بسم الله الرحمـٰن الرحيم

(وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ)

Cytology & Molecular Biology | FINAL 2

Overview & basic Techniques pt.2

Written by : DST

Reviewed by : Abdallah Al-Abdallat

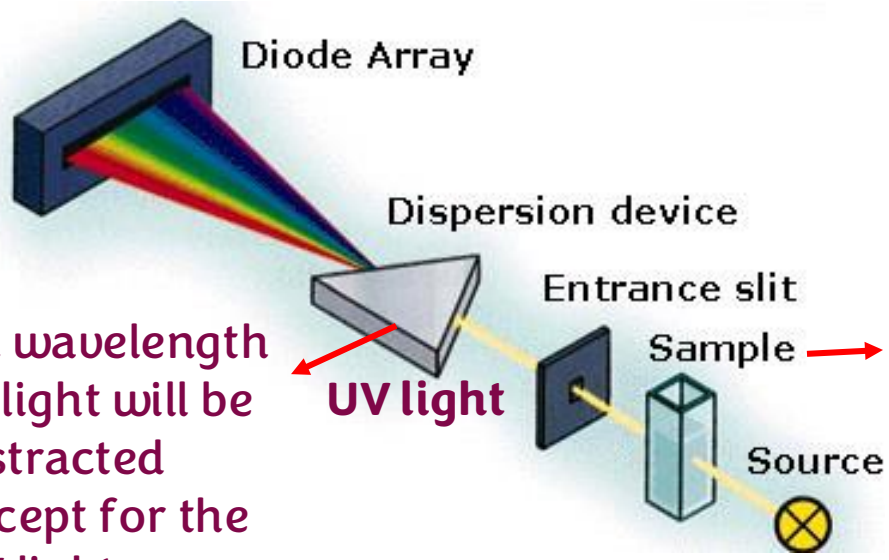
How can we measure how much DNA we have in a sample ?

Light absorbance of nucleic acids

- Aromatic pyrimidines and purines can absorb UV light.
- Using spectrophotometry, the peak absorbance can be measured at 260 nm wavelength. (Spectrometry (Technique) → Spectrometer (Device)).
  - The absorbance of nucleic acids at **260 nm (A260) is constant**
    - **dsDNA: A260 of 1.0 = 50 ug/ml**

What is the concentration of a double stranded DNA sample diluted at 1:10 and the A260 is 0.1?
DNA concentration = 0.1 x 10 x 50 µg/ml
= 50 µg /ml

Hint from the doctor : play with the variables in the equation and test yourself



All wavelength of light will be distracted except for the UV light

DNA then absorbs the UV light

Tells us the amount of light that is absorbed then we transfer this data to concentration

Further explanation :

- DNA does not have a color that we can see, it absorbs the UV light and reflects everything else; that's why we have to stain it.
- 260 nm (A260) –> The max amount of wavelength that DNA can absorb, A=absorption.
- **Concept:**

DNA concentration is directly proportional to its absorbance. If a DNA solution at a certain concentration absorbs a specific amount of light, then halving the concentration will result in half the absorbance.

Example:

Suppose we have a DNA solution with a concentration of 50 µg/mL, and it absorbs 1unit of UV LIGHT. If we dilute this solution to a concentration of 25 µg/mL (half of the original concentration), it will absorb 0.5 units of light. This is because the absorbance is proportional to the concentration, so halving the concentration halves the absorbance.

Spectrophotometry technique illustration (pt.1).

❖Sample Preparation and UV Exposure:

- The DNA sample is placed in a vial and exposed to UV light. Only the UV wavelength reaches the DNA, as other wavelengths are filtered out.
- The DNA absorbs the UV light, and the absorption level is then detected by a sensor.

❖Converting Absorption to Concentration:

- The instrument provides an output indicating the amount of UV light absorbed by the sample.
- This absorption value is then used to calculate the DNA concentration in the sample.

❖Understanding Absorption Standards:

- The instrument is calibrated with known standards. For example:
- Standard Concentration: A DNA concentration of 50 µg/mL will absorb one unit of UV light.
- Half Concentration: If the DNA concentration is 25 µg/mL, it will absorb 0.5 units of light, as half the concentration absorbs half the light, Etc.

❖**Calculating Unknown Concentrations:** For instance, if a DNA sample absorbs 0.1 units of light (one-tenth of the standard), the DNA concentration can be estimated as 5 µg/mL (one-tenth of 50 µg/mL).

❖Handling High DNA Concentrations:

- When samples have very high DNA concentrations, such as 500 µg/mL, the instrument may exceed its measurement limit.
- To address this, the sample is diluted for accurate measurement.

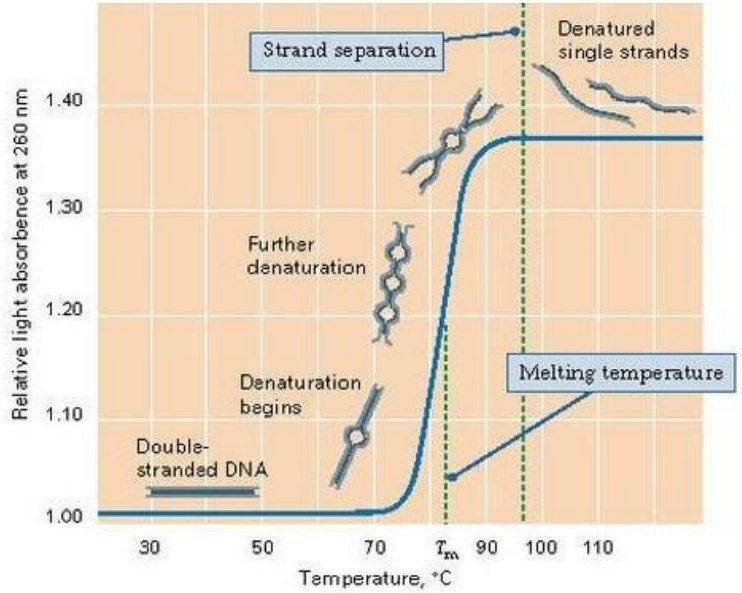
❖Dilution Process Example:

✓ Suppose we have a solution containing DNA at a concentration of 500 µg/mL To dilute this solution at a ratio of 1:10, we take 1 mL of the DNA solution and mix it with 9 mL of water. This will result in a diluted solution with a concentration of 50 µg/mL (since 500 µg/mL ÷ 10 = 50 µg/mL).

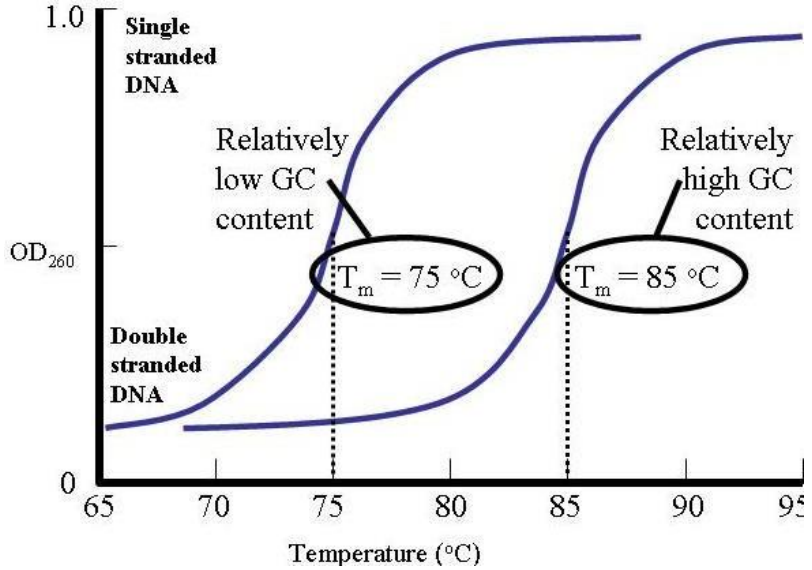
- To use this diluted concentration in further calculations, we must account for this dilution factor (1:10) when applying it in formulas to ensure we obtain the correct final amount of DNA.

❑ Observation of denaturation

- **Denaturation**: loss of the native 3-dimensional conformation due to the breaking of noncovalent interactions within a DNA/Protein.
- In DNA, noncovalent interactions ⇔ hydrogen bonds.
- Hydrogen bonds interactions are reversible.
- The 2 strands can revert to the original state if the conditions are returned to normal (discussed later).



