Lab III-7

Simulated DNA Separation by Gel Electrophoresis

Equipment and Materials

You'll need the following items to complete this lab session. (The standard kit for this book, available from *www.thehomescientist.com*, includes the items listed in the first group.)

Materials from Kit

- □ Goggles
- □ Agar
- □ Beaker, 250 mL
- □ Graduated cylinder, 100 mL
- □ Glycerol
- □ Pipettes
- □ Reaction plate, 96-well
- □ Ruler
- □ Stain: Hucker's crystal violet
- □ Stain: Methylene blue
- □ Stain: Safranin O
- □ Stirring rod
- □ Thermometer

Materials You Provide

- □ Gel electrophoresis apparatus (from III-6)
- □ Measuring spoons
- □ Microwave oven
- □ Soda bottle (2 liter, clean and empty)
- □ Sodium chloride (table salt)
- □ Sodium bicarbonate (baking soda)
- □ Toothpicks
- □ Water (distilled or tap; see text)

Background

Gel electrophoresis is conceptually similar to chromatography, but with a slightly different goal. Ordinarily, we use chromatography to separate different compounds from a mixture. With DNA gel electrophoresis, the goal is to separate DNA fragments of different sizes and masses, which are produced by treating a DNA specimen with *restriction enzymes* to cleave it at known locations into fragments.

To imagine the shape and structure of DNA, think of a standard ladder, with two sides and rungs joining them. Twist the tops of the sides of the ladder to form two counter-rotating, interlocked spirals, with the rungs still joining them. In DNA, the sides of the ladder are made up of molecules of a sugar called 2-deoxyribose, with those sugar molecules bonded together by phosphate groups. The rungs, called *base pairs*, are formed by bonded pairs of four amino acid bases:

called *adenine* (abbreviated A), *cytosine* (C), *guanine* (G), and *thymine* (T). Adenine bonds only with thymine, forming an *AT base pair*, and cytosine only with guanine, forming a *CG base pair*.

To visualize the complexity of DNA, imagine that your ladder, rather than having only a few rungs, has millions or billions of rungs. Genomic human DNA, for example, has about 3.2 billion base pairs. Untwisted and stretched out, a single strand of genomic human DNA would form a “ladder” about 2.4 µm wide and 2 meters long, with 3.2 billion rungs.

> The number of base pairs in genomic DNA is unrelated to the complexity of the organism. For example, while honeybee DNA has only about half as many base pairs as human DNA (1.77 billion versus 3.2 billion), the fruit fly has about 130 million, and E. coli bacteria about 4.6 million, the marbled lungfish has about 130 billion base pairs—more than 40 times the human count—and DNA from one species of amoeba has 670 billion base pairs.

Restriction enzymes function like tiny scissors, cutting the DNA into fragments for subsequent separation by gel electrophoresis. In effect, a restriction enzyme “looks for” a specific sequence of base pairs, such as 3 AGG'CCT. When the restriction enzyme “finds” that specific sequence, it cuts the DNA at that point.

Because that specific short base sequence inevitably occurs many times in any DNA molecule, treatment with that one restriction enzyme cuts that DNA molecule into many fragments. Because repeated occurrences of that base sequence may be relatively close together or far apart on the DNA molecule, the fragments are of differing lengths, typically from hundreds to thousands of base pairs.

The specific size distribution of the DNA fragments obtained by treating DNA with a particular restriction enzyme varies according to the positions of the target base sequence in that particular DNA specimen. So, for example, treating your DNA with a particular restriction enzyme results in a different distribution of fragment sizes than treating your lab partner's DNA with the same restriction enzyme. The distribution of fragment sizes in your DNA specimen is unique to you, just as everyone else's distribution is unique to them.

Once the DNA has been cut into fragments by the restriction enzyme, gel electrophoresis is used to analyze the DNA by producing a map of the fragment sizes present in the specimen. A sample of the fragmented DNA is placed in a small well in a gel, which is then immersed in a buffer solution and subjected to DC electric current. Because DNA fragments are negatively charged, they are attracted to the positive electrode, which is positioned at the far end of the gel from the wells that contain the DNA solution.

