Chromatography

Electrophoresis Technique

E.Learning By

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Michael Tswett , Russian botanist (1872–1919), inventor of chromatography

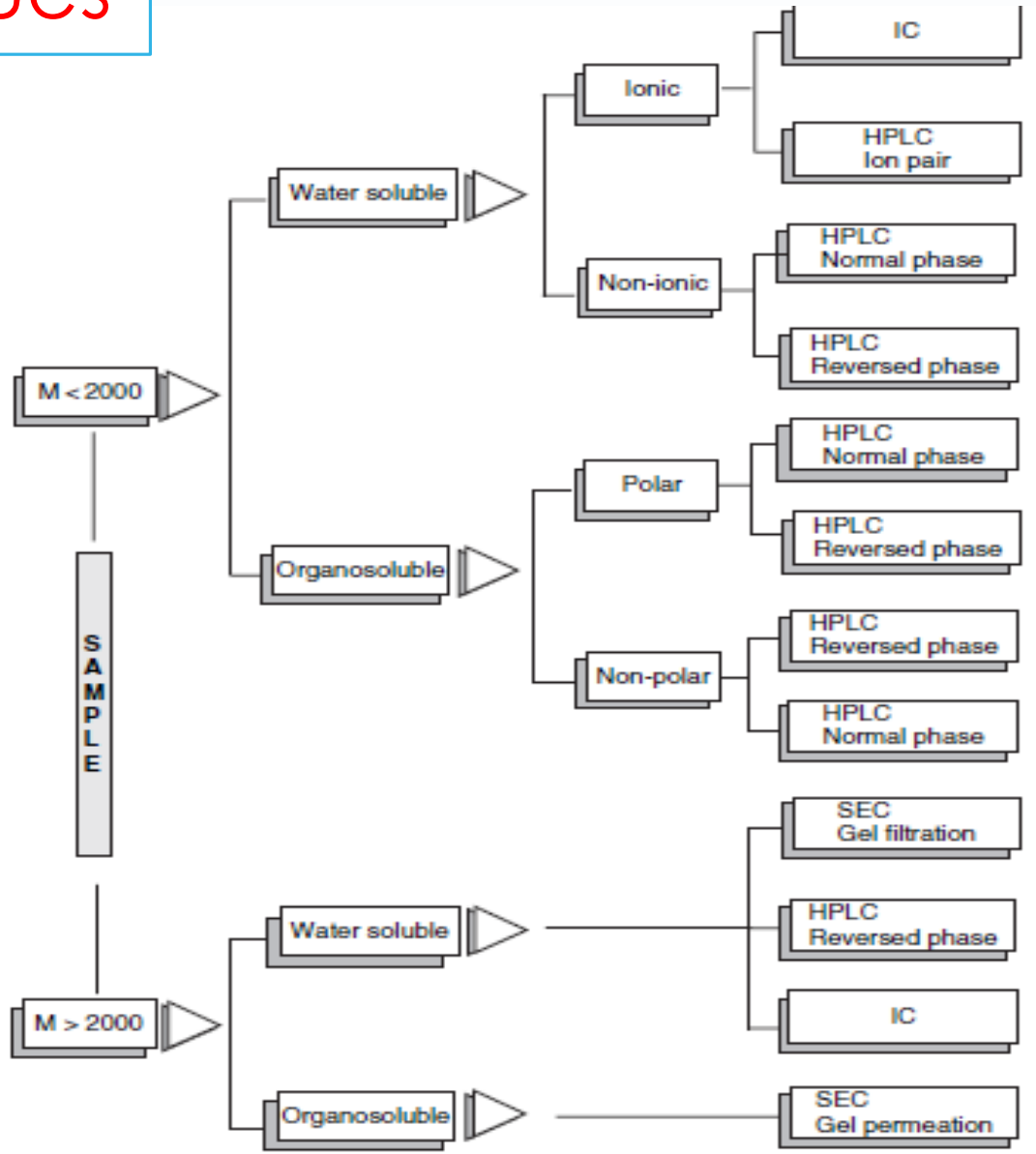
- In 1901, invented adsorption chromatography during his research on **plant pigments**. He separated different colored **chlorophyll and carotenoid pigments** of leaves by passing an extract of the leaves through a column of calcium carbonate, alumina, and sucrose, eluting them with **petroleum ether/ethanol** mixtures
- The International Union of Pure and Applied Chemistry (IUPAC) has drafted a recommended definition of chromatography:

Is a physical method of separation in which the components to be separated are distributed between two phases, one of which is (stationary phase), while the other (**the mobile phase**) moves in a definite direction .

- The stationary phase is usually in a column, but may take other forms, such as a planar phase (flat sheet).

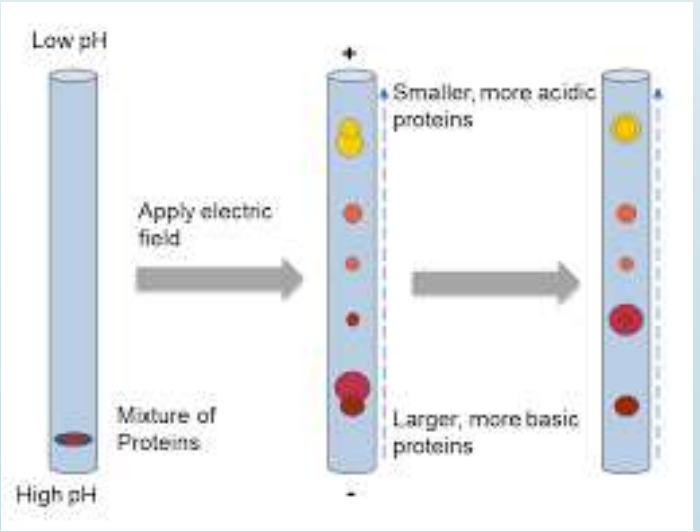
Chromatographic techniques

- This diagram represent Selection guide for all of the different chromatographic techniques with liquid mobile phases.
- **The choice of technique is chosen as a function of the molar mass, solubility and the polarity of the compounds to be separated.**



Introduction:

- Electrophoretic techniques are generally used for separation of charged analytes. Charged analytes move in electrolyte solutions when an **electrical field is established.**
- Separation is obtained if the charged analytes have different migration velocity. The electrolyte solution is most commonly a mixture of weak acids and bases in water.
- Electrophoretic techniques are widely used in ***Biochemistry***, especially for separation of nucleotides and proteins.



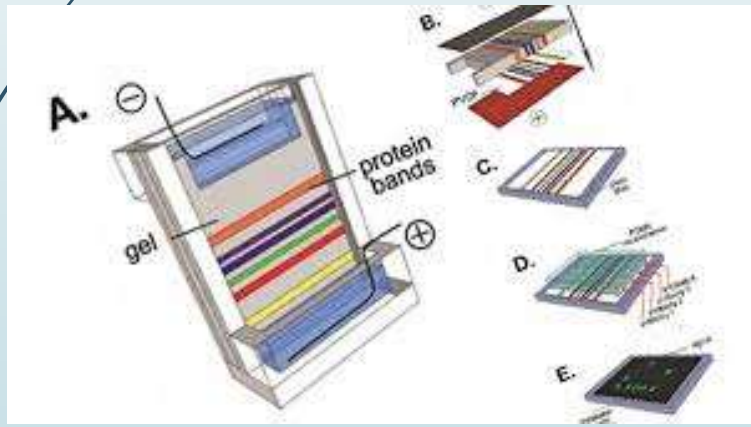
- The electrophoretic techniques can be divided into three main groups:

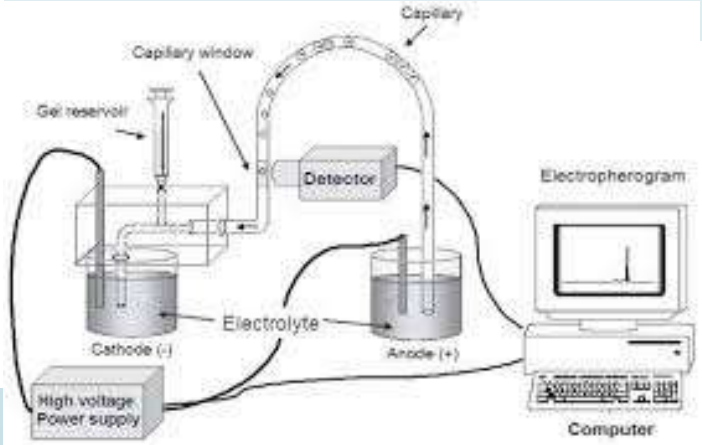
  a. the traditional gel electrophoretic techniques.

