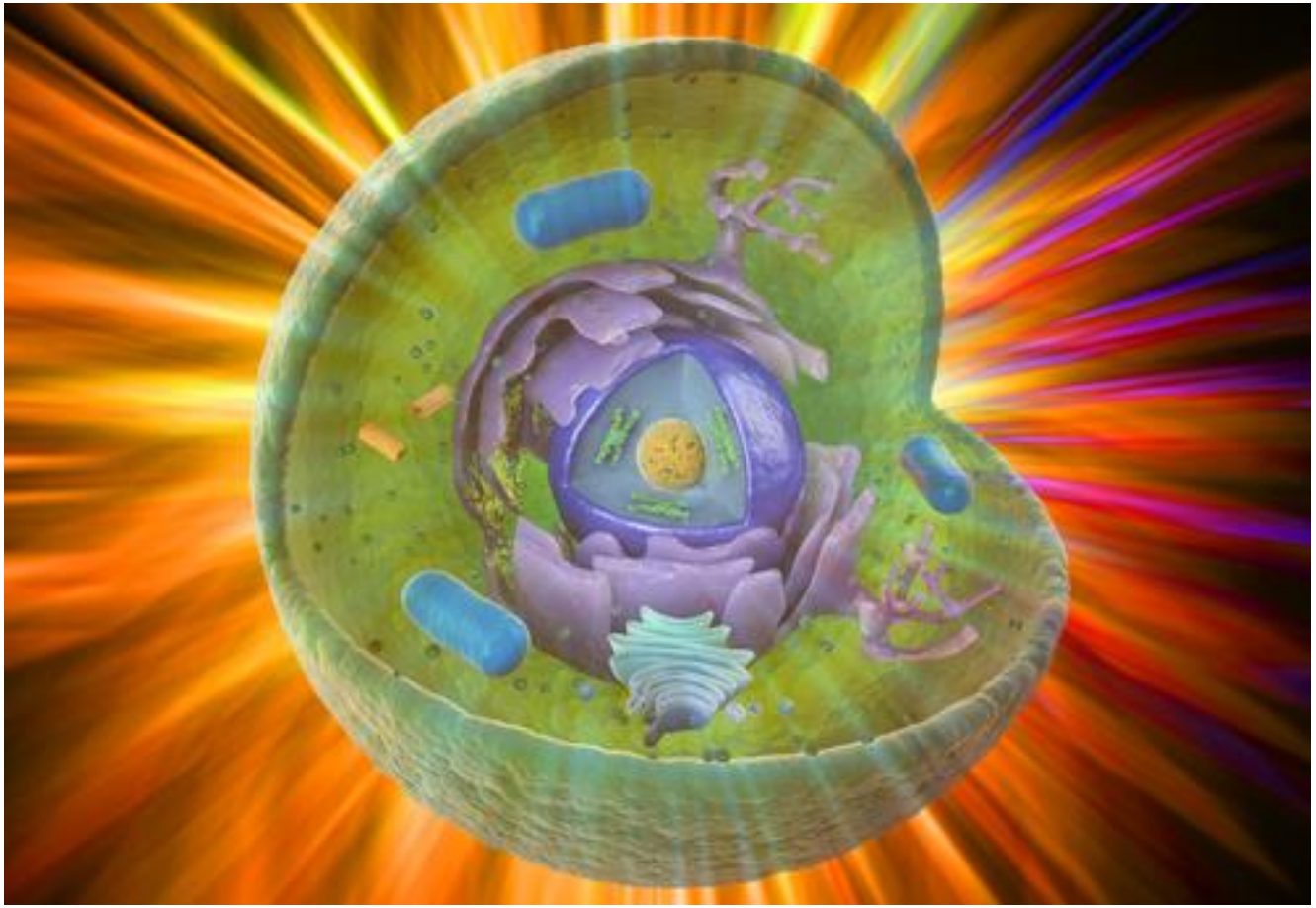
The first isolation of DNA was done in 1869 by Friedrich Miescher.

Miescher isolated various phosphate-rich chemicals, which he called nuclein (now nucleic acids), from the nuclei of white blood cells in 1869.

The significance of the discovery, first published in 1871.

Later, Friedrich Miescher raised the idea that the nucleic acids could be involved in heredity.

DNA Extraction



DNA Extraction

Samples

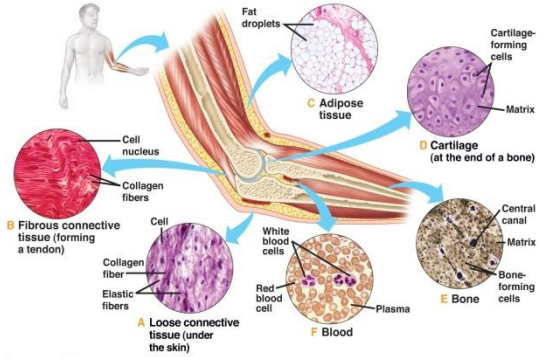
Blood (EDTA)