The gel selectively retards the migration of the DNA fragments toward the positive electrode. Small DNA fragments pass through the gel relatively unhindered, and so move toward the positive electrode quickly. Larger fragments move proportionally more slowly, because the gel provides more resistance to their progress. If the current is applied until the smallest fragments just reach the end of the gel nearest the positive electrode, larger fragments are strung out along the length of the gel, with the largest fragments barely clear of the well where they originated. The positions of the various fragments provide a graphical map of the fragment size distribution in the specimen.

But DNA fragments are colorless, so it's impossible to track the progress of the electrophoresis visually. For that reason, a *marker dye* is added to the DNA specimen before the electrophoresis run. The marker dye is chosen on the basis of how fast it migrates through the gel. By using a marker dye that moves about the same speed as the smallest DNA fragments of

interest, electrophoresis can simply be discontinued when the visible marker dye approaches the positive electrode, at which point the smallest DNA fragments have made about the same amount of progress through the gel.

At this point, the only visible change to the gel is a band of marker dye near the positive electrode end. To visualize the bands of colorless DNA fragments, they're stained using dyes that are selectively attracted to the fragments. The most common stain used in professional laboratories is ethidium bromide, which bonds to the DNA fragments and fluoresces under ultraviolet light to reveal the DNA fragments as bright bands against the dark background of the gel.

We originally designed this lab session to use real DNA, real restriction enzymes, and so forth, but we decided that the cost of doing it that way was much too high—particularly for many home schoolers—relative to the educational benefit. Restriction enzymes, for example, are expensive and must be shipped frozen, which significantly adds to costs. Similarly, separating DNA effectively requires agarose gel rather than agar gel, and agarose in small quantities typically sells for $2 or $3 per gram. Ethidium bromide is expensive, costly to ship, and a deadly poison. There are alternative visualization stains available that are much cheaper and safer to handle, but they are also greatly inferior in resolution. And so on.

Fortunately, it's not necessary to use real DNA to observe how DNA separations are done with gel electrophoresis. You can do so using the apparatus you built in the preceding lab session, some materials from the kit, and some common household items.

> If you would like to delve further into gel electrophoresis and DNA analysis, one of the best sources of equipment and supplies is Edvotek (*http://www.edvotek.com*). Edvotek packages many different kits designed to illustrate various aspects of DNA analysis in a classroom setting.

Procedure III-7-1: Prepare running buffer

Running buffer is used to make up the gel initially, and later to immerse the gel while doing the electrophoresis run. The amount of running buffer you need depends on the size of your electrophoresis apparatus, the size of the gels you make, and the number of runs. Fortunately, running buffer is very cheap to make up, so you can make it in excess and discard any that's left over when you complete this lab session. Actually, for our simulation we'll use a very simple running buffer that wouldn't work very well with real DNA but is suitable for separating dyes.

1. Rinse out the 2 L soda bottle thoroughly and fill it to the 2 L level with water.

   > For a real DNA separation, we'd use distilled or deionized water to make up the running buffer and we'd use different salts but for this simulation most tap water will work fine. If your tap water is extremely hard or has much iron in it, use distilled water. Otherwise, tap water should work.

2. Add 1/8 teaspoon of table salt and a heaping tablespoon of baking soda to the soda bottle, cap it, and invert it several times to dissolve the solids. Invert the bottle every few minutes for half an hour or so to make sure the solution is homogeneous.

Procedure III-7-2: Prepare and cast the gel

1. If you have not done so already, calculate the volume of agar gel you need to cast your gel. Multiply the length and width of the gel chamber in centimeters by the desired thickness of the gel to determine how many cubic centimeters (mL) of gel you'll need. For example, if your gel

chamber is 7 cm by 10 cm, and you want a 1 cm thick gel, you'll need (7 * 10 * 1) = 70 cubic centimeters = 70 mL of gel.

2. Transfer that volume of room-temperature running buffer to the beaker.
3. Stir in about 2 g (1/2 to 5/8 teaspoon) of agar powder per 100 mL of running buffer. (The exact amount of agar is not critical.)

   The kit contains 10 grams of agar powder, which is sufficient to make up 500 mL of 2% agar gel. Agar gel is used both in this lab and in lab VII-2. If you need more agar, you can use agar that is sold by specialty grocery stores and some Chinese and Japanese restaurants. If you use agar from the grocery store, we recommend using a 3% to 4% concentration as a starting point.

