

Polymerase chain reaction (PCR)

Ahmed Salem, MD, PhD, FRCR

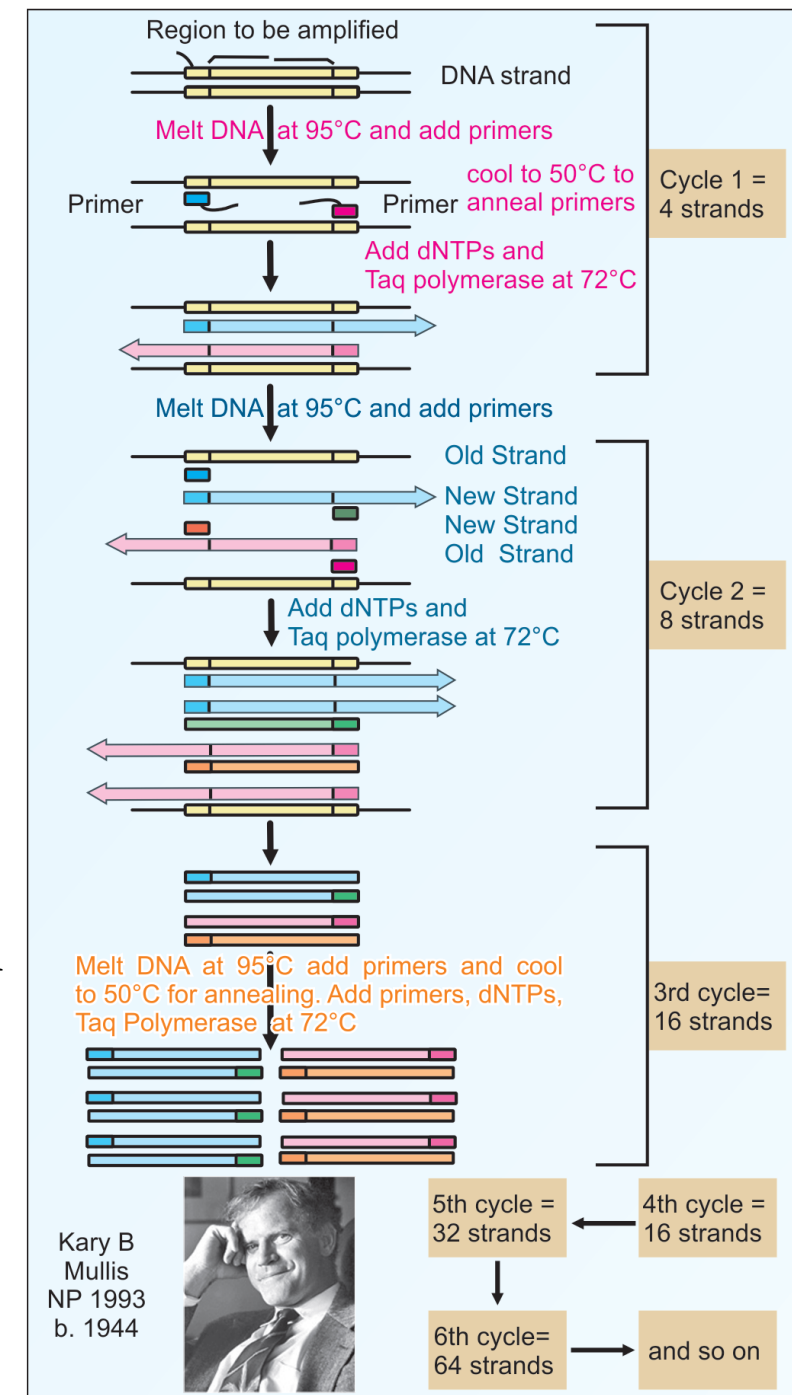
Some slides from Dr Walaa

Polymerase chain reaction (PCR)

- Kary Mullis invented this method in 1983 → awarded Nobel prize in 1993
- PCR is an *in vitro* DNA amplification procedure
 - Millions of copies of a particular sequence of DNA can be produced
 - Takes only a few hours
 - It is like xerox machine for gene copying