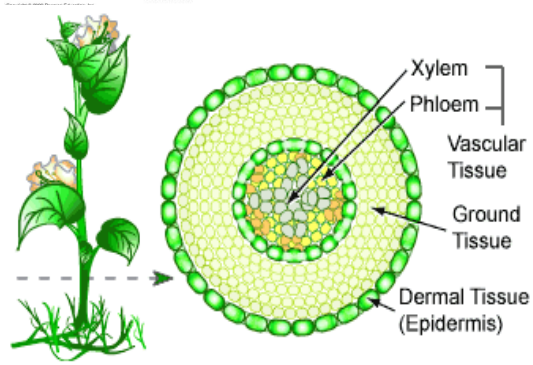
Blood spotted on filter paper

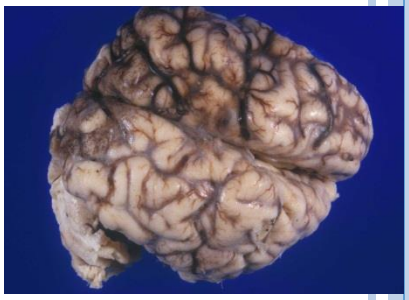
Ticks

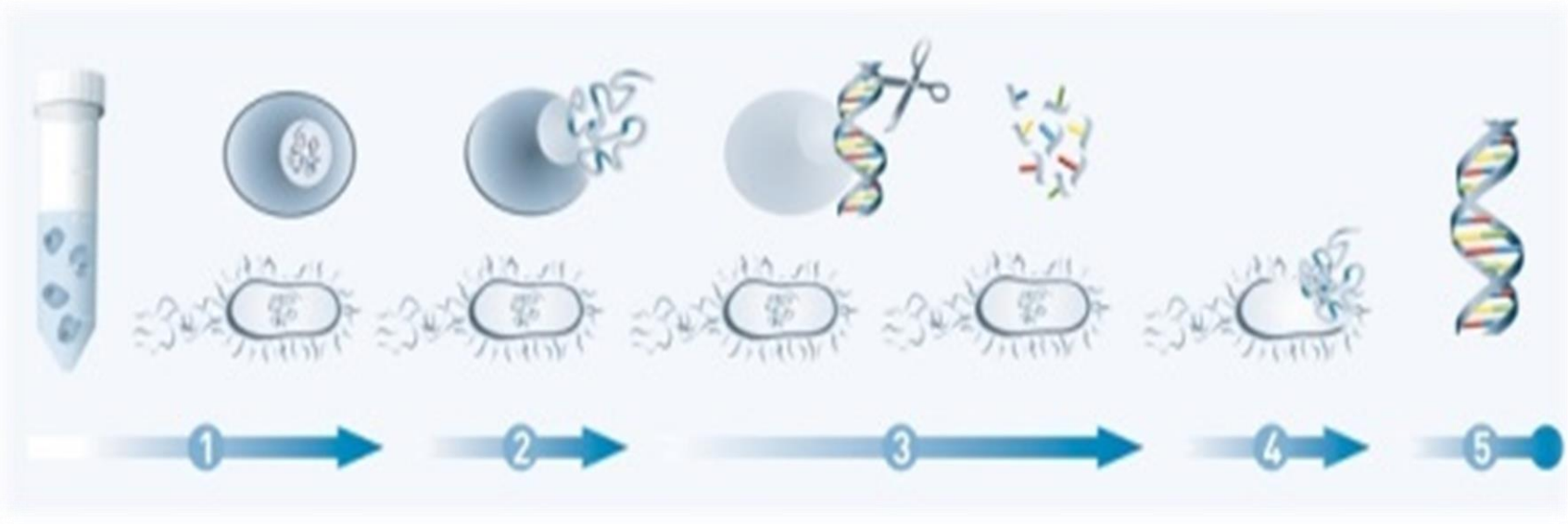
Plants

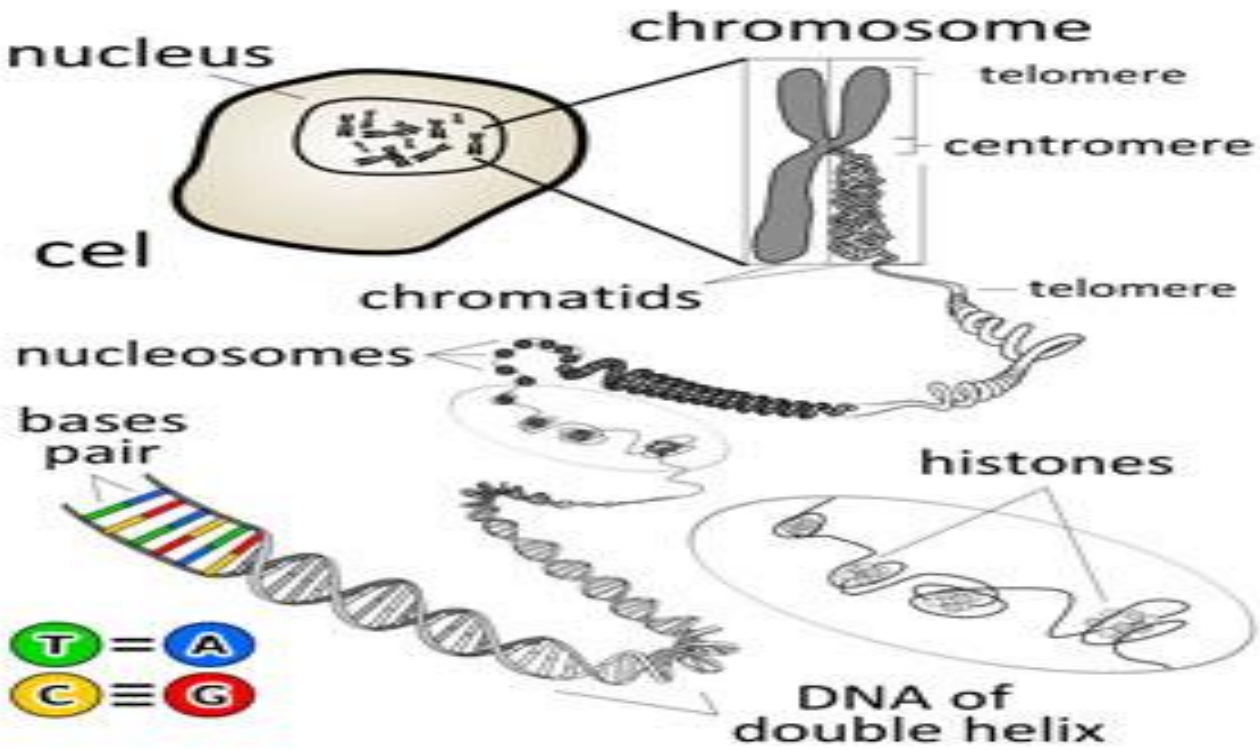
Tissue----------ect.



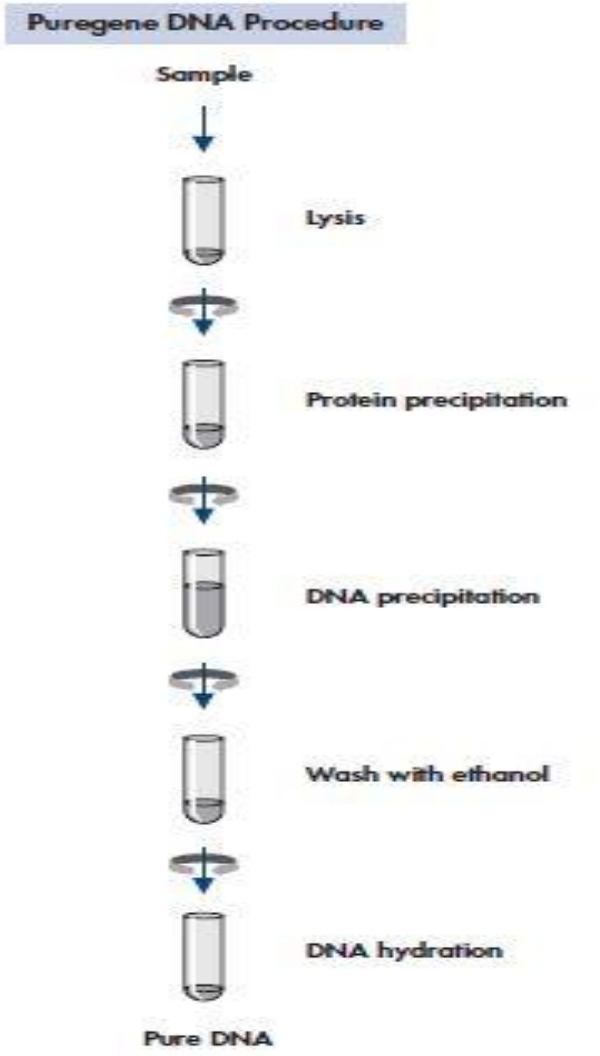


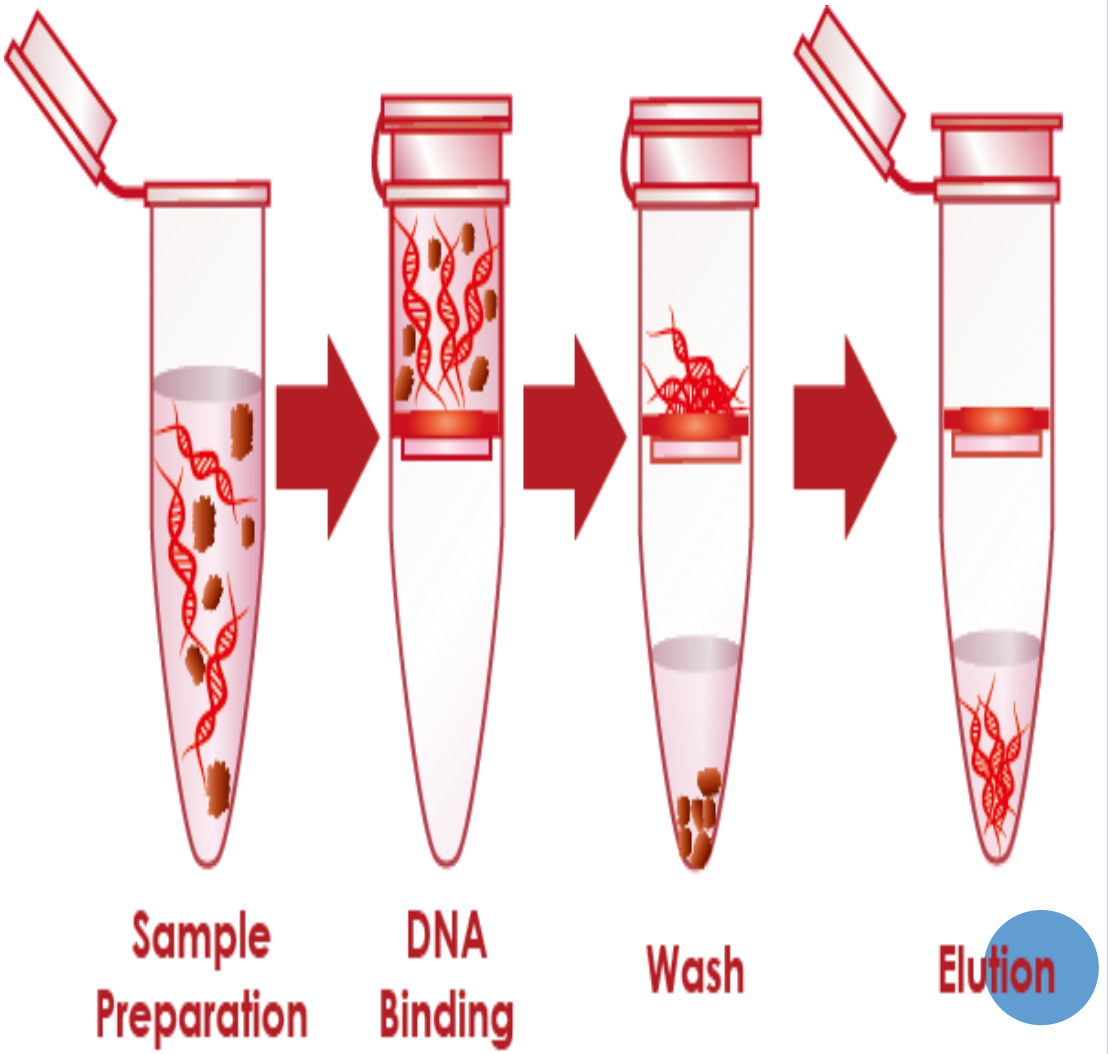



1
2
3
4
5


nucleus
chromosome
telomere
centromere
cel
chromatids
telomere
nucleosomes
bases
pair
histones
T = A
C ≡ G
DNA of
double helix

