Introduction to Electrophoresis

(Exercise provided by Leslie Snider, Mira Costa College; first modified by Ann Wright and Joni Black, Hartnell College)

a) Introduction

Electrophoresis is a common laboratory technique. Its purpose is to separate the individual molecules in a mixture of molecules. Recall that chromatography also separates mixtures of molecules from each other (you used paper chromatography to separate a mixture of pigment molecules in food coloring). Electrophoresis can separate molecules much better than paper chromatography, even molecules that are physically and chemically very similar to each other.

Electrophoresis is the separation of charged molecules (molecules with ionic charges) using an electric field. The basic concept is that the mixture of molecules to be separated is placed between a positively charged electrical wire (the "positive electrode") and a negatively charged electrical wire (the "negative electrode"). Based on the concept that opposite electrical charges attract each other, molecules with positive ionic charges will migrate toward the negative electrode and molecules with negative ionic charges will migrate toward the positive electrode.

The molecules that are to be separated are first placed into a gelatinous substance (the "gel"). One function of the gel is merely to hold the molecules while they are being separated. The other function of the gel is to act as a sieve or strainer that allows the smaller molecules to migrate faster than the larger ones. This allows electrophoresis to separate molecules based on size as well as ionic charge.

An electric power supply provides the electricity to the gel. The researcher sets the voltage and the current that the power supply delivers. A complete discussion of electricity is beyond the scope of this handout, but for a thorough understanding of electrophoresis it is important to know some basic electrical concepts. Electricity is a flow of electrons. The voltage is the force that is applied to the electrons (how hard they are being pushed). The current is the amount of electrons that are flowing. (The units of current are amps or milliamps). If the voltage is held constant (for example, at 100 volts) the amount of current will depend on the resistance to electricity of the material that the current is moving through. For example, pure water has high resistance to electricity, so there will be a low current. But if you add salt to the water, the water's resistance decreases so the current will increase. In general, metals and solutions with ions have low resistance (high current), whereas non-metals and solutions without ions have high resistance (low current).

Agarose gel electrophoresis of DNA

DNA fragments (which are often prepared by cutting larger pieces of DNA with restriction enzymes) are usually separated in agarose gels. Agarose is a natural polysaccharide derived from agar, which is a gelatinous substance found in seaweed. Agarose is supplied as a dry white powder. When an agarose gel is needed, the agarose powder is mixed with buffer and water and then melted in a microwave oven (the agarose powder will not mix with the buffer and water unless it is melted). After the mixture cools, the agarose hardens into a semi-solid gel. The concentration of agarose in the gel usually varies from 0.5% to 2%. (See the next page for a review of concentration units).

An agarose gel is soft and somewhat delicate, but it can withstand the gentle handling. These gels, however, are not rigid enough to support their own weight vertically, so agarose gel electrophoresis is performed in a horizontal gel box. The gel contains small holes called wells for loading the DNA mixture into the gel.

Because the backbone of all DNA molecules contains negatively charged phosphate groups, all DNA molecules have a uniform negative ionic charge. When DNA samples are placed into an agarose gel and exposed to an electric current, all the DNA fragments will migrate toward the positive electrode.

The agarose gel acts as a molecular sieve, so smaller fragments will migrate faster and farther through the gel than larger fragments. The rate of migration, however, is **not** a linear relationship. In other words, a DNA piece that is 2 times smaller than a larger DNA piece does **not** migrate twice as fast, and a DNA piece that is 3 times smaller than a larger piece does **not** migrate three times as fast. The actual differences in migration rates of are based on the logs of the DNA fragment sizes. For example, a DNA piece that is 2 times smaller than a larger DNA piece will migrate 1.3 times faster than the larger piece. This stems from the fact that the log of 2 is 0.3.

As the DNA pieces migrate toward the positive electrode, they separate into groups (called "bands") based on their size. The band that migrates the fastest contains smallest DNA size in the mixture, the band that migrates just behind that contains the second smallest DNA size, etc. Therefore the number of bands is equal to the number of different DNA sizes in the original mixture. All the bands that come from one well are called a "lane" of the gel.

The bands of DNA are not visible to the naked eye, so after the electrophoresis is complete the gel is stained, commonly with ethidium bromide or with other dyes such as methylene blue, to make the DNA bands visible.