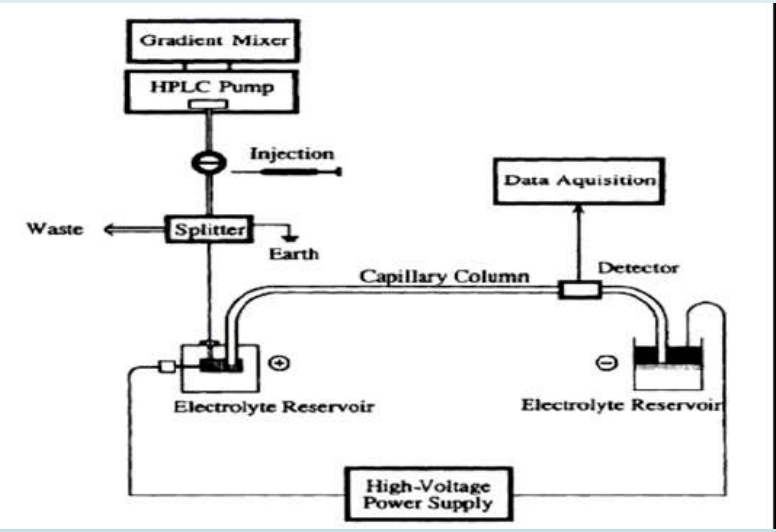
  b. capillary electrophoresis (CE).

  c. potential-driven chromatography (electrochromatography) techniques.

- Again , The term electrophoresis is used for the process where charged species **(ions)** in an **electrolyte solution** move under the influence of an **electric field.**







Main Principle

- Electrophoretic separation is obtained if the charged analytes have different migration velocity u.
- An ion with the charge q will be subjected to a force F=q.E in an electric field, with field strength **E=V/L**, where **V** is the potential applied and **L** is the distance between the anode and the cathode ,see this figure 1 below.

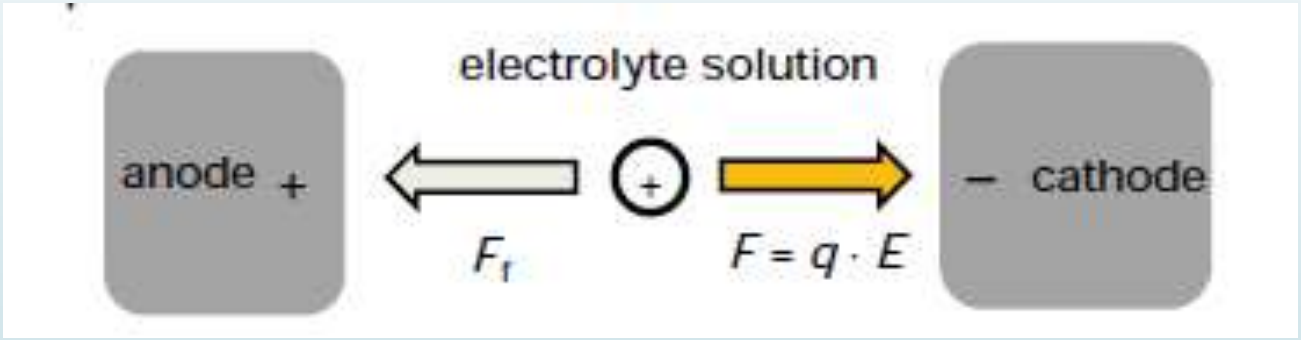
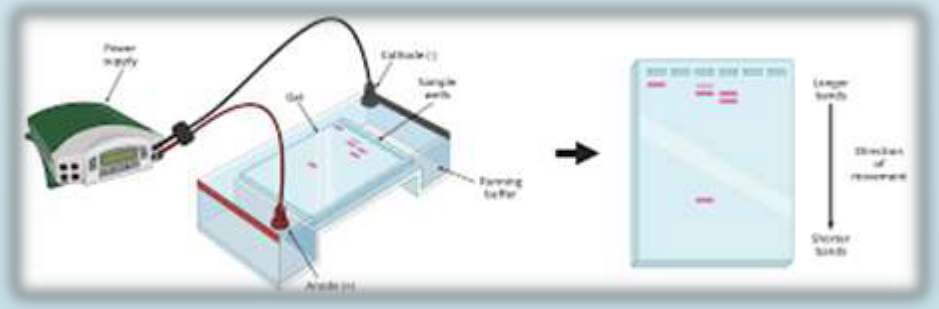


Figure: 1. Forces acting on a charged species in an applied electric field.

- Due to the force, the ion will be accelerated for a very short time until the **frictional force** from the electrolyte **solution Ff** is equal to F. When F_f=F, the ion will move (migrate) at a constant velocity in the electrolyte solution.

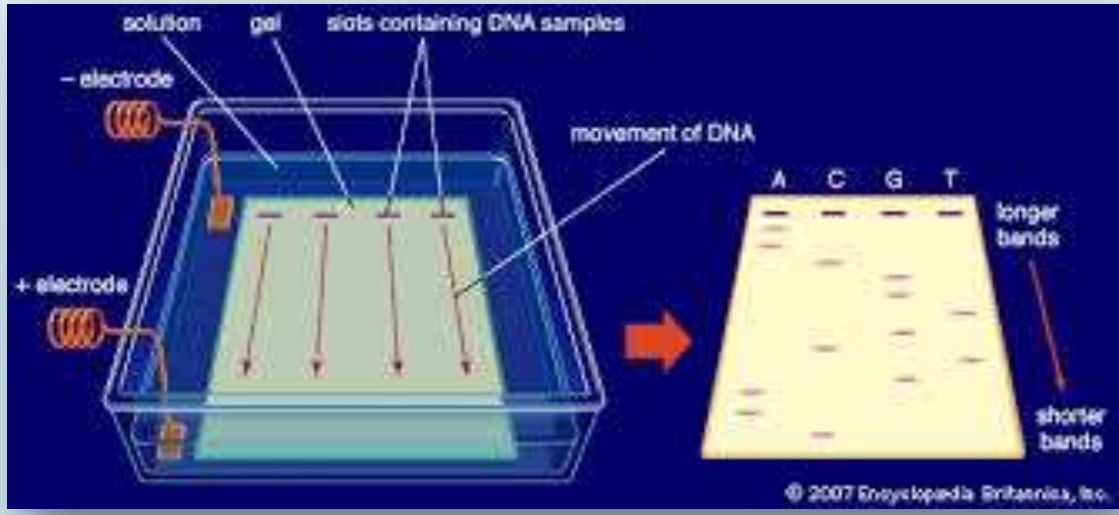
- The frictional force F_f is given by **Stokes' law =F_f = 6πηru**
- Where η is the viscosity of the electrolyte solution and r is the radius of the ion.
- When **F=Ff,** the migration **speed u** can be found by:
- **U =qE/ 6 π r η , This equation shows that the migration velocity u**
- increases with increasing ion charge **q,**
- decreases with increasing ion radius **r,**
- increases with increasing field strength **E,** and
- decreases with increasing temperature, since the viscosity **η** decreases



Gel Electrophoresis Techniques

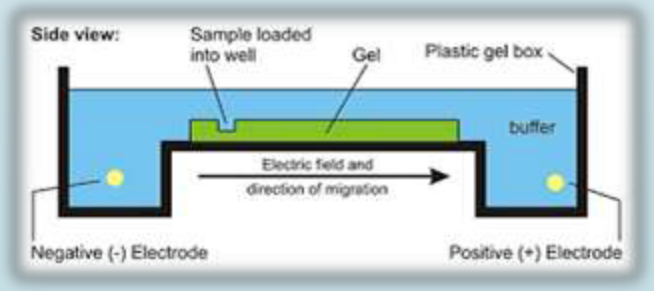
- In electrophoretic separation techniques, as opposed to the chromatographic techniques, there is no mobile phase or stationary phase.
- In zone electrophoresis (a kinetic process), the migration length (L=u.t) is proportional to the applied voltage (υ= μ E) and time, and separation of analytes with different charge/size ratio **(q/r**) is obtained.

  μ =electrophoretic mobility
- Electrophoretic separation can also be carried out using techniques **isoelectric focusing (IEF)** (equilibrium process).
- Zone electrophoresis and isoelectric focusing are the techniques mostly used for analytical separations.



So ..What is gel electrophoresis?

- Gel electrophoresis is a technique commonly used in laboratories to separate charged molecules like DNA, RNA and proteins according to their size.
- Charged molecules move through a gel when an electric current is passed across it.
- An electric current is applied across the gel so that one end of the gel has a positive charge and the other end has a negative charge .
- The movement of charged molecules is called migration.
- Molecules migrate towards the opposite charge. A molecule with a negative charge will therefore be pulled towards the positive end vise versa .

