# Innovating Science®

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## Teacher's Manual

# Electrophoresis: Agarose Gel Separation of Dyes

IS 3011

ALDON®

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# *Next Generation Science Standards*

**MS-PS2-3. Ask questions about data to determine the factors that affect the strength of electric and magnetic forces.**

[Clarification Statement: Examples of devices that use electric and magnetic forces could include electromagnets, electric motors, or generators. Examples of data could include the effect of the number of turns of wire on the strength of an electromagnet, or the effect of increasing the number or strength of magnets on the speed of an electric motor.]

PS2.B: Types of Interactions

- Electric and magnetic (electromagnetic) forces can be attractive or repulsive, and their sizes depend on the magnitudes of the charges, currents, or magnetic strengths involved and on the distances between the interacting objects.

***Aligned to the Next Generation Science Standards (NGSS)****

# Electrophoresis:
# Agarose Gel Separation of Dyes
**IS 3011**

## INTRODUCTION

Electrophoresis is a large, and perhaps intimidating, word to describe what is in actuality a very simple process. In order to understand electrophoresis as a procedure it is helpful to first understand electrophoresis as a word. The prefix *electro-* is derived from the Latin *electrum* and refers to electricity. The suffix *-phoresis* is a Greek word that means the act of being carried. When combined, the word electrophoresis basically means that something is being carried by electricity. While the concept behind electrophoresis may be simple, the procedure is one of the most valuable and powerful tools in the field of biotechnology. Before the advent of electrophoresis, many of the procedures commonly performed in molecular biology today simply would not have been possible.

Electrophoresis is a separation tool. It allows scientists to separate a mix of biological molecules for identification and/or further manipulation. Primarily electrophoresis is used to separate DNA and proteins but electrophoresis may be applied to many different substances.

**What Is Required for Electrophoresis?**

The process of electrophoresis requires four components:

- a sample to be separated
- a medium used to separate the sample
- a source to provide electrical current
- something to carry the electrical current in order to move the sample across the separation medium

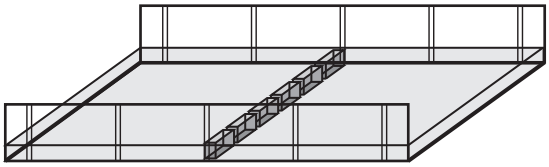
The sample to be separated is usually some kind of macromolecule. A macromolecule is a very large molecule that is usually assembled from many smaller molecules. In the world of biochemistry the four most common macromolecules are nucleic acids (DNA and RNA), proteins (including enzymes), carbohydrates (starches and complex sugars), and lipids (such as fats and waxes). While at first glance it may seem odd to refer to things like DNA and proteins as large molecules, after all cells are quite small and these are components that fit in cells making them even smaller, the fact is that when compared to many other biological molecules these substances are in fact huge.

Macromolecules are often polymers. A polymer (from the Greek *poly* - many, *meros* - parts) is a macromolecule composed from a few smaller units that are very similar to each other (if not identical). These smaller units are called monomers (from the Greek *mono* - single, *meros* - parts). DNA for example is composed from four basic monomers – adenine, cytosine, thymine, and guanine. While these four components are quite small, a DNA molecule may be composed of tens of millions or hundreds of millions of these four molecules, linked together in various combinations. Proteins on the other hand are composed from basic units called amino acids. It is the order that these basic amino acids link together that defines both the structure and function of proteins.

The medium used to separate the sample will vary based on the sample being separated. One of the more common substances used is agarose and as it is the medium being used in this activity it is the one we will focus on here. Agarose is a gelatin-like material that is extracted from seaweed. When extracted and dried it is a white powder but when added to liquid and heated it dissolves (melts) above 85°C. At this point, the hot agarose solution can be poured into a mold and once it cools enough, it solidifies in the shape of the mold with a consistency very similar to solidified gelatin. As it solidifies, it forms a microscopic network of pores throughout. Much like a sponge has a network of pores which allows the sponge to soak up water, the agarose too has a network of pores but these pores are microscopic. The microscopic pores in the agarose are what allow it to function as a separation medium.