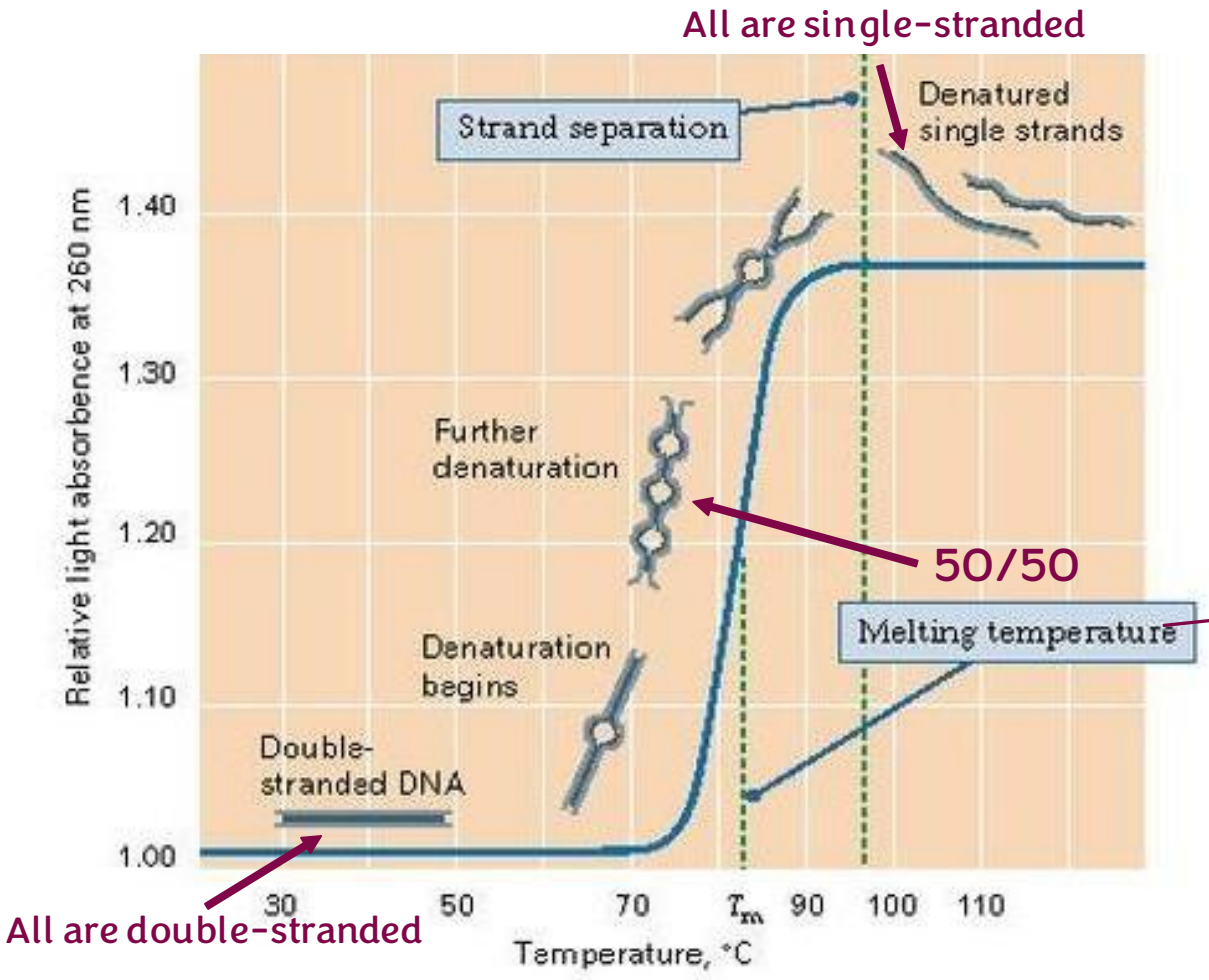
- The transition temperature or melting temperature (Tm).
- Factors influencing Tm
  - Length
  - G·C pairs
    - Hydrogen bonds
  - pH
  - Salts and ions (Cations only)
  - Destabilizing agents (alkaline solutions, formamide, urea)



SEE detailed explanation of the points above on slide 5

❖ Just like proteins, DNA can be denatured

- Heat can disturb the hydrogen bonding between base pairs.



- For 2 different DNAs, the denaturation occurs at different temperatures depending on strength of interactions of the hydrogen bonds.

- We usually compare DNAs by their T_m, which is the temperature where 50% of the DNA molecule is double stranded and 50% has already denatured –is single stranded.

The melting temperature indicates the strength of the connection between the two strands of DNA.

❑ Factors Influencing Denaturation

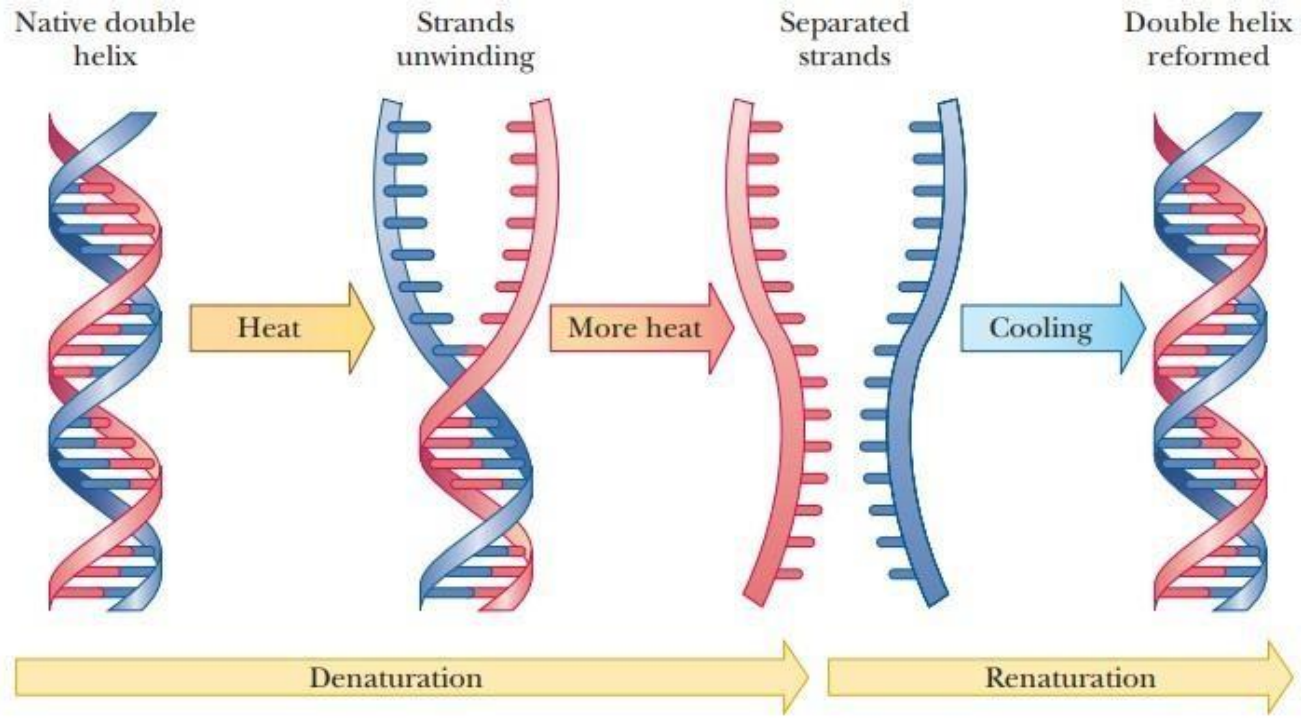
- These 3 makes DNA require higher temperature (energy) to denature:

1. Length
   - More base pairs = more H-bonds = stronger connection = more energy needed.
2. G-C content
   - 3 H-bonds per bp (unlike A-T which has 2) = stronger connection = more energy needed.
     - Ex: 2 DNAs with same length but one with more G-C content, that DNA will be much strongly connected.
3. Salty conditions:
   - In DNA, repulsion occurs due to the negative charges of phosphates in the backbone; in salty conditions, cations (such as Mg^{2+}) can stabilize the (-) charges, making it stronger, and thus more energy is needed for separation.

- Other conditions cause DNA to denature:
  - Extreme pH (especially alkaline pH); affects the hydrogen bonds. (acidic pH leads
  - Destabilizing agents (formamide, urea) eliminate hydrogen bonds.

