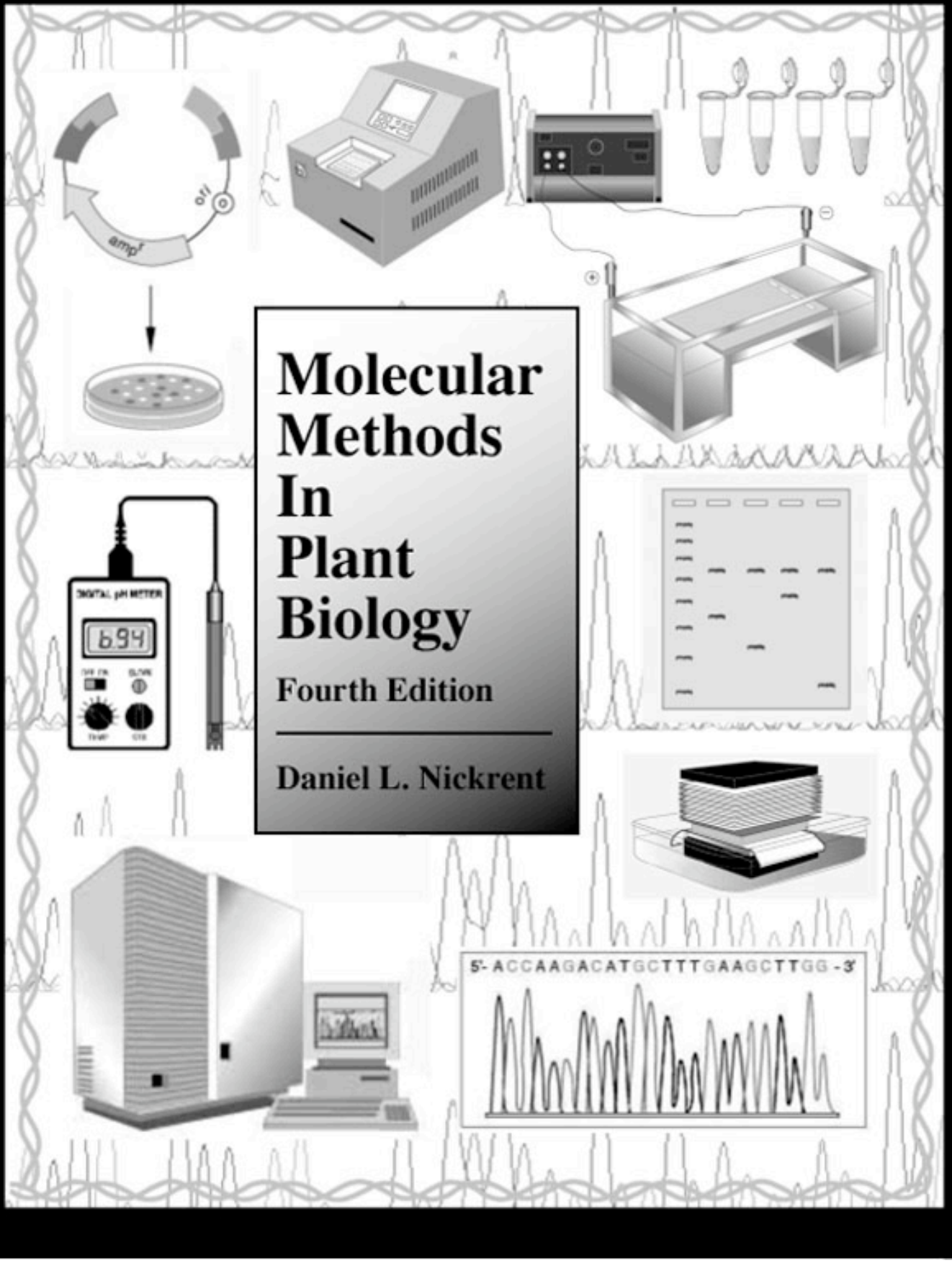

Molecular Methods In Plant Biology

Fourth Edition

Daniel L. Nickrent

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Isozyme Electrophoresis

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STARCH GEL ELECTROPHORESIS OF PLANT TISSUE

Tissue Collection

The species of organism being studied, the tissue type being used, and the number of individuals needed to adequately address the problem are all considerations that must be addressed early in an electrophoretic study. It is desirable to use, whenever possible, diploid vegetative material such as leaves, young shoots, coleoptiles, cotyledons, etc. because other ploidy levels (haploid, triploid, tetraploid) complicate interpretation and Emit various genetic analyses of the isozyme data. There are ways to use non-diploid tissue for certain applications. For example, I use triploid seed endosperm to generate allele frequency data for systematic assessments, however the direct counts of the number of heterozygotes are not representative of the diploid population level. I have also conducted isozyme studies of haploid algae and fungi and used phenetic and cladistic analyses of band phenotypes (presence and absence type data).

An absolute requirement for electrophoretic work is live plant tissue. Some enzymes may be "reconstituted" from dried material, but these represent a tiny subset of the enzymes routinely utilized in allozyme studies. The proper method of collection will vary with the species being worked with. For example, young leaves of greenhouse gown plants are ideal for enzyme extraction. It is often better to obtain young, expanding leaves vs. older ones because of lowered amounts of secondary compounds (more on these later) and because the amount of water per unit volume is lower (i.e. more cytoplasm, less vacuole). For collection, simply remove leaves from individual plants, place them in labeled plastic bags, and put them in a cooler with some ice until returning to the lab. At this point, the leaves can be extracted for protein or frozen in the ultralow freezer.

Unfortunately, freezing plant tissue prior to enzyme extraction can not be used for all plants. In another lab, oak leaves are used but certain enzymes are lost upon freezing (and subsequent thawing). This is generally undesirable because with plants one must frequently strive to maximize the number of resolvable enzyme systems. The sampling methodology for the oak leaves involves placing the leaf in a plastic bag after being wrapped in a moist paper towel. The leaves are kept on ice (in the field) and refrigerated once returned to the lab. Leaves so stored have shown good enzyme activity as long as three weeks after harvest. The disadvantage of this method is that one must really scramble to get the fresh leaf material run before the tissue dies. This can be a real problem if large numbers of individuals must be processed in a short time span. Other leaf tissue, such as peppers *(Capsicum)* actually show better enzyme activity if the leaves are frozen prior to enzyme extraction. A peculiarity of the pepper plants is that certain enzymes can not be detected unless the leaves are harvested before about 10:00 AM. Overall enzyme activity and resolution is also better if leaves are obtained at this time. It is not entirely clear how this diurnal cycle influences enzyme expression, however it may partly involve an overall "start up" of metabolic activity early in the day that utilizes sugars produced the day before. With dwarf mistletoe seed endosperm, seeds are frozen after being germinated in a growth chamber and enzyme activity remains quite good for over two years at -70 °C. The lesson from the oak, pepper, and mistletoe examples is that for an electrophoretically "naive" organism, one must start from the experience of others working with different plant groups and try a variety of methodologies to determine which are optimal.

The sample size needed is often a consideration at the onset of an electrophoretic study. This again varies with the organism in terms of the availability of individuals of a species or population. When sampling forest trees for isozyme analysis, Brown and Moran (1981) wanted to know the minimum number of trees required to ensure that the difference (d) between the sampled allele frequency and the actual population frequency is exceeded on less than 5% of occasions. This can be expressed by the formula:

$$n = \frac{(1 + 3F)^2}{8(1+F)d^2}$$

$$= 0.2\ d^{-2}, \text{ assuming } F < 0.10$$

where n is the number of trees and F is the inbreeding coefficient. For d = 0. 1, a sample of at least 20 trees is needed. A sample of 10 trees from each of two populations will detect gene frequency differences between them of 0.2 at the 5% level of significance. For populations that show only weak differentiation in gene frequencies, larger sample sizes will be needed.

Identification of separate genetic individuals is difficult in plants that reproduce vegetatively or by apomixis. What may appear to be separate shoots above ground may actually be components of one large clone connected by a rhizome or root system below ground level. A normal sampling strategy would not include excavation of each individual to determine who was connected to whom! This situation occurs all too frequently in plants (examples include trees such as *Populus* to herbs such as *Lobelia, Aster, and Podophyllum).*

An alternate strategy for sampling a common, wide ranging species would be to obtain larger numbers of individuals that are separated by geographic distances great enough to make physical connection an impossibility. The statistical analysis of the data will then require an alteration of the "scale" of population dimensions, e.g. one population may be all individuals sampled within a county (artificial partitioning) or within a river valley (geophysical partitioning). A possible problem with this strategy is that allelic similarities detected among distant individuals within the "artificial population" may be due to existing gene flow or to historical factors.

Enzyme Extraction - General Considerations

As mentioned above, a variety of tissue types can be used for extraction. Certain isozymes may be tissue specific, may show subcellular compartmentalization, or may be differentially active during various developmental stages of the plant's life cycle. For these reasons, it is strongly suggested that one choose a particular tissue type and stage of development and stay with this type throughout an isozyme study. For example, if leaves are used, always choose leaves of approximately the same dimensions, from the same position on the stem, and, if sampled from natural populations, during the same growth phase during a season. As mentioned with the peppers, even time of day may be a factor to consider.

The methods of homogenizing plant tissue to obtain enzymes for electrophoresis are quite variable and depend upon the species of plant used and the tissue type. For example, megagametophyte tissue of balsam fir (Jacobs et al. 1984) gave excellent enzyme activity across many loci when extracted in distilled water. More often, a buffer is used to maintain a particular pH. One of the major problems encountered by researchers who first utilized plant tissue for electrophoresis involved dealing with secondary compounds, specifically phenolics, which bind to proteins and adversely affect their activity. Many recipes have been developed which deal with these compounds. Table 1 gives some commonly used components and their function.

