CONDUCTING ELECTROPHORESIS: BACKGROUND INFORMATION

When DNA is digested by restriction enzymes, the result is a DNA solution containing DNA fragments of varying sizes. The number of fragments and the sizes of the fragments depend on the restriction enzyme used and the size of the original DNA molecule. In order to determine what the DNA fragment sizes are, it is necessary to: (1) separate the fragments by size; (2) have some way to visualize the DNA; and (3) have a standard to which the fragments can be compared. The first is accomplished by separating the DNA using agarose gel electrophoresis.

Electrophoresis is the movement of a charged molecule in an electrical field. A charged molecule will migrate toward the electrode of opposite charge. Since DNA molecules are negatively charged, they will migrate toward the anode (positive electrode).

If electrophoresis were done without a solid matrix (e.g., agarose), the rate of migration would be determined strictly by the charge:mass ratio of the molecules. Since DNA is a repeating polymer, DNA molecules of all sizes have the same charge:mass ratio. (If an electrical current is applied to DNA in a buffer solution alone, the DNA molecules would all migrate toward the anode at the same rate.) Thus, it is necessary to add a matrix such as agarose or acrylamide to act as a sieve and separate the DNA molecules based on their size.

The choice of matrix, agarose or acrylamide, is determined by the sizes of the molecules to be separated. Acrylamide is used primarily to separate proteins and small DNA molecules (under 1000 base pairs). Agarose is the matrix used to separate most DNA molecules.

Agarose is a polysaccharide (from algae) that can be dissolved in hot water. As the agarose solution cools, it solidifies to form a matrix of gelatin-like consistency. The matrix contains pores through which the DNA molecules must pass. The size of the pores, and hence the sizes of the DNA molecules that can be separated on the gel, is determined by the concentration of the agarose solution. For example, large DNA molecules (>10,000 base pairs) can best be separated on a 0.3% agarose gels (e.g., larger pores), whereas small DNA molecules (100-3000 base pairs) would separate with better resolution on a 2.0% agarose gel (e.g., smaller pores). In the experiments in this kit, 0.9% agarose gels will be used to separate the DNA molecules.

As these 0.9% gels are prepared, a comb is placed in the gel at the end closest to the cathode (negative electrode). After the agarose solution has solidified, the comb can be removed, leaving small holes or *wells* in the gel into which the samples will be loaded. The DNA samples are mixed with a loading buffer that contains glycerol and a tracking dye. The glycerol adds density to the samples, assuring that they will stay in the wells when loaded. The tracking dye usually contains a dye like bromphenol blue, a small molecule that migrates through the gel at a position approximately equivalent to a DNA fragment of 300 base pairs, or Orange G, which migrates through the gel at a position approximately equivalent to a DNA fragment of 50 base pairs. The dyes serve two functions. They makes it easier to see the samples while the wells are being loaded and, since the dye can be seen as it migrates through the gel, it can be used to estimate how far the DNA has migrated in the gel.

When it is time to load and run the gel, the gel is covered in buffer, the comb carefully removed, and the samples loaded into the wells. A standard solution consisting of DNA fragments of known sizes is loaded into an adjacent well. The lid is placed on the gel box, the gel box is connected to a power supply, and an electrical current is passed through the gel.

The DNA molecules immediately begin to migrate toward the anode, with smaller molecules migrating more rapidly than larger DNA molecules (Figure 1).

It is necessary to have some method for visualizing the DNA in the agarose gel. In the research lab, a stain called ethidium bromide is frequently used. Ethidium bromide can be added to the gel and buffer solutions or the gel can be post-stained in an ethidium bromide solution. Either way, the dye is inserted between the stacked bases of DNA and glows bright orange when the gel is exposed to UV light. Unfortunately, ethidium bromide is a mutagen and hazardous to handle, and thus is not suited for use in most classrooms.