❑ Denaturation versus Renaturation

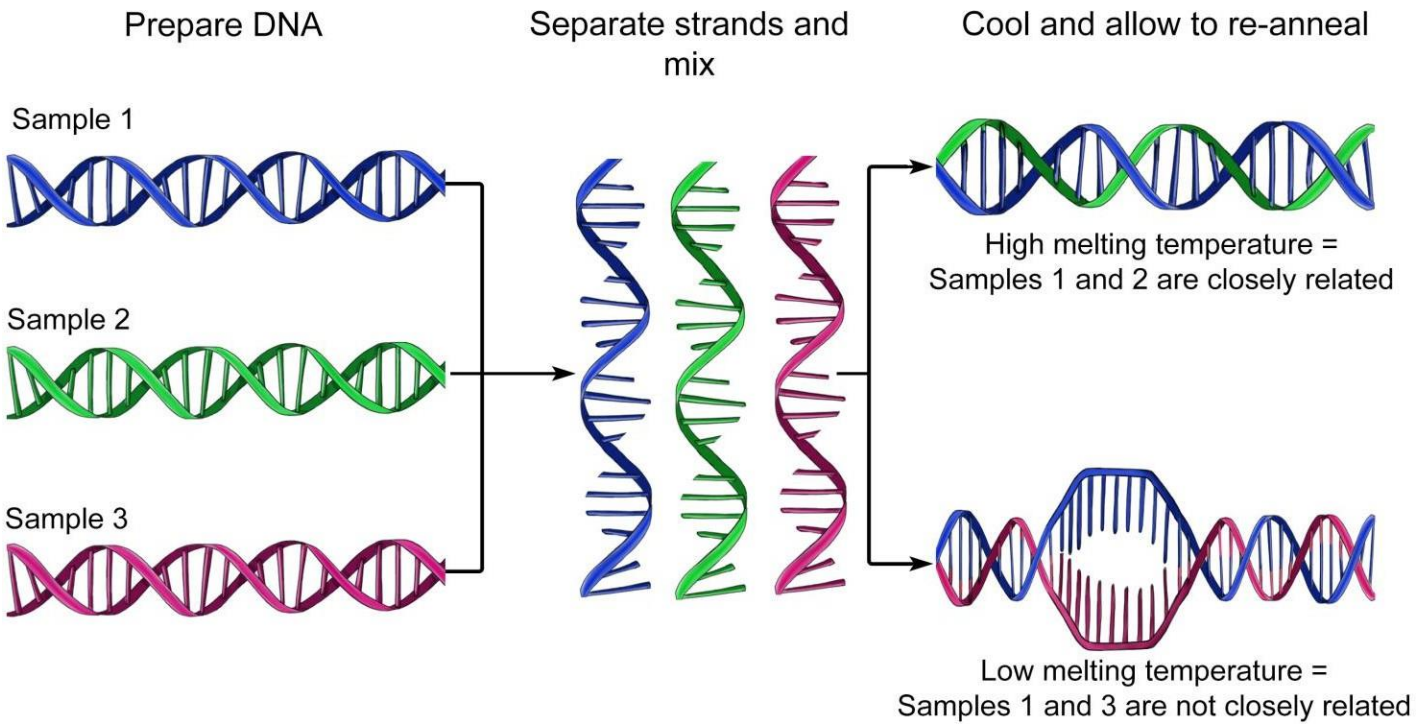
- DNA strands that were separated by denaturation can join forming a double- stranded helix again.
- This is possible because both strands are **complementary** and would favor the "renaturation".
- For renaturation to occur, the denaturing factor should be removed.



■ **FIGURE 9.19 Helix unwinding in DNA denaturation.** The double helix unwinds when DNA is denatured, with eventual separation of the strands. The double helix is re-formed on renaturation with slow cooling and annealing.

- ✓ Raising the temp. = Breaking of hydrogen bonds (denaturation)
- ✓ Lowering the temp. = Reformation of hydrogen bonds (renaturation)

❑ Denaturation versus Hybridization



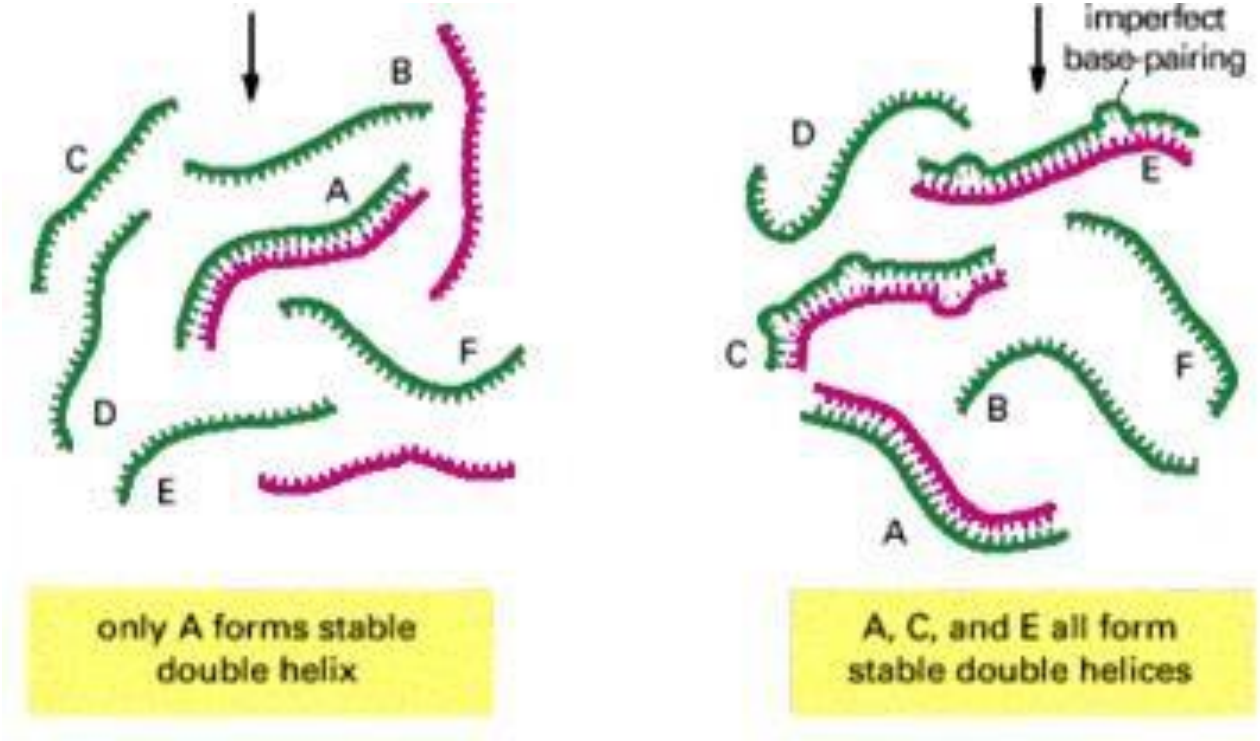
- When denaturing agents are removed, any 2 DNA strands can join forming a double-stranded DNA as long as both strands are complementary, regardless of their origin.
- When 2 strands from different origins unite, it is called "**hybridization**".

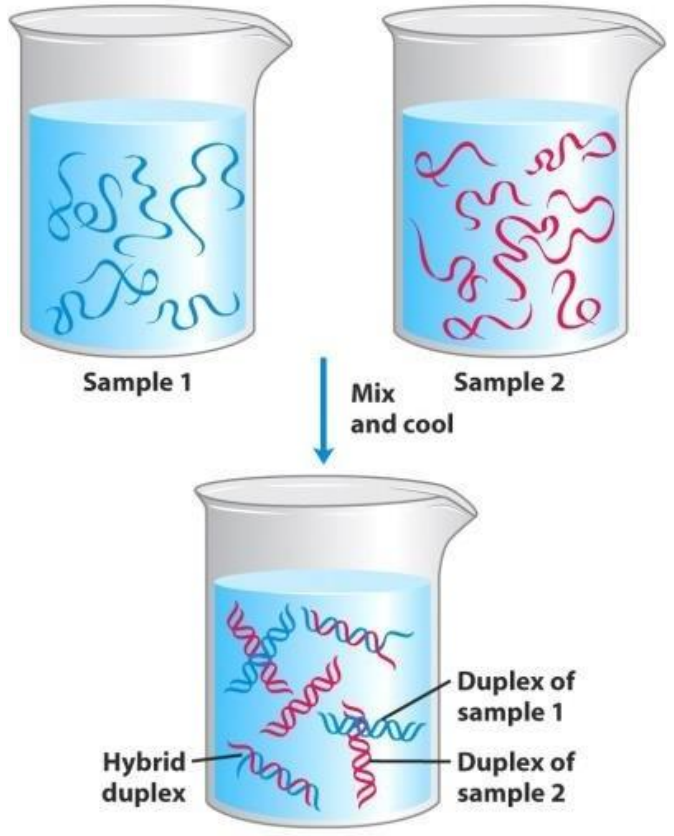
- As seen in the lower image, hybridization can occur between complementary segments of DNA strands as long as there are enough hydrogen bonds between their complementary bases, even if not all nucleotides are complementary; this is called "imperfect hybridization".

- No exact percentage; it depends on the DNA fragments (G-C content and other factors).
- G-C are stronger that A-T.

