WEEK #4: GALL FLY EVOLUTION I. PROTEIN ELECTROPHORESIS AND ENZYME STAINING AND *DROSOPHILA* GENETICS I. CROSSING FRUIT FLIES

Project Goals

In this lab, you will learn to use the technique of cellulose acetate electrophoresis to separate proteins. You will perform this technique on gall fly larvae from two different sites. You will stain your gel for a particular enzyme, and use the results to determine the genotypes of your gall fly larvae. Data from the entire class will be pooled, and you will analyze the results in a formal scientific report.

You will also be crossing fruit flies (*Drosophila melanogaster*) to learn about their genetics. You will receive a handout describing this part of the lab.

Introduction

Basic Biology of the Goldenrod-Gall Fly System

As you remember from lab last week, the gall flies we are interested in infect the goldenrod *Solidago altissima* in the Carleton Arboretum and at McKnight Prairie. *S. altissima* is a perennial plant; while the above-ground portions of its stem dies back over the winter, an underground stem system of rhizomes is maintained, and it grows new above-ground stems from the rhizomes in the spring.

Adult gall flies, *Eurosta solidaginis*, emerge from the previous year's galls in late May and early June (Fig. 1). The gall flies are quite small, only around a cm long. Adult flies only live for about ten days (Uhler, 1951); in that time, they mate and lay eggs. Mating takes around forty minutes. Approximately fifteen minutes after mating, females puncture leaf buds on young goldenrod stems to lay their eggs (Uhler, 1951), a process called oviposition. One egg is laid at a time, but a female probably lays dozens of eggs (Uhler, 1951).

Gall fly eggs hatch within four to seven days, and the larvae burrow into the growing stem (Uhler, 1951). Galls are first apparent three weeks later; the galls are produced by the plants in response to plant growth regulators (auxins) produced by the larvae. Some combination of these auxins and the chewing activity of the larva causes the goldenrod plant to form a thick sphere of plant tissue around the larva (Abrahamson and Weis, 1997). This thickening of tissue produces the galls you observed and collected in lab last week. Only one gall fly larva is present in each goldenrod gall. Three weeks after the gall is apparent, it reaches its maximum size (Weis and Abrahamson, 1985). Inside the gall, the gall fly larva undergoes three larval stages (each larval stage is called an "instar" in insects) (Fig. 1). Between each of these stages, the insect molts (sheds its outer layer to allow for growth in size). The larva feeds off of the interior surfaces of the plant gall. In the late summer or early fall, during the third larval stage (third instar), the larva burrows an almost-complete exit tunnel through the gall wall; only the very outside layer of the gall is left intact (Uhler, 1951) (Fig. 1).

The third instar larva spends the winter in the central cavity of the gall, in a condition of dormancy called "diapause." In early spring, the larva pupates: it forms a pupal case and rearranges its entire body plan from a grub-like creature to that of a fly. This metamorphosis is quite amazing, and involves the complete disassembly of existing tissues and organ systems and the formation of completely new structures.

In spring, the gall fly emerges from its pupal case and breaks through the last unopened part of its exit tunnel.

Other Players in the System

The goldenrod and gall fly system is not limited to just these two organisms. There is a fascinating web of interaction involving organisms who take advantage of the gall system by preying on the gall fly larvae, using their galls for protection, or some combination of these two. Chickadees and woodpeckers both peck holes in goldenrod galls to eat the larvae; you probably noticed these large holes

