A Guide to

Radish Electrophoresis

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GRINDING

Welcome to the wonderful world of grinding radish buds! There are several methods of grinding buds, but I will primarily be using the "spot plate" method. Feel free to customize this procedure to your own specifications!

Collect your buds [NOTE: with violets, you can use buds, fresh leaves, seeds, or CL fruits]

1. Take a sharpie, some paper, and a bunch of centrifuge tubes (in a bag) over to the greenhouse - preferably in the early morning before the greenhouse bay gets too hot (Rule of Thumb: if you're sweating in the greenhouse, it probably is not the best of times to collect buds). Start to label the centrifuge tubes with the sharpie (if they are not labeled already).
2. Collect buds from your desired plants, taking care to remove only the side buds and not the main meristem. Fill the centrifuge tube with buds until it is about 1/3 to 1/2 full. Remember...it is always better to have too many buds, rather than too little (you can always throw buds out if you have too many). Make sure the tube cap is tightly closed, since it will often pop open in the bag and spill all of your buds out.
3. After you fill each tube, record the tube number and the plant ID on your piece of paper. As you fill up the centrifuge tubes with buds, make sure that the tubes are out of direct sunlight (i.e. place them deep inside the bag).
4. After you are finished collecting, take the tubes back to the lab and place them in the refrigerator while you get ready for the next step.

Prepare grinding materials

1. The next step is to get all your materials together before you actually start to grind. The most important thing that you need is the extraction buffer. You can either use some frozen buffer stored in the frig (in centrifuge tubes in box labeled "radish extraction buffer"), or you can make up your own fresh solution. If you choose the latter, use the following formula to make enough grinding buffer for about 60 tubes (you can freeze the extra):

   7.5 mls 0.2M Tris-HCl pH 8.0
   - left-hand side of large glass-front refrigerator

   7.5 mls water

   22.5 mg DTT (dithiothreitol - Sigma D9163)
   - kept in refrigerator in the starch bottle filled with desiccant

   - mix all of the above in a 50 ml beaker. Try to make sure that this extraction buffer is always cold, especially when you are about to apply it to the buds! [For violets, I always use the sorghum grinding buffer.]

2. Now you need to collect together all of the equipment that you will need:

   spot plates (3) - stored on top shelf of glassware cabinet
   test tube - to grind with

sand
gloves
water squirt bottle with beaker
pipette with bulb
forceps
tissues to wipe forceps off with
wicks (about 1/8" wide and 1/2" long)
petri dishes with a labeled Whatman's paper in each
tray filled with ice - tray is above the incubator and ice is from Dr. Crawford's lab

The grind

The grinding process is best done in the fume hood, but it can also be done in a well-ventilated room, if necessary (since there is no mercaptoethanol, it doesn't stink very much).

1. The first step in the actual grinding of buds is to put your gloves on and place a pinch of sand in each of the wells on the spot plate. Place all of the spot plates immediately on top of the ice in the ice tray so they can cool down.
2. Remove your bag o' buds from the frig and start to place the buds from each vial in the corresponding numbered well on the spot plates. After you have finished this, remove the first spot plate from the ice and place it on a paper towel directly in front of you.
3. Add approximately 3-4 drops of the cold (but not frozen) extraction buffer to each of the first four wells on your spot plate. Then, starting with the first well, start to grind the bud tissue with your test tube until it is mostly liquid and there are no large clumps of plant goop. If necessary, add another drop or two of extraction buffer to the well as you grind. After you are finished with the first well, wash off your test tube over a large beaker using the squirt bottle filled with water. Dab your test tube on the paper towel to remove any excess moisture and then start on the next well.
4. Repeat the process until you are finished with all four wells. Now you are ready to work on the next four wells in the same manner. Continue to work on four wells at a time until you are finished with all wells on the spot plate. At this time, put the plate back onto the ice to keep the extracts cool and select the next spot plate to use.
5. Continue this entire process until you have completely ground all the plant tissue you originally collected.

