# Lab 5

# Agarose Gel Electrophoresis

**A. Expt. #8: Agarose Electrophoresis of Plasmid Digests & PCR Samples**
**B. Expt. #9: Inoculation of Bacterial Cultures-II**

## Introduction

One of the main techniques used in molecular biology and biochemistry is gel electrophoresis. This technique is used as a relatively fast, easy and economical method for separating proteins or fragments of DNA on the basis of size and/or charge. There is a wide variety of electrophoresis techniques that are used for either preparative or analytical purposes. In this course you will use native agarose gel electrophoresis to analyze the restriction digest of your plasmid DNAs.

The fundamental theory behind electrophoresis is to place the sample in a gel-like material (usually agarose or acrylamide) and expose the gel to an electric current. The negatively charged phosphate groups on the nucleic acids confer an overall negative charge, making the DNA molecules migrate to the positive electrode (anode) during electrophoresis. As the sample migrates to the positive electrode, it has to pass through the pores of the gel. Molecules will be separated on the basis of size, in which smaller molecules more easily migrate through the gel and will therefore run faster than larger molecules. The mobility of a DNA molecule is inversely proportional to the logarithm of its molecular weight. It is therefore possible to find out how big a fragment of DNA is by analyzing its mobility in a gel.

Gels can be poured in a variety of shapes, sizes, and pore sizes. The choice of gel size and type primarily depends on the type and size of the molecules that are being separated. Agarose gels are run horizontally and submerged in a tank of buffer, whereas acrylamide gels are run vertically with the gel sandwiched between two glass plates. A gel with a large pore size (low percentage agarose or acrylamide) will efficiently separate (resolve) molecules of high molecular weight (long DNA fragments). Unfortunately such large pore size gels are not good for resolving small molecular weight DNAs because they separate too much and run as diffuse bands. High percentage gels, with small pore sizes, are more appropriate for resolving small molecular weight DNA fragments into sharp bands. Agarose gels have lower resolving power than acrylamide but have a greater range of separation. Agarose gels are good for separating DNA fragments in the range of 200 bp to 50 kb in length. Acrylamide gels are better for high resolution of short DNA fragments. The table at the top of the next page lists several agarose and acrylamide gel concentrations and the effective range of separation of DNA fragments. The migration position of the tracking dyes xylene cyanol (XC) and bromophenol blue (BPB) in different acrylamide gels are also shown. These dyes are very useful in both agarose and acrylamide gels to get an estimate of how far the DNA fragments have migrated (see next page).

| Agarose (%[w/v]) | Range (kb) |
|---|---|
| 0.3 | 5-60 |
| 0.7 | 0.8-10 |
| 1.2 | 0.4-6 |
| 2.0 | 0.1-2 |

| Acrylamide (%[w/v]) | Range (bp) | Dye migration XC | Dye migration BPB |
|---|---|---|---|
| 3.5 | 1000-2000 | 460 | 100 |
| 5.0 | 80-500 | 260 | 65 |
| 8.0 | 60-400 | 160 | 45 |
| 12.0 | 40-200 | 70 | 20 |

Maniatis, p. 6.37

# A. Expt. #8: Agarose Gel Electrophoresis of Digests & PCR Rxns.

## I. <u>Prepare the agarose gel</u>.

1. **Each <u>group</u> should prepare two liters of 1X TBE containing 0.5 μg/ml ethidium bromide.**
   The 10X TBE stock is located in bottles on the lab prep bench. Each group should have a 10 mg/ml stock of ethidium bromide (stored in your styrofoam box in the refrigerator). Prepare this two-liter solution of 1X TBE / EtBr in a 2-liter size, orange-capped plastic container. (Don't forget to label the contents of the container with tape and indicate your group number. This TBE / EtBr solution will be used in the following steps. You will also need this solution for future experiments, so store your container of TBE / EtBr above your bench.

   **REMEMBER, EtBr IS A POTENTIAL MUTAGEN.** ALWAYS WEAR GLOVES WHEN WORKING WITH EtBr. IF YOU HAVE ANY QUESTIONS OR CONCERNS, TALK TO ONE OF THE INSTRUCTORS.

