ADVANCED ELECTROPHORESIS

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1. EXPERIMENT OBJETIVE

The aim of this experiment is to introduce students to the knowledge of **electrophoretic theory** and to familiarize themselves with the procedures involved in **agarose gel electrophoresis** to separate biological molecules.

In this case, real DNA fragments will be used, molecular weight markers where it will be possible to observe how the smaller fragments migrate more quickly through the agarose gel and genomic DNA due to its high molecular weight will migrate slowly.

It will also be introduced in the techniques of visualization of the DNA fragments in the agarose gel, using a method developed by **BioTed** and non-toxic unlike the traditional methods used (ethidium bromide).

2. BACKGROUND INFORMATION

Agarose gel electrophoresis is an analytical procedure used in several areas of biotechnology, both in research laboratories, as biomedical and forensic. Of the existing types of electrophoresis, agarose gel electrophoresis is the most common and most widely used method. It is a separation method frequently used to analyze DNA fragments generated by restriction enzymes, PCR, etc., and is a convenient analytical method to determine its size in a range of 500-30,000 base pairs. It can also be used to separate other biomolecules such as dyes, RNAs and proteins.

There are different types of agarose gel electrophoresis but **horizontal electrophoresis** is the most used to separate DNA molecules into agarose. Other types, such as **vertical electrophoresis**, are used to separate proteins and use polyacrylamide gels.

The horizontal electrophoresis apparatus is essentially a box with electrodes at each end, these electrodes are platinum because it has a very good electrical conductivity, because the platinum electrodes are expensive and fragile care must be taken when handling electrophoresis equipment.

The separation medium is an **agarose gel**, the agarose is a polysaccharide derived from the agar. Originally from algae, agarose is highly purified to remove all impurities. It is extracted from the same alga that is used to obtain the agar used in microbiology and that used in food. It is a non-toxic substance but should not be ingested.

The gel is performed by dissolving the agarose in a buffer and this mix takes to boiling point. The solution is then cooled to about 55°C and added to the mold to make the gel. A comb with wells is placed in the gel to form the wells where the samples will be loaded.

After the gel has solidified, the gel is placed in the electrophoresis unit and immersed with a suitable buffer to carry out the electrophoresis. The unit has a positive electrode at one end and a negative electrode at the other end. Samples are prepared with a loading buffer containing glycerol to give them density and thus are kept in the well without departing. The samples are loaded into the wells with a micropipette.

A power supply is connected to the electrophoresis apparatus and an electric current is generated. The charged molecules of the sample will enter the gel through the wall of the load well. Molecules with a negative charge will migrate to the positive electrode (anode) while molecules with a positive charge will migrate to the negative electrode (cathode). The buffer serves as the conductor of electricity and controls the pH, which is important for the stability of biological molecules. As the DNA has a negative charge at neutral pH it will migrate through the gel towards the positive electrode during electrophoresis.

If electrophoresis is performed with dye samples simulating DNA fragments, migration of several molecules can be visualized directly on the gel during electrophoresis and does not require subsequent gel staining. However, small dye molecules may be susceptible to diffusion out of the gel, so it should be monitored when complete separation of the dyes occurs.

The agarose gel contains microscopic pores that act as a molecular sieve separating the molecules according to their charge, shape and size. These characteristics along with buffer conditions, gel concentration and voltage, will affect the mobility of the molecules in the gel.

The separation occurs because the smaller molecules pass through the pores of the gel more easily than the larger ones. If the sizes of 2 fragments are similar or identical, they will migrate together in the gel. If the genomic DNA is digested several times, there will be a wide range of fragments that will produce a "***smear***" on the gel. Linear DNA fragments will migrate faster through the gel.

Two molecules with the same molecular weight and shape, will migrate faster the higher the charge. The molecules that bind more strongly to the agarose will migrate more slowly. The mobility of molecules during electrophoresis can also be influenced by the concentration of the agarose gel, the higher agarose concentration the electrophoresis will last longer.

For DNA molecules to be visible on the agarose gel, staining is required since the DNA itself is colorless. The most common DNA staining method uses **ethidium bromide** because of its high sensitivity. Ethidium bromide is a mutagen and must be handled very carefully. An ultraviolet light source (UV transilluminator) is also required for visualization. Currently there are other dyes, different companies have developed different types of dyes to increase sensitivity and mutagenic power much lower than ethidium bromide.

In our case we will use a NO TOXIC method that will allow us to visualize the DNA fragments of blue color, they will be able to be observed during the electrophoretic process but a subsequent staining will be necessary to be able to observe all the bands.

3. EXPERIMENT COMPONENTS

COMPONENT		STORE
10x Concentrated electrophoresis buffer	2 x 50 ml	
Agarose	1 x 1.75 gr	
Micropipette 20 µl	1	
Tips rack	1	
Samples microtubes	7	at 4ºC
DanaBlue 0.1%	1 x 400 µl	
FlashBlue 0.75x	1 x 125 ml	

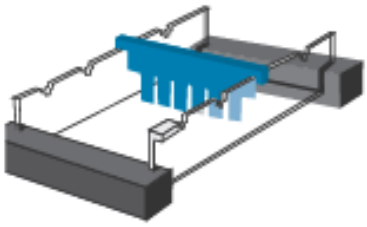
Add 450 ml of distilled water to each 10x Electrophoresis Buffer container to make 2 x 500 ml of 1x Electrophoresis Buffer which is the Working Buffer.