Usually DNA fragments of known sizes are loaded into one well of the gel. These provide a positive control (to show that the gel is separating DNA fragments correctly) and also serve as DNA size controls to make a standard curve (a graph of DNA size vs. distance traveled). Using the standard curve, the size of any DNA piece in the gel can be calculated from its distance traveled.

Concentration terms used in gel electrophoresis

The concentration of agarose in the gel is usually given in concentration units called Percent Concentration (%). Percent concentration means grams of solute per 100 ml solution. For agarose gels, the agarose is the solute and the volume of the gel is the solution volume. For example, a 1% agarose gel means a gel that contains 1 gram of agarose per 100 ml of gel solution. And a 3% agarose gel contains 3 grams of agarose per 100 ml of gel volume.

Percent concentrations can also be used for gel volumes that are not 100 ml. The general equation for finding the percent of an agarose gel is this:

$$\text{Percent of agarose gel} = \frac{\text{Grams of agarose}}{\text{Volume of gel}} \times 100$$

Here is an example: What percent is an agarose gel that has a volume of 50 mL and that contains 2 grams of agarose? The answer is that the gel is a 4% gel:

$$\frac{2 \text{ grams}}{50 \text{ mL}} \times 100 = 4\,\%$$

Here are two more examples. What % is a 200 mL gel that has 5 grams of agarose? What % is a 20 mL gel that has 8 grams of agarose? The answers are 2.5% and 40%, respectively.

When you are doing an experiment that involves agarose gel electrophoresis, you usually begin making the agarose gel solution in a flask. To make the gel solution in a flask, you add three things to the flask:

(1) Dry agarose powder
(2) High concentration buffer
(3) Water

But how much of each of these do you add to the flask?

To calculate how much dry agarose powder to add to the flask, you need to know the volume of the agarose gel that you are making and also the percent of the agarose gel. The experimental procedure that you are following usually tells you the volume and the percent of the gel that you need. When you know the volume and percent of the gel that you are making the equation below is used to calculate how many grams of agarose are needed to make the gel:

$$\text{Grams of agarose} = \%\text{ gel concentration} \times \frac{\text{Required volume of gel}}{100}$$

To verify that you can use this formula correctly, answer this question: How many grams of agarose are needed to make 50 ml of 0.6% gel? The answer is 0.3 grams.

After the correct weight of agarose powder is weighed out and placed into a flask, the agarose powder in the flask is mixed with water and high concentration buffer. But what volume of water and what volume of high concentration buffer are added to the agarose powder in the flask?

Let us begin by discussing what volume of high concentration buffer that needs to be added to the flask. The concentration of a buffer is often given using a concentration unit called X (which is pronounced “X” or “times”). The final concentration of the buffer in the flask (after it has been mixed with the agarose powder and the water) is always 1X concentration. 1X concentration is sometimes called the "normal" concentration of the buffer. However, buffers are usually supplied at a concentration that is higher than 1X, such as 10X (10 times normal concentration) to 50X (50 times normal concentration) because it is more efficient to store and ship buffers at high concentrations. The equation below is used to calculate what volume of the high concentration buffer you need to add to the flask:

$$\text{Volume of high Concentration buffer} = \frac{\text{Volume of gel}}{\text{X of high concentration buffer}}$$

For example, if you had a 20X high concentration buffer and you are making an 500 mL agarose gel, the equation gives you 25 ml. This means you should add 25 ml of the 20X buffer to the flask that has the agarose powder. To confirm that can do the buffer calculation correctly, answer these questions: How much 10X buffer do you add to the flask if you are making a 150 mL agarose gel? The answer is 15 mL of 10X buffer. How much 30X buffer do you add to the flask if you are making a 40 mL agarose gel? The answer is 1.3 mL of 30X buffer.

The last of the three ingredients that you add to the flask is water. What volume of water do you add? The equation below allows you to calculate the volume of water that you add to the flask:

$$\text{Volume of water} = (\text{Volume of gel}) - (\text{Volume of high concentration buffer})$$

As examples, if the gel volume is 500 mL and you added 25 mL of high concentration buffer to the flask, you would need to add 475 mL of water to the flask. If the gel volume is 40 mL and you added 1.3 mL of high concentration buffer to the flask, you would need to add 38.7 mL of water to the flask.