Table 1. Plant Enzyme Extraction Buffer Components and Their Function

Component	Function
ascorbate (0.1 - 0.3 M)	reduces quinones, phenols
bovine serum albumin (0.1 %)	binds fatty acids
butyl alcohol	removes phenolics
dimethylsulfoxide (DMSO) (10%)	freeze stabilization
diethyldithiocarbamate (DIECA) (0.02 M)	competes with phenolase for copper ions; thiol reduces quinones; inhibits dehydrogenase activity
EDTA (Ethylenediamine tetraacetate)	chelating agent; binds divalent cations
germanium dioxide (0.01 M)	binds o-diphenols, acts as a substrate inhibitor
ß-mercaptoethanol (0.1 - 1 %)	sulfhydryl reducing agent
polyethylene glycol (PEG) (I%)	freeze stabilization
polyvinylpyrrolidone (PVP-40; soluble)	binds phenolics
2-phenoxyethanol (1%)	removes phenolics
sodium metabisulfite (0.02 M)	phenolase condensation to a non-reactive phenolase OH-sulfite compound
Tris -HCl (0.1- 0.2 M)	pH stabilization (7.0 -8.5)

In addition to the components shown in Table 1, many other ingredients have been added to extraction buffers such as sucrose, NAD, NADP, pyridoxal 5'-phosphate, Tween 80, cysteine-HCl, dithiothreitol, and Tergitol. Cardy, et al. (1981) report the homogenization of samples in 16.7% sucrose and 4% ascorbic acid selectively inactivates the cytosolic forms of MDH which facilitates the identification of mitochondrial forms. Oxidized nucleotide coenzymes such as NAD, in combination with ß-mercaptoethanol, helps to stabilize plant dehydrogenase activity (Hassig and Schipper, 1975). Detergents such as Tween possibly help solubilize membrane bound proteins. It is clear that experimentation is necessary to determine an appropriate extraction protocol for a given plant, however methodologies developed for one taxon often work for related taxa.

If it is possible, tissue samples should be ground immediately before loading onto the gels. It is possible to store homogenates frozen, preferably at -60 to -100 ˚C. Some taxa can be stored in this fashion for days or even weeks, yet others show considerable enzyme activity loss with storing frozen for even a few days. Again, storing capacity must be determined empirically. Samples are usually absorbed onto filter paper wicks (Whatman #3M) to facilitate loading onto the gels. Other methods include placing a comb into the liquid gel which forms a "slot" after the gel solidifies. Liquid samples are then transferred to the slot with a pipet.

Enzyme Extraction Protocols

Protocol using a Homogenizer

The extraction methods mentioned above may not work for very tough plant tissue. To adequately disrupt these tissues, grinding on liquid nitrogen and/or homogenization with a motorized system (e.g. the Brinkman Polytron) has proven especially useful. This method works very well for dwarf mistletoe *(Arceuthobium)* stems and conifer needles. The Polytron can also be used to grind softer plant tissues (such as leaves) directly without the liquid nitrogen step. As always, some experimentation with the extraction buffer may be necessary to maximize enzyme yield from a particular plant species.

For most isozyme studies and for most plant tissues, the best possible results are obtained when young plant tissue is extracted immediately after it is harvested. Since this is not always practical, measures to ensure freshness should be taken. One method is to harvest whole plants (for small species) or parts of plants (e.g. leaves), seal them in ziplock sandwich bags, label the bags (permanent marking pen), and place the samples on ice (or under refrigeration) until ready to extract protein. The amount of tissue used for extraction will vary depending on availability and the volume of the vessel you choose to bring the tissue in. Generally 1 - 2 g of fresh tissue is all that is needed.

Before starting the extraction procedure, you will need to make up the extraction buffer. Listed below are two extraction buffer recipes that work well with a wide variety of plants:

"Microbuffer" (Werth 1985)

Molarities of Components Sigma Amount in 100 ml

0.2 M Trizma base	T- 1 503	2.42 g
7 mM sodium borate, 10-hydrate, (= sodium tetraborate or borax)	B-9876	0.26 g
2 mM sodium bisulfite (= sodium metabisulfite)	S-9000	80 mg
50 mM ascorbic acid, sodium salt	A-7631	0.99 g
4 mM diethyldithiocarbamic acid	D-0632	68 mg
0.4 mM EDTA, disodium salt	ED2SS	13 mg
pH the solution to 7.5 with conc. HCl		
Add just prior to use:		
0.17 % ß-mercaptoethanol	M-6250	0.17 ml
10 % (w/v) polyvinylpyrrolidone (PVP-40)	PVP-40T	10.0 g

The microbuffer is exceptionally versatile and has been used successfully for many flowering plants such as *Acacia, Primula, Silene, Draba, Aster, Populus, Quercus, Asclepias* and many others. The P & C #3 buffer is used for dwarf mistletoe (*Arceuthobium*) tissue mainly because it seems that the added detergents (Tween and Tergitol) and the B.S.A. take care of lipids and triglycerides (respectively). The NAD in the buffer may also improve banding for enzymes such as MDH and PGI.

The buffers can be stored refrigerated for several days or frozen for several weeks, however, **DO NOT** freeze them with the PVP-40. This should be stirred into the buffer just before use. I add the p-mercaptoethanol just before use as well, mainly to keep the odor from escaping during storage. After stirring the PVP-40 into the buffer, it will still take some time to go into solution. I usually allow 30 minutes time and place the buffer in the refrigerator to get cold. Do not store the extraction buffer with PVP for more than one day.

Before getting started, you will need the following equipment:

1. Homogenizer. Brinkman Polytron: the 1.0 cm generator can be used for volumes as low as 3.0 ml. For smaller tissue samples, the Tissue Tearor (Biospec Prod., Inc.) can be used.

2. Liquid nitrogen tank with nitrogen (optional - see below)

3. Mortars (9.3 X 6.7 cm), pestle, spoonulas (**optional**)

4. Coolers with ice

5. Grinding/centrifuge tubes.

For the Polytron, I use non-breakable 46 ml polypropylene tubes (2.85 X 10.35 em, Fisher # 05430-20). A tube with slightly smaller outside diameter such as a polyallomer tube (25.3 X 88.9 mm, Fisher 05-566-5M) or a Pyrex glass tube with the same dimensions will give better results since there will be less space between the inside wall of the tube and the homogenizer generator. If you have an appropriate rotor to take these tubes, your samples can be spun without transferring to another size tube.

For the Tissue Tearor. Use glass test tubes (1.0 X 7.5 cm).

6. Centrifuge and rotor. You will need to spin the samples at ca. 10,000 G at 4 ˚C, so a refrigerated centrifuge is required (with fixed angle or swinging bucket rotor).

7. Plastic beakers (600 ml to 1.0 liter sizes) for ice bath and rinse water.

8. Squirt bottle(s)

9. 1.5 ml microfuge tubes and rack

When you have the centrifuge precooled to 4 ˚C and the PVP has gone into solution, you are ready to begin grinding the plant material. Use liquid nitrogen only if the plant material is too tough or fibrous for homogenization directly.

1. To grind the tissue with liquid nitrogen, add 1-2 g of plant material to the mortar. Cover the tissue with nitrogen (mortar ca. 1/2 full). Grind until all the nitrogen evaporates and the material is reduced to a fine powder. Quickly transfer the powder to the centrifuge or test tube using a spoonula. It is important to not let the powder begin to thaw before buffer is added. Add a volume of ice cold buffer that is approximately equal to the amount of powder you have in the tube (by eye, works out to be 3-4 ml). You may use a little more or less depending upon taste and later results.

2. If you do not grind the plant material on nitrogen, cut the leaves into small pieces and place in the bottom of the centrifuge or test tube. As above, add an equal volume of ice cold buffer.

3. Homogenize the sample with buffer. Move the tube up and down gently and stop if excessive foaming occurs. Buffers that contain detergents are of course more prone to foaming. Homogenization should not require more than ca. one minute. If longer times are used, intermittently cool the sample on ice. When the sample is completely homogenized it should be consistent in texture (no large "chunks"). Cool the sample briefly by immersing the tube in an ice bath. If a different size of centrifuge tubes are to be used, pour the contents into the other tube. Store the tube on ice until enough tubes accumulate to spin. Remember, as you work, record the number of the centrifuge tube in your log to identify each sample.

4. Clean the homogenizer generator after each sample by immersing it in a beaker of water. Turn it on for a few seconds to clear away debris. Rinse the generator with distilled water and wipe clean with a paper towel.

5. Place the tubes in the centrifuge, balanced tubes opposite each other. Spin the samples at 10,000 rpm for 15 minutes at 4 ˚C.

6. Label as many 1.5 ml plastic microfuge tubes as you will need. Store in a rack in the freezer until later.

7. When the spin is complete, remove the tubes from the centrifuge, check your log to match the sample number with the tube number, and quickly pour off the supernatant into a microfuge tube. The spinning step should separate cellular debris from the liquid fraction such that the supernatant is relatively clean (i.e. no chunks). If you find that separation was not complete, try spinning longer or at higher speeds. I have found that better separation occurs with liquid nitrogen ground material than with material that was homogenized directly.

8. Cap the tubes and place the samples in a freezer. The best results are obtained with colder storage temperatures (-70 to -100 ˚C).

Whether one can successfully store samples at ultralow temperatures depends on the organism and the enzyme system and must be determined empirically. For example, aspartate amino transferase (= glutamate oxaloacetate transaminase or GOT) does not appear to store well frozen, at least for several dicots we have tried. One can, of course, absorb the homogenate directly onto filter paper wicks and load the gels directly to avoid problems with freezing the sample. The ability to freeze the sample is an advantage, however, since it does not require that the grinding and gel run be done on the same day.

Alternate Extraction Protocols

Often very simple extraction methods result in good enzyme resolution; hence, experiment with the simple procedures before launching into the more complicated ones. For example, many workers simply add an equal volumes of plant tissue and buffer to a small chilled mortar (or depressions of porcelain spot plates), grind, and then absorb the grindate onto filter paper wicks through Miracloth. Sometimes acid washed sand is added which acts as an abrasive and facilitates grinding.

For fairly soft plant tissue, the ground-glass homogenizer tube method is quite effective. Again, approximately equal volumes of plant material and buffer are added to the cold tubes and the glass pestle is placed in the rubber tube sleeve of the homogenizer. The material is ground while keeping the tube immersed in an ice bath. Usually this solution must be centrifuged to separate cellular debris (ca. 15 mins at 4 ˚C and 15,000 rpm). The supernatant is poured into cold, labeled vials and stored at -60 to -100 ˚C.