Preparing the wicks

1. Starting with the first well on the first spot plate, use your forceps to place three wicks in the well. Continue placing three wicks into all of the other wells.
2. Let the plant extract soak up into the wicks - you may need to apply slight pressure to some of the wicks if they are not fully soaked after a few minutes. If there is still a significant amount of plant extract remaining in the wells, you can add more wicks.
3. Now it is time to prepare your wicks for cold storage. Starting with the first well on the first spot plate, with your forceps place each wick into the appropriately numbered location on the paper in the petri dish. It may often be necessary to first blot the wick on a paper towel to remove any excess moisture or plant goop. Continue the process until

all sets of wicks in all of the wells are placed in their appropriate locations in the petri dishes.

4. As soon as possible, place a section of saran wrap on top of the petri dish containing the wicks, being careful not to disrupt the wicks and cause them to touch one another. Take the lid of the petri dish and gently place it on top of the saran wrap, sandwiching the saran wrap on top of the wicks so that they do not move. Be sure to tape the petri dish lids together with masking tape and then label the entire petri dish with labeling tape. Do this for all three (or more) petri dishes and place all dishes inside a zipped-lock baggie that is labeled with the date, our lab name, and the run name. Make sure that you always hold the petri dishes horizontal as the wicks may still be able to slide around.

Storing the wicks

1. Now you need to place the petri dishes in the ultracold.
2. CLEAN UP YOUR MESS

MAKING THE GEL

If you are going to be doing an overnight run, it is best to make your gel early in the morning of the run. Because PGM requires histidine for activation, it is best to use histidine-citrate gels for PGM (and PGI if needed). Making the gel is pretty easy, and here are some steps and hints to help you along:

Preparations

The first step is to assemble your equipment together. For one gel, make sure you have the following on hand:

1 filtering flask with vacuum arm
1 volumetric flask
vinyl gloves
orange fire-proof gloves
large spatula
plastic weigh boat
rubber stopper that fits filtering flask (located above sink)
vacuum tube attached to sink faucet - make sure it is readily available
100 ml graduated cylinder
500 ml graduated cylinder
Bunsen burner and matches
gel mould - deep
disposable pipette with bulb (for sucking up bubbles)
paper towels

Mixing the histidine-citrate gel ingredients

1. The first step is to put on your gloves and measure out **32 g** of potato starch in the weigh boat (use the spatula if necessary). Gently pour the starch into the filtering flask, making sure that the arm of the flask is pointed upwards so the starch will not get stuck there. Try to make sure that the starch goes straight down to the bottom of the flask - the less starch on the walls of the flask, the better.
2. Measure out **43 mls** of the histidine-citrate buffer (pH 6.0) into the 100 ml graduated cylinder. Pour the buffer into the 500 ml graduated cylinder and add enough distilled water until **300 mls** is reached.
3. Pour approx. **75 mls** of the buffer solution into the filtering flask containing the starch. Make sure you pour the solution *gently* into the flask so that the starch doesn't splash up on the sides. Pour the remaining buffer solution into the volumetric flask.
4. Pull the Bunsen burner apparatus out towards the center of the table, turn on the gas, and light the burner using matches. Adjust the flame until is barely seen and place the volumetric flask on the burner to boil.
5. While you are waiting for the solution to boil, it is time to get everything else ready. First, slowly mix the starch-buffer solution in your filtering flask by swirling it *gently*

until you do not see any clumps of starch on the flask bottom. Next, place your gel mould on the counter near the sink and make sure you have a pipette with bulb lying close by.

6. As your buffer solution just begins to boil (small bubbles starting to appear), make sure your orange protection gloves are nearby and start to gently swirl your starch solution to make sure all of the starch is resuspended (otherwise you will get clumps in your gel).