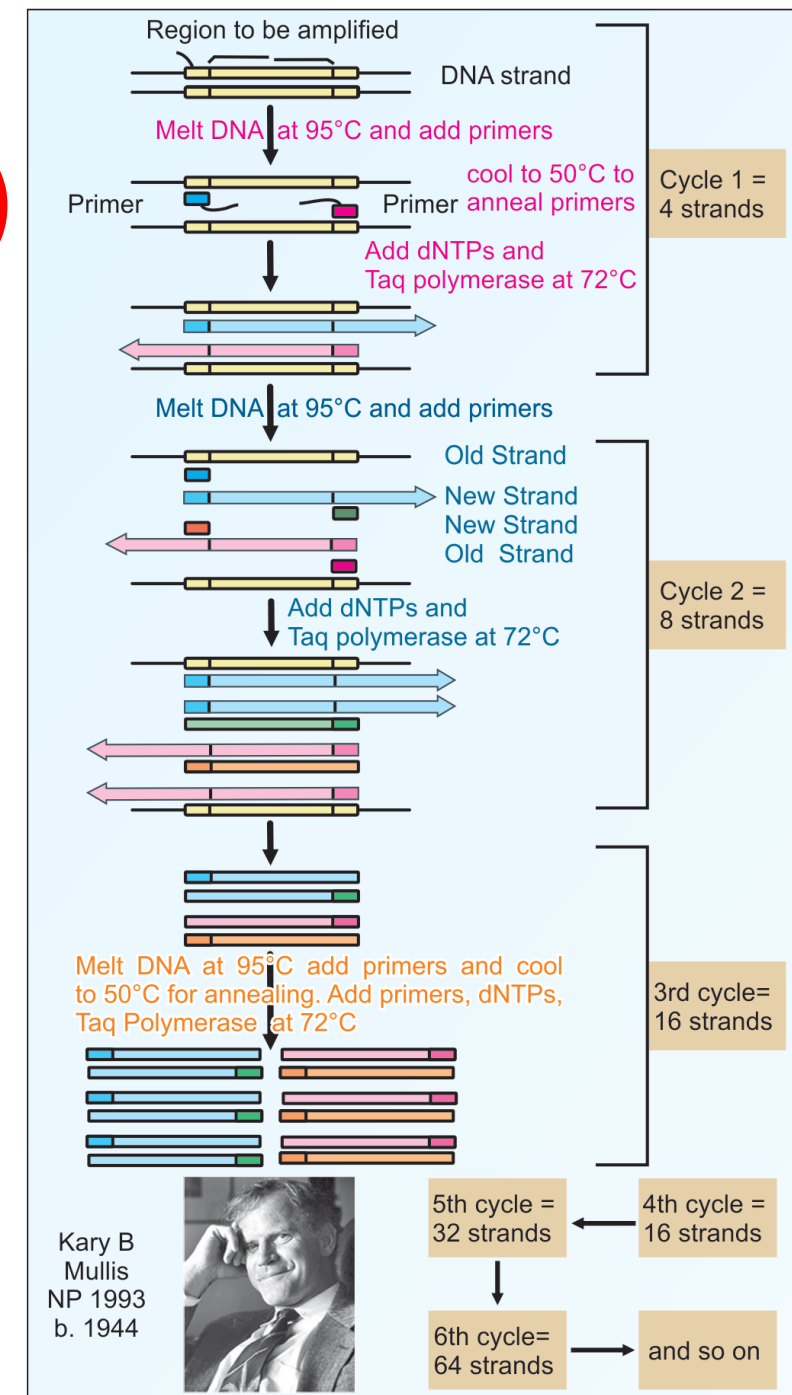
Steps for PCR

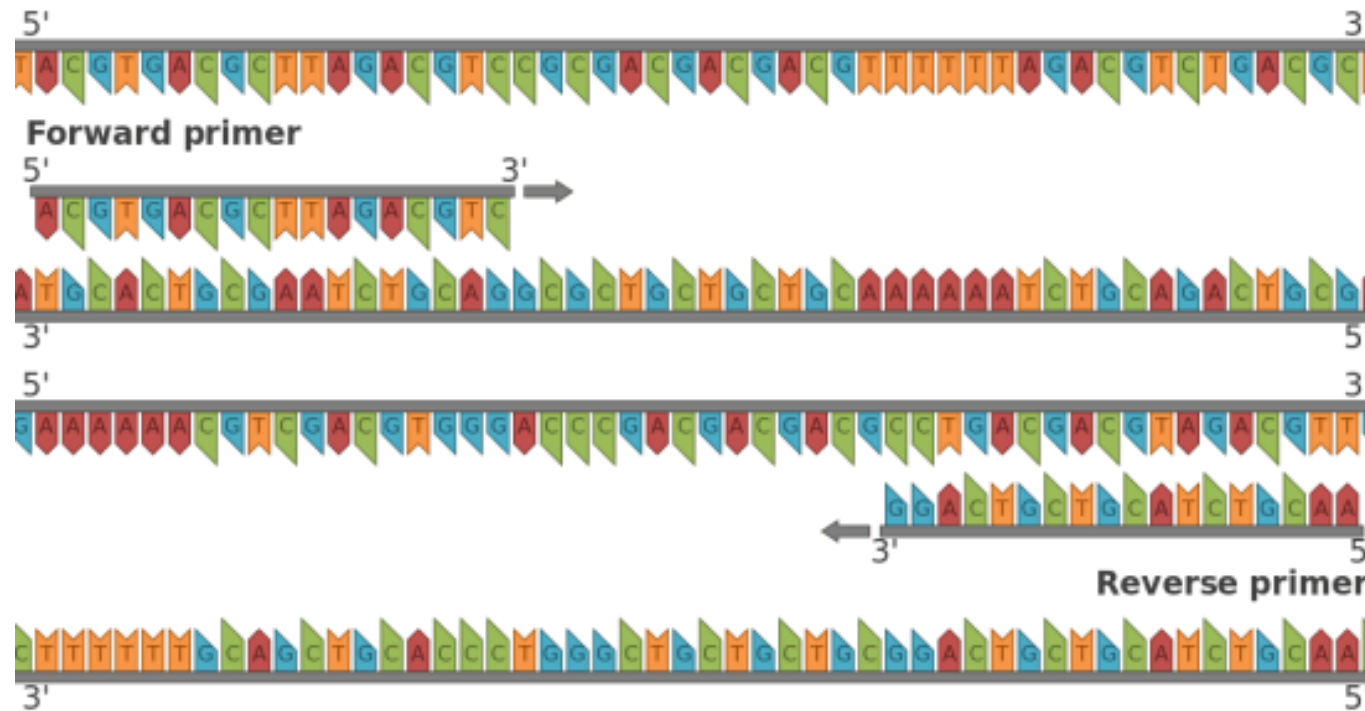
- The flanking sequences of the gene of interest should be known
 - Two **DNA primers** of about 20–30 nucleotides with complementary sequence of the flanking region are synthesized
- The reaction cycle has the following steps:
 - **Step 1: Separation (Denaturation):** DNA strands are separated (**melted**) by heating at 95°C for 15sec-2min
 - **Step 2: Priming (Annealing):** The primers are **annealed** by cooling to 50°C for 0.5-2min
 - The primers hybridize with their complementary single stranded DNA produced in the first step



Steps of PCR (continued 1 of 2)

- **Step 3:** New DNA strands are synthesized by **Taq polymerase**
 - This enzyme is derived from bacteria *Thermus aquaticus*
 - The polymerase reaction is allowed to take place at 72°C for 30sec
 - In presence of dNTPs (all four deoxy ribonucleotide triphosphates)
- → Both strands of DNA are now duplicated