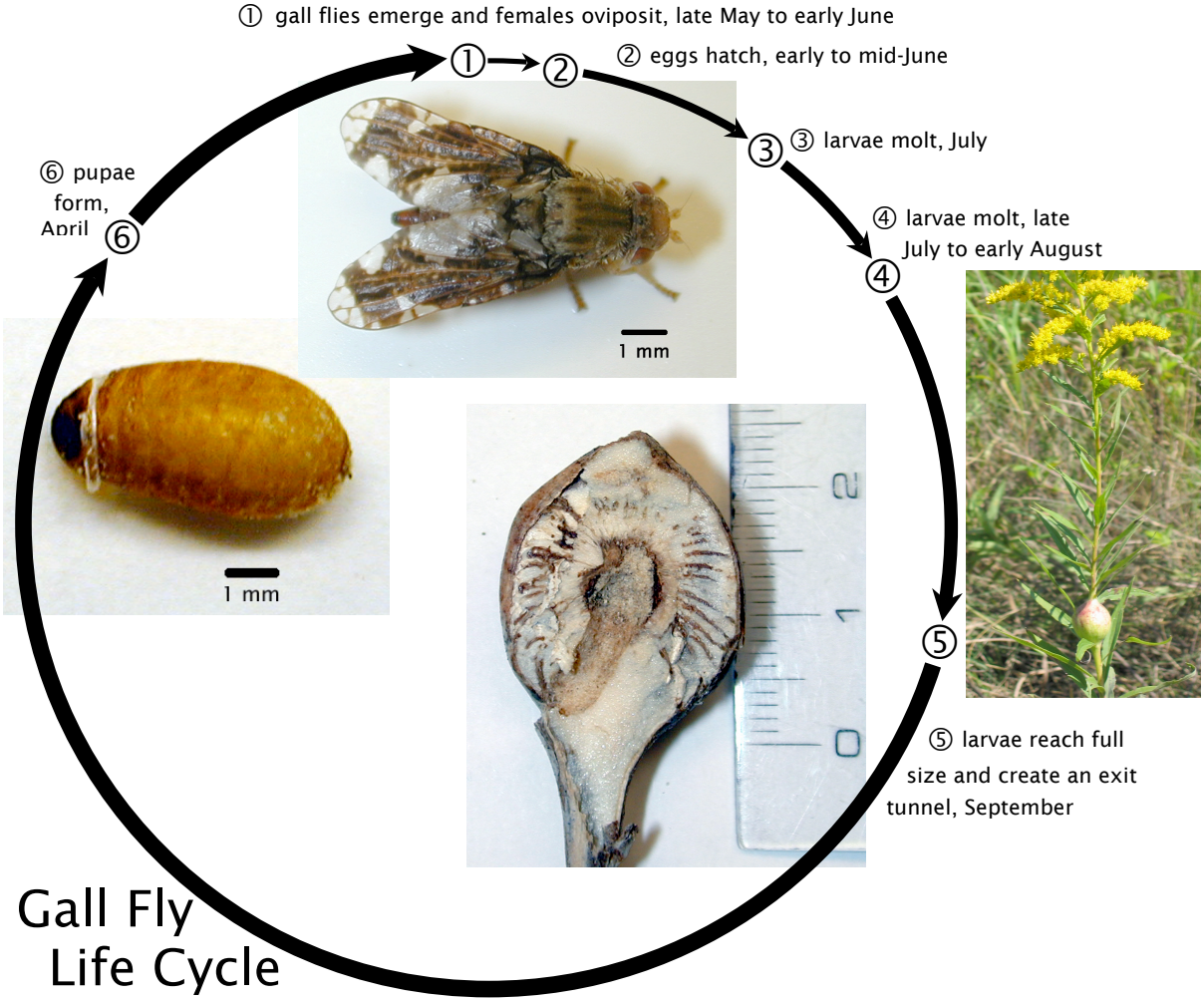

Figure 1. The life cycle of the goldenrod gall fly, Eurosta solidaginis. Dates (based on Uhler, 1951) are approximate, and will vary depending on weather and geographic location. The photographs, clockwise from top, are of an adult gall fly, a goldenrod with gall, a gall opened to show the exit tunnel (scale in cm), and a gall fly pupa.

last week in the Arb. There is a species of beetle (*Mordellistena unicolor*) which seems to use the gall for protection of its larva, and the beetle larva often (but not always) kills the gall fly larva inside the gall (Abrahamson and Weis, 1997).

Two species of the wasp genus *Eurytoma* are parasitoids of the *Eurosta* gall fly larvae. *Eurytoma obtusiventris* lays its eggs in the gall fly egg or young gall fly larva before gall formation begins. The wasp larva hatches in the gall fly larva, but does not have any adverse effect until the fall, when the wasp larva causes the gall fly larva to pupate prematurely. Once the gall fly pupates, the wasp larva lives off the gall fly pupa, consuming it. The other wasp species, *Eurytoma gigantea*, actually injects its eggs into a gall after the gall is fully formed. This type of wasp larva burrows in and consumes the gall fly larva.