For very small samples (such as individual seeds, ovules, seedling leaves, etc.), I find that grinding the tissue with a stainless steel pestle in wells formed in an acrylic block works very well. A Contorque or Delrin spinning homogenizer can be used with the pestle. The blocks contain either 20 or 36 wells. The smaller wells can accommodate the plant tissue (samples as small as 5 mg) and ca. 2050 μl of extraction buffer. The larger wells can be used with as much as 150-200 μl of extraction buffer.

Various plastic and teflon pestles are now being marketed by biological suppliers that will fit snugly into a 1.5 ml microfuge tube. We have had good luck with extracting small leaf disks (ca. 10 disks cut out with a paper hole puncher) using ca. 200-400 μl of extraction buffer and this type of pestle. Since the inside walls of the tube are smooth, very little "grinding" takes place and the tissue is actually squeezed between the wall and the pestle. It is best to start the grinding by pushing all of the tissue to the bottom of the tube before starting the pestle spinning.

Gel Buffers

Electrode and Gel Buffers

A tremendous variety of buffers have been used for starch gel electrophoresis. They vary in the type of buffering agent (phosphate, citrate, Tris, etc.), pH, molarity, running times, storage times, and other features. The only way to determine the appropriate buffer for an organism is to test it directly. A logical approach is to sample buffers reported in the literature that have proven effective with organisms related to yours.

Methodological papers that describe electrophoretic techniques and that list buffers and staining protocols include: Shaw and Prasad (1970) [note several errors in this reference outlined in Soltis et al.], Selander et al. (1971), Siciliano and Shaw (1976), Harris and Hopkinson (1976) [humans], Conkle et al. (1982) [conifers], Soltis et al. (1983) ferns], Soltis and Soltis (1989), Tanksley and Orton (1983), Cardy et al. (1981) [maize], Cardy and Beversdorf (1984) [soybean], Cheliak and Pitel (1984) [conifers], Pitel and Cheliak (1984) [conifers], and Micales (1986) [fungi].

Although many different buffers exist, we have found that only a few different ones are required to sufficiently resolve a large number of enzymes for widely divergent organisms. In an isozyme study, the ideal situation would be that all enzymes resolve equally well on one buffer system. This situation is never fully realized since enzyme mobility and activity varies from buffer to buffer. Given this, one wishes to minimize the number of buffer systems and maximize the number of different enzymes that can be obtained from each gel. Three different buffers will most often provide the range of conditions needed to resolve a large group of enzymes.

The milliamperage (and/or voltage) suggested below each recipe will vary based upon the physical dimensions of the gel setup you are using. Thicker (and larger) gels take more voltage to derive the same milliamperage as a smaller gel. We generally monitor the progress of a gel run by a bromophenol blue marker dye. We stop the run when the dye reaches the anodal sponge. For better protein migration, one can also run the dye into the sponge for at least a half hour.

The "Heavy Duty" Buffers

In this category I include the lithium hydroxide, tris-borate, and tris maleate buffers. These comprise discontinuous and continuous types of buffers. Continuous buffers have identical gel and electrode components (at the same pH and molarity) and are renowned for their long running times (i.e. over 8 hours). Depending upon the organism, of course, the lithium gels (and variants such as "Ridgeway") are excellent for enzymes such as PGM, GOT, PGI, TPI, and LDH (animals).

Lithium Hydroxide (Selander et al. 1971)

Solution A (used "straight" as electrode buffer)
(0.029 M lithium hydroxide, monohydrate; 0. 19 M boric acid; pH w/o adjustment ca. 8. 1)

LiOH-H20	1.20 g/liter
Boric acid	11.89 g/liter

Solution B
(0.05 M Tris; 8.3 mM citrate; pH 8.4)

Citric acid (free acid)	1.6 g/liter
Tris (Trizma) base	6.2 g/liter

pH to 8.4 using 6.0 N NaOH. To make the gel buffer, mix I part of solution A with 9 parts of B. We run the gel at ca. 75 ma. Run times are ca. 4 - 5 hours.

Ridgeway Buffer (Ridgeway et al. 1970)

Solution A (used straight as electrode buffer
(0.06 M Lithium hydroxide, monohydrate; 0. 3 M boric acid; pH 8. 1)

LiOH	2.52 g/liter
Boric acid	18.55 g/liter

pH is normally between 8.1 and 8.2 without adjustment

Solution B
0.03 M Tris, 5 mM citric acid; pH 8.1

Tris (Trizma) base	3.63 g/liter
Citric acid (free acid, anhydrous)	0.96 g/liter --or -- 1.05 g citric acid monohydrate

pH is normally between 8.0 and 8.1 without adjustment

To make the gel, mix 1 part of solution A with 9 parts of solution B.
We run the gel at ca. 50 - 75 ma. Running time is ca. 4 hours.

Tris/EDTA/Borate (Ayala et al. 1972)

0.084 M Tris, 8.7 mM boric acid, 7.7 mM EDTA; pH 9.1

Tris (Trizma) base	10.20 g/liter
Boric acid	0.54 g/liter
EDTA (Na_2 salt)	0.29 g/liter

Use conc. HCI (ca. 0. 1 ml) to pH to 9.1

This is a continuous buffer, so it is used undiluted for making the gel and for the electrode box.
Run times are generally long (8 - 12 hours) at maximum milliamperage.

Poulik (Selander et al. 197 1)

Electrode Buffer
(0.3 M boric acid; pH 8.2)

Boric acid	18.55 g/liter

Gel Buffer
(0.076 M Tris, 5 mM citric acid; pH 8.7)

Tris (Trizma) base	9.21 g/liter
Citric acid (anhydrous)	1.05 g/liter

Run at ca. 75 ma. Running time ca. 4 hours

Tris-Maleate (Selander et al. 197 1)

Electrode Buffer
(0.1 M Tris, 0.1 M maleic acid, 0.01 M EDTA, 0.01 M $MgCl_2$)

Tris (Trizma) base	24.20 9/liter
Maleic acid	23.20 g/liter
EDTA (Na_2 salt)	7.44 g/liter
$MgCI_2 \cdot 6H_20$	4.06 g/liter

Use 10 M NaOH 9 (ca. 25.9 ml) to pH to 7.4, bring vol. to 1.0 liter.

Gel **Buffer**
Use 1 part of electrode to 9 parts of water.

Run gel at ca. 75 - 100 ma. Running time is ca. 5 hours.

The "Light Duty Buffers"

In this category I include the tris-citrate, histidine, phosphate, and citrate-morpholine buffers. I call these "light duty buffers" because most have low molarity gel buffers, prepared by diluting the electrode buffer with water. It is this group of buffers that provides the best resolution for enzymes such as 6-PGDH, G-6-PDH, IDH, MDH, ACO, PX, PGI, and many others.

C & T (Clayton and Tretiak 1972)

Electrode Buffer
(0.04 M citric acid)

Citric acid, anhydrous 7.68 g/liter or--
Citric acid, monohydrate 8.40 g/liter

pH to 6.1 using N-(-3-aminopropyl)-morpholine (ca. 10 ml/liter)

Gel **Buffer**
(2 mM citric acid)

The original recipe simply dilutes the electrode buffer 1: 19 with water to prepare the gel buffer. This buffer is then ca. pH 6.0. We have found that by changing the pH (with morpholine) to 6.5 (watch out - it only takes a couple of drops!), better resolution occurs for many enzymes such as MDH, 6PGDH, etc.

Run gel at ca. 50 ma or less since running time is short (ca. 3 hours). Be sure to check cathodal slice for enzyme activity.

Many types of histidine-citrate buffers have been reported. Number 7 listed below has been used extensively by Cardy's group for soybean isozymes. Labs working with conifer megagametophytes (such as Guries, U of Wisconsin) use the histidine-citrate buffer reported by Fildes and Harris (1966). We find the histidine buffers behave much like the citrate-morpholine buffer in terms of their ability to resolve isozymes.

NOTE: There are many types of histidine available, e.g. both L- and D-stereoisomers, monohydrate or anhydrous, and as a free base or an HCI salt. L-histidine-HCl monohydrate is the cheapest form followed by L-histidine, free base. Be sure to check the molecular weight of the form to determine the proper amount for a particular molarity.

Histidine-citrate (Cardy et al. 1981)

Electrode Buffer
(0.065 M L-histidine, 7 mM citric acid; pH 6.5)

L-histidine, free base (M.W. 155.6)	10.09 g/liter
Citric acid, anhydrous	1.34 g/liter

pH to 6.5 with citric acid

Gel Buffer
(0.016 M L-histidine, 2 mM citric acid; pH 6.5)

Dilute 1 part of electrode with 3 parts of water

Run gel at ca. 50 ma. Gel running time is ca. 4 hours.

Histidine-citrate (Fildes and Harris 1966; #1 1 in Soltis et al. 1983)

Electrode Buffer
(0.41 M citric acid; pH 7.0)

Citric acid ($Na_3 \bullet 2H_20$) 17.6 g/liter

pH to 7.0 with 0.41 M citric acid (anhydrous; M.W. 192.1 - 78.8 g/liter)

Gel Buffer
(5 mM L-histidine; pH 7.0)

L-histidine -HCl monohydrate	1.05 g/liter	----**OR** ---
L-histidine, free base		0.776 g/liter

pH to 7.0 using 0.1 N NaOH

Run gel at ca. 50 ma. Running time ca. 3- 4 hours.

A wide variety of tris-citrate buffers can be seen in the literature (such as in Soltis et al. 1983). Most modifications have only slight variations in the molarity of the tris, citrate, and/or pH. The only way to assess which is best for your organism is to experiment. The following has proven especially useful for a number of plant species examined in our lab:

Tris-Citrate 7.5 (Soltis et al. 1983)

Electrode Buffer
(0.223 M Tris, 0.086 M citrate; pH 7.5)

Tris (Trizma) base	27.00 g/liter		
Citric acid (free acid)	16.52 g/liter	or	18.06 g citric acid monohydrate

pH to 7.5 using 6.0 N NaOH (takes about 40 ml)

Gel Buffer
(8 mM Tris, 3 mM citric acid; pH 7.5)

Dilute 35 ml of electrode buffer to 1.0 liter.

Run at ca 50 ma. Running time is ca. 4 - 5 hours.

Phosphate 6.7 (Selander et al. 1971)

Electrode Buffer
(0.138 M potassium phosphate, 0.062 M NaOH; pH 6.7)

KH_2PO_4, monobasic	18.78 g/liter
NaOH (pellets)	2.48 g/liter

Adjust pH to 6.7 with 4 N NaOH

Gel Buffer

Dilute 1 part electrode with 19 parts water.