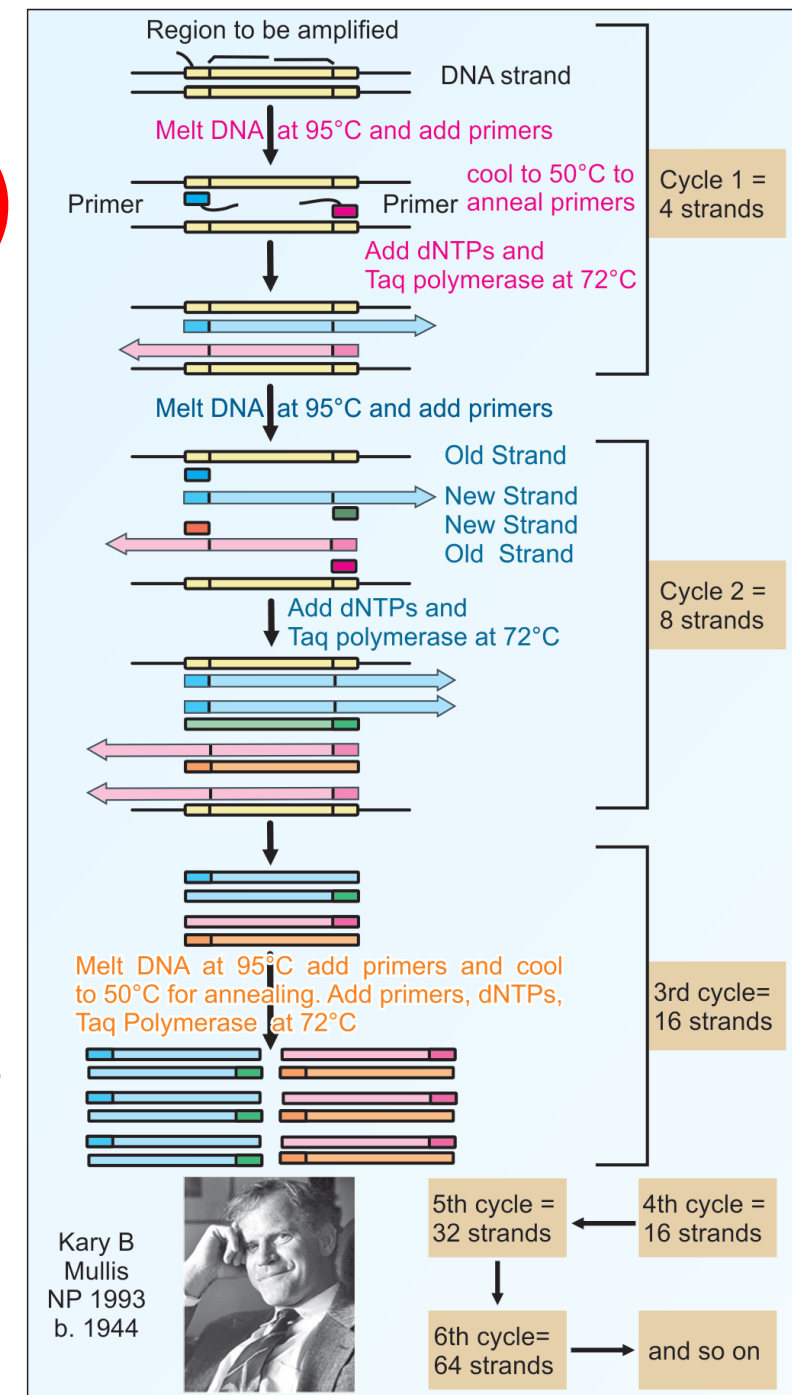




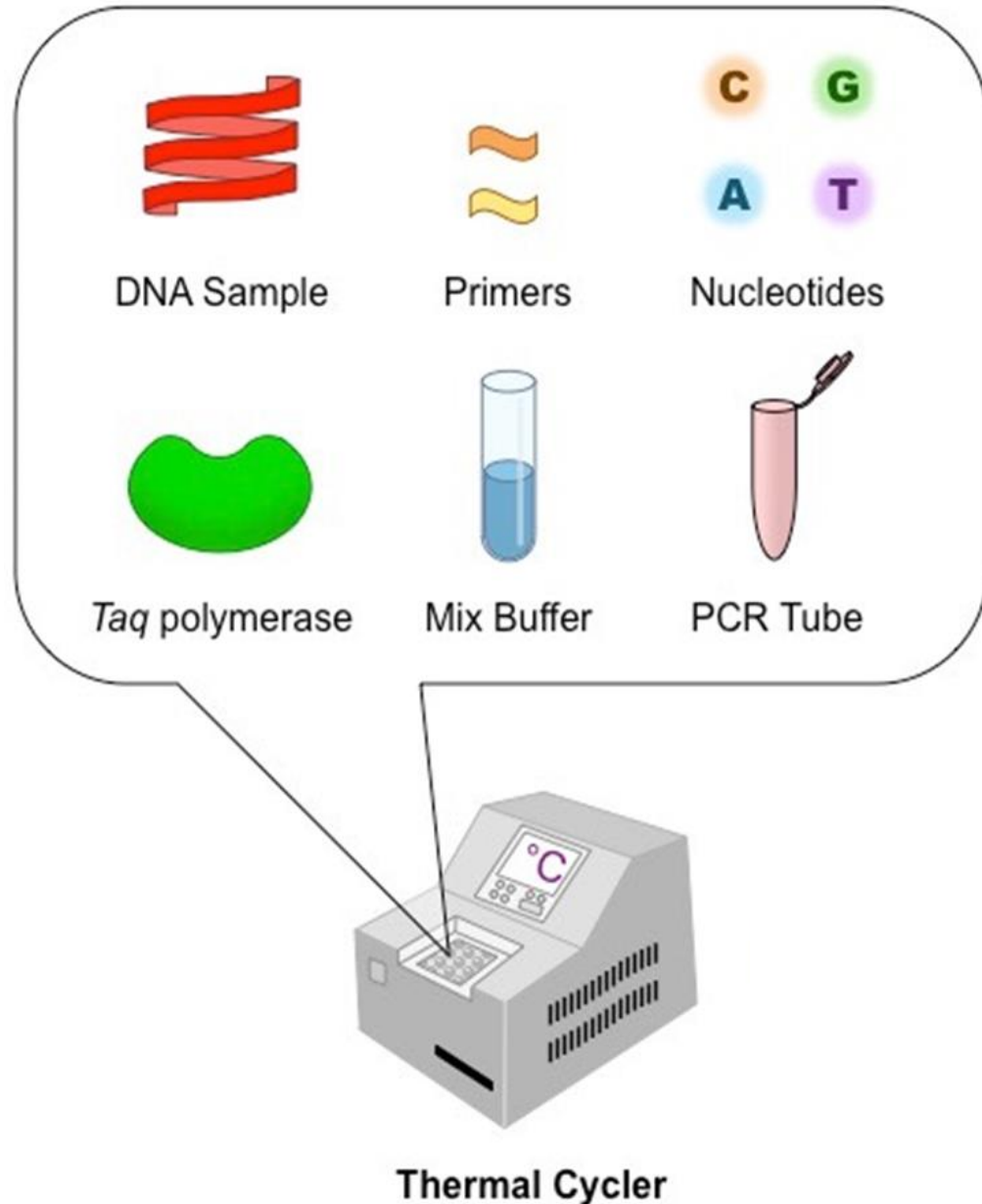
- Forward primers anneal to the antisense strand of the double-stranded DNA, which runs from 3' to 5' direction, whereas reverse primers anneal to the sense strand of the double-stranded DNA, which runs from 5' to 3' direction.

Steps of PCR (continued 2 of 2)

- **Step 4: Generation of copies**
 - Steps of 1, 2 and 3 are **repeated**
 - In each cycle, the DNA strands are doubled
 - → in 20 cycles → 1 million times amplifications
 - These cycles are repeated by automated instrument (**Thermal cycler**)
- **Step 5: Test for DNA in sample**
 - → DNA hybridization technique or Southern blot analysis with a suitable probe



PCR Components



Modification of PCR

- According to new modifications in technology, all the three cycles of PCR are performed at 63°C for 60 minutes
- The melting of DNA is achieved, not by high temperature, but by enzymes
- This will reduce the cost of the test
- Result will be available within hours

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 → 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 (short tandem repeat (STR) analysis)**

- PCR used to produce many copies of specific STR sequences
 - Primers used in the PCR are designed to attach to either end of the STR sequence of interest

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.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 Histocompatibility 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

Reverse Transcriptase PCR (RT-PCR)