These predators affect the gall size in *S. altissima*. Small galls seem to be less subject to attack from birds, while *Eurytoma gigantea* is less effective at injecting its eggs into large galls (Weis and Abrahamson, 1985). Remember that one of the differences between the galls in the Arb and those at McKnight is that the galls in the Arb are smaller on average. One hypothesis for this size difference is that because woodpeckers and chickadees are rare are at McKnight, there is no advantage to having small galls.

We will not be investigating any of these interactions directly in lab this term, but you need to be aware of them, partly because of their effects on the difference betweeen Arb and McKnight gall flies. More importantly for the lab today, some (perhaps many) of your galls will contain wasp or beetle larvae, and we do not want to include those in our data set.

What Do We Want to Know?

The lab you're doing this week will address two questions about the gall fly population in the Arb and at McKnight.

1. Is there variation in the population of gall flies? You'll look to see if there is more than one form of the protein phosphoglucomutase (PGM) present in the population.

2. Are the gall flies from the Arb different from the gall flies from McKnight? Asking the question another way, if there is variation in the population, is one form of the protein more common in gall fly larvae from one site than from the other? Re-read the gall fly section from last week's lab to remind yourself of why we are asking this question.

What Protein Are We Looking At?

The protein we will be investigating is phosphoglucomutase (PGM). This is an enzyme (note the "-ase" ending), which means it catalyzes chemical reactions, helping them occur faster than they would otherwise happen. (There are non-enzymatic proteins in cells as well, which serve structural functions.)

In cells, PGM is involved in the process of utilizing glycogen (a large sugar storage molecule) for the purpose of making energy in the cell. It converts glucose 1-phosphate to glucose 6-phosphate (Fig. 2) so that the glucose can be used by other enzymes (which can only work on the glucose 6-phosphate form).

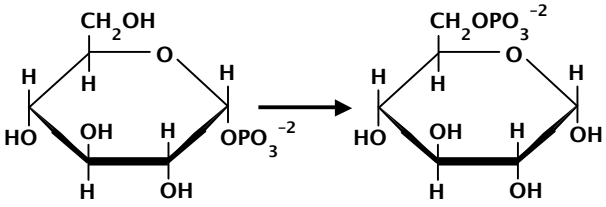

Figure 2. The reaction catalyzed by the enzyme phosphoglucomutase (PGM). Keep in mind that this enzyme can catalyze the reaction either direction; the arrow is drawn to indicate the direction which normally occurs in cells.

Why Look at Proteins for Genetic Differences?

The most direct way to look for genetic differences in gall flies from the two different sites would be to sequence gall fly DNA. However, sequencing is a time-consuming and expensive process for each organism analyzed.

Protein electrophoresis is a well-accepted means of determining something about the genetic makeup of organisms. Using this technique makes it feasible to analyze data from many organisms quickly.

Remember that each protein in an organism is coded for by a gene; in our case, the PGM protein is coded by a gene we can call the PGM gene. The PGM gene is the particular region of DNA with the sequence of DNA bases that code for the sequence of amino acids in the PGM protein. Remember also that in diploid organisms there are **two** copies of each gene, on separate chromosomes (one from each parent).

So how will we find out if there are differences in the proteins? We will run the proteins from a single larva out on a special type of gel, which will separate different forms of proteins based on their biochemical properties. If there is a difference in the DNA sequence which changes the amino acid sequence of the protein, this may cause enough of a biochemical difference in the protein that it will run a different distance on the gel.

When we run our gels, we will put *all* the proteins from the larva on the gel. We won't be able to see any of them while the gel is running. After the gel has finished running, we'll stain it for PGM. If both (remember, these are diploid animals) the copies of the PGM gene are identical, we would expect all the PGM molecules in the organism to be identical, and we should see a single band on the gel for that larva. If the two genes for PGM are not identical in a larva (i.e. if the organism is heterozygous) in a way that affects the biochemical properties of PGM, we would expect to see two bands on the gel for that larva.