DNA Extraction





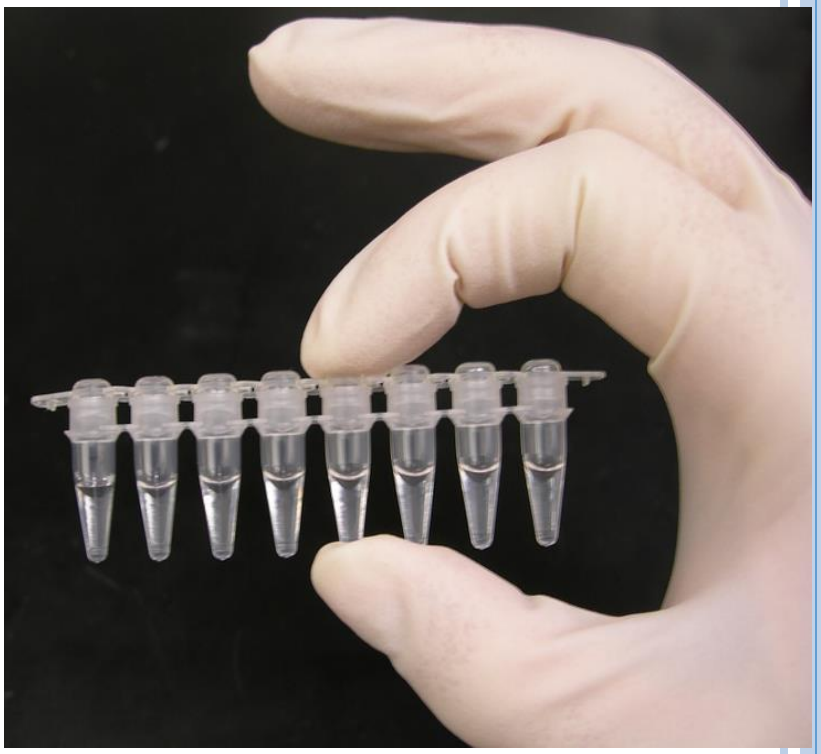
What is PCR?

- **The polymerase chain reaction (PCR) is a fast and inexpensive technique used to "amplify" - copy - small segments of DNA.**
- **Sometimes called "molecular photocopying,"**
- **Because we usually need a significant amounts of a sample of DNA are necessary for molecular and genetic analyses, studies of DNA.**

Polymerase Chain Reaction (PCR)

Key components

1. DNA
2. Primers (Reverse and forward)
3. dNTPs
4. *Taq* polymerase (enzyme of Thermus aquaticus 1967)
5. Buffer



Template DNA:

An adequate amount of template DNA is between 0.1 and 1 µg of genomic DNA for a total reaction mixture of 100 µl.

Larger template DNA amounts usually increase the yield of non-specific PCR products.

Taq DNA polymerase:

- **The DNA polymerase, known as 'Taq polymerase isolated from Thermus aquaticus bacterium.**
- **The enzyme can withstand the high temperatures needed for DNA-strand separation, and can be left in the reaction tube.**
- **Higher Taq DNA polymerase concentrations than needed may cause synthesis of non-specific products.**

dNTPs:

The concentration of each dNTP (dATP, dCTP, dGTP, dTTP) in the reaction mixture is usually 200 µM.

These concentrations must be checked as being equal, because inaccuracies will increase the degree of misincorporation.

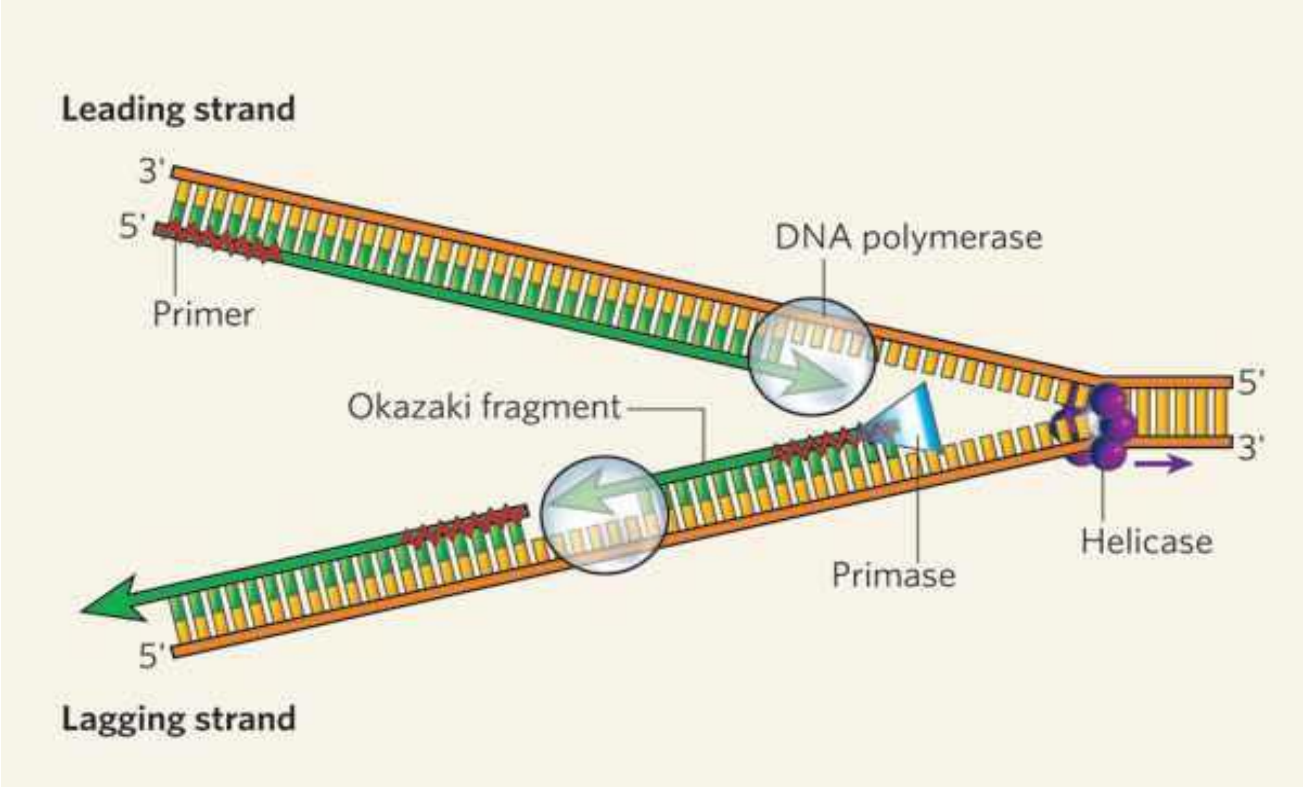
Primer: It is a strand of nucleic acid that serves as a starting point for DNA replication.

They are required because the enzymes that catalyze replication, DNA polymerases, can only add new nucleotides to an existing strand of DNA

The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand

5-TCGAATATGCCGGATTC ⟶

3-AGCTTATACGGCCTAAGTTAGCTAGCTTGCA



Primers:

(1) PCR primers should be 10-24 nucleotides in length.

(2) The GC content should be 40%-60%.

(3)The primer should not be self-complementary or complementary to any other primer in the reaction mixture, to prevent primer-dimer and hairpin formation.

(4) Melting temperatures of primer pairs should not differ by more than 5°C, so that the GC content and length must be chosen accordingly.

(5) The melting and annealing temperatures of a primer are estimated as follows:

If the primer is shorter than 25 nucleotides, the approximate melting temperature is calculated with the formula: Tm = 4 (G + C) + 2 (A + T).

(6) The annealing temperature should be about 5°C lower than the melting temperature.