After all three ingredients (agarose powder, high concentration buffer, and water) have been added to the flask, the flask is briefly heated in a microwave oven. The heating

melts the agarose powder and allows it to dissolve into the rest of the gel solution in the flask.

b) Casting a 0.8% Agarose Gel

1) Obtain a clean, dry casting tray and place the rubber dams over the open ends. The volume of this gel will be 30 ml.

2) Place the "comb" (well-forming device) firmly into the notches so that it sits firmly and evenly across the base of the casting tray. The comb goes in the notch near the end of the tray, not in the notch in the middle of the tray.

3) You will need to prepare 30 ml of 0.8% agarose gel for today's experiment. Calculate how many grams of agarose, how many ml of 50X TAE buffer, and how many ml of water you will need to prepare this gel. You may have to review the previous section of this handout to do the calculations. **Show your instructor your answers before you prepare the gel.**

Grams of agarose needed: _______ g.

ml of 50X TAE needed: _______ mL.

ml of water needed: _______ mL.

4) After your instructor has approved your answers, you will be given the agarose (pre-weighed) in a flask. You must add the correct volumes of 50X TAE and deionized water. Use the p1000 micropipette (with disposable tip) to add the 50X buffer. Use a graduated cylinder to add the water.

5) Melt the agarose powder by heating the flask in the microwave for one minute at 50% power.

6) Remove the flask (wearing an insulated glove) and swirl the contents, looking carefully for any undissolved clumps of agarose. If there are any, microwave the agarose for 30-second bursts until all of the agarose is dissolved.

7) Observe the mark on the side of the flask. If detectable evaporation has occurred, bring the solution up to the original volume with deionized water, using a wash bottle.

8) Allow the agarose to cool to approximately 50-55ºC. To do this, let the flask sit at room temperature. After approximately 5 minutes, start touching the flask. When it is very warm to the touch but you can stand to pick it up, it is at 50-55ºC. **If you pour the gel while it is too hot you might permanently warp the casting tray. Ask your instructor for a second opinion if you are not sure of the temperature.** If you wait too long and the agarose hardens, you can always melt it again.

9) To pour the gel: make sure the dams and comb are in place and the tray is level and out of your way. (Once it is poured, you don't want to move the gel until it has hardened.) Swirl the melted agarose gently (to prevent bubbles) and pour it into the tray in one slow smooth motion. Make sure it completely covers the bottom of the tray.
10) Allow the agarose to harden completely; this will take about 20 minutes. Do not remove the comb until you are ready to load the samples (in activity e).

11) While it is hardening, read sections c and d.

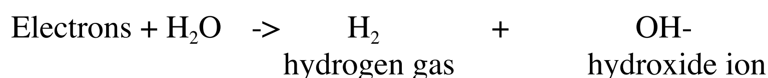
c) Electricity and Electrophoresis Solutions

Recall that the agarose powder was mixed with TAE buffer when you prepared your gel. You will also use TAE buffer to submerge your gel after it has hardened. You may wonder why buffers are used in electrophoresis instead of plain water. There are two main reasons for using buffers instead of plain water in electrophoresis.

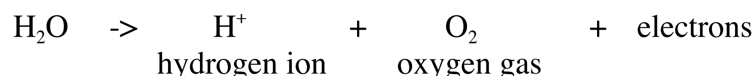
1) **Buffers contain positive and negative ions.** The ions in the buffer (which are sometimes called "salts" or "electrolytes") are what allow the electricity to flow through the gel. Without ions, the molecules you load into the gel would not migrate.

2) **Buffers prevent pH changes in the gel.** The electricity that flows into and out of the gel is actually a stream of electrons. The electrons enter at the cathode and exit at the anode. The electrons interact with the water molecules in the buffer to cause the following two chemical reactions:

At the cathode (the negative or black lead) this reaction occurs:



At the anode (the positive or red lead) the following reaction occurs:



The hydrogen gas and the oxygen gas that are produced by these reactions can be seen as bubbles that form at the black and red leads, respectively. But consider the ions being produced in the above two reactions. Remember that H^+ ion is acidic and OH^- is basic. Because of these ions, a difference in pH can develop at the two ends of the box. The cathode end can become basic and the anode end can become acidic.