- It is the method used to amplify, isolate or identify a known sequence from RNA library
 - 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
- Instead of Taq polymerase described above, Tth polymerase from *Thermus thermophilus* may be used
- This enzyme has both DNA polymerase and reverse transcriptase activities at high temperature
 - This allows both cDNA synthesis from mRNA followed by PCR amplification
- In ordinary PCR, DNA is detected; that DNA could be from a living or non-living organism
 - But in reverse PCR, mRNA is detected; that means, it is derived from a **living organism**
- Presence of HIV RNA in blood can be detected as early as 4 weeks after infection (HepC RNA can be seen in 1-2 weeks)

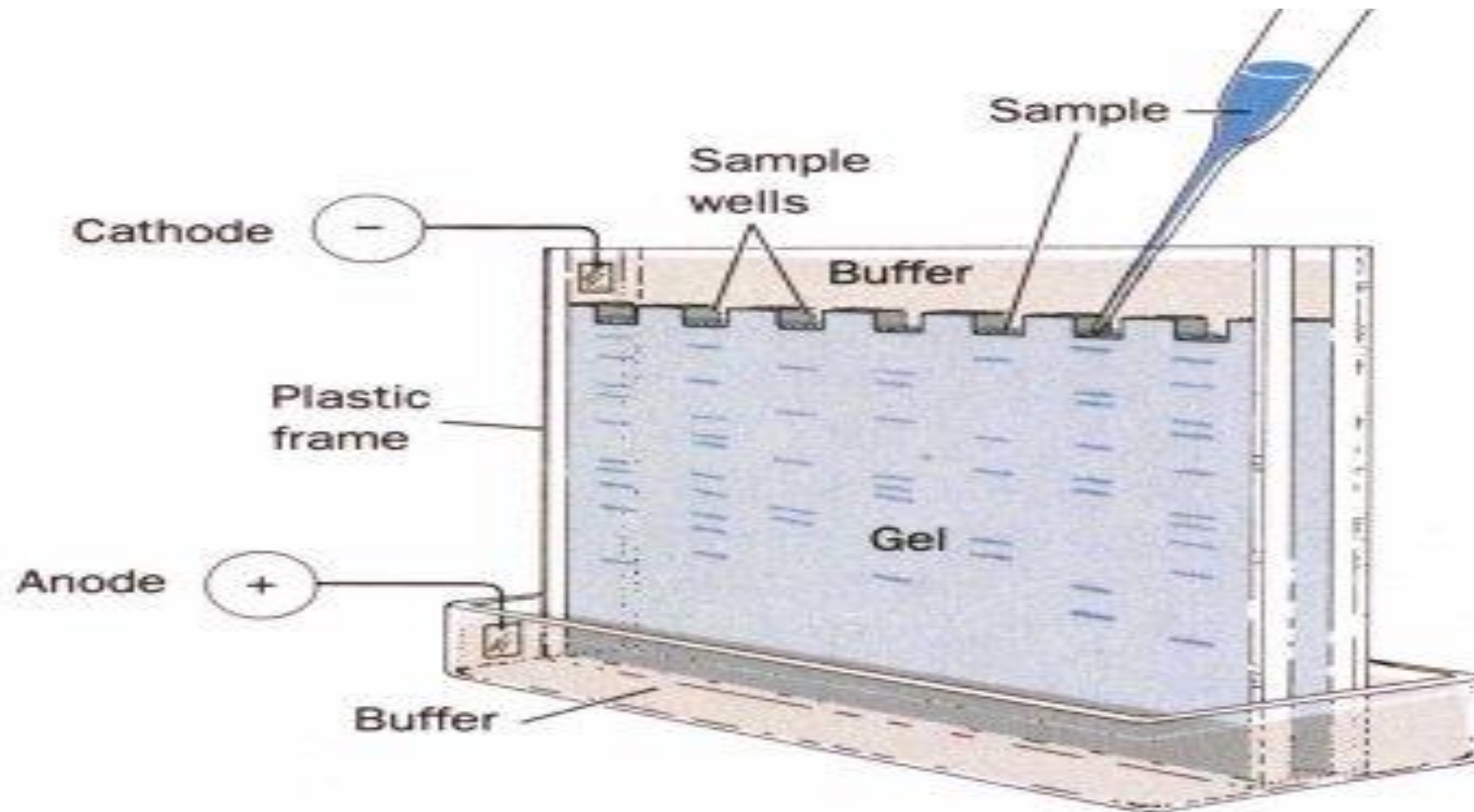
Quantitative PCR (Q-PCR)