MgCl2 concentration: **Because Mg2+ ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of MgCl2 has to be selected for each experiment.**

- **Too few Mg2+ ions result in a low yield of PCR product, and too many will increase the yield of non-specific products.**
- **The recommended range of MgCl2 concentration is 1 to 3 mM,under the standard reaction conditions specified.**

POLYMERASE CHAIN REACTION (PCR)

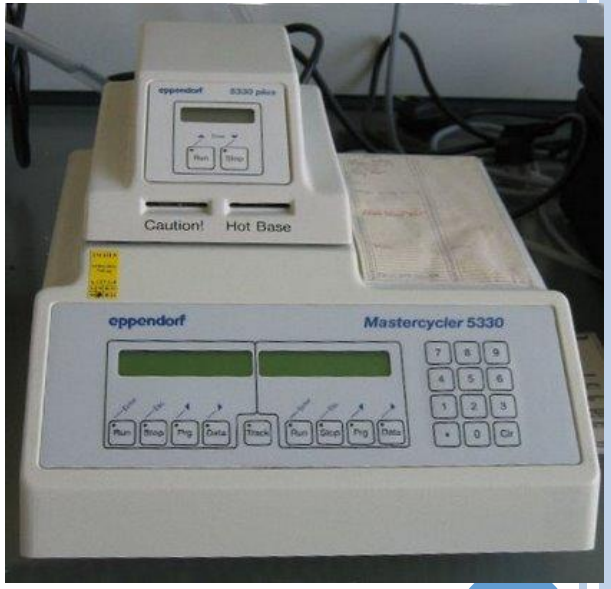
Laboratory requirements

Thermal cycler (PCR machine)

Denaturation at 94°C.

Annulling at 50:60°C.

Extension at72°C.



PCR Three steps

1. **Separating the Target (DNA-Denaturation):**

During the first step of PCR, the DNA is heated to more than 90 degrees Celsius (194 degrees Fahrenheit) to separate the double-stranded DNA into two separate strands.

The high temperature breaks the relatively weak bonds between the nucleotides that form the DNA code.

PCR Three steps

2. Binding Primers to the DNA Sequence – Annealing:

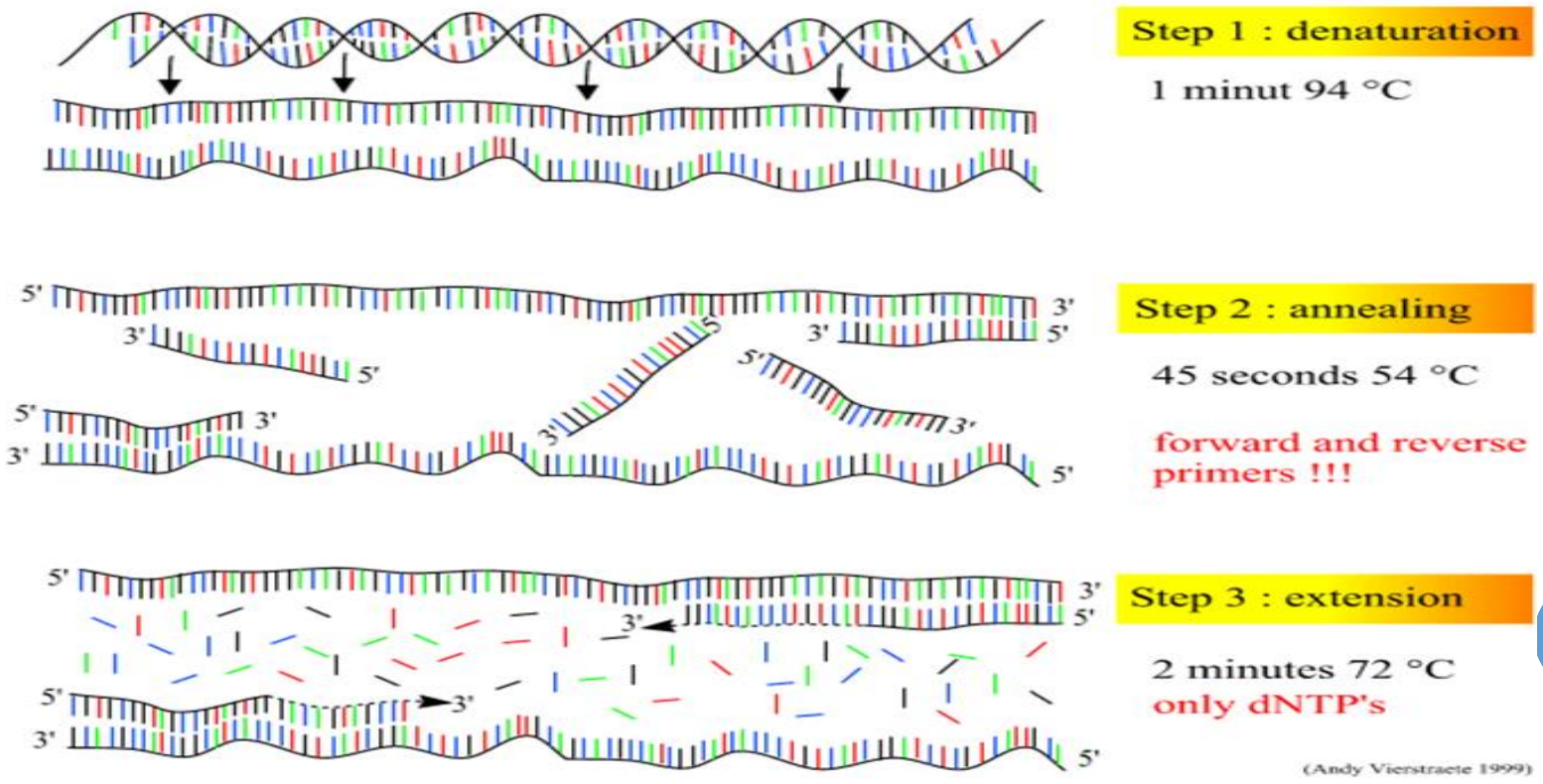
- **PCR does not copy the all of the DNA in the sample.**
- **It copies only a very specific sequence of genetic code, targeted by the PCR primers.**
- **The primers bind to the beginning of the sequence that will be copied, marking off the sequence for step three.**
- **During step two, the tube is cooled and primer binding occurs between 40 and 60 degrees Celsius (104 – 140 degrees Fahrenheit).**