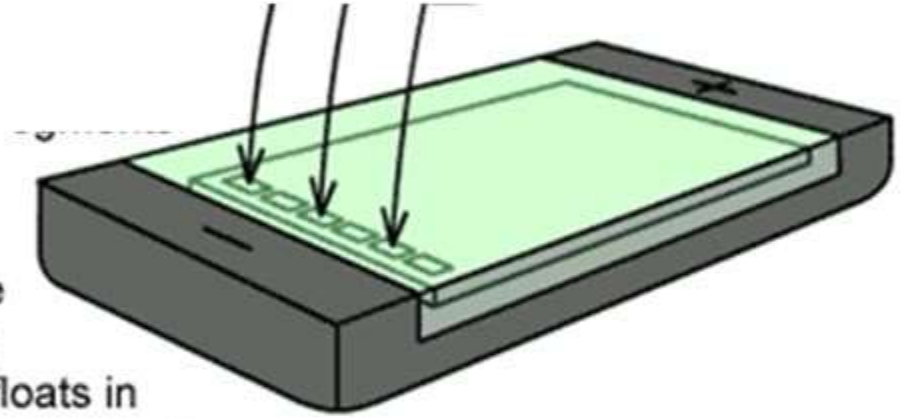


- The gel consists of a permeable matrix, a bit like a sieve, through which molecules can travel when an electric current is passed across it.
- Smaller molecules migrate through the gel more quickly and therefore travel further than **larger fragments** that migrate **more slowly** and therefore will travel a shorter distance. As a result the molecules are separated by size.

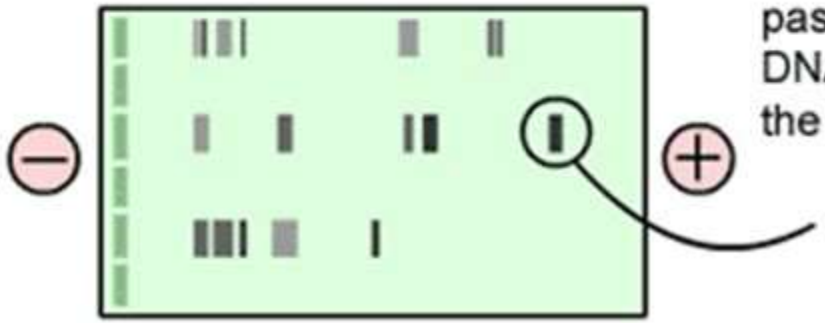
How can Gel Electrophoresis Separate DNA , RNA or even Proteins ?

- Electrophoresis can be use to distinguish DNA fragments of different lengths.
- DNA is negatively charged, therefore, when an electric current is applied to the gel, DNA will migrate towards the positively charged electrode.
- See the figure in the next slide :

DNA (or RNA) samples loaded into wells



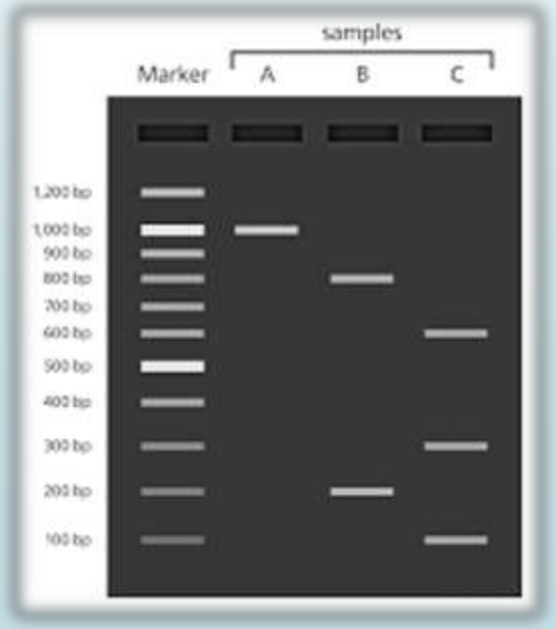
2. DNA segments are loaded into wells in a porous gel. The gel floats in a buffer solution within a chamber between two electrodes.



3. When an electric current is passed through the chamber, DNA fragments move toward the positively-charged cathode.

4. Smaller DNA segments move faster and farther than larger DNA segments.

- Shorter strands of DNA move more quickly through the gel than longer strands resulting in the fragments being arranged in order of size.
- The use of dyes, fluorescent tags or radioactive labels enables the DNA on the gel to be seen after they have been separated. They will appear as bands on the gel.
- A DNA marker with fragments of known lengths is usually run through the gel at the same time as the samples (ladder).
- By comparing the bands of the DNA samples with those from the DNA marker, you can work out the approximate length of the DNA fragments in the samples.



How is gel electrophoresis carried out? The procedure

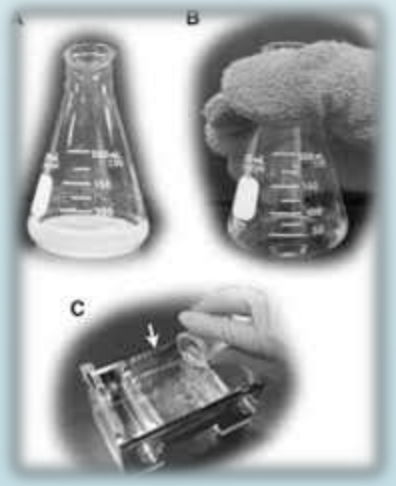
- Preparing the gel
- Agarose gels are typically used to visualize fragments of **DNA.**
- The concentration of agarose used to make the gel depends on the size of the DNA fragments you are working with.
- The higher the agarose concentration, the denser the matrix and vice versa.
- Smaller fragments of DNA are separated on higher concentrations of agarose whilst **larger molecules require a lower concentration of agarose.**

Agarose Gels

- Agarose (a natural linear polysaccharide derived from agar) powder dissolves in boiling water, forming a gel when cooled to 35–43 °C.
- A concentration of about **1% agarose** is commonly used.
- The agarose gels have generally much larger pore size and lesser mechanical strength than the polyacrylamide gels.
- The pore size of the agarose gels can easily be varied.

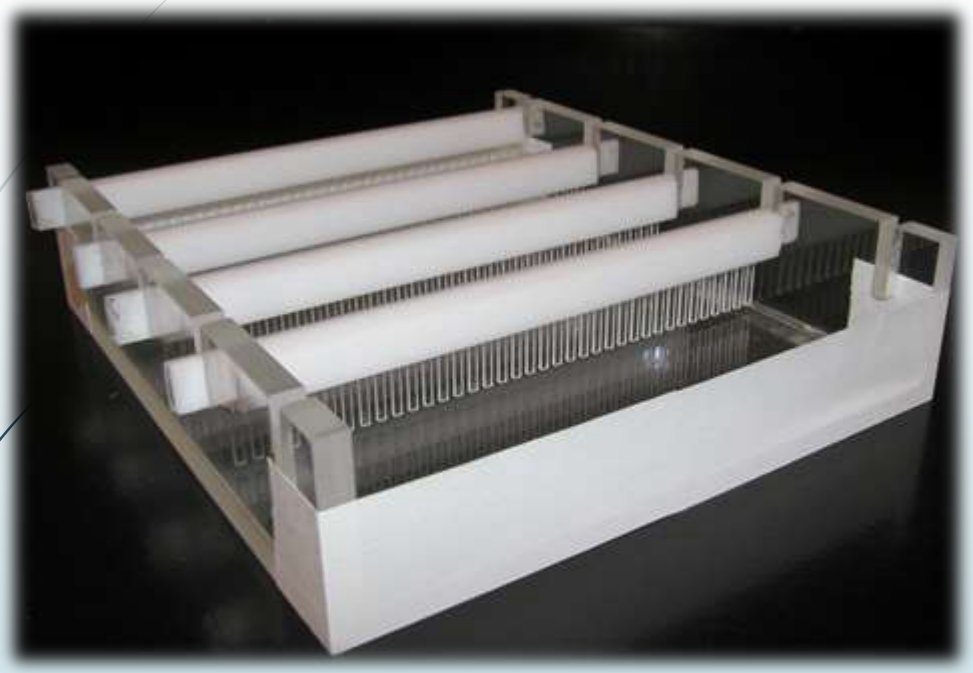
1.To make a gel

- agarose powder is mixed with an electrophoresis buffer and heated to a high temperature until all of the agarose powder has melted.
- The molten gel is then poured into a gel casting tray and a "comb" is placed at one end to make wells for the sample to be pipetted into.
- Once the gel has cooled and solidified (it will now be opaque rather than clear) the comb is removed.
- Many people now use pre-made gels.
- The gel is then placed into an electrophoresis tank and electrophoresis buffer is poured into the tank until the surface of the gel is covered.
- The buffer conducts the electric current. The type of buffer used depends on the approximate size of the **DNA fragments** in the sample.