In order for the agarose to separate the sample there has to be a method of placing the sample into the solidified agarose. When the agarose solution is heated and liquefied it is poured into what is called a gel casting tray. The gel casting tray is the mold to hold the agarose as it solidifies. The gel casting tray is a rectangular or square mold. The agarose solution is poured into the gel casting tray to a desired thickness, usually several millimeters, and allowed to sit and cool. Before the agarose cools, however, one more component is added called a gel comb. A gel comb is a structure that has several “teeth.” The gel comb is constructed in a manner that allows each end of it to slip firmly onto the outside of the gel casting tray across the width of the gel. The teeth of the gel comb are submerged in the liquefied agarose across the gel casting tray. Once the agarose has solidified, the gel comb is removed. This leaves a series of small compartments called wells across the solidified agarose gel. These wells will eventually have the samples added to them.

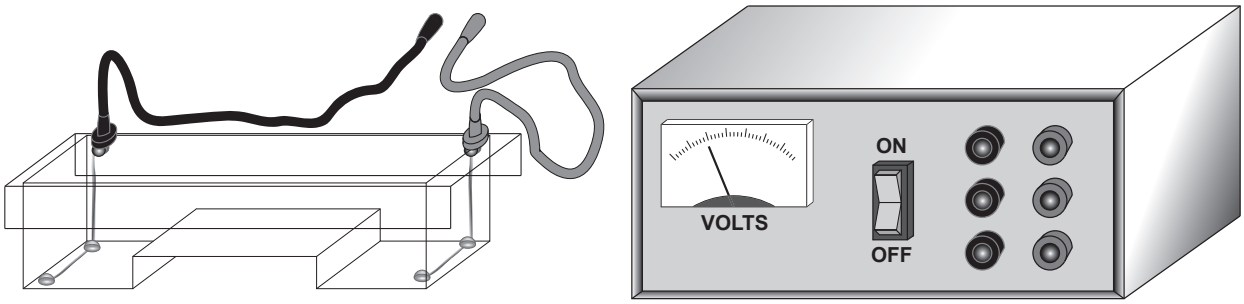
**Figure 1**
**Agarose Gel with Wells in a Gel Casting Tray**



In order to separate the sample, the sample must be moved across the agarose gel. This is where the last two components of electrophoresis come in. In electrophoresis, the sample is driven by electricity. To provide electricity, the system is hooked up to some sort of power source. The power source may range from something as simple as a battery (or batteries) to very complex and expensive specialized power supplies that plug into a wall outlet and give the user a great degree of control over parameters such as voltage and current. Regardless of the cost and sophistication, all power supplies are designed to do one thing - provide electrical current.

To carry the current provided by the power supply, a special chamber is used. The chamber is large enough to hold the agarose gel and has electrodes on either side. On one side is a positive electrode (anode) and on the opposite side is a negative electrode (cathode). The power supply is attached to the chamber and the electrodes carry the current into the chamber. A solution called a buffer is added to the chamber and the buffer serves a couple of purposes. It carries current across the chamber and it helps protect the samples being separated by providing an environment that will not damage the samples (such as a specific pH or salt balance).

**Figure 2**
**Electrophoresis Chamber and Power Supply**




Once all of the individual components of the electrophoresis process are understood, it is possible to examine them as a whole and see how they work in concert to achieve separation.