- ✦ Used to measure the **quantity** of a PCR product (commonly in real-time) QRT-PCR
 - ✦ Determines whether a sequence is present or not, and if present the number of copies in the sample 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

Identification of the PCR product

- Agarose gel electrophoresis is an important technique in molecular biology
 - It is routinely used for analysis of PCR products
 - Step for analysis of specific **RNA** and **DNA** fragments by **Northern** and **Southern** blots
- Gel electrophoresis is used to separate mixtures of DNA, RNA, or proteins according to molecular size
 - The molecules are pushed by an electrical field through a gel
 - Small molecules will travel a greater distance than will a larger molecules
- The electrical 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

Electrophoresis



DNA ladder

Samples

1500 bp

1000 bp

700 bp

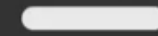
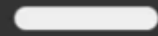
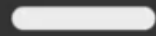
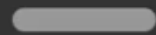
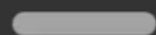
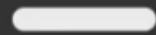
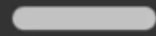
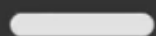
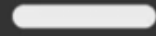
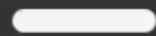
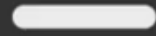
500 bp

350 bp

200 bp

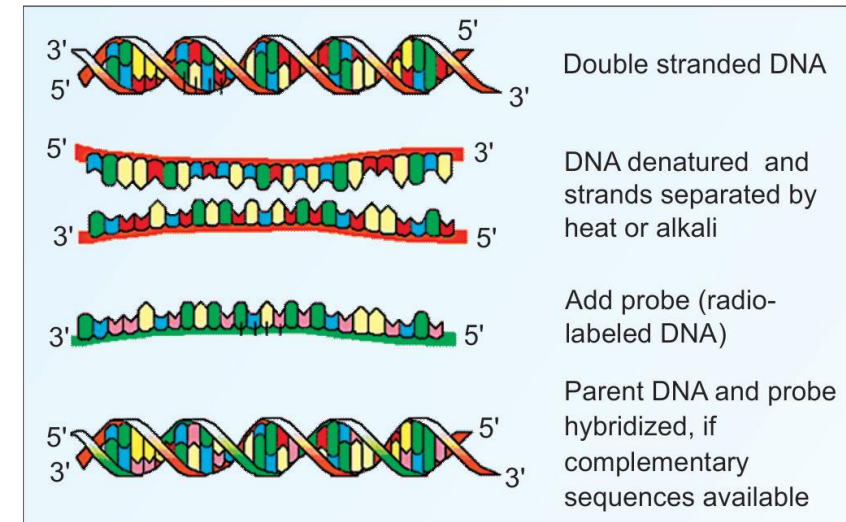
150 bp

50 bp



Hybridization and blot techniques

- **Hybridization (or annealing)**: A probe containing a complementary sequence binds a single-stranded sequence of a target DNA
- **Probes**
 - **Def:** single stranded piece of DNA, labeled (either with radioisotope or with non-radioactive label), the nucleotide sequence of which is complementary to the target DNA



