Polymerase chain reaction
PCR

By

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PCR

- PCR is an **in vitro DNA amplification** *procedure in which* millions of copies of a particular sequence of DNA can be produced within a few hours.
- The flanking sequences of the gene of interest should be known.
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Material required

1-**Target DNA** .

2- Two synthetic oligonucleotide primers:

These primers are complementary to the end of each strand of target DNA to be amplified. The selection of primer requires the knowledge of the flanking sequences of the gene of interest.

- **Two DNA primers** of about 20- 30 nucleotides with complementary sequence of the flanking region can be synthesized.(just at the edges of the region to be copied)

3- Heat stable DNA polymerase:

This enzyme is derived from bacteria Thermus acquaticus that can tolerate high temperatures. Therefore the enzyme is not denatured at high temperature. This polymerase is not denatured even at temperature around 95°C.

4- All four deoxyribonucleotide triphosphates (dNTPs).

Technique steps

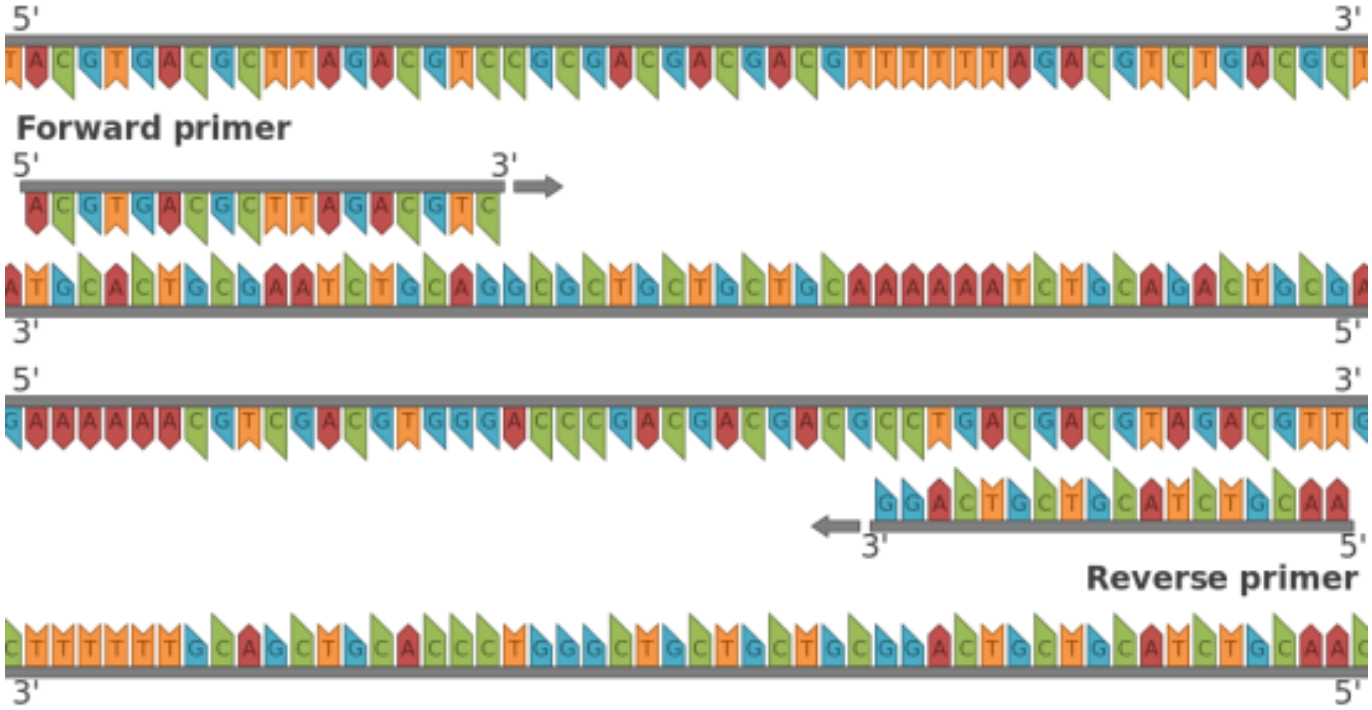
Step 1: Separation (Denaturation):

DNA strands are separated (melted) by heating at 95°C for 15 seconds to 2 minutes.

Step 2: Priming (Annealing):

The reaction mixture is cooled to about 50-65°C for about 1-3 minutes. The temperature chosen for cooling is usually about 2-3 below **Primer melting temperature (Tm).**

- The melting temperature (T_m) of an oligonucleotide is the temperature at which 50% of the oligonucleotide is duplexed with its perfect complement and 50% is free in solution.
- The primer melting temperature (Tm) can be defined as the temperature at which half of the primers dissociate from the template DNA.



•**Forward primers anneal to the antisense strand of the double-stranded DNA, which runs from 3′ to 5′ direction, whereas reverse primers anneal to the sense strand of the double-stranded DNA, which runs from 5′ to 3′ direction**.