Run gel at 120 volts for 5 hours.

Starch Gels: Preparation, Electrophoresis, Slicing & Staining

Sources of Starch

The hydrolyzed potato starch we previously used in our lab came from Sigma Chemical Co. (S-4501). Sigma purchases this starch from Connaught Lab. (P. 0. Box 1755, Willowdale, Ontario, Canada, M2N 5T8). A new company, founded by Kerry McEntire (Starch Art, P.O. Box 268, Smithville, TX, 78957) is now producing an excellent (and more economical) starch for electrophoresis.

Being a biological product, different lots of starch will have different electrophoretic characteristics. We generally use 14% gels (w/v), however many labs go as low as 12%. The only disadvantage here is that the slices may be more difficult to handle. Remember to weigh out starch on a triple-beam balance, not a Sartorius scale where the fine powder can sift into the works causing all sorts of problems.

Cooking a Starch Gel

Prior to starting, get the following items ready:

- Ice in small coolers for the vacuum pump traps
- Electrode and/or gel buffers
- Gel molds
- Starch
- 1.0 liter flask(s) [as many as there are gels to pour]
- Bunsen burner
- Spatula
- 500 ml graduated cylinder(s) [as many as there are gels to pour]
- Plastic wrap

A number of methods exist to cook starch gels. These fall into three categories: the "traditional" method, the hotplate method, and the microwave method. The non-traditional methods involve heating ca. 2/3 of the gel buffer (either in a microwave oven or on a hotplate) and then adding this hot buffer to a slurry composed of the starch and the remaining 1/3 of the buffer. Considerable agitation is necessary during this mixing process to ensure lump-free gels. The mixture is then heated over a flame (or again in the microwave) until boiling. We find that the most consistent results can be obtained with the traditional method (outlined below), however you may wish to experiment with the others.

1. Set up a vacuum pump (or sink style aspirator if you have enough water pressure) with at least two traps (one liter side-arm vacuum flasks) on ice. The traps will keep the hot vapors from the starch solution from condensing inside the oil chamber of the pump.

2. Weigh out the starch and add to 1.0 liter Erlenmeyer flasks using the large plastic funnel. Use masking tape to label the flask for the particular buffer.

3. Using a 500 ml graduated cylinder, measure the appropriate volume of gel buffer, noting any dilutions (with dH_20) that are required by certain recipes. Pour the buffer into the flask, cover the mouth with your palm, and vigorously shake the solution. This mixes the starch and buffer and breaks any lumps that may have formed with the starch.

4. Light the bunsen burner (full blast) with the striker and retrieve one pair of ZETEX or Tericloth gloves (safe replacement for Asbestos gloves) from the refrigerator freezer. Heat the solution over the burner with continuous swirling. Switch from one hand to the other when one arm becomes tired. After about two minutes of heating the starch grains will burst and the solution will change character

becoming thicker and more translucent. Continue shaking (now a lateral back and forth motion; you should hear a "plop, plop" sound) until the gel starts to boil. When the gel boils it will become slightly thinner.

5. To degas the gel, start the vacuum pump, place the stopper over the mouth of the flask, and bend the rubber tubing over to seal the system. Gently shake the flask while degassing; try not to let starch get sucked into the tubing. When the starch solution settles and most of the small bubbles have disappeared, slowly release the rubber tubing. Quickly, pour the starch into the mold (from the center outward). 350 ml of starch/buffer after cooking is enough to slightly overfill the molds.

6. Remove any foreign matter (clumps of starch, paper, etc.) that may have gotten into the solution using a spatula. Do not try this any later than 30 seconds after pouring the gel (it is already starting to solidify at this point). Large bubbles should also be removed if they are in the area of the gel that will be cut and stained. Small bubbles near the top are OK (the top slice is discarded anyway).

7. Remove the label from the flask and place it on the outside of the gel mold.

8. Immediately fill the flask with hot water and let the water run through the flask for about 1-2 minutes. This helps make later washing easier. If you must dispose of molten starch (say a mistake is made or there is excess starch in the flask after pouring), it is much better to pour the excess onto a paper towel and let the solution solidify before tossing it in the trash. Conversely, starch still in suspension can be dumped down the sink.

9. When all gels for the day are poured, thoroughly clean the side-arm trap flasks, stoppers, and rubber tubing using hot water. Leave the parts disassembled so they will dry.

10. When a gel has cooled to room temperature (test by placing hand over gel), wrap it with plastic wrap. Wrapping helps prevent excessive evaporation prior to running the gel. Gels must be "aged" for at least one hour after cooling for proper running. Gels should not be run if left for over one day.

11. When convenient, rinse the sponges with deionized water and fill the buffer chambers with the appropriate electrode buffer. The chambers should be filled to ca. 1/4 inch from the top (with the electrode apparatus in place). The buffer height can be adjusted later after the gel has been set in place.

12. Place the wrapped gels on top of their appropriate electrode buffer chambers. Leave in the dairy case for at least an hour to cool.

Loading the Samples on Gels

In past years we would dip individual filter paper wicks into sample vials and then apply these to the cut surface of the gel. Our new method is much faster and results in very evenly spaced samples on the gel. The equipment was designed to complement our existing 350 ml gel mold dimensions (168 X 156 X 10 mm), however these ideas could be modified for molds of any size.

Our method of loading involves the use of paper wick combs. Each comb contains ten 5.8 X 18 mm wicks or eighteen 2.8 X 19 mm wicks (Whatman 3MM chromatography paper) held together by a doubled-over piece of "time tape". Two interdigitating combs are used such that a total of 20 or 36 samples can be run per gel. The spacing of the wicks is kept constant while the comb is being assembled by using a plexiglass template. We chose the 9.0 mm space between wicks because this is the center to center distance between microtitre plate wells which hold the sample homogenates. We recently had plexiglass sample plates manufactured that have the proper spacing between wells, thus avoiding the use of microtitre plates.

Most of our plant enzyme homogenates are stored in microfuge tubes at ultralow temperatures. Part of the frozen homogenate (e.g. 25- 200 μl, depending on the well size and the number of "dips" you need) is transferred to the plexiglass sample plates using a small metal scoop. The samples are kept on ice during this procedure so that they can be returned to the ultracold still partially frozen. This allows very long (> 3 years) storage with no detectable loss of enzyme activity. If very small samples (e.g. seeds) are used, the tissue can be homogenized directly in the wells and then either stored frozen or absorbed immediately on filter paper wicks. The odd numbered samples (i.e. 1, 3, 5 ... 17) are loaded into the top row of wells and the even numbered samples (2, 4, 6 ... 18) are loaded into the bottom row. The samples will be positioned sequentially on the gel after the combs are interdigitated. When the homogenate has thawed (keep cold on an ice pan after this), the combs are inserted into the wells to absorb the sample. We use a bromophenol blue marker dye (0.1%) in the last well.

Retrieve the gel from the dairy case and remove the plastic wrap. It should be cold to the touch and somewhat firm. Trim any excess starch from the edges of the gel mold with an X-Acto knife. Choose the best position for your origin (ca. 3.0 - 4.0 cm from the cathodal end). Using the straightedge of a ruler and an X-Acto knife, make a straight, perfectly vertical incision from one side to the other. Make sure the knife is held perpendicular to the gel and that the point reaches the bottom. Gently lift out the backslice and return it to the mold leaving a space at the origin.

Remove the odd numbered comb from the sample wells. Gently blot off the wicks on a paper towel but be careful not to cross contaminate between samples. Apply the comb to the cut gel surface such that wick #1 is ca. 2 mm in from the edge. Make sure the wicks reach the bottom of the gel. The wicks should stick to the cut surface thus allowing the comb to "stand" upright. Repeat this process for the even numbered comb. Be especially careful when interdigitating it between the comb already on the gel. Push the backslice forward against the origin and massage out any air bubbles below the front or backslice. Be sure there is good contact at the origin by gently pushing the front and backslice together.

The gel is covered with plastic wrap (gently accommodating the combs) and is then placed on the electrode box. You will note that the time tape "backbones" of the two combs project up from the surface of the gel. This may appear to be a problem since the samples would be disturbed if the cooling pan of ice were placed on at this time. We only put the ice on <u>after</u> we have run the samples into the gel and pulled the combs (ca. 15-20 minutes). To keep pressure on the sponges during this initial time, we fill two plastic stain boxes with ice and position one over each sponge. After the wicks are removed, the aluminum ice pan is placed on the gel for the rest of the run.

Lithium hydroxide and Tris-EDTA-Borate gels tend to heat up during running. This results in a bowed front and sometimes gel shrinkage severe enough to pull the gel apart at the origin. If this happens, electrical continuity between the anode and cathode is interrupted and the gel stops running. To prevent this (for slab gels), a plexiglass spacer bar can be inserted between the gel and the gel mold at the anodal end.

<u>Electrophoresis</u>

The gel mold is always placed in the buffer chamber assembly with the cathodal end (negative, black taped plug) to the left and the anodal end (positive, red plug) to the right. The sponges we use are cellulose sponge cloth (Magla Product, Irvington, NJ, 07111). These are positioned about 3-4 cm onto the gel and then into the buffer chamber between the electrode apparatus and the mold. The top of the gel is always covered with the plastic wrap to prevent electrical conduction between the two sponges via the aluminum pan.

We use Fisher Model FB 105 power supplies. The "ON-OFF" power switch is on the left of the unit and the voltage selection knob is in the middle. The switch on the right selects either milliamps or volts (we usually monitor the former). For starch gels, you will probably need the unit set to the "Hi"

range (lower switch).

As a general rule, it is wise to keep the milliamperage below 50. This will prevent any unnecessary heating of the gel during running. Be cognizant of any peculiarities when turning up the voltage. If you get voltage but no milliamperage, then no current is running through the gel. Check to make sure you have turned on the correct number power supply for the gel you are using. If the voltage and milliamperage values are very discordant, you should question whether you have prepared the gel correctly. Generally, to obtain 50 milliamperes, between 250 and 350 volts should be used (depending of course on the gel buffer). Keep in mind that most gels show a decrease in milliamperes as the gel run proceeds. The voltage can be adjusted (if desired) along the way to compensate for this change.