4. EXPERIMENT PROCEDURES

4.1 Agarose gel preparation

A) Mold preparation

Take the mold to make the gels and close the ends with the stops so that the agarose does not go out. Then place the comb to form the wells.



B) Agarose gel preparation

1.b) Use a 100 ml beaker or erlenmeyer to prepare the gel solution.

2.b) **For 7 x 7 cm gels**: Add **32 ml of 1x electrophoresis buffer** plus **0.30 g of agarose**, stir the mixture to dissolve the agarose clumps.

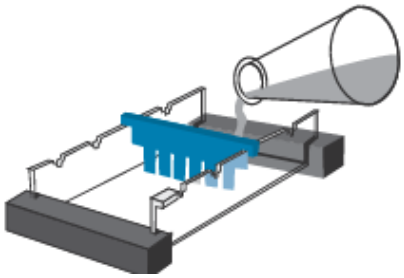
For 7 x 10 cm gels: Add **42 ml of 1x electrophoresis buffer** plus **0.40 g of agarose**, stir the mixture to dissolve the agarose clumps.

Make sure the 450 ml of distilled water has been added to the 10x Electrophoresis Buffer

3.b) Heat the mixture to dissolve the agarose. The fastest method is the use of a microwave, a heating plate can also be used, in both cases, in order for the agarose to dissolve **the solution must be brought to boiling point**. The final solution should appear clear without apparent particles.

4.b) **Cool** the agarose solution to about 55°C (to accelerate the process can be cooled by placing the container under a water tap and shaking). If there is excessive evaporation of the liquid, add electrophoresis buffer.

5.b) Add the agarose solution to the mold.

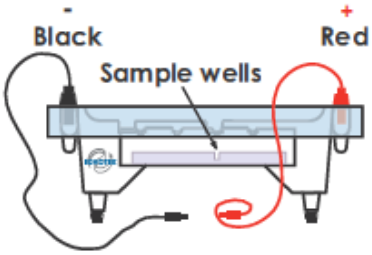


6.b) Allow the gel to solidify. To accelerate the process, the gel can be planted and then put it in a refrigerator (if the electrophoresis is performed the next day, keep the gel at 4ºC).

C) Gel preparation for electrophoresis

1.c) After the gel has solidified carefully remove the stops.

2.c) Place the gel in the electrophoresis chamber correctly oriented with the wells closest to the negative pole (black color).



3.c) Fill the electrophoresis chamber with **300 ml of 1x electrophoresis buffer**. ***The electrophoresis buffer can be used for 2 electrophoresis practice. Once the electrophoresis is finished, store this used buffer in a different container; don't mix a electrophoresis buffer new with one used buffer.***

4.c) Ensure that the gel is completely covered with tampon.

5.c) Remove the comb that has formed the wells very carefully to do not break any well.

6.c) Proceed to the load of the gel and carry out the electrophoresis.

4.2 Gel load and electrophoresis

Note: If you are unfamiliar with loading agarose gels, it is advisable to practice load before performing the experiment, or carry out the complete experiment before doing it with the students.

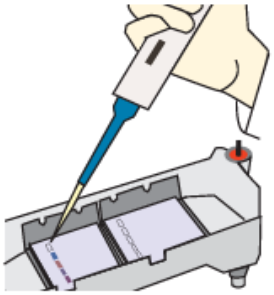
A) Electrophoresis samples

Check the volume of the all samples. Sometimes small drops of the sample may be on the walls of the microtubes. Make sure that the entire amount of sample is uniform before loading the gel. Centrifuge briefly the sample microtubes, or tap microtubes over a table to get the entire sample in the bottom of the microtube.

1.a) Four different samples presented in 4 tubes of a different color each are supplied, loading the samples in the following order:

WELL	SAMPLE	DESCRIPTION
1	GREEN	Molecular weight marker
2	LILAC	Genomic ADN
3	RED	Genomic ADN
4	BLUE	Molecular weight marker

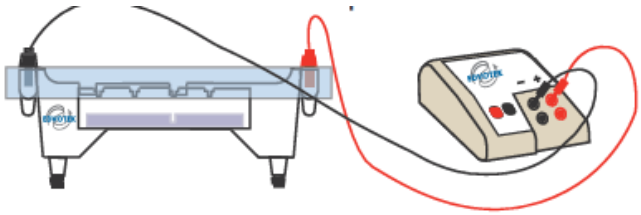
2.b) Load 20 microliters of each sample, using the fixed volume micropipette with a pipette tip supplied.



B) Carry out electrophoresis

1.b) After the samples have been loaded, place the electrophoresis apparatus cover on the electrode terminals carefully.

2.b) Insert the plug of the black cable into the black input of the power supply (negative input). Insert the red cable plug into the red input of the power supply (positive input).