2. **Melt 70 ml of 1% agarose gel in 1X TBE buffer in the microwave.**
   A 1% gel contains 0.7 g of agarose in 70 ml of 1X TBE / EtBr. Add the agarose to 70 ml of 1X TBE / EtBr stock in a 250 ml bottle. Swirl to briefly suspend the agarose and make sure it is dispersed. Make sure the cap is loose so that pressure will not build in the bottle when it is heated. Heat the mixture for 2 min at full power. Wearing an oven mitt, periodically take out the bottle and swirl it to mix any agarose that has not melted. **Be Careful! — The bottle is very hot and may boil over when mixed.** (To reduce the possibility of boil-over, never microwave a bottle that is more than 50% full.) Never hold the bottle from above, since the rising steam may burn your hand. While swirling the bottle, look for undissolved agarose that appears as clear specks in the solution. If necessary heat the mixture again for 1 minute to completely dissolve the agarose. <u>Let the agarose slowly cool to about 60°C before pouring. This should take about 20 minutes.</u>

3. **Pour the cooled agarose into the gel casting box with two 14-well combs in place and let it harden for approximately 30 minutes.**
   Before adding the agarose, make sure the gel apparatus is clean, dry, and on a level part of the bench. Make sure the gel tray is in the pour position in the gel box and that the comb is the proper height above the tray. If the comb is too close to the tray, the bottoms of the wells may rip when the comb is removed causing the sample to spread over the bottom of the tray under the gel. **Make sure the agarose is not too hot or it will warp the tray.** If there is not a good seal of the tray in the pouring position it is wise to pipet a small amount of agarose around the edges and let it cool. This will seal any cracks so that the gel does not leak when the remaining agarose is added. Rinse out your agarose bottle with tap water when you are finished and keep it at your bench for future use. DO NOT put solidified or liquid agarose gel in the sink!!! This will clog the sinks. Always dispose of agarose in the biohazard trash cans.

## II. Prepare samples from your PCR reactions.

You will also be analyzing your PCR reactions on this agarose gel. Transfer 10 μl of each PCR sample from last week's Expt. #7 to a fresh 1.7 ml microfuge tube and add water and gel loading dye as specified below.

| | |
|---|---|
| $H_2O$ | 8 μl |
| 10X Gel loading dye | 2 μl |
| PCR reaction sample | 10 μl |
| | =20μl |

## III. Prepare uncut DNA samples for agarose gel electrophoresis.

How can you tell whether the enzyme has cut or not? The best way is to include on your gel a comparison lane containing uncut vector, i.e., vector DNA that has not been treated with a restriction enzyme. Prepare an uncut control sample for each vector in the following way:

| | |
|---|---|
| Sterile $dH_2O$ | 8 μl |
| 10X Gel Loading Dye Mix | 2 μl |
| Miniprep Plasmid DNA | 10 μl |
| | = 20 μl |

## IV. Load your agarose gel.

1. **When the gel has hardened, remove the tray and place it in the box in the electrophoresis position.**

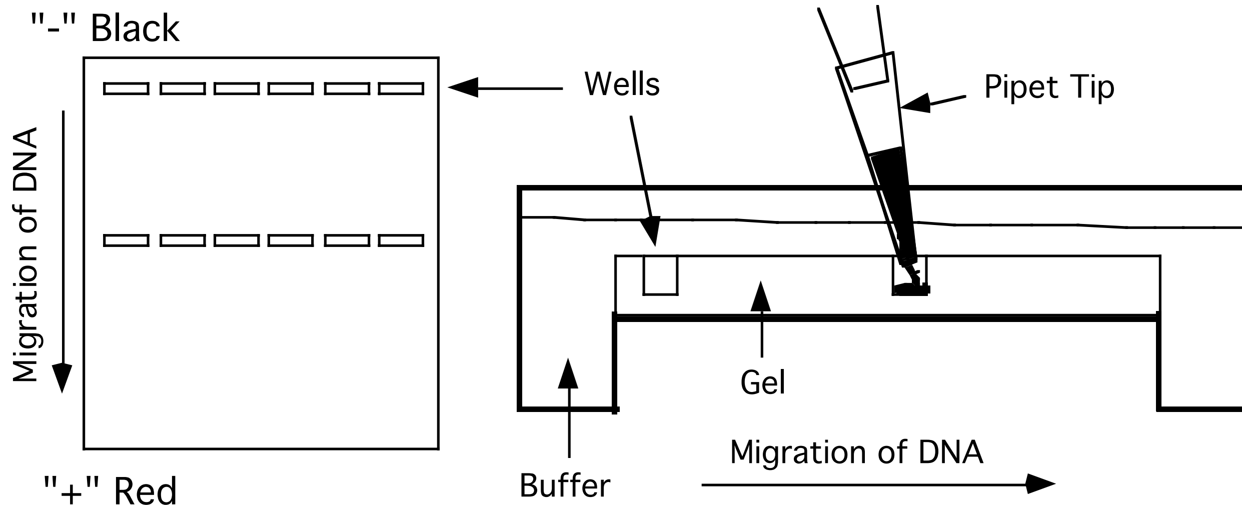
2. **Fill up the gel box with 1X TBE / EtBr running buffer so that the gel is submerged under approximately 1 inch of running buffer. Gently pull the combs out.**