These pH changes could damage the molecules migrating through the gel and cause them to migrate in abnormal directions. To prevent pH changes, buffers are used in gel electrophoresis. Recall that a buffer is any substance that stabilizes the pH of a solution. In other words, buffers prevent pH changes.

d) Practice loading samples into the wells of rubber "agarose gel"

All members of your group should practice using a micropipette to load samples into the wells of an agarose gel.

(1) Obtain a rubber practice gel in a small plastic box, a microcentrifuge test tube containing a blue dye, a p20 micropipette, and several disposable tips for the pipette.

(2) Use tap water to cover the rubber gel.

(3) Pipette 8 ul of the blue dye into each well of the rubber gel. Try to get all 8 ul of the dye into each well without spilling any onto the surface of the gel.

Some tips for loading the wells without spilling are given below:

(a) When loading the sample into the gel, hold the micropipette with two hands. One hand is on the handle of the micropipette. The other hand is near the tip of the micropipette, to keep the tip steady.

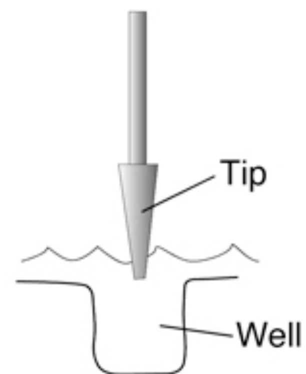
(b) Keep both elbows on the bench top for added steadiness.

(c) The tip of the micropipette should **not** be inserted into the well. Keep the tip at the top of the well.

(d) To deliver the sample into the well, push down slowly and steadily on the plunger to the plunger's second stopping point.

(e) After delivering the sample into the well, do **not** release the plunger until after you have withdrawn it from the buffer.

Hold the pipette with two hands



Keep the tip at the top of the well, not in the well.

e) Agarose Gel Electrophoresis of Food colors

1) After your agarose gel has hardened, remove the comb by gently pulling it straight up and away from the gel. Run a spatula carefully along the interface between the gel and the rubber dam at each end. Carefully remove the rubber dams, keeping the gel on the tray and level. Then place the gel (<u>and the tray it is on</u>) into the platform in the box. Place the gel so that the wells are closest to the black electrode.

2) Add approximately 250 ml of the 1X TAE buffer to the box. (The buffer for the gel box has already been made at 1X concentration by the technician. You do not need to make it by diluting a stock solution). Pour the buffer into one end of the box, allowing it to wash over the gel and into the other end. The gel should be completely, but barely, submerged in the buffer. Note that the gel was prepared using the same buffer at the same concentration (1X TAE). Be sure that the wells in the gel are filled with buffer. Move the gel box next to a power supply now (because you can't move the gel after you have loaded samples into it).

3) Each group of students should now obtain a clean empty microcentrifuge tube, a microcentrifuge tube with a mixture of food colors, a micropipette (2 – 20 ul), and some disposable tips for the micropipette.

On the front desk is a tube with loading dye. Transfer 5 ul of the loading dye into your empty tube. If you need help using the micropipette, please ask your instructor. The loading dye contains three chemicals: A buffer, a dye molecule called bromophenol blue, and glycerol. Because the bromophenol blue is a negatively charged molecule, it migrates toward the positive electrode during electrophoresis. Its purpose is to provide a visual marker of how far the molecules in the wells have migrated. (Recall that agarose gel electrophoresis is usually used to separate mixtures of DNA molecules, and that DNA molecules can't be seen on the gel. This is why a visible marker is usually needed). The glycerol is denser than water. Its purpose is to cause the samples to sink to the bottom of the well. Having the sample resting at the bottom of the well minimizes loss of sample by diffusion into the TAE buffer.

4) Transfer 45 ul of the food color mixture into your microcentrifuge tube with the 5 ul of loading dye. Mix the loading dye and the food color by pipetting gently up and down, avoiding introducing bubbles. This mixture will be called the "sample".

5) Each member of your group should now load one 8 ul sample into a well.

- Set micropipette to 8 ul. Be sure you have placed a disposable tip on the micropipette before you proceed.
- Use the same pipetting technique you practiced on the rubber gel:
  - a) Hold the pipette with two hands
  - b) Both elbows on the benchtop
  - c) Pipette tip above the well (not into the well)
  - d) Push the plunger slowly and steadily down to the second stop
  - e) Keep the plunger fully depressed until you withdraw the pipette

• Repeat on each well until all wells have been loaded. If you miss a well or spill the sample, try again. Be sure each member of the group practices using the micropipette correctly and loading sample cleanly into the wells.