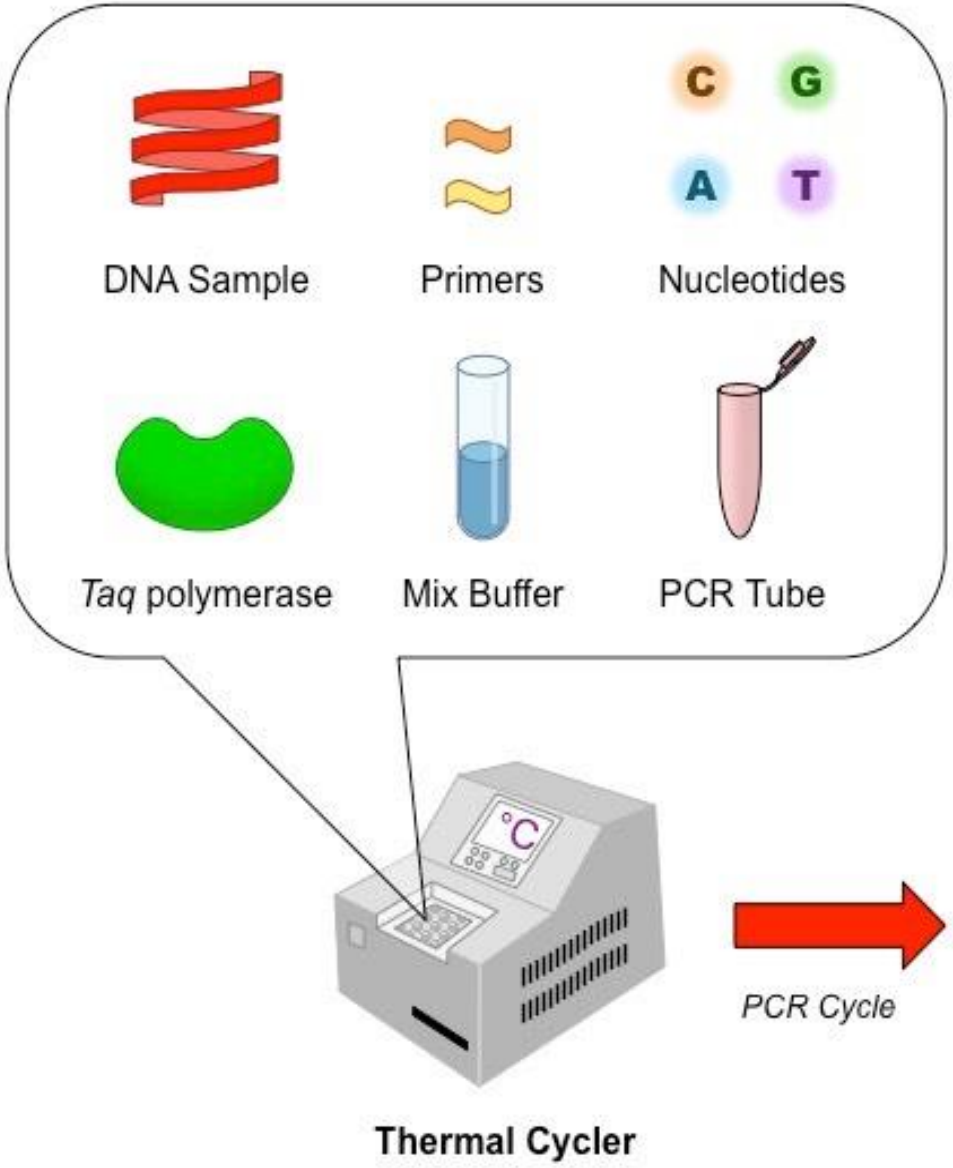
Step 3: Polymerization (extension) (elongation):

New DNA strands are synthesized by **Taq polymerase.** The polymerase reaction is allowed to take place at 72°C for 30 seconds in presence of dNTPs (all four deoxy ribonucleotide triphosphates).

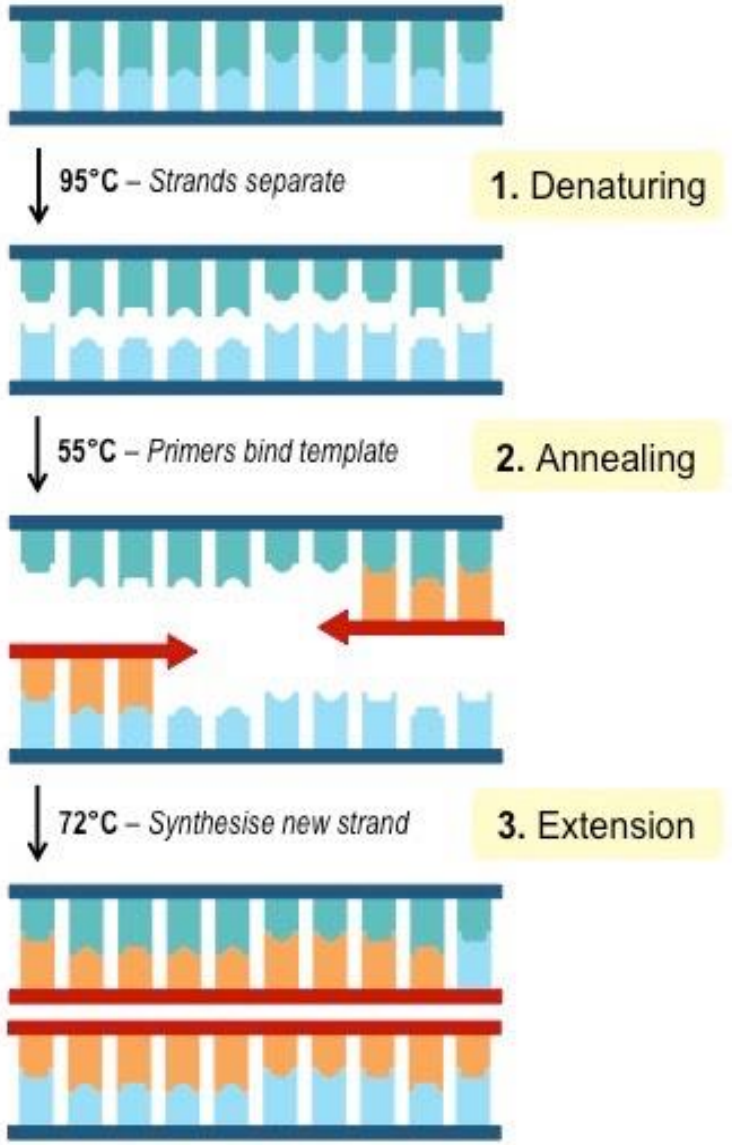
4-The steps of 1,2 and 3 are **repeated.**

In each cycle, the DNA strands are doubled. These cycles are generally repeated by automated instrument, called (**Tempcycler or thermalcycler).**