3. **Load the DNA samples (all are approx. 20 μl) into wells using a P-20 pipetman (see diagram on next page). In addition, load two lanes each of 20 μl of pre-made DNA**

**size standards known as 1Kb Plus DNA Ladder (see p. 5-7). Follow the loading order on the next page!**
Be careful not to stick the pipet tip through the well! Dipping the tip just below the top of the well is the objective. The loading dye is more dense than the electrophoresis buffer and will make your DNA sample sink. Also, expel the DNA solution **gently** and slowly. If you expel too quickly your sample may be forced out of the well. As little as 2 ng of DNA in a band can be detected with EtBr stain. Overloading (too much DNA per band) can cause smearing and trailing of the DNA.

**Here is the recommended order for loading your various samples on your agarose gel. This will allow easier interpretation of your results.**

| Lane # | Sample |
|---|---|
| 1 | 1 Kb Plus DNA Ladder Size Standard |
| 2 | Uncut Plasmid DNA “A” |
| 3 | EcoR I Digested Plasmid DNA “A” |
| 4 | PCR of Plasmid DNA “A” |
| 5 | Uncut Plasmid DNA “B” |
| 6 | EcoR I Digested Plasmid DNA “B” |
| 7 | PCR of Plasmid DNA “B” |
| 8 | Uncut Plasmid DNA “C” |
| 9 | EcoR I Digested Plasmid DNA “C” |
| 10 | PCR of Plasmid DNA “C” |
| 11 | Uncut Plasmid DNA “D” |
| 12 | EcoR I Digested Plasmid DNA “D” |
| 13 | PCR of Plasmid DNA “D” |
| 14 | 1 Kb Plus DNA Ladder Size Standard |




4. **After loading the gel, attach the lid to the gel box and make sure the leads are connected in the proper places (DNA migrates to the red-colored anode lead) and apply a voltage of 80-90 V.**
After you have turned on the gel, make sure that bubbles are coming from the wire electrodes, indicating that there is current in the gel. Take note of the current (in mA) that your power

supply is registering and write the value in your notebook. Check the gel after a couple of minutes to make sure the dyes are running in the proper direction.

5. **When the dyes have migrated an appropriate distance, turn off the power supply, and remove your gel.**

   The appropriate distance for running DNA samples is very flexible and depends on the sample you are analyzing. You will learn how far to run samples through experience. For now, ask the instructors for help.

6. **Take a photo of the gel for your notebook.**
   Immediately after you print out a photo of your gel, tape it in your lab notebook with transparent and label all of the lanes with a fine Sharpie marker. Also label your name and the date that you took the picture in case it falls out of your notebook. Try to label it as neatly as possible so that anyone (especially your instructors!) can read and interpret the gel. Each lane should be numbered, and the contents of each lane should be noted near the picture for easy reference.