❑ Hybridization

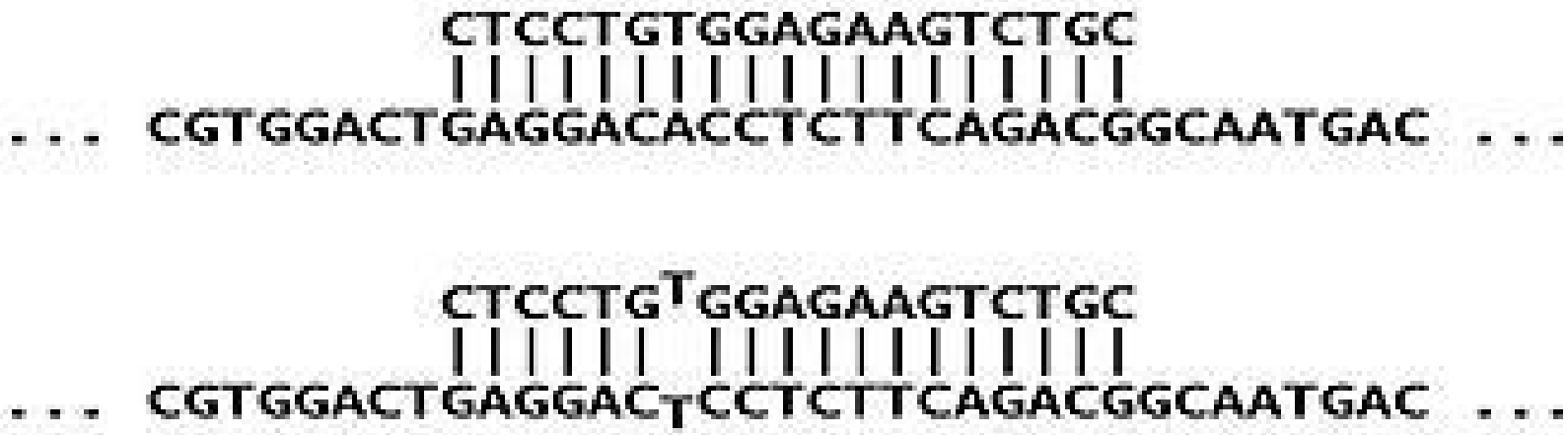
- DNA from different sources can form double helix as long as their sequences are compatible/complementary (hybrid DNA).
- Hybridization can be imperfect, which makes it less stable (but we can promote stabilizing it – the DNA – by lowering the temperature, increasing salt concentration, etc).





❑ Hybridization can be non-specific

When there are enough H-bonds formed between partially complementary bases



Hybridization can be controlled by changing the temperature, ionic strength of solutions, GC content, etc.

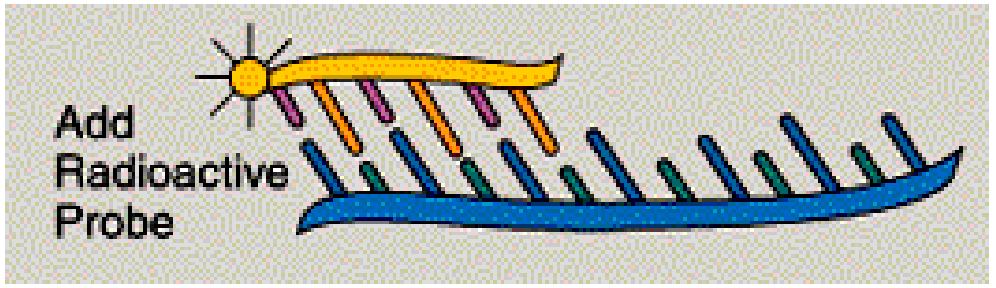
❑ Hybridization techniques Based on the use of probes.

- Hybridization reactions can occur between any two single-stranded nucleic acid chains provided that they have complementary nucleotide sequences

- Hybridization reactions are used to detect and characterize specific nucleotide sequences

❑ Probes (Oligonucleotides)

Oligo: Short sequence of DNA (or RNA)
DNA is more often because it is more stable



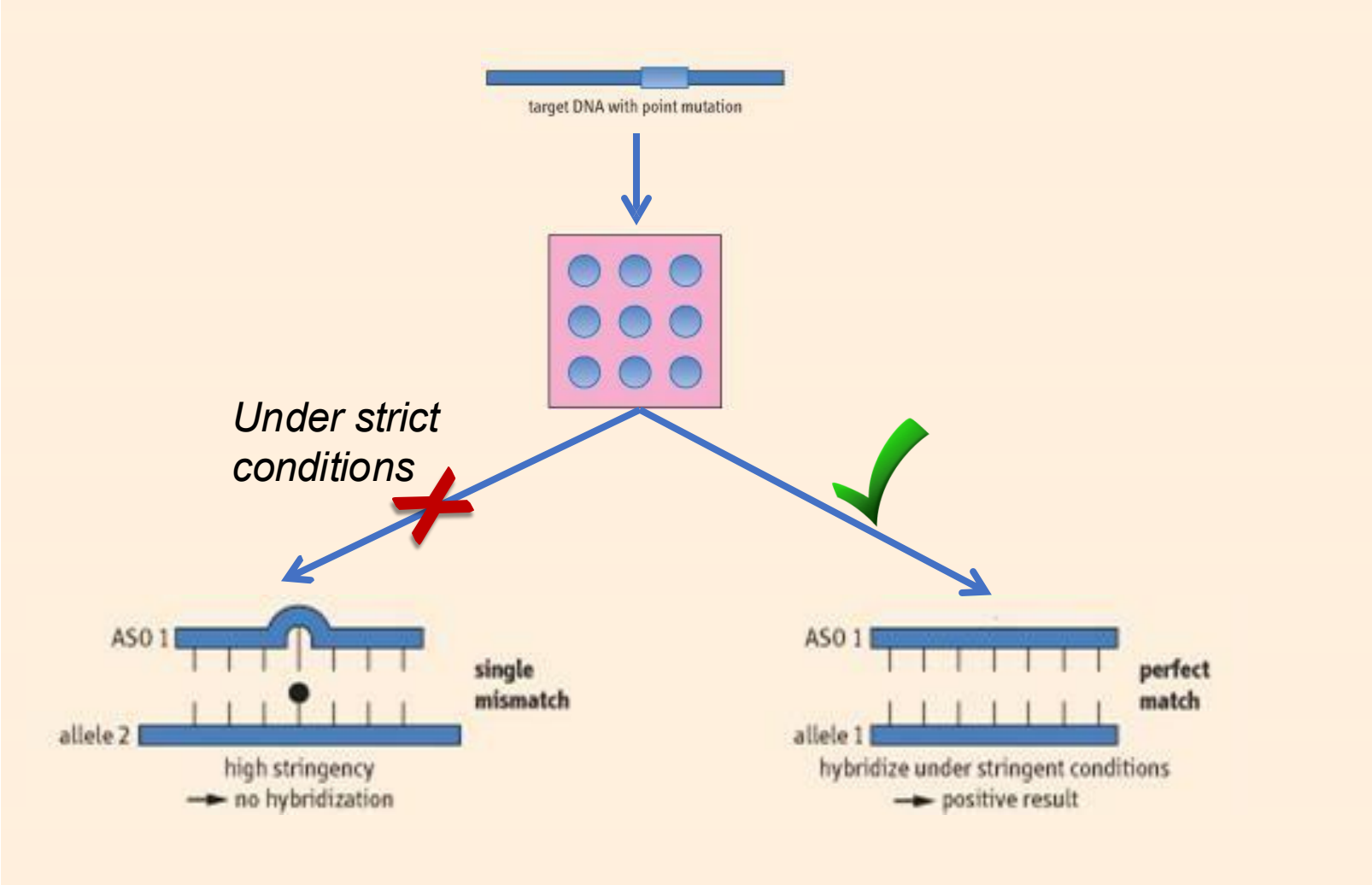
- A probes is a short sequence (almost **20 nucleotide long** (not 20 BP)) of single stranded DNA (an oligonucleotide) that is complementary to a small part of a large DNA sequence.
- Hybridization reactions use labeled DNA probes to detect larger DNA fragments.

✓ This can happen because the probe will pair with the portion of the DNA strand (by binding in an anti-parallel fashion) that is complementary to the probe (even if the probe is RNA, uracil can pair with adenine just like thymine does); a carefully designed probe must be used such that it is complementary to the segment of interest in the DNA.

✓ Labeled meaning the probe itself will emit a signal; when it binds, this signal (radioactive or fluorescent) is detected by a by certain instruments.