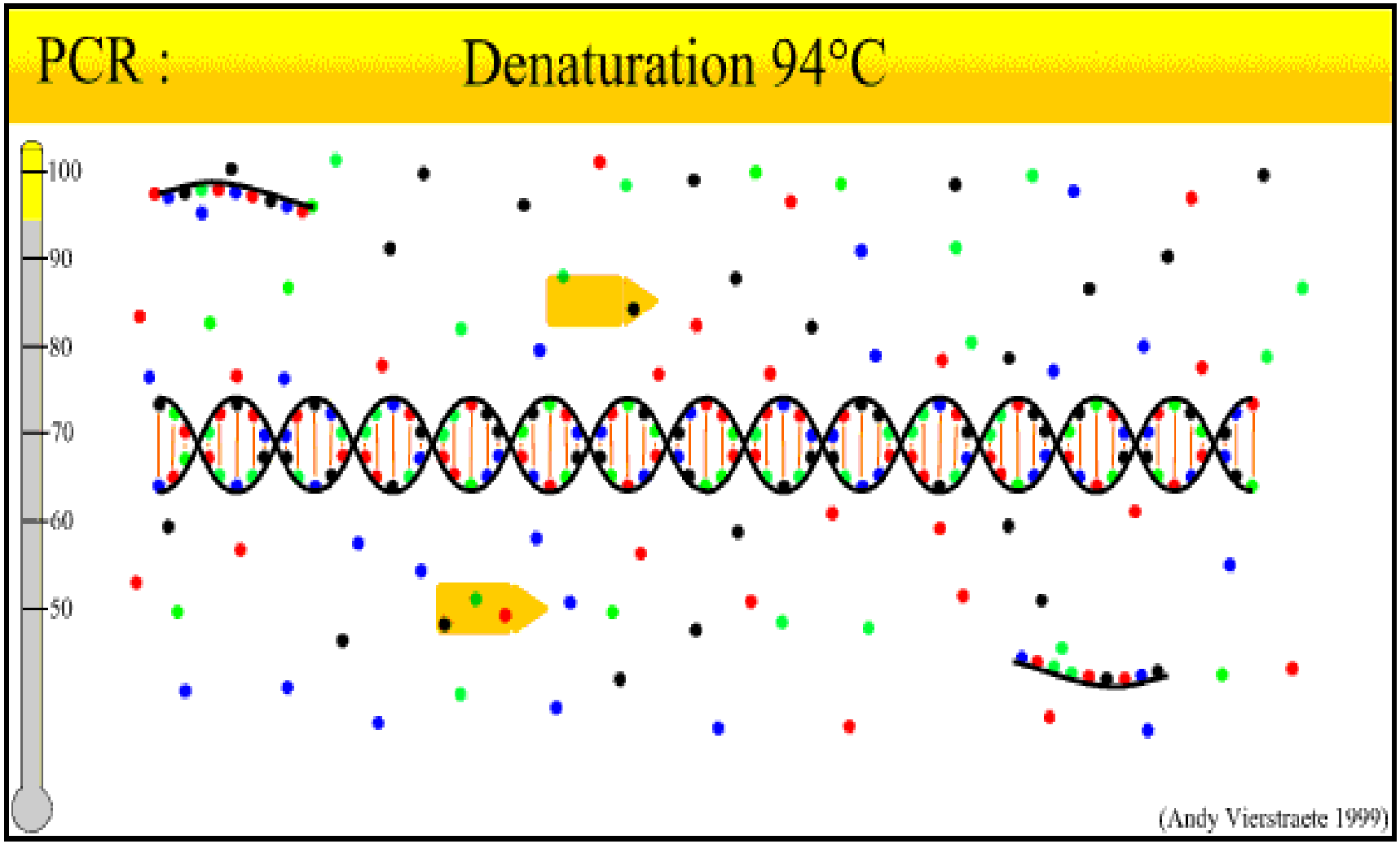
PCR Three steps

3. Making a Copy – Extension:

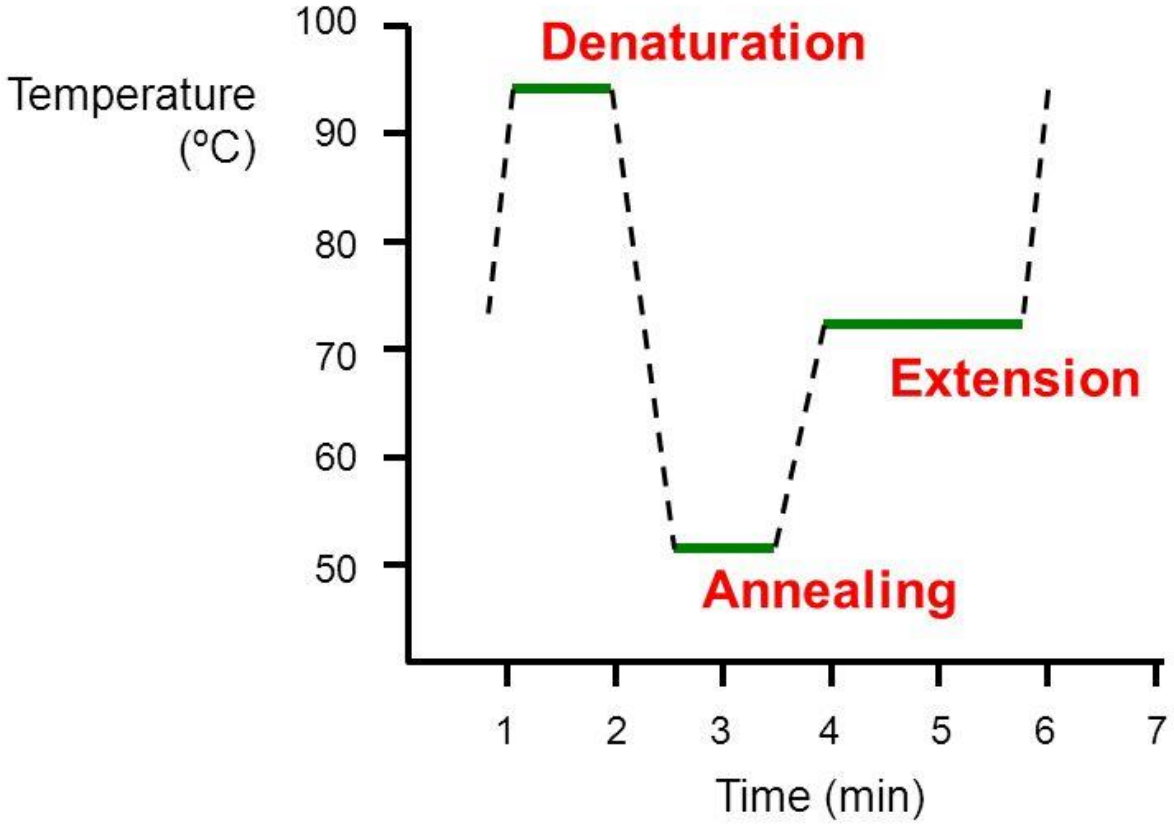
- **In the third phase of the reaction, called extension, the temperature is increased to approximately 72 degrees Celsius (161.5 degrees Fahrenheit).**
- **Beginning at the regions marked by the primers, nucleotides in the solution are added to the annealed primers by the DNA polymerase to create a new strand of DNA complementary to each of the single template strands.**
- **After completing the extension, two identical copies of the original DNA have been made.**

- **Usually, the extension step is performed at 72°C and a 1-min extension is sufficient to synthesise PCR fragments as long as 2 kb (kb = kilobase = 1000 bp). When larger DNA fragments are amplified, time is usually extended by 1 min per 1000 bp.**

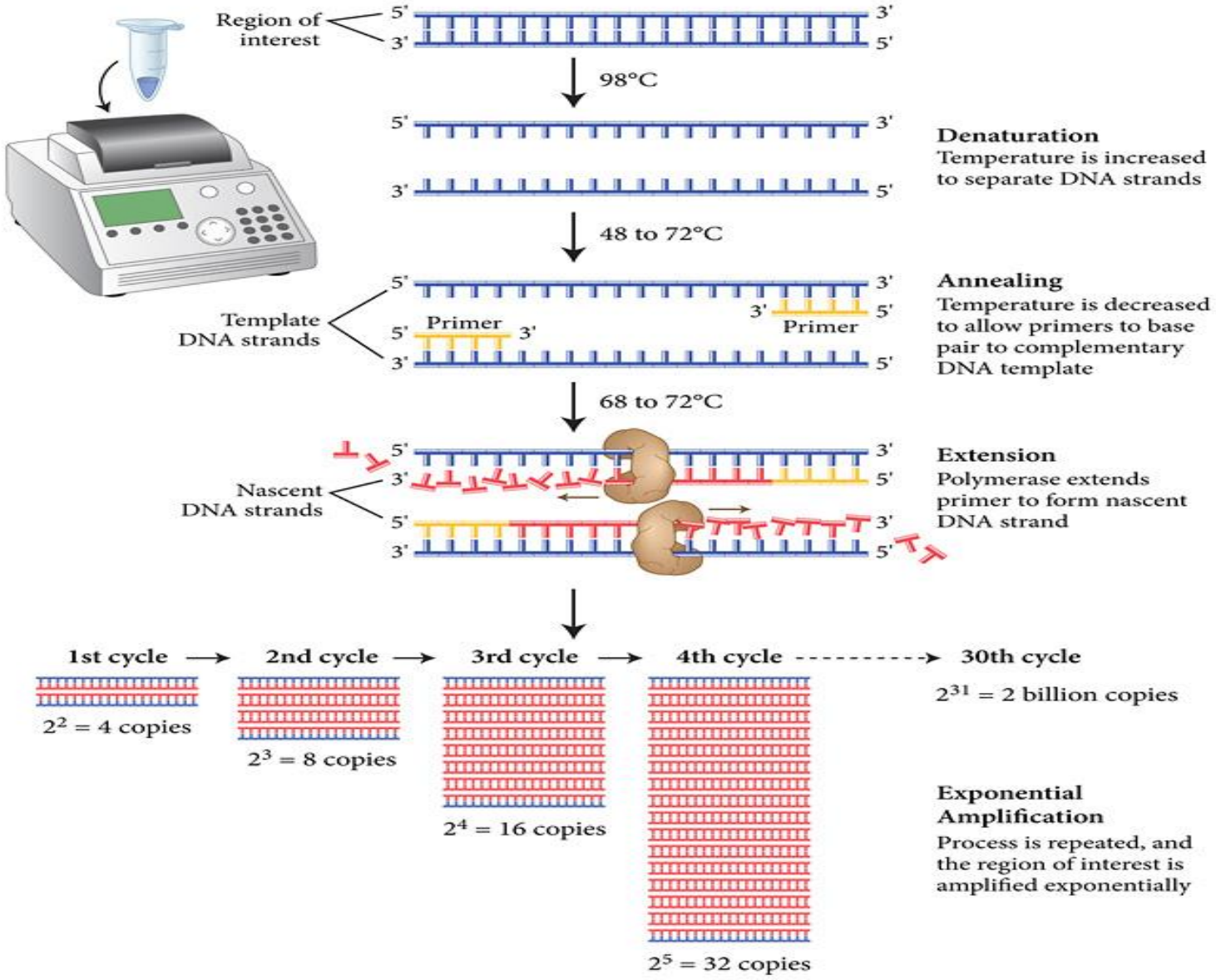



PCR : Denaturation 94°C
100
90
80
70
60
50
(Andy Vierstraete 1999)