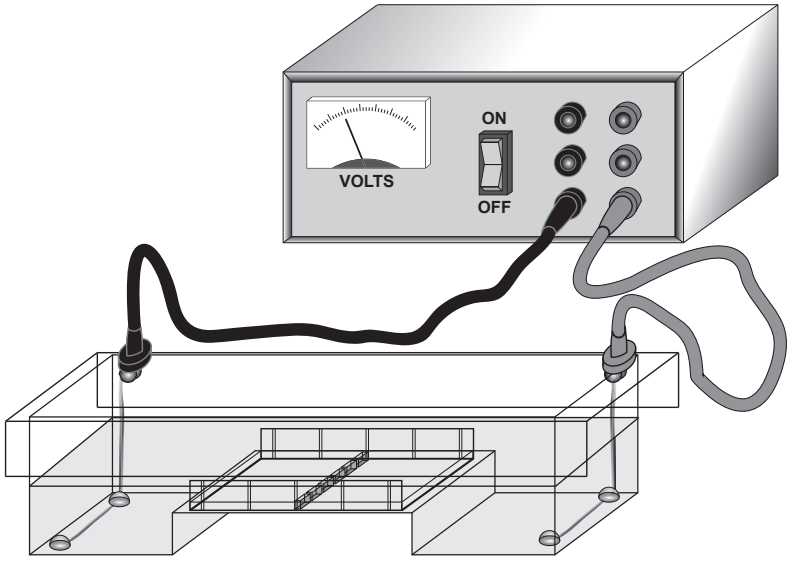
## How Does Electrophoresis Work?

An agarose gel, on a gel casting tray, is placed into the electrophoresis chamber. The agarose gel contains a series of wells because of the gel comb used during the pouring of the gel. A buffer solution is added to the chamber until the agarose gel is completely submerged. After addition of the buffer, both electrodes are submerged as well.

After the gel is submerged in buffer, the samples can be placed in the wells. Before the samples are loaded, however, a small amount of loading dye is added to them. The loading dye is a solution that contains some dye and a high concentration of either sucrose or glycerin and it serves several purposes. The sucrose or glycerin, in high enough concentration, gives the sample a higher density than the buffer in the chamber. Higher density liquids sink when added to lower density liquids. This causes the samples to sink down into the wells and stay there. Without the loading dye the samples may simply float out of the wells and into the buffer in the chamber. The dye in the loading dye gives an indication of how long the gel should be exposed to electrical current as well as provides visual confirmation that the sample is down in the well. Most molecules, say DNA for example, cannot be seen when they are being separated. The dye molecules in the loading dye are much smaller than the molecules of interest and the importance of this will be expanded upon shortly.

Once all of the samples are loaded into the wells of the gel, a lid is placed on the chamber and the power supply and the chamber are connected to each other. The power supply is then turned on and electrical current is introduced into the chamber through the electrodes submerged in buffer. Electrical current running through a liquid buffer can be quite dangerous. It should be noted that electrophoresis chambers are designed in a manner that if the lid to the chamber is removed once the power is applied it will break the circuit and stop current from flowing through the buffer.

**Figure 3**
**Electrophoresis Chamber Containing Gel and Buffer Attached to Power Supply**



Before exploring what occurs after the current is applied there are a couple of concepts that need to be understood:

- First, molecules may have a charge. In fact, in order for electrophoresis to work, the molecules in the samples must have a charge. Staying with the example of DNA, DNA molecules are negatively charged. Remembering the phrase “opposites attract,” it can be concluded that negatively charged molecules that are exposed to an electrical current will be attracted to the positive electrode. Conversely, positively charged molecules will be attracted to the negative electrode. For this reason a gel casting tray usually offers a selection of places the gel comb can be placed to form wells in the gel. If all of the molecules in the sample to be separated have the same charge, the wells can be placed at the end of the gel. If the sample has some molecules that are positively charged and some molecules that are negatively charged, the wells should be placed in the center along the length of the gel.

- Second, the properties of the agarose gel will vary based on the amount of agarose used in the solution prepared to pour the gel. Agarose gel solutions are prepared with a specific percentage of agarose (usually anywhere from as little as 0.6% to as much as 3.0% agarose). The higher the percentage of agarose, the smaller the pores that will form as the agarose solidifies into a gel. The size of the pores in the agarose ultimately affects the size of the molecules that can be effectively separated on the gel.