PCR Components



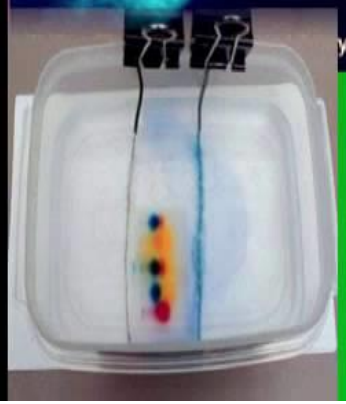
PCR Process (ONE Cycle)



Identification of the PCR product

- Agarose gel electrophoresis is a basic and essential technique in molecular biology. It is routinely used for analysis of PCR products. It is the first step for analysis of specific DNA and RNA fragments by northern and Southern blots.
- Gel electrophoresis is used to separate mixtures of DNA, RNA, or proteins according to molecular size. In gel electrophoresis, the molecules to be separated are pushed by an electrical field through a gel that contains small pores.
- Small DNA molecule will travel a greater distance through the gel than will a larger DNA molecule.
- As previously mentioned, gel electrophoresis involves an electrical field; in particular, this field is applied such that one end of the gel has a positive charge and the other end has a negative charge. Because DNA and RNA are negatively charged molecules, they will be pulled toward the positively charged end of the gel.

What is gel electrophoresis?