Interesting note

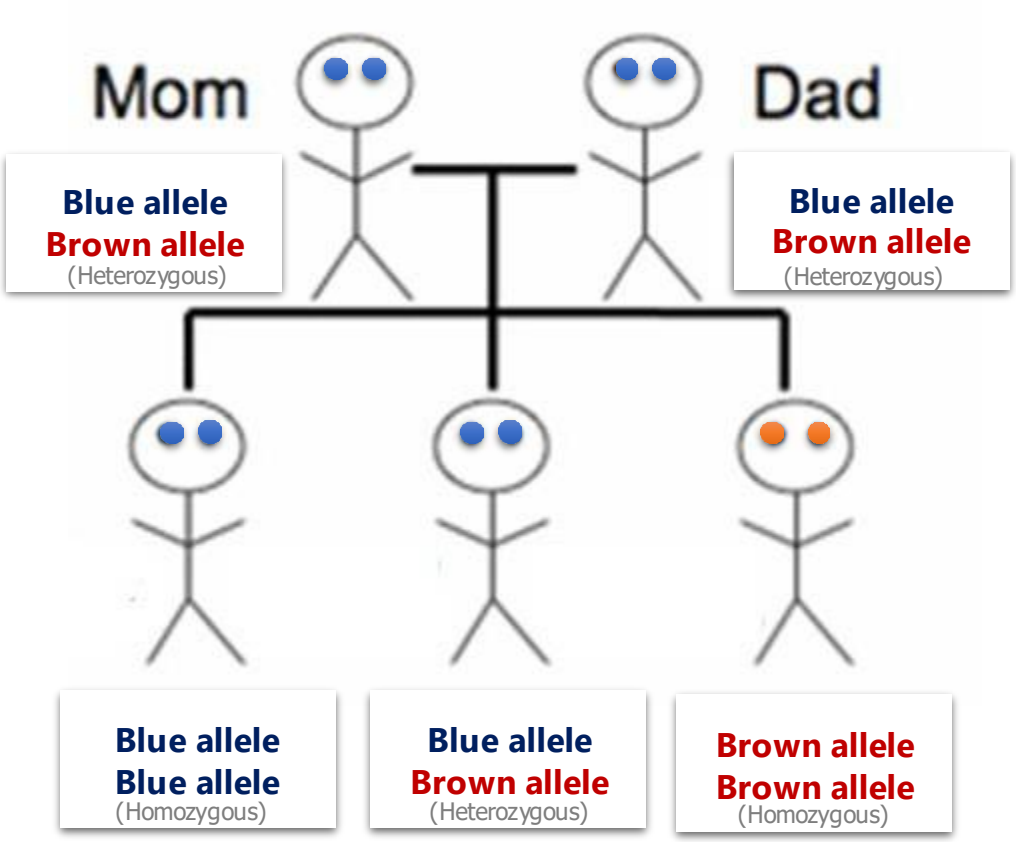
- A combination of a strand of a DNA and a strand of an RNA is possible, IF THEY ARE COMPLEMENTARY FOR EACH OTHER (OBVIOUSLY “U” RATHER THAN “T” FOR RNA)



However, also imperfect probe hybridization can occur (logically it is less common because the short nature of probes mean that they bind with less H-bonds) if enough pairing strength is present to stabilize this interaction.

❑ Concepts to know…

➢ Pedigree (the family tree below)

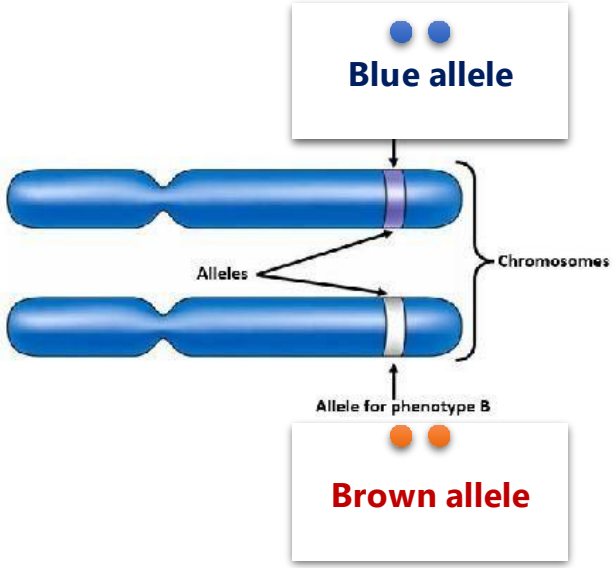


- ❖ All humans have the same genes responsible for eye color (HYPOTHETICAL BTW), but they may carry different alleles for these genes (e.g., some people have an allele for blue eyes, while others have an allele for brown eyes).
  One of the two alleles can be dominant over the other, meaning it determines the visible characteristic, or phenotype, of eye color.

- ❖ Suppose there's a gene for eye color;
  - ➢ The mother has one blue allele on one chromosome and a brown allele on the other chromosome, making her heterozygous for the eye color gene.
  - ➢ The child with two blue alleles is homozygous for the blue allele for the eye color gene.
  - ➢ The child with two brown alleles is homozygous for the brown allele for the eye color gene.

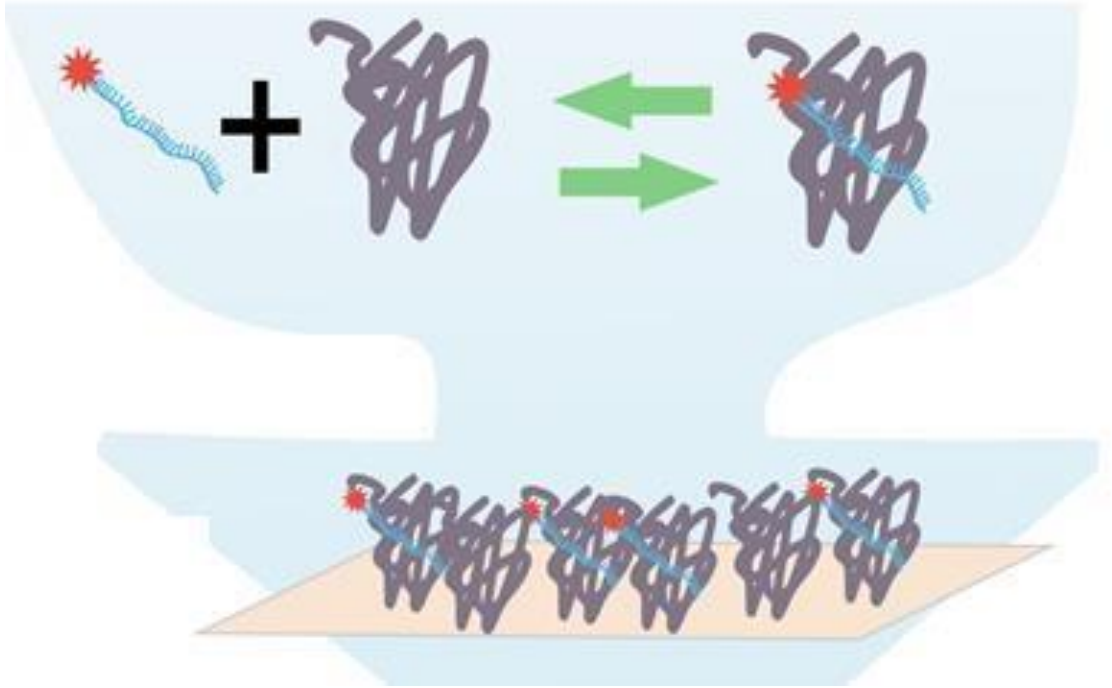
❑ Concepts to know…

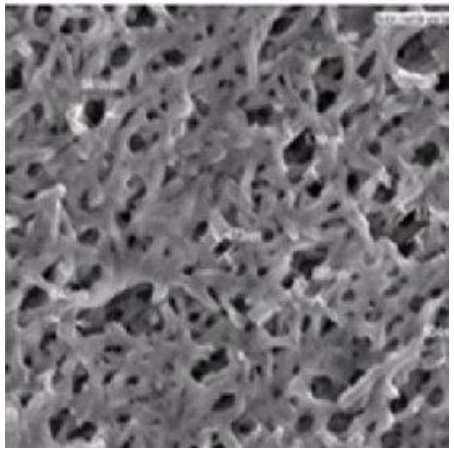
- **Alleles:** a specific variant or form of a gene
  Dominant (سائد) vs. Recessive (متنحي)
  Homozygous vs. Heterozygous



❑ Dot blot (first technique)

(blot = stain)





- **This is a technique that informs us if a specific sequence that is complementary to a probe of a known sequence exists in a larger DNA.**
- **DNA is bound to a solid support, and a labeled probe is added. If binding occurs, the sequence exists.**

❖ When we take a sample from someone, such as a blood sample, and isolate, purify, or extract the DNA (all these terms mean the same thing), We don't just isolate a single DNA molecule, instead, we isolate DNA from millions of cells, in other words we are extracting millions of DNA molecules in one sample. For simplicity, however, we represent this with just one DNA molecule.

❖ If we add a probe to this DNA molecule and the probe is complementary to any part of the DNA, it will bind to it, (hybridize to it). This causes that specific part of the DNA to light up, not the entire molecule – only the section where the probe is bound.

❖ Think of it like a person wearing a hat with a light on it. In a dark room, you see the light moving, but not the person directly. Yet, you know the person is there because of the lighted hat. Similarly, when a probe binds to a DNA molecule, it illuminates that specific part, creating a detectable signal.

❑ More explanation on the previous slide:

- We take a membrane and add DNA to it – millions of DNA molecules. The DNA attaches to the membrane. Next, we add a probe, and anything that isn't bound is washed away. If the probe binds to the DNA (hybridizes with it), that specific part will light up, indicating the presence of the probe. This tells us that a sequence complementary to the probe exists within the DNA.