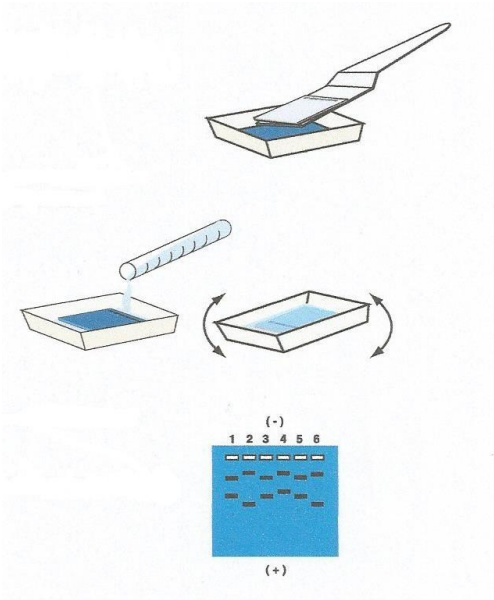
3.b) Set the power supply **at 75 volts (30 minutes) or 150 volts (20 minutes)**. **Watch that the dyes do not come out of the gel.**

4.b) After 10 minutes the separation of the dyes will begin to be observed.

5.b) After the electrophoresis is finished, **turn off the power supply**, disconnect the cables and remove the cover.

6.b) Place the gel in a white light transilluminator (if not available, a sheet of white paper may also be used). For a correct visualization of the bands you must go to the next section which is the staining of the gel to display all the bands correctly.

4.3 Staining of agarose gel



1. Do not stain the gels in the electrophoresis cell.

2. Place the gel in a container with **100 ml FlashBlue 0.75x**, so that it is completely covered.

3. Incubate for 10 minutes. Increasing the staining time will lead to a subsequent greater number of washes with water to remove excess dye.

4. After the staining step, you can store 100 ml FlashBlue 0.75x for other stains.

5. Place the container with the gel under a tap of water and let the water run until the water does not turn blue. Hold the gel in order not to lose it. Fill the container with water.

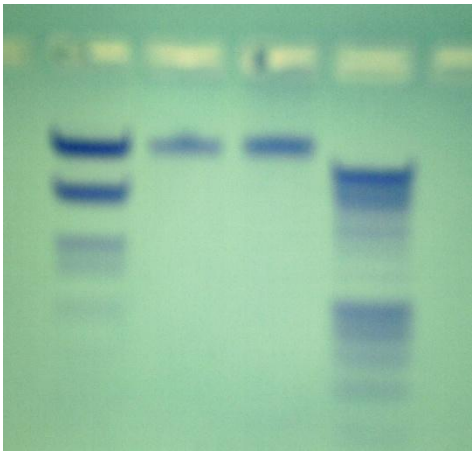
6. Carefully remove the gel from the container and examine the gel in a white light transilluminator (if not available, a white sheet may be used). In this step the bands will be appreciated but the gel will have a very intense blue color that will not allow appreciating the bands well.

7. Perform several washes with agitated water if it is possible. You will notice how the bands become more visible and the blue background color descends.

8. If it still has a very deep background color, it is possible to leave it overnight in water and the next morning observes the gel.

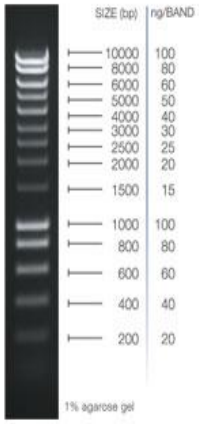
5. PRACTICE RESULTS

M1 1 2 M2



M1

M2



M1: Molecular weight marker, in the attached figure you can see the size of the DNA fragments

M2: Molecular weight marker, in the attached figure you can see the size of the DNA fragments

1 and **2**: Genomic DNA extracted from human saliva.

6. QUESTIONS AND ANSWERS ABOUT THE PRACTICE

A series of questions can be asked of students about the practice:

1. **What conclusion can be drawn from the experiment?**

   That the genomic DNA has a size of 20-25 Kb (25,000 base pairs) comparing to what height it is with respect to the markers of molecular weight. That the genomic DNA is intact, since it appears as a single intact band, if it were degraded it would see a "smear" all that well down would look blue.

2. **What is genomic DNA?**

   It is the DNA that is found in our chromosomes and is where all the information is.

3. **Based on what does agarose electrophoresis separate molecules?**

   Electrophoresis separates molecules based on their size, charge and shape.

4. **Explain the migration according to the load.**

   Molecules that have a negative charge will migrate towards the positive pole; while the positively charged molecules will migrate towards the negative pole.

5. **What would happen if distilled water was used instead of the Electrophoresis Buffer in the electrophoresis chamber or in the agarose gel solution?**

   As the distilled water does not contain ions, the conductivity of the fluid and the mobility of the molecules to migrate in the gel are reduced. The buffer serves as the conductor of electricity and controls the pH, which is important for the stability of biological molecules.

6. **To which electrode will go the DNA?**

   As the DNA has a negative charge at neutral pH it will migrate through the gel towards the positive electrode during electrophoresis.

For any further questions or queries, please contact us info@bioted.es