After the current is applied, the system begins to do its job. The first thing of note is the formation of bubbles at the electrodes submerged in the buffer. This is a sign that electricity is making it into the chamber. The charges that originate from the electrodes begin to take effect. Each electrode attracts molecules with a charge opposite of that particular electrode. However, the samples encounter resistance as they are down in the wells of the agarose gel. They cannot simply run to the electrodes, they must make their way through the agarose gel, or more accurately the pores in the agarose gel.

Smaller molecules have an easier time making their way through the pores of the agarose gel than larger molecules. Being smaller they are able to weave their way through much more efficiently whereas larger molecules find it more difficult to move and therefore take more time to work their way through the agarose. After a set amount of time, the system is turned off. In the case of a visible substance, this would be when the fastest moving sample reaches one end of the agarose gel. In the case of a sample that cannot be readily seen, this would be when the loading dye reaches the end of the agarose gel. In the case of visible samples the results can be analyzed immediately but in the case of non-visible samples the agarose gel must be treated with a special stain depending on the nature of the sample, such as a special stain for DNA molecules or a special stain for proteins.

## Objectives

- Learn the components involved in the process of electrophoresis.
- Understand the role of each component as it applies to the entire process of electrophoresis.
- Perform the electrophoresis process on several individual dyes and two mixtures of dyes.
- Analyze the resulting agarose gel to determine which dyes are contained in the two dye mixtures.

## Materials Included in the Kit

Prepared agarose, 2.0%, 200ml
TBE buffer, 5X, 500ml
Agarose dye marker set, 1.0ml each
- Crystal violet
- Orange G
- Xylene cyanol
- Malachite green
- Dye mixture #1
- Dye mixture #2

## Materials Needed but not Supplied

Agarose electrophoresis chamber
Electrophoresis power supply
Micropipettes w/micropipette tips, 10µl
Microwave or hot water bath
Distilled or deionized water

## Safety

Safety goggles
Gloves
Lab apron

## *Pre-lab Preparation*

### Prepare 1X TBE Working Buffer

1. The provided TBE buffer is in a 5X concentrated form and must be diluted using distilled or deionized water prior to use. The volume of 1X TBE buffer needed will vary depending on the volume required for your electrophoresis chambers. Consult the table below to determine the amount of concentrated buffer and water to be used to prepare various volumes of 1X TBE buffer:

| Volume 1X TBE Buffer Required | Volume 5X Buffer Concentrate | Volume Distilled or Deionized Water |
|---|---|---|
| 100ml | 20ml | 80ml |
| 250ml | 50ml | 200ml |
| 500ml | 100ml | 400ml |
| 750ml | 150ml | 600ml |
| 1000ml | 200ml | 800ml |
| 1500ml | 300ml | 1200ml |
| 2000ml | 400ml | 1600ml |
| 2500ml | 500ml (entire bottle) | 2000ml |

2. In a large enough container for the volume of 1X TBE buffer being prepared, combine the 5X buffer concentrate and water. Mix thoroughly. Label the container “1X TBE Buffer.”

### Prepare 2% Agarose Gels

**Note:** *Always handle hot agarose with heat protective gloves. The activity will require each group to load six samples on an agarose gel. Be sure to use a proper gel comb when pouring the agarose gels.*

Microwave Method

1. Set up gel casting trays according to the instructions provided by the manufacturer of your electrophoresis equipment.
2. **Loosen the cap** on the bottle of prepared agarose.
3. Place the bottle of prepared agarose in a microwave and heat on high for one minute.
4. Wearing heat protective gloves, remove the bottle of prepared agarose from the microwave.
5. Gently swirl the bottle. It may be helpful to squeeze the sides of the bottle after the first interval to break up the prepared agarose a little. This will help the agarose melt faster and more evenly.
6. Repeat, microwaving in one minute intervals, until the prepared agarose is completely melted.