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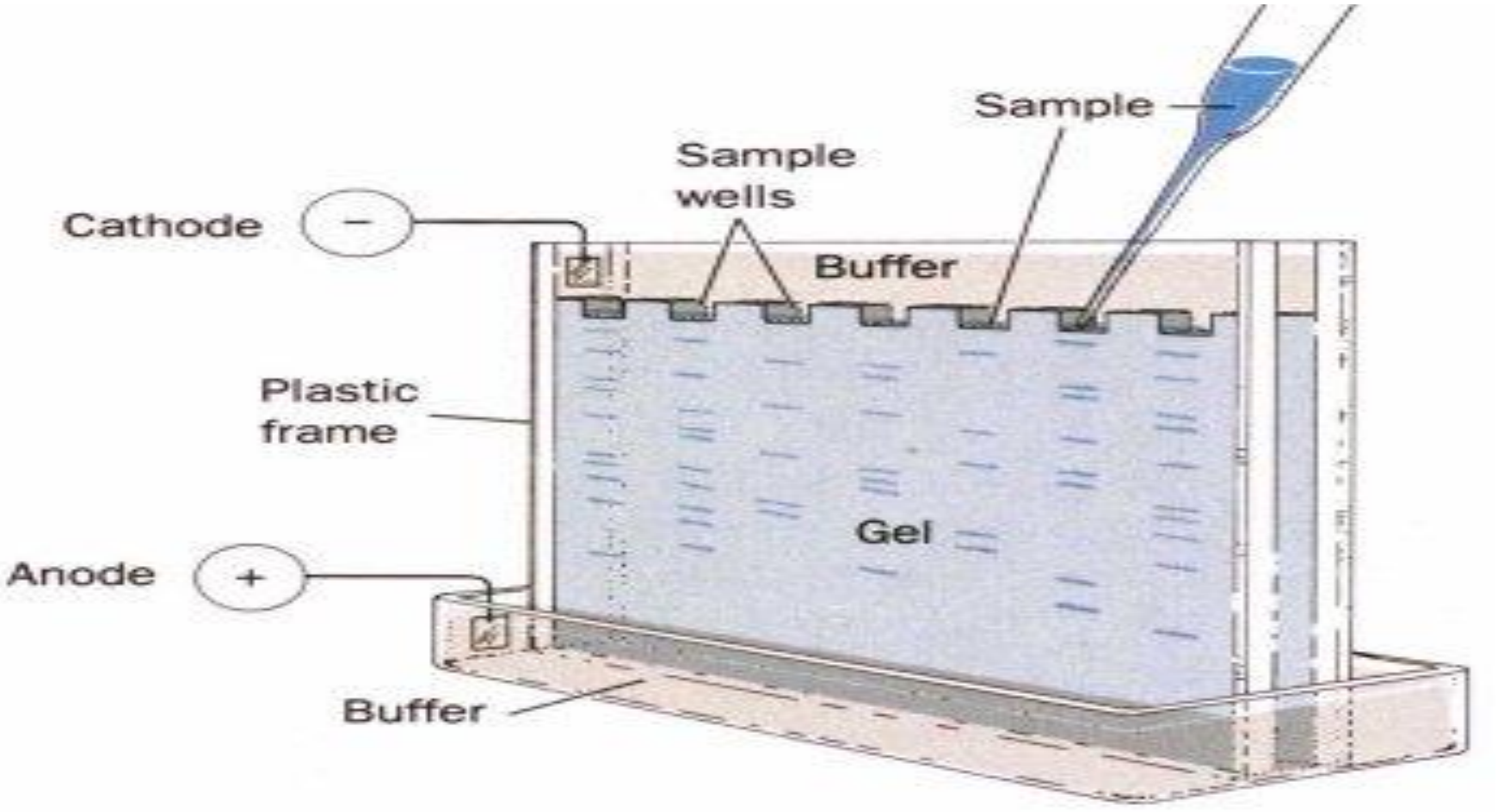
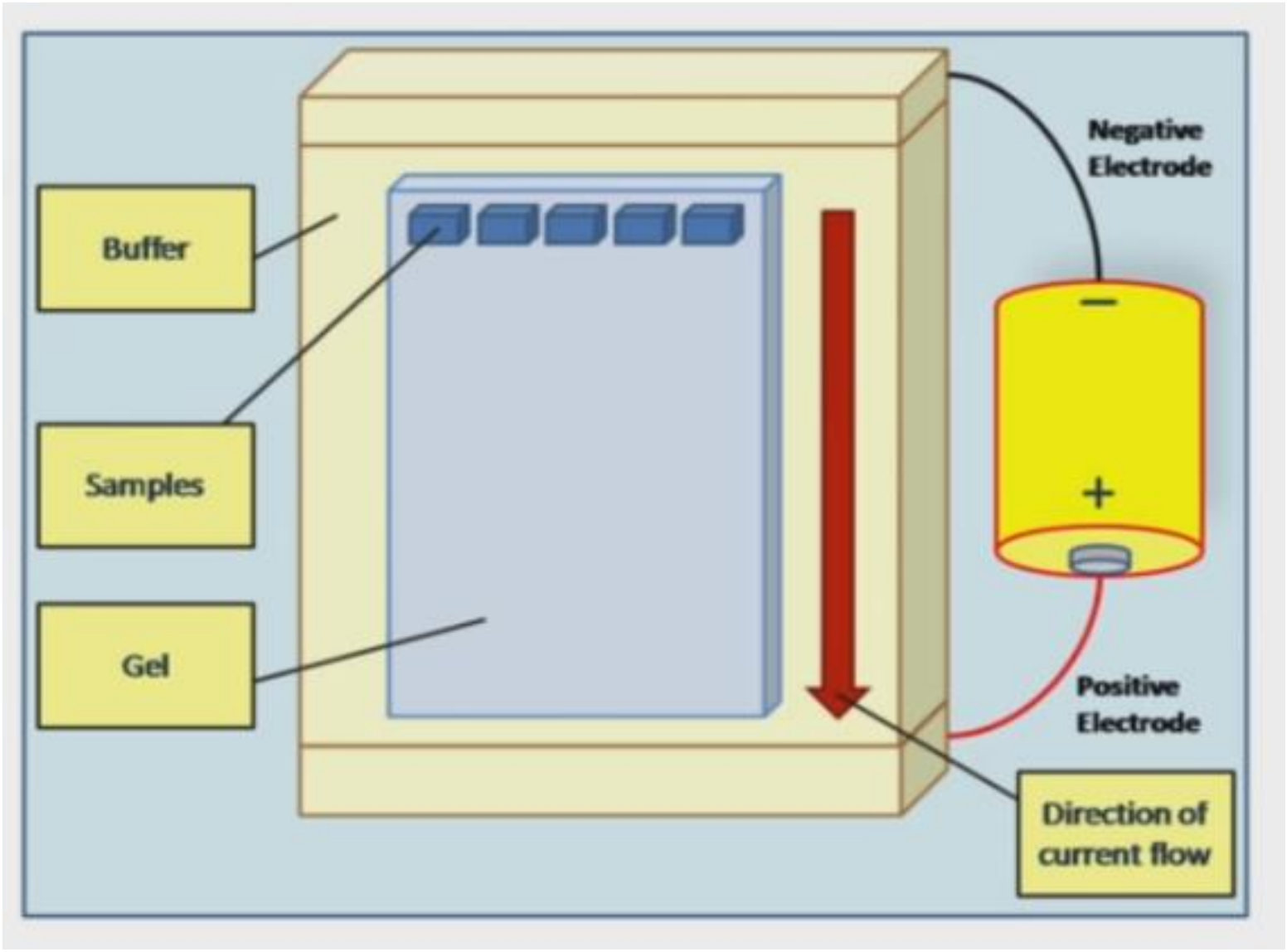
