UNIT 8: **Separation of Dye Molecules using Agarose Gel Electrophoresis**

Laboratory Objectives

This exercise is intended to acquaint you with the principle of electrophoresis. After completion of this lab, you should know:

1. Components of electrophoresis apparatus
2. What is agarose and what is agarose gel
3. Components required to run a agarose gel electrophoresis
4. How agarose gel electrophoresis works; *i.e.*, how a mixture of dye molecules in different sizes are separated on agarose gel.
5. Relationship between size of molecules and migration distance on an agarose gel.
6. Factors affecting mobility of molecules through agarose gel

Introduction

Electrophoresis is a common procedure used in molecular biological laboratories to separate molecules based on the fact that charged molecules in solution will migrate in an electrical field. Electrophoresis entails placing the sample to be analyzed on the end of a gel prepared of agarose, starch, or polyacrylamide and a suitable buffer system. An electrical current is passed through the gel and under its influence the molecules present in the sample move through the gel matrix. The rate at which a molecule moves through the gel depends upon the temperature and pH of the gel, the concentration of the gel matrix, voltage of electrical current applied, and the size, shape and electric charge of the molecule. If a mixture of samples that move at different rates is electrophoresed, a series of bands appear in the gel at the position where the molecules have moved after a time. (See Figure 8-1).

**Agarose Gel**

Agarose is a mixture of carbohydrate polymers and the agarose gel is a matrix (porous lattice) of the carbohydrate polymers filled with water. In aqueous solutions, hydrogen bonding between polymers causes a gel to form at certain temperatures. Agarose has the melting temperature higher than gelling temperature. General purpose agarose forms gel around 35-40°C but melt at about 95°C; however, the gelling and melting temperatures can be affected by the concentration of the agarose.
FIGURE 8-1. Separation of DNA fragments based on their sizes using agarose gel electrophoresis.

FIGURE 8-2. An agarose gel showing relative migration rates of four commonly used tracking dye in agarose gel electrophoresis.
During dissolution, the agarose powder disperses in the water (at cooler temperatures), then it becomes hydrated (at moderate temperatures) and finally melts or dissolved (at high temperatures). If the temperature is too high before it becomes dispersed in the water, a shell of hydrated and/or gelled agarose can prevent rapid penetration of more water and can significantly slow dissolution. Sometimes, this is not easy to see, and the agarose may appear to have dissolved. If cast at this point the gel will be inconsistent and will be virtually useless for good separation of molecules. Therefore, melting of agarose should be started with water at room temperature.

Migration of Molecules Through Agarose Gel

When electric current is applied through the gel and an electrical potential (voltage difference between plus and minus poles) is established, negatively charged molecules migrate toward the positive (red wire); positively charged ones toward the negative (black wire). Under the electrical potential, charged molecules must pass through the pores in the lattice and move toward the electrode. Larger molecules will be slowed down more than smaller molecules because the smaller molecules can pass through the holes faster. As a result, a mixture of large and small molecules that has been run through an agarose gel will be separated by size.

Factors Affecting Mobility of Charged Molecules in Agarose Gel

The mobility of molecules in agarose gel depends on several factors. By understanding these factors, you can optimize separation of molecules for your purpose. Important among these factors are:

Agarose Concentration

You can resolve different sizes of molecules by using agarose at different concentrations. Higher concentrations of agarose facilitate separation of small molecules, while low agarose concentrations allow resolution of larger molecules.

Voltage Applied

As the voltage applied to a gel increased, charged molecules migrate faster. However, if larger molecules carry more net charges than smaller ones, they migrate proportionally faster than small molecules. Therefore, as higher voltage is applied, difference in distances migrated by small molecules and large molecules become smaller; thus, the resolution gets poorer.

Buffer

For agarose gel electrophoresis, a buffer solution must be used for both gel preparation and for running electrophoresis. The purpose of using buffer is:

1. to provide ions to support conductivity; i.e., ions in the buffer facilitate the flow of electrons (electric current). If deionized water is used instead of buffer, there will be no migration of charged molecules. Conversely, if you mistakenly use a buffer stock (concentrated buffer solution) without dilution, too much current will generate enough heat to melt the agarose gel.