   You can substitute unflavored gelatin from the grocery store **for dye separations only**. (Using gelatin for DNA separations results in a horrible mess.) Use double the amount of gelatin suggested on the package, otherwise following the procedure described for agar. Run gelatin gels at no more than 63 VDC and keep a careful eye on progress. If the gelatin overheats it will melt and may cause a short in the apparatus.

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4. Carefully heat the liquid in the microwave just until it begins to foam slightly. Keep a very close eye on the beaker as you heat it.

   The first time we made up 100 mL of agar in the microwave, we figured that one minute would be about right to get the stuff hot but not yet boiling. We intended to heat it for that one minute and then blip it for 5 or 10 seconds more at a time until it boiled.

   At about the 50 second mark, the contents of the beaker erupted volcanically, with agar foam flowing up out of the beaker and down its side. We opened the door immediately, but we still ended up with 25 mL of our agar on the microwave tray.

   The moral here is that if you use the microwave to heat your agar, do so in very short bursts. You can keep an eye on the actual temperature by using the thermometer (carefully) as a stirring rod. It's not actually necessary for the agar solution to boil. Getting it up near the boiling point but not actually boiling the solution is a good way to prevent messy accidents.

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5. Using a towel or oven mitt to protect your hand from the heat, remove the agar liquid from the microwave and set it aside to cool. While the agar cools, set up your gel electrophoresis apparatus on a level surface. Verify that the tips of the comb do not contact the bottom of the gel casting tray.
6. Once the agar has cooled to the point where it feels very warm or hot to the touch (about 50 to 60 °C), pour the agar liquid into the gel casting tray to a depth of roughly 1 cm. If there are any bubbles, use the stirring rod to eliminate them. While the agar is still hot and flowing freely, place the comb near one end of the casting tray with its teeth immersed in the liquid agar.
7. Allow the agar to cool and gel completely. Once it has set up, carefully remove the comb. Try to avoid tearing the agar gel when you do so. The goal is to have a solid, flat gel with a series of small neat holes near one end. Remove the ends from the gel casting tray.

Procedure III-7-3: Load and run the dye specimens

If you want sharper separations with agar gels, you can pre-run the gel. That is, run the gel normally, but without loading specimens in any of the wells. After 15 minutes or so, disconnect the power, remove the gel tray from the running buffer, load the specimens, and re-run the gel. The pre-run clears out a lot of the gunk that's present in agar (but not agarose) and allows the agar to function more like actual agarose.

A pre-run is not necessary for this lab session. If you do a pre-run, you'll probably find that you get much sharper separation of the dyes, but for our purposes simply measuring from the well to the center of the dye cloud is good enough.

1. Transfer nine drops of Hucker's crystal violet stain to one well of the 96-well reaction plate, nine drops of methylene blue to a second well, and nine drops of safranin O to a third well. To a fourth well, transfer three drops each of these three stains.

   We'll use the reaction plate and plastic pipettes rather than attempting to transfer the stains directly from their bottles to the wells in the gel because the former method is much more precise.

2. Transfer one drop of glycerol to each of the four wells containing dyes. Use a clean toothpick to stir each well until the dye solution and glycerol is thoroughly mixed.
3. Use a clean pipette to transfer sufficient the Hucker's crystal violet dye and glycerol mixture to one well of the gel to fill the well about half full. (Depending on the size of your wells, one drop may suffice.)
4. Repeat the preceding step to transfer the methylene blue, safranin O, and mixed stain mixtures to three vacant wells of the gel.

   If you have a fifth well in your gel, you can place the individual stains in wells 1, 3, and 5 and the mixed stains in both wells 2 and 4 to make it easier to compare migration distances.

5. Carefully place the gel tray centered in the outer container (electrophoresis tank). Gently pour sufficient running buffer into the electrophoresis tank to fill it to a level just above the top of the gel tray. The goal is to immerse the gel tray completely in running buffer.
6. Verify that the aluminum foil electrodes are in place on the electrophoresis tank. Connect the red alligator clip lead to the electrode nearest the well end of the gel tray and the black alligator clip lead to the other electrode.
7. Connect the free end of the red alligator clip lead to the exposed positive terminal on your 9V battery stack.
8. Carefully (**shock hazard!**) connect the free end of the black alligator clip lead to the exposed negative terminal on your 9V battery stack.