Pouring the gel

1. Wait until your buffer solution is definitely boiling (large bubbles now appearing and steam starting to vent out the top of the flask). Don your orange gloves, give your starch solution one last swirl and take both the boiling buffer solution and your starch solution over to the sink. Slowly invert the volumetric flask containing the boiling solution into your filtering flask while slowly swirling the whole contraption.
2. Once all of the boiling solution is in the filtering flask, take it back over to your Bunsen burner and swirl the flask over the flame for about a minute or two (to make sure everything is nice and melted).
3. Turn off the gas and bring your filtering flask back to the sink area. In whatever order you prefer, put the rubber stopper on the flask, hook up the vinyl tubing to the flask arm, and turn on both faucets full blast to create a vacuum inside the flask. You should try to do these steps as quickly as possible so the solution doesn't cool off too much. Remember....if it takes too long, you can usually reheat your solution over the burner again and start over.
4. Wait about one to three minutes (this may vary) until all of the small bubbles on the flask sides have been sucked up. When there are no more small bubbles and only large bubbles on the surface, it is time to end the vacuuming process. Pull the rubber stopper off the flask, turn off the faucets, and pull off the tubing from the flask arm (actually you can do this in any order you like). Still wearing the orange gloves, take the flask over to your gel mould.
5. Gently pour the hot gel solution lengthwise in three long strips into the gel mould. If there is any solution left in the flask, pour it into the corners and along the sides of the mould. Remaining solution can then be pored into the gel center.
6. Quickly place the empty flask in the sink and start to run hot water into it.
7. Turning back to the gel, take your pipette with bulb and suck out any bubbles or clumps that you see. Remember that you have about a minute to do this after you pour the gel - otherwise the pipette will start to leave imprints in the gel. Dispose of pipette in broken glass container.

Storing the gel

1. Leave your now perfect gel on the counter to cool off for the next hour or two - the gel will turn from opaque to white as it cools.
2. Once your gel has sufficiently cooled and leaves no imprint when touched, you can cover it with saran wrap and place it in the refrigerator. *The gel will remain good for about a day or two.*

RUNNING THE GEL

If you are going to run your gel overnight, you need to start working on it around 4:00 to 4:30 pm or whatever you like (keeping in mind that the gel must run for 15 to 16 hours). The following guidelines are the ones that I have found yield the best results.

Preparing the gel

1. The first step is to retrieve your frozen wicks from the ultracold. Set them on the counter to warm up.
2. Take your refrigerated gel out and remove the saran wrap covering. Remember to place the saran wrap close by since you will be using it again.
3. Take a paper towel and place it on top of the gel. Lightly press down on the towel to absorb any excess moisture. This will give you a better grip on the gel and make it easier to handle.
4. Run a small flat spatula along the inside edges of the gel to help loosen it from the sides of the mould. Trim off any excess gel that has dripped over the sides.
5. Turn the gel length-wise so that its longest side is parallel to the edge of the counter. Place a plastic ruler on the edge of the gel that is nearest to you and make a straight length-wise cut with the flat spatula one ruler's width away from the gel side. Gently pull aside the edges of the gel at the cut to make sure that the gel is indeed separated.

Loading the gel

1. Now using forceps, start to load the wicks into the gel. Starting about one centimeter from the left edge, start to individually place the wicks into the cut, making sure that the wicks are flush against the mould bottom. Try to make sure that there is at least a 2mm separation between wicks (else the plant juices will blend together and you'll get a garbled mess on your gel). There should be room on the gel for about 30 wicks.
2. Last of all, place a bromophenol blue tracking dye wick on the extreme right side of the gel (it can be right on the edge).
3. Get a wooden stick and gently insert it in between the mould side and the bottom edge of the gel (nearest to the cut). Next, insert a straw in between the mould side and the wooden stick. This will make keep your enzymes running in a straight line and will ensure that the current will run uninterrupted throughout the gel.
4. Place the saran wrap back over the top of the gel and place the gel in the refrigerator while you do the next step.