**Note:** *The exact time required to completely melt the agarose will vary by microwave. Simply continue in one minute intervals until the prepared agarose is completely melted. If, during any interval, it appears the agarose is boiling over (coming out of the top of the bottle), stop the microwave and allow the bottle to sit for a few seconds before removing it from the microwave.*

7. Pour the liquefied agarose into the gel casting tray. Use the amount of agarose recommended by the manufacturer of your electrophoresis equipment. Most agarose gels are poured a few millimeters thick.
8. Place a gel comb in the center of the tray containing the agarose. This activity will require wells in the center of the gel.
9. Allow the gel casting tray to sit completely undisturbed until the agarose has completely solidified (usually approximately 20-30 minutes, depending on the amount of agarose poured).
10. After the agarose has completely solidified, gently remove the gel comb.

Hot Water Bath Method

1. Set up gel casting trays according to the instructions provided by the manufacturer of your electrophoresis equipment.
2. **Loosen the cap** on the bottle of prepared agarose.
3. Place a glass beaker, taller than the bottle of prepared agarose, on a hot plate.
4. Place the bottle of prepared agarose in the beaker. Add water to the beaker until the level of water is just above the level of agarose in the bottle.
5. Turn the hot plate on and heat until the water in the beaker is boiling.
6. Allow the bottle of agarose to stay in the boiling water until the material in the bottle is completely melted.
7. After the agarose is completely melted, turn off the hot plate and remove the bottle from the beaker wearing heat protective gloves.
8. Pour the liquefied agarose into the gel casting tray. Use the amount of agarose recommended by the manufacturer of your electrophoresis equipment. Most agarose gels are poured a few millimeters thick.
9. Place a gel comb in the center of the tray containing the agarose. This activity will require wells in the center of the gel.
10. Allow the gel casting tray to sit completely undisturbed until the agarose has completely solidified (usually approximately 20-30 minute, depending on the amount of agarose poured).
11. After the agarose has completely solidified, gently remove the gel comb.

## *Instructor's Notes*

Instead of pouring the agarose gels and diluting the TBE buffer in advance you may choose to have students perform these steps as part of the lab. If so, be aware that this will add a significant amount of time required to complete the exercise. The amount of time it takes for the electrophoresis run will vary by voltage and the length of the gel.

If pouring the agarose gels in advance, it can be done up to 2-3 days prior to performing the exercise. If the gels are poured in advance, leave them in the gel casting tray and place in a sealed bag. Add a couple of milliliters of 1X TBE buffer to the bag before sealing to keep the gels moist.

Be sure students are familiar with all electrophoresis equipment and the proper use of micropipettes before conducting the lab. The activity requires the loading of small quantities of dye samples (10 microliters) into the wells of an agarose gel. While electrophoresis equipment contains many safety features to minimize risk during the electrophoresis procedure, be sure to caution students that they will be working with a conductive liquid and electricity. Electrophoresis chambers should be on a level surface with no spilled liquid of any kind in the vicinity. All wires, plugs, etc. should be inspected and should be dry before any connections are made and any power is supplied to the electrophoresis chamber. Do not use any equipment that appears damaged (frayed or exposed wires, cracked chambers, etc.) for the electrophoresis procedure. Always refer to the manufacturer's instructions for the proper use and handling of equipment.

## *Chemical Disposal*

Dispose of all chemicals used in this activity in accordance with local and state regulations.