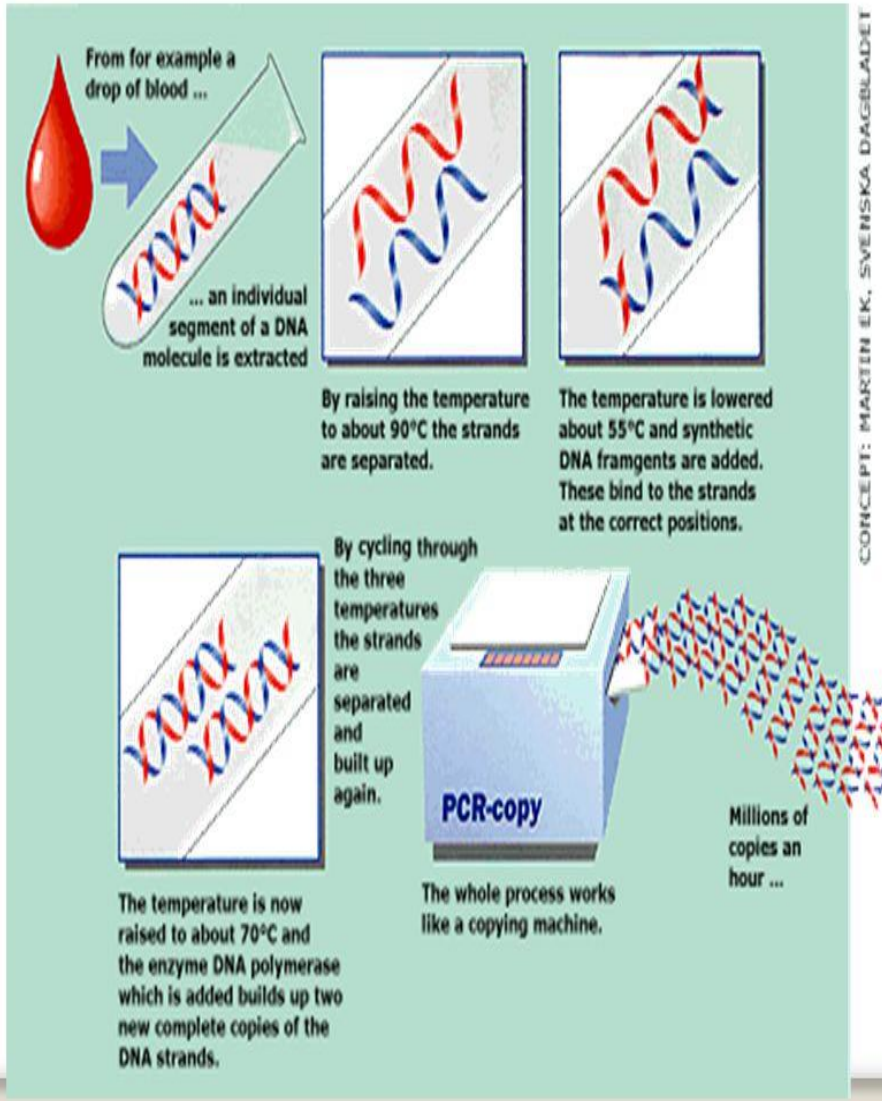
PCR is a dance with 3 steps



Adapted Brown 9.6


Region of interest
5'
3'
3'
5'
98°C
Denaturation
Temperature is increased to separate DNA strands
48 to 72°C
Annealing
Temperature is decreased to allow primers to base pair to complementary DNA template
Template DNA strands
Primer
Primer
68 to 72°C
Extension
Polymerase extends primer to form nascent DNA strand
Nascent DNA strands
1st cycle
2nd cycle
3rd cycle
4th cycle
30th cycle
2^2 = 4 copies
2^3 = 8 copies
2^4 = 16 copies
2^5 = 32 copies
2^{31} = 2 billion copies
Exponential Amplification
Process is repeated, and the region of interest is amplified exponentially

PCR Steps



PCR Cycles	Target Copies
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
15	32,768
20	1,048,576
25	33,554,432
30	1,073,741,842

GEL ELECTROPHORESIS

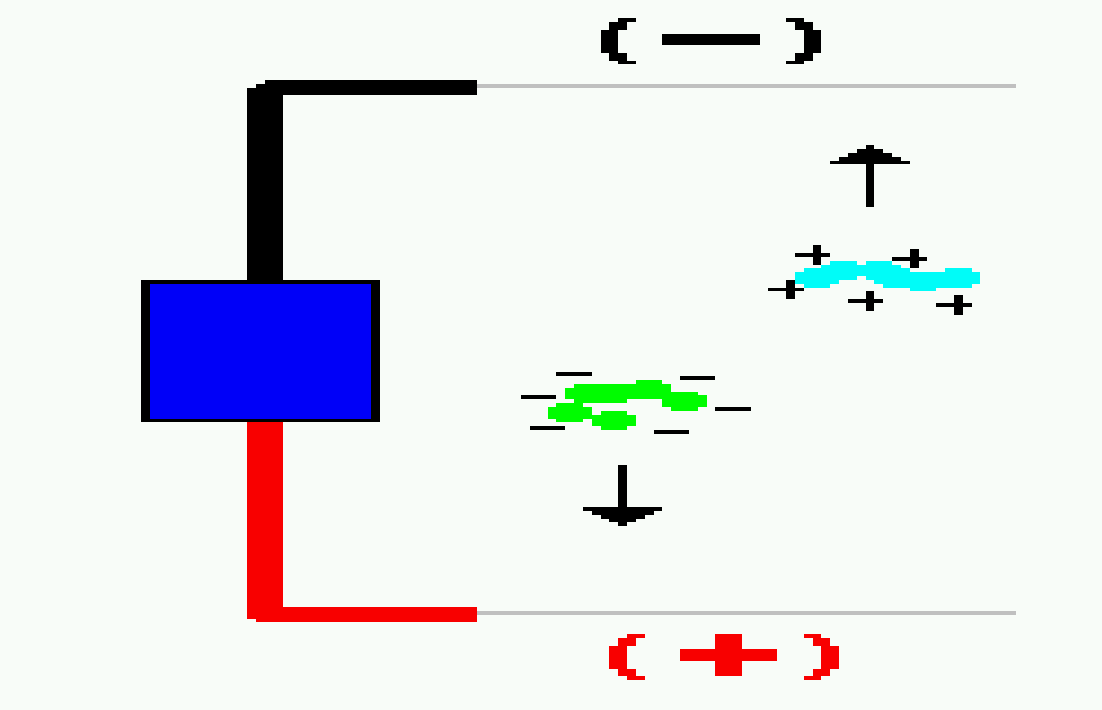
This phenomenon is called Sieving

Nucleic acid and Proteins' molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances.

Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel.

"Electrophoresis“

Refers to the *Electromotive force* (EMF) that is used to move the molecules through the gel matrix.