Preparing the gel rig

1. Take the gel rig out of the large refrigerator, remove the sponges, and carefully rinse the rig out in the sink. *CAUTION: the carbon rods are very brittle and likely to break if mishandled.* Rinse the sponges out multiple times to remove any excess ions from the last run.

2. Pour histidine-citrate buffer (kept in large refrigerator in 2 liter bottle) into each of the two wells until they are both about 2/3 full. If you run out of the histidine-citrate buffer, you can make some more according to the following recipe:

   20.2 g histidine (free base) - in dry chemical cabinet
   1800 ml H_2O

   - Add stirring rod and mix well.
   - Titrate to pH 6.0 with solid citric acid (monohydrate) - on shelf above balances
   - Add 200 ml of H_2O to bring solution to 2 mls
   - Refrigerate immediately (has a shelf life of 1 month)

3. Immerse one sponge in each well until they are well saturated.
4. Remove gel from refrigerator, and fold saran wrap so that about one inch of gel is exposed on each length-wide side. Make sure that saran wrap is not touching the wicks.
5. Place the gel mould on top of the gel rig so that the wicks on orientated towards the left side of the gel rig. Carefully pull up the sponge in each well and drape it onto the exposed section of the gel. Press gently on the sponge and smooth out any air bubbles that may prevent direct contact in between the sponge and the gel.
6. Make sure that each sponge is in direct contact with both the gel and the buffer solution (along the entire length of the sponge). If needed, add more buffer to the gels to bring the level up to about 3/4 full.

Running the gel

1. Carefully carry the gel rig and gel over to a walk-in cooler. Gently place the rig on one of the shelves and orientate it so that the red and black banana clips are next to the power leads (i.e. carbon rids sticking out of the gel rig).
2. Clip on each banana clip onto its adjoining power lead (black clip should be on the left, red on the right). Double-check to make sure that the black clip is near the portion of the gel containing the wicks and the red clip is on the opposite end. Remember that the enzymes generally have a negative charge and will run towards the positive (red) end of the gel (and we want them to run the full length of the gel).
3. Turn on the Power switch. Next, make sure that the powerpack is set to constant VOLTAGE.
4. Push the Start button and wait a few seconds until some numbers start to appear on the digital readouts.
5. Using the dial or buttons, adjust the VOLTAGE (not the AMPS) to **210 Volts** and the corresponding amps should be around 0.029. The dial is a bit touchy - you may have to turn it a while before numbers start to register and then the dial becomes very sensitive - the numbers will zoom up with only a slight turn.

   NOTE: Remember that although the amps will fall as time goes on, the voltage will be constant - this is most likely to prevent burning of your gel (e.g. a constant

current of 0.029 would damage your gel). Here is an example of a really good run that I had:

Volts	Amps	Amps (end)	Start Time	End Time	Total Time
210	0.029	0.008	4:30 pm	8:35 am	16 hours

Problems: I have found that the power pack can be very temperamental, so here are some hints and suggestions to help you past trouble spots:

- *Numbers do not register on the readouts after you hit the "Start" button (or numbers are very low - e.g. 0.005 volts).*
  - either (1) your carbon rod is broken somewhere (check inside the rubber stopper), or (2) the sponges are not in direct contact with the gel and the buffer solution.
- *After you adjust the constant voltage to 210, the amps are lower than 0.027-0.029.*
  - check the following in the order presented until the problem is fixed:
    a. the wells are 3/4 full of buffer
    b. there is direct contact between the sponges and the buffer
    c. double-check the pH of the buffer (should be 6.0)
    d. mix the buffer solutions in the two wells by pipette
    e. the carbon rods are intact and well-formed (not flaky)
    f. switch gel rigs
  - if none of these suggestions work, then you may want to either adjust the voltage up to 220 volts (but not any higher or you'll burn the gel) or just run the gel longer.