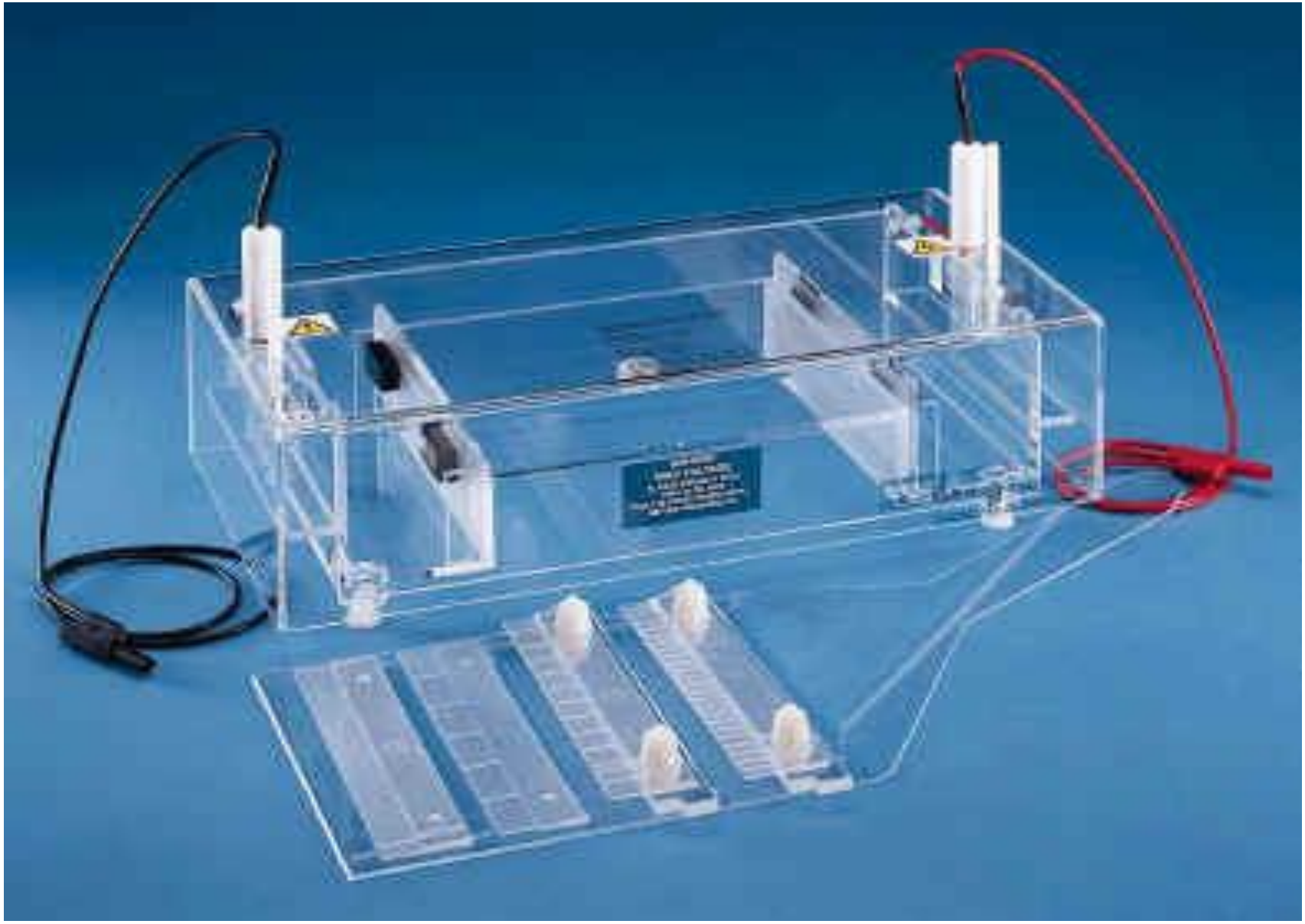
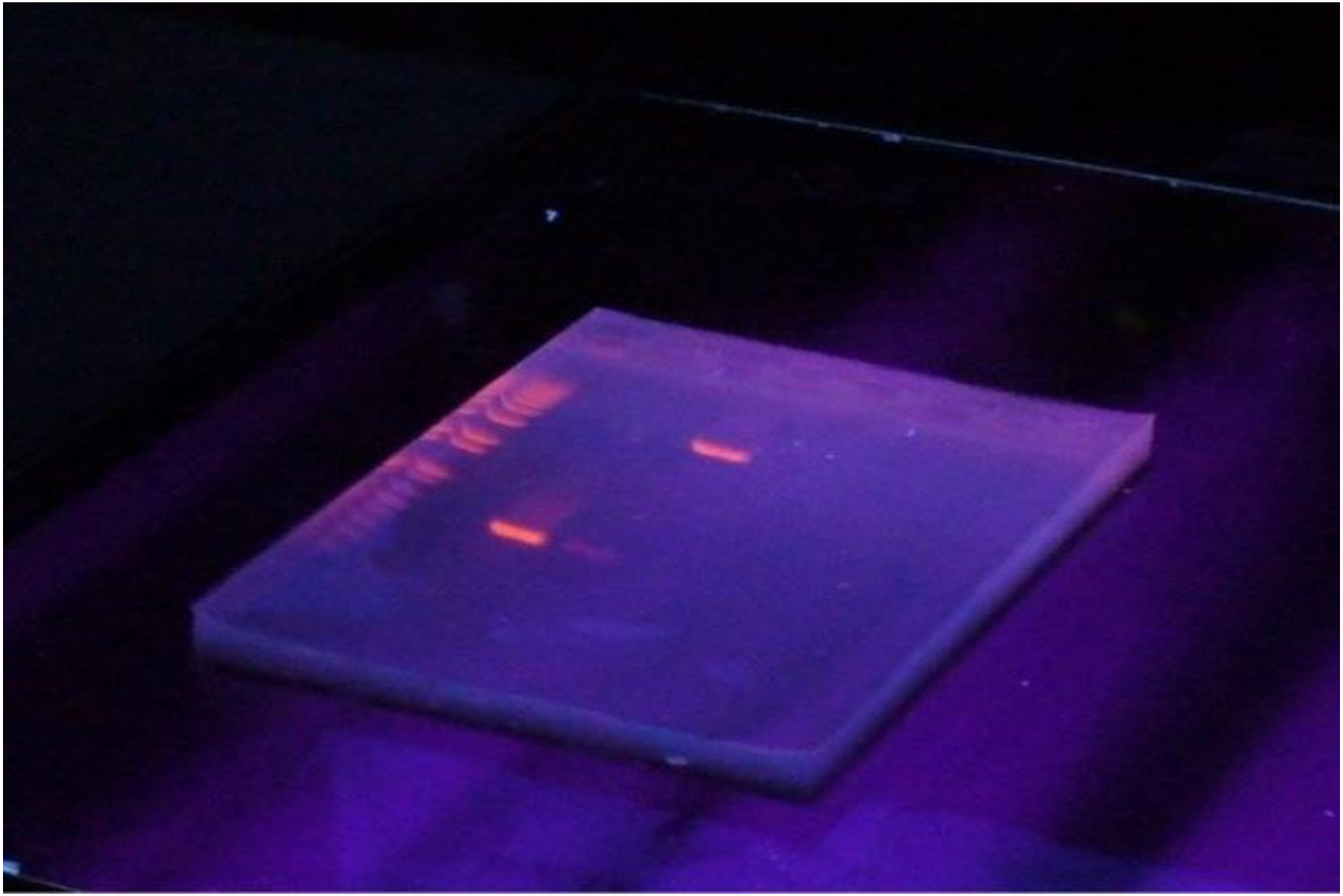
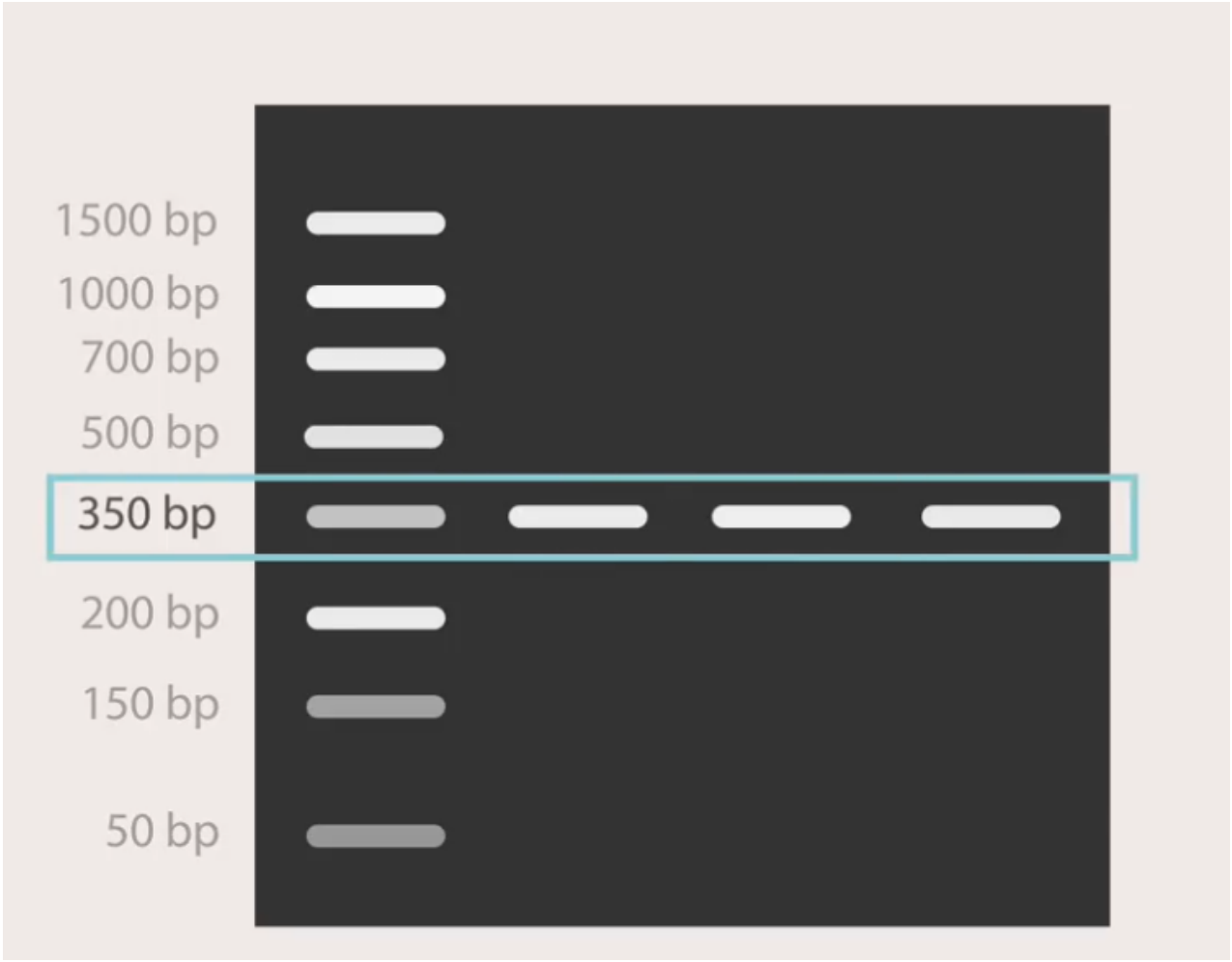
Electrophoresis