   Do not allow the red and black leads to contact each other directly!

   A short circuit is very bad news. Depending on the voltage of the battery stack, the best you can hope for is a show of sparks and destruction of your battery stack. It's possible that the clips will weld together, that the wires will overheat and vaporize, and that the insulation will catch fire.

9. Observe the gel until some migration of the dyes is visible. If all is well, all dyes should be migrating toward the far end of the gel. If instead they are migrating toward the near end of the gel, the polarity is reversed. Switch the positions of the black and red leads.

Depending on the voltage you use, there may be enough voltage exposed on the running buffer surface to give you a severe shock. Never touch the apparatus (and particularly the running buffer) while power is connected to the apparatus. You can tell it's working because bubbles appear at the positive electrode.

10. Continue observing the gel until one of the dyes has migrated nearly to the far end of the gel. Depending on the size, type, and concentration of the gel and the voltage of the battery stack, this may require anything from a few minutes to a couple of hours. When the farthest advanced dye has nearly reached the far end of the gel, disconnect both leads from the electrodes and the battery stack. Allow the gel to cool to room temperature before handling it.
11. For each well, measure the distance from the middle of the well to the middle of the corresponding dye streak. Record these values in your lab notebook.
12. Calculate the distances each dye migrated as ratios to the dye that advanced farthest. For example, if dye #1 migrated 10.0 cm, dye #2 7.8 cm, and dye #3 5.3 cm, record the ratios for dye #1 as 1.00, dye #2 as 0.78, and dye #3 as 0.53.

This calculation is similar conceptually to the R_f value used in paper chromatography. The difference is that in paper chromatography the baseline is the distance that the solvent front advanced. With gel electrophoresis, there is no solvent front, so we use the distance that the farthest-advanced dye migrated as the baseline value.

Review Questions

Q1: The kit also contains eosin Y stain and Sudan III stain. Why did we not use these stains in our gel run? (Hint: research acidic, basic, and neutral dyes. Second hint: as applied to stains, these terms refer to ionization rather than pH.)

The three stains we used are all basic stains, which means the chromophore (coloring component) is present in the cation. Eosin Y is an acidic stain, which means the chromophore is present in the anion, which in turn means that eosin Y migrates toward the opposite electrode. Sudan III is a neutral stain, which means it does not ionize (the coloring component is present in a neutral molecule). Because the Sudan III molecule is not ionized, it is attracted to neither electrode and merely remains in the well.

Q2: Could any of the three stains we used be used as marker dyes if we were doing actual DNA electrophoresis? Why or why not? If not, what common dyes, stains, and pH indicators could be used as marker dyes?

None of the three stains we used could be used as marker dyes in DNA analysis, because all have the opposite polarity of DNA. That is, the DNA would migrate toward one electrode while all of these three stains migrated toward the other. Among the common dyes, stains, and pH indicators that could be used as DNA marker dyes are bromocresol green, bromocresol purple, bromophenol blue, eosin Y, m-cresol purple, o-cresol red, orange G, phenol red, and xylene cyanol.

Q3: The gram molecular masses of crystal violet, methylene blue, and safranin O are 407.979, 319.85, and 350.84 g/mol, respectively. Based on those molecular masses, what ratios of migration distances would you expect?

Methylene blue is the lightest molecule and therefore the one that should migrate fastest. If migration distance correlates directly to molecular mass, we would expect the ratio for safranin O to be (319.85/350.84 = 0.91+) and for crystal violet to be (319.85/407.979 =

0.78+). So, if methylene blue migrated 10.0 cm, we would expect safranin O to migrate 9.1+ cm and crystal violet to migrate 7.8+ cm.

Another way to look at this is that we would expect the products of the molecular masses and distances migrated to be the same for all three molecules:

methylene blue – 319.85 * 10.0 = ~ 3200

safranin O – 350.84 * 9.1+ = ~ 3200

crystal violet – 407.979 * 7.8+ = ~3200

Actual results will vary because migration ratios are affected by both the concentration of the gel and the makeup of the running buffer.