Removing the wicks

1. Run the gel at the above settings for approximately 10 - 15 minutes. Then turn **off** the power and carefully remove all of the wicks with forceps.
   NOTE: Make sure that the power pack is turned OFF whenever you will be touching the gel rig!
2. Adjust the saran wrap to cover the portion of the gel you just removed the wicks from. Lay a half-filled zip-lock baggie (with H_2O) on top of the gel. Get a cold pack from the freezer and place it on top of your bag of water on the gel. Once everything is in place, shut the frig door and turn the Power back on.
3. Turn off the lights in the lab and go home to relax.

SLICING THE GEL

After about 15 to 16 hours of running your overnight gel, it is time to slice it and prepare the gel for staining. The first thing you should notice is that the tracking dye should have run off your gel and the right well should be blue-colored (if not, then the gel may not have run properly). After turning off the power pack at the appropriate time, follow these steps:

Preparing for slicing

Make sure that you have the following items on hand:

gel slicer
plexiglass gel slicing board
glass plate
flat spatula
water squirt bottle
plastic staining bowl
nearby trash can

Slice the gel

1. Remove the gel mould from your rig and pull off the saran wrap (keep it nearby since you'll not finished with it yet). Take the gel over to a counter and remove the wooden stick and straw at the bottom (these can be washed and reused).
2. With your flat spatula, carefully pry up the bottom section of the gel that was in between the wicks and the wooden stick (the portion that is about one inch wide). Throw this portion away, leaving the majority of the gel still in the mould.
3. Cut a small notch in the upper right hand corner of your gel with the flat spatula, so that you will know which side of the gel is which.
4. Place your gel slicing board directly in front of you on the counter and make sure that it is perfectly clean. Use the flat spatula to gently pry up one corner of your gel and insert your hand underneath. Gently move your hand lengthwise down the gel and insert your other hand so that eventually you are holding the gel in both hands.
5. Carefully place the gel length-wise on the gel slicing board. Carefully smooth down the gel and try to remove any air bubbles. You may need to carefully hold the gel and slicing board above your head (but make sure that the gel doesn't slide off while you're doing this).
6. Next put the slicing board back on the counter and place a glass plate on top of the gel (so that the gel is now sandwiched between the slicing board and the glass plate).
7. While applying slight pressure to the top of the glass plate, place the gel slicer on the far end of the slicing board and gently pull the slicer towards you, thereby cutting the gel. Remember that this doesn't have to be one smooth motion - at any time you can stop and readjust your hands, before continuing again.
8. Remove the glass plate and gently pry up the top gel layer with the flat spatula. Insert your hands underneath until you are holding the layer in your hands. Place it on the glass plate. Pry up the edge of the new slice that is still on the slicing board.

Transfer the new slice to a glass plate (I usually don't use this bottom slice, but I like to keep it handy in case none of the other slices come out).

9. Return the original layer from the first glass plate back to the slicing board. Repeat the slicing process.
10. Squirt a little water into your original gel mould. Remove the top gel layer again from the slicing board and transfer the newest slice (underneath) to the original gel mould. Reposition the gel slice in the mould until it is surrounded on three sides by the mould walls. Place a wooden stick on the fourth side to create a dam. This gel will be stained for PGI. Place the mould in the refrigerator.
11. Return the original layer back onto the slicing board and repeat the slicing procedure. After you have successfully sliced the gel once again, place the original layer back on a glass plate and wrap it up with your saran wrap (store in the frig). Squirt a little water into the plastic staining bowl and transfer your newest slice to the bowl. Store in the refrigerator if you will not be staining right away. This gel slice will be stained for PGM.
12. Clean up all of your equipment!

You are now ready to begin the staining process.

STAINING THE GEL

We will be staining our histidine-citrate gel for both PGI and PGM. By now you should have one gel slice in the original gel mould, one slice in a plastic staining bowl, and the rest of the gel (along with the first, bottom slice) stored in the frig.