After 20 to 30 minutes, or until the bromphenol blue marker dye has migrated into the gel, the power can be turned off and the combs removed from the gel. To ensure good contact of the front and back slice of the gel, the plexiglass spacer bar can be placed between the gel and the gel mold (anodal end) at this time (optional).

Continue the electrophoresis noting the voltage, milliamperage, and condition of the ice in the pans. Run the marker dye out ca. 10 cm from the origin or until the marker dye touches the sponge. When ready to take the gel down, turn the power knob to "OFF". The gel is now ready for slicing.

During the time the gels are running, it is good practice to weigh out all chemicals and to dispense the buffers into the boxes for all enzymes to be stained. The most common stain box buffer is 0.2 M Tris-HCI pH 8.0 which is easily dispensed using the repipetter. All stain boxes have also been labeled with drafting tape with information on (1) the enzyme system (three letter abbreviation), (2) the gel run number (yours), (3) the gel buffer, and (4) the date and/or your initials. If all of this is prepared ahead of time, the slicing and staining can proceed smoothly and rapidly.

Slicing Gels

Although starch gels are fairly tough, care should be taken when removing the gel from the mold and during slicing. Most proteins are negatively charged and move towards the anode, however in an electrophoretically naive organism, it is not known *a priori* how certain enzymes will behave. Until determined otherwise, both the front and backslice of the gel should be taken for staining.

The procedure used in our lab for cutting and slicing gels uses the gel slicer apparatus in the accompanying figure and outlined below:

1. Using the width of a stain box, measure the gel, still in the mold, for dimensions that will fit into the box. Cut away the anodal portion of the gel and the required portion of the backslice so that both pieces will fit into the box. Cut a thin strip away from the right hand side of the gel (where the marker dye migrated). This will allow you to start slicing the gel on a freshly cut surface. Cut off the upper left hand comer of the front slice and the lower left hand comer of the backslice. These notches will allow you to identify the left and right sides of the gel slice should it get flipped over.

2. Place the gel (front and backslice, if needed) onto a clean paper towel and blot off excess moisture. Move the gel piece(s) to the bottom plate of the slicer. Pat the gel to remove air pockets that may get trapped underneath.

3. Add one spacer strip to each side of the bottom plate.

4. Check the tightness of the wire on the cutter, wipe the wire with a moist paper towel, and then, pressing firmly but gently downward, run the wire along the two spacer strips the full length of the gel. This should be an even motion, not so fast as to make the gel "jump" when the wire enters or leaves the gel.

5. Add another spacer strip to each side of the bottom plate and continue slicing. For a 1.0 cm thick gel you should be able to get ca. 7 slices (not counting the top and bottom). We generally do not use the top slice, however the bottom slice can be used in a pinch. Some workers report a certain order of slices that must be allotted to particular stain boxes but the reasons for this are not entirely clear.

6. The slice is then placed in a stain box, notch to the left and the buffer swirled to wet the slice evenly.

Staining Gels

Methods to stain for a large number of enzymes are known. Many of these are commonly used in plant isozyme studies (Table 2) and others are more exotic, having been developed using human or animal tissue. The staining agent for different enzyme systems is varied, ranging from the standard tetrazolium stains (NBT, MTT, and PMS) to fluorescent stains. Be aware that may of the substrates and stains used in this part of the electrophoretic procedure are toxic or carcinogenic. Care must be taken to avoid contacting these chemicals. It is advisable, especially when handling stained gels, to wear gloves.

As mentioned, the stain boxes should have been labeled in advance. The recipe card for a particular enzyme is placed near the stain box so that reagent amounts can be followed explicitly. The dry chemicals that were weighed out previously can now be removed from the small desiccator in the freezer. When a large number of different solids are to be weighed out, the weigh paper is folded, taped shut, labeled, and stored in the desiccator.

Chemicals are usually added in the order given on the card, substrates first, stains last. Do not pour dry chemicals or stains directly on the gel; tip the stain box and pour the chemical in the buffer, mixing well afterwards. Many substrates are dispensed as liquids (e.g. glucose-1,6-diphosphate, malic acid, and $MgCl_2$) using appropriate size pipettes. Incidentally, mouth pipetting is a matter of person choice; for absolute safety, the pipetter (top drawer, island) should be used, especially if one is unsure about the reagent. Always use the pipetter with strong acids, bases, carcinogens (e.g. LAP substrate), or toxic chemicals! Most of the tetrazolium dyes (and others such as Fast Blue BB salt used for GOT staining) are light sensitive, especially after being put in solution. For this reason the serum bottles with some of these liquids are covered with black tape. After the addition of these stains (using syringes) to the boxes, it is important to avoid any unnecessary exposure to light. This is accomplished by placing the box in the incubator (37 ˚C) or in a drawer for room temperature staining (e.g. ADH, PGI which come up very quickly). Some stains, such as catalase, result in the gel turning blue and the areas of enzyme activity remaining white. This "negative" staining can be very subtle and care must be taken to monitor the progress of the blue color reaction as soon as the staining reagent (here potassium iodide) is added. It is probably a good idea to place the stain box on the light table so that a photograph can be taken when contrast is optimal.

Other methods have been used to apply the substrates and stains to gels including agar overlay. This is used primarily to conserve expensive reagents since the total amounts of substrates and stains needed may be 1/2 to 1/3 the amount required with the above methods. The techniques and recipes for using agar overlays can be found in O'Malley, et al. (1984), Soltis, et al. (1983) and Soltis & Soltis (1989).

Table 2. Enzymes Typically Employed in Plant Starch Gel Electrophoresis

Enzyme Name	Abbreviation	E.C.#	No. Loci	Subcellular Localization
	MONOMERS			
1. Acid Phosphatase (& dimers)	ACP	3.1.3.2	1-3	various
2. Aconitase	ACO	4.2.1.3	1-3	c, mt
3. Adenylate Kinase	ADK	2.7.4.3	1-2	c, p
4. α -Amylase	α-AMY	3.2.1.1	1	
5. ß-Amylase	ß-AMY	3.2.1.2	1	
6. Diaphorase (& dimers, tetramers)	DIA	1.6.2.2	1-4	c, p, mt
7. Esterases (& dimers)	EST	3.1.1.-	3-10	c
8. α-Galactosidase (& dimers)	a-GAL	3.2.1.22	1-3	c, p
9. Leucine Amino Peptidase	LAP	3.4.11.-	1-2	
10. Mannose Phosphate Isomerase	MPI	5.3.1.8	1	
11.Peptidase	PEP	3.4.3.1	4 or >	
12. Peroxidase	PRX	1.11.1.7	2-many	c, cell wall
13.Phosphoglucomutase	PGM	2.7.5.1	2	c, p
	DIMERS			
14. Alcohol Dehydrogenase	ADH	1.1.1.1	1-3	c
15. Aspartate Amino Transferase	AAT	2.6.1.1	1-4	c, p, mt, mb
(=Glutamate Oxaloacetate Transaminase)	GOT			
16. Fructose-1,6 diphosphatase	F-1,6 DP	3.1.3.11	2	c, p
17. Glucose-6-Phosphate Dehydrogenase	G-6-PDH	1.1.1.49	2	c, p
18. ß-Glucosidase	ß-GLU	3.2.1.21	1	c
19. Isocitrate Dehydrogenase (NADP)	IDH	1.1.1.42	1	c
20. Malate Dehydrogenase (NAD)	MDH	1.1.1.37	3 or >	c, mt, mb
21.6-Phosphogluconate Dehydrogenase	6-PGDH	1.1.1.44	2	c, p
22. Phosphoglucoisomerase	PGI	5.3.1.9	2	c, p
(= Glucose Phosphate Isomerase)	GPI			
23.Superoxide Dismutase (& tetramers)	SOD	1.15.1.1	3	c, p, mt
(=Tetrazolium Oxidase)	TOX			
24. Triose Phosphate Isomerase	TPI	5.3.1.1	2	c, p
	TETRAMERS			
25. Aldolase	ALD	4.1.2.13	2	c, p
26. Catalase	CAT	1.11.1.6	1	mt
27. Glyceraldehyde-3-Phosphate Dehydrog.	G-3-PDH	1.2.1.12	3	c, p
28. Lactate Dehydrogenase	LDH	1.1.1.27	1	c
29. Malic Enzyme	ME	1.1.1.40	1	c
30. Fumarase	FUM	4.2.1.1	1	mt
31. Sorbitol Dehydrogenase	SDH	1.1.1.14	1	c
	HEXAMERS			
32. Glutamate Dehydrogenase	GDH	1.4.1.2	1	c

Photographing Gels

Gels should be monitored closely after placing the stain boxes in the incubator. Some enzyme activity will begin to appear within one minute for certain systems. If it is determined that staining is occurring so quickly as to be impractical, the stain boxes can be placed in a dark drawer at room temperature. When more than one species is present on a gel, enzyme activity may differ between them, hence staining intensity will vary at particular points in time. For these gels it is wise to take a series of at least two or three photographs to capture each species at its optimal staining intensity and resolution. The gel can be removed from the box, placed on the glass plate on the light box, photographed, and then returned to the stain box for additional staining. It is important not to leave the gel set on the light box (with the light on) for very long because the tetrazolium stains turn dark blue more quickly when exposed to the light.

Determining what is optimal staining for a particular system is largely a matter of experience. Overstaining results in bands that become so intense that they become "spots", often obscuring adjacent bands with slightly different migration distances. It is desirable to photograph the gel when it has reached a "peak" of staining intensity where intensity and resolution are both very good. If the gel requires no further staining time, it is rinsed in tap distilled water and photographed (with the numbered acetate strip and its stain box label below). If the gel is very wet and shiny, it should be blotted with a paper towel to avoid reflections from the room lights.

Because of the cost of black and white prints, our lab uses inexpensive color print film (e.g. KodaColor Gold). Exposures are generally correct using the camera's light meter. An exception involves photographing UV fluorescent stains (such as esterase and stains that use 4-methylumbeliferyl substrates). For these, you will need to use a yellow filter over the camera lens with the room completely dark. Illuminate the bands with either a transilluminator or a hand-held U.V. lamp (long wave only). I have experimented with the "Auto" exposure feature on my camera, but generally do a manual exposure of ca. 2.0 seconds at f-2.8.