If we see multiple forms of the PGM protein on our gel, we can name those protein forms based on the distance they run from the wells ("slow" or "fast"). The faster form of the protein must have a slightly more negative charge than the slow form of the protein, since it is moving more quickly to the positive electrode. This difference in the proteins is due to a difference in the genes coding for them; while both genes code for the PGM protein, one version of the gene must be slightly different (perhaps even by only one base pair). We can call the version of the gene which codes for the fast

form of the protein the fast, or "F" allele, and the version of the gene which codes for the slow form of the protein the slow, or "S" allele. Throughout the lab, you will need to understand this distinction between the proteins on the gel and the alleles of the gene.

In a given population, there may be one allele of a gene present, two different alleles, or several different alleles. If two organisms have the same allele of the PGM gene, we would expect them to both have a band at the same distance from the well on a gel. By looking at a gel with protein from several organisms (one organism in each lane), we will gain information about the genetic variation in the population.

It is important to recognize that the differences we may see on our gel probably do not correspond to functional differences in the organism. While the substitution of a negative amino acid for a neutral one may change how the protein runs on a gel, it may not have any effect on how the protein works in a cell. It is unlikely that PGM is involved in determining gall size. We are using the natural variation of the PGM gene in the population to serve as a potential marker of difference, not because we suspect that PGM functions differently in flies from one site versus the other.

How Does This Gel Differ from an Agarose Gel?

The gels we are running this week are made of cellulose acetate, not agarose. The basic idea of the electrophoresis technique remains the same: you will still separate molecules based on charge, by putting them on some matrix (agarose or cellulose acetate) and applying a charge to the system (using the gel boxes and power supplies). Cellulose acetate, unlike agarose, does not separate molecules based on size; this is not a problem for us, because we expect our molecules to be very similar in size anyway.

Unlike DNA, which is all very negatively charged, proteins can have slightly different rates of movement through the gel based on small differences in amino acids. Imagine two forms of the same protein with one amino acid difference. This type of difference has potentially dramatic effects on function, but in this case, let's imagine a situation where the amino acid change does not affect the overall function of the protein (maybe because it is not located close to crucial parts of the protein). If the differing amino acids differ in charge (one is slightly less negative than the other), then the behavior of the proteins on a gel will be different. The protein with the slightly-less-negative amino acid will probably not move toward the positive electrode at the same rate as the protein containing the more negatively charged amino acid: the presence of the slightly-less-negative amino acid will cause that protein molecule to move more slowly.

Does this mean that we will be able to distinguish all differences between the two copies of the gene which codes for PGM? Absolutely NOT. Using this technique, we will never be able to distinguish between versions of the gene which differ but make the same amino acids. We won't even be able to distinguish between different forms of the protein containing different amino acids, if the difference in amino acids does not affect the biochemistry of the protein enough to alter its rate of movement through the gel. Despite these limitations of this approach, it is a very good way to get a quick look at the molecular-level variation present in a population.

How Did We Choose This Enzymes?

We used the information from the scientific paper by Waring et al. (1990), who looked at many different proteins in gall flies from two different species of goldenrods. Waring and her colleagues studied some larvae from Minnesota, and found some variability in the PGM protein. This information made PGM a good candidate to look at in lab this term. Remember that our first question in this lab is still to determine if there really is variation in our population in the Arb.

How Will We Find Just the Proteins We Want?

There are two parts to the detection systems we'll use for these enzymes. First, we will use what we know about the reaction catalyzed by PGM to provide conditions where only PGM will be active. This means we will choose a very specific reactant that interacts only with PGM (see page 4-10). Second, we will tie this reaction to a reaction which produces a color change (see page 4-10). The first reaction is biological: it is the sort of reaction which occurs in the cells of the organism. The first part of the system ensures we are only looking at the enzyme we are interested in. The second part of the system is not biological: it is based on our knowledge of chemistry and what will change colors nicely. The second reaction allows us to visualize

where the first reaction occurred, and allows us to see where the enzyme is on the gel.

References

Abrahamson, W. G., and A. E. Weis. 1997. Evolutionary Ecology Across Three Trophic Levels: Goldenrods, Gallmakers, and Natural Enemies. Princetown University Press, Princeton, NJ.

Uhler, L. D. 1951. Biology and ecology of the goldenrod gall fly, Eurosta solidaginis (Fitch). Cornell University Agricultural Station Memoir 300:1-51.