2. to establish pH so that molecules can carry a net negative charge, which is the driving force for migration of molecules in the agarose gel electrophoresis. The optimal acidity for electrophoresis for separation of molecules is the pH where the molecules take the highest electrical charge.
Activity A: Preparation of Agarose Gel

Exercise.  
Separation of Dye Molecules Using Agarose Gel Electrophoresis

In this exercise, you will electrophorese four different dye samples using 1% agarose gel. These dyes are frequently used as a ‘tracking dye’ in gel electrophoresis of nucleic acids (DNA, RNA) and proteins. Tracking dyes are used to ‘track’ sample molecules on the gel, which are not visible.

Activity A: Preparation of Agarose Gel

Weighing and Melting Agarose

1. Add 25 ml electrophoresis buffer into a clean agarose melting vessel. You will use a 100-ml wide-mouth media bottle.

2. To weigh agarose, create an X fold lines on a 3” x 3” weighing paper by folding it from corner to corner as follows:
   2a. First, fold from corner A to corner B. Unfold paper.
   2b. Next, rotate it and fold from corner C to corner D. Unfold.
   2c. You now have an X on your paper.

3. Weigh out 0.20 g of agarose on the weighing paper with diagonal fold lines and add slowly (sprinkle) into the bottle with swirling to prevent forming clumps.

4. Swirl the bottle gently until the agarose powder suspends in the buffer. You should see no clump of agarose powder.

5. Cover the mouth of the bottle containing agarose suspension with a cap. **DO NOT TIGHTEN THE CAP** as vapor pressure might dangerously rupture the bottle while the solution boiling.

6. Place a small reference mark with a permanent maker on the glass wall (not on the labeling area) of the bottle containing the agarose suspension at the level of the liquid. If evaporation occurred during boiling and the liquid level falls below the reference line, water should be added to bring the volume of the liquid back to the starting level.

7. Let the suspended agarose sit at least 1 minutes at room temperature to hydrate the agarose.

8. Place the bottle containing agarose suspension loosely covered with a cap into the microwave oven.

9. Set the power to medium and the timer for 5 minutes.

10. Heat the agarose suspension for one (1) minutes or until it starts boiling.

**CAUTION!** Do not expose yourself to microwaves by looking through the microwave oven window. Always wear protective gloves. The bottle containing hot molten agarose can cause severe burns if allowed to contact skin. Additionally, molten agarose can suddenly boil over when swirled. Do not hold the bottle with the mouth of the bottle aimed toward yourself or others.

11. Stop the microwave oven by opening the door, and using protected gloves swirl the bottle gently to suspend the undissolved agarose. Place the bottle back into the microwave and heat it again.
12. Repeat heating and swirling every 30-45 seconds (or, every time the solution starts boiling) until all of the small translucent agarose particles are dissolved. The agarose should be absolutely clear without undissolved agarose particles, which often appear as clear speckles that are hard to detect.

13. With the cap still in place, but not tightened, place the bottle containing completely molten agarose in a 65°C water bath and wait until the temperature equilibrates. You may let the bottle stand at room temperature to cool it down with occasional swirling and check the temperature using a thermometer. **However, the thermometer can be a source of contamination and this will not be used in this lab.** If you can hold the bottle comfortably in your hand, the molten gel is ready for you to pour on the gel tray.

**CAUTION! Never pour hot agarose solution on the gel tray. Hot agarose may cause the gel tray and comb to warp and/or craze and will significantly decrease the lifetime of the tray. Also, warped gel tray will result in a gel with uneven thickness and sample wells of uneven depth.**

**Casting Gel**

14. While the molten agarose gel cools down, set up a gel caster as follows:

14a. Place the Mini-Gel Caster on a level surface (Choose a location where the gel caster won’t be disturbed by other activities while the gel solidifies).

14b. Disengage and slide the movable wall to the open position by turning and lifting the cam peg upward.

14c. Place the open end of the gel tray against the rubber seal of the fixed wall of the gel caster.

14d. Slide the movable wall against the edge of the gel tray (Figure 8-3).

![Figure 8-3. Sealing the gel tray using Mini-Gel Caster to cast an agarose gel.](image-url)
14e. To seal the open tray ends, engage the cam peg by turning and pressing down simultaneously. When the cam peg has dropped into place, turn the peg in either direction until resistance is felt. This will seal the edges of the tray for gel casting.

14f. Place the leveling bubble on the gel tray and level the gel tray using the leveling feet in the Mini-Gel Caster. (Note: You must level the gel tray, not the gel caster.)