Fixing Gels

It is standard practice in this lab to fix any gels that showed enzyme staining. The reason for this is that sometimes accidents happen with the photography and one then has not lost the information on the gels. Also, when the gels are being scored, it is often desirable to look at a particular gel again to check determinations. Many workers score their gels immediately after photography. This requires, however, that one can designate alleles as certain numbers or letters and this is not always possible, especially early in a study. Given a good quality 3.5 X 5 print, relative mobility designations can be made from measurements taken directly from the picture. After the film is printed, the gels can be discarded. If one uses Polaroid photographs (more expensive!), the results are known immediately.

Two fixatives are used in our lab: 5:5:1 (Methanol, Water, Acetic Acid) and 50% Glycerol. The glycerol fixative results in less fading of the bands, hence on can use glycerol for any of the gels (but it is more expensive). Table 3 lists some commonly stained enzyme systems and the fixative that can be used for them:

Table 3. Enzyme Systems and Fixatives

50% Glycerol			5:5:1	
ACO	PGI	ALD	ACP	LDH
ADH	PGM	GSR	DIA	ME
FDP	PRX	TPI	EST	MDH
G-6-PDH	TPI		FUM	PEP
6-PGDH	SKDH		GDH	MDR
HXK	IDH		GOT	LAP

A general rule of thumb is that any gel which was stained with MTT should be fixed in 50% glycerol; those stained with NBT or any of the Fast stains should be fixed in 5:5: 1. Gels will be completely fixed in about 3-5 hours and are usually taken out of the stain boxes and wrapped in plastic wrap the next day. Some enzyme systems (such as total protein and possibly LAP) undergo destaining in the 5:5:1 solution and the bands may actually look sharper than they would without this treatment.

Scanning for Enzyme Activity: An Example Protocol

For a person just beginning an isozyme study of a group of plants, a useful procedure to begin with is called an enzyme scan. The scan simultaneously compares and contrasts a number of variables involving the extraction buffers, the gel buffers, the enzyme systems, and even the condition and type of tissue being used. The following protocol is provided as an example of what a typical gel run scan might look like.

Let's assume that the gels we run can accommodate 21 slots, be they enzyme samples extracted from the plant tissue or marker dyes. Let's also assume that Table 4 constitutes the combinations of extraction buffers, extra components and plant tissue conditions we wish to use:

Table 4. Sample Extraction Protocol

Slot	Buffer	Added Ingredients	Tissue Condition
1.	Hepes, pH 7.0	None	Fresh Leaves
2.	Hepes, pH 7.0	ß-Mercaptoethanol, PVP	Fresh Leaves
3.	Hepes, pH 7.0	ß-Mercaptoethanol, PVP	Frozen Leaves
4.	Tris-Ascorbate, pH 7.2	None	Fresh Leaves
5.	Tris-Ascorbate, pH 7.2	ß-Mercaptoethanol, PVP	Fresh Leaves
6.	Tris-Ascorbate, pH 7.2	ß-Mercaptoethanol, PVP	Frozen Leaves
7.	Bromphenol Blue Marker Dye (marks where to cut off first 1/3)		
8 to 13.	Same as 1 through 6		
14.	Bromphenol Blue Marker Dye (marks where to cut off second 1/3)		
15 to 20.	Same as 1 through 6		
21.	Bromphenol Blue Marker Dye (last lane - trim off small strip)		

Essentially, loading one gel in this fashion is like loading three identical smaller gels owing to the repeated samples. If this same loading scheme is used for two additional gels, then in effect nine gels have been produced. These can be considered separate gels because each larger gel will be cut into three equal pieces later. Given a conservative estimate of five good slices per gel, a total of 45 slices will be obtained for all three gel buffers. Three slices, one from each gel buffer, will be stained in each stain box, hence a total of fifteen enzyme systems can be scanned across six different extraction protocols and three gel buffers.

Since there is considerably more slicing involved during a scan than during a normal gel run, it is important to consider the extra time involved with this step, especially if you are working alone. Gels should not be left at room temperature (even in the box buffers) for more than a few minutes and should not be left in the buffers for long periods of time without the substrate and stain ingredients. During a scan, it is desirable to have all three or four different gel buffer pieces in the box at the time the substrates are added. The ideal situation would be if all the gels ran for the same length of time and could be cut and positioned in the boxes within a short time period. Gel running times vary considerably, hence they should be staggered to allow cutting at approximately the same time. Alternately, a gel can be turned off to allow the others to catch up. While off, make sure there is plenty of ice in the enamel pan above the gel. When one gel has been cut and positioned in the boxes, store the boxes in the dairy case while the next gels are being cut.

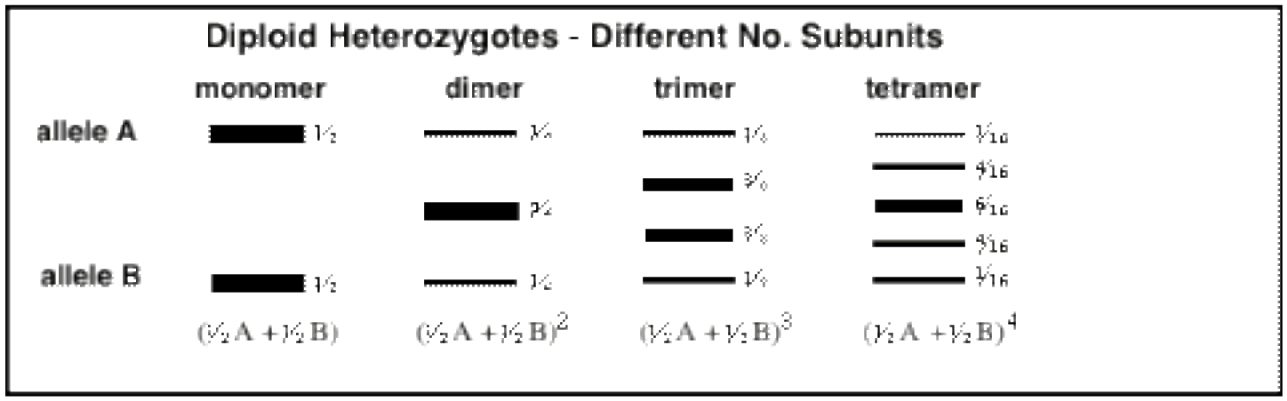
Scoring Starch Gels

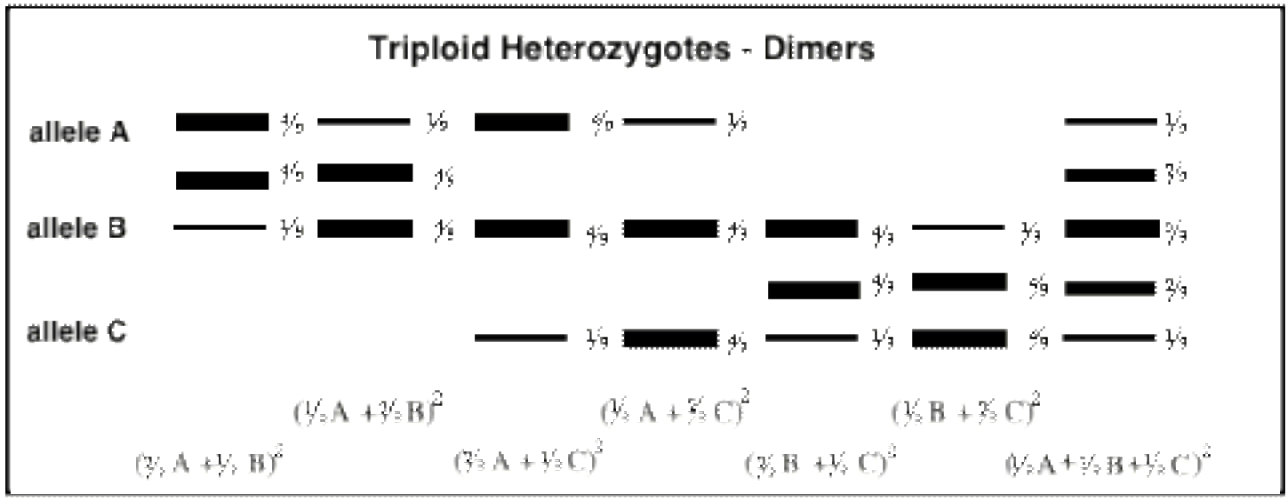
The interpretation of the bands resulting from electrophoresis and subsequent staining is commonly referred to as "scoring" a gel. Some experience is needed to accurately interpret the often large number of bands present on one gel, especially those with multiple loci such as MDH that can form interlocus heterodimers. The best way to start is to look at results of the analysis of other organisms in which the methodology has already been worked out to the point where band intensity and resolution is already good. Such results can be seen in published articles or better yet by observing gels directly. The diagram on the next page illustrates the types of patterns seen for different quaternary structure (monomers, dimers, trimers, tetramers) as well as different ploidy levels of the organism. A good summary of enzyme band interpretation can be found in Kephart (1990).