6) After all the samples have been loaded, it is time to connect your electrophoresis box to the electrical power supply. Steps a – f below are the procedure for doing this.

a) Place the lid on your electrophoresis tray. The lid has a red wire and a black wire connected to it.

b) Obtain an Edvotek power supply. Plug your lid's red and black wires into a matching colored pair of sockets on the power supply.

c) Turn on the power supply by toggling the big on-off switch to "on".

d) The screen should show time, volts, and current. Press the "Mode" button until the screen shows an arrow next to the volts. By selecting volts with the arrow, you are programming the power supply to deliver a constant voltage to the gel.

e) Use the up and down arrow buttons next to the right of the display to set the power supply for 70 volts, then press the "Run/Stop" button to start the electrophoresis.

f) To confirm that the electrophoresis is working properly, look to see that bubbles are forming at the anode and cathode wires. Do you recall from section (c) what gases these bubbles are and how they are formed?

The gels should run for about 30 minutes to separate the food colors.

7) After about 30 minutes turn down the voltage, turn off the power supply, and unplug it. Remove the gel from the gel box. The food color dyes should be separated into separate bands. If the sample had contained DNA pieces (instead of dye molecules) the DNA pieces would have been separated, but they would not be visible. Record the distance that each dye molecule migrated (in mm) in data table 3.

After you have filled in the data table in section (e) below, you can throw your gel into the trash but be sure you save the casting tray.

Do not remove the buffer from the gel box. Just leave your gel box with the buffer in it on the counter at the back of the room, The laboratory technician will clean the gel box for you.

Wash out the rubber practice gel with water and put it back where you got it.

Don't throw away any of the microcentrifuge tubes, but do throw away all of the pipette tips.

e) Data table

Dye color:	Distance migrated (mm):

f) Review Questions

1) Electrophoresis can separate molecules based on the ionic charges of the molecules. Describe why electrophoresis makes differently charged molecules migrate differently.

2) If all the molecules in a mixture have similar ionic charges, agarose gel electrophoresis can separate molecules based on their sizes. Describe why agarose gel electrophoresis makes different sized molecules migrate at different speeds.

3) One DNA piece is 20 base pairs in size. Another DNA piece is 40 base pairs in size. Another piece is 100 base pairs in size.

a) Which piece will migrate the fastest? ______

b) The faster piece of DNA will/won't (←circle one word) migrate twice as fast as the slower piece of DNA.

4) Why do DNA molecules always migrate toward the positive electrode? Your answer must include naming a functional group found on all DNA molecules.

5) Calculate the % concentration of each of the following:
  a) 3 grams of agarose in 100 ml of solution: _____
  b) 10 grams of agarose in 200 ml solution: _____
  c) 10 grams of agarose in 30 ml solution: _____

6) How many grams of agarose do you need to make 50 ml of 2% agarose? _____

7) Calculate the amount of 40X buffer that you need to make each of the following:
  a) 300 ml of 1X: ______
  b) 20 ml of 1X: ______
  c) 4000 ml of 1X: ______

8) In each of the answers to problem (7), how much deionized water would you need to prepare the 1X buffer?

9) If a student melted an agarose solution using the microwave (in the exact same way you did) but didn't wait for it to cool before pouring the gel, what could go wrong?

10) Why are ions found in all electrophoresis buffers?

11) The solution that is put into the gel and the gel box is called TAE buffer. Define "Buffer":

12) Why are buffering molecules found in all electrophoresis buffers? What causes pH changes in gels and what exactly would go wrong if no buffering molecules were present?

13) For each electrode (positive and negative) state what gas is formed, what ion is formed from water, and what pH (acidic or basic) are created.

	Positive electrode	Negative electrode
Gas:		
Ion:		
pH:		

14) When you are drawing liquid into the micropipette, the plunger is pushed down to the first/second (circle one) stop. When you deliver the liquid out of the micropipette, the plunger is pushed down to the first/second (circle one) stop.

15) Explain why the loading dye contains glycerol and bromophenol blue.

16) If we were casting a 0.8% gel with a volume of 50 ml, how much 50X TAE would we use? ______ How much agarose would we use? _______ How much water would we use? ______