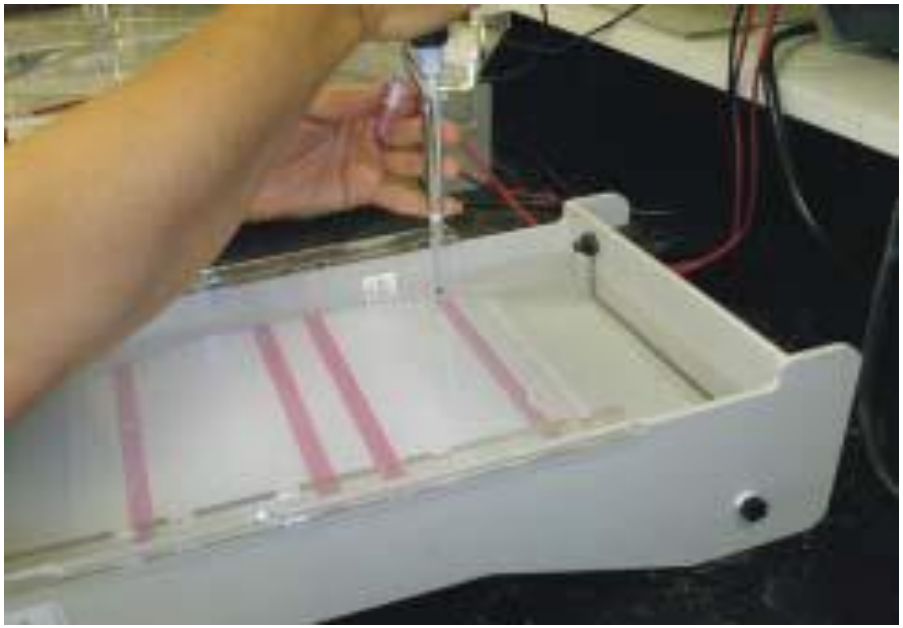


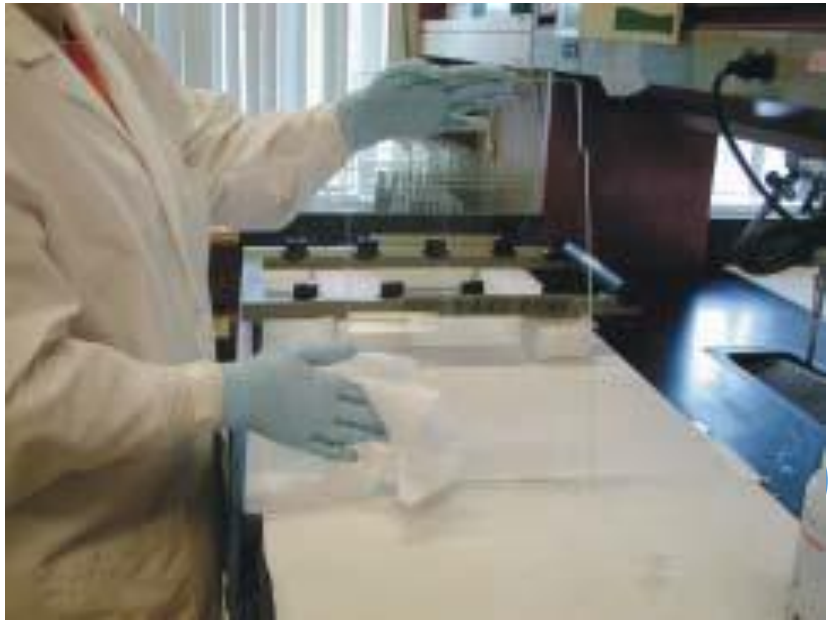
Electrophoresis is a technique used for sorting of macromolecules molecules based on size and charge.

- The gel is placed in an electrophoresis chamber, which is then connected to a power source.
- The electric field consists of a negative charge at one end which pushes the molecules through the gel and a positive charge at the other end that pulls the molecules through the gel.

Electrophoeresis

Depending on the size of the PCR bands produced and the discrimination needed, band visualization can be accomplished through either a regular, horizontal agarose gel or a vertical acrylamide sequencing gel.





Method For Electrophoresis

Prepare agarose gel
Melt, cool and add Ethidium Bromide. Mix thoroughly.

⇩

Pour into casting tray with comb and allow to solidify

⇩

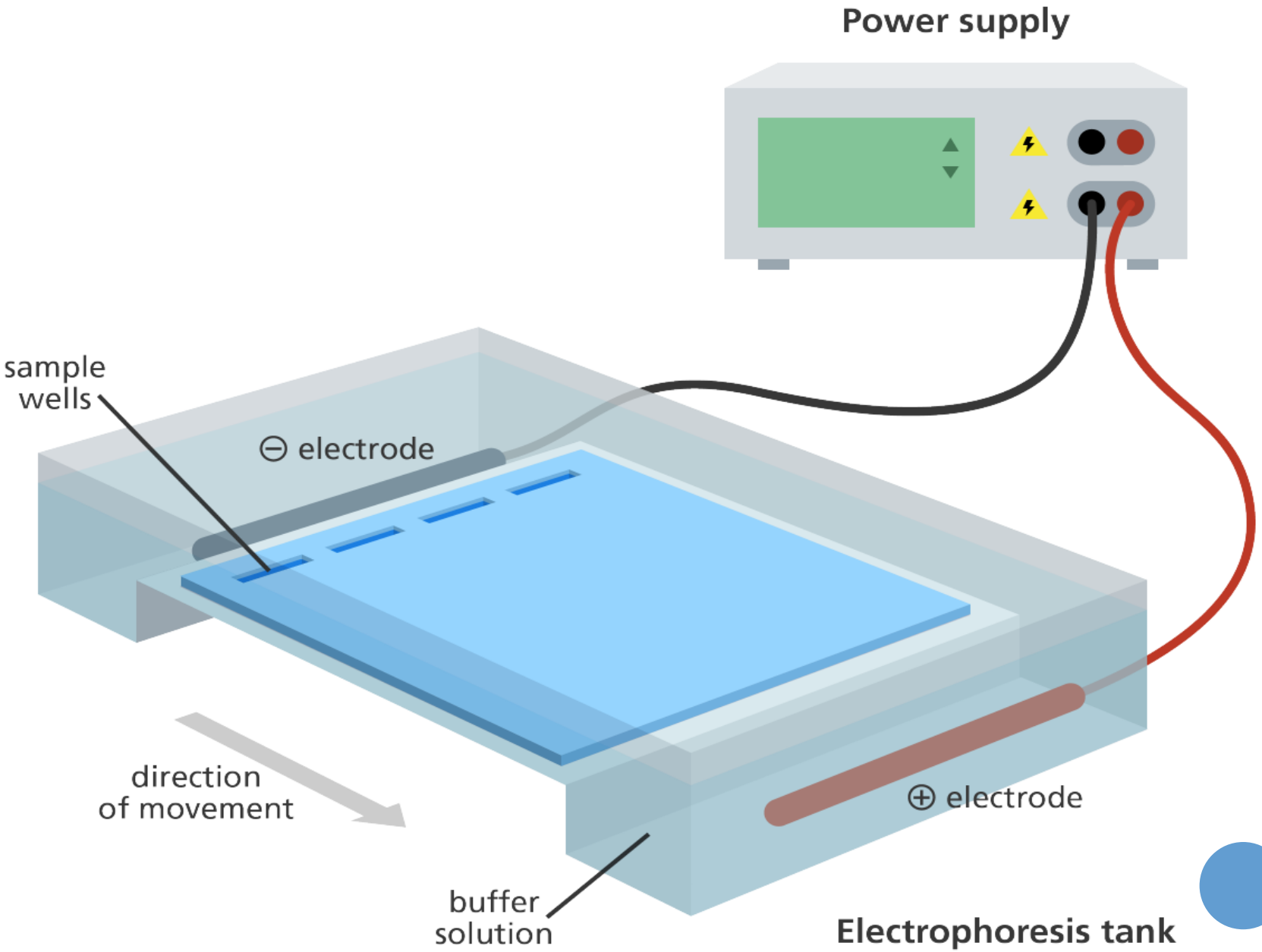
Add running buffer, load samples and marker

⇩

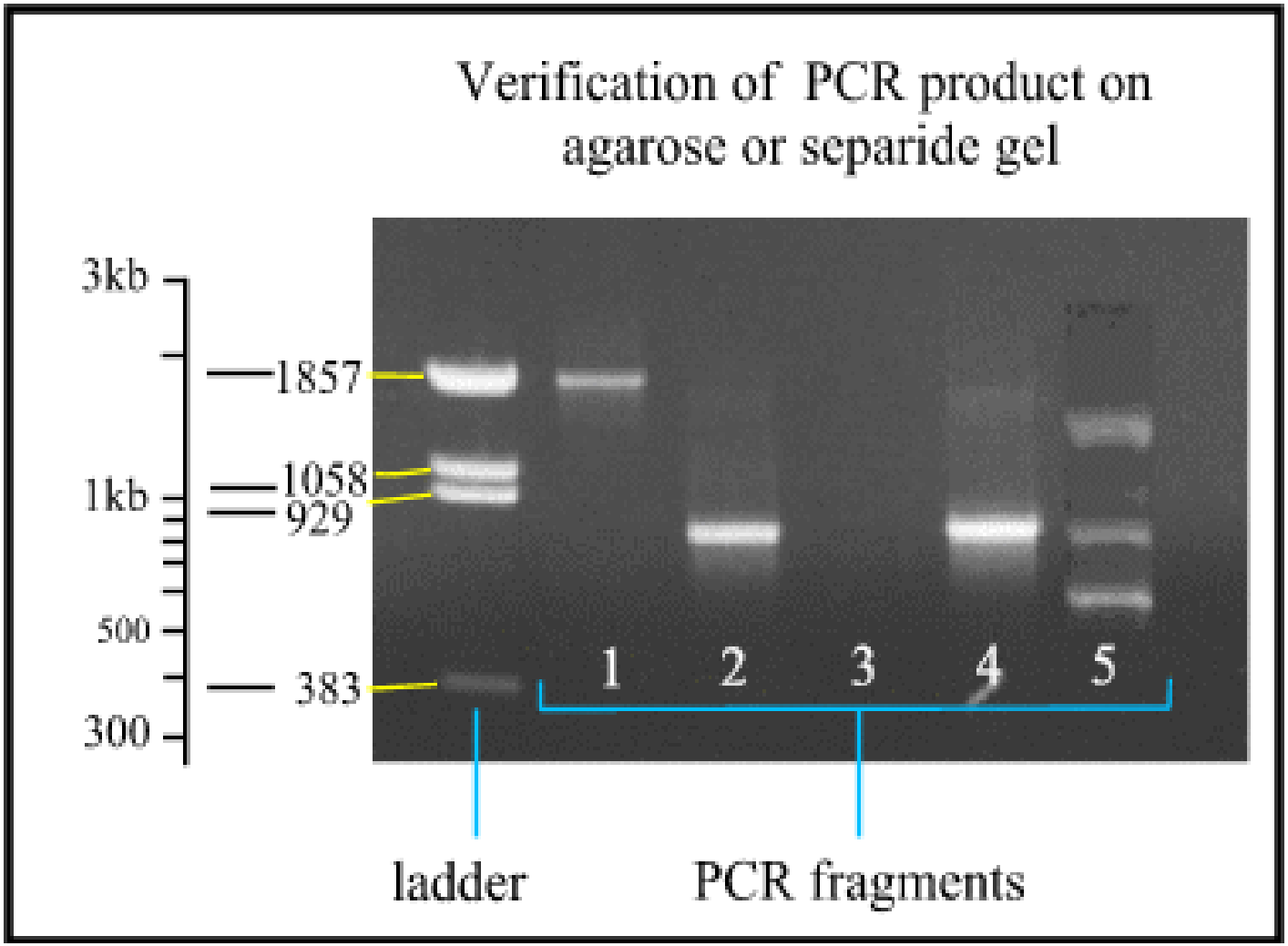
Run gel at constant voltage until band separation occurs