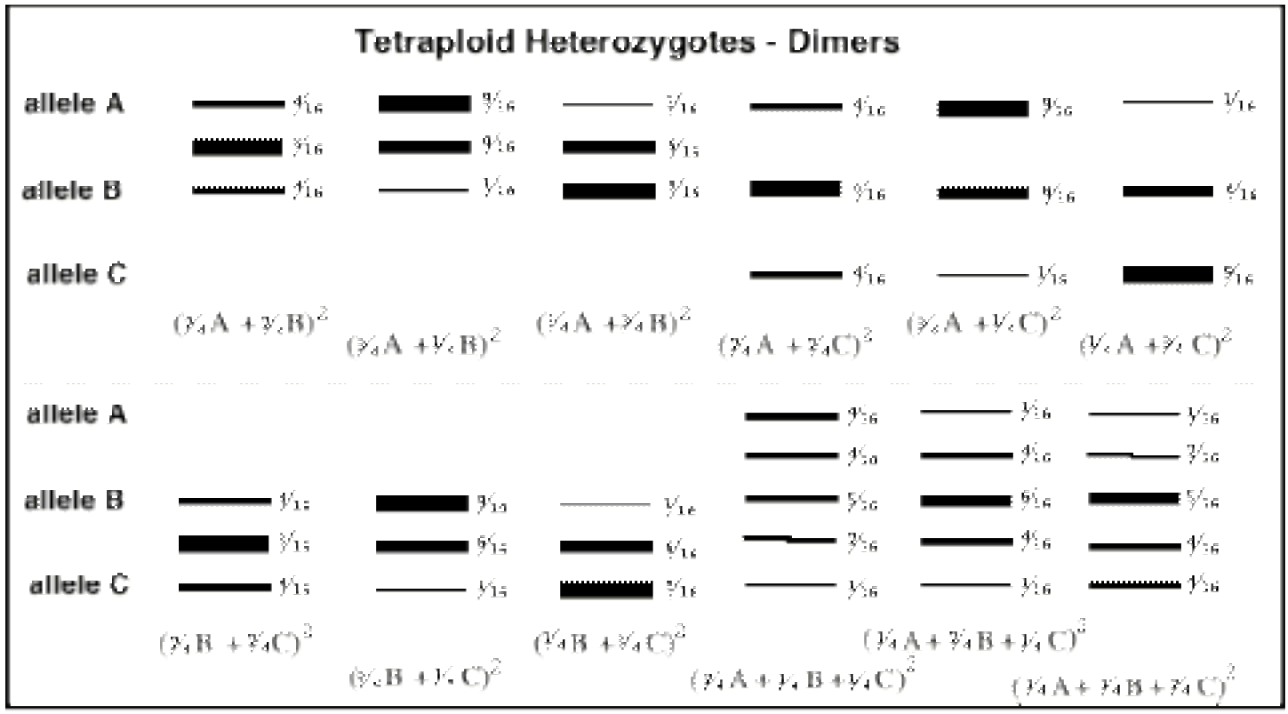
There are a variety of ways to record the alleles visualized on the gels. One method is to assign a letter to each allele starting with the most anodal one and proceeding towards the cathodal end. The problem with this method is that upon discovering a new allele, reassignment of all the other alleles is required. For this reason, many workers utilize relative mobility (RM) numbers for each allele. Here, all alleles at a locus are permanently assigned a number which represents a percentage of the migration distance of a known standard loaded onto every gel. The standard can be any readily obtainable organism that gives interpretable banding across all loci stained for the organisms under study. It is convenient to use one out of the group of species being studied for the standard.

Caliper measurements of band migration distance (from the origin) just after photography combined with values obtained from the black and white prints obtained later gives the most accurate RM values. Unfortunately, electrophoresis is not perfect and variations in the gel front will inevitably be transferred to the band positions. The most common malady is a bowed front caused by uneven cooling of the gel. Even with an uneven front, one can score the gel by noting the "trends" in the front (and homozygote band positions).

A useful program for the analysis of isozyme data is BIOSYS: A computer program for the analysis of allelic variation in genetics (Swofford and Selander 1981). Relative mobility numbers must be converted to alphabetic allele designations for input into the program. The data can be input either as individual genotypes or as genotype frequencies (useful for polyploids).


Diploid Heterozygotes - Different No. Subunits
monomer
dimer
trimer
tetramer
allele A
allele B
(½ A + ½ B)
(½ A + ½ B)²
(½ A + ½ B)³
(½ A + ½ B)⁴


Triploid Heterozygotes - Dimers
allele A
allele B
allele C
(⅔ A + ⅓ B)²
(⅓ A + ⅔ B)²
(⅔ A + ⅓ C)²
(⅓ A + ⅔ C)²
(⅔ B + ⅓ C)²
(⅓ B + ⅔ C)²
(⅓ A + ⅓ B + ⅓ C)²


Tetraploid Heterozygotes - Dimers
allele A
allele B
allele C
(¾ A + ¼ B)²
(¾ A + ¼ B)²
(½ A + ½ B)²
(¾ A + ¼ C)²
(½ A + ½ C)²
(¼ A + ¾ C)²
(¾ B + ¼ C)²
(¼ B + ¾ C)²
(½ A + ¼ B + ¼ C)²
(¼ A + ¾ B + ¼ C)²
(¼ A + ¼ B + ¾ C)²

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D. L. Nickrent -- 1/15/91
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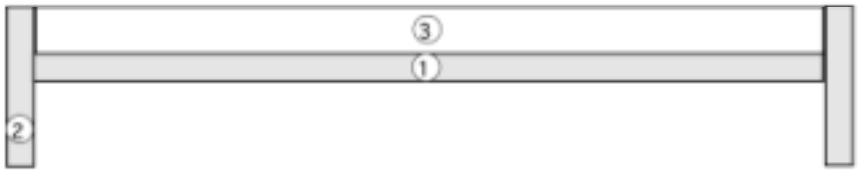
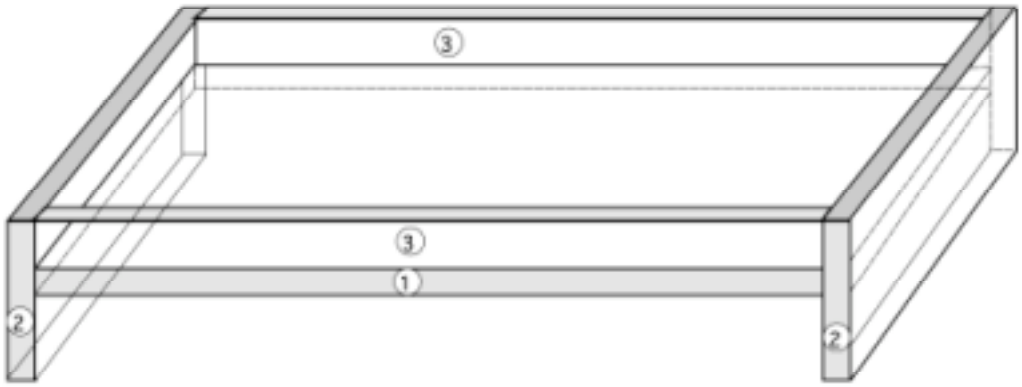
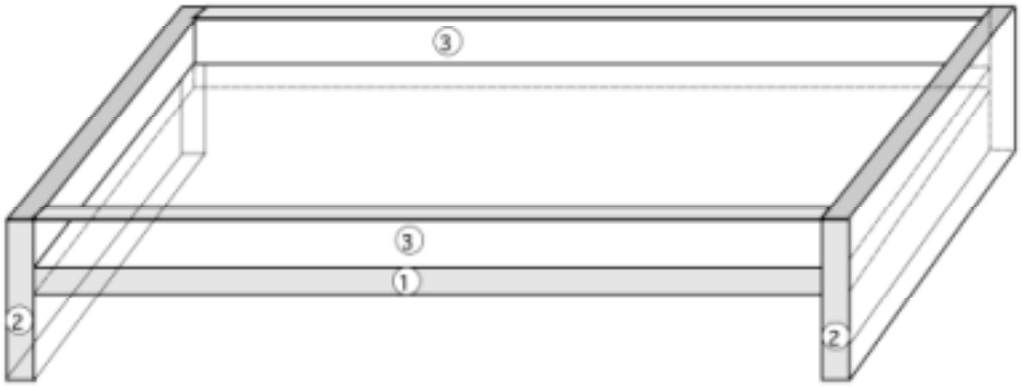
Gel Plate - side view





Dimensions

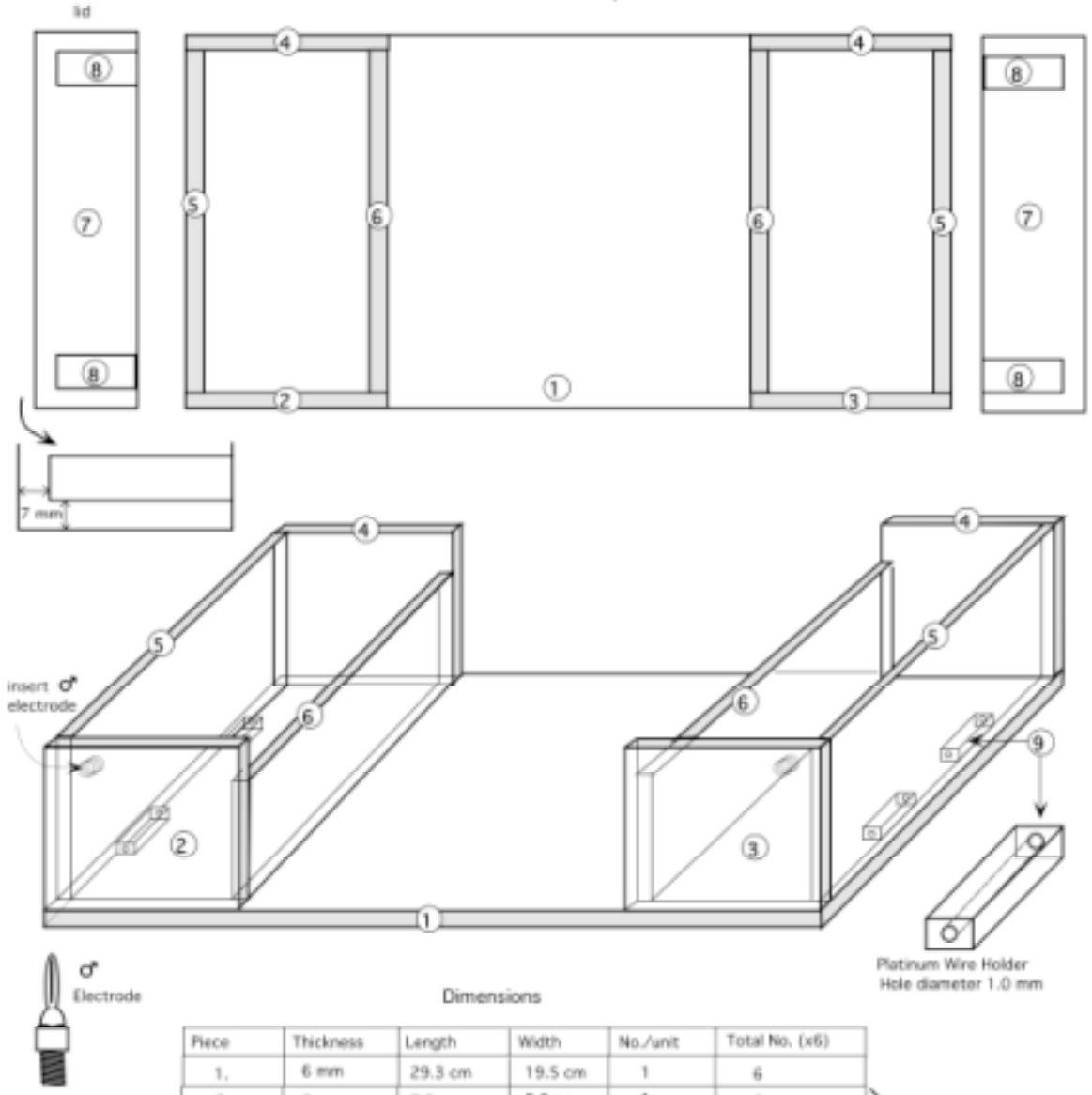
Piece	Thickness	Length	Width	No./unit	Total No. (x6)
1.	6 mm	16.8 cm	18 cm	1	6
2.	6 mm	18.0 cm	3.5 cm	2	12
3.	6 mm	16.8 cm	1.0 cm	2	12