Buffer
Samples
Gel
Negative Electrode
−
+
Positive Electrode
Direction of current flow






1500 bp
1000 bp
700 bp
500 bp
350 bp
200 bp
150 bp
50 bp

Reverse Transcriptase PCR (RT-PCR)

- Refers to utilization of mRNA for the formation of target DNA using **reverse transcriptase enzyme**.
- Essentially normal PCR is preceded by reverse transcription (to convert the RNA to cDNA). This is widely used in expression mapping, determining when and where certain genes are expressed.
- **Presence of HIV RNA in blood can be detected as early as 4 weeks after infection.**

- There are three types of HIV tests: **antibody tests**, **antigen/antibody tests**, and **nucleic acid tests (NAT)**.
- An **antibody test** looks for antibodies to HIV in your blood or oral fluid.
- An **antigen/antibody test** looks for both HIV antibodies and antigens.
- A **NAT** looks for the actual virus in the blood.

- **Can an HIV test detect the virus immediately after exposure?**
- No HIV test can detect HIV immediately after infection. That's because of the window period—the time between HIV exposure and when a test can detect HIV in your body. The window period depends on the type of HIV test. A nucleic acid test can usually detect HIV the soonest (about 10 to 33 days after exposure).

- If you've been exposed to hepatitis C, it takes about 1-2 weeks for viral particles (called HCV RNA) to be found. Hepatitis C antibodies appear after RNA is detectable and can take 3-12 weeks to appear.

Quantitative PCR (Q-PCR)

- Used to measure the quantity of a PCR product (commonly in real –time) QRT-PCR. It quantitatively measures starting amounts of DNA, cDNA or RNA.

- Quantitative real time PCR has a very high degree of precision.

- QRT-PCR methods use fluorescent dyes or DNA probes to measure the amount of amplified product in real time.

Clinical Applications of PCR

- **1. Diagnosis of bacterial and viral diseases:**

  In early phases of tuberculosis, the sputum may contain only very few tubercle bacilli, so that usual acid fast staining may be negative. But PCR could detect even one bacillus present in the specimen. Any other bacterial infection could also be detected similarly. The specific nucleotide sequences of the bacilli are amplified by PCR and then detected by Southern blot analysis.

- Reverse PCR is widely used in the diagnosis of viral infections like **Hepatitis C**, and **HIV**.

- **2. Medicolegal cases:**

❖ **DNA profiling**

- Modern-day DNA profiling is also called **short tandem repeat (STR) analysis.** It uses the polymerase chain reaction (PCR)to produce many copies of specific STR sequences.
- In STR analysis the primers used in the PCR are designed to attach to either end of the STR sequence of interest.

- Short tandem repeats (STRs) are short tandemly repeated DNA sequences that involve a repetitive unit of 2-6 bp with the number of repeats varying among individuals, making STRs effective for human identification purposes.

3. Diagnosis of genetic disorders:

The PCR technology has been widely used to amplify the gene segments that contain known mutations for diagnosis of inherited diseases such as sickle cell anemia, beta thalassemia, cystic fibrosis, etc.

4. PCR is especially useful for prenatal diagnosis of inherited diseases, where cells obtained from fetus by amniocentesis are very few.

5. Cancer detection:

PCR is widely used to monitor residual abnormal cells present in treated patients. Similarly identification of mutations in oncosuppressor genes such as p53, retinoblastoma gene, etc. can help to identify individuals at high risk of cancer

6. Fossil studies:

DNA can be isolated and PCR amplified from fossils and is used to study evolution by comparing the sequences in the extinct and living organisms.