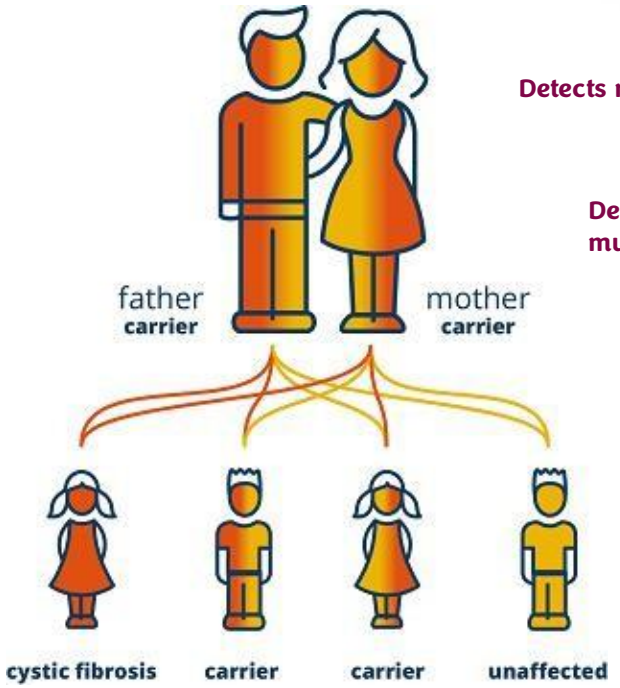
- This doesn't indicate the entire DNA sequence; but only shows that there is a specific nucleotide sequence in the DNA that is complementary to the probe.

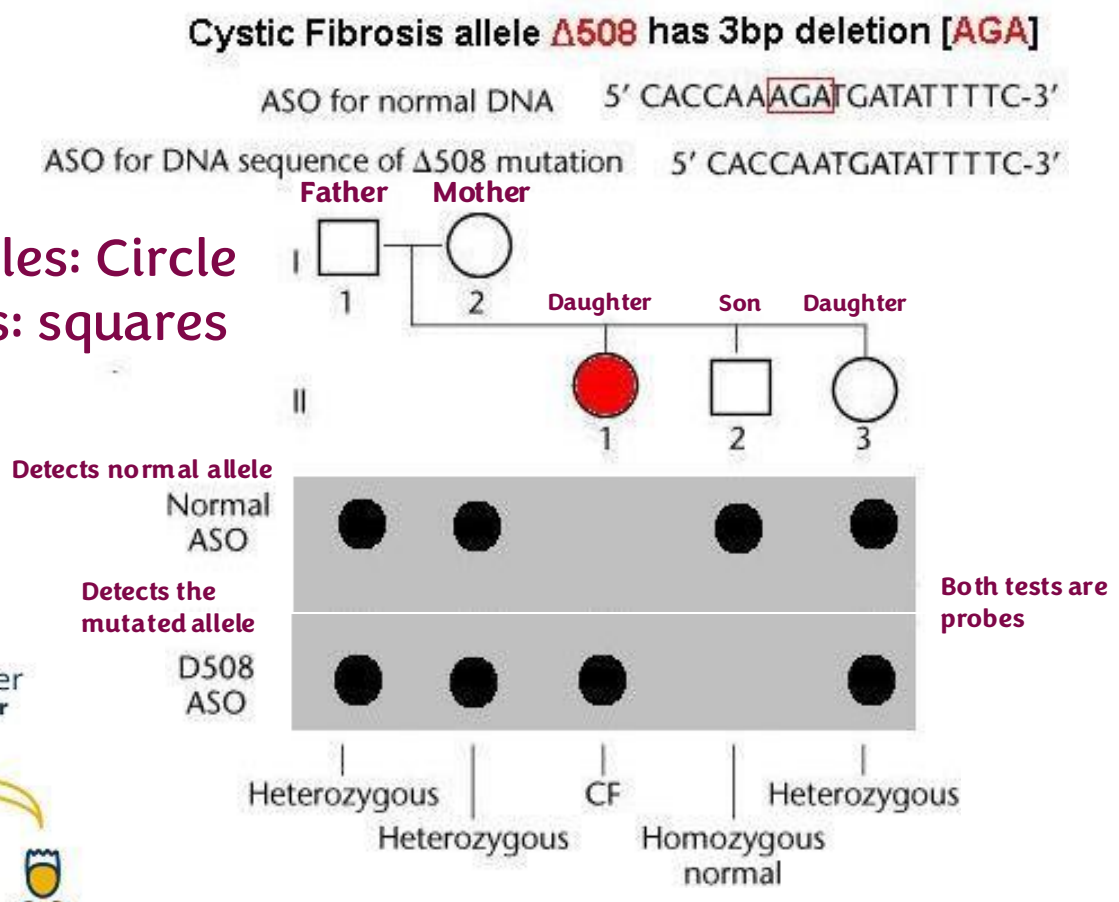
❑ Disease detection by ASO (Cystic fibrosis) (second technique)

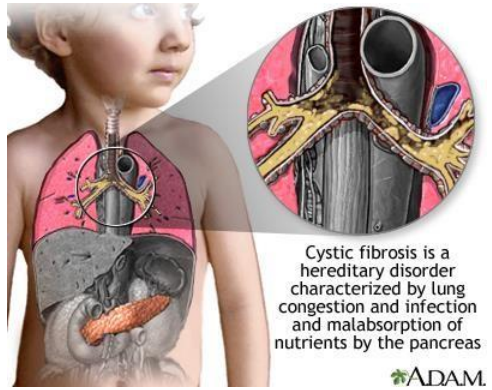
ASO: Allele-specific oligonucleotide

The whole genomic DNA is spotted on a solid support (a membrane) and hybridized with two ASO's, one at a time.

- Females: Circle
- Males: squares







- Diseased child from non-carrier (and not diseased of course) parents may mean the child is adopted.

The 5 columns above each represents 1of the 5 members of this family

- If a member has both tests positive they are heterozygous (carriers of cystic fibrosis)
- If a member has only one positive they are homozygous
  (they either have cystic fibrosis or completely free of the mutated allele)

❑ More explanation on the previous slide:

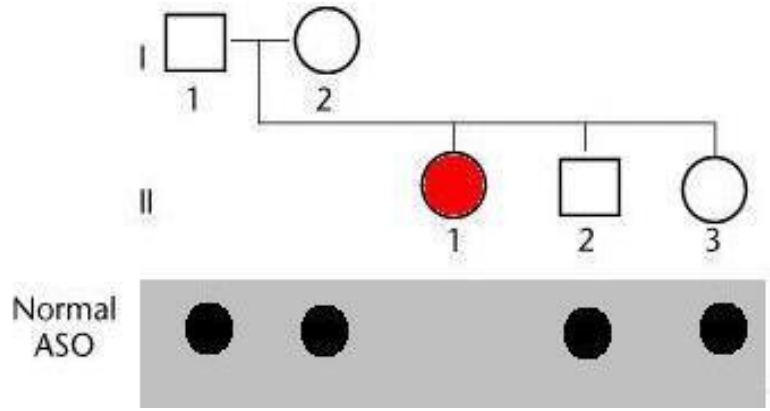
- Certain diseases can be detected , such as cystic fibrosis, using a dot blot technique. Cystic fibrosis is a genetic disorder that affects the lungs, making it difficult for affected individuals to breathe. The disease is primarily caused by a mutation, and to develop cystic fibrosis, the mutated gene must be on both chromosomes(homozygous for the mutated gene).
- In cystic fibrosis, the mutation is usually a deletion mutation. Normally, a person would have a specific DNA sequence, However, in individuals with cystic fibrosis, part of this sequence (A, G, A) is missing from both chromosomes. This deletion results in the individual being homozygous for the mutated allele. If a person has two normal alleles (no deletion), they are homozygous for the normal allele. If someone has one normal allele and one mutated allele, they are heterozygous.
- To detect this, two probes are used: one that binds to the normal allele and another that binds to the mutated allele (the allele with the deletion).
- We then take DNA samples from each family member. In the diagram, squares represent males, and circles represent females. The family members are arranged to show their relationships. We spot each individual's DNA on a membrane, labeling spots for the mother, father, and each child. Finally, we add the first probe that detects the normal allele.
- A similar approach is used for the mutated allele (using a different probe).