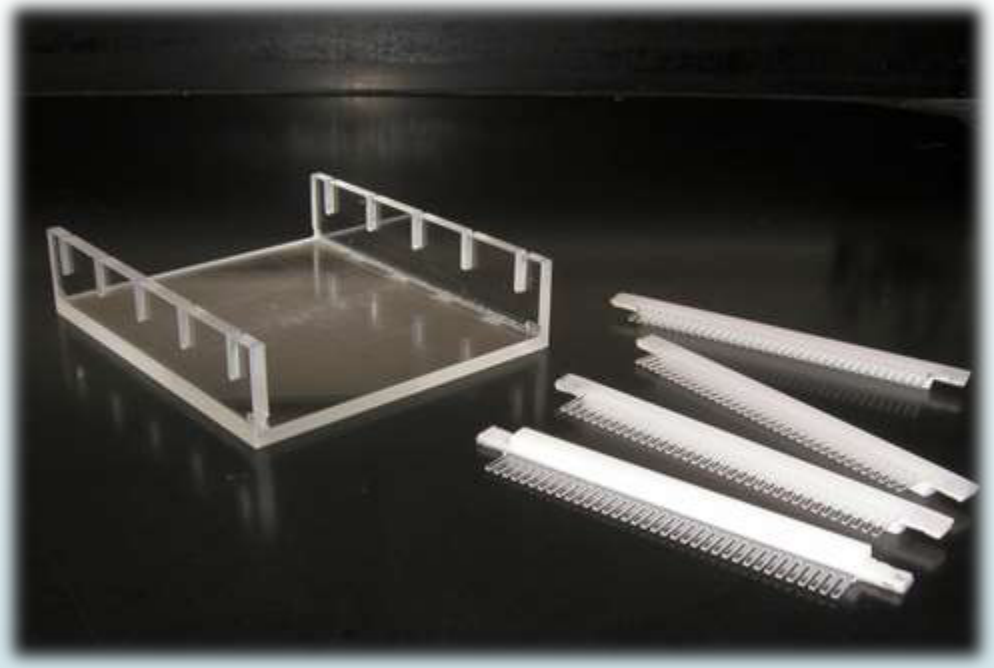


Electrophoresis Equipment

Preparing the Casting Tray



Gel casting tray & combs



Seal the edges of the casting tray and put in the combs. Place the casting tray on a level surface. None of the gel combs should be touching the surface of the casting tray.

Agarose

Buffer Solution

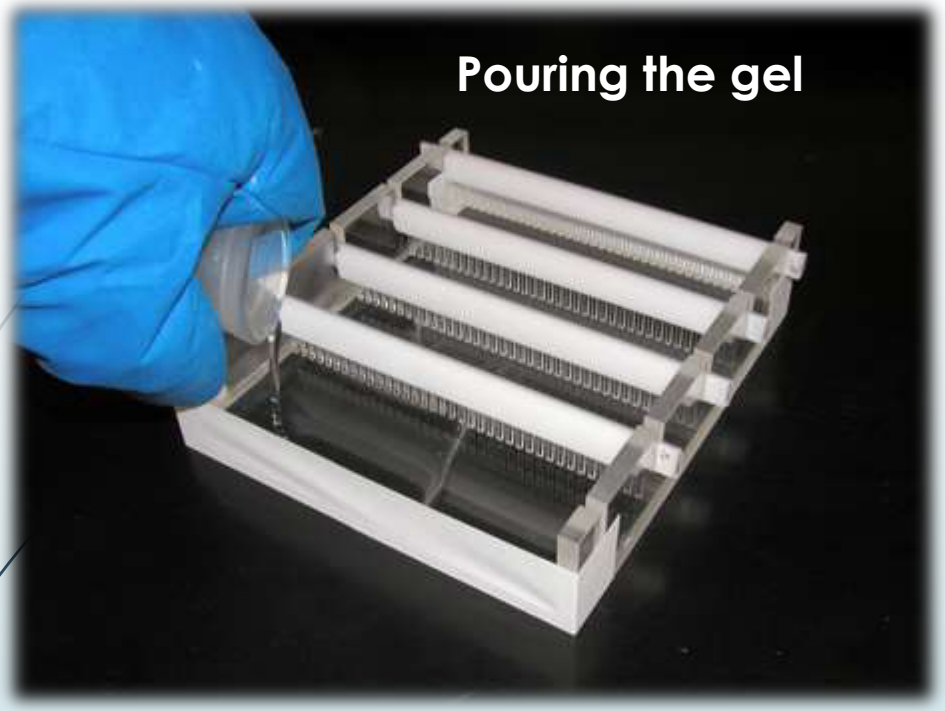
Combine the agarose powder and buffer solution. Use a flask that is several times larger than the volume of buffer.

Melting the Agarose

Agarose is insoluble at room temperature (left). The agarose solution is boiled until clear (right).

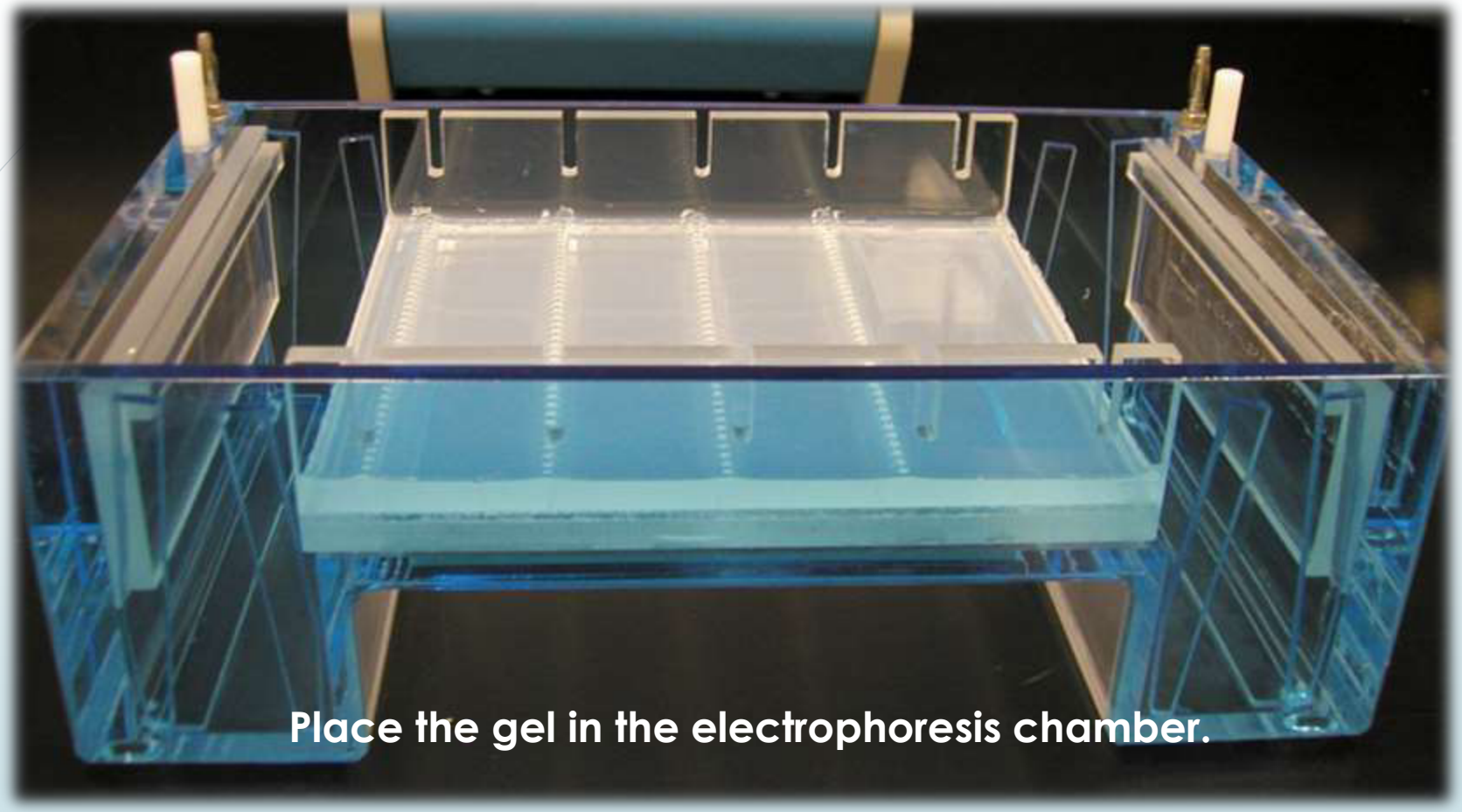
Note : Gently swirl the solution periodically when heating to allow all the grains of agarose to dissolve.

***Be careful when boiling - the agarose solution may become superheated and may hurt your hand if it has been heated too long in a microwave oven.