# Procedure

## *Materials Needed per Group*

1 Pre-cast 2% agarose gel on gel casting tray
Agarose electrophoresis chamber
Electrophoresis power supply
Micropipette w/micropipette tips

## *Shared Materials*

1X TBE buffer
Crystal violet
Orange G
Xylene cyanol
Malachite green
Dye mixture #1
Dye mixture #2

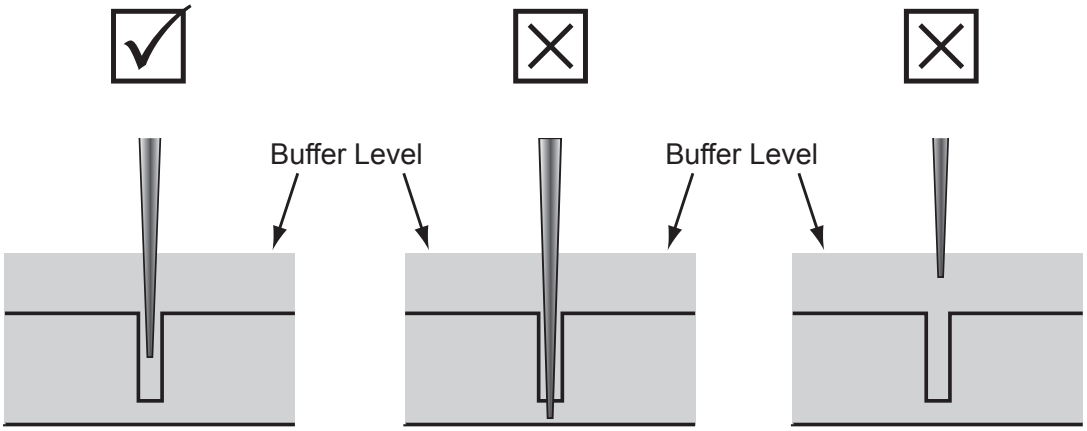
## *Safety*

Safety goggles
Gloves
Lab apron

**Note to Students:** *Your instructor may have poured your agarose gel and diluted your TBE buffer in advance. If this is the case, continue with the directions below. If not, follow your instructor's directions for the procedures of preparing agarose gels and diluting TBE buffer concentrate.*

1. Place a pre-cast 2% agarose gel in your electrophoresis chamber. The wells should be in the center of the agarose gel.
2. Slowly add 1X TBE buffer to one side of the electrophoresis chamber. Once enough TBE buffer has been added to reach the level of the agarose gel on one side of the chamber stop and continue by adding buffer to the other side of the chamber. Continue to slowly add buffer until the entire agarose gel is completely submerged and the buffer is at a level of 2-3mm above the top of the gel.

**Note to Students:** *Be sure you are familiar with the use of a micropipette before proceeding. If you do not know how to use a micropipette, consult your instructor for explanation. When loading a well in an agarose gel, you should position the very end of the micropipette tip beneath the surface of the buffer and just below the top of the well. Be very careful not to insert the tip too deep into the well or you may poke through the bottom of the well with the pointed end of the micropipette tip. This may cause your sample to leak out of the bottom of the well. After the tip is in place in the well, slowly add the sample to the well. Remember that the samples contain loading dye so you should see each sample sink down to the bottom of the well. Adding the sample too quickly may create a current in the well that may wash your sample out of the well.*




**Correct** - pipette tip below the top of the well but not poking through the bottom of the well

**Incorrect** - pipette tip poking through the bottom of the well

**Incorrect** - pipette tip above the top of the well

3. Using a micropipette, add 10µl (microliters) of each dye sample to the wells in your agarose gel in the following order:

   Well #1 – Crystal violet
   Well #2 – Orange G
   Well #3 – Xylene cyanol
   Well #4 – Malachite green
   Well #5 – Dye mixture #1
   Well #6 – Dye mixture #2

4. After the samples are added to all of the wells, be very careful not to jar or shift the electrophoresis chamber in any way. Examine the area around the electrophoresis chamber for any spilled liquids on or around the chamber. If there are any, carefully wipe them up with paper towels.

5. Carefully and gently place the lid on to the electrophoresis chamber properly. Be sure to do this gently so as not to disturb the buffer and loaded samples in the chamber.