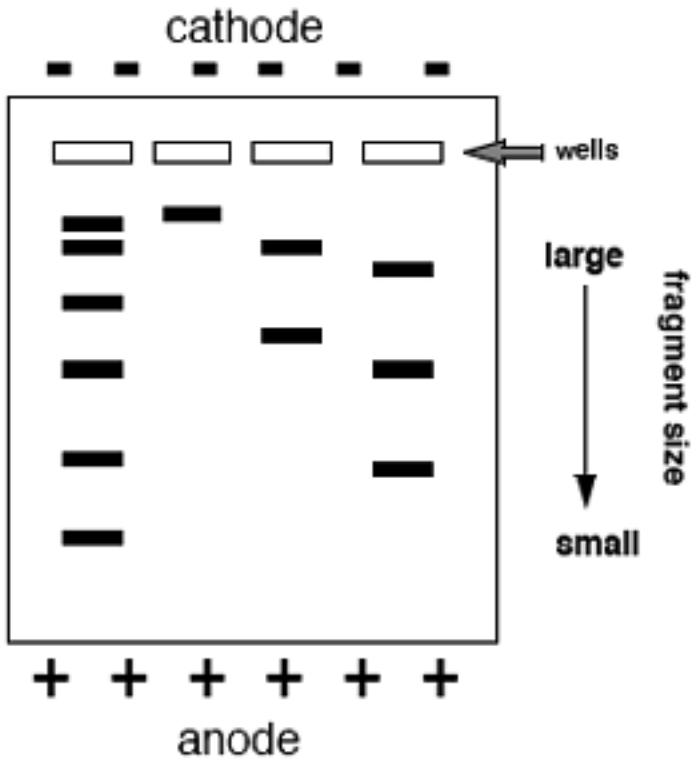


Figure 1. Agarose gel electrophoresis of DNA fragments. The negative electrode and gel loading wells are at the top of the figure. The DNA fragments migrate toward the positive electrode. In this graphic, Lane 1 (from left) represents a standard (DNA ladder), Lane 2 represents uncut sample DNA, and Lanes 3 and 4 represent restriction enzyme digests of sample DNA.

In the following procedures, either methylene blue or Bio-Safe is used as a post-stain for DNA. Although they are not as sensitive as ethidium bromide, they are less hazardous. The DNA bands appear blue on a clear background and the migration of the fragments can be measured. Using the migration distances of the DNA fragments in the standard, a standard curve can be generated and the sizes of the DNA fragments from the experimental restriction digests can be calculated (see below for details).

ANALYZING DATA FROM AN AGAROSE GEL

Analysis procedures can be as simple as determining the guilty party (or cat) if you followed a crime scene scenario. If you wish to go further with the analysis, you may have your students measure the migration of the DNA bands, plot a standard curve, and determine the sizes of the DNA fragments from the enzyme digests. A final step, also optional, is to use the data to create a simple map of the plasmid.

Procedure: Constructing a standard curve and determining the sizes of the DNA fragments from the enzyme digests

1. Cover the light box with plastic wrap or place the gel in a plastic sandwich bag. Place the gel on the light box.

2. First look at the lane containing the 1 kb Plus DNA ladder. Compare it to a picture of the bands with their known sizes. To figure out which band is which, look at the figure, and find the two bands that are 1650 and 2000 base pairs. Notice that these two bands are separated from the other bands, and rather easy to find.

3. Find these two bands on the stained gel and measure their migration from their point of origin in the gel, i.e., from the well. Measure from the bottom the well to the foremost edge of the stained band. Be certain to measure each from the same point, e.g., from the bottom of the well each time, not the bottom one time and the top of the well the next. Record the base pair size of the band and its migration distance.

4. Working up (toward larger DNA fragments) and down (toward smaller DNA fragments) from the 1650 and 2000 bp bands, measure and record the migration of the other bands in the DNA ladder.
   The large bands will be too close together to be measured accurately, and the smaller bands may have migrated off the bottom of the gel. Remember, if you have run the gel until the dye has reached the bottom of the gel, then anything smaller than dye (50 bp for Orange G or 300 bp for bromphenol blue) will have run off the bottom of the gel.

5. Measure the migration of the bands in the experimental lanes and record the migration distances.

6. Create a standard curve using the data from the 1 kb DNA ladder. Graph the migration distance of the DNA fragments (x-axis) against the size of the DNA fragments (y-axis) on semi-log graph paper.

7. Connect the points to form a line.

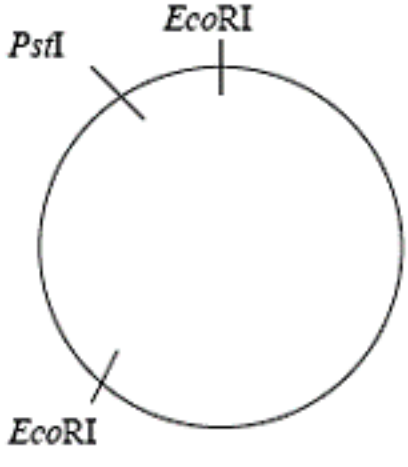
8. To determine the size of an enzyme-digested DNA fragment, find where the migration distance of the DNA fragment intersects the standard curve. Draw a line from this point to the y-axis. Where this line meets the y-axis is the size of the fragment.