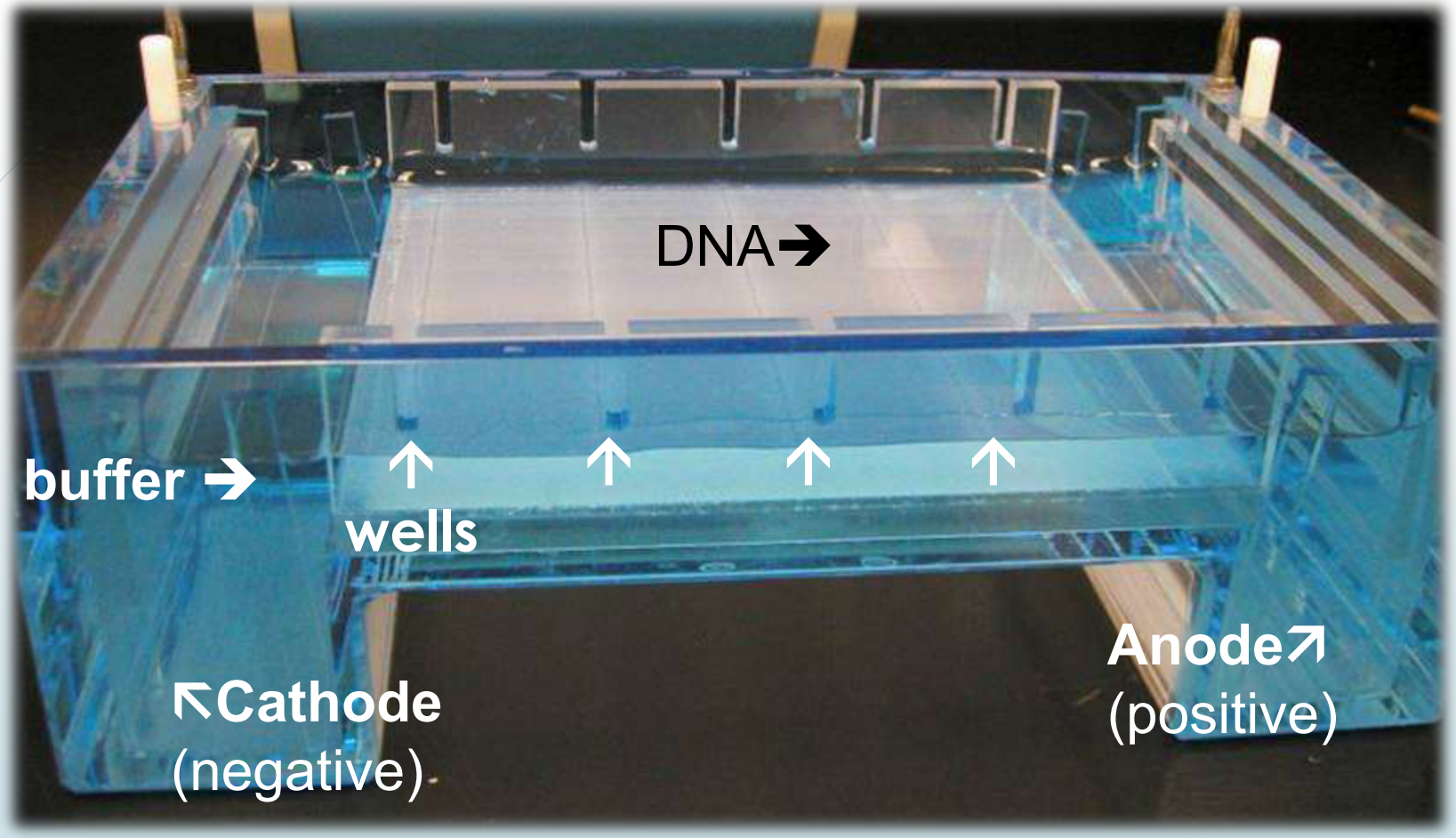


Allow the agarose solution to cool slightly (~60°C) and then carefully pour the melted agarose solution into the casting tray. Avoid air bubbles.

When cooled, the agarose polymerizes, forming a flexible gel. It should appear lighter in color when completely cooled (30-45 minutes). Carefully remove the combs and tape.



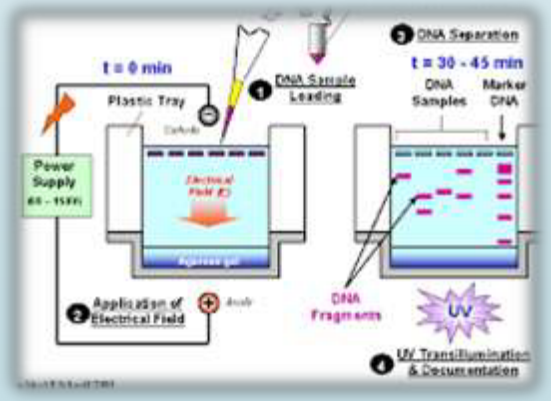
Place the gel in the electrophoresis chamber.

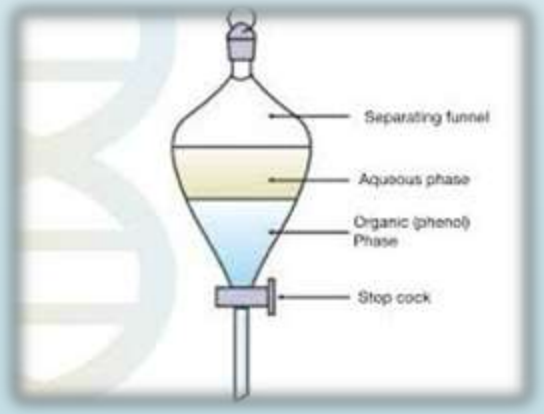


Add enough electrophoresis buffer to cover the gel to a depth of at least 1 mm. Make sure each well is filled with buffer.

2.Preparing the DNA for electrophoresis

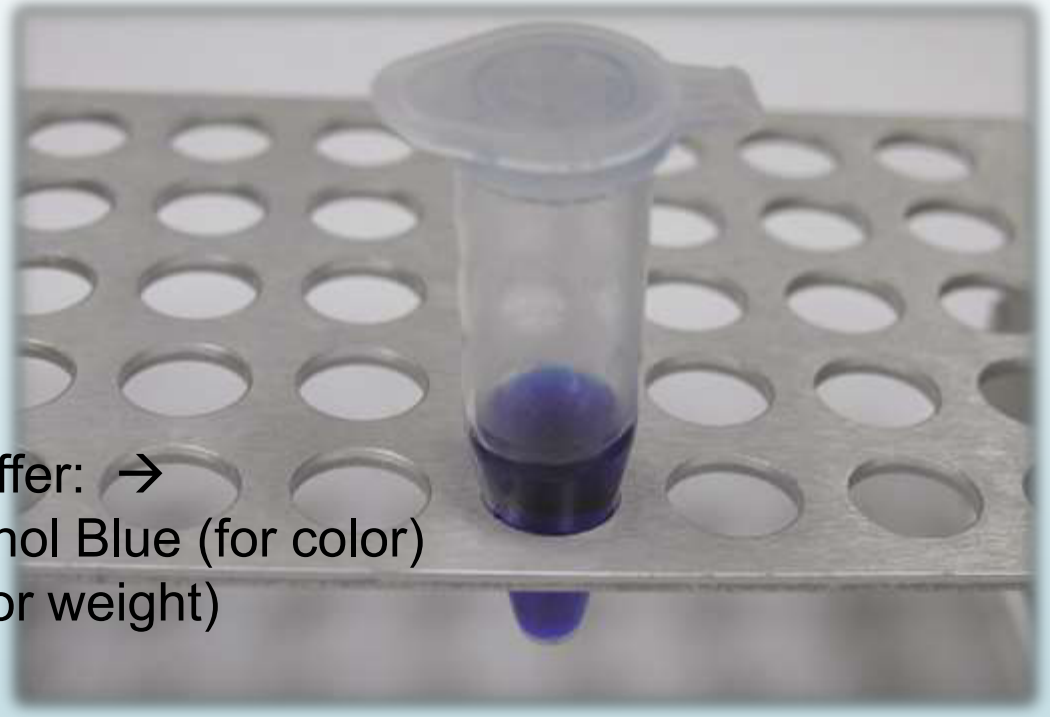
- A dye is added to the sample of **DNA** prior to electrophoresis to increase the viscosity of the sample which will prevent it from floating out of the wells and so that the migration of the sample through the gel can be seen.
- A DNA marker (also known as a size standard or **a DNA ladder**) is loaded into the first well of the gel. The fragments in the marker are of a known length so can be used to help approximate the size of the fragments in the samples.
- The prepared DNA samples are then pipetted into the remaining wells of the gel.
- When this is done the lid is placed on the electrophoresis tank making sure that the orientation of the gel and positive and negative electrodes is correct (we want the DNA to migrate across the gel to the positive end).





Sample Preparation

Mix the samples of **DNA** with the 6X sample loading buffer (w/ tracking dye). This allows the samples to be seen when loading onto the gel, and increases the density of the samples, causing them to sink into the gel wells.