Southern blot

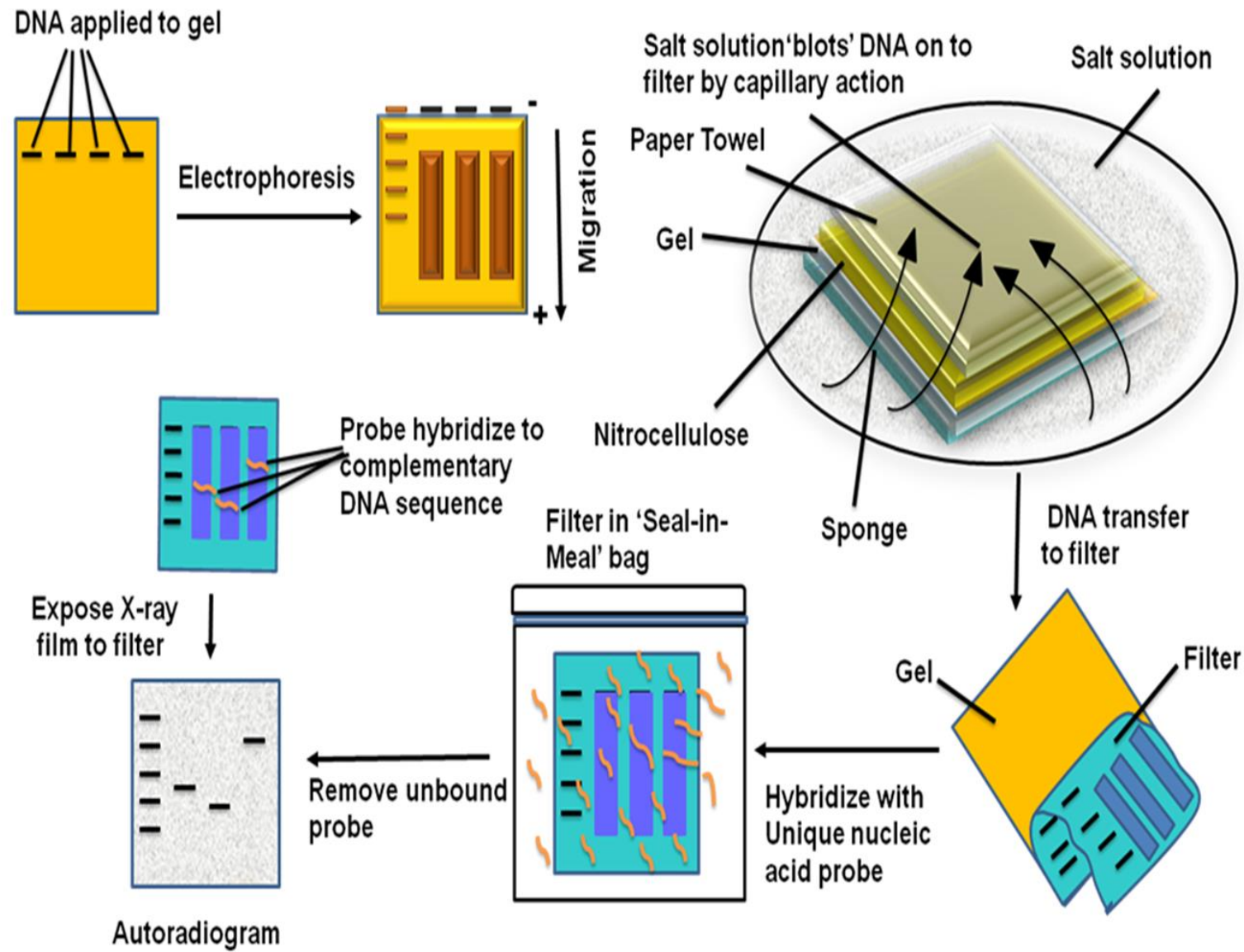
- It is based on the specific base pairing properties of complementary nucleic acid strands
- This technique is therefore based on **DNA hybridization**
- The blot technique was developed by Edwin Southern in 1975
- This is used to detect a **specific segment of DNA**

Sothern Blot

- DNA is isolated from the tissue. It is then fragmented by **restriction endonucleases**
- The cut pieces are **electrophoresed** on agarose gel
 - Treatment with NaOH denatures 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 on the **nitrocellulose** membrane
 - An exact replica of the pattern in the gel is reproduced on the 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

Southern blot

- The radioactive **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
 - 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



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
 - There will be RNA-DNA hybridization
- This is used to detect the gene expression in a tissue

Western Blot

- 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

Fluorescence in situ hybridization (FISH)

- Histology section or single cells may be treated with specific probes tagged with fluorescence and seen under microscope
- DNA/mRNA of interest can be viewed in the context of the tissue morphology
- Examples of applications of FISH are:
 - assessment of gene rearrangements in leukemia
 - amplification of HER2/neu in breast cancer