14g. After leveling, place the comb into the slot closest to the end of the gel tray on the fixed wall side of the gel caster. Make sure the comb is set properly on the notches on both sides of the gel tray. (Note: If the comb placed on the movable wall side, it will be difficult to remove the comb or disengage the cam peg as the gel comb and the cam peg lever will contact each other.)

15. When you are ready to pour the gel, bring your bottle containing molten gel to your gel casting area.

16. Without letting the bottle sit on the table too long, slowly pour the molten agarose (<60°C) onto the gel tray to avoid forming bubbles (See Figure 8-4). If you let the gel sit and solidify, you will need to re-melt using the microwave and cool it to <60°C.

17. Remove any air bubbles from the agarose on the tray using a clean micropipette tip.

18. Immediately after pouring the gel, wash the bottle and cap with plenty of running water before the agarose solidifies. DO NOT pour molten agarose into the drain without water. It will solidify in the drain pipe to clog it.

19. Allow the gel to cool and solidify for 15-20 minutes at room temperature. As it “gels,” it will turn opaque (cloudy). The gel must be completely solidified at room temperature before the comb is removed or the wells may be improperly formed and the sample bands distorted.

NOTE: Do not place an agarose gel in a refrigerator in an attempt to speed up solidification. The agarose gel must be completely solidified at room temperature before being placed in the refrigerator for curing or storage; otherwise, the texture of the gel will become uneven and migration of dye molecules in the gel will be hindered.

NOTE: Do not disturb the gel casting setup until the gel completely solidified as it may result in a gel of uneven thickness and/or texture. Also, if you disturb or remove the comb, the wells will collapse or deform necessitating you to prepare a fresh gel.
Activity B: Practicing Sample Loading

While the gel solidifies, practice loading samples in the wells of a gel. Each student should practice loading sample repeatedly to be able to load samples for experiment comfortably.

1. Obtain the following items:
   1a. an electrophoresis chamber designated for practice only.
   1b. a practice gel tray and a slab of practice gel.
   1c. a vial or tube containing sample for practicing gel loading.

2. Place a practice gel in its tray on the platform in the middle of the electrophoresis chamber unit with the well side away from you.

3. Add about 275 ml of tap water into the buffer chamber.

4. Adjust the water level in the chamber so that the gel should be completely submerged in the water about 1 mm below the water level.

5. Using a gel-loading tip, load 20 μl of practice sample into each well on your practice gel. For practice, you may use a tip repeatedly as long as the sample solution does not adhere on the inside wall of the tip interfering with accurate measurement.

   To load a sample:
   5a. With your own most comfortable posture, use two hands to keep the micropipette steady over the gel.
   5b. Expel any air in the pipette tip before loading the sample.
   5c. Position the pipette tip under the surface of the buffer and over the well (or only a millimeter, or so below the top edge of the well).
   5d. Slowly expel the sample into the well.
   5e. Be careful not to insert the pipette tip into the well and punch through the bottom of the well.

6. Immediately after mastering gel loading techniques, return all the practice items including the practice gel and the practice loading buffer to the supply station.

Activity C: Running Agarose Gel Electrophoresis

Removing Gel Comb from the Solidified Agarose Gel

1. When the gel completely solidifies, remove the gel tray containing solidified agarose gel from the gel caster and transfer to the electrophoresis chamber unit.

   1a. To remove the gel tray from the caster, disengage the cam peg by turning and lifting upward.
   1b. Before moving the wall of the gel caster, carefully slide the gel tray along the rubber gasket to break the seal between the solidified agarose and gasket. If the solidified gel is stuck on the gasket, the gel wells will be ripped when the wall of the gel caster is pulled away.

2. Place the agarose gel in its tray onto the leveled electrophoretic base (platform in the middle of the electrophoresis chamber unit) so that the sample wells are near the cathode (negative pole; black) and away from you.

3. If the electrophoresis chamber is empty, pour about 275 ml of electrophoresis running buffer (0.5X TBE; Tris-borate-EDTA, pH 8) into the buffer chamber.

4. Adjust the buffer level in the chamber so that the gel completely submerged in the buffer about 1 mm below
5. Remove the gel comb, by slowly and carefully lifting one end of the comb slightly (1 mm or so) and then the other. Repeat this rocking motion until the comb is free of the gel. If the comb is pulled quickly, vacuum may be created in the wells and wells may collapse.