6. Before proceeding, be sure the electrophoresis power supply is unplugged. Examine the ends of the red and black cords coming off of the electrophoresis chamber. Be sure they are dry. Connect the red plug to the red terminal of the power supply and connect the black plug to the black terminal. Have your instructor examine your setup before proceeding.

7. Once you have your instructor's approval, plug the electrophoresis power supply in and turn the unit on. If necessary, adjust the voltage of the power supply to the voltage specified by your instructor.

**Note to Students:** *After turning the power supply on, you should see bubbles coming off of the electrodes in the electrophoresis chamber. This means the system is carrying an electrical current. If you do not bubbles forming on the electrodes, turn off the power supply and unplug it. Make sure the lid is properly in place and the black and red cords are firmly attached to the proper terminals of the power supply. Plug the power supply back in and turn the power supply on again. If you still don't see bubbles forming on the electrodes, call your instructor for help.*

8. Observe the agarose gel in the chamber, after a couple of minutes, you should see evidence that the dyes are starting to leave the wells and migrate into the agarose gel.

9. Allow the electrophoresis procedure to continue, observing periodically. Allow the process to proceed until the fastest moving dye reaches one end of the agarose gel.

10. When the fastest moving dye has reached the end of the agarose gel, turn the power supply off and unplug it from the wall. Detach the black and red plugs from the terminals of the power supply. Wait approximately one minute after unplugging the power supply before proceeding.

11. After approximately one minute, remove the lid from the electrophoresis chamber. Remove the agarose gel, on the gel casting tray, from the electrophoresis chamber. **Caution:** *depending on the voltage used for the electrophoresis procedure, the buffer may be hot.*

12. Examine your finished agarose gel and sketch a representation of the gel in the Data Analysis section of the lab. Be sure to try and note how far each dye moved as well as indicate the color of each dye.

13. Clean up all materials according to your instructor. Be sure to wash your hands with soap and water before leaving the lab.

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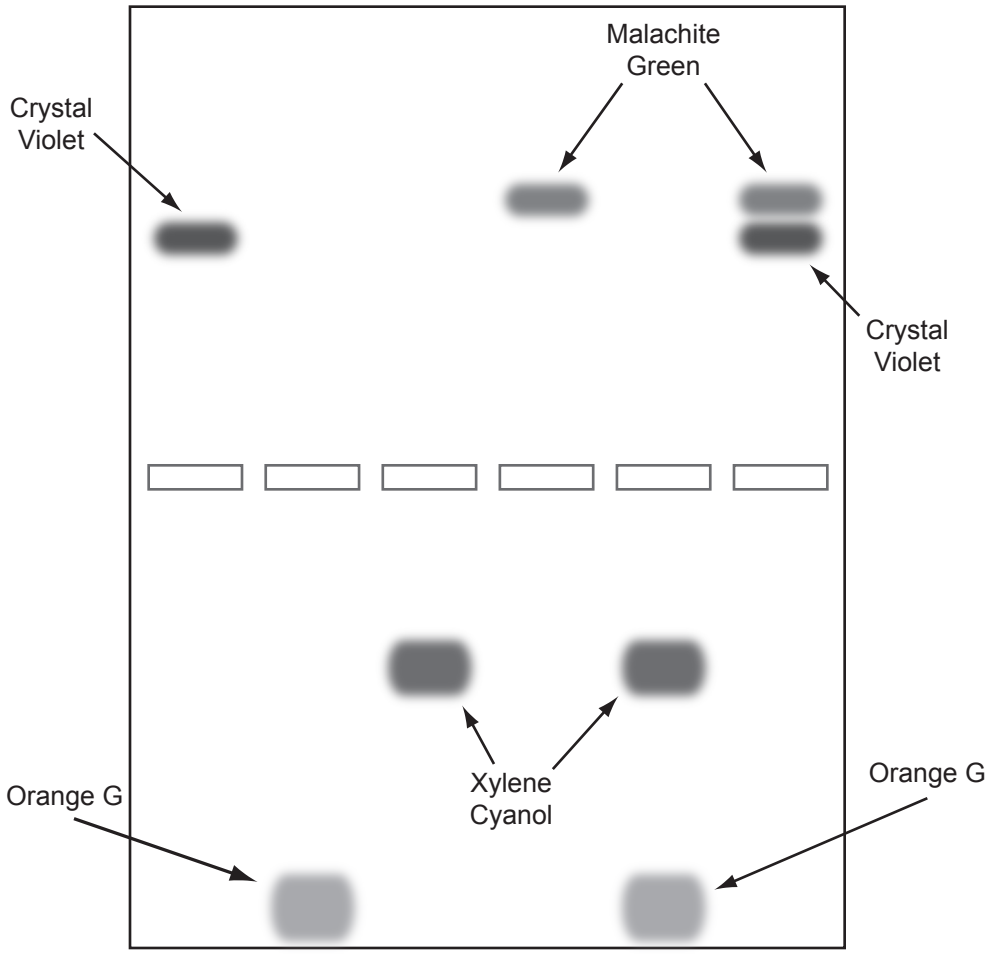
| Name: | Instructor: |
|---|---|
| Date: | Class/Lab Section: |