Cystic Fibrosis allele Δ508 has 3bp deletion [AGA]

ASO for normal DNA 5′ CACCAAAGATGATATTTTC-3′

ASO for DNA sequence of Δ508 mutation 5′ CACCAATGATATTTTC-3′

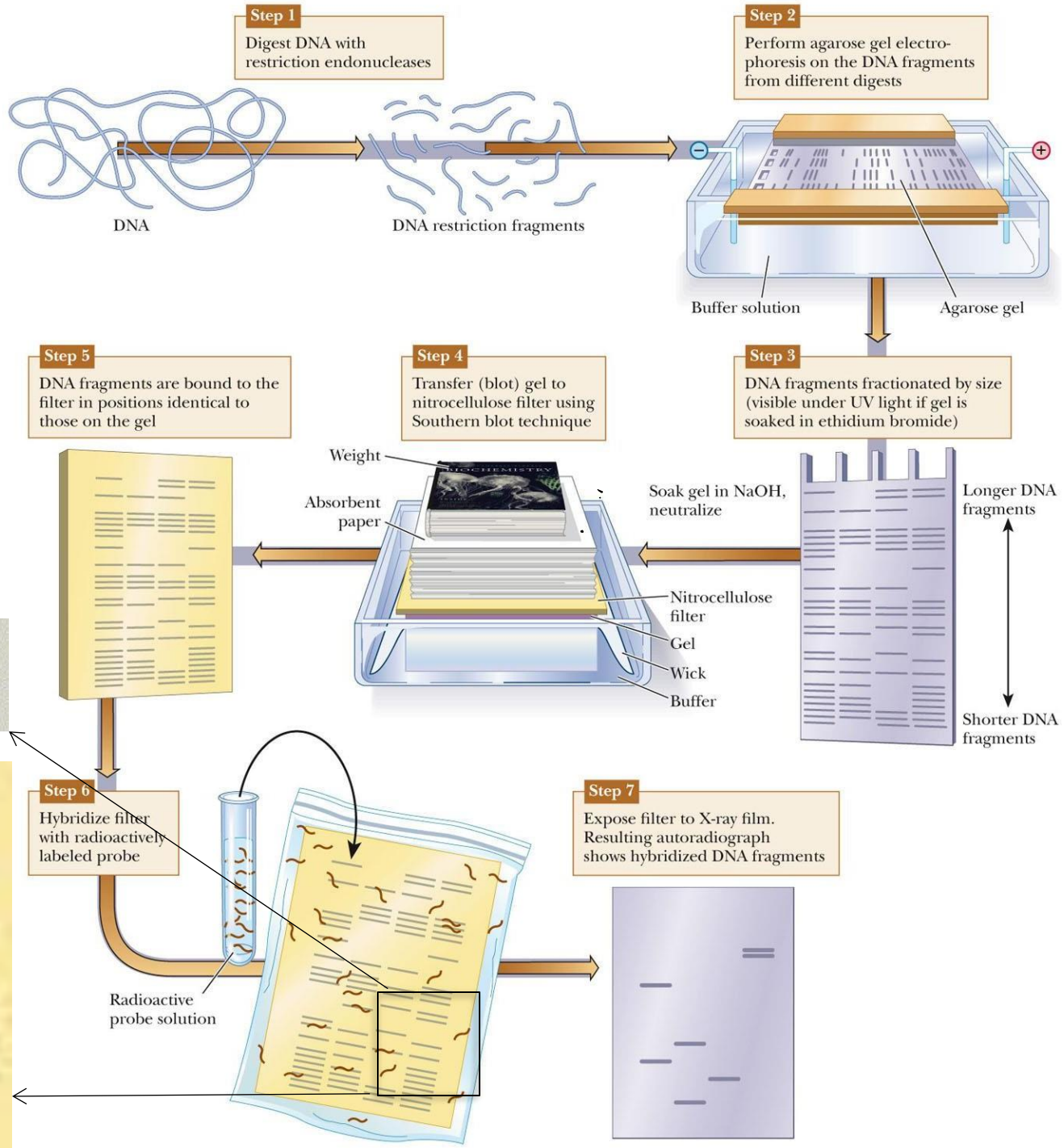
Some might ask whether the probe for the mutated allele could accidentally bind to the normal allele due to partial sequence similarity; To prevent this, we adjust factors like salt concentration and temperature to ensure that only perfect hybridization occurs.

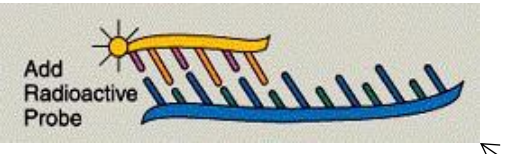


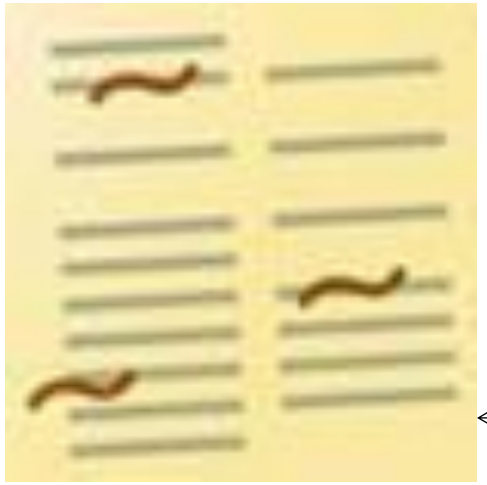
❑ Southern blotting (third technique)

- This technique is a combination of DNA gel electrophoresis and dot blotting

- Used to detect:
  - the presence of a DNA segment complementary to the probe
  - the size of the DNA fragment