7.Quantification of gene expression.

8.Tissue typing for transplanting, by PCR and detection of genetic variants.

- A very specific set of genes is examined when DNA testing is used for tissue typing. On chromosome 6 resides a large set of genes in the so called “Major Histocompability Complex,” or MHC. These genes are very polymorphic (different) between individuals, and they code for the production of specific glycoprotein antigens located on the surface of many cells called the “human leukocyte antigens” or HLA.
- It is these antigens that allow our immune system to “recognise” our own organs and tissues from those of another individual. These antigens have the ability to provoke an immune system response that results in organ or tissue rejection if the tissue looks foreign.
- In tissue typing, the genes for a number of different HLA molecules are carefully compared between donor and recipient to ensure that they are as similar as possible to minimise the chance of a rejection.

9.In sex determination of embryos, also useful to detect sex-linked disorders in fertilized embryos.

HYBRIDIZATION AND BLOT TECHNIQUES

PROBES

- **How can the DNA sequence of interest be picked out of a mixture of thousands or even millions of irrelevant DNA fragments?**

- The answer lies in the use of a probe (a single-stranded sequence of DNA or RNA of variable length used to search for its complementary sequence and can be radioactively or fluorescently labeled to allows its binding to be visualized).

Hybridization of a probe to DNA fragments

- The utility of probes hinges on the phenomenon of hybridization (or annealing) in which a probe containing a complementary sequence binds a single-stranded sequence of a target DNA.
- ssDNA, produced by alkaline denaturation of dsDNA, is first bound to a solid support, such as a nitrocellulose membrane. The immobilized DNA strands are prevented from self-annealing, but are available for hybridization to an exogenous, radiolabeled, ssDNA probe.

Blotting techniques

- **Southern Blot Technique:**

- It is based on the specific base pairing properties of complementary nucleic acid strands. This technique is therefore based on **DNA hybridization.**
- The blot technique was developed by EM Southern in 1975. This is used to detect a **specific segment of DNA in the whole genome.**

- DNA is isolated from the tissue.
- It is then fragmented by **restriction endonucleases.**
- The cut pieces are **electrophoresed on agarose gel.** It is then treated with NaOH to denature the DNA, so that the pieces become single-stranded.
- This is then **blotted (adsorbed)** over to a nitrocellulose membrane. The single-stranded DNA is adsorbed in the nitrocellulose membrane.

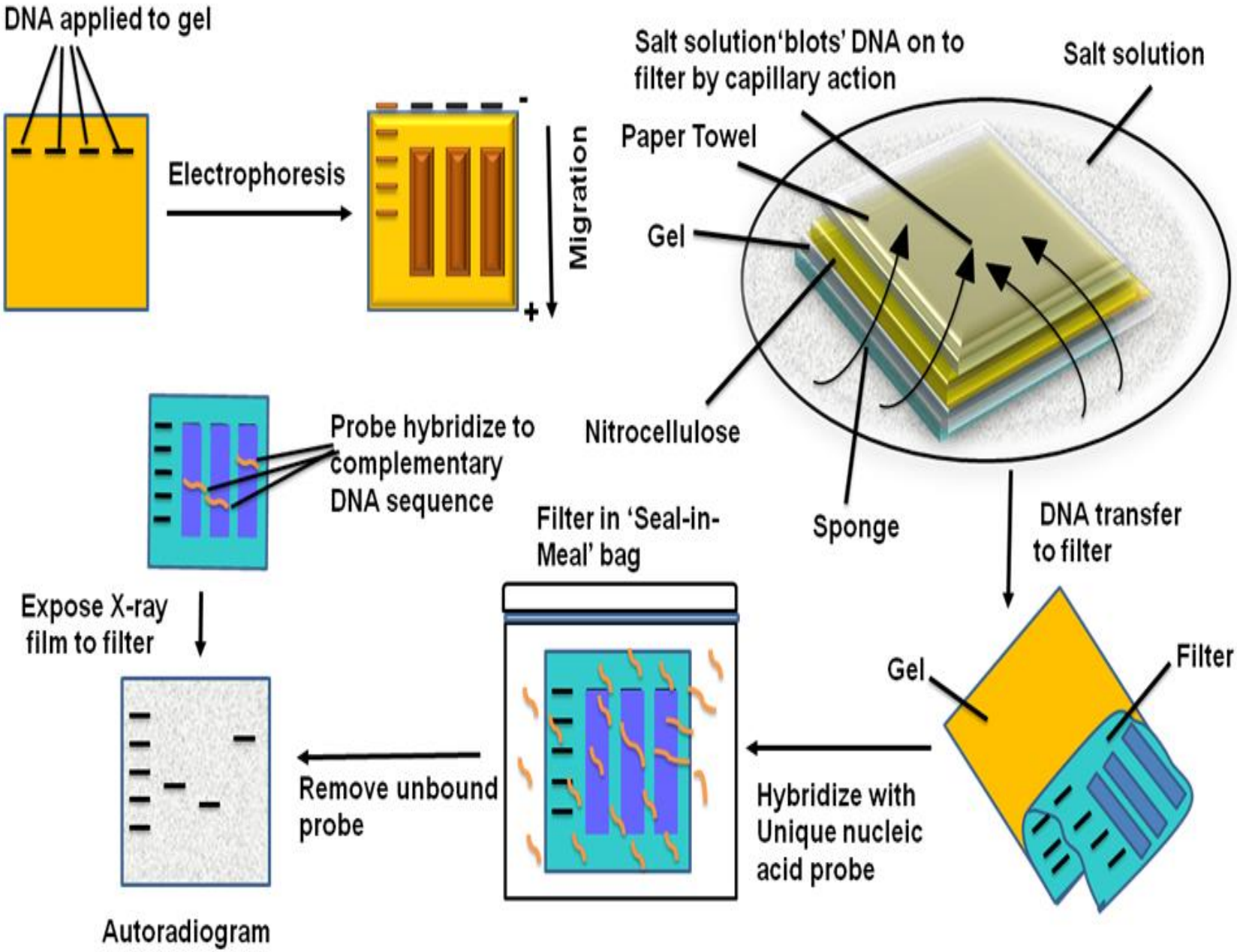
- The DNA is then fixed on the membrane by baking at 80°C. There will be many DNA fragments on the membrane, but only one or two pieces contain the target DNA.