Waring, G. L., W. G. Abrahamson, and D. J. Howard. 1990. Genetic differentiation among host-associated populations of the gallmaker Eurosta solidaginis (Diptera: Tephritidae). Evolution 44:1648-1655.

Weis, A. E., and W. G. Abrahamson. 1985. Potential selective pressures by parasitoids on the evolution of a plant-herbivore interaction. Ecology 66:1261-1269.

Experimental Procedures

Dissecting Galls to Remove Larvae

1. Find out from your lab instructor how many galls from each site your lab group should dissect. Save any extra galls for use in later lab sections. Someone in your group should fill your ice bucket with ice before you start the dissections.

2. Opening a gall
   a. Locate a sharp knife (plastic handle) and the gall you wish to open.

   b. Cut into the gall approximately a quarter of an inch depth all the way around the "equator" of the gall. **Do not cut all the way through the gall with your knife.**

   c. Carefully insert the bottom part of the blade of your spatula (wooden handle) into the groove you cut and even more carefully twist the blade to pry or pop the gall open. You may have to try this at a couple of places around the edge of the gall. Do not use the sharp knife for this step.

   Ask your lab instructor or TA if you have any questions about this procedure.

3. Now that your gall is open, you need to determine if you have a gall fly larva inside. Your gall might contain
   - nothing
   - *Eurytoma obtusiventris* (parasitoid wasp) larva
   - *Eurytoma gigantea* (parasitoid wasp) larva
   - *Mordellistena unicolor* (beetle) larva
   - *Eurosta solidaginis* (gall fly) larva

   There are diagrams and descriptions of the different larvae in lab. Be sure that you have a *Eurosta solidaginis* larva before proceeding.

4. Place the *Eurosta solidaginis* larva in a microfuge tube using the forceps at your bench. Label the tube with your name and the collection site. Keep the tube in your ice bucket.

5. Repeat these steps as needed, placing each larva in its own microfuge tube. Since you will have more than one larva from each site, label each tube with a number to distinguish them.

Homogenizing larvae to release proteins

6. Now that you have found larvae, you need to break open the cells of each larva to release the proteins into solution. Add 50 μl of water to the larva in each tube.

7. Place one clean pestle into each microfuge tube. Initially, press down very gently to begin the process. Turn the handle and move it up and down firmly to homogenize (grind) the larval tissues until the mixture in the tube is smooth (or "homogenous"). Keep the tubes on ice for this procedure, and leave the tubes on ice when you are done. **Do not throw away the pestles**.

   This procedure kills the larvae; if you have concerns about participating in this part of the experiment, please contact your lab instructor before lab.

Preparing and Running the Cellulose Acetate Gel

8. Your instructor and TA will help coordinate this process. Each lab group will run one gel: the two lab groups sitting across from each other will share a gel-loading system, and will need to take turns loading their gels.

9. Begin by setting up your gel box to run this different type of gel. **You should wear gloves for the remainder of the lab activities today.**

   a. Locate 10 rectangular sheets of filter paper.

      These should already be cut to the proper size.

   b. Put the correct buffer (0.025 M Tris-glycine, pH 8.5, for PGM) in your gel box so that there is enough in each well to submerge the electrodes, but none over the "bridge" in the center part of the gel box (the place where the gel rests). See Fig. 3.

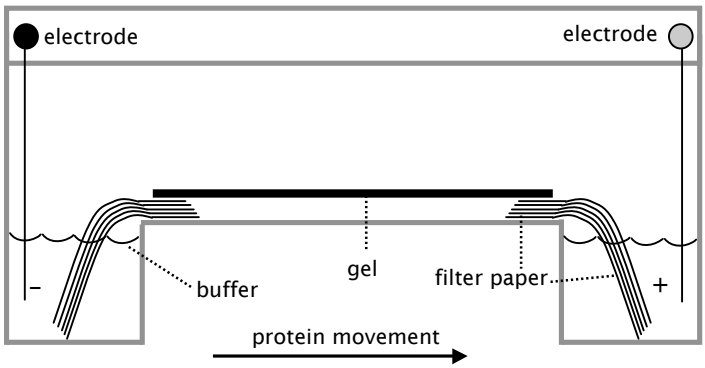

Figure 3. Gel box for cellulose acetate gel electrophoresis, side view.

   c. Take a stack of 5 pieces of filter paper, and dip them in one end of your gel box. Make sure the filter paper stack is completely wet with buffer. Place the stack so that approximately a half inch to an inch or so rests on the "bridge" and the rest hangs down into the end of the gel box. The filter paper pieces should take up the width of the bridge; try rotating them 90° if they seem too narrow.