⇩

View DNA on UV light box and show results

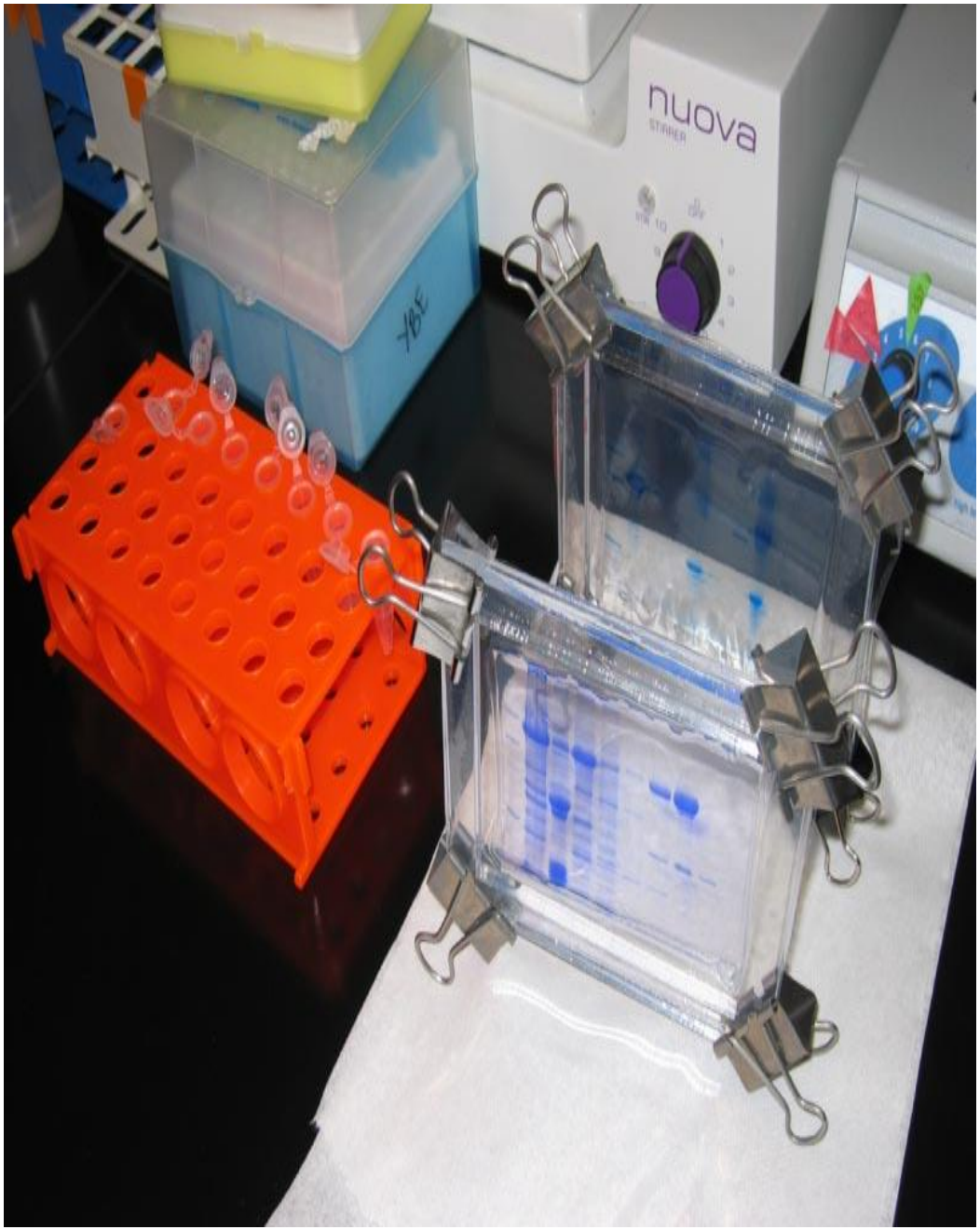

Power supply
sample wells
⊖ electrode
direction of movement
⊕ electrode
buffer solution
Electrophoresis tank

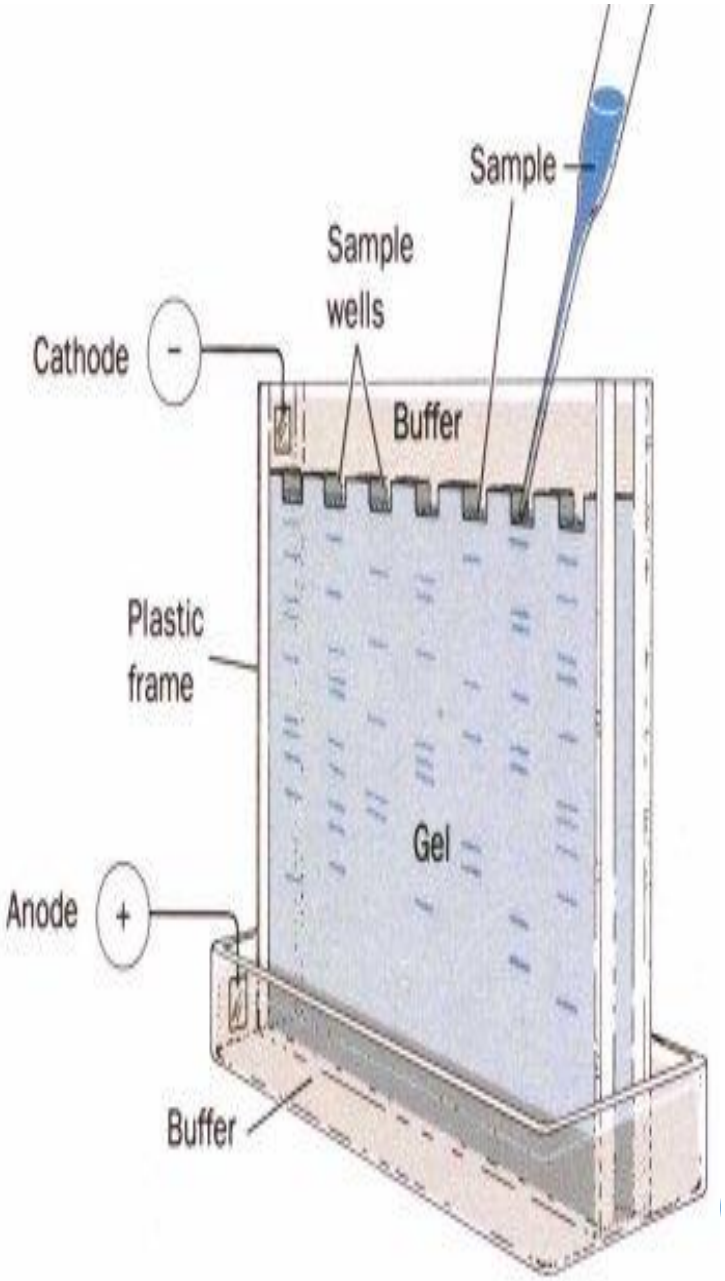
Agarose gel electrophoeresis



POLYACRYLAMIDE (PAGE)

- ***In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins.***
- ***Traditional DNA sequencing techniques used polyacrylamide gels to separate DNA fragments differing by a single base-pair in length so the sequence could be read.***
- ***In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp.***


nuova
STIRRER


Sample
Sample wells
Cathode
−
Buffer
Plastic frame
Gel
Anode
+
Buffer

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(00201004477501)
amiraelhosary@yahoo.com