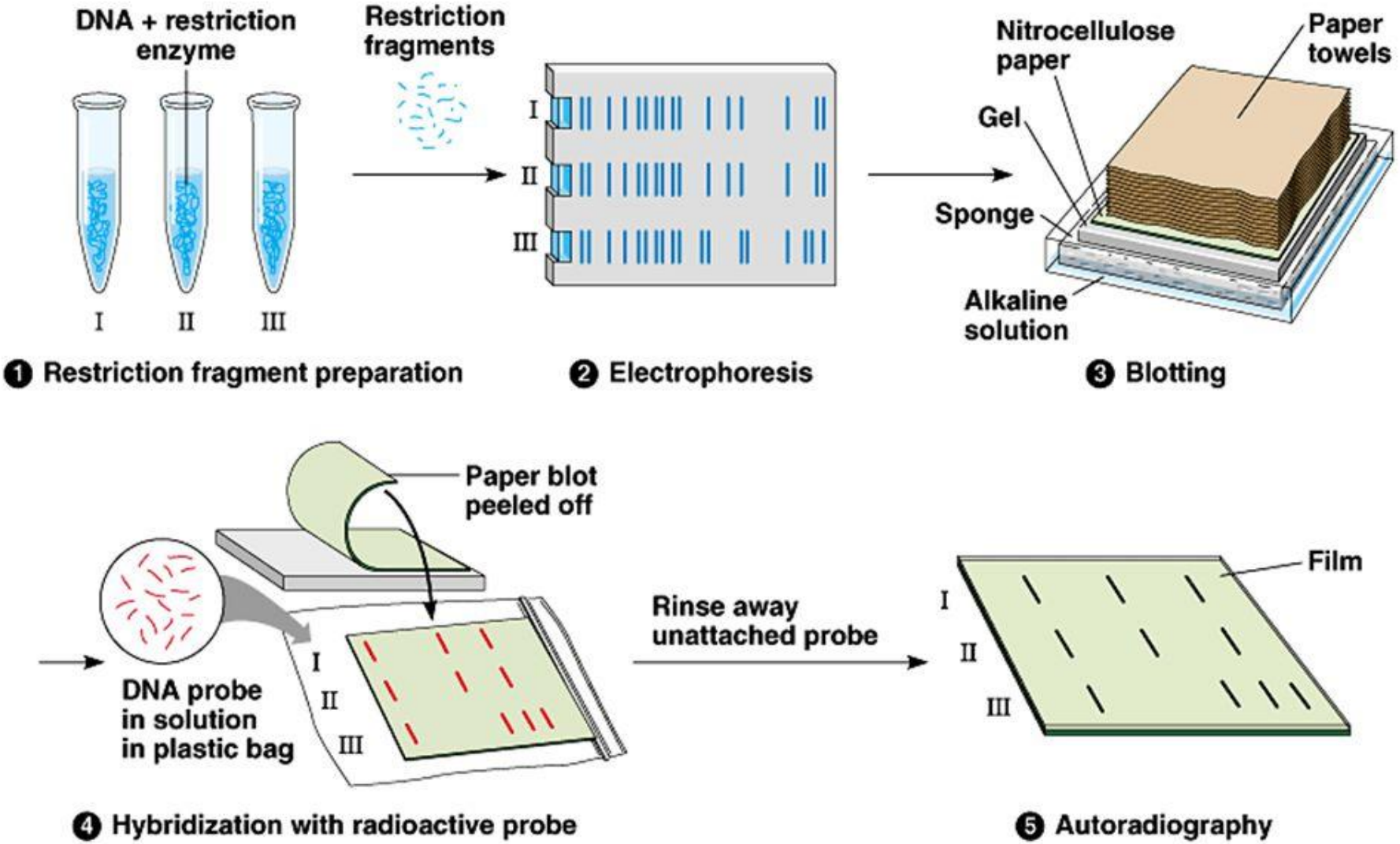
- The radio active **DNA probe is placed over the membrane.** If the target genes are present in the host DNA, the probe will detect the complementary nucleotide sequence in the host DNA. So the probe is hybridized to the particular pieces of host DNA.

•The membrane is then thoroughly **washed to remove excess probes.**

• **An** X-ray plate is placed over the membrane in the dark for a few days. The radiation from the fixed probe will produce its mark on the X-ray plate. This is called **autoradiography.**

• **Mutant genes** such as HbS, cystic fibrosis, DMD, PKU as well as presence of viral DNA (hepatitis virus B and C) can be identified by this method.


DNA applied to gel
Electrophoresis
-
+
Migration
Salt solution'blots' DNA on to filter by capillary action
Salt solution
Paper Towel
Gel
Nitrocellulose
Sponge
DNA transfer to filter
Gel
Filter
Hybridize with Unique nucleic acid probe
Filter in 'Seal-in-Meal' bag
Remove unbound probe
Probe hybridize to complementary DNA sequence
Expose X-ray film to filter
Autoradiogram



Northern Blotting for Identifying RNA:

- The Northern blot is used to demonstrate **specific RNA.** The total RNA is isolated from the cell, electrophoresed and then blotted on to a membrane. **This is then probed with radioactive cDNA (RNA-DNA hybridization) or RNA.**
- This is used to detect the gene expression in a tissue

Western Blot Analysis for Proteins

- In this technique, proteins (not nucleic acids) are identified.
- The proteins are isolated from the tissue and electrophoresis is done. The separated proteins are then transferred on to a nitrocellulose membrane.
- After fixation, it is probed with radioactive **antibody and autoradiographed.**
- This technique is very useful to identify the specific protein in a tissue, thereby showing the expression of a particular gene.