      These will serve as wicks; all the current going through your gel will pass through the wicks.

   d. Repeat with the remaining 5 pieces of filter paper at the other end of the gel box.

   e. Make sure there is no moisture on the bridge between the stacks of filter paper; use a Kimwipe to dry the bridge if necessary.

10. Load your sample plate:

    When your group has finished homogenizing and prepared your gel box, you are ready to begin the process of loading your gel. You will need to take turns with the group across from you at your bench.

    a. Load 10 µl of each sample into the "sample plate" at your lab bench. Be careful not to let a sample get in more than one well. Avoid using lanes 1 and 12 on the outside edges of the gel.

       This plate is just a special reservoir for the samples until they are ready to be loaded on the gel. It looks like a plastic version of the gel you ran last week, with a line of wells down the middle.

    b. One of your wells should be filled with a sample from a different lab group in the lab. (Use lane 1 or 12 for this sample.)

       This will allow us to check for consistency between lab groups: if one group sees no variation, they will still know how their data compare to other groups.

    c. Use the top section of the data sheet on page 4-9 to record which sample is placed in each well.

    d. As you continue with the next steps, cover the sample wells with the glass slide at your bench so that the samples do not evaporate.

11. Locate the equipment for gel loading:

    a. Locate the large beaker which contains one or more **gels** soaking in the buffer you're using for your gel.

    b. Locate the **aligning base** at your bench (this is made of the same type of plastic as the sample well plate, and says "cathode loading" next to a line).

    c. Locate the **gel applicator** at your bench. This looks something like a stamper, with a small handle and a wide base. Instead of a stamp image on the base, there is a row of what look like staples.

       Be extremely careful with the gel applicator: it is fragile and expensive, and very precise. Those "staples" will pick up a tiny amount of fluid from the sample wells, and when pressed into the gel, will actually cut tiny wells while inserting the sample into the well.

    d. Locate the **round filter paper** at your bench. You will use this to blot the gel and the gel applicator.

12. Load your gel:

    Read through this set of instructions before starting the process; you'll need to move efficiently.

    a. Remove the slide from your sample plate. **Prime the gel applicator** by setting it into the slots on the sample plate. Depress and release the plunger **four** times. Replace the slide on the sample plate.

    b. Immediately following step 12a, **blot the gel applicator** by setting it on a piece of filter paper and depressing the plunger once. You can then leave the applicator sitting on the filter paper, or in the slots of the sample plate.

    c. Immediately following step 12b, one member of your lab group (wearing gloves) should carefully **remove one gel** from the beaker containing the proper buffer.

    d. Immediately following step 12c, **blot the gel**, gently but thoroughly on both sides, on the filter paper. Do not rub the gel.

    This is tricky. You do not want the gel to completely dry out before you get it loaded, but you also do not want any standing fluid left on the gel. Try to work quickly.

    These gels are much, much thinner than the agarose gels we used two weeks ago. We purchase them pre-made; each gel is already mounted to a piece of plastic to help keep it intact. When you look at the gel, the plastic side is much shinier than the side the gel is on.

    e. Immediately following step 12d, place the **gel**, DULL SIDE UP, **on the aligning base**, with one edge aligned with the line that says "cathode loading." Center the gel between the slots which hold the gel applicator. Check that it is centered by placing the gel applicator in the slots (but don't press down yet).

    f. Immediately following step 12e, remove the glass slide from the sample plate. **Load the gel applicator**: place it in the slots of the sample plate, and depress and release the plunger **four** times. Replace glass slide to cover the wells of the sample plate.

    g. Immediately following step 12f, **load the gel** by placing the loaded applicator in the slots of the aligning base and depressing the plunger for **ten seconds**.