6X Loading Buffer: →

- Bromophenol Blue (for color)
- Glycerol (for weight)

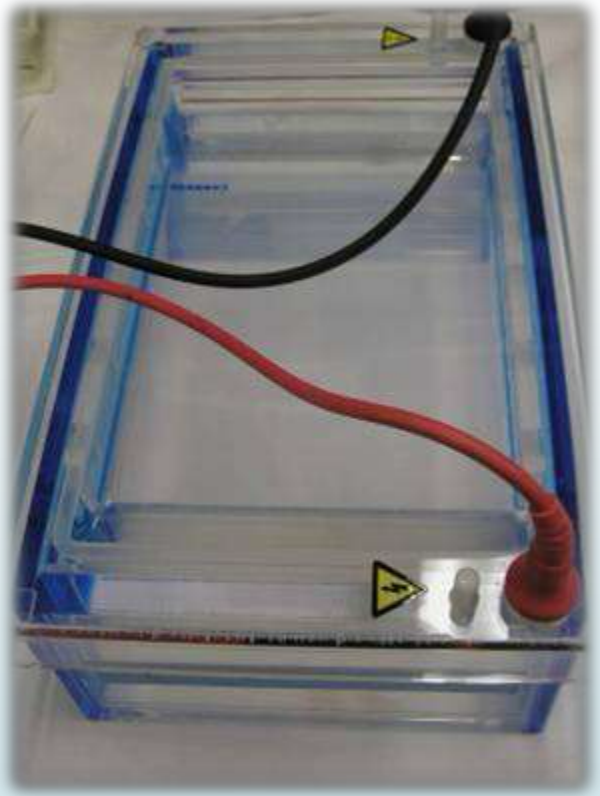
Loading on the Gel

Carefully place the pipette tip over a well and gently expel the sample. The sample should sink into the well. Be careful not to puncture the gel with the pipette tip.

3.Separating the fragments

- The electrical current is then turned on so that the negatively charged DNA moves through the gel towards the positive side of the gel.
- Shorter lengths of DNA move faster than longer lengths so move further in the time the current is run.
- The distance the DNA has migrated in the gel can be judged visually by monitoring the migration of the loading buffer dye.
- The electrical current is left on long enough to ensure that the DNA fragments move far enough across the gel to separate them, but not so long that they run off the end of the gel.

Running the Gel



Place the cover on the electrophoresis chamber, connecting the electrical leads. Connect the electrical leads to the power supply. Be sure the leads are attached correctly - DNA migrates toward the anode (red). When the power is turned on, bubbles should form on the electrodes in the electrophoresis chamber.

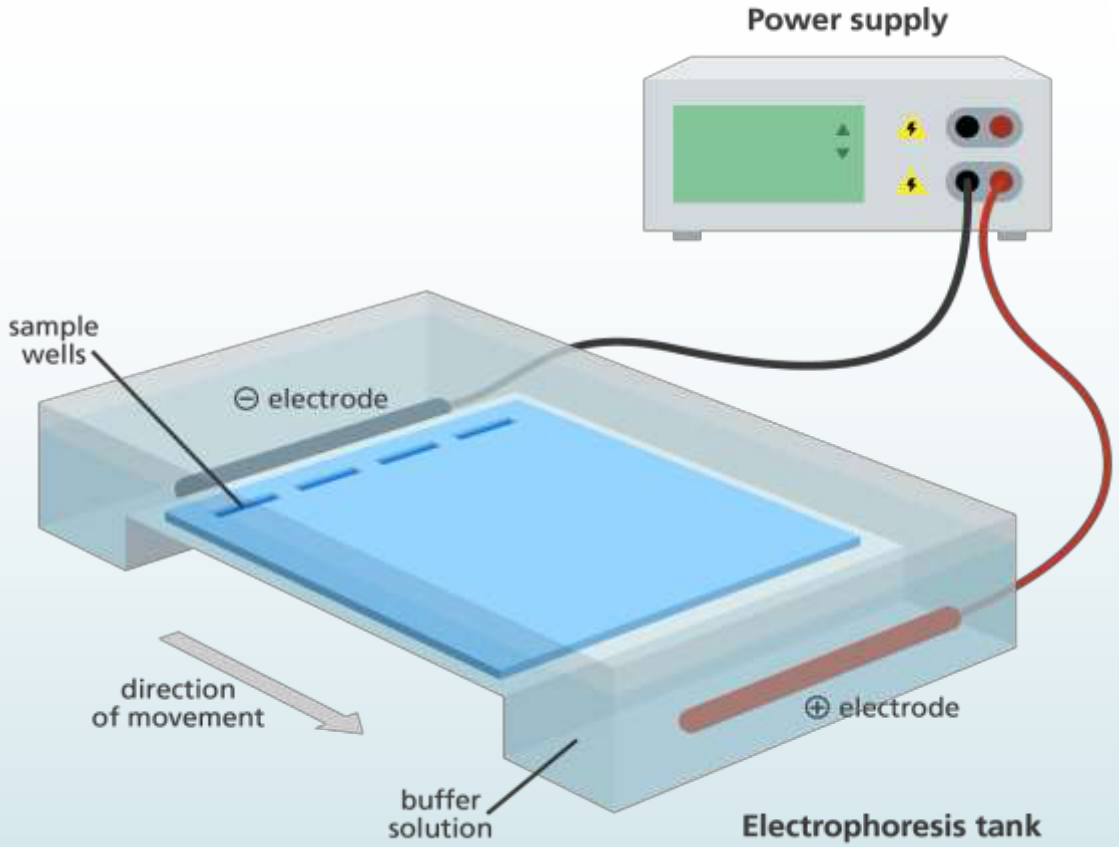
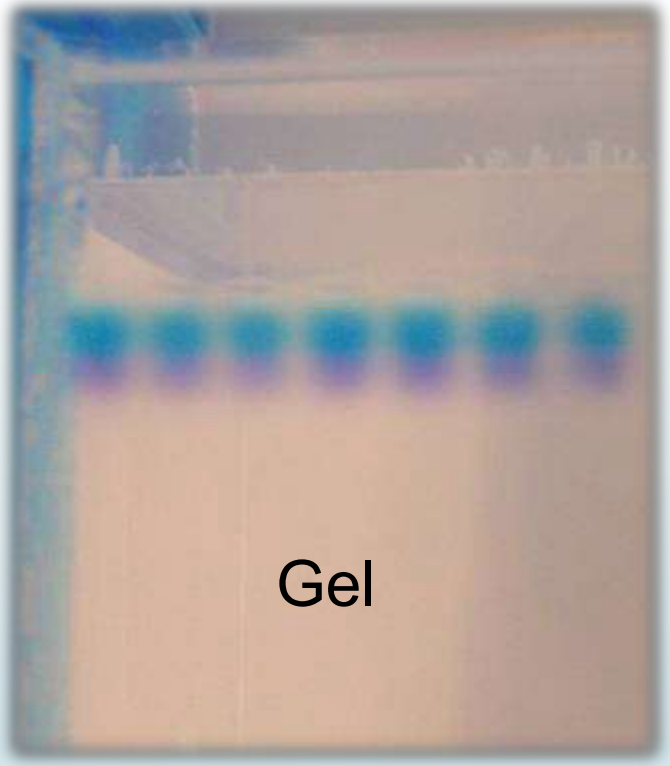


Figure (2): Illustration of DNA electrophoresis equipment used to separate DNA fragments by size. A gel sits within a tank of buffer. The DNA samples are placed in wells at one end of the gel and an electrical current passed across the gel. The negatively-charged DNA moves towards the positive electrode

Cathode
(-)