**NOTE:** When the gel is completely solidified, the comb may be removed and the gel can be used immediately or cured for 15-20 minutes, with the comb left in place, in the refrigerator. Gels can be stored for about a week before use. For short-term gel storage (overnight), the comb is left in place and the tray containing the gel and comb is moistened with buffer, wrapped in plastic wrap, and stored overnight in the refrigerator. For longer than overnight storage, the gel should be stored in the same buffer solution in a tightly sealed container to avoid desiccation during storage. The gel comb can be left in position or removed.

### Loading Dye Samples on the Gel

6. Obtain dye samples.

7. **Using a fresh gel loading tip each time**, load 10 μl of each of the four dye samples into each well on your gel in the order indicated in Table 8-1.

<table>
<thead>
<tr>
<th>Sample Well #</th>
<th>Dye Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dye Mixture</td>
</tr>
<tr>
<td>2</td>
<td>Bromophenol blue</td>
</tr>
<tr>
<td>3</td>
<td>Phenol red</td>
</tr>
<tr>
<td>4</td>
<td>Orange G</td>
</tr>
<tr>
<td>5</td>
<td>Dye Mixture</td>
</tr>
<tr>
<td>6</td>
<td>Bromophenol blue</td>
</tr>
<tr>
<td>7</td>
<td>Phenol red</td>
</tr>
<tr>
<td>8</td>
<td>Orange G</td>
</tr>
</tbody>
</table>

### Running Agarose Gel Electrophoresis

8. Place the lid on the electrophoresis chamber carefully so as not to disturb the sample. To attach the lid correctly, match the red and black banana jacks with the red and black banana plugs of the cell.

9. Connect the electrophoresis chamber to the power supply by inserting the molded two-prong plugs into the power supply's high voltage output jacks.

10. Press the Power switch that is located on the right side of the power supply unit. Press the side labeled “I” on the switch.

11. Press the Constant key (**Const**) to select constant voltage.

12. Use the Scroll key to enter 100 volts.

13. Press the Run key (with running person icon) to start the run.

14. Electrophorese the samples until the fastest dye front reaches 4/5 of the distance between the wells and the opposite end of the gel. Do not let the dye front migrate out of the agarose gel.

15. Stop the run by pressing the Run Key.
16. Press the Power switch to turn off the power supply and unplug the black/red power cords from the power supply unit.

17. Remove the top of the electrophoresis chamber, and remove the gel tray containing the gel. *Be careful, the gel is very slippery and easily broken.*

**Imaging the Gel**

1. Remove liquid from the sides and bottom of the gel tray using a piece of paper towel.
2. Place the gel on its tray on a piece of white paper.
3. Take a photograph of your gel image per group to use in your lab report.

**Wrapping Up Lab Session 1**

1. Discard used gel in the trash, thoroughly rinse the gel tray under running tap water, and then place it vertically on the rack placed on the sink-top.
2. Leave the used electrophoresis buffer in the chamber as it can be used for another run after replacing a portion of it with fresh buffer.
3. If instructed to empty the electrophoresis chamber to replace the exhausted buffer, decant the used buffer into the drain and rinse the lid and the electrophoresis chamber with tap water and place them on a piece of paper towel to dry them.

**Analyzing Results**

Measure the distance traveled by the dyes and record the results in Table 8-2. Measurements are typically done from the leading edge of the sample well to the leading edge of the dye band.

<table>
<thead>
<tr>
<th>Sample Well #</th>
<th>Dye Samples</th>
<th>Distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dye Mixture</td>
<td>, ,</td>
</tr>
<tr>
<td>2</td>
<td>Bromophenol blue</td>
<td></td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>Orange G</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Dye Mixture</td>
<td>, ,</td>
</tr>
<tr>
<td>6</td>
<td>Bromophenol blue</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Phenol red</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Orange G</td>
<td></td>
</tr>
</tbody>
</table>
Q 8-1. Place dye samples in order of migration rate (fastest first, slowest last):

Q 8-2. Which dye molecule is the largest in size assuming the net charges are the same or similar to each other.

Q 8-3. Did dye samples, each loaded in two separate wells traveled the same distance? If not, Explain any possible reasons why not.

Q 8-4. Identify each component in the dye mixture.