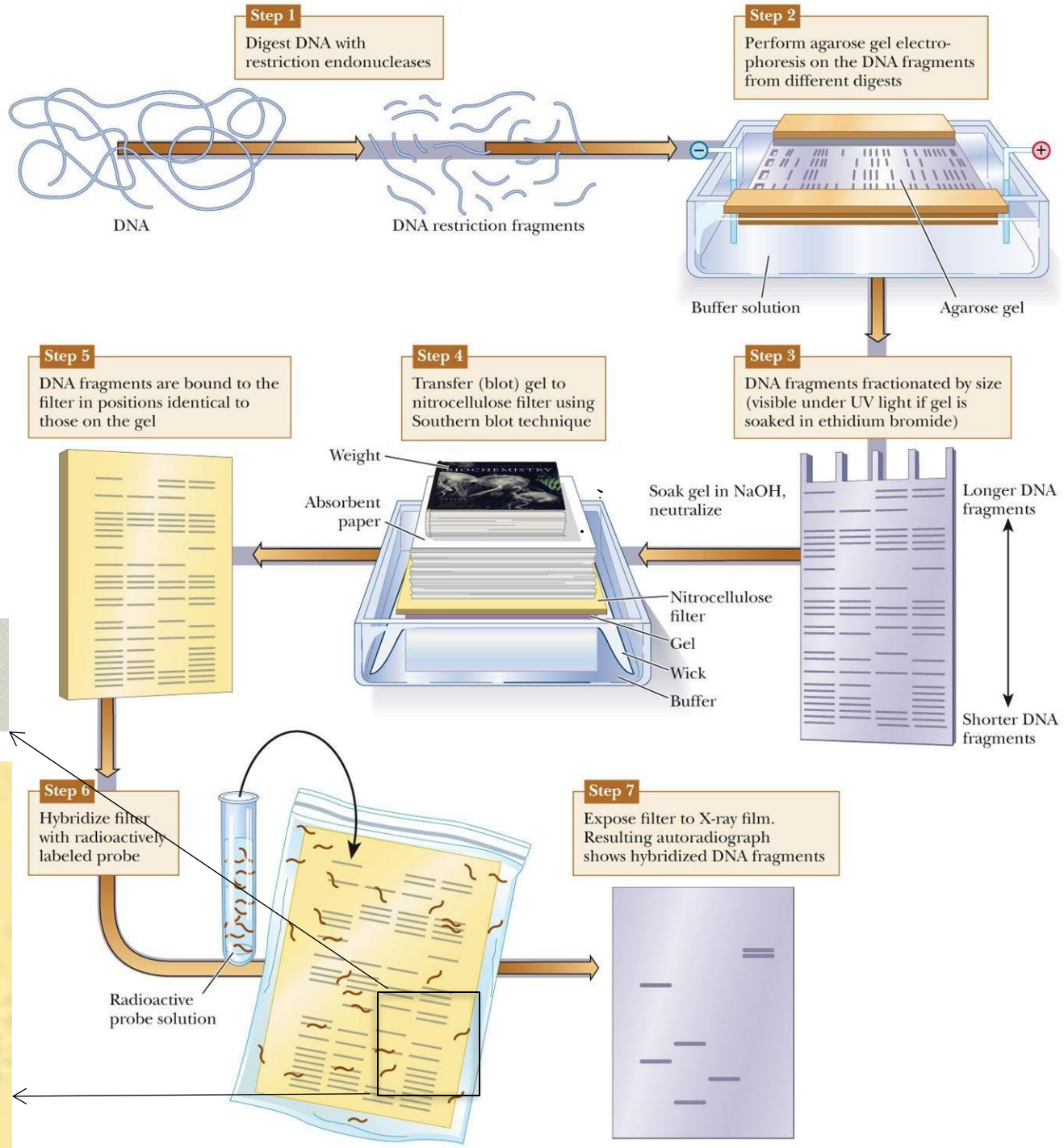


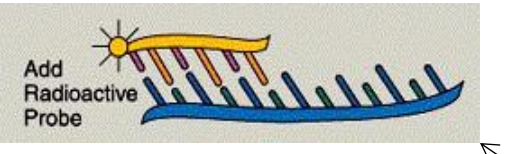


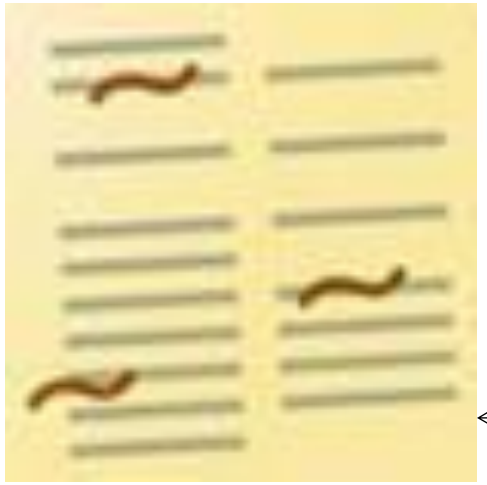


1. DNA is extracted from cells or organisms and cut into smaller fragments using restriction enzymes. These fragments are separated by size using gel electrophoresis, forming a pattern on the gel that serves as a replica of the DNA.
2. The DNA fragments are transferred from the gel to a membrane, creating an identical replica that is easier for probes to access.

Continued next page…





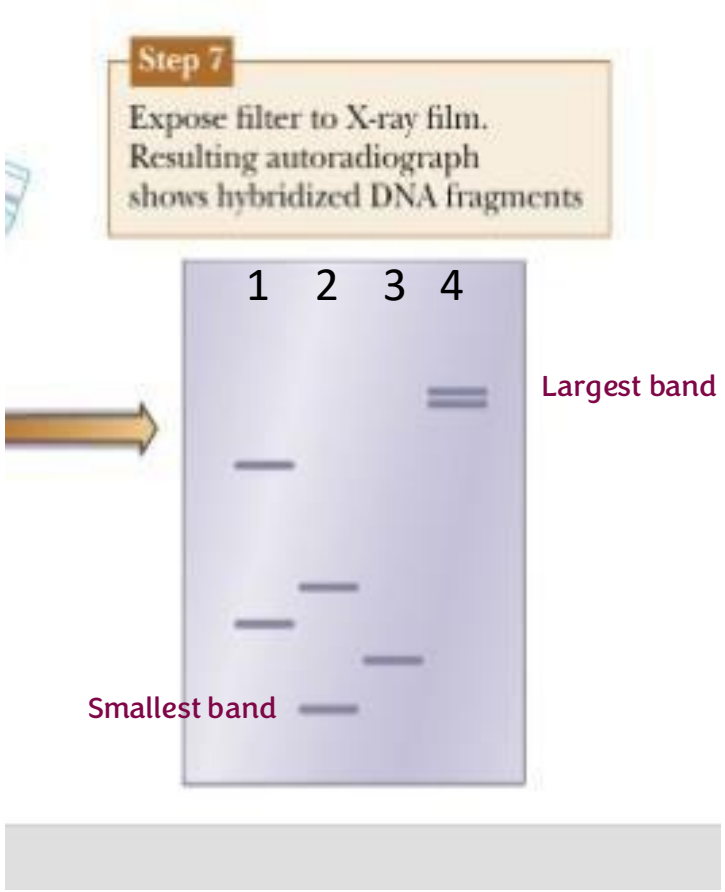


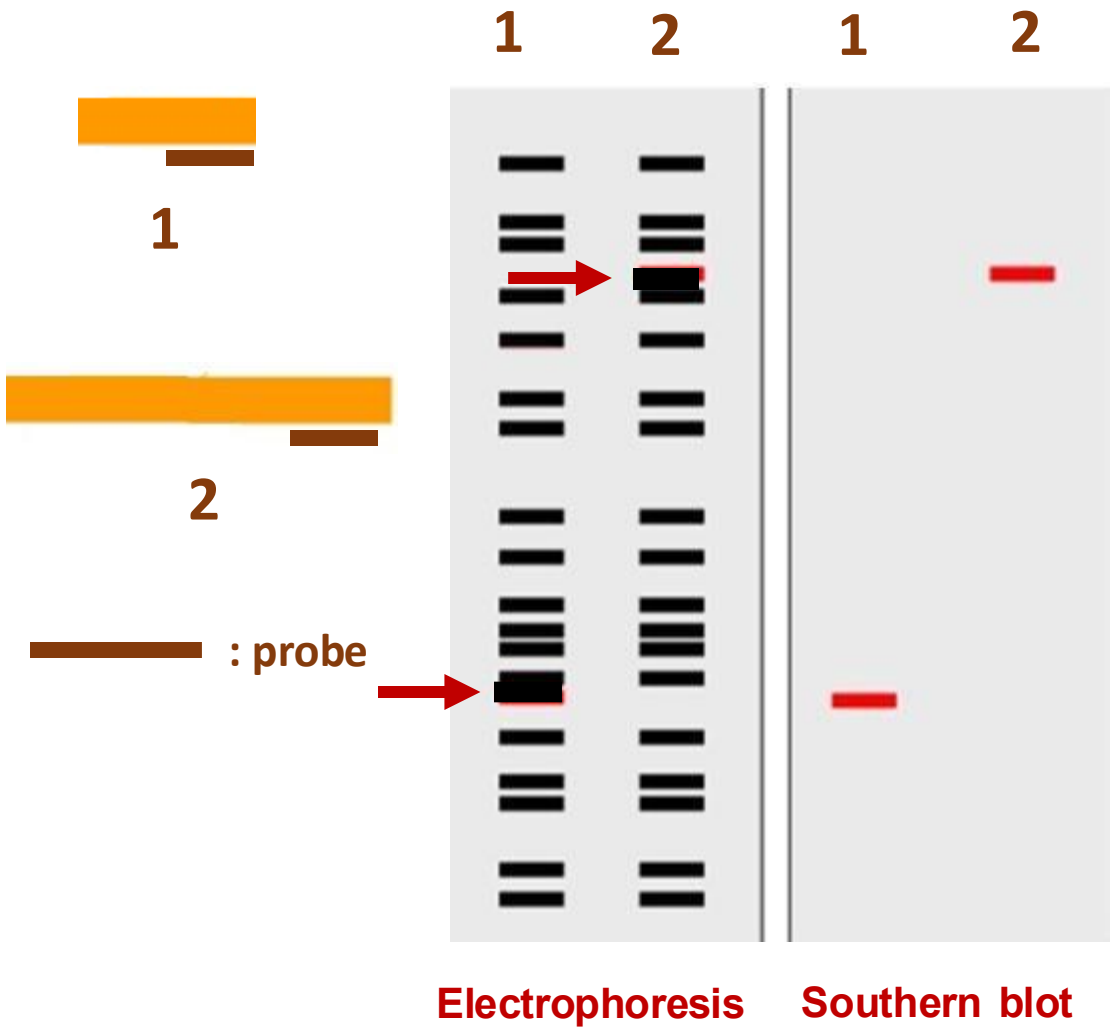
3. A labeled DNA probe is added to the membrane. Fragments containing sequences complementary to the probe will hybridize with it.
4. Unbound probes are washed out, leaving only the probe bound to complementary DNA. We then look for a signal to identify the hybridized fragments.

➢ Information Obtained

I. First, we determine whether the sample contains DNA complementary to the probe.
II. Second, we determine the size of the hybridized DNA fragment based on its position on the membrane.

- In the figure, there are four DNA samples. The information we can collect from this is the sizes of the DNA bands and whether these bands contain sequences complementary to the probe. The four samples are not identical, as the band patterns differ. Note: the sizes of the bands do not reflect the size of the probe, because the DNA fragments are separated before the probe is added. The probe is applied to the membrane, not the gel, because it is easier for the probe to access the DNA fragments. This is why the DNA is first transferred from the gel to the membrane.





- ✓ Gel electrophoresis will be performed for two samples, resulting in multiple DNA fragments appearing as bands. The fragments will then be transferred to a membrane, and a probe will be added to bind to specific bands.

- ✓ In Sample 1, the probe will bind to a smaller DNA fragment, while in Sample 2, it will bind to a larger fragment. The probe will bind to the same target site in both samples, but the difference in fragment size will be observed.

Resources

- http://www.sumanasinc.com/webcontent/animations/content/gelelectrophoresis.html

- Watch this….very important

رسالة من الفريق العلمي:

For any feedback, scan the code or click on it.

Corrections from previous versions:

Versions	Slide # and Place of Error	Before Correction	After Correction
V0 → V1	Slides **2-5** were added		
V1 → V2			