DNA
(-)
↓

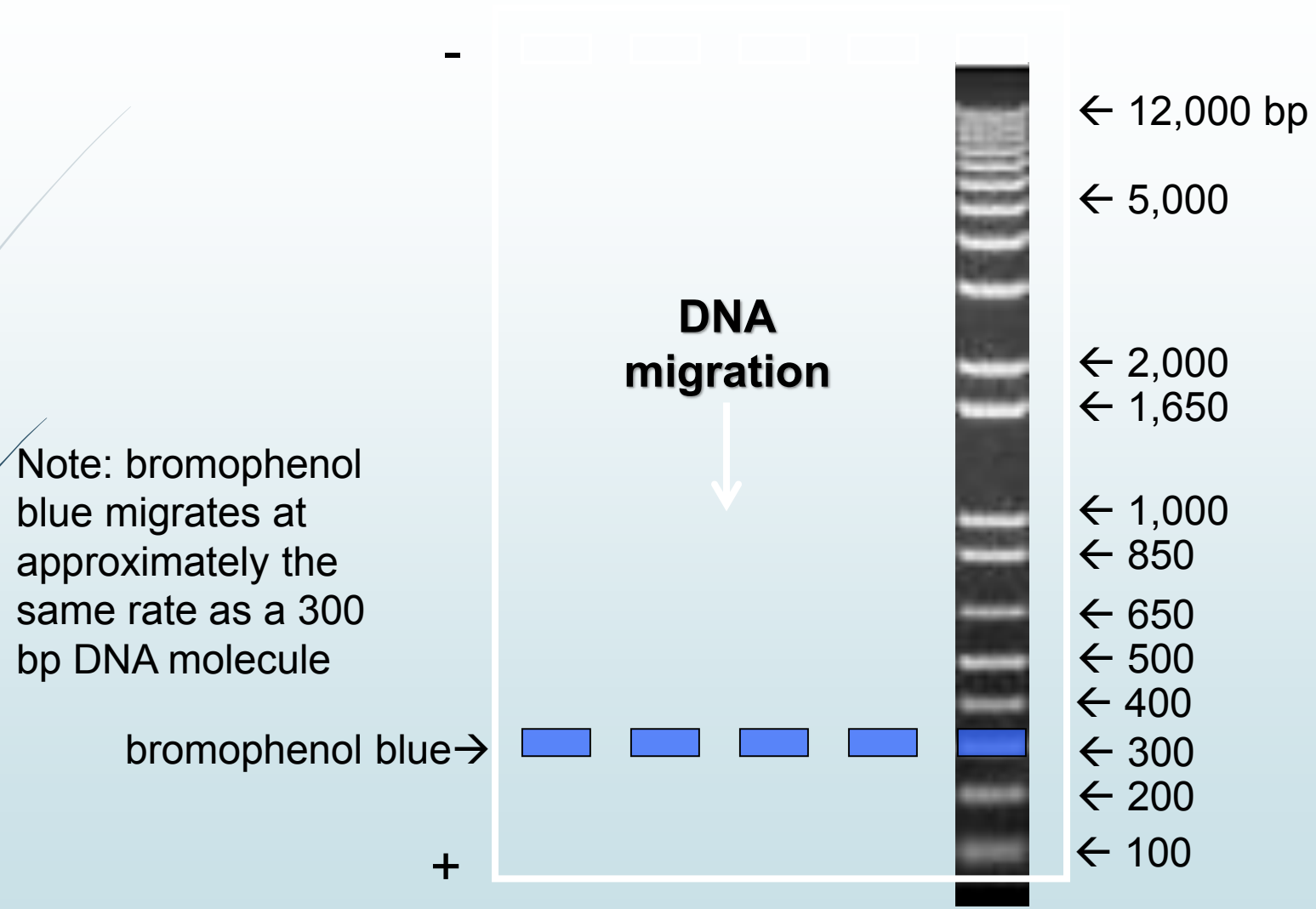


← wells
← Bromophenol Blue

Anode
(+)

After the current is applied, make sure the Gel is running in the correct direction. Bromophenol blue will run in the same direction as the DNA.

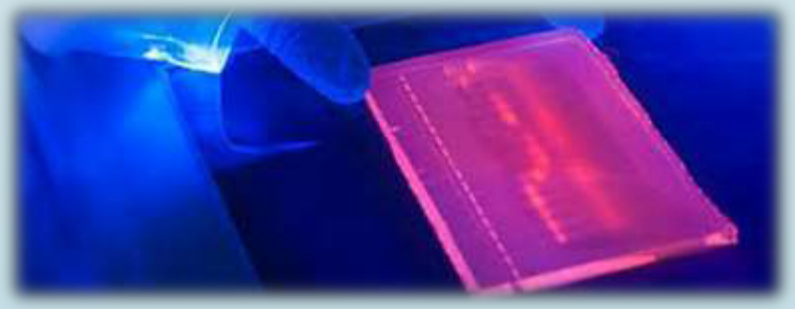
DNA Ladder Standard



Inclusion of a DNA ladder (DNAs of know sizes) on the gel makes it easy to determine the sizes of unknown DNAs.

4.Visualizing the results

- Once the DNA has migrated far enough across the gel, the electrical current is switched off and the gel is removed from the electrophoresis tank.
- To visualize the DNA, the gel is stained with a fluorescent dye that binds to the DNA, and is placed on an **ultraviolet transilluminator** which will show up the stained DNA as bright bands.
- Alternatively the dye can be mixed with the gel before it is poured.
- If the gel has run correctly the banding pattern of the DNA marker/size standard will be visible.
- It is then possible to judge the size of the DNA in your sample by imagining a horizontal line running across from the bands of the DNA marker. You can then estimate the size of the DNA in the sample by matching them against the closest band in the marker.

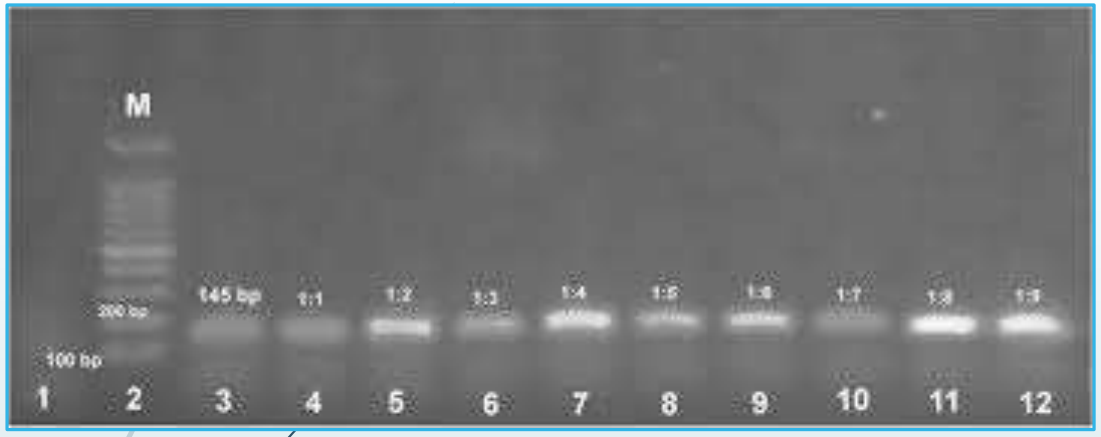


Staining of gel

Stain	Use	Detection limit[a] (ng)
Amido black	Proteins	400
Coomassie blue	Proteins	200
Ponceau red	Proteins (reversible)	200
Bis-1-anilino-8-Naphthalene sulphonate	Proteins	150
Nile red	Proteins (reversible)	20
SYPRO orange	Proteins	10
Fluorescamine (protein treated prior to electrophoresis)	Proteins	1
Silver chloride	Proteins/DNA	1
SYPRO red	Proteins	0.5
Ethidium bromide	DNA/RNA	10

[a] These limits of detection should be regarded as approximate since individual proteins may stain more or less intensely than average.

Table 2. Commonly used stains for biopolymers after electrophoretic separation in agarose or polyacrylamide gels.