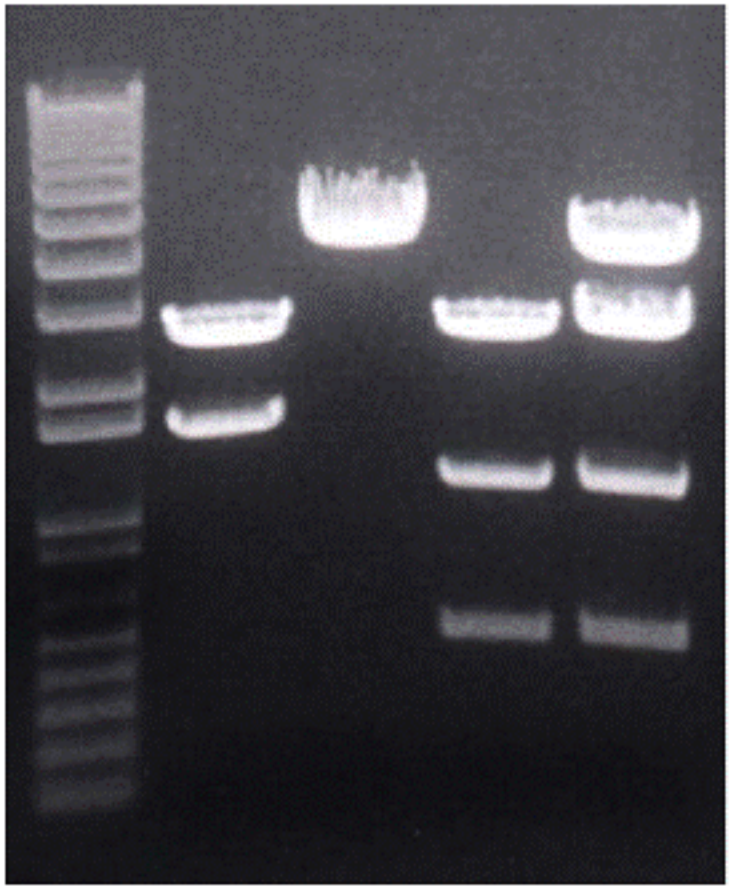
SIZE OF DNA FRAGMENTS GENERATED BY RESTRICTION DIGESTS OF pHOKIE PLASMID DNA

fragment	size (in basepairs)
intact pHokie	4200
*Eco*RI digest	2700 1500
*Pst*I digest	4200
*Eco*RI & *Pst*I double digest	2700 1100 400
Crime scene sample (mixture of *Pst*I digest and *Eco*RI & *Pst*I double digest)	4200 2700 1100 400

Sample gel and plasmid map

Lane 1 DNA ladder
Lane 2 *Eco*RI digest
Lane 3 *Pst*I digest
Lane 4 *Eco*RI & *Pst*I double digest
Lane 5 Crime scene sample





Procedure for DNA Fingerprinting

1. Obtain a precast agarose gel with SYBRsafe stain and a comb added. Each gel will be in a ziplock bag.
2. Remove the precast gel with a comb from the ziplock bag
3. Carefully remove the comb and casting gates from the gel.
4. Use 5 µl microliter pipettes to load 5 µl of each sample tube into separate wells in the gel. Be sure that the micropipettor tip is below the surface of the buffer and just above the center of each well that you load. CHANGE
5. Leaving an empty lane on right, load the samples into the wells in the order left to right shown below: L 1 2 3 CS

L = ladder DNA (White sample tube labeled L - standardized control sample
1 = (Yellow sample tube labeled X) Suspect Bob Smith, former thief
2 = (Purple sample tube labeled Y) Suspect Jim Dale, boyfriend
3 = (Blue sample tube labeled Z) Suspect Pam, wife of victim
CS = (Red/pink sample tube labeled E) crime scene evidence DNA

6. Once the wells are loaded, place the gel in the casting tray into the electrophoresis chamber
7. Pour enough SB buffer into the gel box so that the gel is completely covered (approximately 0.5-1.0 cm coverage over the top level of the gel).
8. Once the wells are loaded, put the top on the gel box by connecting red electrode to red chamber connection and black electrode to black chamber connection.
9. Connect the electrical cables or leads to the electrode connection on the chamber and into the connection outlet on the power supply connecting red to red and black to black.
10. Plug in the power supply and turn the unit to the desired voltage at 125 volt setting. This voltage should be preset on the power supply.
11. Run until the ladder well fingerprint is one centimeter from the bottom of the gel. At this point, the current can be turned off and the leads (cables) disconnected. Also unplug the power supply. This will take about 30 to 40 minutes. Begin monitoring the progress of the movement of the samples after 20-25 minutes.
12. Remove the casting tray from the gel box. Carefully slide your gel off the casting tray into a ziplock bag that is labeled for your group. The extra SB buffer can be reused by other classes. Please ask for directions on the procedure for recycling the SB buffer from the gel box.
13. Obtain UV goggles to wear to protect your eyes throughout this next step of the procedure. Take the gel in the ziplock bag to the designated area of the lab where you can view it under UV light.
14. Place your gel in its ziplock bag onto the UV light source and observe the results. Sketch these results on your worksheet
15. Dispose of your gel in its ziplock bag in a labeled chemical disposal container.
16. Upon completion of the lab
- dispose of designated materials in the appropriate places.
- leave equipment as you found it.
- check that your work station is in order.
- wash your hands.