## DATA ANALYSIS



| Name: | Instructor: |
|---|---|
| Date: | Class/Lab Section: |

## DATA ANALYSIS

Questions

1. Based on your examination of your agarose gel what dyes were contained in dye mixture #1?

**The finished gel should show the dye mixture #1 contained the dyes xylene cyanol and orange G.**

2. What dyes were in dye mixture #2?

**Students should see crystal violet and malachite green in dye mixture #2.**

3. Based on your results, what conclusions can you draw with regards to the electrical charge of the molecules for each of the dyes? Explain your answer.

**Based on the results displayed on the agarose gel, two of the dyes, xylene cyanol and orange G, are negatively charged as they migrated toward the positive electrode (anode). The other two dyes, crystal violet and malachite green, are positively charged as they migrated toward the negative electrode (cathode).**

4. Why were the wells placed in the center of the agarose gel as opposed to the one end of the agarose gel?

**The samples used in this activity had dyes that were both negatively and positively charged. If the wells were at one end of the agarose gel, as opposed to the center, two of the dyes would rapidly move off of the gel, depending on which end of the gel the wells were placed at.**

| Name: | Instructor: |
|---|---|
| Date: | Class/Lab Section: |

## DATA ANALYSIS

5. When separating DNA, the wells are placed at one end of the agarose gel and not in the center. Why do you think this is? What end of the agarose gel do you think the wells should be placed at? Why?

**With DNA, the molecules, regardless of size, are all charged negatively. By placing the wells at the end, the DNA molecules can run along the entire length of the agarose gel and separate. Since the DNA is negatively charged, the molecules will be attracted to the positive electrode, therefore the wells should be at the end of the agarose gel that will be nearest the negative electrode when the electrophoresis procedure begins.**

6. After running the electrophoresis procedure with the dyes you were able to remove your agarose gel and observe the results immediately. When performing the electrophoresis procedure on other biomolecules, such as DNA or proteins, the resulting gel from the electrophoresis process must be stained with a special stain, depending on the molecules being separated. Why do you think this is?

**While the dyes used in this activity are readily visible after the procedure is complete, materials such as proteins or DNA are not naturally colored and therefore cannot be readily observed after electrophoresis. The molecules must be stained with a special stain (which will vary depending on the type of molecule) to observe the molecules trapped in the pores of the gel.**

7. What is purpose of loading dye? Why is it added to samples?

**Loading dye serves two purposes: it is very dense and causes the samples to sink and stay in the wells during the loading process. It also contains a dye that moves along the gel to provide indication of when to stop the electrophoresis process, since many times the samples being separated cannot be seen as the electrophoresis process is occurring.**