Staining preparations

1. Remove two vials of Glucose-6-phosphate dehydrongenase (G-6-PDH or "enzyme in a tube") from the door of the freezer. Let these slowly warm up to room temperature as you complete the remaining steps. Also remove the Erlenmeyer flask containing solidified agar from the refrigerator.

Staining for PGM

I prefer to make the stain for PGM first since this requires about one hour to stain adequately (PGI just requires about 10-15 minutes). The recipe that I use for PGM is from the Wendel and Weeden chapter of the Soltis and Soltis book. The recipe is as follows:

50 ml	Tris-HCl, 50 mM, pH 8.5	[in refrigerator]
50 mg	$McCl_2$	[in dry chemical cabinet]
≈ 160 mg	Glucose-1-phosphate, Na_2 salt	[in freezer]
10 mg	NAD	[in freezer]
20 units	G-6-PDH (enzyme)	[one tube]
20 drops	MTT	[in refrigerator]
4 drops	PMS	[" "]

1. Mix up the stain in the order given in a 50 ml beaker. Before you are ready to add the MTT and PMS, take your gel slice (in the staining bowl) out of the frig. Add the last two ingredients to your beaker and then quickly swirl the mixture together before gently pouring it on your gel.
2. Quickly place your gel out of the light to incubate (in the oven or in a drawer). Remember that the MTT and PMS are light-sensitive and will turn an ugly brown if left out in the light for too long.
3. Bands should start to faintly appear in about 15 minutes, but adequate staining will require at least one hour. Check periodically to make sure that the gel does not overstain.

Staining for PGI

The staining recipe that I use for PGI is the same one as in Bob's instructions. It is as follows:

10 ml	Tris HCl, 50mM, pH 8.0	[in large frig - left side]
4 mg	NAD	[in freezer]
15 mg	Fructose-6-phosphate	[in freezer - upper shelf]

20 units	G-6-PDH (enzyme)	[one tube]
20 drops	MTT	[in frig door]
4 drops	PMS	[" "]
10 ml	hot agar solution	[in frig]

1. Mix up the first four ingredients in the order given in a 50 ml beaker. At this time, remove the gel slice (in the original gel mould) from the refrigerator and place it on the counter.
2. Take the flask of solidified agar and heat it in the microwave until boiling (about 50 seconds, depending on amount). Take a very small 10 ml beaker and place it on the counter next to your gel slice.
3. Add the MTT to your beaker and place the beaker in a dark drawer. Before you add the PMS, make sure that the agar is still liquid with no clumps in it at all (if necessary, zap it in the microwave for a few more seconds).
4. As quickly as possible, pull the agar from the microwave, add the PMS to your staining solution, pour the hot agar into your 10 ml beaker and dump that into your staining solution.
5. Swirl the mixture a few times and slowly pour it on your gel slice. As soon as the staining solution starts to set up (about 5 seconds) put the gel in a dark drawer to incubate (otherwise, the entire gel will stain dark).
6. Gel should be adequately stained in about 10-15 minutes.

Scoring the gels

You must score the gels as soon as possible since they will continue to stain unless fixed (see below). It may also be helpful to draw a picture of the bands. To assist in scoring, keep the following in mind:

PGM a.) a monomer, so heterozygotes will have two bands & homozygotes one band.
b.) this enzyme has three different loci:

Locus	Location	# alleles
1	bottom	3
2	middle	4
3	top	?

PGI a.) a dimer, so heterozygotes will have three bands & homozygotes one band.
b.) this enzyme has only one locus with four possible alleles

Fixing the gel

Gels that have been stained in a bath (like PGM) can be fixed for preservation. Gels should be soaked overnight in a 30% ethanol solution in a staining bowl. The following morning, the gel can be wrapped in saran wrap and stored in the frig indefinitely.