    Again, these gels do not have wells in them; the special applicator just inserts the samples into the gel in a straight line; it basically makes all the wells as it loads all the samples, in one step.

    h. Immediately following step 12g, **transfer the gel to the gel rig**. Place it DULL SIDE DOWN, shiny side up, on top of the wicks. The wells should be closer to the negative (black) electrode. The wells should not touch the wicks.

    There should be no buffer going across the surface of the plastic gel box bridge; this way all the current is forced through the gel. You can run your finger across the back of the gel right above the wicks to make sure there is good contact along the length of the gel on both sides.

13. **Run the gel** at **200 volts** for **fifteen minutes**. Attach the lid of the box and plug in the leads. Turn the power supply on and set it to 200 volts.

    This type of gel does not use a dye, so we will not be able to check the progress of the gel. You can look for bubbles in the buffer in your gel box to make sure that the current is running.

Staining Gels

14. While your gel is running, you should get the stain vial for your enzyme from the freezer and allow it to thaw.

15. Identify what last minute additions need to be made to your stain (see page 4-10 for details), thaw them, and add them to your stain vial. Swirl to mix.

    You may want to locate the warm agar, but do not remove it from the hot block at this point, and do not add it to your stain yet.

16. When your gel is ready to stop, turn off the power supply and unhook the electrodes. Leave the lid on to keep the gel moist.

17. In this step, you want to move very efficiently. Read these instructions through before beginning the process; make sure everything is ready to go before you start.

    a. Remove the gel from the gel box and place it (right side up, so you'll have to invert it—remember it is upside down on the wicks!) in a square plastic box.

b. Pour one tube of warm agar into your stain vial, and swirl gently—only a couple of times, since it will harden VERY quickly—to mix completely.

c. Immediately pour the entire contents of the stain container over the top of your gel. Make sure the area BELOW your wells is covered with stain.

You want to complete this procedure before the gel has time to dry out. The stain should all (or almost all) remain on top of your gel; pour very carefully. Probably, the stain will cover the entire gel; it is okay if the area above the wells is not completely covered, but try to cover the area below the wells. Then, don't jostle the box as the agar cools and hardens. The idea is that the agar will keep the stain where it belongs, on top of the gel.

18. Let the gel stain for 2 minutes, but consult your TA or instructor before stopping the staining if bands are not appearing on your gel yet. To stop the staining process, gently slide the agar off the surface of the gel into the trash (not into the sink!) and rinse the gel off the gel off gently with cool tap water. Leave the gel submerged in water until you have photographed it.

Watch your timing carefully, and do not leave the stain on the gel too long; it will keep getting darker, and the bands will blur together. You can use your gloved fingertip to nudge the stain from a corner of the gel, and it should loosen and slide off.

Record Your Results & Interpret Your Gel

29. Record your results by taking photos of your gel with the camera (your lab instructor or TA will help you with this).

20. Put your gel back in water in the plastic box. make sure the box is labeled, then give it to your TA. The TAs will dry the gels and take care of storing them.

21. To interpret your results, look for the bands on the gel. How many bands are in each lane? Did they all move down the gel the same distance from the wells? Are there any obvious heterozygotes? How many different protein forms are there for PGM? Discuss your results as a lab group and with your lab instructor or TA.

22. As part of your data interpretation, measure and record the distance each band moved from the well. (You do not need to do this for each lane, just for each group of bands located at a different distance from the well.) We'll use this information in our discussion next week.

23. Enter your results onto the data sheet on page 4-9 We will have copies of this form available in lab. Both sides of the lab bench should report their results together on a single form and turn it in to their lab instructor, so we can quickly compile the class results.

After Thursday's labs, we will provide you with the class results for analysis. Next week's discussion in lab should help you check your analysis and prepare for your lab report.

Gel Record Bio 125 Protein Electrophoresis Lab

Lab Section(circle one): Tues AM Tues. PM Weds. Thurs. AM Thurs. PM

Lane	Source (Arb or McK.)	Lab Partners	Lane	Source (Arb or McK.)	Lab Partners
1	_____	_____	7	_____	_____
2	_____	_____	8	_____	_____
3	_____	_____	9	_____	_____
4	_____	_____	10	_____	_____
5	_____	_____	11	_____	_____
6	_____	_____	12	_____	_____

PGM Results

1	2	3	4	5	6	7	8	9	10	11	12

Draw gel results (band pattern) in the space above.