Dimensions

Piece	Thickness	Width	Length	No./unit	Total No. (x8)
1.	6 mm	18 cm	20 cm	1	8
2.	6 mm	3.5 cm	18 cm	2	16
3.	6 mm	1 cm	20 cm	2	16

Gel Electrode Box -- Top View



Dimensions

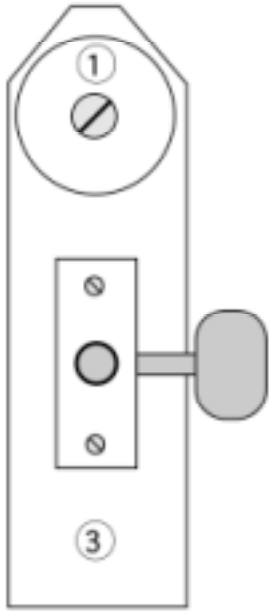
Piece	Thickness	Length	Width	No./unit	Total No. (x6)
1.	6 mm	29.3 cm	19.5 cm	1	6
2.	6 mm	7.0 cm	5.0 cm	1	6
3.	6 mm	7.0 cm	5.0 cm	1	6
4	6 mm	7.0 cm	5.0 cm	2	12
5	6 mm	18.3 cm	5.0 cm	2	12
6	6 mm	18.3 cm	4.0 cm	2	12
7	6 mm	19.5 cm	5.0 cm	2	12
8	6 mm	4.3 cm	1.0 cm	4	24
9	1 cm ?	2.0 cm	1.0 cm	4	24

only difference is hole is on the left side of #2 & right side of #3

Need ca. 20 cm of 0.5 mm diam. platinum wire for each side X 2 = 40 cm per gel rig. X 6 gel rigs = 240 cm

Gel Slicer -- Side View

Gel Slicer -- End View



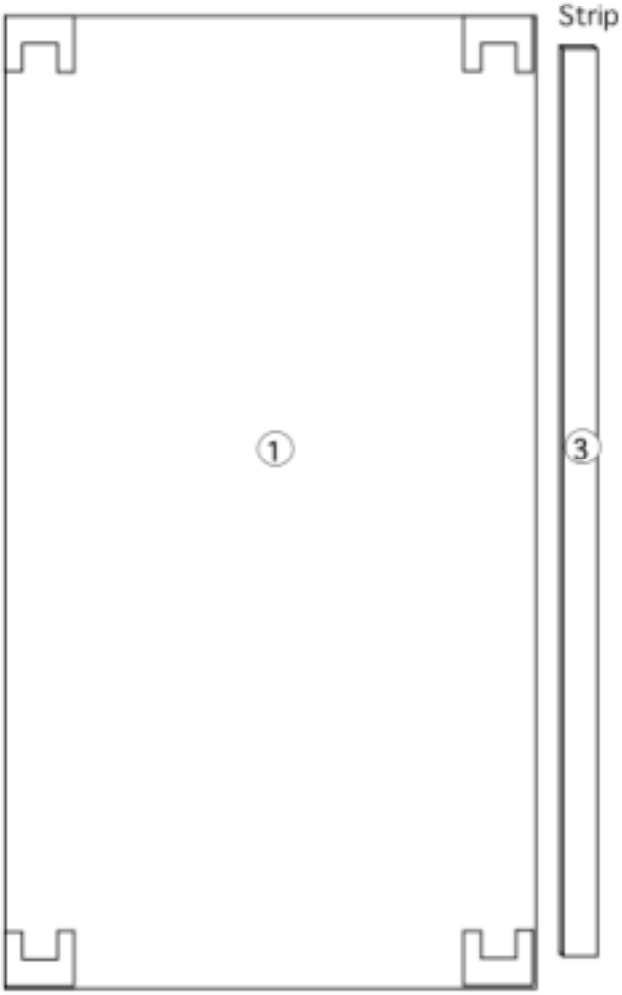
Dimensions - Metric

Piece	Thickness	Length	Width	No./unit	Total No. (x2)	
1.	2.54 cm	16.5 cm	---	1	2	Rod
2.	1.27 cm	9.525 cm	3.175 cm	2	12	taper end
3.	1.27 cm	9.525 cm	3.175 cm	2	12	taper end

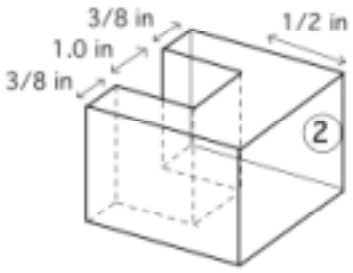
Dimensions - Inches

Piece	Thickness	Length	Width	No./unit	Total No. (x2)	
1.	1.0 diam.	6 1/2	--	1	2	Rod
2.	1/2	3 3/4	1 1/4	1	2	taper end
3.	1/2	3 3/4	1 1/4	1	2	taper end

Gel Slicer Base -- Top View



End Piece



Dimensions - Metric

Piece	Thickness	Length	Width	No./unit	Total No.	
1.	0.66 cm	35.5 cm	21.0 cm	1	1	base
2.	2.5 cm	2.5 cm	4.4 cm	4	4	end piece
3.	1.1 mm	33 cm	2.5 cm	24	24	strip

Dimensions - Inches

Piece	Thickness	Length	Width	No./unit	Total No.	
1.	1/4	14	8 1/4	1	1	base
2.	1	1	1 3/4	4	4	end piece
3.	1/24	13	1	24	24	strip

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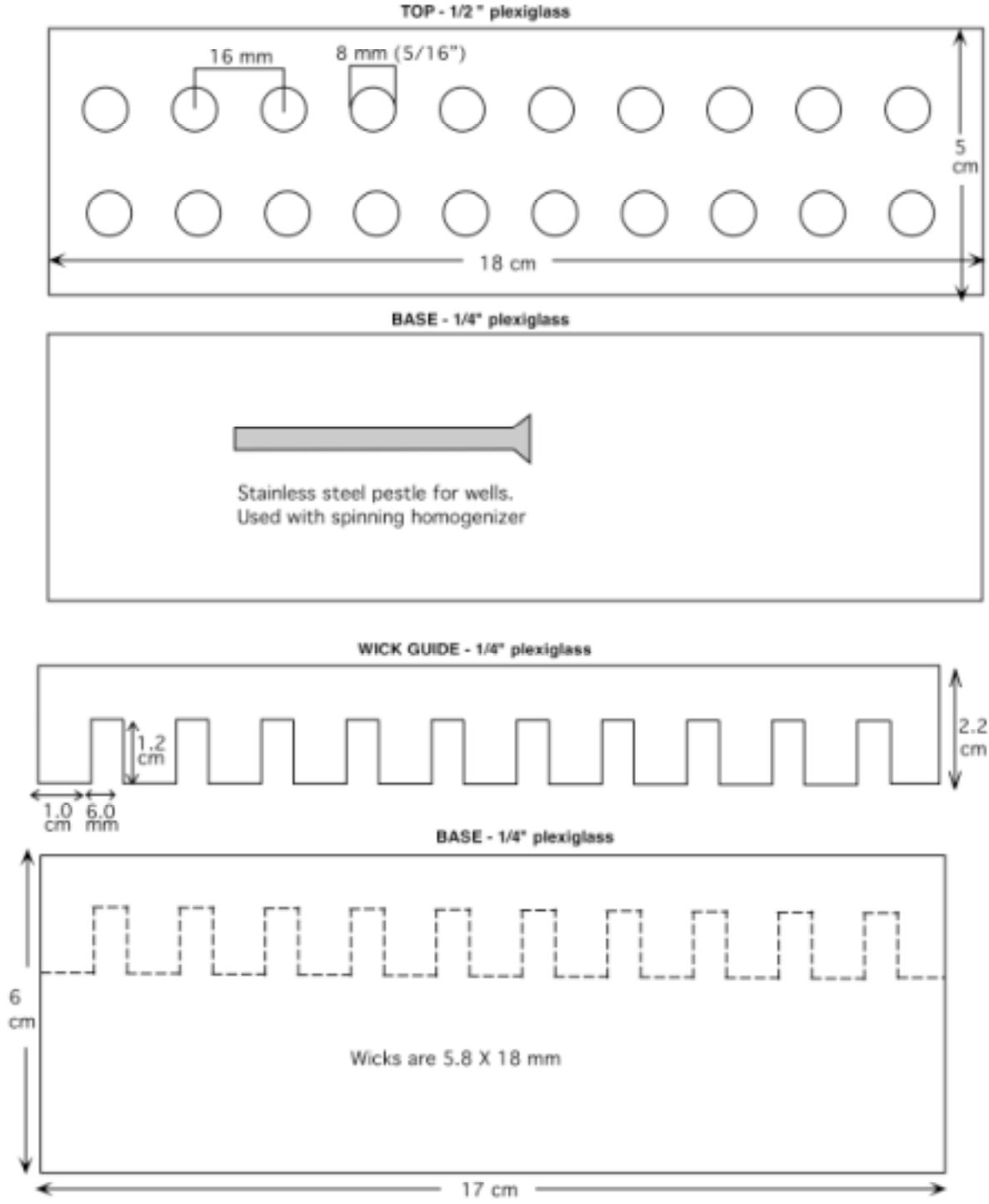

36-Well Mold and Starch Gel Comb Guide