M
145 bp
100 bp
1
2
3
4
5
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7
8
9
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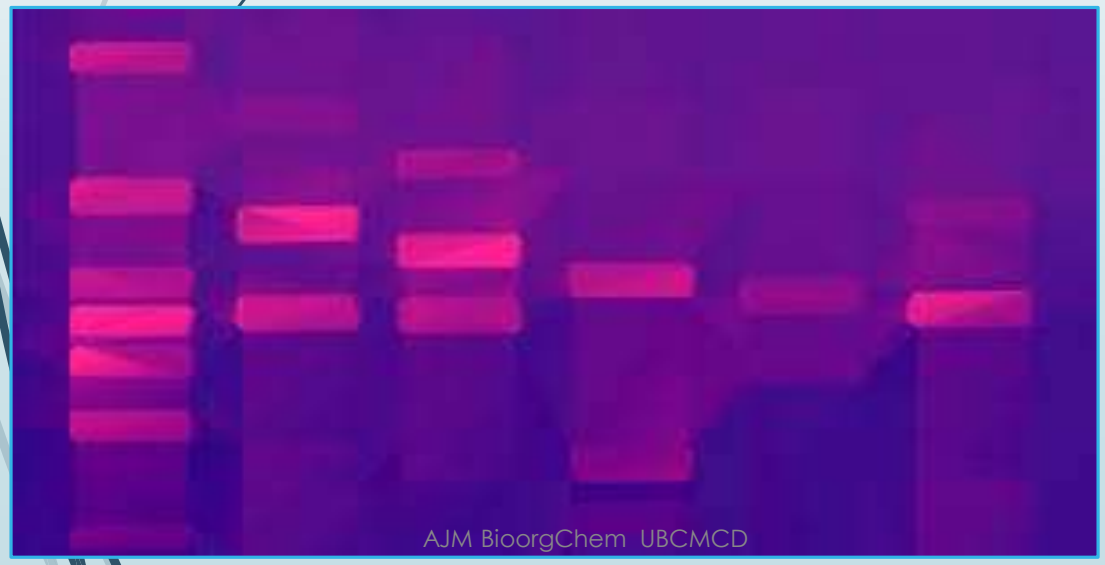
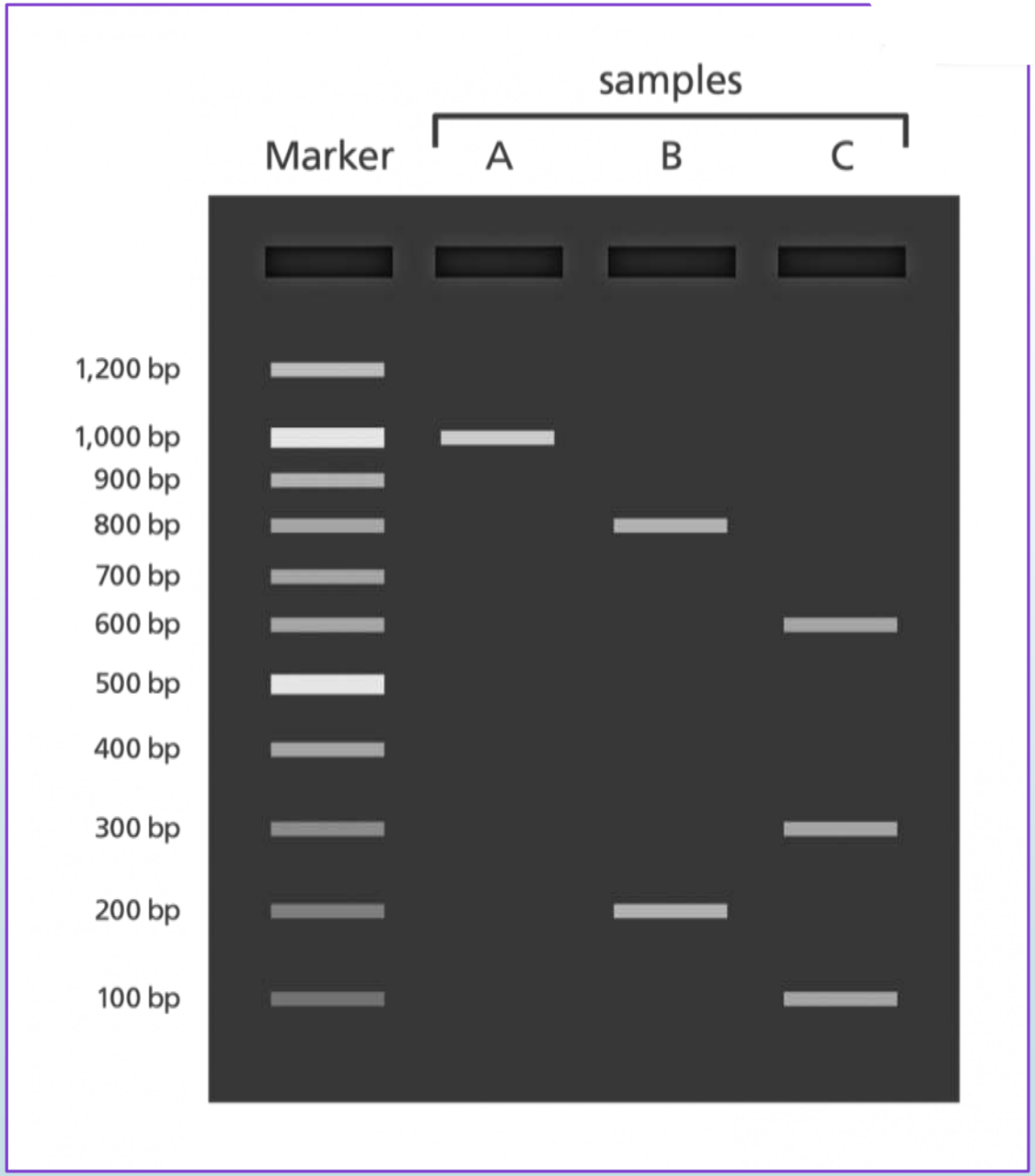
11
12


AJM BioorgChem UBCMCD


samples
Marker
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B
C
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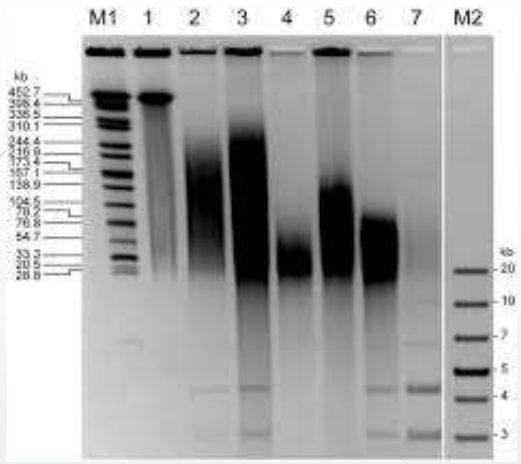
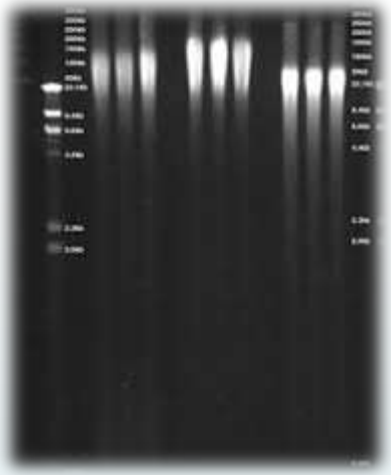
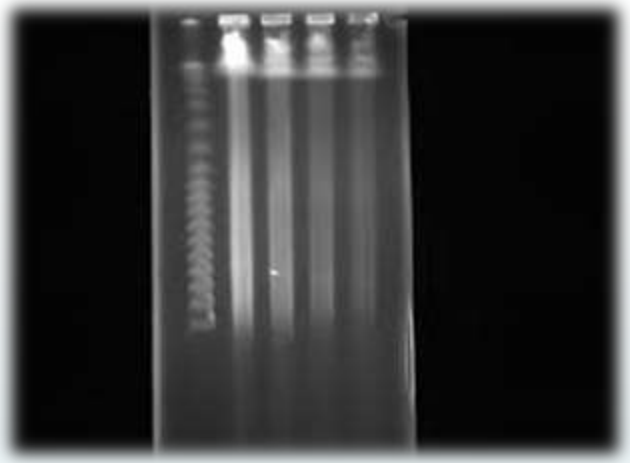
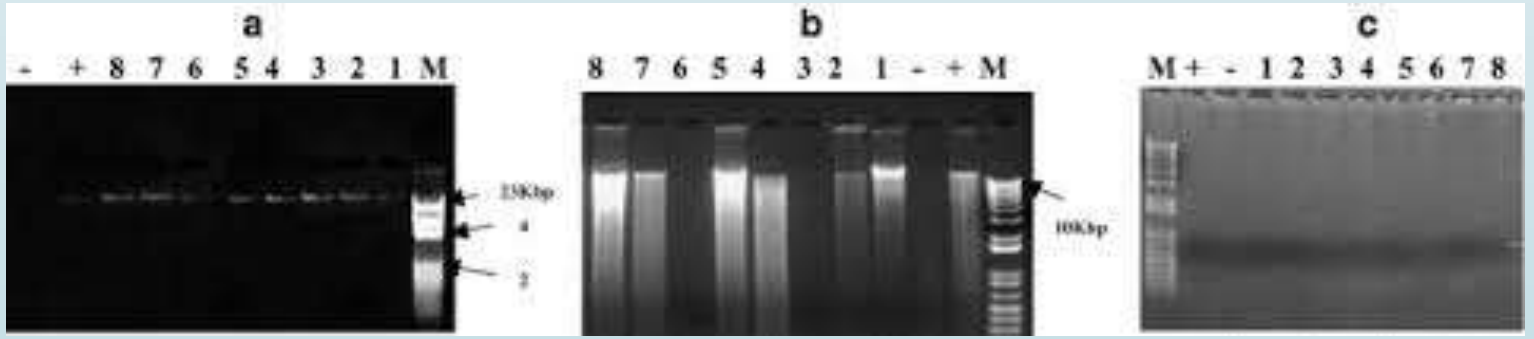
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp







Examples for separation By Gel Elec.



What about separation of Proteins ?

…. Next lecture

Questions : H.W

1.Can we used polyacrylamide in stead of Agarose in separation of DNA ? And Why ?

2. Why we used the Ladder during the separation of DNA ?

3. What is the aim of staining after Gel electrophoresis process ?

4. The small DNA moving faster in gel than the larger one ?

5. We load this sample with buffer solution, why we used buffer ? Can we used distilled water instead buffer ?

6. With Agarose GE we can separate DNA based only the length (size) easily and without thinking of charge ? Why ?

For more information you ,Read the textbook :Chromatography basic principles by Elsa Lundanes, Leon Reubsaet and Tyge Greibrokk , 2018 ,Wiley-VCH , Chapter 6

Or Chapter 8 in the Textbook : Chemical Analysis , Modern Instrumentation Methods and Techniques , by (**Francis Rouessac and Annick Rouessac)** Wiley ,2017 .

GOOD LUCK