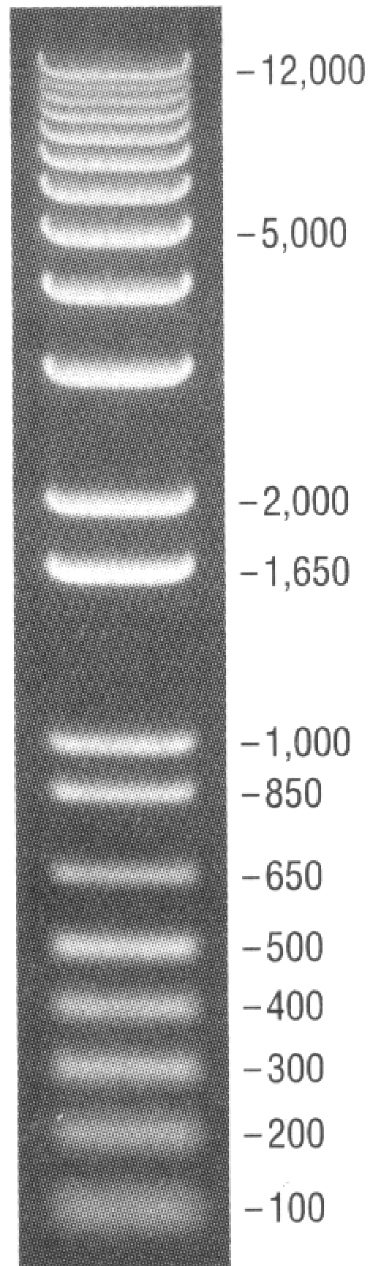
## V. Interpret your restriction digests and PCR reactions

Now that you have taken a photograph of your agarose gel it is time to interpret the data that you obtained.

1. **Determine the size and number of DNA fragments that result from each restriction digest or PCR reaction.**
   By comparing the distance that your fragments have migrated relative to the DNA standards (see p. 5-7 for a photograph of the 1 Kb Plus DNA Ladder), you can determine the approximate size (in nucleotide pairs) of your fragments. In the four digest lanes make sure you can identify which fragments are the plasmid DNA backbone and which fragments are the DNA inserts. How do the sizes of the PCR products (in the four PCR sample lanes) compare with the sizes of the restriction digest inserts?

2. **Draw a circular map of each of your plasmids showing the location of your insert.**
   Indicate the size of the DNA inserts. Indicate the positions of the restriction enzyme sites in the vector and, if present, in the insert. Do all of your plasmids contain inserts?

# "1 Kb Plus DNA Ladder" Size Standard



# **Questions:**

1) Are your PCR results consistent with the results of your restriction digests?

2) Would you expect to see more than one band in the PCR reaction? Explain.

3) Were there any ambiguities in your digestion results? For example, did you observe any partial digests or failures to digest? If no digestion occurred, what are some reasons for this?

# B. Expt. #9: Inoculation of Bacterial Cultures-II

This is the same protocol used in Expt. #4 in Lab 2. Each student will inoculate six more overnight cultures for preparing plasmid DNA in the next lab period.

1. **Each person should prepare a 15 ml solution of LB with 100 μg/ml ampicillin.**
   Each person should prepare 15 ml of LB-amp solution with sterile LB broth and sterile 100 mg/ml Ampicillin stock in a sterile 50-ml conical tube. (Remember that you saved your aliquot of 100 mg/ml Ampicillin from Expt. #4 in your freezer box.)

2. **Into each of six sterile snap culture tubes, add 2 ml of LB + 100 μg/ml ampicillin solution.**
   Each student will inoculate six cultures. Remove the cap from a sterile snap-cap culture tube. Work quickly to minimize contact of the tube with the possibly contaminated air. Use a sterile pipette to transfer 2 ml of LB-amp into the tube.

3. **Before inoculating your media with an *E. coli* colony it is critical to label your tubes!**
   Be sure to name these new cultures unique names from those that you grew up in Expt. #4 (see p. 2-12 for instructions on naming clones.)

   To avoid confusing these six new cultures with those started in Expt. #4, it is best to give these new cultures unique names that are consistent with your existing numbering scheme. For example if your first set of four clones were labeled 2A-1, 2A-3, 2A-5, and 2A-7, it would be a good idea to label these six new clones as 2A-9, 2A-11, 2A-13, 2A-15, 2A-17, and 2A-19. (See Supplement #2 as an example.)

4. **Inoculate media with a bacteria colony.**
   Once your tubes are labeled, inoculate each tube with a **single white** bacterial colony by touching a sterile wooden stick to the colony, making certain that some of the cells have been transferred to the stick, and then dipping the stick into the liquid and shaking it a bit. Replace the tube's cap as soon as inoculation is complete.

5. **Grow overnight at 37ºC with aeration.**
   Place your group's newly inoculated cultures in a culture tube rack (with your group number on it) on the prep bench. At the end of today's lab one of the instructors with place your cultures in the shaking 37ºC incubator for overnight growth. The next morning your saturated cultures will be taken out of the shaking incubator and returned to your culture tube rack and placed in your refrigerator until next week.